Nonenzymatic Glucosylation of Serum Proteins in Diabetes Mellitus

KAY F. MCFARLAND, EDWARD W. CATALANO, JAMES F. DAY, SUZANNE R. THORPE, AND JOHN W. BAYNES

SUMMARY

The extent of nonenzymatic glucosylation of serum protein in control and diabetic subjects was measured by a chemical procedure using thiobarbituric acid. A mean value of 0.81 (±0.21 SD) nmol glucose per milligram serum protein was observed in the control group. Diabetics displayed elevated levels of glucosylated serum proteins, up to 4 nmol glucose per milligram protein. Glucosylation of serum protein correlated strongly with fasting blood sugar (r = 0.71), percent hemoglobin A_1 (r = 0.79), and percent glucosylated albumin (r = 0.99). There was no overlap between control and diabetic groups, i.e., within 3 SD of the mean of controls. These studies indicate that the assay for glucosylated serum protein appears to be an especially sensitive indicator of the degree of hyperglycemia in diabetes. DIABETES 28:1011–1014, November 1979.

The identification of glucosyl derivatives of a variety of proteins, including hemoglobin,[1] albumin,[2–4] collagen,[5] erythrocyte membrane proteins,[4] and lens crystallins,[6] has established that nonenzymatic glucosylation is a common posttranslational modification of proteins in vivo. Prolonged elevation of blood glucose in diabetes is known to cause an increase in levels of nonenzymatically glucosylated hemoglobin[1] and albumin.[3,7] The concentration of glucosylated hemoglobin correlates well with fasting and mean and highest daily glucose levels[8,9] and is useful as an index of diabetes' control.[8–11]

It has been hypothesized that nonenzymatic glucosylation of body protein, occurring at enhanced rates during hyperglycemia, may contribute to the progressive, chronic complications of diabetes.[12] Although direct correlations between increased glucosylation of proteins and the pathophysiology of diabetes cannot yet be made, alterations in physical and biochemical properties of proteins subsequent to glucosylation have been described. Thus, glucosylated hemoglobin has an increased oxygen affinity and decreased sensitivity to 2,3-diphosphoglycerate,[13–15] glucosylated crystallins show both a decreased solubility and enhanced tendency to aggregate compared with the native forms,[6] and glucosylated albumin is less soluble than the unglucosylated form (unpublished data).

The recent observations that several classes of human and rat serum proteins are subject to nonenzymatic glucosylation in vitro[2,3] and that levels of glucosylated albumin are elevated in poorly controlled diabetics[7] suggest that serum proteins, in general, should be more heavily glucosylated during diabetic hyperglycemia. In this paper, we present evidence to show that (1) the glucosylation of serum protein is increased in diabetes and (2) the degree of elevation is proportional to the degree of hyperglycemia.

METHODS

Blood was collected from 20 normal controls and 29 diabetic patients after an overnight fast. Controls, ages 22–38 yr, had fasting serum glucose levels less than 110 mg/dl, and, after a 75-g oral glucose load, glucose values were less than 195 mg/dl and 140 mg/dl after 1 and 2 h, respectively. The diagnosis of diabetes mellitus had been established in the 5 male and 24 female diabetic patients, ages 21–81 yr, by documentation of two or more fasting serum glucose levels of greater than 140 mg/dl. All diabetics were being treated as outpatients at Richland Memorial Hospital: 17 were insulin treated, 5 were treated by diet alone, and 11 were taking oral hypoglycemic agents. Control of hyperglycemia in these patients ranged from excellent to poor, as judged by fasting serum glucose and hemoglobin A_1 (HbA_1) measurements (see Figure 2).

Glucose measurements were done by the method of Trinder[16] using an automated glucose oxidase/peroxidase procedure. Serum protein concentrations were determined by the manual Biuret procedure (Sigma Chemical Company). Glycohemoglobins, as % HbA_1, were determined in

From the Departments of Chemistry, Obstetrics and Gynecology, and Pathology, University of South Carolina, and the Richland Memorial Hospital, Columbia, South Carolina.
Address reprint requests to Dr. John W. Baynes, Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208.
Received for publication 30 July 1979.

duplicate using Isolab's Fast Hemoglobin Test System;[17] the coefficient of variation between replicate samples was 3.5%. The extent of glucosylation of serum protein was determined by a modification of the thiobarbituric acid assay of Flückiger and Winterhalter,[18] in which nonenzymatically bound glucose is released as 5-hydroxymethylfurfural (HMF) and is quantitated colorimetrically. Briefly, serum aliquots (0.1 ml) were diluted to 1.0 ml and mixed with 0.5 ml 1M oxalic acid, then hydrolyzed for 4.5 h at 100°C. These modified hydrolysis conditions released the maximum amount of HMF from serum protein and released >80% of ^{3}H-glucose as HMF from ^{3}H-glucose–labeled albumin prepared as previously described.[4] HMF release was quantitated by measuring the absorbance at 443 nm after reaction with thiobarbituric acid.[18] The absorbance increase was proportional to sample volume, up to 0.25 ml serum, in the assay. An absorbance of about 0.1 was obtained for a 0.1-ml sample of normal control serum in this assay. Assays were run in duplicate, and results were averaged. The coefficient of variation between replicates was 2.5% throughout the range of values tested. A normal serum control was included with each batch of samples assayed. The extent of glucosylation of serum protein was calculated as nanomoles HMF per milligram protein (Biuret) using glucosylated human albumin as standard. Since ketoamine derivatives of protein are reducible by $NaBH_4$ to the nonreactive deoxyglucitoyl-protein adduct, each sample was assayed against its own $NaBH_4$-reduced blank.

Percent glucosylated albumin was also measured by the thiobarbituric acid procedure, after purification of the albumin on Affi-Gel Blue.[19] Briefly, 0.3 ml serum was diluted to 1.0 ml with buffer A: 0.15 M NaCl in 0.01 M KH_2PO_4, pH 7.8. This solution was applied to a 2.0-ml Affi-Gel Blue column, and the column was washed with 20 ml of buffer A. Albumin was eluted with 1.5 M NaCl, and 1-ml fractions were collected. Fractions containing albumin were pooled and diluted with H_2O to 5 mg/ml, i.e., $A_{279} = 2.65$ ($A^{0.1\%}_{279} = 0.531$ for human serum albumin[20]). One milliliter of this solution (5 mg albumin) was mixed with 0.5 ml 1M oxalic acid and was assayed as described above for serum protein. The coefficient of variation between replicates for this entire procedure was 3%.

Standard statistical methods were employed to determine means, standard deviations, and correlation coefficients used for analyzing and comparing the test data on the normal and diabetic subjects.

RESULTS

The results of assays for glucosylation of serum protein, carried out on normal and diabetic subjects, are presented in Figure 1. A mean value of 0.81 (±0.21 SD) nmol HMF per milligram serum protein was observed in the normal population, the distribution being skewed toward lower values. There was no overlap between the normal and diabetic groups (normal range = 0.63–1.31, diabetic range = 1.54–3.97), and the lowest value for any diabetic subject was greater than 3 SD above the mean of normals.

Figures 2A and 2B show that the extent of serum protein glucosylation correlates strongly with both fasting serum glucose ($r = 0.71$, $P < 0.001$) and % HbA_1 ($r = 0.79$, $P < 0.001$) in the diabetic population. The elevation in glucosylated serum protein is, therefore, proportional to the degree of hyperglycemia, as was revealed by fasting glucose and glycohemoglobin measurements.

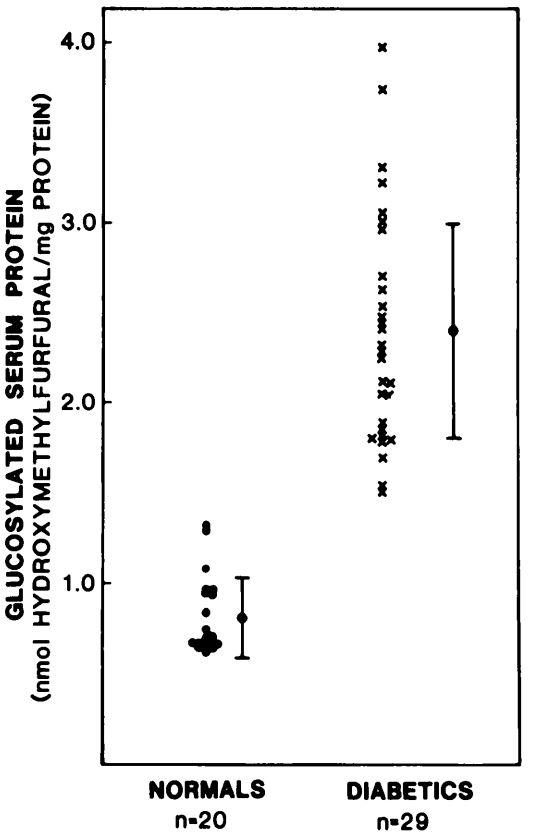

FIGURE 1. Scattergram of levels of glucosylated serum proteins in diabetic and normal nondiabetic control subjects.

The correlation between glucosylation of albumin and total serum proteins is shown in Figure 2C. A strong correlation is observed in both the normal and diabetic populations ($r = 0.99$, $P < 0.001$). Although the elevation in total protein glucosylation is proportional to that of glucosylated albumin, only about 60–70% of this increase is attributable to albumin, as was determined by chromatography of diabetic sera on Sephadex G-200 (unpublished data). A proportionate increase in glucosylation was demonstrable by thiobarbituric acid assay in the regions of elution of IgG (150,000 mol wt) and IgM/α_2-macroglobulin (~800,000 mol wt) from these columns.

DISCUSSION

Because of the slow rate of glucosylation of hemoglobin[1] and the 120-day average life span of erythrocytes, levels of glycohemoglobins are indicative of the time-averaged blood glucose concentration over a period of several weeks; in contrast, individual plasma glucose levels reflect minute-to-minute changes in glucose homeostasis. Glycohemoglobin levels are useful for assessing the level of control in insulin-dependent diabetics in whom day-to-day fluctuations in plasma glucose concentrations are large, whereas plasma glucose levels are an accurate measure of control in stable diabetics. The significant correlation between fasting glucose, glycohemoglobins, and glucosylated serum protein in diabetic subjects is evidence that glucosylation of serum protein may serve as another index of the level of glycemia. Because of the shorter half-life of

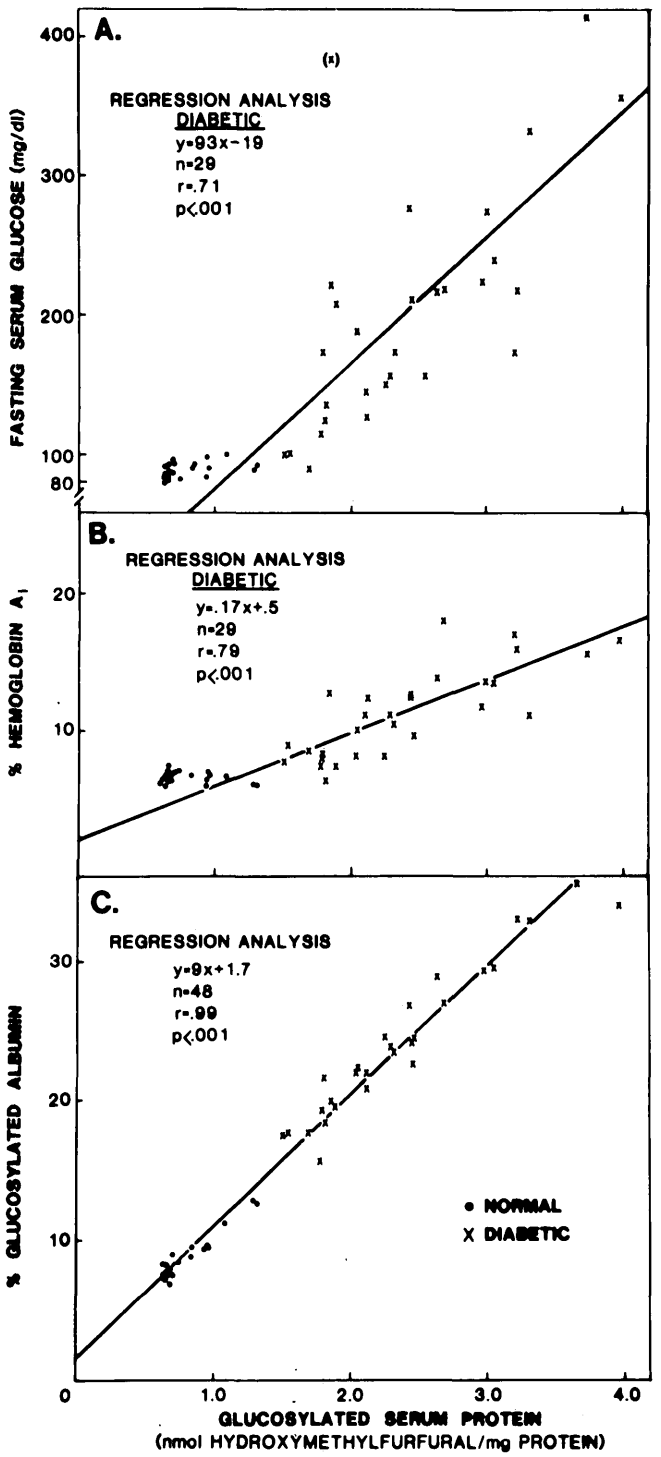

FIGURE 2. Correlations between levels of glucosylated serum proteins and (A) fasting serum glucose, (B) percent hemoglobin A_1, and (C) percent glucosylated albumin.

serum proteins compared with hemoglobin, changes in serum protein glucosylation should be observed more rapidly in response to changes in diabetes' control and may indicate the status of glycemia during the previous 1–2-wk period.

A strong correlation between % HbA_1 and % glucosylated albumin in poorly controlled diabetics has been reported previously.[7] The data in Figure 2C show, in addition, that there is excellent agreement between the assays for glucosylation of albumin and total serum protein in both normal and diabetic populations. The assay for serum protein glucosylation, however, is technically simpler, i.e., does not require purification of albumin, and appears to be an equally valid indicator of the degree of hyperglycemia. In preliminary experiments, we observed that the measurement of serum protein glucosylation is not affected by time of day or previous food intake. An overnight fast is not required of the patient, and samples drawn, for example, at various times before or during a glucose tolerance test yield statistically identical results. If the thiobarbituric acid assay used here for research purposes can be automated for routine use, it may have clinical application for assessing the degree of glucose intolerance and control of hyperglycemia in diabetes.

It is remarkable that, with respect to measurements of serum protein glucosylation, there is no overlap between the normal control and diabetic patient populations (Figure 1). Even in diabetics who appear to be in good control, based on measurements of fasting serum glucose or % HbA_1, there is evidence for enhanced nonenzymatic glucosylation of serum protein (Figure 2A and B). This assay would appear, therefore, to be an especially sensitive indicator of glucose intolerance and hyperglycemia. Other proteins in cell membranes and the extracellular matrix throughout the body are undoubtedly also subject to glucosylation at enhanced rates during hyperglycemia. How this process affects, in detail, the functional properties of membranes and the regulation of cellular metabolism remains to be established. As specific glucosylated proteins are identified and as changes in their properties are studied, the relationship between nonenzymatic glucosylation and the pathophysiology of diabetes will be clarified.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the Class of 1982 of the University of South Carolina School of Medicine for participating in this study as controls and to Ms. Georgia Lloyd for able technical assistance.

This work was supported, in part, by USPHS research grant 1 R01 AM 19971 and biomedical research support grant 5S07 RR 07106 and by a research grant award from the Susan Greenwall Foundation and the American Diabetes Association.

REFERENCES

[1] Bunn, H. F., Gabbay, K. H., and Gallop, P. M.: The glycosylation of hemoglobin: relevance to diabetes mellitus. Science *200*:21–27, 1978.

[2] Day, J. F., Thorpe, S. R., and Baynes, J. W.: Non-enzymatically glucosylated albumin: *in vitro* preparation and isolation from normal human serum. J. Biol. Chem. *254*:595–97, 1979.

[3] Day, J. F., Thorpe, S. R., and Baynes, J. W.: Non-enzymatic glucosylation of rat serum proteins *in vitro* and *in vivo*. Fed. Proc. *38*:418, 1979.

[4] Bailey, A. J., Robins, S. P., and Tanner, M. J. A.: Reducible components in the proteins of human erythrocyte membrane. Biochim. Biophys. Acta *434*:51–57, 1976.

[5] Robins, S. P., and Bailey, A. J.: Age-related changes in collagen: the identification of reducible lysine-carbohydrate condensation products. Biochem. Biophys. Res. Commun. *48*:76–84, 1972.

[6] Stevens, V. J., Rouzer, C. A., Monnier, V. M., and Cerami, A.: Diabetic cataract formation: potential role of glycosylation of len crystallins. Proc. Natl. Acad. Sci. USA *75*:2918–22, 1978.

[7] Guthrow, C. E., Morris, M. A., Day, J. F., Thorpe, S. R., and Baynes, J. W.: Enhanced nonenzymatic glucosylation of human serum albumin in diabetes mellitus. Proc. Natl. Acad. Sci. USA *76*: in press, 1979.

[8] Gonen, B., Rubenstein, A. H., Rochman, H., Tanega, S. P., and Horwitz, D. L.: Hemoglobin A_1: an indicator of the metabolic control of diabetic patients. Lancet *2*:734–36, 1977.

[9] Arye, L.-V., and Vanderlaan, W. P.: Glycohemoglobins and glucose tolerance. JAMA *241*:912–14, 1979.

[10] Koenig, R. J., Peterson, C. M., Jones, R. L., Saudek, C., Lehrman, M., and Cerami, A.: Correlation of glucose regulation and hemoglobin A_{Ic} in diabetes mellitus. N. Engl. J. Med. *295*:417–20, 1976.

[11] Gabbay, K. H., Hasty, K., Breslow, R., Ellison, C., Bunn, H. F., and Gallop, P. M.: Glycosylated hemoglobins and long term blood glucose control in diabetes mellitus. J. Clin. Endocrinol. Metab. *44*:859–64, 1977.

[12] Renold, A. E., Mintz, D. H., Muller, W. A., and Cahill, G. F.: Diabetes mellitus. *In* The Metabolic Basis of Inherited Disease, fourth edition. Stanury, J. B., Wyngaarden, J. B., and Fredrickson, D. S. Eds. New York, McGraw-Hill, 1972, pp. 80–109.

[13] Bunn, H. F., and Briehl, H. F.: The interaction of 2,3-diphosphoglycerate with various human hemoglobins. J. Clin. Invest. *49*:1088–95, 1970.

[14] Ditzel, J., Anderson, H., and Daugaard-Peters, N.: Oxygen affinity of hemoglobin and red cell 2,3-diphosphoglycerate in childhood diabetes. Acta Paediatr. Scand. *64*:355–61, 1975.

[15] McDonald, M. J., Bleichmann, M., Bunn, H. F., and Nobel, R. W.: Functional properties of the glycosylated minor components of human adult hemoglobin. J. Biol. Chem. *254*:702–07, 1979.

[16] Trinder, D.: Determination of glucose in blood using glucose oxicase with an alternative acceptor. Ann. Clin. Biochem. 6:24–27, 1969.

[17] Abraham, E. C., Huff, T. A., Cope, N. D., Wilson, J. B., Bransome, E. D., and Huisman, T. H. J.: Determination of the glycosylated hemoglobins (HbA_1) with a new microcolumn procedure. Diabetes 27:931–37, 1978.

[18] Flückiger, R., and Winterhalter, K. H.: *In vitro* synthesis of hemoglobin A_{Ic}. FEBS Lett. *71*:356–60, 1976.

[19] Travis, J., and Pannell, R.: Selective removal of albumin from plasma by affinity chromatography. Clin. Chim. Acta *49*:49–52, 1973.

[20] Peters, T., Jr.: Serum albumin. *In* The Plasma Proteins, second edition. Putnam, F. W., Ed. New York, Academic Press, 1975, pp. 133–81.