Vol. 95, No. 2, 1980
July 31, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 765-769

INCREASED GLYCOSYLATION OF GLOMERULAR BASEMENT MEMBRANE COLLAGEN IN DIABETES

Margo P. Cohen,* Enrique Urdanivia, Maria Surma, and Van-Yu Wu

Wayne State University School of Medicine
540 East Canfield, Detroit, Michigan 48201.

Received June 16,1980

SUMMARY

Basement membrane was purified from glomeruli isolated from normal and streptozotocin-diabetic rats. After extraction of non-collagen protein with 8M urea, the extent of glycosylation in glomerular basement membrane collagen was determined with a specific colorimetric reaction that detects carbohydrate in ketoamine linkage with proteins. The level of glycosylation of glomerular basement membrane collagen purified from diabetic rats was significantly greater than that in non-diabetic animals. Increased basement membrane glycosylation may alter structure-function relationships of the capillary filtration barrier.

The demonstration that hemoglobin undergoes post-ribosomal non-enzymatic glycosylation in vivo (1) was followed by reports that other proteins such as albumin (2,3), erythrocyte membrane proteins (4), lens crystallins (5,6) and aortic collagen (7,8) are also subject to non-enzymatic glycosylation. The interaction of glucose and protein is a slow, non-enzymatic process that occurs via ketoamine linkage, and involves free amino groups at the N-terminus or ε-amino groups of lysine residues (9,10). It is a condensation reaction in which the degree of glycosylation is proportional to the ambient glucose concentration (2). Thus increased levels of glycosylation of several proteins are found in the diabetic state with attendant hyperglycemia (8,10-14), and it has been postulated that non-enzymatic glycosylation of various proteins may be pathogenetically related to the chronic complications of diabetes (15). To date, however, elevated glycosylation of specific proteins concerned with diabetic microangiopathy and in specific tissues typically involved with the microvascular complications of diabetes has not been described. We now report that the level of non-enzymatic glycosylation of glomerular basement membrane collagen purified from rats with streptozotocin-diabetes is significantly increased

* To whom requests for reprints should be addressed.

0006-291X/80/140765-05$01.00/0

MATERIALS AND METHODS

Glomeruli were isolated from male white rats as previously described (16), and the basement membranes purified by osmotic lysis followed by sequential extraction with detergents (17,18). The purified preparations of glomerular basement membrane were extracted overnite at 4°C with 8M urea containing protease inhibitors (25mM EDTA, 10mM N-ethylmaleimide, 1mM benzamidine HCl and 1mM phenylmethylsulfonylflouride) to solubilize non-collagen protein (19). The urea insoluble material, which contains the basement membrane collagen components, was solubilized in 0.1N sodium hydroxide at 50°C for 30 minutes. Samples were neutralized with 0.1N HCl before analysis for ketoamine-linked hexose.

Diabetic rats were killed 3-4 weeks after the induction of diabetes by intraperitoneal injection of streptozotocin, 65 mg/kg. Streptozotocin was given when the rats weighed ≈120-150 grams, and non-injected litter mates served as controls. Renal cortical tissue from 4-5 animals in each group was pooled for each experiment.

Glycosylation of basement membrane collagen was estimated by measuring the chromogen produced by the reaction of 5-hydroxymethylfurfural (HMF) with thiobarbituric acid (TBA). The TBA reaction appears to be specific for the detection of carbohydrate bound by ketoamine linkage to proteins, and depends on the generation of HMF from the carbohydrate moieties upon heating under acid conditions (20). We employed the method described by Fluckiger et al (20) as adapted by Yue et al (11). Pure 5-hydroxymethylfurfuraldehyde (Sigma Chemical Company) was used as standard. Protein was measured by the method of Lowry et al (21) using albumin as standard. Hydroxyproline was measured by the method of Rojkind and Gonzalez (22). Amino acid analysis was performed on a Beckman Model 118BL single column analyzer packed with W-2 resin and eluted with a three buffer system.

RESULTS AND DISCUSSION

Table 1 presents data for the experimental animal groups. Diabetic animals were markedly hyperglycemic and manifested typical untreated insulin deficient diabetes. Despite weight loss and peripheral wasting, including diminution of subcutaneous connective tissue, renal cortical and glomerular mass were preserved in diabetic animals. The proportion of total basement membrane protein representing collagen, calculated as μmoles hydroxyproline per mg of membrane protein, was also preserved in diabetic animals.

TABLE 1 - Experimental Animal Data

Animal Group	Body Wt.(gm)	Glucose (mM/l)	Cortex (gm)
Non-diabetic	342 ± 12	<8.0	1.149 ± .06
Diabetic	214 ± 16	18.17 ± .78	1.049 ± .03

Results represent mean ± SEM from 4 experiments, in each of which renal cortex from 4-5 animals per group was pooled for purification of basement membrane.

TABLE 2 - Amino Acid Composition, in Residues/1000, of Rat Glomerular Basement Membrane Collagen

Amino Acid	Urea Soluble	Urea Insoluble
3-hydroxyproline	ND	2
4-hydroxyproline	ND	69
Aspartic acid	88	75
Threonine	60	42
Serine	84	53
Glutamic Acid	121	89
Proline	73	79
Glycine	118	237
Alanine	89	75
Half-cystine	18	5
Valine	46	22
Methionine	9	9
Isoleucine	38	21
Leucine	71	54
Tyrosine	22	16
Phenylalanine	34	25
Hydroxylysine	2	22
Histidine	25	15
Lysine	37	22
Arginine	57	52

ND - not detected

Treatment of purified glomerular basement membrane solubilizes glycosaminoglycans (23) and non-collagen protein which is devoid of hydroxyproline and contains only 2 residues/1000 amino acid residues of hydroxylysine (see Table 2). The glomerular basement membrane which remains insoluble in urea has the general compositional features of basement membrane collagen including high levels of hydroxyproline and hydroxylysine, relatively low levels of arginine and alanine, and the presence of cystine. The urea-insoluble fraction of glomerular basement membrane thus contains all the collagen components.[1]

Table 3 compares the liberation of HMF from glomerular basement membrane collagen purified from normal and streptozotocin-diabetic rats. The amount of HMF, calculated both as nmoles/mg protein and as nmoles/μmole hydroxyproline, generated from diabetic glomerular basement membrane collagen was significantly increased compared to that liberated from non-diabetic samples. The amount of HMF released represents ketoamine-linked hexose in basement membrane collagen;

1. The collagen components of glomerular basement membrane resemble procollagen and contain α-chain like segments associated with non-collagenous sequences. To avoid confusion, we use the term glomerular basement membrane collagen to refer to material that is insoluble in urea and contains all of the hydroxyproline.

TABLE 3 - Hydroxymethylfurfural (HMF) Generation from Glomerular Basement Membrane Collagen

Basement Membrane	nmoles HMF / mg protein	nmoles HMF / μmole OH-Proline
Non-Diabetic	6.105 ± .545	14.89 ± 1.33
Diabetic	13.815 ± 1.911*	32.89 ± 4.55*

Results represent mean ± SEM of four experiments in each of which glomerular basement membrane collagen was purified from renal cortex pooled from 4-5 animals/group.

* $p < 0.05$

although other sugars may attach to protein (24) and mannose can also be converted to HMF and react with thiobarbituric acid (13), the marked hyperglycemia of the diabetic rats suggests that this increase in collagen glycosylation reflects non-enzymatic interaction with glucose.

Identification of specific proteins subject to non-enzymatic glycosylation and determination of the effect of increased glycosylation on the structure and function of those proteins involved in the microvascular sequalae of diabetes are essential to the understanding of the relationship between hyperglycemia and microangiopathic complications of diabetes. Glycosylation of hemoglobin affects its oxygen affinity (25,26), and non-enzymatic glycosylation of α-crystallins in lens cultures in vitro causes aggregation and precipitation of the crystallins (5). These phenomena, however, are not typically classified as diabetic microangiopathic lesions and despite arguments incriminating tissue hypoxia resulting from the increased oxygen affinity of glycosylated hemoglobin as causative in diabetic microangiopathy (27), physiologic evidence makes this possibility unlikely (13). While the effect of increased glycosylation of glomerular basement membrane collagen must remain speculative at present, consideration of the function of this extracellular matrix offers intriguing possibilities. Glomerular basement membrane serves as a size and charge-selective filtration barrier (28). Alteration in its electrochemical properties via glycosylation of amino groups or interference with molecular packing via ketoamine linkage of ε-amino groups of lysine and hydroxylysine with consequent decreased availability of these amino acids for collagen cross-linking could respectively com-

promise the charge or size selective nature of the filtration barrier. Such changes would lead to proteinuria, the clinical hallmark of diabetic nephropathy, and loss of capillary integrity with increased permeability at other microvascular sites such as the retina. The effect of increased basement membrane glycosylation on the function and metabolism of this extracellular matrix warrants further investigation.

ACKNOWLEDGMENTS

Supported in part by NIH Grant No. AM-26435, the American Diabetes Association, and the Affiliated Internists Corporation.

REFERENCES

1. Bunn, H.F., Hamey, D.N., Kamin, S., Gabbay, K.H. and Gallop, P. (1976) J. Clin. Invest. 57:1652-1659.
2. Day, F.J., Thornburg, R.W., Thorpe, S.R. and Baynes, J.W. (1979) J. Biol. Chem. 254:9394-9400.
3. Dolhofer, R. and Wieland, O.H. (1979) FEBS Lett. 102:282-286.
4. Bailey, A.J., Robins, S.P. and Tanner, M.J.A. (1976) Biochim. Biophys. Acta 434:51-57.
5. Stevens, V.J., Rouzer, C.A., Monnier, V.M. and Cerami, A. (1978) Proc. Natl. Acad. Sci. USA 75:2918-2922.
6. Cerami, A., Stevens, V.J. and Monnier, V.M. (1979) Metabolism 28:431-437.
7. Robins, S.P. and Bailey, A.J. (1972) Biochem. Biophys. Res. Comm. 48:76-84.
8. Rosenberg, H., Modrak, J.B., Hassing, J.M., Al-Turk, W.A. and Stohs, S.J. (1979) Biochem. Biophys. Res. Comm. 91:498-501.
9. Bunn, H.F., Haney, D.N. Gabbay, K.H. and Gallop, P.M. (1975) Biochem. Biophys. Res. Comm. 67:103-109.
10. Monnier, V.M., Stevens, V.J. and Cerami, A. (1979) J. Exp. Med. 150:1098-1107.
11. Yue, D.K., Morris, K., McLennan, S. and Turtle, J.R. (1980) Diabetes 29: 296-300.
12. Guthrow, C.E., Morris, M.A., Day, J.F., Thorpe, S.R. and Baynes, J.W. (1979) Proc. Natl. Acad. Sci. 76:4258-4261.
13. Bunn, H.F., Gabbay, K.H. and Gallop, P.M. (1978) Science 200:21-27.
14. Gabbay, K.H., Sosenko, J.M., Banuchi, G.A., Mininsohn, M.J. and Fluckiger, R. (1979) Diabetes 28:337-340.
15. Renold, A.E., Mintz, D.H., Muller, W.A. and Cahill, G.F. Jr. (1978), in The Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S., eds.) pp. 80-109, McGraw-Hill Book Co., New York.
16. Cohen, M.P. and Vogt, C.A. (1975) Biochem. Biophys. Acta 393:78-87.
17. Carlson, E.C., Brendel, K., Hjelle, J.T. and Meezan, E. (1978) J. Ultrastruct. Res. 62:26-53.
18. Cohen, M.P. and Surma, M. (1979) J. Biol. Chem. 255:1767-1770.
19. Cohen, M.P., Wu, V.Y. and Surma, M.L. (1980) Submitted for publication.
20. Fluckiger, R. and Winterhalter, K.H. (1976) FEBS Lett. 71:356-360.
21. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, J.R. (1951) J. Biol. Chem. 194:265-275.
22. Rojkind, M. and Gonzalez, E. (1974) Analyt. Biochem. 57:1-7.
23. Cohen, M.P. (1980) Biochem. Biophys. Res. Comm. 92:343-348.
24. Dolhofer, R. and Wieland, O.H. (1978) FEBS Lett. 85:86-90.
25. McDonald, M.J., Bleichman, M., Bunn, H.F. and Noble, R.W. (1979) J. Biol. Chem. 254:702-707.
26. Ditzel, J., Andersen, H. and Peters, N.D. (1975) Acta Pediatr. Scand. 64: 335-361.
27. Ditzel, J. (1976) Diabetes 25 (Suppl.2):832-838.
28. Brenner, B.M., Hotstetter, T.H. and Humes, H.D. (1978) New Engl. J. Med. 298:826-833.