Normalizing Glycated Albumin Reduces Increased Urinary Collagen IV and Prevents Renal Insufficiency in Diabetic *db/db* Mice

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Increased excretion of type IV collagen accompanies the accumulation of mesangial matrix, which leads to compromise in the glomerular filtration surface area, during the development of diabetic nephropathy. We postulated that the response of urinary collagen IV would be useful in evaluating possible treatment strategies to arrest the nephropathic process while still at a reversible stage. To test this hypothesis, we examined the effect of a small molecule (22CPPA) that inhibits the formation of glycated albumin, which is causally linked to the pathogenesis of diabetic nephropathy, on collagen IV excretion, albuminuria, and renal function in *db/db* mice. Compared to nondiabetic *db/m* mice, *db/db* animals showed markedly increased urinary collagen IV and albumin, significantly elevated serum glycated albumin and creatinine concentrations, and a significantly reduced creatinine clearance. Treatment of *db/db* mice with test compound, which normalized glycated albumin concentrations, significantly lowered collagen IV and albumin excretion and ameliorated the fall in creatinine clearance and the rise in serum creatinine despite persistent hyperglycemia. The findings indicate that reduction of elevated collagen IV excretion in diabetes reflects a salutary influence on developing glomerulosclerosis, and that glycated albumin has an important nephropathogenic role that can be therapeutically addressed independent of glycemic status.

RECENT STUDIES have demonstrated that increased excretion of type IV collagen is a useful marker of developing diabetic nephropathy in both humans and rodents with diabetes mellitus.[1-7] Whereas microalbuminuria, an established parameter of early renal dysfunction in diabetes, appears to represent leaky nephrons,[8-11] elevated urinary collagen IV excretion is believed to reflect renal overproduction of this extracellular matrix protein and the associated mesangial expansion that leads to glomerular occlusion and loss of filtration function.[6,7] In a previous study, we showed in *db/db* mice that measurable increases in collagen IV excretion coincided with morphometric evidence of glomerular mesangial matrix expansion, and suggested that assessment of urinary collagen IV in response to intervention strategies may aid in the evaluation of potential therapies to arrest the development of diabetic nephropathy.[7] In the present study, we examined the effect of treatment of diabetic *db/db* mice with a small molecule that inhibits the formation of glycated albumin, a glucose-modified protein that is causally linked to the pathogenesis of diabetic nephropathy,[12-19] on the urinary excretion of type IV collagen and albumin and on the compromise in filtration function.

The impetus for this study derived from experimental findings indicating that (1) glycated albumin stimulates the expression of transforming growth factor (TGF)- β1, the TGF-β type II signaling receptor and extracellular matrix proteins, and activates protein kinase C(PKC)-β and extracellular signal-related kinase (ERK), in glomerular mesangial and endothelial cells[12-15,20]; (2) reducing the burden of glycated albumin in vivo can beneficially influence the development of diabetic nephropathy and retinopathy[16-19,21]; and (3) the nonenzymatic glycation of albumin can be inhibited by certain drugs such as diclofenac and aspirin that render susceptible lysine ϵ-amino groups in the protein inaccessible for glycation.[22-26] For the current study, we synthesized a small molecule of the 2-(phenylamino) phenylacetic acid structural class that was able to impede the condensation of glucose with albumin but was essentially devoid of the cyclo-oxygenase (COX) inhibitory activity possessed by these anti-inflammatory agents, thereby eliminating possible confounding of results due to inhibition of COX enzymes. We report that chronic administration of this compound significantly reduces urinary collagen IV and albumin excretion, and prevents renal insufficiency in *db/db* mice.

MATERIALS AND METHODS

Experimental Animals

Male diabetic *db/db* mice and age-matched nondiabetic *db/m* mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were weighed and hyperglycemia was confirmed in the diabetic mice after arrival in our laboratory. Animals were provided food and water ad libitum, weighed weekly, and the persistence of hyperglycemia in *db/db* mice and normoglycemia in *db/m* mice during the course of the experiment was documented. Blood was obtained from the retro-orbital sinus for measurement of glucose, creatinine, and glycated albumin and 24-hour urine samples were collected for measurement of albumin, type IV collagen, and creatinine at the baseline, midpoint, and completion of the experimental protocol. All animal experimentation was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Treatment Protocol

At age 12 weeks, *db/db* animals were divided into 2 groups, one of which received test compound (15 mg/kg body weight), administered twice per day by gavage for a total of 7 weeks, the other serving as diabetic control. Dosing was selected on the basis of results from preliminary experiments with variable dosages and from stoichiometric relationships between the 50% inhibitory concentration (IC_{50}) of albumin glycation (see below) and the serum concentration of albumin in the mouse. Nondiabetic *db/m* and *db/db* control mice received daily saline gavage for the same 7-week period.

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Table 1. Experimental Animal Data

Age	Group	Body Weight (g)	Glucose (mg/dL)	Kidney Weight (mg)
12 weeks	*db/m*	24.3 ± 0.2	82 ± 12	
	db/db control	42.7 ± 0.6*	260 ± 20*	
	db/db treated	42.3 ± 0.6*	275 ± 30*	
16 weeks	*db/m*	30.0 ± 0.8	132 ± 10	
	db/db control	49.8 ± 0.9*	426 ± 48*	
	db/db treated	50.3 ± 1.7*	471 ± 23*	
19 weeks	*db/m*	30.7 ± 0.6	106 ± 12	373 ± 7
	db/db control	45.8 ± 0.9	678 ± 37*	448 ± 23*
	db/db treated	46.2 ± 2.2	695 ± 42*	399 ± 30†

NOTE. 12-week values are baseline (pretreatment). *db/m* and *db/db* control, saline gavage; *db/db* treated, 15 mg/kg/d test compound by gavage; n = 6 in each experimental group.

*$P < .05$ *v db/m*.

†$P < .05$ *v db/db* control.

Preparation and Analysis of Test Compound

The test compound, designated 22CPPA, was synthesized via condensation of 2-bromophenylacetic acid with a chloro-substituted aniline and purified through filtration, organic extraction, and crystallization by described techniques.[27] It migrated as a single spot on silica gel thin-layer chromatography, exhibited appropriate aromatic and acid protons on nuclear magnetic resonance, and had the correct composition on elemental analysis ($C_{14}H_{12}$ Cl NO_2), with a molecular weight of 261.71 and a melting point of 116°C. This compound lacks the 2,6 dichloro substitution required for inhibition of COX enzymes.[27] We confirmed low COX inhibitory activity by incubating COX-1 purified from ram seminal vesicles in the presence of cofactors, substrate, and 0-1000 μmol/L of compound and measuring the peroxidase activity according to the vendor's instructions (Cayman Chemical, Ann Arbor, MI). The IC_{50} for COX inhibition of 22CPPA was greater than 700 μmol/L, compared to an IC_{50} of 1 μmol/L for diclofenac assayed by the same procedure. The ability of 22CPPA to inhibit the nonenzymatic glycation of albumin in vitro was tested by incubating 0 to 1,000 μmol/L of compound with serum albumin for 2 days at room temperature in the presence of 50 mmol/L glucose in buffered saline, and determining the amount of glycated albumin formed. This was accomplished by processing the reaction mixtures through Sephadex G-25 desalting columns (Amersham, Piscataway, NJ) to remove free glucose and compound, followed by application to phenylboronate agarose (PBA) (Perkin Elmer, Norwalk, CT) to separate nonglycated (unbound) from glycated (bound) albumin. The albumin concentration in the bound fraction, eluted with 0.3 mol/L sorbitol, was measured by immunoassay with anti-human albumin-specific antibody.[28] The IC_{50} for inhibition of albumin glycation by test compound in this assay was approximately 35 μmol/L.

Analytical and Immunoassay Procedures

Glucose was determined by the glucose oxidase method and creatinine by the picric acid colorimetric procedure (Sigma Chemical, St Louis, MO). To ensure accurate measurements of creatinine, samples were deproteinated with acid tungstate and the alkaline picrate reagent was added after adsorption of the creatinine to Fuller's earth. Urine albumin and type IV collagen were measured with competitive enzyme-linked assays that have been described previously and that have been validated with respect to specificity, sensitivity, and reproducibility.[7,12,16,17] Serum concentrations of glycated albumin were measured after affinity chromatography on PBA as described above, with determination of the albumin concentration in the 0.3-mol/L sorbitol elution by immunoassay specific for mouse albumin.[7,12,16,17]

RESULTS

The *db/db* diabetic mutant mouse develops hyperglycemia associated with obesity and insulin resistance a few weeks after birth, and exhibits pronounced glomerular mesangial expansion by age 16 weeks.[16,17,29-31] General characteristics of the experimental animals used in the present study conformed with those known to be associated with this model (Table 1). The diabetic *db/db* mice were obese and hyperglycemic at the start and conclusion of the study. Mean body weight was greater in *db/db* mice compared to *db/m* controls throughout the experimental period, although diabetic animals stopped gaining weight at about age 16 weeks, consistent with entry into an insulin-deficient catabolic state as reported in this rodent model.[30,31] The treatment protocol did not affect body weight or blood glucose; since the test compound binds to serum albumin and does not react with free glucose, no effect on metabolic status was expected. The compound was well tolerated by the mice and had no obvious toxicity. Kidney weights at the end of the study were significantly greater in diabetic controls compared to nondiabetic animals, but the mean kidney weight was significantly less in *db/db* mice that received test compound (Table 1). Due to obesity, the kidney to body weight ratio was lower in *db/db* compared to *db/m* mice (9.8 ± 0.5 *v* 12.1 ± 0.2 mg/kg/body weight) but showed significant diminution in *db/db* mice receiving test compound (8.8 ± 0.6; $P < .05$), consistent with prevention of nephromegaly.

Serum concentrations of glycated albumin were approximately 40% higher in diabetic compared to nondiabetic mice at baseline, reflecting exposure to a hyperglycemic milieu.[32] Glycated albumin concentrations were normalized in diabetic mice that received test compound (Fig 1). This sustained reduction in glycated albumin concentrations occurred despite persistent and marked elevations in blood glucose (Table 1), and indicates an inhibition of nonenzymatic glycation in vivo during the residence time of the protein in the circulation, which is approximately 3 to 5 days in the mouse.[33]

Albumin excretion was significantly increased in *db/db* control compared to nondiabetic mice throughout the experimental period (Fig 2). Diabetic mice receiving test compound showed a significant reduction in albumin excretion, with values at 25% to 30% of baseline after four and seven weeks of treatment (Fig

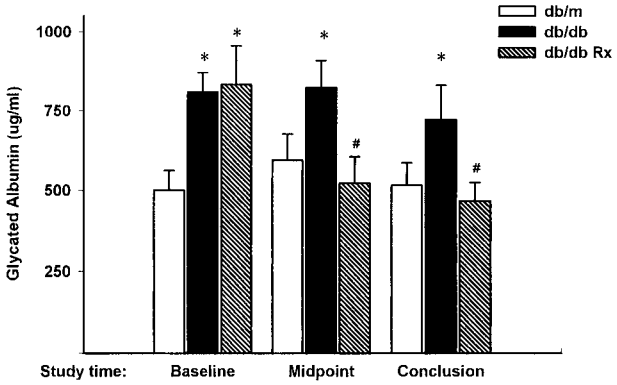

Fig 1. Serum glycated albumin concentrations at age 12, 16, and 19 weeks (initiation, midpoint, and conclusion of the experimental protocol) in nondiabetic and diabetic control mice and in diabetic *db/db* mice receiving test compound. Experimental animal groups as in legend to Table 1. Glycated albumin measured as described in the text. *$P < .05$ *v* nondiabetic; #$P < .05$ *v* diabetic control.

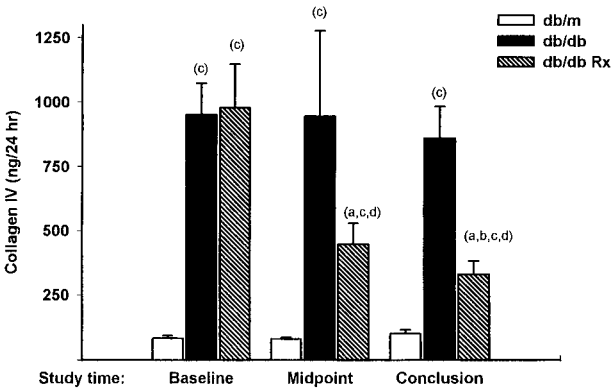

Fig 3. Urinary collagen IV excretion at age 12, 16, and 19 weeks (initiation, midpoint, and conclusion of experimental protocol) in nondiabetic and diabetic control mice and in diabetic mice receiving test compound. (a) $P < .05$ *v* baseline in same experimental group; (b) $P < .05$ *v* midpoint in same experimental group; (c) $P < .05$ *v db/m* at same age; (d) $P < .05$ *v db/db* control at same age.

2). The absence of a significant difference in the mean body weight in treated versus control diabetic mice supports the interpretation that the decrease in albumin excretion was not due to caloric deprivation, although the lack of complete normalization of albumin excretion is consistent with the continued severe hyperglycemia.

The urinary excretion of type IV collagen was significantly greater in *db/db* compared to *db/m* mice at age 12 weeks (baseline), as has been reported,[7] and remained significantly elevated in *db/db* control animals throughout the experimental period (Fig 3). The *db/db* mice receiving test compound showed significant reductions in urinary collagen IV after 4 and 7 weeks of treatment (Fig 3), with values at 46% and 34% of baseline levels, respectively.

Diabetic *db/db* mice have been shown to develop compromised filtration function, manifest by a reduction in creatinine clearance and a rise in serum creatinine.[7,18,34,35] These changes were also observed in the present study (Fig 4). Serum creati-

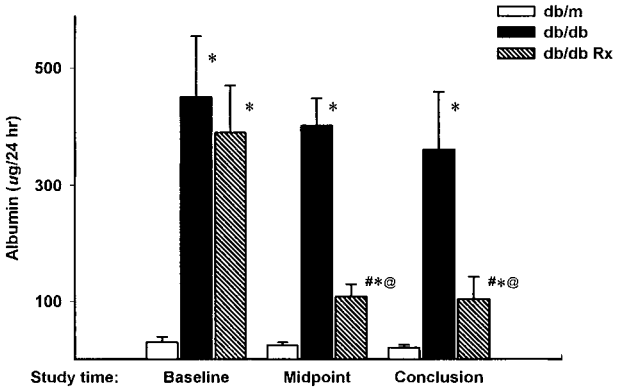

Fig 2. Urine albumin excretion measured at age 12, 16, and 19 weeks (initiation, midpoint, and conclusion of the experimental protocol) in nondiabetic and diabetic control mice and in diabetic *db/db* mice receiving test compound.*$P < .05$ *v db/m* at same age; #$P < .05$ *v* baseline in same experimental group; @$P < .05$ *v db/db* control at same age.

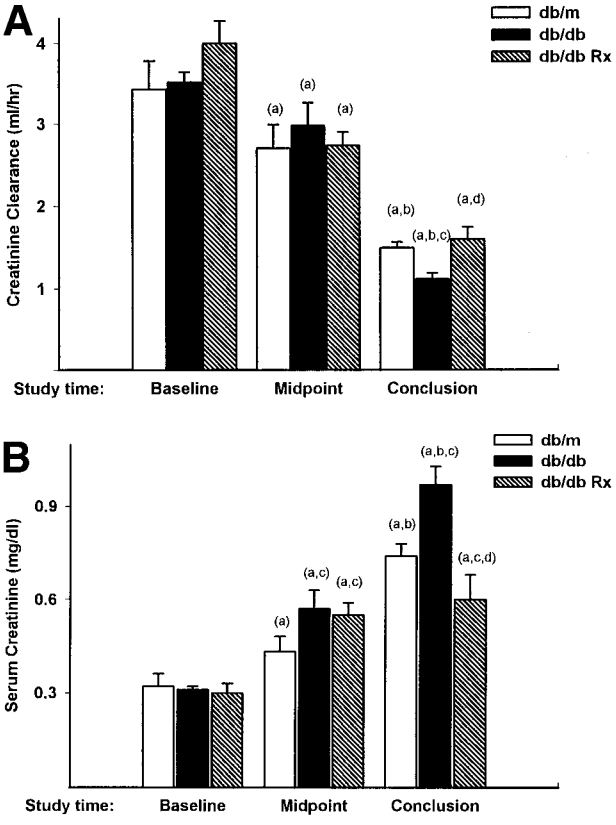

Fig 4. (A) Creatinine clearance and (B) serum creatinine at age 12, 16, and 19 weeks (initiation, midpoint, and conclusion of the experimental protocol) in nondiabetic and diabetic control mice and in diabetic mice receiving test compound. (a) $P < .05$ *v* baseline in same experimental group; (b) $P < .05$ *v* midpoint in same experimental group; (c) $P < .05$ *v db/m* at same age; (d) $P < .05$ *v db/db* control at same age.

nine increased and creatinine clearance decreased with age in nondiabetic animals as well, but serum creatinine was significantly higher and creatinine clearance was significantly lower in *db/db* controls compared to *db/m* mice at the conclusion of the study period. In contrast, creatinine clearance in *db/db* mice receiving test compound was significantly greater than that in *db/db* controls at the end of the study, and did not differ from that in nondiabetic *db/m* mice of the same age (Fig 4). Similarly, serum creatinine was significantly lower in treated *db/db* animals compared to *db/db* controls at the end of the study, and was less than that in nondiabetic *db/m* mice of the same age (Fig 4).

DISCUSSION

The present results demonstrate that the urinary excretion of collagen IV, which becomes elevated as renal involvment progresses in the *db/db* mouse, can be significantly reduced by administration of a compound that inhibits the nonenzymatic glycation of albumin in vivo. We have previously shown that this increase in urinary collagen IV: (1) is not accompanied by changes in the serum concentration of collagen IV; (2) reflects the renal overproduction of this extracellular matrix protein in diabetic nephropathy; (3) is an indicator of diabetic renal disease entering a phase of compromised filtration function; and (4) is observed in human diabetes as well as in the *db/db* mouse model.[6,7,36] These observations prompted the suggestion that measurement of urinary collagen IV may be a useful marker to evaluate the ability of potential therapies to arrest the development of diabetic nephropathy while it is still at a reversible phase. The experimental findings reported herein support this postulate. Notably, the reduction in urinary collagen IV in treated diabetic mice was accompanied by an amelioration of the fall in creatinine clearance and rise in serum creatinine observed in untreated *db/db* controls.

These data also support the hypothesis that albumin modified by Amadori glucose adducts plays an important role in the development of diabetic renal disease, and does so independent of the direct influence of hyperglycemia. Indeed, the effects of glycated albumin on glomerular cell biology, which are observed with physiologic (5.5 mmol/L) glucose concentration, mimic those induced by high ambient glucose concentration.[12-15,20,37-41] Treatment of *db/db* mice with test compound, which normalized serum glycated albumin concentrations, significantly reduced albuminuria and collagen IV excretion and arrested the fall in creatinine clearance and rise in serum creatinine even though the animals remained markedly hyperglycemic. It is worth noting that the treatment protocol was initiated at a stage of elevated collagen IV excretion but before measurable compromise in filtration function, consistent with the notion advanced by the results of our previous study[7] that the former heralds the latter and marks an expanding glomerular matrix that has not yet overtly diminished the filtration surface area. In that study, we showed that a rise in collagen IV excretion coincided with an increased renal cortical expression of type IV collagen mRNA as well as with significant mesangial matrix expansion on glomerular morphometry.

The observation that urinary albumin excretion was significantly reduced in treated *db/db* mice is of interest in view of the report implicating increased glycated albumin in perm-selective changes in the glomerular basement membrane that underlie albuminuria early in the course of diabetes.[42] The lowering of urinary albumin in association with normalization of serum glycated albumin lends credence to this hypothesis. Further, the decrease in albumin excretion cannot be ascribed to inhibition of COX activity, which can modulate pathological renal hemodynamics contributory to diabetic nephropathy,[43] since the test compound has very low affinity for COX enzymes and has no effect on COX activity at the dose administered.

The biological properties of glycated albumin that contribute to renal pathology in diabetes are believed to be triggered through ligand receptor systems. Cells in culture selectively bind glycated albumin dose-dependently, saturably, and as a bifunctional ligand with a plasma membrane site that recognizes glucose and another site in plasmalemmal vesicles that recognizes albumin domains.[44-48] Calnexin serves as one of the receptor proteins for Amadori-modified glycated albumin.[49] Presumably, ligand receptor binding in renal glomerular cells is instrumental in triggering activation of the molecular mediators TGF-β1, PKC-β and ERK, but direct examination of the links between these events is required. Nevertheless, the results of the present study show that inhibiting the formation of glycated albumin with a compound that impedes the ability of glucose to condense with susceptible lysine amino groups, regardless of contemporaneous glycemic status, has the potential to beneficially influence the development of abnormal renal function in diabetes despite imperfect control of blood glucose concentrations.

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