Urinary Excretion of Glycated Protein Determined with a Specific Radioimmunoassay

Chizuko Ukita, Mitsugishi Nishikawa, Akira Shouzu, and Mitsuo Inada

We developed a simple and highly sensitive RIA for glycated protein (GP), and used it to measure GP in serum and urine from 15 normal controls and 30 diabetics (14 with urinary excretion rate of albumin, Ualb <15 μg/min, group A; nine with 15≤ Ualb ≤150 μg/min, group B; and seven with Ualb >150 μg/min, group C). The mean serum concentration of GP was above normal in all groups of diabetics, and the mean glycation ratios of serum protein (SGP) were higher in groups B and C than in normal subjects. Urinary concentrations of GP also were increased in groups B and C, although the glycation ratio of urinary protein (UGP) was decreased in group C. Consequently, the selectivity of urinary excretion of GP (UGP/SGP) was significantly decreased in group C. Moreover, there was a significant difference in the mean values of selectivity between groups of patients with various degrees of retinopathy. We suggest that measurements of serum and urinary GP are useful to evaluate the progression of diabetic complications.

Addional Keyphrases: diabetes · nephropathy · retinopathy · glucitol-lysine residues

Nonenzymatic glycation of hemoglobin, lens crystallines, collagen, albumin, and other serum proteins occurs in normal individuals and, to a greater extent, in diabetic subjects (1–8). Because diabetic nephropathy might result from the glycation of structural glomerular proteins, some investigators have determined the urinary excretion of glycated protein (GP) in diabetics (9–11).

However, there is still a paucity of information about the urinary excretion of GP, because the previous procedures for determining GP were rather difficult and not very sensitive. We therefore developed a simple, highly sensitive RIA for GP, with which we measured the glucitol-lysine residues of glycated protein reduced in vitro. We used this method to investigate the renal handling of GP in normal subjects and in diabetic patients.

Materials and Methods

Preparative Procedures

GP. Proteins were glycated and reduced by use of techniques similar to those described by Curtis and Witztum (12). In the present paper, the proteins glycated in the presence of the reducing agent are termed reduced glycated proteins (RGlc-prot), whereas those glycated in the absence of the reducing agent are termed nonreduced glycated protein (NRGlc-prot). Reduced glycated human serum albumin (RGlc-HSA) was prepared by incubating 100 mg of HSA (Sigma Chemical Co., St. Louis, MO) with 700 mg of D-glucose in the presence of sodium cyanoborohydride (NaCNBH3), 0.64 mol/L, in 5 mL of phosphate-buffered saline (PBS; 50 mmol/L, pH 7.4) at room temperature for seven days. This solution was acidified by adding 400 μL of glacial acetic acid to stop the reaction, and then was dialyzed against distilled water at 4 °C for 24 h. Reduced glycated bovine low-density lipoprotein (RGlc-LDL) was prepared by a similar method from LDL (United States Biochemical Corp., Cleveland, OH). Reduced glycated hippuryl-lysine (RGlc-lysine) was kindly donated from Otsuka Assay Labs., Tokushima, Japan (13, 14). Control HSA, bovine serum albumin (BSA), and LDL were prepared by a similar method, with NaCNBH3 but without glucose.

Immunization and labeling. Three New Zealand White rabbits (mean body weight, 1.4 kg) were injected subcutaneously with 2 mg each of RGlc-HSA emulsified in complete Freund’s adjuvant. At two-week intervals they received subcutaneous booster injections of 2 mg of RGlc-HSA, and the antisera was obtained.

RGlc-LDL was labeled with 125I by the Chloramine T method of Hunter and Greenwood (15). 125I-labeled RGlc-LDL was purified by gel chromatography on a 1.0 × 30 cm column of Sephacryl S-200 (Pharmacia, Uppsala, Sweden) with PBS containing 16 μmol of gelatin and 3.1 mmol of sodium azide per liter.

Subjects

We studied 15 normal subjects and 30 diabetic patients. The diabetic patients were divided into three groups according to their urinary excretion rates of albumin (Ualb): group A, 14 patients with Ualb <15 μg/min; group B, nine with Ualb between 15 and 150 μg/min; and group C, seven with Ualb >150 μg/min. In all patients in groups A and B, urinary protein was qualitatively negative by the Uro lab sticks (Miles Sankyo). Twenty of the patients were receiving insulin therapy: group A, seven; B, six; and C, seven.

Study protocol. In the morning after an overnight fast, 90-min urine collections were performed; at the midpoint of this period, sera were obtained. Serum and urine samples were stored at −20 °C until the assay.

Assay Procedure

We determined serum and urinary concentrations of GP as follows. Mix 100 μL of serum or urine with 2 mL
of 51 mol/L ethanol reagent. Centrifugate at 2300 × g for 10 min, then dissolve the precipitate in 400 μL of 40 mmol/L phosphate buffer, pH 8.0. Divide the samples into two 200-μL aliquots: use one aliquot to determine protein concentration (we used a commercial kit from Bio-Rad Labs., Richmond, CA) with crystallized BSA as the standard. Incubate the other aliquot with 0.8 mg of sodium borohydride (NaBH₄) at 37 °C for 30 min. After adding 50 μL of 4.2 mol/L acetic acid solution and 150 μL of 1 mol/L sodium hydroxide reagent, dilute the solution with 40 mmol/L phosphate buffer, 500-fold for serum, twofold for urine.

For the RIA, mix 100 μL of anti-RGlc-HSA antiserum (initial dilution 2000-fold, final dilution 10 000-fold), 100 μL of 125I-labeled RGlc-LDL, 100 μL of RGlc-lysine as the standard or sample, and 200 μL of 40 mmol/L phosphate buffer containing 7.2 μmol of BSA per liter (pH 8.0). Incubate at 4 °C for 24 h, then add 100 μL of 50-fold-diluted normal rabbit serum (Daichi Radioisotope, Tokyo, Japan) and 100 μL of 10-fold-diluted rabbit anti-goat immunoglobulin and incubate at 4 °C for 24 h. Centrifuge at 2300 × g at 4 °C for 30 min, then count the radioactivity of the precipitate (we used a well-type scintillation counter from Packard, Meriden, CT).

We measured urinary albumin by the specific RIA described previously (16). Glycated hemoglobin (HbA1c) was measured by HPLC (17). The mean HbA1c value of 200 normal subjects was 5.8 (SE 0.02)% of total hemoglobin. To measure fructosamine concentrations, we used a fructosamine kit (Roche, Basel, Switzerland); normal values were ≤2.9 mmol/L.

Statistical Analysis

All values were presented as mean ± SE. The differences between the means of the two groups were analyzed by Student’s t-test, and correlation coefficients were calculated by the least-squares method.

Results

Assay Performance

Figure 1 shows the standard curve in the present study and the cross-reactivities with various materials. The antiserum reacted with all kinds of RGlc-proteins studied, e.g., RGlc-lysine, RGlc-HSA, and RGlc-LDL, but not at all with NRGlc-HSA, HSA, BSA, LDL, control HSA, control BSA, control LDL, mannitol, sorbitol, or lysine.

We found a significant (P < 0.05) difference for the values of B/T % (quadruplicate determinations) between 0 and 0.5 ng of RGlc-lysine/tube. Therefore, the minimal detectable quantity was about 0.5 ng of RGlc-lysine/tube, or 15 pmol/L for a 0.1-mL sample. The dilution curve of serum sample diluted with assay buffer paralleled the standard curve for RGlc-lysine. The mean analytical recovery rate was 89.2% and 99.0% for serum and urine, respectively. Intra-assay studies gave CVs of 9.1% and 11% at GP concentrations of 98 and 290 pmol/L, respectively (n = 6). We found interassay CVs of 14% and 12% at GP concentrations of 95 and 230 pmol/L, respectively (n = 5), for samples assayed within three months of storage.

The following data were obtained by assaying within three months of sample storage at −20 °C. Without ethanol treatment, adding glucose at 4.7–19 mmol/L to serum samples gave values of 0.3–1.7 μmol/L for RGlc-lysine; adding glucose, 0–0.28 mol/L, to urine samples gave RGlc-lysine values of 0.46–71 μmol/L. However, treating the samples with ethanol, 51 mol/L, gave essentially constant RGlc-lysine values of 0.61–0.83 μmol/L, irrespective of the amount of glucose added.

The correlation between serum concentrations of GP (RGlc-lysine, μmol/L) measured by our RIA (y), and by the fructosamine kit (x) (1-deoxy-1-morphofruuctose, mmol/L) was significant and positive: y = 295x – 501 (r = 0.88, P < 0.01, n = 30).

Measurement of Serum and Urinary GP

Serum concentrations of GP (RGlc-lysine) in normal subjects ranged from 210 to 600 μmol/L, averaging 370 (SE 30). Although values for serum concentrations of GP in the diabetic groups showed a wide scatter, the mean serum concentration of GP in each group was significantly (P < 0.01) higher than that in normal subjects: group A, 610 (SE 80) μmol/L; group B, 840 (SE 130) μmol/L; and group C, 680 (SE 100) μmol/L. The mean serum concentrations of GP per mole of protein—namely, the glycation ratios of serum protein (SGP)—were significantly (P < 0.01) higher in group B [10 (SE 2) mol of RGlc-lysine per mole of protein] and C [9.0 (SE 1.3) mol/mol protein] than in normal subjects [5.7 (SE 0.6) mol/mol protein] (Figure 2, left panel). However, no significant difference was found between the mean RGlc-lysine value in normal subjects and that in group A [7.9 (SE 1.1) mol/mol protein] (Figure 2, left panel). HbA1c was not measured in all patients, but the mean value in group A was 7.9 (SE 0.6) % (n = 10); in group
tients was unaffected by insulin therapy. However, patients with simple diabetic retinopathy (n = 11; five each in groups A and B, and one in group C) had a significantly (P < 0.01) lower ratio (1.5 ± 0.2) than those without retinopathy (n = 13; nine in group A, four in group B), 3.5 (SE 0.6). Moreover, the selectivity ratio in the patients with proliferative-type diabetic retinopathy (six in group C), 0.50 (SE 0.11), was significantly (P < 0.01) lower than that in those with simple diabetic retinopathy. On the other hand, HbA1c concentrations showed no significant correlation with the progression of retinopathy.

**Discussion**

In the present study, the antiserum produced with RGlc-HSA antigen bound well all kinds of RGlc-prot, which suggests that the antiserum recognized mainly glucitol-lysine, because all kinds of RGlc-prot had glucitol-lysine as the glucose adduct, unlike NRGlc-prot. However, RGlc-HSA is believed not to exist in vivo (18). Therefore, for estimating GP by the RIA, we had to reduce samples by using NaBH₄ in advance. In addition, because free glucose in serum or urine might lead to falsely high measurements of GP, we effectively eliminated the interference from free glucose by precipitating the proteins with ethanol.

Many papers have been reported about serum GP measured by RIA (13, 14), as well as by the thiobarbituric acid (TBA) method (7, 19) and the fructosamine method (20). The maximum value of SGP from nondiabetics was three or seven times as high as the minimal value in their reports (7, 19). This finding is comparable with our result that the maximum value was about three times as high as the minimum value. But, because of the low sensitivity in these assays, a concentrating process was necessary, especially for the measurement of urinary GP (10, 21). In the present RIA, the minimal detectable quantity, 0.5 ng of RGlc-lysine per tube, was low enough for measuring GP not only in serum but also in unconcentrated urine. Because glucitol-lysine is thought to be the major borohydride-reducible adduct in urine (22), the UGP estimated by the present RIA probably reflects nearly all the GP in the urine. In addition, the dilution curve was parallel with the standard curve, reproducibility was reliable for three months of storage of the samples, and there was a good correlation between SGP measured by our RIA and by the fructosamine kit. These data validate the use of the present RIA to measure serum and urinary GP.

The urinary excretion of glycated albumin has been measured by the TBA method (10, 21) or by HPLC (11). These methods are time consuming for multiple samples. Using the RIA in this study, one can assay many samples at one time. Moreover, with the present RIA, which measures all glycated molecules as well as albumin, the metabolism of GP can be investigated more extensively than with methods (17) in which albumin is isolated before glycated albumin is measured by HPLC. In diabetic patients with normal urinary albumin excretion, renal clearance of nonglycated albumin vs the
clearance of glycated albumin was significantly increased, whereas in patients with increasing urinary albumin excretion, the ratio was reduced (11). Kverneland et al. (11) suggested that in normo-albuminuric diabetics, nonenzymatic glycation of structural glomerular proteins occurred, and in increased albuminuric patients (in the early stages of diabetic renal disease), structural changes were thought to result from loss of anionic charges, owing to reduced heparan sulfate content in glomerular basement membranes.

However, urinary GP measured by RIA has not yet been reported so far as we know, and the clinical significance of urinary GP is still unclear. Gragnoli et al. (9) determined urinary GP by the TBA method and demonstrated that in the stage of normoalbuminuria, urinary excretion of GP was increased through an increase of UGP (glycation ratio of urinary protein). In the present study, the urinary concentration of GP was significantly higher in the diabetics in groups B and C than in normal subjects and the diabetics in group A, probably because of the increase in the total urinary protein excretion.

In our study, as urinary albumin excretion increased, the UGP/SGP ratio, which reflected the selectivity of urinary excretion of GP, tended to decrease to <1. The electrical charge and pore size of basement membranes are reportedly the major factors determining the filtration of proteins through the glomerular wall (23, 24). The pore size was thought to be large in group C. A progressive decrease in UGP in group C probably resulted from severe renal damage, as judged by the degree of proteinuria. Ghiggeri et al. (10) demonstrated that the mean urinary glycated albumin/serum glycated albumin ratio was about 8 in normal subjects and tended to decrease to about 1 in diabetic patients whose urinary albumin excretion was increased (as determined by the TBA method). We also previously reported a similar tendency (21). The reason why the ratio was <1 in the present paper is unclear, but the difference might have resulted from the methods of measurement (specificity of the epitope of the antisera), the difference between glycated albumin and GP, and the variety of the subjects studied.

In the diabetic patients, we found a significant positive correlation between SGP and HbA1c, but not between the selectivity ratio and HbA1c. HbA1c showed no significant correlation with the progression of retinopathy. On the other hand, the selectivity ratio tended to decrease as diabetic nephropathy developed and as urinary albumin excretion increased. Moreover, there was a significant difference in the mean values of selectivity between groups of patients with various degrees of retinopathy. Therefore, perhaps the progression of retinopathy cannot be attributed to glycemic control alone, and the values for UGP/SGP (selectivity) are more indicative of the progression of retinopathy than is HbA1c.

References