SUGGESTED MECHANISM FOR THE SELECTIVE EXCRETION OF GLUCOSYLATED ALBUMIN

The Effects of Diabetes Mellitus and Aging on This Process and the Origins of Diabetic Microalbuminuria

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Changes in renal function are usually found early in the course of diabetes mellitus and include microscopic albuminuria (45–200 mg protein/24 h) (1), polyuria (>4 liters/24 h), and an increase in glomerular filtration rate (GFR)[1] (2). These early functional changes are unaccompanied by histological changes and could well reflect the increase in plasma osmolality associated with diabetic hyperglycemia (3). In 1983, Williams et al. (4) described the selective excretion of glucosylated albumins by the mammalian kidney; i.e., the percent of nonenzymatically glucosylated (NEG) albumin in the urine of normal rats was significantly higher than the percent of glucosylated albumin in plasma. We call this preferential (urinary) excretion of glucosylated albumin "editing." In addition, we use the phrase "editing ratio" to describe the ratio of the percent glucosylation of urine albumin to the percent glucosylation of plasma albumin. In 1984, Ghiggeri et al. (5) also reported increased glucosylation of urinary albumin in humans. Moreover, they observed a relative reduction of editing in patients with diabetes mellitus.

In the present study we further examine the process of editing as a function of diabetes and age in both man and rat. We describe the actual time course for the reduction of editing after the onset of chemically induced diabetes in the rat. Furthermore, we offer a molecular/physiological explanation for the editing of albumin populations by the normal nephron and suggest a pathophysiological mechanism for the reduction of editing found in diabetes. We also show that this process is entirely reversible with insulin though not with the aldose reductase inhibitor, Sorbinil. In addition, we distinguish the reduction of editing found in diabetes from a more gradual decline of editing that we find to accompany aging in both man and rat.

Materials and Methods

Experimental Subjects.   Human subjects without evidence of diabetes mellitus or hypertension were identified among Los Alamos employees and their children. Males ($n = 14$) ranged in age from 6 to 58 yr and females ($n = 8$) from 5 to 43 yr. The mean age of control subjects was 33 ± 16. Diabetic subjects (Type I) attended a summer camp organized by the American Diabetes Association in New Mexico. Diabetic males ($n = 13$) ranged in age from 10 to 24 yr, and females ($n = 12$), from 10 to 37 yr with a mean of 16 ± 9 (see Table I). None of the diabetic subjects were hypertensive. All human subjects provided a casual urine, 1 ml of blood (heparinized), and informed consent (given by parents for minors). There was no difference in editing ratio between a casual urine sample and a 24-h collection in two humans and six rats. In all studies of editing, a casual urine was used. In human subjects, we evaluated the relationship between percent glucosylation of plasma and percent glucosylation of urinary albumin as a function of either age or diabetes.

In animal experiments, we used Sprague-Dawley, Fischer-344, or Wistar rats. We induced chemical diabetes in Sprague-Dawley rats (6) and monitored the time course for the reduction of editing of glucosylated albumin as a function of the kinetics of onset of hyperglycemia. Fischer-344 rats served as a model (7) in which we studied age-related loss of renal editing, also found in human subjects. In nondiabetic Wistar rats we studied the effects of mannitol diuresis on GFR, urinary albumin content, and the status of editing.

Streptozotocin (Stz) and Sorbinil Administration.   Male Sprague-Dawley rats (150–200 g) received 50 mg i.p. of Stz per kilogram of body weight (6). Sorbinil (a gift from Dr. Nancy Hutson, Pfizer, Groton, CT) was administered (20 mg/kg) by oral gavage (8). Animals were kept in metabolic cages (Nalgene, Rochester, NY) for collection of 24-h urines. Tail vein blood was used to measure plasma glucose, glucosylated albumin, and HbA_1C.

Plasma glucose was measured spectrophotometrically (OD 650 nm) with toluidine (9). Diabetes was also evaluated by quantitating the glucosylation of plasma albumin and hemoglobin (10). Animals with plasma glucose concentrations >400 mg/dl, HbA_1C values >7%, and plasma albumin glycosylation values >2.8% were classified diabetic for this study.

Separation of Glucosylated and Unmodified Albumin.   Urines (3 ml) were exhaustively dialyzed (4°C) against distilled water, lyophilized, and reconstituted in 250 µl of column buffer (250 mM ammonium acetate containing 50 mM $MgCl_2$ and 0.02% sodium azide, pH 8.0). Heparinized blood was centrifuged and the plasma was exhaustively dialyzed (4°C) against distilled water. Urine and plasma samples (0.05–0.5 mg protein) were added to a (0.8 × 4 cm) Glycogel B (Pierce Chemical Co., Rockford, IL) column (11) in column buffer. Nonglucosylated proteins were readily eluted with (20 ml) column buffer. Retained glucosylated proteins were then eluted with 5 ml of 200 mM sorbitol in 50 mM Tris-HCl (pH 8.5) for those albumin samples assayed by RIA (12). Alternatively, glucosylated albumin was eluted with 200 mM citrate buffer (pH 4.5) for assay with bromocresol green (10). Accuracy and reproducibility of Glycogel B chromatography were tested by processing known amounts of glucosylated or unmodified albumins or defined mixtures. We recovered >99% of the protein applied to the column. In addition, the unbound fraction contained no detectable glucosylated albumin and the bound fraction contained only glucosylated albumin. The coefficient of variation ($n = 6$) for a given sample was <2%.

Quantitation of Albumin.   Urine albumin was quantitated by RIA (12) with rabbit anti-human albumin (12) and goat anti-γ-globulin antisera (Boehringer Mannheim Diagnostics, Inc., Houston, TX). With [125I]human serum albumin as standard, the RIA gave albumin values that were linear from 12.5 to 250 ng, and indistinguishable values with both glucosylated and unmodified albumins. Plasma albumin was quantitated by spectrophotometric measurement (OD, 630 nm) of the complex formed with bromocresol green (10).

Determination of the Isoelectric Point (pI) of Glucosylated Albumins.   Fatty acid-free human serum albumin (Sigma Chemical Co., St. Louis, MO) was glucosylated for 6 d (22°C) in the presence of 100 mM glucose in PBS with 0.02% sodium azide. Glucosylated and

TABLE I

Human Subjects

Subject category	n	Age	Sex (M/F)	Duration of diabetes	Serum creatinine	Urine creatinine
		yr		*yr*	*mg/dl*	*mg/dl*
Nondiabetics	22	5–58 (33 ± 15)*	14:8	—	0.71 ± 0.41	114 ± 67
Diabetics	25	10–37 (16 ± 9)	13:12	1–21 (9 ± 7)	0.70 ± 0.17	90 ± 38

* Numbers in parentheses indicate mean age ± SD.

unmodified albumins were separated on Glycogel B and dialyzed against distilled water (4°C).

Plasma albumin from normal and diabetic rats was purified with Sepharose CL-6B (13). SDS-PAGE (14) of these preparations gave a single band with an apparent M_r of 68 × 10^3, which comigrated with native albumin. Glucosylated and unmodified albumins were separated on Glycogel B, dialyzed, and their pIs were determined in acrylamide gels (6 × 130 mm) with pH 3–10 pharmalyte (Pharmacia Fine Chemicals, Piscataway, NJ) (15).

Renal Function. Clearance studies were carried out with minor modifications of a published method (16). Wistar rats (200–300 g) were anesthetized (1 mg inactin/kg, i.p.) and a jugular infusion catheter was inserted. All infusion solutions contained [^{3}H]inulin (8 μCi/ml). The ureter was ligated as it left the bladder, and a bladder cannula was inserted for urine collection into preweighed tubes. Blood was collected from the cut tail at the middle of each clearance period for determination of Na^+, K^+, and hematocrit. Femoral vein blood was analyzed for percent glucosylation of plasma albumin and plasma osmolality. Plasma and urine osmolalities were measured in a vapor pressure osmometer (model 5100C; Wescor Inc., Logan, UT).

2 h after the start of a saline infusion (1 ml/h), baseline samples were collected. Because of low urinary protein concentrations and flow rates in the premannitol infusion periods, 1 h collection periods were needed. At the end of two collections, infusion solutions were switched to 5% mannitol in saline with an infusion rate of 5 ml/h. The duration of the collection periods was subsequently reduced to 30 min. Additional 30-min collections were made until a new, steady state was reached as indicated by two periods of equivalent urine volume. In the mannitol infusion experiments GFR was calculated as inulin clearance (17) and expressed as ml/min/kg body weight. In other experiments GFR was also calculated as creatinine clearance (18). Plasma and 24-h urine creatinines were determined spectrophotometrically by measuring OD at 500 nm with alkaline sodium picrate (19).

Urinary volume (ml) was estimated as the weight of the collected urine in grams. Samples of blood were centrifuged and the plasma samples were separated. For all renal studies, [^{3}H] was quantitated by scintillation counting and GFR was calculated from the count rates. Na^+ and K^+ (in plasma and urine) were measured by flame photometry.

In other experiments, 2 h of mannitol infusions were followed by infusion of normal saline. Additional urine samples were collected at 30-min intervals to achieve a new steady-state value. GFR, percent urinary glucosylated albumin, and diuresis were monitored as above.

Results

Reduction of Renal Editing of Glucosylated Albumins in Patients with Diabetes Mellitus. We found no significant differences in plasma and urinary creatinine concentrations between normal and diabetic subjects, whose plasma creatinine concentrations were <2 mg/dl (Table I). Our study of human diabetics was confined to Type I diabetes. We observed an apparent reduction in the prefer-
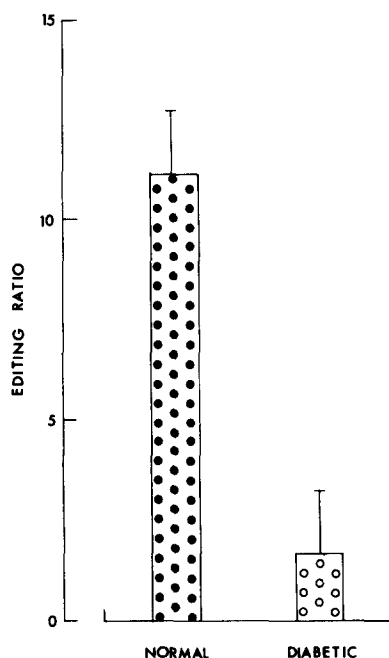
15

10

EDITING RATIO

5

0

NORMAL   DIABETIC

FIGURE 1. Reduction of the editing ratio in human diabetes mellitus. Editing ratio in 10 controls (age range 10–35 yr) and 25 diabetics (age range 10–37 yr) was calculated as described in the text. The SEM is given for both groups.

ential excretion of glucosylated albumin in urine as evidenced by a >50% fall in editing ratio (Fig. 1) in both sexes, varying in age from 10 to 37 yr. Our data were in good agreement with data from groups A and B of Ghiggeri et al. (5), in which all subjects had an albumin excretion rate <50 mg/24 h. We found a strong inverse correlation between the fall in the percent urinary albumin glucosylation (i.e., loss of editing) and the degree of microscopic albuminuria (Fig. 2). The correlation coefficient (20) between loss of editing and microscopic albuminuria was −0.42 in our diabetic human subjects ($p < 0.05$). Data for 24-h urine collections in Stz diabetic rats exhibited an even stronger (inverse) correlation ($r = -0.89$) between the level of microscopic albuminuria and the reduction in editing ratio (please see below). There was no correlation between the duration of diabetes and loss of editing, during the first 10 yr of disease ($r = -0.127$, $n = 25$). Beyond this point, the editing ratio is likely to be influenced by both age and possibly the longer term renal effects of diabetes. We also note a strong inverse correlation between the percent glucosylation of plasma albumin and the relative loss of editing (Fig. 3).

Renal Editing in Diabetes: Editing Loss Correlates with the Development of Hyperglycemia. The phenomenon of editing is readily observed in the nondiabetic 150 g Sprague-Dawley rat (editing ratio = 15 ± 1, $n = 8$) and is markedly reduced 3 wk after Stz induction of diabetes (editing ratio = 4 ± 0.8, $n = 8$). Our studies of human diabetics (and those of Ghiggeri et al. [5]) clearly demonstrate a reduced level of editing associated with hyperglycemia. Such studies, however, do not examine the rate at which editing is reduced in relation to the onset of diabetes mellitus. We gave Stz to 150-g male Sprague-Dawley rats and measured plasma glucose and editing levels daily. We observed a very rapid
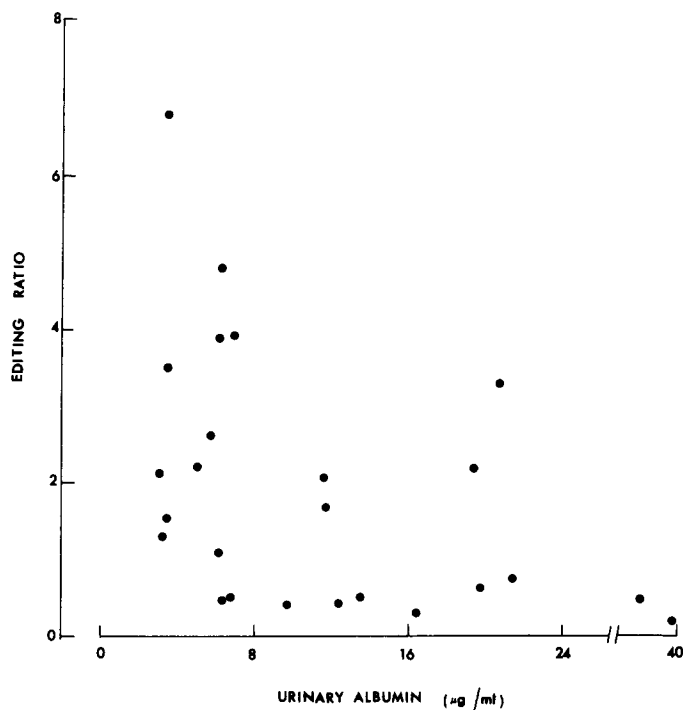
FIGURE 2. Inverse correlation of editing ratio and microscopic albuminuria in diabetic humans. The editing ratios ($n = 25$) were calculated and plotted against urinary albumin concentrations ($r = -0.42$, $p < 0.05$).

reduction in editing after the injection of Stz which is virtually complete by day three (Fig. 4). The kinetics of the decline in editing follow closely the development of hyperglycemia (Fig. 4) and also the development of polyuria, glucosuria, and microscopic albuminuria but do not follow a more gradual increase in the percent glucosylation of plasma albumin (not shown).

Mechanism(s) of Loss of Editing.   We then evaluated a series of possible mechanisms to explain the loss of editing observed in chemical diabetes mellitus. This loss of editing does not result from Stz toxicity. The effect persists as long as hyperglycemia persists, and well after the 24-h period when the signs of Stz toxicity in liver (e.g., degranulation of rough endoplasmic reticulum, and mitochondrial swelling) (21, 22) have subsided. Occasionally, a Stz injected rat (especially when the intraperitoneal injection route is used) will exhibit transient hyperglycemia. We have studied such a rat (Fig. 5) and found that the transient elevation in blood glucose is accompanied by a transient (and fully reversible) reduction in editing. Moreover, in rats with fully established diabetes (>4 wk duration) and a marked reduction in editing, the administration of insulin in quantities sufficient to normalize plasma glucose levels (2 U of zinc protamine insulin per diem, subcutaneously) was followed by restoration of a normal editing ratio by treatment day 9 (Fig. 6).

Possible Involvement of the Sorbitol Pathway.   The polyol pathway is markedly accelerated in poorly controlled diabetes (23). Increased rates of glucose reduc-
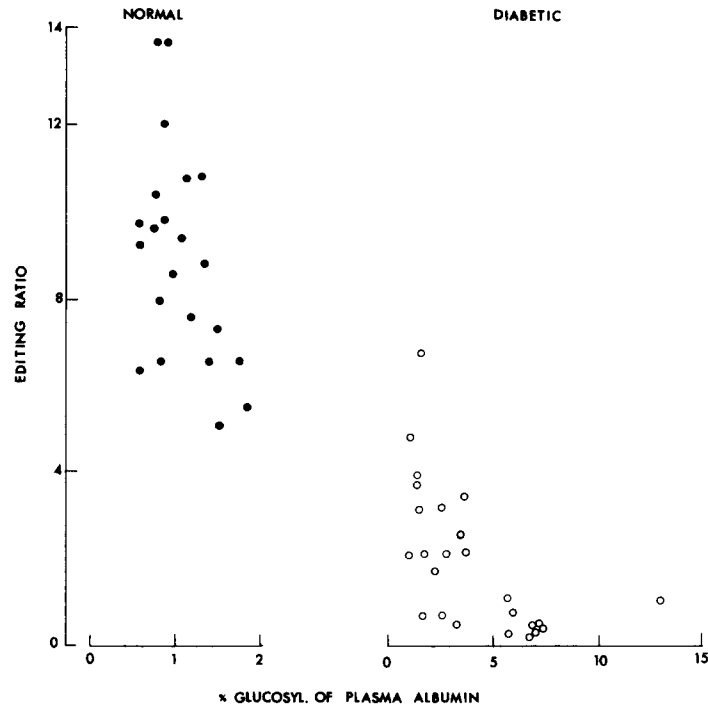
FIGURE 3.   Relationship between editing ratio and the percent glucosylation of plasma albumin in man. Editing ratios were determined in control ($n = 22$) and diabetic ($n = 25$) subjects and plotted against percent plasma albumin glucosylation. A different scale is used for the percent plasma albumin glucosylation in normal and diabetic subjects.

tion by aldose reductase are associated with sorbitol accumulation and other metabolic changes in red cells and lens (24). The polyol pathway has been implicated in diabetic peripheral neuropathy (25) and cataract formation (26). We have administered the aldose reductase inhibitor Sorbinil concomitantly with Stz injection and on a daily basis, thereafter. Sorbinil treated diabetic rats show a reduction in editing which occurs with the same kinetics and to the same extent as observed in animals treated with Stz alone (Fig. 4).

Mannitol Diuresis and Recovery Studies.   We also studied the possible role of osmotic diuresis by using mannitol infusion. Since mannitol is not a reducing sugar (27), effects observed with mannitol cannot be attributed to rapid NEG of protein amino groups. In the course of mannitol induced diuresis in nondiabetic Wistar rats (within 60–90 min after the initiation of mannitol infusion), we observed (a) the expected increase in urine volume, (b) an increase in GFR, (c) the appearance of microscopic albuminuria, and (d) a marked reduction in editing ratio (Fig. 7). We note that during mannitol diuresis there is a strong inverse correlation ($r = -0.44$, $n = 39$, $p < 0.01$) between loss of editing and the observed increase in GFR (Fig. 8). The observed reduction in editing ratio with mannitol infusion does not correlate with urine osmolality, urine or plasma Na^+ or K^+, or the hematocrit (data not shown). Cessation of the mannitol infusion was followed
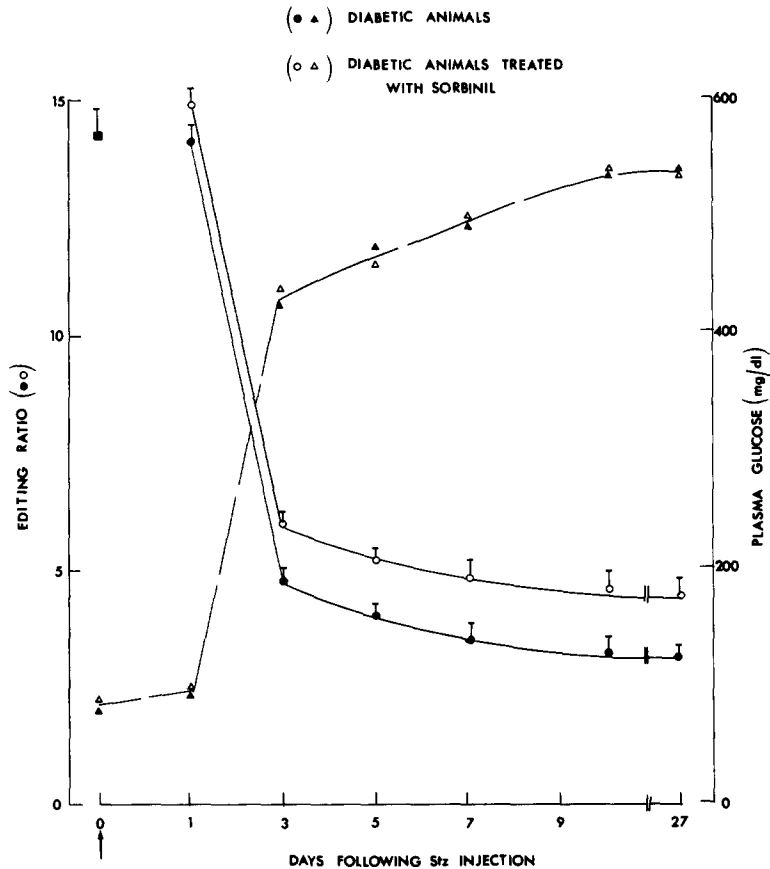
FIGURE 4. Kinetics of the reduction of editing after Stz injection; failure to prevent this change with Sorbinil. The editing ratio for Stz-treated animals ($n = 4$) was determined at different time intervals after Stz injection. The average editing ratio of six control rats before induction of diabetes is shown as the closed square. The editing ratio for Stz-injected, Sorbinil-treated rats ($n = 4$) is also shown at varying time intervals, after Stz injection. The average plasma glucose levels (△, ▲) are also shown for both groups ($n = 8$).

by reversal of osmotic diuresis, normalization of GFR, and a concomitant recovery of editing ratios to levels observed prior to infusion (Table II).

Decline of the Editing Ratio with Aging.   The preferential excretion of glucosylated albumin by the human kidney was initially studied in nondiabetic subjects where we consistently observed editing ratios of 13–16 (ages 5–15 yr). The average glucosylation of urinary albumin in nondiabetic subjects between 10 and 15 yr of age was 15% while the glucosylation of plasma albumin in these subjects was often <1%. The capacity of the human kidney to preferentially excrete glucosylated albumin gradually diminished with age (Fig. 9 and Table III). By the sixth decade, editing ratios as low as five were observed. While age had a marked effect upon editing ratios, gender did not significantly influence the preferential renal excretion of glucosylated albumin. We found robust editing in both young males and young females as well as an age-dependent (gradual) decline of editing in both sexes.
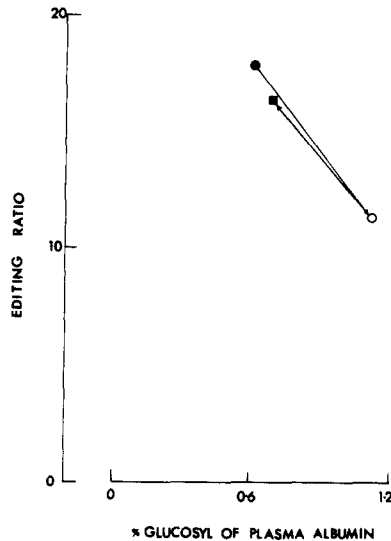
FIGURE 5. Reduction and recovery of editing ratio after an Stz injection. These data were obtained in a rat that developed transient hyperglycemia after Stz administration, and then recovered euglycemia in 10 d. The editing ratios were plotted against percent plasma glucosylation. (●) Baseline value; (○) value at the height of hyperglycemia (5 d after injection of Stz); (■) recovery value (12 d after injection of Stz).

Decline of Editing in Aging was Unaccompanied by Microscopic Albuminuria. A striking difference was observed between the reduction of editing found in diabetes and that found with aging. The presence of microscopic albuminuria is a pathophysiological feature of the diabetic kidney (1) that correlates strongly with the reduction of editing found in diabetes (Fig. 2). However, in the absence of diabetes, intrinsic renal disease, and any known systemic disease process, the aging mammalian kidney exhibits a significant loss of editing ratio in the absence of microscopic albuminuria (Table III). Thus, the pathophysiology and molecular basis for the loss of editing are likely to be different in aging and diabetes mellitus. The absolute values for the percent glucosylation of plasma and urine albumin in normal subjects (at different ages) and diabetic subjects are given in Table III. Examination of these data reveals that the reduction in editing ratio in diabetes reflects the combined effects of an increase in the percent glucosylation of plasma albumin and a decrease in the percent glucosylation of urinary albumin. In contrast, the decline in editing ratio in aging primarily reflects a reduction in the percent glucosylation of urinary albumin since increases in percent glucosylation of plasma albumin are modest.

Decline of Editing in Aging in a Variety of Species. In the nondiabetic rat, the magnitude of the editing ratio is a function of species and age. The 4-mo-old Wistar rat exhibits the remarkable editing ratio of 35, the Fischer rat a ratio of 5, and the Sprague-Dawley rat is intermediate (Table IV). We also attempted to identify an experimental system to study the reduction of editing which appears as a function of aging in human populations. In the Fischer-344 rat, we found that 3-mo-old males exhibit an editing ratio of 14, while in 24-mo-old male Fischer-344 rats, the editing ratio has fallen below 5 (Table IV). In the rat, as in man, this decline in editing which accompanies aging was not associated with microscopic albuminuria (Fig. 10). It is interesting that the decline in editing which develops gradually over a 50-yr period in humans appears to take place on an accelerated schedule within the shorter lifespan of the Fischer-344 rat.
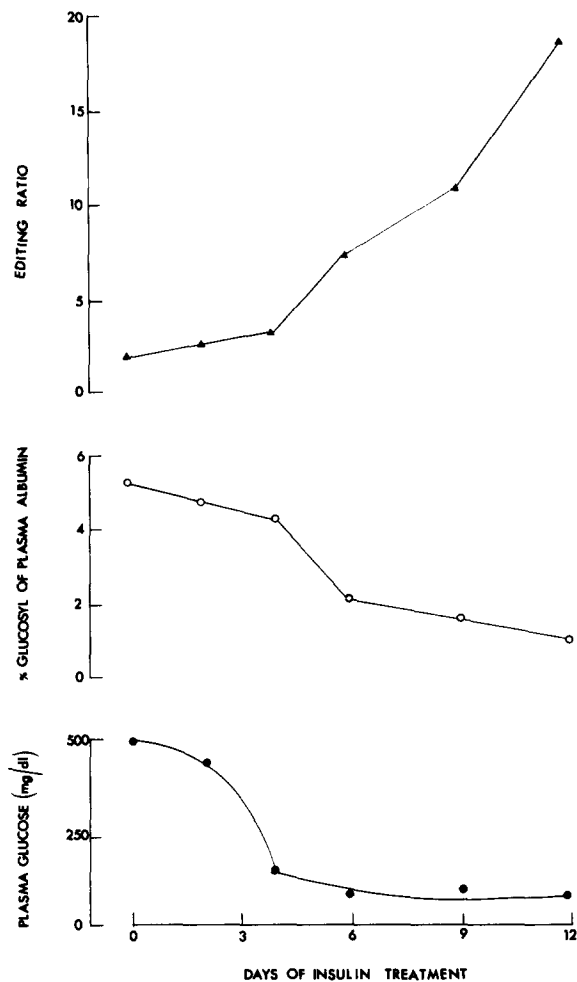
FIGURE 6. Recovery of physiological editing ratio by Stz diabetic animals after insulin treatment. Sprague-Dawley rats ($n = 4$) with 4 wk of uncontrolled diabetes were injected daily with 2 U of zinc protamine insulin (subcutaneously). Editing ratios were also calculated as were plasma glucose and percent glucosylation of plasma albumin. All were plotted against days of insulin treatment.

Relationship Between GFR (24 h) Microscopic Albuminuria and Editing Ratios in Aging and Diabetes. To further study the putative relationship between GFR, microalbuminuria, and editing ratios, we measured GFR (as creatinine clearance) in control and Stz diabetic rats and young (2 mo) and old (15 mo) Sprague-Dawley rats (Fig. 10). In each of these animals we also measured the editing ratio and the 24-h excretion of albumin in the urine. In Stz-diabetic animals, the reduction in editing is associated with an increase in creatinine clearance and microscopic albuminuria. On the other hand, the decrease in editing in aging is associated with no significant change in GFR or microalbuminuria (Fig. 10).

Cationized Albumin. The term cationized albumin, is used by Ghiggeri and coworkers (28, 29) to designate a class of albumin molecules which they found in the plasma (but not in the urine) of fewer than half of all diabetics studied. These albumin molecules bind to Con A–Sepharose suggesting glucosylation, and yet they exhibit pIs that are more positive than 4.7, which is the pI of native albumin. The chemical modification which produces cationized albumins is
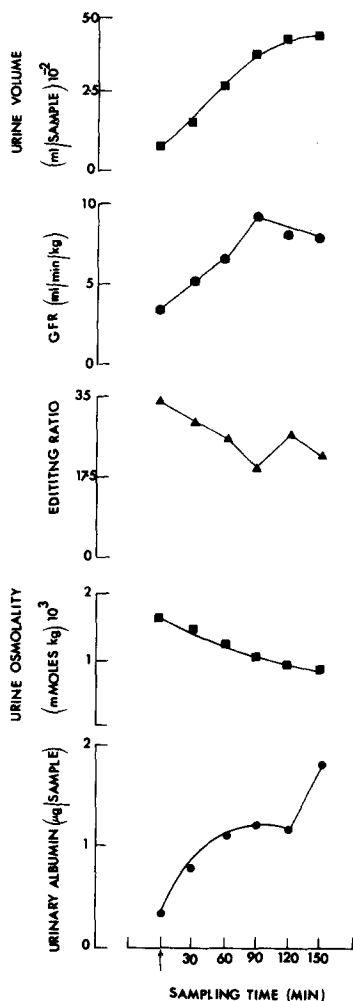
FIGURE 7.   Effect of mannitol induced diuresis on GFR and editing ratio of nondiabetic Wistar rats. Nondiabetic Wistar rats were anesthetized with Inactin and infused with a solution of mannitol. Timed samples of urine and blood were collected and urine volume, GFR, editing ratio, osmolality, and total urinary albumin were determined. The data shown are representative of one of seven animals giving comparable results.

unknown. They do not appear to contribute directly to the phenomenon of editing since they do not appear in human urine (28). Moreover, we have found that these materials are not simply a result of NEG of albumin, a process that consistently yields protein with pIs below 4.7 (Fig. 11). These values reflect the fact that for each molecule of glucose attached to albumin there is a loss of a single positive change at physiological plasma pH. We have not found cationized albumins in the plasma of our diabetic rats, all of whom lose editing in a striking way. In addition, in the study of Ghiggeri et al. (28) no correlation is attempted between the attenuation of editing and the presence of cationized albumins.

Discussion

The data presented demonstrate that glucosylated albumin is selectively excreted by the normal mammalian kidney. The selective editing of albumin populations declines very gradually (over a 50-yr period) with age and very
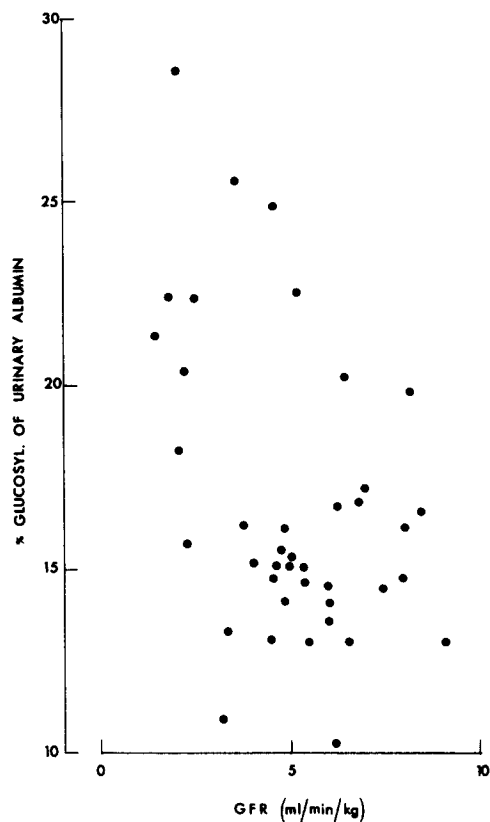
FIGURE 8. Correlation between GFR and percent glucosylation of urinary albumin during mannitol diuresis in nondiabetic Wistar rats. Data were obtained at each of the different times during the mannitol diuresis and GFR and editing ratios were compared at each time for seven animals. There was a strong inverse correlation between these parameters with an r value of -0.44 ($n = 39$).

TABLE II

Reversal of Reduction of Editing after Recovery from Mannitol Diuresis

Condition	Editing ratio urine/plasma	GFR (ml/min/kg body weight)
Baseline saline infusion	34.0	1.93
Mannitol diuresis	20.5	5.23
Recovery from mannitol diuresis	32.5	1.06

rapidly (with kinetics that follow the onset of hyperglycemia) in diabetes. Paradoxically, although the percent glucosylation of plasma albumin is increased by about fivefold in diabetes mellitus, there is a concomitant and marked reduction in the percent glucosylation of urinary albumin. Thus, it is not possible to explain the microscopic albuminuria observed in diabetes mellitus in terms of an exaggeration of the selective excretion of glucosylated albumin normally found in the absence of diabetes. Conversely, we suggest that the loss of editing in diabetes is a dilutional consequence of microscopic albuminuria (see below).

The molecular mechanisms that promote this reduction in editing are clearly different in diabetes and aging. The rapid reduction of editing that follows the

FIGURE 9.   Decline in editing as a function of age in human subjects. Percent glucosylation of both plasma and urinary albumin was measured for individuals ranging in age between 5 and 58 yr.

onset of diabetes is invariably accompanied by microscopic albuminuria and a conspicuous increase in GFR, while these factors are absent in the decline of editing that appears as a function of age. Our data suggest a model for the reduction of editing in diabetes that is based on the phenomenon of microscopic albuminuria as well as our experiments with mannitol diuresis. The infusion of mannitol into the jugular vein of the nondiabetic male Wistar rat reliably induces an increase in GFR, microscopic albuminuria, and loss of editing (see below).

The first report of the selective excretion of glucosylated albumin by the mammalian kidney appeared in 1983 and focused upon the Sprague-Dawley rat (4). More recently, Ghiggeri and coworkers have described the selective excretion of glucosylated albumin in normal individuals and a reduction of this capability in diabetes (5). We note here that loss of editing thus far, is observed in patients with Type I diabetes mellitus. Loss of editing appears to be a consequence of hyperglycemia and especially the increase in GFR which accompanies hyperglycemia. However, studies in Type II patients will be needed to confirm this expectation.

We believe that the proposal of Ghiggeri et al. (5) that the phenomenon of editing is best explained in terms of preferential glomerular filtration of glucosylated albumin is untenable. Glucosylated albumins found in the urine had an even lower pI (hence more negative charge) than native albumin at the pH of the glomerular filtrate (7.4). Thus the glomerular filtration apparatus with its negatively charged podocytes heavily endowed with sialated podocalyxin (30) should be less permeable to glucosylated than unmodified albumins. Ghiggeri et al. have, in addition, suggested that the attenuation in editing of glucosylated

TABLE III

A Listing of the Individual Values for Plasma (g/dl) and Urine (μg/dl) Albumin Levels Illustrating Our Findings for Both Nondiabetic and Diabetic Human Subjects

Subjects	Age	Plasma albumin			Total	Urinary albumin		
		Total	Glucosy-lated	Glucosy-lation		Glucosy-lated	Glucosyla-tion	Editing ratio
	yr	*g/dl*	*g/dl*	*%*	*μg/dl*	*μg/dl*	*%*	
Nondiabetic								
1	6	ND	ND	ND	621	103	16.6	ND
2	10	4.6	0.050	1.08	190	27	14.1	13.0
3	15	ND	ND	ND	320	50	15.6	ND
4	18	4.3	0.040	0.93	280	36	12.8	13.7
5	27	4.2	0.033	0.78	328	37	11.3	14.5
6	29	4.8	0.048	1.00	322	32	9.9	9.9
7	29	4.7	0.061	1.29	625	69	11.0	8.5
8	31	4.3	0.033	0.77	993	109	10.9	14.1
9	31	4.2	0.045	1.07	205	32	15.6	14.5
10	31	3.9	0.054	1.38	112	14	12.5	9.0
11	31	4.6	0.041	0.89	559	63	11.3	12.6
12	32	4.5	0.054	1.20	445	57	12.8	10.6
13	52	4.6	0.032	0.69	392	25	6.4	9.2
14	53	4.3	0.034	0.79	329	17	5.1	6.4
Diabetic								
1	10	4.8	0.14	2.9	1,345	21	1.56	0.53
2	11	4.5	0.31	6.9	945	49	5.18	0.75
3	11	5.1	0.34	6.6	945	49	5.18	0.78
4	11	4.9	0.25	5.1	661	36	5.44	1.06
5	12	5.5	0.31	5.6	2,139	96	4.48	0.80
6	13	5.0	0.09	1.8	1,965	22	1.12	0.62
7	13	5.2	0.17	3.2	470	40	2.03	0.63
8	14	4.5	0.24	5.3	3,987	56	1.40	0.26
9	17	4.9	0.11	2.2	3,665	55	1.50	0.68
10	18	5.6	0.35	6.2	1,635	34	2.08	0.33
11	24	4.9	0.10	2.0	1,170	41	3.50	1.75
12	24	4.1	0.09	2.2	2,065	155	7.50	3.40
13	29	5.0	0.34	6.8	670	23	3.43	0.50
14	31	5.7	0.37	6.5	1,230	36	2.92	0.45

Significance (p values) for age vs. percent plasma albumin glucosylation: $r = -0.39$; $n = 12$, $p = > 0.05$, not significant; age vs. percent urine albumin glucosylation: $r = -0.86$, $n = 14$, $p < 0.001$; age vs. editing ratio: $r = -0.623$, $n = 12$, $p < 0.05$.

albumin in diabetes is explained by a reduction in the filtration of glucosylated albumin. We must disagree with this model for the following reasons:

If the site of editing were in fact the glomerulus, then editing should be strongly enhanced in diabetes rather than diminished, since the amount of glucosylated albumin presented to the glomerulus is increased by a factor of 4–5. Furthermore, if the locus of editing were the glomerulus, and editing represented a preferential filtration of glucosylated albumin that somehow is reduced in diabetes, then the absolute quantity of glucosylated albumin that is excreted in diabetes should also diminish. In fact, the total glucosylated albumin in the urine is markedly increased (51.6 ± 3 μg/24 h in four diabetic rats vs. 12.3 ±

TABLE IV

Editing Ratio as Influenced by Species and Age

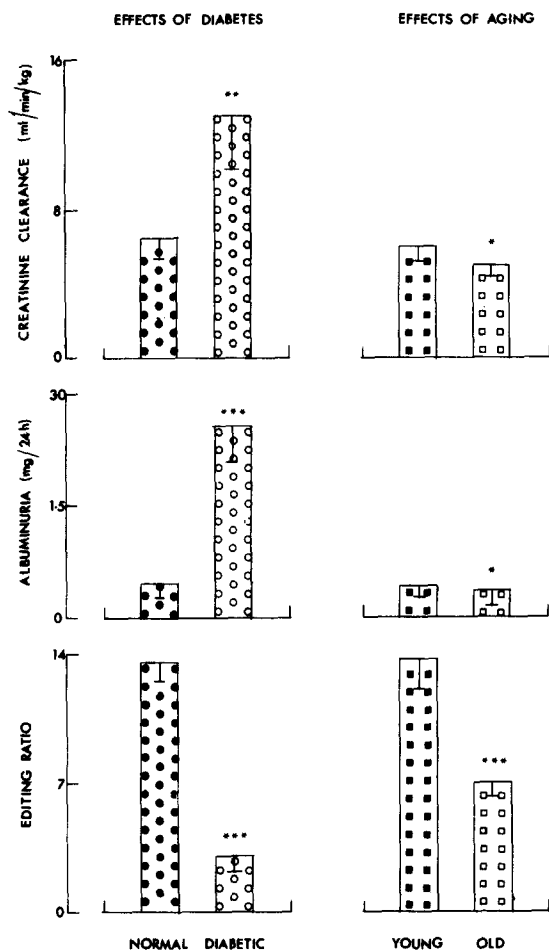| Species	Age	Editing Ratio
Sprague-Dawley Rat	1 mo	18
	3 mo	16
	12 mo	15
Fischer-344	3 mo	14
	2 yr	<5
Wistar	4 mo	35
Human	5–10 yr	14–16
	50–60 yr	4–7

FIGURE 10. Relationship between GFR, albuminuria and editing ratio in diabetes and aging. Creatinine clearance, 24 h albuminuria and editing ratios were determined in nondiabetic young (2 mo) and old (15 mo) and nondiabetic vs. diabetic Sprague-Dawley rats (3 mo). Data are mean and standard error for three to five animals in each group. ***, $p < 0.001$, **, $p < 0.02$, and *, not significant.

1.6 μg/24 h in four control rats) even though the percent glucosylation of urine albumin and the editing ratios are, in fact, markedly diminished in the diabetic animals. We note that the increased filtration of unmodified (pI 4.7) and glucosylated albumin (pI 4.5) in diabetes mellitus could also be due to net decrease in the negative charge of the filtration apparatus. Significant reduction
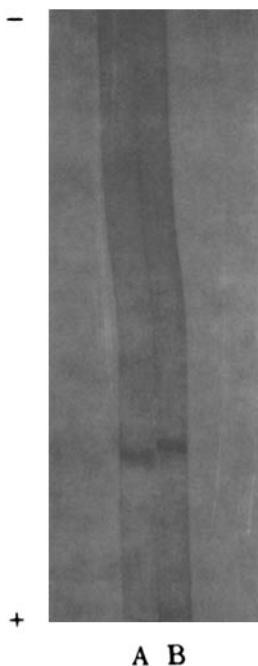
FIGURE 11. Determination of isoelectric point for glucosylated human serum albumin. Data represent the relative mobilities of both (in vitro) glucosylated (A) and unmodified (B) human serum albumin. The apparent pIs of unmodified and glucosylated albumins were determined by extrapolating their observed electrophoretic mobilities onto a "standard curve" using calibration proteins with known pIs. The standards used were: amyloglucosidase (3.5), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin (6.85, 7.35), lentil lectin (8.15, 8.45, 8.65) and bovine pancreas trypsinogen (9.30).

A B

in glomerular sialic acid (31) and glycosaminoglycan (32, 33) content is found in diabetic animals.

As an alternative explanation of reduced editing based on filtration mechanisms, we turn to the striking increases in microalbuminuria and GFR which are well documented in the human diabetic (1, 2). It is clear that associated with these increases there is a corresponding increase in the delivery of unmodified albumin into the proximal tubule. Moreover, micropuncture studies indicate that the proximal tubule normally reabsorbs almost all of the unmodified albumin presented to it (34). We suggest that it is the proximal tubule that is the site of editing and that selectively excludes glucosylated albumin from the process of reabsorption.

In this view the proposed failure of the proximal tubule to reabsorb glucosylated albumin might reflect a change in the local surface charge of albumin (35) since each attached glucose molecule neutralizes a positive charge associated with lysine or the NH_2-terminal (36). This idea is indirectly supported by the observation that the net charge of proteins plays a contributing role in their reabsorption by the proximal tubule. Chemically increasing the positive charge for a given macromolecule increases the extent of its reabsorption (37). Alternatively, the glucosylation of albumin could obfuscate its recognition by specific albumin binding sites on the luminal surface of proximal tubular epithelial cells. Such binding sites might depend upon the recognition of one or more lysine groups that are modified by NEG and/or a specific albumin conformer that is lost as a consequence of NEG, or some combination of the above. We suggest that it is the proximal tubule that, in diabetes, is overwhelmed by the influx of albumin, i.e., its maximal reabsorptive capacity is exceeded (38).

In this scenario, the diabetic nephron allows an increased amount of unmodified albumin to escape into the urine and to dilute the glucosylated albumin which is simply not reabsorbed either in normal or diabetic individuals. This relative dilution by unmodified albumin is the cause of the apparent loss of editing. This apparent reduction of editing does not appear because glucosylated albumin is not being filtered by the diabetic glomerulus. Rather, it is seen because unmodified albumin is now appearing in the glomerular filtrate in larger than normal amounts. Thus, two consequences of increased renal plasma flow and increased GFR appear in the diabetic nephron. These are the appearance of microscopic albuminuria and a concomitant reduction in editing ratio. These are acute consequences of the diabetic state and are rapidly reversible when blood glucose levels are normalized by insulin (Fig. 6). In addition, reduction of editing was also seen to be reversible in three other examples encountered in this study. One of our control human subjects exhibited an attenuated editing ratio of 5, at 3 mo after the delivery of a normal infant. This subject recovered an editing ratio of 14 at 7 mo postpartum. There was no evidence of maternal hyperglycemia at any time during or after this pregnancy. We also observed reversible attenuation in editing in a Stz-diabetic animal with transient hyperglycemia (Fig. 5). Finally, the mannitol diuresis experiments in nondiabetic Wistar rats also demonstrated a rapidly reversible reduction in editing (Table II). These acute changes are quite different (in temporal character and reversibility) from the chronic changes suggested by Brenner et al. (39) (gradual long-term destruction of the glomerulus) to be associated with an increase in GFR, and possibly increased deposition of protein in the mesangium.

We note that some of our diabetic patients are old enough to encounter age-related declines in editing ratio. In each of these patients (18–34 yr of age), however, the editing ratio was without exception (1.3 ± 1.1, $n = 6$) much more reduced than in their age-matched (nondiabetic) counterparts (11.8 ± 1.8, $n = 6$). Moreover, when we compared our diabetics of <5 yr duration with those of >15 yr duration, we found examples of both moderate and severe editing declines in each of the groups. As indicated above, the severity of the decline in editing correlated with the severity and not the duration of diabetes. These and our other findings support the concept that the editing changes are a manifestation of the increased GFR and microalbuminuria rather than changes which gradually occur over many years of diabetes. Clearly, a rapid and marked decline in editing (which is fully reversible) can be observed within days of the induction of Stz diabetes in our murine subjects.

It is important to note that as a consequence of NEG, a significant amount of protein refolding can occur (40). We have observed striking changes in the polymerization capacity of tubulin (41), and in the function of calmodulin with respect to activation of its target enzymes and its binding of Ca^{2+} (42) all as a consequence of NEG. Analogous changes in the folding of albumin as a consequence of NEG may occur as evidenced by changes in the way it is recognized by different cells. Endothelial cells avidly micropinocytose NEG albumin and rigorously exclude unmodified albumin (43). Vlassara et al. (44) have observed that glucosylated myelin basic protein was preferentially ingested by peritoneal macrophages. In contrast, pulmonary macrophages ingest unmodified albumin

more rapidly that NEG albumin (45). The data presented here strongly suggest that the proximal tubular epithelium avidly reabsorbs unmodified albumin and rigorously excludes NEG albumin. When taken together these data support the hypothesis that NEG can change folding and/or function and/or the recognition of proteins.

In the case of plasma albumin, there appears a consistent theme of retaining unmodified albumin within the vascular compartment and rapidly expelling NEG albumin from the circulation. This is also seen from the fact that while unmodified albumin has a half-life of >2 wk (46, 47), after insulin therapy elevations in glucosylated albumin disappear within 6 d (Fig. 6). Clearly, the escape of NEG albumin from the systemic circulation is far more rapid than the clearance or turnover of unmodified albumin which is estimated at 17–19 d (46, 47). Our data are in agreement with that of Morris and Preddy (48) who found a circulating half-life of ^{125}I-glucosylated albumin of ~6 d in alloxan diabetic dogs. In contrast, Day et al. (49) report a 48 h half life for [^{125}I] glucosylated albumin in diabetic rats. These studies also find the surprising half-life of 48 h for nonglucosylated ^{125}I-albumin. However, Garlick and Mazer (46) published an estimate of 17 d for the half-life of human serum albumin. We believe that our experimental approach is both accurate and reliable because we have measured the kinetics of disappearance of endogenously formed unlabeled glucosylated albumin by sensitive RIA. Introduction of ^{125}I into exogenous albumin carries the risk of loss and/or exchange of label and/or accelerated removal of the labeled protein from the circulation. Our finding of rapid disappearance of glucosylated albumin from the circulation is consistent with increased rates of deposition of albumin in the basement membrane of the diabetic capillary (50, 51) and consistent with the findings of Williams et al. (43) of increased micropinocytic ingestion of glucosylated albumin by endothelium.

The mannitol infusion experiments provide an independent way of producing an increase in GFR, presumably in association with the plasma volume expansion that follows infusion of hypertonic solutions. The mannitol associated reduction in editing is principally correlated with the increase in plasma osmolality, GFR, and the associated microalbuminuria.

Our model for editing and its loss in diabetes is thus supported by the following ideas and/or observations: (a) The increase in GFR in diabetes presents an increased amount (two- to threefold) of albumin to the proximal tubule; (b) since ~90% of filtered albumin is normally reabsorbed (52) (i.e., the tubule is working near saturation), it seems reasonable to suggest that increased albumin presented in diabetes or mannitol diuresis exceeds the maximal reabsorptive capacity of the tubule. This provides a simple explanation for the microalbuminuria of early diabetes; (c) the total amount of glucosylated albumin per 24 h was actually increased in the urine of our diabetic rats, when the editing ratio was attenuated. This is also seen in human diabetics in the data of Ghiggeri et al. (5), especially in groups A and B. This relationship is obfuscated in group C, presumably because of the increased age of the subjects; (d) in mannitol diuresis, loss of editing appears with the same kinetics as microalbuminuria; (e) we consistently observe an inverse relationship between the editing ratio and microalbuminuria.

The gradual decline of editing with age is clearly different than the rapid

reduction in diabetes, since in the normal aging kidney, no albuminuria is seen to accompany the loss of editing (Fig. 10). Moreover, GFR tends to diminish with age (Fig. 10 and reference 53) in contrast to the increase in GFR in early diabetes and with mannitol diuresis. While the fall in editing ratio in aging is primarily associated with a decrease in percent glucosylation of urine albumin, in diabetes mellitus the reduction in editing ratio is associated with both an increase in the percent plasma albumin glucosylation and a decrease in urinary albumin glucosylation.

In conclusion, the data presented here strongly suggest that the reduction in editing ratio in diabetes is a reversible phenomenon and primarily results from the differential handling of unmodified and glucosylated albumins by the proximal tubule, and the inability of the proximal tubule to reabsorb the increased amounts of unmodified albumin presented to it in diabetes. Definitive proof of this model must await micropuncture study of the fate of glucosylated and unmodified albumin in the proximal tubule. Our data also suggest that the microalbuminuria of early diabetes is a feature of the (reversible) increase in GFR and inability of the proximal tubule to effectively reabsorb the increased quantities of albumin which are filtered.

Summary

In previous studies in the Sprague-Dawley rat, Williams and coworkers (4) reported the phenomenon of selective urinary excretion of glucosylated albumin (editing, i.e., the percent glucosylation of urinary albumin is more than that of plasma albumin) by the mammalian kidney. Ghiggeri and coworkers (5) subsequently found that the extent of editing is reduced in human diabetics. Moreover, the reduction in editing in diabetes correlates inversely with levels of microalbuminuria. We also find reduction in the extent of editing in diabetic humans. We find a striking inverse correlation not only with the magnitude of microalbuminuria but also with the extent of plasma albumin glucosylation. In contrast, we found little correlation between the reduction in editing and the duration of diabetes in human subjects. Stz induced diabetes in the Sprague-Dawley rat is associated with a striking and rapid reduction in editing which develops virtually with the same kinetics exhibited by the appearance of hyperglycemia. This loss of editing is rapidly reversed by daily administration of insulin but not by aldose reductase inhibitors.

Mannitol infusion in anesthetized Wistar rats resulted in an increase in urine volume, GFR, and microalbuminuria, and was also accompanied by a marked reduction in editing. This reduction was rapidly reversed by a cessation of mannitol infusion. We propose here that glucosylated albumin (in contrast to unmodified albumin) is not reabsorbed by the proximal tubule, and thus, is preferentially excreted in the urine. We postulate that the increase in GFR which emerges as a consequence of increased plasma osmolality in diabetes mellitus delivers more albumin to the proximal tubule than can be reabsorbed. This results in a dilution of excreted glucosylated albumin molecules by excreted unmodified albumin, which appears as the early microscopic albuminuria of diabetes. Paradoxically, the fall in apparent editing is accompanied by an absolute increase in the total quantity of glucosylated albumin excreted.

In contrast, we found that editing of glucosylated albumin by the normal kidney is found to gradually decline as a function of age *without* the appearance of microalbuminuria. This suggests that a different mechanism operates to produce the loss of editing seen with aging in man, and as clearly (but in a shorter absolute time intervals) in the Fischer-344 rat.

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