Effect of Glycation of Low-Density Lipoprotein on the Immunological Determination of Apolipoprotein B

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Non-enzymatic glycation of low-density lipoprotein (LDL) may contribute to the premature atherogenesis of patients with diabetes mellitus. To assess whether glycation of apolipoprotein B, the predominant protein of LDL, interferes with the ability to immunologically quantify this protein, we prepared and purified glyated LDL by incubating normal plasma samples with high concentrations of glucose. Although both the plasma and the LDL specimens incubated with glucose contained significantly more glycated protein than control specimens, the quantitative interaction of an apolipoprotein B-specific antibody with glycated vs nonglycated LDL was not significantly different. We conclude that apolipoprotein B can be accurately quantified immunologically despite the presence of clinically excessive degrees of LDL glycation.

Additional Keyphrases: diabetes mellitus · atherogenesis · immunonephelometry

Non-enzymatic glycation of proteins is thought to be a primary initiating event in the pathogenesis of many of the clinical complications of long-standing diabetes mellitus (1). In particular, glycation of apolipoprotein B, the major apolipoprotein of low-density lipoprotein (LDL) has been shown to occur in vitro (2, 3) and in vivo (3) after prolonged hyperglycemia. Involvement of this glycated LDL in the accelerated atherogenesis characteristic of prolonged diabetes has been suggested by reports of both impaired recognition of glycated LDL by the LDL receptor (4) and increased cholesterol ester accumulation by monocyte/macrophage cells exposed to glycated LDL (5). Because concentrations of apolipoproteins A and B in serum may be better predictors of atherogenic risk than is the typical cholesterol or lipoprotein analysis (6), more clinical laboratories have begun offering apolipoprotein measurements, predominantly by immunological methods with use of commercial apolipoprotein-specific antibodies.

To assess whether non-enzymatic glycation of LDL apolipoprotein B had any quantitative effect on the interaction of this protein with the antibody used for its clinical determination, we used an immunonephelometric assay to compare the amounts of apolipoprotein B in LDL purified from plasma samples that had been pre-incubated with or without high concentrations of glucose. We found that, although the LDLS from the plasma specimens incubated with glucose were significantly more glycated than the control LDLS, this degree of glycation had no significant effect on the quantitative immunological determination of apolipoprotein B.

Materials and Methods

Specimens: Plasma was sampled from 10 donors with no clinical evidence of diabetes. These samples included five outdated units of fresh-frozen plasma, three fresh specimens obtained after therapeutic plasma exchange from patients with chronic inflammatory demyelinating polyneuropathy, one fresh specimen obtained after therapeutic plphlebotomy from a patient with polycythemia vera, and one fresh specimen from a healthy laboratory technologist. After dialysis against isotonic saline containing 1 mmol of EDTA per liter to remove the preservatives and anticoagulants required for plasma storage, the 10 plasma specimens were each divided into two identical 15-mL aliquots. To one of each pair of 10 specimens we then added concentrated glucose (210 g/L solution) to give a final concentration of 9.0 g/L; the other received no addition. All speci-

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mens, with or without added glucose, were incubated for seven days at 37°C, then were dialyzed against isotonic saline containing 1 mmol of EDTA per liter to remove excess glucose. Plasma specimens were then stored at 4°C until further analysis.

Preparation of LDL: LDL was prepared by density-gradient ultracentrifugation as described by Kelly and Kruski (7), NaBr solutions being replaced by equal-density solutions of KBr. Each 12.5-mL ultracentrifuge tube containing 4.0 mL of plasma was centrifuged in a Beckman SW40 rotor at 26 300 rpm for 16 to 24 h at 4°C. The LDL, visible as a yellow band migrating about 3 mL of the way down the tube, was harvested with a Pasteur pipette (approximately 1.5 mL of LDL per tube) and stored at 4°C in its high-salt buffer.

Electrophoresis: For electrophoresis on agarose gels we used commercially prepared gel plates (Helena Laboratories, Beaumont, TX) as described in the manufacturer’s instructions. Proteins were detected by staining with Amido Black, and lipoproteins by staining with Fat Red 7B. The concentrations of cholesterol and total protein in the plasma and LDL specimens were determined by using an Ektachem 700 analyzer (Kodak, Rochester, NY) and the appropriate dry-film slides (8).

Fructosamine assays: We measured fructosamine in plasma in a Cobas Mira centrifugal analyzer, using reagent (nitro blue tetrazolium), fructosamine calibrator solution, and fructosamine standards provided in a commercial kit (Rotag; Roche Diagnostic Systems, Nutley, NJ). The analyzer was programmed as recommended by the kit manufacturer (20 mL of sample, 50 mL of diluent, 200 mL of reagent; measurements at 550 nm after 10 and 15 min). The assay was calibrated before each test run, and each plasma specimen was analyzed at least in duplicate.

We measured fructosamine in purified LDL specimens in a Cobas Mira analyzer programmed to deliver 75 mL of sample, 20 mL of diluent, and 200 mL of NBT reagent. Measurements were made at 550 nm after 2.5 and 20 min at 37°C. We confirmed the linearity of the assay curve in the range of 0.1 to 3 mmol of fructosamine per liter, using a 3 mmol/L calibrator solution serially diluted into the 1.019 kg/L density-gradient buffer. The assay was calibrated before each test run with the 3 mmol/L calibrator solution diluted to about 0.2 mmol/L, and each LDL specimen was analyzed in triplicate.

Apolipoprotein B assay: Concentrations of apolipoprotein (apo) B were determined by an automated immunonephelometric assay on a Behring nephelometer with polyclonal apo B-specific rabbit antibody provided in a commercial kit (Behring Diagnostics, La Jolla, CA). We calibrated the assay at least once every eight days with standards provided by Behring, and before each test run the apo B concentrations of control serum specimens from two independent sources (Behring, and Bio-Rad Labs., Richmond, CA) were confirmed.

Statistics: We evaluated the fructosamine and total-protein measurements by the paired Student’s t-test to determine the significance of differences between samples incubated with or without glucose. Significance was defined as a P value of <0.05 in the two-tailed t-test.

Results

We used plasma from 10 nondiabetic patients for in vitro glycation reactions, isolation of LDL, and determination of LDL-specific glycation and recognition of apo B antibody. Before incubation with or without glucose, we confirmed the absence of significant in vivo protein glycation in any of these samples by measuring plasma fructosamine, a quantitative means of assessing protein glycation based on the reactivity in alkaline medium of ketamine groups with nitro blue tetrazolium (9). Each of the 10 plasma specimens had a fructosamine concentration well below the cutoff of 2.7 mmol/L exhibited by >95% of nondiabetics (Table 1). After a seven-day incubation with glucose, however, there was a significant (P <0.001) increase in the extent of protein glycation in the samples incubated with glucose [mean fructosamine 3.7 (SD 0.4) mmol/L] as compared with those incubated without glucose [mean fructosamine 1.8 (SD 0.5) mmol/L] (Table 1). In contrast, there was no significant difference (P >0.35) in protein glycation between specimens incubated for seven days with no added glucose [mean fructosamine 1.8 (SD 0.5) mmol/L] and those same specimens before incubation [mean fructosamine 1.8 (SD 0.2) mmol/L]. The increased fructosamine in specimens 3, 4, and 5 after incubation without added glucose probably

| Table 1. In Vitro Glycation of Plasma Proteins |
|--------------------------|--------------------------|--------------------------|
| Patient | Before incubation | With glucose | Without glucose |
| 1 | 2.00 | 4.28 | 1.86 |
| 2 | 1.80 | ND | 1.67 |
| 3 | 1.44 | 3.76 | 2.16 |
| 4 | 1.95 | 4.39 | 2.61 |
| 5 | 1.70 | 3.98 | 2.43 |
| 6 | 1.61 | 3.50 | 1.32 |
| 7 | 1.92 | 3.52 | 1.31 |
| 8 | 2.14 | 3.40 | 1.55 |
| 9 | 1.80 | 3.12 | 1.32 |
| 10 | 1.96 | 3.51 | 1.61 |

For fructosamine, the average of at least two determinations is shown, the maximum inter-run CV being 8.0%. For protein, a single determination was done. Because the plasma specimens incubated with glucose were diluted by the addition of 0.043 volume of concentrated glucose whereas the control specimens received no additional volume, the protein and fructosamine values in this table for the “without glucose” specimens represent the measured concentration divided by the dilution factor 1.043, and rounded to the nearest decimal place. ND, not determined.

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effected protein glycation enhanced by prolonged incubation in medium containing a low, but physiological, glucose concentration (400–1000 mg/L). The decreased fructosamine in specimens 7, 8, and 9 after the control incubation probably resulted from nonspecific losses of glycated and nonglycated proteins during dialysis. Each of the 10 plasma specimens treated with glucose had fructosamine values in the range expected for poorly controlled diabetics (2.8 mmol/L), compared with none of the control plasma specimens in this range (Table 1). These higher fructosamine values in the samples treated with glucose could not be attributed to a significant difference in total protein concentration between these pairs of samples (mean protein concentration with glucose, 45 (SD 7) g/L; without glucose, 43 (SD 6 g/L); P >0.05; Table 1). Again, the low concentrations of total protein in all of these samples probably resulted from nonspecific protein losses during dialysis. The significant difference in fructosamine between these sample pairs was therefore clearly the result of an accelerated rate of non-enzymatic glycation in the presence of high concentrations of glucose.

To assess whether this increase in protein glycation in the plasma specimens treated with glucose resulted in specific glycation of LDL, we purified LDL from each of the 10 plasma samples by ultracentrifugation. Agarose gel electrophoresis of these LDLs revealed a single species after staining for either protein or lipid (Figure 1), implying that the purified LDL was free of contaminating protein and lipoprotein. The increased electrophoretic migration of these LDLs (into the pre-β region in the lipid gels and the α-β interzone in the protein gels) was not characteristic of LDL from fresh plasma (Figure 1, lane 14). This alteration in LDL mobility probably resulted from accelerated oxidative degradation, lipolysis, or albumin-binding during prolonged incubation at 37 °C. We noted this characteristic increase in gel mobility only in LDLs purified from plasma incubated at 37 °C for seven days, and not from fresh plasma (data not shown). Similar degradative changes in LDL have been documented after prolonged storage at 4 °C (10) and the resulting LDL, like ours, had increased electrophoretic mobility.

Table 2 shows the results of replicate determinations of the extent of glycation in the 20 LDL samples, expressed as reactivity in a modified fructosamine assay. Concentrations of total cholesterol were also determined for each of these LDLs, and to control for variability in the quantitative yield of LDL per preparation, the data are expressed as millimoles of fructosamine per gram of LDL cholesterol. For each of the 10 LDL pairs, the relative extent of glycation of the sample exposed to glucose was greater than that of its paired control, the mean increase being 37% (0.136 to 0.186 mmol/g). Statistical analysis of these pairs revealed a highly significant (P <0.001) increase in glycation of the samples incubated with glucose as compared with those without glucose. Apo B is the predominant protein of LDL (11), so we conclude that apo B is a specific target of the non-enzymatic glycation that occurs in the presence of in vitro hyperglycemia.

To determine whether glycation of apo B altered the interaction between the apo B antibody and its antigen, we measured the amount of immunoreactive apo B in each of the paired LDL specimens, using an immunonephelometric assay. Again, the data are presented as a ratio relative to LDL cholesterol and they revealed that the measured concentrations of apo B in the LDL samples exposed to glucose were not significantly different from those of their paired controls (P >0.4; Table 2). Thus, despite an average 37% increase in glycation of apolipoprotein B in the LDLs incubated with vs without glucose, the antibody used to immunologically quantify this protein interacted normally with its antigen binding site.

Discussion

Using a modified fructosamine assay to quantify protein glycation, we have shown here that prolonged exposure of plasma to high concentrations of glucose results in a specific non-enzymatic glycation of LDL apo B, and that this modified apo B can interact with apo B-specific antibodies to the same extent as does nonglycated apo B. Other studies have demonstrated the presence of glycated LDL after in vivo or in vitro hyperglycemia by use of methods such as m-aminophenyl boronate affinity chromatography (12, 13), radioimmunoassay with antibodies specific for glucosamine adducts (14), glucosamine mass measurement in an amino acid analyser (3), the trinitrobenzenesulfonic acid assay (4), or reduction of glucosamine adducts with tritiated sodium borohydride (3, 15). In comparison, our methodology took advantage of the ability of fructosamine groups to reduce nitro blue tetrazolium in alkaline medium into a colored product (9). Application of this fructosamine assay to fractions of purified LDL allowed a quantitative assessment of the extent of glycation of apo B, the major protein of LDL. The absence of other contaminating proteins in our LDL preparations (Figure 1) eliminated a major limitation of the fructosamine assay, namely the preferential reactivity of the more abundant protein species in heterogeneous mixtures (12, 16).

Because non-enzymatic glycation of intra- and extravascular proteins probably contributes to many of the life-threatening complications of long-standing diabetes (1), a role for glycated LDL in promoting the accelerated atherogenesis characteristic of diabetics would be an attractive
pathological model. In support of such a model, glycated LDL has been found to be recognized less efficiently than control LDL by the apo B-specific LDL receptor (3, 4, 17). Furthermore, glycated LDL, when incubated with monocytes/macrophages, stimulates the rates of cholesterol ester synthesis relative to control LDL (5, 18). LDL is not, however, the only protein to become functionally altered upon glycation, because non-enzymatic glycation of serum albumin results in both a specific protein conformational change and a decreased binding affinity for bilirubin and fatty acids (19). A model thus arises where glycated LDL, poorly recognized by the classic LDL receptor, accumulates to high extracellular concentrations and is cleared via alternative LDL receptors (such as the monocyte/macrophage scavenger receptor). These alternative LDL receptors stimulate accelerated rates of cholesterol ester synthesis and may therefore lead to the transformation of macrophages into the lipid-laden foam cells characteristic of early atheromatous fatty streaks. Like glycated LDL, LDL modified by chemical processes such as oxidation or acetylation is also preferentially recognized by alternative LDL receptors (20), implying a common atherogenic pathway for LDL damaged by various means.

Although our patient group failed to include any diabetics, exposure of normal plasma to high concentrations of glucose resulted in specimens with degrees of protein glycation (mean fructosamine 3.7 mmol/L) exceeding those of even the most poorly controlled groups of diabetics (21–23). Unless there is some fundamental difference in the distribution of protein glycation in vivo vs in vitro, these data imply that, even at the highest degrees of LDL glycation likely to be encountered in a clinical setting, there is no impairment of the interaction between apo B and the antibody used for its quantitative assessment. Immunological assays should therefore be an accurate means of quantifying apo B concentrations, even in poorly controlled diabetics. The concentration of apo B may represent a superior index of atherogenic risk than standard cholesterol or lipoprotein assays (6), so it may be especially important to follow apo B concentrations in high-risk diabetic patients, either before or during treatment of hyperlipidemia.

The inability of polyclonal antibody to distinguish glycated from nonglycated apo B may reflect a true inability of protein side-chain chemical modification to alter the interaction between antibody and antigen. Alternatively, it is possible that either the extent of protein glycation is not sufficient to cause a visible effect on antibody binding or the antibody itself lacks the avidity or specificity required for the differential effect to be measurable. Clinically, however, these alternatives are of little practical consequence, because degrees of glycation greater than those produced here are unlikely to be encountered, and because, in most commercially available apo B immunoassays the polyclonal antibodies are similar to those used here. Despite this inability of commercial reagents to distinguish glycated from nonglycated apo B, the different biochemical characteristics of these two related species suggest distinct in vivo recognition mechanisms that may contribute to the overall pathogenesis of hyperglycemia.

References
2. Sasaki J, Cottam GL. Glycoleyation of human LDL and its
n a previous comparison study of "dry chemistry" desktop analyzers, the ChemPro 1000 (Arden Medical Systems) was one of several instruments found suitable for field use. We have now evaluated the linearity, accuracy, and precision of the ChemPro 1000, according to NCCLS Document EP 0-P. We also compared results with those by the SMAC Technicon) and the Nova 9 (Nova Biomedical) for electrolytes, serum urea nitrogen, and ionized calcium in field and laboratory environments. The precision (CV) of the ChemPro was within acceptable ranges for dry chemistry desktop analyzers for all analytes tested. This instrument is a suitable and reasonable alternative to manual chemistry or to large, automated instrumentation in a field environment.


A Clinical Chemistry Analyzer Evaluated by NCCLS Guidelines for Use in a Military Field Laboratory Unit

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The ability to perform fundamental chemistry analyses in the field is essential to the mission of a military medical laboratory unit. The standard manual chemistry methods require water-based reagents, which are bulky and difficult to transport and keep from damage during mobilization. The methodology is laborious and requires specially trained personnel. Current automated analyzers are impractical in a field setting. Recently, the development of "dry chemistry" desktop analyzers for physicians' offices has allowed decentralization of some laboratory services to provide timely results for diagnostic or management decisions while the patient waits (1-5). Doos et al. (6) examined the suitability of several portable dry chemistry desktop analyzers in a mobile army field laboratory. In that patient-comparison study, all of the instruments tested were found to be "field suitable." Although it did not have applications for all of the required analytes, the ChemPro 1000 (Arden Medical Systems, St. Paul, MN) was the instrument of choice because of its ease of use and low requirement for temperature-sensitive, fragile, or bulky consumables. We undertook the present study specifically to evaluate linearity, accuracy, and precision in a field laboratory setting, with the evaluation based on the guide-