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Micropinocytic ingestion of glycosylated albumin by isolated microvessels: Possible role in pathogenesis of diabetic microangiopathy

(endocytosis/diabetes mellitus/fluorescence)

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ABSTRACT **Microvessels isolated from rat epididymal fat exhibit differential vesicular ingestion rates for unmodified and nonenzymatically glycosylated rat albumin. While unmodified rat albumin is excluded from ingestion by endothelial micropinocytic vesicles, glycosylated albumin is avidly taken up by endocytosis. Interaction of albumin and glycosylated albumin with endothelium was studied with a double-label fluorescence assay of micropinocytosis. When glycosylated albumin was present at a concentration of 6% with respect to total albumin (the level found in "non diabetic" serum), only glycosylated albumin was ingested. At higher concentrations of glycosylated albumin (those found in diabetic serum), both albumin and glycosylated albumin are ingested. Glycosylation of endothelial membrane components results in stimulated ingestion of glycosylated albumin, persistent exclusion of unmodified albumin, and unaltered micropinocytic ingestion of native ferritin. These results indicate that nonenzymatic glycosylation of serum albumin may result in rapid vesicle-mediated extravasation of albumin. Chronic microvascular leakage of glycosylated albumin could contribute to the pathogenesis of diabetic microangiopathy.**

Molecular "languages" shape the complex interactions that support life processes. Unique symbols, fidelity of transcription, and rules of grammar are features of the genetic code (1). Specificity of recognition and informational content are also apparent in the interactions of hormones with receptors (2), antigens with antibodies (3), and substrates with enzymes (4). In each of these examples, accurate recognition of a unique molecular conformation is crucial for the underlying process. Moreover, lapses in the fidelity of recognition can produce catastrophic results for the systems involved.

Endothelial micropinocytosis provides a bidirectional large pore conduit for the transendothelial transport of macromolecules. Another form of molecular language is manifest in the interactions of such molecules with the caveolar (plasmalemmal vesicle) membrane components of endothelial cells. The use of this molecular language in the process of recognition-dependent endocytosis provides a discrimination function for transendothelial transport. There results a striking heterogeneity in the rates of transendothelial vesicular transport of a variety of serum components (5–8).

The reasons for these differences must be sought in specific interactions between each of the components and putative recognition sites in lumenal endothelial membranes. Such sites are situated within or adjacent to the stomata of caveolae and actively regulate the process of adsorptive endocytosis. Serum albumin appears virtually excluded from ingestion by micropinocytic vesicles (5). This exclusion cannot be attributed solely to the molecular dimensions or the net charge of albumin. Ferritin, which is larger and of similar net charge, is readily ingested by endothelial vesicles. The fine molecular details that govern these recognition processes, and thus modulate transendothelial escape rates, are not yet known. There is, however, a teleological assessment of these observations. Because lumenal albumin contributes the major fraction of macromolecular osmotic pressure, increased extravasation of albumin would result in edema.

Disease of the microcirculation involving the retina and glomerulus is of principal importance for the morbidity of long-standing diabetes mellitus (9). The mechanisms and prevention of the long-standing complications of microangiopathy remain unclear. Recent studies in the diabetic dog and human confirm a long-standing clinical suspicion that optimal control of plasma glucose levels will prevent microangiopathy (10–12). These findings and recent insight into the process of nonenzymatic glycosylation suggest that hyperglycemia per se may be of direct importance in the pathogenesis of diabetic complications.

Interest in nonenzymatic glycosylation was stimulated by studies of hemoglobin A_{1c}, which can be present at more than 3 times the normal concentrations in the poorly controlled diabetic (13, 14). These data have focused attention on this molecule as a putative long-term integrator of plasma glucose levels (15). Nonenzymatic glycosylation provides a glucose-linked mechanism for the direct covalent modification of a variety of proteins. We have studied the reaction of nonenzymatic glycosylation of rat serum albumin and endothelial membrane proteins *in vitro*. Because the glycosylation of endothelial membrane proteins is carried out with D-[6-^{3}H]glucose and intact endothelium, in this instance both enzymatic and nonenzymatic mechanisms are likely to operate in the covalent attachment of glucose to protein. Moreover, we have examined the possibility that significant functional consequences on microvascular transport might accrue from the modification of proteins by covalent insertion of glucose.

We have confirmed the finding that serum albumin is readily glycosylated *in vitro* (16). Moreover, the insertion of 1 or 2 mol of glucose per mol of albumin has profound consequences for the biological behavior of this protein. There is a >1000-fold increase in the ingestion of glycosylated albumin by micropinocytic vesicles of endothelial cells. The presence of glycosylated albumin also stimulates the ingestion of unmodified albumin. Finally, the glycosylation of endothelial cell membrane proteins stimulates the ingestion of glycosylated but not "unmodified" albumin.

These findings, when viewed in the context of the early "leakiness" of diabetic retinal capillaries (17), suggest the possibility that both the early and the chronic changes in the diabetic mi-

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Abbreviations: MDPF, 2-methoxy-2,4-diphenyl-3(2*H*)furanone; CF-P_i/NaCl, divalent cation-free phosphate-buffered saline.

crocirculation could, in part, reflect inappropriate extravasation of albumin (or other plasma proteins).

MATERIALS AND METHODS

Materials. 2-Methoxy-2,4-diphenyl-3(2*H*)furanone (MDPF) was the kind gift of Walter Scott, Hoffmann–La Roche. Mithramycin was kindly provided by Nathan Belcher of Pfizer.

Isolation of Capillary Endothelium. Microvessels were isolated by a modification of the method of Wagner and Matthews (18). The distal two-thirds of epididymal fat pads from (≈300-g) male Sprague–Dawley rats were dissected, minced, and suspended (two pads per Erlenmeyer flask) in 15 ml of Dulbecco's (19) divalent cation-free, phosphate-buffered saline (CF-P_i/NaCl) containing collagenase (type II, Sigma) at 2 mg/ml bovine serum albumin (fatty acid-free, Sigma) at 2 mg/ml, and a Teflon-coated magnetic stir bar. The flasks were incubated for 40 min at 37°C with gentle stirring. The slurry was then centrifuged (100 × *g*, 7 min, 4°C) and adipocytes and supernatant were decanted. The vascular pellet was resuspended with 5.0 ml of CF-P_i/NaCl and centrifuged at 100 × *g* for 5 min. The pellet was resuspended in 45% (vol/vol) Percoll (Pharmacia) in CF-P_i/NaCl and centrifuged (23,000 × *g*, 15 min, 4°C). This Percoll gradient centrifugation results in the separation of microvessels, contaminating blood cells, and large blood vessels. The band of microvessels was removed and washed twice with CF-P_i/NaCl. The final vascular pellet was resuspended in Dulbecco's phosphate-buffered saline (19) and contained predominantly capillary endothelium as observed by phrase-contrast and electron microscopy (18).

Preparations of Proteins and Fluorescent Conjugates. Rat albumin was purified from fresh serum as described by Dolhofer and Wieland (15). Albumin was free of fatty acid (20) and not glycosylated (21). Glycosylated albumin was prepared according to the procedure of Day *et al.* (16), and it was found to contain 1–2 mol of glucose per mol of albumin (21).

Proteins were conjugated with rhodamine-B isothiocyanate (Sigma) according to the procedure of Landel (20). Proteins were conjugated with MDPF according to the procedure of Weigele *et al.* (22). All conjugates were purified by dialysis in 50,000 molecular weight cut-off tubing (Spectrapor, Fisher) against two changes of 100 vol of 0.02 M phosphate-buffered saline, pH 7.1, and subsequent chromatography on Sephadex G-25. Conjugation with fluorochrome did not alter the electrophoretic mobility of albumin, glycosylated albumin, or ferritin in $NaDodSO_4$/polyacrylamide gel electrophoresis. The dye-to-protein ratio for either fluorochrome was not changed by nonenzymatic glycosylation. Rat albumin was iodinated to a specific activity of 1700 cpm/μg of protein with lactoperoxidase.

Measurement of Micropinocytosis. Micropinocytic ingestion of proteins by endothelial cells was quantitated by the procedure of Wagner *et al.* (23). The quantity of protein ingested during a 30-min incubation was analyzed in an Aminco SPF-500 spectrofluorometer or by liquid scintillation spectroscopy (24) and expressed as protein ingested per μg of endothelial cell DNA. The fluorescence yield of fluorochrome–protein conjugates was not quenched by various admixtures of endothelial cell sonicates in each sample (23).

Preparation of Endothelial Cell Plasma Membranes. Microvessel plasma membranes were prepared from freshly isolated fat microvessels. Microvessels were resuspended in 20 vol of 10 mM Tris·HCl (pH 7.6) and hypoosmotically swollen for 15 min at ice temperature. After gentle homogenization with a Teflon-on-glass homogenizer [0.004 inch (0.1 mm) clearance] and centrifugation at 500 × *g* for 30 seconds to remove debris, membranes were partitioned on a discontinuous sucrose gradient (116,000 × *g* for 60 min). Membranes that sedimented at the interface between 0.87 and 1.16 M sucrose were harvested and washed once with Tris·HCl. The resultant membrane pellet was enhanced 100-fold with respect to 5′-nucleotidase, alkaline phosphatase, and the Na^+, K^+-ATPase (25). Less than 10% of the original levels of cytochrome oxidase and glucose-6-phosphatase were observed. Electron microscopic examination revealed this material to consist predominantly of membrane sheets and apparent pinocytic vesicles.

Glycosylation with D-[6-^{3}H]Glucose. Proteins and endothelial membranes were incubated with D-[6-^{3}H]glucose (New England Nuclear, lot 1141-108) at 37°C for appropriate times. The extent of protein glycosylation was determined by liquid scintillation counting of purified proteins. Recent data have shown that specific lots of tritiated glucose contain a radioactive contaminant that covalently labels proteins, thereby producing overestimation of the extent of tritiated glucose binding (26). The rates and specificity of glycosylation of albumin and microvessel membranes were determined to be identical using either D-[6-^{3}H]glucose or D-glucose with subsequent quantitation of the extent of glycosylation by thiobarbituric acid (20) or NaB^3H_4 methods (27). These data indicate that the sample of D-[6-^{3}H]glucose used in these experiments was free of those contaminants that have been found to cause overestimation of nonenzymatic glycosylation.

Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed by using 6.5% polyacrylamide tube

Table 1. Vesicular ingestion of albumin by isolated microvessels

Albumin		Albumin concentration during ingestion		Vesicular ingestion in 30 min†	
Glycosylation,* %	Label	mg/ml	μM	ng (pmol) of protein per μg of DNA	μg of protein per g of microvascularized fat
0	^{125}I	10	145	ND	ND
0	Rhodamine-B	10	145	ND	ND
0	MDPF	11.5	167	ND	ND
100	Rhodamine-B	10	145	871.0 ± 15.0 (12.6)	70.5 ± 1.21
100	MDPF	11.5	167	1020.0 ± 200.0 (14.7)	82.5 ± 16.2

* Glycosylation was determined by thiobarbituric acid assay (21) and carboxymethylcellulose affinity chromatography; 100% glycosylated albumin contains 1–2 mol of covalently bound glucose.

† Ingestion data are given ±SD. Each fat capillary endothelial cell contains 7.1 ± 0.1 pg of DNA (30). DNA content of endothelial cells isolated from microvascularized fat of known weight was determined by mithramycin fluorescence enhancement. Vesicular ingestion per μg of DNA was converted to vesicular ingestion per g of microvascularized fat. ND indicates not detected: <0.1 ng of protein in 30 min per μg of DNA or <0.1 μg of protein in 30 min per g of microvascularized fat.

Table 2. Vesicular ingestion of glycosylated albumin in the presence of unmodified albumin

Albumin	Fluorochrome	Albumin concentration during ingestion* mg/ml	Albumin concentration during ingestion* % of total albumin	Vesicular ingestion in 30 min† ng (pmol) of protein per μg of DNA	Vesicular ingestion in 30 min† μg of protein per g of microvascularized fat
Glycosylated	MDPF	1.54	6	60.9 ± 10.0 (0.8)	4.92 ± 0.8
Unmodified	Rhodamine-B	24.1	94	ND	ND
Glycosylated	MDPF	4.61	18	151.6 ± 15.6 (2.1)	12.3 ± 1.3
Unmodified	Rhodamine-B	21.0	82	131.9 ± 14.9 (1.9)	10.7 ± 1.2
Glycosylated	MDPF	7.69	30	390.0 ± 42.9 (5.6)	31.6 ± 3.5
Unmodified	Rhodamine-B	17.93	70	110.9 ± 39.5 (1.6)	9.0 ± 3.2

* The amounts of glycosylated rat albumin selected for *in vitro* endothelial ingestion experiments are those routinely encountered in normal humans (6%), well-controlled (18%), and poorly controlled (30%) diabetics (32).

† Ingestion data are given ±SD. ND indicates not detected: <0.1 ng of protein in 30 min per μg of DNA or <0.1 μg of protein in 30 min per g of microvascularized fat.

gels with a 4.5% stacking gel and the discontinuous buffer system of Laemmli (28). Proteins were stained with Coomassie blue R-250, and lipids with oil red O (29).

RESULTS

Differential Ingestion of Albumin. Isolated microvessels excluded rat serum albumin (purified to homogeneity) from micropinocytic ingestion (Table 1). A similar exclusion was reported previously by Wagner *et al.* (5), using isolated rat epididymal fat endothelium and bovine serum albumin. Exclusion is not a result of the conjugation of albumin with fluorescent dyes, because MDPF, rhodamine, and ^{125}I-labeled proteins behaved identically. However, when glycosylated rat serum albumin was incubated with isolated endothelial cells, this material was avidly ingested over a 30-min period (Table 1).

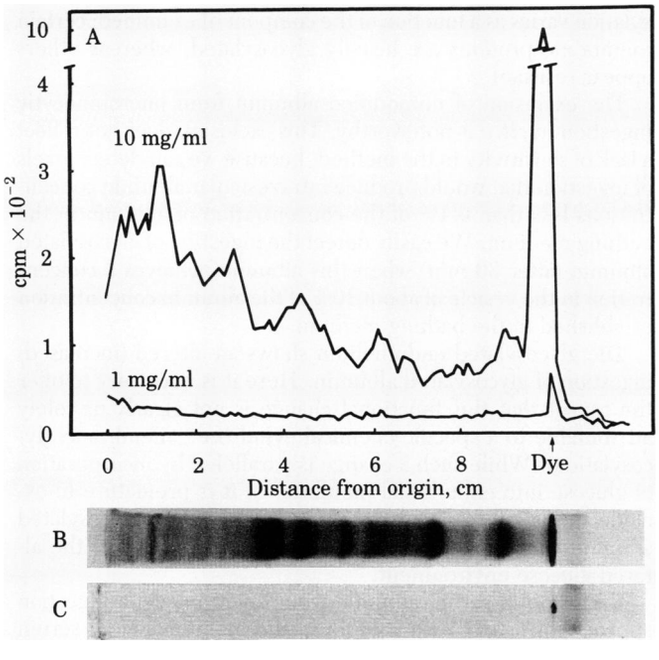

FIG. 1. Glycosylation of microvessel plasma membrane components. Isolated microvessels were incubated with D-[6-^{3}H]glucose for 1 hr at 37°C at final glucose concentrations of 1 mg/ml and 10 mg/ml. Plasma membranes were then isolated and 120 μg of protein was analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. Identical gels were sliced into 1-mm sections and analyzed for radioactivity (*A*), stained for protein with Coomassie blue R-250 (*B*), or stained for lipids with oil red O (*C*).

Micropinocytic Ingestion of Glycosylated and Unmodified Albumins. When glycosylated and unmodified albumins are conjugated to separate dyes with discrete resolvable fluorescence spectra, the extent of micropinocytic ingestion of both types of albumin can be simultaneously and independently measured (5, 31). Because albumin is present in the serum in both the glycosylated and unmodified form, we varied the ratio of glycosylated to unmodified albumin presented to isolated endothelium *in vitro*.

As shown in Table 2, 6% glycosylated albumin was ingested in the presence of 94% unmodified albumin. Under these conditions, glycosylated albumin did not stimulate ingestion of unmodified albumin, while unmodified albumin did not exclude glycosylated albumin from ingestion. Raising the relative concentration of glycosylated albumin to 18% resulted in increased ingestion of glycosylated albumin and a striking increase in the ingestion of unmodified albumin. A further increase in the relative amounts of glycosylated albumin (from 18% to 30%, Table 2) produced no further stimulation of the ingestion of unmodified albumin.

Glycosylation of Endothelial Plasma Membrane Components. When microvessels were incubated with D-[6-^{3}H]glucose for 1 hr, a concentration-dependent glycosylation of plasma membrane components was observed (Fig. 1). Comparison of the extent of glycosylation (radioactivity) with the corresponding Coomassie blue staining pattern of proteins indicates that the quantity of glucose bound is not directly related to the amount of each component (as indicated by staining intensity). Some proteins appear resistant to glycosylation, whereas others are highly susceptible. Lipid staining of identical NaDodSO$_4$ gels of glucose-treated endothelial plasma membranes revealed a minor band coincident with the dye front and a major diffuse band beyond the dye front (Fig. 1). Labeling of components in this region by D-[6-^{3}H]glucose suggested that membrane phospholipids might also be nonenzymatically glycosylated in a concentration-dependent manner.

Glycosylation of Endothelial Membrane Components: Effects on Protein Ingestion. Incubation of isolated microvessels with glucose did not alter the ability of microvessels to exclude albumin from micropinocytic ingestion (Table 3). The ingestion of glycosylated albumin, however, is markedly stimulated by prior incubation of endothelium with glucose. While glycosylation appears to increase the recognition of glycosylated albumin

Table 3. Effect of glycosylated microvessels on protein ingestion

Protein	Albumin or ferritin concentration during ingestion		Endothelial cell glucose treatment,* mg/ml	Vesicular ingestion in 30 min†	
	mg/ml	μM		μg (pmol) of protein per μg of DNA	mg of protein per g of microvascularized fat
Albumin	13.0	188.4	1	ND	ND
	13.0	188.4	5	ND	ND
	13.0	188.4	10	ND	ND
Glycosylated albumin	26.4	382.6	1	1.546 ± 0.64 (22.4)	0.125 ± 0.05
	26.4	382.6	5	2.567 ± 0.52 (37.2)	0.208 ± 0.04
	26.4	382.6	10	1.790 ± 0.29 (25.9)	0.145 ± 0.02
Ferritin	23.5	49.0	1	43.36 ± 6.9 (72.2)	3.51 ± 0.56
	23.5	49.0	5	41.92 ± 16.6 (69.8)	3.40 ± 1.30
	23.5	49.0	10	42.40 ± 10.5 (70.6)	3.43 ± 0.80

* In these experiments, we have selected a 60-min glucose incubation. This is a brief exposure to glucose as compared with the continuing exposure that the diabetic endothelium would undergo to fluctuating glucose concentrations *in vivo*. We chose 60 min because previous experience indicated that, by 180 min, the endothelium begins to exhibit an uncharacteristic macropinocytic (ATP-dependent) endocytosis (unpublished observations). In order to preserve our focus on characteristic, physiological endothelial micropinocytosis, the shorter time period is needed. For this reason, we selected the extreme "diabetic" conditions of 5 and 10 mg of glucose per ml (as well as the normal 1 mg/ml). Because the extent of protein nonenzymatic glycosylation appears to be a linear function of both time and glucose concentration, by utilizing higher glucose levels and shorter times, we are simulating a situation of more moderate glucose concentration and longer times of exposure. Even those endothelia that are exposed to the extreme situation of 10 mg of glucose per ml retain full micropinocytic capacity as demonstrated by their unaltered ingestion of native ferritin.

† Ingestion data are given ±SD. ND indicates not detected: <10 ng of protein in 30 min per μg of DNA or <1 μg of protein in 30 min per g of microvascularized fat.

by caveolae, the rate of microvesicular endocytosis of native ferritin is unchanged (Table 3). Thus, membrane glycosylation appears to selectively affect the interaction between proteins and caveolar membrane components rather than the process of pinocytic shuttling.

DISCUSSION AND CONCLUSION

The rates and extent of nonenzymatic glycosylation of proteins *in vivo* and *in vitro* are a function of sugar concentration, temperature, and pH (21). The data presented show that nonenzymatic glycosylation of serum albumin profoundly changes the manner in which albumin interacts with endothelial cell plasma membranes. Unmodified albumin is virtually excluded while glycosylated albumin is ingested avidly by isolated endothelial cells. When glycosylated albumin is incubated with endothelial cells in the presence of unmodified albumin, at a concentration ratio observed in unmodified serum, only glycosylated albumin is ingested. Increasing the relative concentration of glycosylated albumin results in the ingestion of unmodified albumin as well. Moreover, the glycosylated albumin is ingested even more avidly by endothelial cells that have themselves been glycosylated. Details of the conformational basis for this enhanced ingestion of albumin are unknown. Oligomerization of albumin has been described (33). It is possible that nonenzymatic glycosylation of albumin could increase the formation of oligomers. Increased dimerization could possibly result in the observed endocytosis of unmodified albumin in the presence of increased concentrations of glycosylated albumin; it is also possible that the strikingly enhanced ingestion of glycosylated albumin reflects increased oligomer formation.

The data show that serum albumin, endothelial membrane proteins, and apparently endothelial membrane phospholipids are susceptible to modification by the covalent attachment of glucose. Under our experimental conditions, the glycosylation of albumin *in vitro* by glucose is clearly nonenzymatic. However, because the glycosylation of endothelial membrane components is carried out with intact cells, enzymatic mechanisms are functioning as well. Stevens *et al.* (34) have reported that monophosphorylated monosaccharides exhibit a striking increase in their capacity to nonenzymatically glycosylate proteins. This suggests that enzymatic phosphorylation of glucose (e.g., with hexokinase) by endothelial cells could contribute to an increase in the nonenzymatic glycosylation of membrane proteins by glucose 6-phosphate. It is also not possible to exclude a direct enzymatic attachment of glucose to endothelial membrane proteins. It is noteworthy that the extent of glycosylation varies as a function of the component examined: certain membrane proteins are heavily glycosylated, whereas others appear resistant.

The exclusion of unmodified albumin from micropinocytic ingestion *in vitro* is noteworthy. This exclusion does not reflect a lack of sensitivity in the method, because we can detect levels of ingestion that would produce intravesicular albumin concentrations less than 0.1% of the concentration of albumin in the bathing medium. We easily detect the ingestion of glycosylated albumin (after 30 min) when this albumin achieves a concentration in the vesicle of about 10% of the albumin concentration established in the bathing medium.

The glycosylated endothelium shows an altered (increased) ingestion of glycosylated albumin. Here it is necessary to offer the caveat that this functional change is not clearly or solely attributable to a specific chemical event (i.e., membrane glycosylation). While such a change is paralleled by incorporation of glucose into endothelial membranes, it is premature to exclude the possibility that the altered ingestion of glycosylated albumin could reflect some metabolic consequence of the altered glucose environment.

Exclusion of serum albumin from micropinocytic ingestion appears consistent with its role in the maintenance of serum osmotic pressure. Of the numerous circulating proteins that contribute to the lumenal oncotic pressure, it is highly significant that serum albumin is the only protein that does not contain a carbohydrate. We suggest that the intravascular localization of albumin (i.e., its exclusion from vesicular ingestion) could result from its lack of a carbohydrate moiety. Recognition-dependent endocytosis of glycoconjugated serum and extralysosomal proteins have been previously reported (35, 36). Cap-

illary endothelial cells may also exhibit recognition for other glycoproteins in addition to glycosylated albumin.

Serum albumin is known to bind to a wide variety of molecular species, and it may thereby influence the kinetics of their disappearance from the circulation (37–40). In addition, Curry and Michel (41) have suggested that albumin may regulate capillary permeability by selectively binding to the lumenal surface of capillaries. It is possible that such functions of albumin might also be altered by nonenzymatic glycosylation. Escape of glycosylated albumin may occur from continuous capillary endothelium that exhibits vesicular transport. The proposal of vesicle-mediated albumin leakage in retinal microangiopathy might appear inappropriate, however, because unmodified retinal (and brain) capillaries do not exhibit pinocytic activity. It is known, however, that diabetic retinal capillaries do exhibit micropinocytic activity (42), thus providing a mechanism for the escape of glycosylated albumin. While this atypical micropinocytic shuttling might also arise as a consequence of endothelial membrane glycosylation, it could as well result from some independent and unrecognized consequence of the diabetic microenvironment. In any case, vesicular transport of glycosylated albumin could be responsible for the leakage of this protein from the capillaries of the diabetic retina.

Of the earliest lesions in diabetic retinopathy, seen well before gross morphological changes, is a leakiness of fluorescent dyes that are known to bind to albumin (43). In the context of the data presented here, it seems that nonenzymatic glycosylation of albumin and endothelial membrane proteins could contribute to extravasation of albumin in locations that are not ordinarily so exposed. The simple and direct pathway of nonenzymatic glycosylation appears capable of causing changes in specific protein conformation or recognition functions that have important pathogenetic potential in human diabetic states.

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