Nonenzymatic Glycosylation of Basement Membranes

In Vitro Studies

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SUMMARY

Incubation of purified rat glomerular basement membrane (GBM) with [^{14}C]-glucose in vitro resulted in the incorporation of [^{14}C] into acid-precipitable radioactivity in a reaction that was time and temperature dependent. Findings with rat lens capsule basement membrane (LCBM), an anatomically distinct but chemically similar extracellular matrix, incubated for varying times at different temperatures with [^{14}C]-glucose at constant specific activity were similar. Nonenzymatic glycosylation of basement membrane, documented by hydroxymethylfurfuraldehyde generation after incubation with unlabeled glucose, increased in proportion to the ambient glucose concentration over a range of 5–100 mM. Acid-precipitable radioactivity also increased in proportion to [^{14}C]-glucose concentration, although this method overestimated glycosylation about 15-fold at 5–20 mM glucose and 50-fold at 50–100 mM glucose. Coupled with recent in vivo studies, these findings indicate that exposure to increased glucose concentration alters the chemistry of glomerular and other basement membranes. Since accumulation of basement membrane characterizes several of the microangiopathic sequelae of diabetes, the role of increased nonenzymatic glycosylation on the structure, function, and metabolism of basement membrane warrants investigation. DIABETES *30*:367–371, May 1981.

Recent studies have demonstrated that several proteins undergo post-ribosomal, nonenzymatic glycosylation in vivo[1–4] and in vitro.[2,5–7] In this reaction, glucose attaches to proteins via free amino groups at the N-terminus or ξ-amino groups of lysine residues, forming a stable ketoamine derivative.[1,8–12] Since the interaction of glucose with protein is a condensation reaction in which the degree of glycosylation is proportional to the ambient glucose concentration,[2] this process is enhanced in the diabetic state with attendant hyperglycemia. The list of proteins subject to increased glycosylation in human and experimental diabetes is growing, and includes hemoglobin,[13] albumin,[5,14] erythrocyte membrane proteins,[4] lens crystallins,[3,11] and aortic collagen.[15,16]

Glycosylation may alter the physicochemical properties of certain proteins. For example, glycosylation of hemoglobin affects its oxygen affinity,[17,18] and nonenzymatic glycosylation of α-crystallins in lens cultures in vitro causes their aggregation and precipitation.[3] Such findings offer some support to the hypothesis that increased nonenzymatic glycosylation of various proteins, particularly in non-insulin-dependent tissues, is pathogenetically linked to the chronic complications of diabetes.[19] The postulate that glycosylation contributes to the microangiopathic sequelae of diabetes, however, requires evidence that this process affects proteins of specific tissues in which diabetic complications characteristically occur, and that increased glycosylation alters structural or functional properties of proteins in these tissues. Basement membrane is one such tissue that is typically associated with pathologic[20] and functional[21] changes in diabetes, manifested respectively as basement membrane thickening and increased permeability of the filtration barrier. We recently found that the level of nonenzymatic glycosylation of glomerular basement membrane collagen purified from rats with streptozotocin diabetes was significantly greater than that in preparations from nondiabetic animals.[22] We now extend these observations with the present report, which describes the ability of glucose to interact in vitro with purified preparations of glomerular basement membrane. Nonenzymatic glycosylation of basement membrane proteins may be a generalized reaction, since we also found that the anatomically distinct lens capsule basement membrane undergoes glycosylation in vitro.

MATERIALS AND METHODS

Glomeruli were isolated from the renal cortex of adult male white rats by differential sieving through a series of stainless

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steel meshes;[23] with the aid of a dissecting microscope, lens capsules were peeled from the lenses of eyes removed from the same animals. The basement membranes from these tissues were isolated by osmotic lysis and sequential extraction with detergents, as previously described.[24,25] The purified preparations of glomerular (GBM) and lens capsule (LCBM) basement membrane were washed several times with distilled water and lyophilized until use.

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. Basement membrane samples were hydrolyzed in 6 N HCl for 22 h at 110°C in sealed glass ampules that had been previously flushed with nitrogen, evaporated in vacuo, and reconstituted in buffer for amino acid analysis.

Basememt membranes are relatively insoluble in dilute acids or neutral buffers, and solubilization for analytic studies is generally achieved by reduction in sodium dodecyl sulfate or by limited pepsin digestion. Since the effects of these techniques on the integrity of authentic components of basement membrane are not completely elucidated, and since these methods might disturb potential sites of glycosylation, we elected to prepare suspensions of glomerular and lens capsule basement membranes for in vitro studies by homogenization in a buffered salt (0.1 M potassium phosphate buffer, pH 7.4). For each experiment, weighed amounts of lyophilized basement membrane were homogenized in defined volumes of buffer until a fine suspension was achieved. Aliquots were directly pipetted from these suspensions into the individual assay tubes; the parent suspension was agitated just before withdrawal of each aliquot to ensure uniformity. Similar aliquots were taken for measurement of protein by the method of Lowry et al.,[26] using albumin as the standard. Incubations were performed in a total volume of 0.5–1.0 ml containing phosphate buffer (pH 7.4) and 5–100 mM glucose to which U-[^{14}C]-glucose (New England Nuclear, 9 mCi/mM) had been added to obtain constant specific activities. Incubations were conducted at 0°, 25°, and 37°C and continued for 3 and 5 days at which time 0.5 mg of carrier albumin and an equal volume of 20% trichloroacetic acid were added to each assay tube to precipitate protein-bound radioactivity. The precipitates were washed, collected by recentrifugation, and the final pellets were solubilized in 0.3 ml of 0.2 N NaOH and transferred to scintillation vials containing 15 ml of Bray's solution. Nonspecific binding of glucose was determined by adding labeled glucose of the same specific activity and concentration to basement membrane preparations, and directly adding carrier albumin and trichloroacetic acid as described above after 30 min at room temperature. Specific binding was calculated by subtracting the nonspecific binding from the counts precipitated after appropriate incubation.

Since a recent study indicates that contaminants in radiolabeled glucose preparations can bind covalently to proteins,[27] we confirmed that glucose interacted with basement membrane via ketoamine linkage in a series of parallel experiments with varying concentrations of radioactive and cold glucose. Equal aliquots of basement membrane were incubated for 7 days at 25°C with 5–100 mM unlabeled or [^{14}C]-glucose in a total volume of 1 ml. Acid-precipitable radioactivity was measured as described above. The basement membranes from samples incubated with cold glucose were collected by centrifugation and repeatedly washed with large volumes of distilled water to remove free glucose. Samples were then subjected to the thiobarbituric acid reaction, which is specific for the detection of carbohydrate bound by ketoamine linkage to proteins.[9]

RESULTS

Table 1 presents the amino acid composition of the glomerular and lens capsule basement membrane preparations used in these experiments. They have the appropriate general compositional features associated with basement membranes, including high levels of hydroxyproline and hydroxylysine, relatively low levels of alanine, and the presence of cystine.

Radioactivity gradually accumulated in the acid-precipitable protein during incubation of lens capsule (Figure 1) and glomerular (Figure 2) basement membrane with tracer amounts of [^{14}C]-glucose. The interaction of glucose with basement membrane at 25°C was proportional to time during the study period. The amount of acid-precipitable radioactivity also increased in proportion to the amount of lens capsule or glomerular basement membrane protein.

Figure 3 shows the effect of temperature on [^{14}C]-glucose interaction with lens capsule basement membrane. The rate of [^{14}C]-incorporation was temperature dependent and increased from 0 to 37°C, although the rate of increase per degree was greater from 0 to 25°C than from 25 to 37°C. The effect of temperature on [^{14}C]-incorporation into glomerular basement membrane was similar.

Figures 1 through 3 also depict the effect of glucose concentration on [^{14}C]-glucose attachment to basement membranes in vitro. [^{14}C]-glycosylation increased in proportion to the glucose concentration with both lens capsule (Figures 1 and 3) and glomerular (Figure 2) basement membranes. The quantity of hydroxymethylfurfuraldehyde liberated from basement membrane incubated with varying

TABLE 1
Amino acid composition, in residues/1000, of rat glomerular and lens capsule basement membranes (BM)

Amino acid	Glomerular BM	Lens capsule BM
4-Hydroxyproline	48.5	83.7
Aspartic acid	74.6	56.3
Threonine	42.0	37.9
Serine	52.0	45.3
Glutamic acid	99.0	87.4
Proline	73.3	69.0
Glycine	213.1	235.3
Alanine	77.3	44.2
Half-cystine	31.8	24.8
Valine	40.5	36.4
Methionine*	5.0	14.3
Isoleucine	21.7	26.1
Leucine	57.1	53.9
Tyrosine	10.0	12.1
Phenylalanine	27.1	27.6
Hydroxylysine	24.0	30.7
Histidine	20.3	21.0
Lysine	27.4	27.6
Arginine	56.1	40.5

Values uncorrected for hydrolytic losses.
* Sum of methionine and methionine sulfoxide.

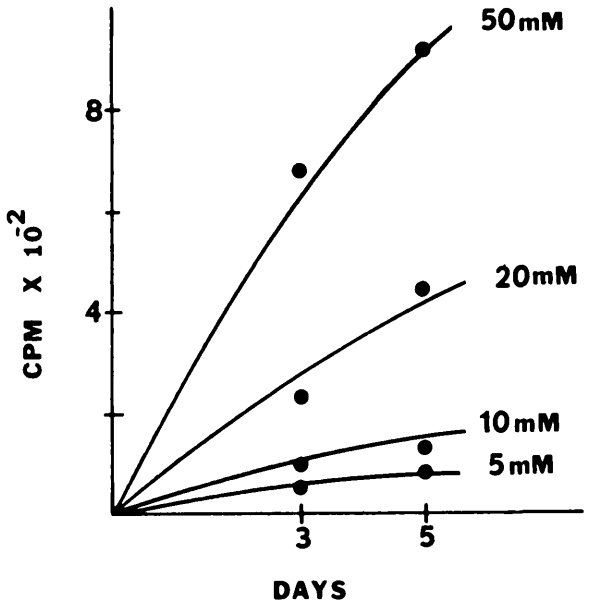
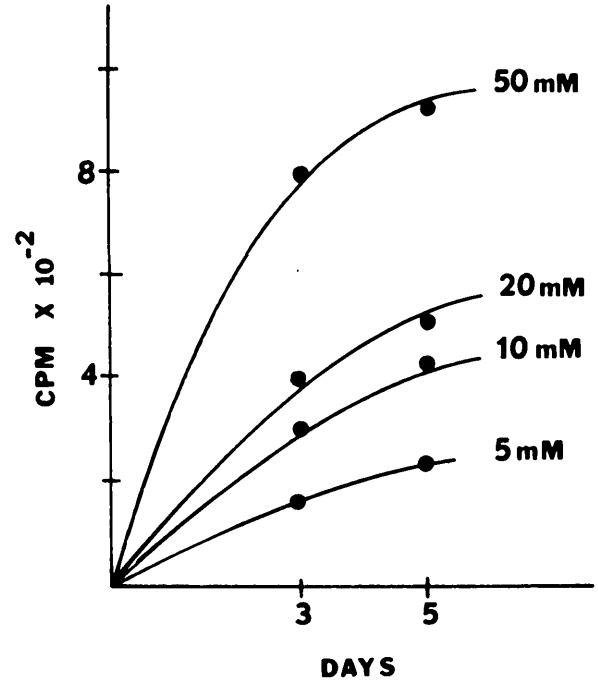


FIGURE 1. Time-dependent formation of acid-precipitable [^{14}C]-glucose radioactivity after incubation with lens capsule basement membrane. Each incubation contained ≃ 80 μg of protein and [^{14}C]-glucose at the concentrations indicated, adjusted to a constant specific activity of 5.5 μCi/mmole. Incubations were performed at 25°C for the specified number of days, at which time protein was precipitated by the addition of carrier albumin and an equal volume of 20% trichloroacetic acid. Data are expressed as precipitated counts in each incubation and are corrected for nonspecific binding.

concentrations of unlabeled glucose increased in proportion to the glucose concentration in the incubation media (Table 2). These results confirm that nonenzymatic glycosylation of basement membranes is glucose dependent and

FIGURE 2. Time-dependent formation of acid-precipitable [^{14}C]-glucose radioactivity after incubation with rat glomerular basement membrane. Each incubation contained ≃ 95 μg protein and [^{14}C]-glucose (specific activity 5.5 μCi/mmole) at the concentrations indicated. Incubations were performed at 25°C for the specified number of days. Acid-precipitable radioactivity was determined as described in the text and in the legend to Figure 1.



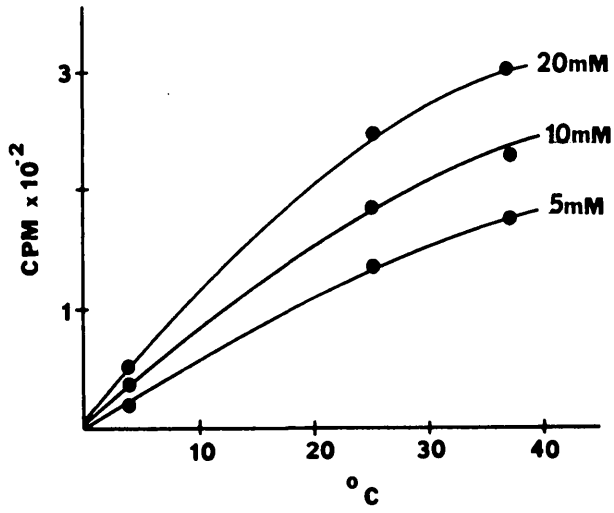


FIGURE 3. [^{14}C]-glycosylation of lens capsule basement membrane as a function of temperature and glucose concentration. Incubations were performed for 5 days at varying temperature with 5–20 mM [^{14}C]-glucose at constant specific activity (4 μCi/mmole). Each incubation contained ≃ 40 μg protein in a total volume of 0.5 ml. Acid-precipitable radioactivity was determined as described in the text.

that attached glucose is in ketoamine linkage. In a parallel series of incubations with equal amounts of basement membrane, calculation of the extent of [^{14}C]-glycosylation (based on specific activity and counting efficiency of the [^{14}C]-glucose) indicated that apparent glycosylation measured with this method was greater than that measured with the thiobarbituric acid reaction (Table 2). Acid-precipitable radioactivity overestimated nonenzymatic glycosylation about 15-fold at 5–20 mM glucose concentration and ≃ 50-fold at 50–100 mM glucose concentration. Thus acid-precipitable [^{14}C]-glucose reflects glycosylation in vitro only when the incubational [^{14}C]-glucose concentration is within the physiologic range and when appropriate correction factors, described above, are considered. These findings support the relevance of the "warning" by Trueb et al.[27] that nonenzymatic glycosylation cannot be estimated solely on the basis of radioactive glucose incorporation.

DISCUSSION

The studies reported here demonstrate that basement membrane proteins undergo glycosylation in vitro. The reaction is slow, nonenzymatic, and is time and temperature depen-

TABLE 2
Nonenzymatic glycosylation of basement membrane in vitro

Glucose (mM)	nmoles HMF	nmoles [^{14}C]	(HMF/[^{14}C]) × 100
5	0.3	4.4	6.8
10	0.6	8.6	6.9
20	1.1	16.1	6.8
50	3.4	178.0	1.9
100	6.5	312.0	2.0

Each incubation contained ≃85 μg glomerular basement membrane protein and either unlabeled glucose or [^{14}C]-glucose (specific activity 4.0 μCi/mmole) in the concentrations indicated. Incubations were performed for 5 days at 25°C. Hydroxymethylfurfuraldehyde (HMF), acid-precipitable radioactivity, and calculation of [^{14}C]-glucose incorporated were determined as described in the text.

dent. It is also dependent on glucose concentration and proceeds at progressively higher initial rates as glucose concentration increases. It appears to be a generic reaction, in that we found that basement membrane purified from two anatomically distinct sites interacts in a similar fashion with glucose in vitro. These results extend our previous observations in which we found that the level of glycosylation of glomerular basement membrane, measured by the thiobarbituric acid reaction, purified from rats with streptozotocin diabetes was significantly higher than that detected in nondiabetic preparations.[22] Thus, basement membrane can be added to the list of proteins that are subject to nonenzymatic glycosylation both in vivo and in vitro and that have increased glycosylation in the presence of high glucose concentration.

We did not examine specific sites at which glycosylation occurs, but available evidence from studies with other proteins indicates that this reaction involves free amino groups at the N-terminus and ξ-amino groups of lysine residues.[1,8,9,11,15] Preferred sites of glycosylation in vitro, however, may differ from those in vivo, as demonstrated by Shapiro et al.,[6] who found that intrachain lysine residues of hemoglobin are more extensively glycosylated in vitro than in vivo. Our previous study indicated that the specific glomerular basement membrane fraction that contains the collagen components is subject to glycosylation in vivo. The chemical similarities between the collagenous fractions of glomerular and lens capsule basement membranes and the interaction of both of these basement membranes with glucose in vitro suggest that the common denominator for nonenzymatic glycosylation resides in the collagen components, with lysine and hydroxylysine as the most likely sites. Some of the lysine sites may reside in noncollagenous domains of the basement membrane procollagen molecules. This does not exclude the possibility that free amino groups of unique noncollagen basement membrane proteins also become glycosylated. Identification of the exact sites of glycosylation of basement membrane proteins will require isolation and characterization of individual and authentic basement membrane polypeptides, an area of investigation that has proved difficult and controversial.

The present findings, coupled with our previous study, provide the first direct experimental evidence that hyperglycemia, via nonenzymatic glycosylation, modifies the chemistry of proteins specifically involved in diabetic glomerulosclerosis. That another basement membrane is subject to nonenzymatic glycosylation offers support for the hypothesis that this process may be a model reaction contributory to microangiopathic complications in other anatomic sites. While the potential biologic consequences of this modification of basement membrane chemistry are speculative at present, current understanding of the physiology and biochemistry of basement membranes allows some insight into the manner in which increased glycosylation in diabetes might alter the function or metabolism of these extracellular matrices. Glycosylation of ξ-amino groups of lysine and hydroxylysine would decrease the availability of these amino acids for collagen crosslinking, while ketoamine linkage of free amino groups could alter electrochemical properties of involved proteins. Such changes could respectively compromise the molecular or electrical integrity of the capillary filtration barrier and lead to increased microvascular permeability. Normal basement membrane has a slow turnover[25,28] and would thus undergo a progressive glycosylation over several weeks that could also, via interference with the efficiency of normal degradative pathways, prolong its biologic half-life and promote its accumulation in the diabetic state.

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