Inhibiting albumin glycation attenuates dysregulation of VEGFR-1 and collagen IV subchain production and the development of renal insufficiency

Margo P. Cohen,1,2 Gregory T. Lautenslager,2 Elizabeth Hud,1,2 Elizabeth Shea,1,2 Amy Wang,3 Sheldon Chen,3 and Clyde W. Shearman2

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Cohen MP, Lautenslager GT, Hud E, Wang A, Chen S, Shearman CW. Inhibiting albumin glycation attenuates dysregulation of VEGFR-1 and collagen IV subchain production and the development of renal insufficiency. Am J Physiol Renal Physiol 292: F789–F795, 2007. First published October 3, 2006; doi:10.1152/ajprenal.00201.2006.—Glomerular cells in culture respond, in a euglycemic milieu, to albumin modified by Amadori glucose adducts (the principal serum glycate protein), with activation of protein kinase C-β1, increased expression of transforming growth factor (TGF)-β1, the TGF-β type II signaling receptor, and the extracellular matrix proteins α1(IV) collagen and fibronectin and with decreased production of the podocyte protein nephrin. Decreasing the burden of glycated albumin in diabetic db/db mice significantly reduces glomerular overexpression of TGF-β1 mRNA, restores glomerular nephrin immunofluorescence, and lessens proteinuria, mesangial expansion, renal extracellular matrix protein production, and increased glomerular vascular endothelial growth factor (VEGF) immunostaining. In the present study, db/db mice were treated with a small molecule, designated 23CPPA, that inhibits the nonenzymatic condensation of glucose with the albumin protein to evaluate whether inhibiting glycated albumin influences the production of VEGF receptors (VEGFRs) and type IV collagen subchains and ameliorates the development of renal insufficiency.

Renal levels of VEGF and VEGFR-1 proteins and serum creatinine concentrations were significantly higher and renal levels of α1(IV) collagen and nephrin proteins and endogenous creatinine clearance values were significantly lower in control diabetic than in age-matched nondiabetic (db/m) mice. These changes were significantly attenuated in db/db littermate mice treated with 23CPPA for nine consecutive weeks. The findings indicate that inhibiting excess nonenzymatic glycation of serum albumin improves renal molecular biology abnormalities and protects against the development of renal insufficiency in the db/db mouse.

GLOMERULAR CELLS IN CULTURE respond, in a euglycemic milieu, to albumin modified by Amadori glucose adducts with activation of protein kinase C-β1 and extracellular signal-regulated kinase, upregulation of the transforming growth factor (TGF)-β system, increased production of extracellular matrix proteins, and reduced expression of the podocyte protein nephrin (4, 6, 12, 13, 15, 19). The relevance of these observations to the pathogenesis of diabetic nephropathy in vivo is supported by results of animal studies, which have shown that treatment of diabetic db/db mice with a small molecule that inhibits the nonenzymatic glycation of serum albumin decreases the glomerular mRNA expression of TGF-β1, prevents depletion of glomerular nephrin (quantified by immunofluorescence), ameliorates mesangial expansion and mRNA overexpression of the extracellular matrix proteins α1(IV) collagen and fibronectin, and lessens the urinary excretion of albumin and type IV collagen (measured as mg/24 h and ng/24 h, respectively) (9, 14). This treatment has also been shown to reduce the increased glomerular immunostaining for vascular endothelial growth factor (VEGF) and renal VEGF protein content (measured by immunobassay) that was found in control db/db mice (9), thereby causally implicating elevated levels of glycated albumin associated with the diabetic state in the abnormal VEGF production found in diabetes.

In the present study, we treated db/db mice with the glycation inhibitor 23CPPA to evaluate whether reducing the burden of glycated albumin modulates expression of VEGF receptors (VEGFRs) and/or the dissociated expression of type IV collagen chains that may be stimulated by VEGFR-1 (7, 22, 26) and whether it influences the development of renal insufficiency, for which the universally accepted sine qua non is a rising serum creatinine. We report that inhibiting the formation of glycated albumin in vivo attenuates the increased renal VEGFR-1 levels and changes in renal α1(IV) collagen subchain production and significantly lessens the rise in serum creatinine in control db/db mice.

MATERIALS AND METHODS

Experimental animals. Male db/db mice and age-matched nondiabetic db/m mice (Jackson Laboratory, Bar Harbor, ME) were provided with food and water ad libitum. Blood was obtained from the retro-orbital sinus, and 24-h urine samples were collected for measurement of the analytes.

Treatment protocol. Diabetic db/db mice were divided into two groups of six animals each: one received 23CPPA, and the other served as the diabetic control. The synthesis, structure, and actions of 23CPPA (2-[3-chlorophenylamino]phenylacetic acid) have been described previously (14). The drug was administered by gavage at 5 mg/kg twice per day for nine consecutive weeks, commencing when the animals were ~9 wk old. Control diabetic and nondiabetic mice received saline gavage twice daily for the same period. The timing of initiation and duration of treatment were based on results of studies that have delineated the evolution of renal functional and pathological changes in this experimental model and the animal ages at which effects of intervention have been demonstrated in the animals (9–11, 14, 42). The dosage of 23CPPA, which significantly reduces serum glycated albumin concentrations without affecting hyperglycemia, corresponded with that employed in previous experiments (9, 14). At the conclusion of the experimental protocol, terminal blood and urine samples were collected, and the kidneys were harvested and snap frozen. The renal cortex was separated by gross dissection and then

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frozen. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

**Analytic methods.** Glucose was determined by the glucose oxidase method and creatinine by the picric acid colorimetric procedure (Sigma-Aldrich, St. Louis, MO). To ensure accurate creatinine measurement, samples were deproteinated with acid tungstate, and the alkaline picrate reagent was added after adsorption of the creatinine to Fuller’s earth. This protocol eliminates interference by confounding noncreatinine chromogens, which may falsely elevate serum creatinine concentrations (20), and yielded values in five random samples containing 0.25–0.50 mg/dl creatinine comparable to those obtained by HPLC analysis according to the method described by Dunn et al. (20). Urinary albumin and type IV collagen were measured by immunospecific ELISA according to the manufacturer’s instructions (Exocell, Philadelphia, PA).

Equal amounts of lysates were prepared by homogenization of the cortex in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% NP-40, proteinase inhibitor cocktail, and 0.5 mM PMSF], centrifuged at 10,000 g for collection of supernatants, and subjected to SDS-PAGE (3–8% gradient; NuPage precast gels, Invitrogen, Carlsbad, CA), and the wet samples were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% nonfat milk in Tris-buffered saline-0.1% Tween 20 and probed overnight at 4°C with primary antibody: anti-VEGF mouse monoclonal, anti-VEGFR-1 and anti-VEGFR-2 rabbit polyclonal (both from NeoMarkers/Lab Vision), anti-α5(IV) collagen (gift of Dr. Jean-François Beaulieu, University of Sherbrooke, Quebec, PQ, Canada), anti-nephrin (gift of Dr. Lawrence Holzman, University of Michigan, Ann Arbor, MI), and anti-α3(IV) collagen human polyclonal (gift of Dr. Michael Madaio, University of Pennsylvania, Philadelphia, PA). After they were washed in Tris-buffered saline-0.1% Tween 20, the membranes were probed with the corresponding secondary antibody conjugated to horseradish peroxidase: sheep anti-mouse or donkey anti-rabbit (both from Amersham Biosciences, Piscataway, NJ) or goat anti-human (Jackson ImmunoResearch, West Grove, PA). Chemiluminescence was developed with Super Signal West Pico substrate (Pierce Biotechnology, Rockford, IL). The membranes were reprobed with β-actin (Sigma, St. Louis, MO) to correct for small differences in loading and efficiency in electrophoretic transfer. Immunoreactive bands were recorded on autoradiography film and quantified with computer-assisted video densitometry.

**RESULTS**

As documented in Fig. 1, the animal data in the present experiments were comparable to those reported in previous studies using animals of the same species from the same vendor (9, 14). The diabetic *db/db* mice were obese, hyperglycemic, and nephromegalic compared with the nondiabetic *db/m* controls. Treatment with 23CPPA did not affect glycemic status but significantly reduced kidney weights in the diabetic mice. Elevated serum concentrations of glycated albumin associated with diabetes were significantly decreased in the 23CPPA-treated animals, confirming the ability of the drug to impede the nonenzymatic condensation of glucose with free amino groups in the albumin protein, despite prevailing hyperglycemia. Urinary excretion of albumin, normalized to urinary creatinine, was elevated in the diabetic compared with the nondiabetic control mice and was significantly lower in the 23CPPA-treated animals. Persistence of an increased excretion of albumin in the diabetic compared with the nondiabetic...
control mice is consistent with the continued marked hyperglycemia in all the db/db mice. Analogous changes in urinary type IV collagen were observed in nondiabetic, diabetic control, and diabetic 23CPPA-treated mice.

Western blot analysis of VEGF and VEGFRs showed increased intensity of VEGF and VEGFR-1, but not VEGFR-2, in the renal cortex from the diabetic compared with the nondiabetic control mice and diminished intensities of VEGF and VEGFR-1, but no change in VEGFR-2, bands in samples from the 23CPPA-treated diabetic mice compared with the diabetic control mice. The relative ratio of VEGF or VEGFR to β-actin band intensity confirmed increases in VEGF and VEGFR-1 in the db/db mice compared with the value in the db/m control mice (which was assigned an arbitrary value of 1.0) that were significantly reduced in the 23CPPA-treated db/db mice. No significant difference was seen in VEGFR-2 levels between the three experimental groups (Fig. 2).

Analysis by immunoblotting of collagen IV subchains in renal cortex by quantification of the immunoreactive bands, calculated as the relative ratio of α3(IV) collagen subchain to β-actin band intensity, indicated a significant reduction of α3(IV) collagen in the db/db mice compared with the db/m controls and partial prevention of this change in samples from the 23CPPA-treated db/db mice (Fig. 3). The mean values for α5(IV) collagen subchain-to-actin ratio were not significantly different in renal cortex from the three experimental animal groups (Fig. 3).

Nephrin protein levels assessed by immunoblotting, which were calculated as the relative ratio of nephrin to β-actin band intensity, were diminished in renal cortex from the db/db mice compared with db/m control mice (which were assigned an arbitrary value of 1). On the other hand, nephrin protein levels were significantly higher in samples from the 23CPPA-treated db/db than the db/db control mice (Fig. 4). These data, which are consistent with results from glomerular nephrin immunofluorescent studies (9), allowed a framework for interpreting differences in renal cortex levels of VEGFR and collagen subchain proteins between the experimental groups (see Discussion).

The primary end point for evaluating the effect of the drug on the development of renal insufficiency was the serum creatinine concentration. At the conclusion of the experimental period, creatinine values in the control diabetic mice were approximately twice those in the nondiabetic animals and were significantly less in the 23CPPA-treated diabetic mice (Fig. 5). Calculation of creatinine clearance as another marker for renal insufficiency and as a relative comparative index between groups showed a significant reduction in the db/m mice compared with the db/m controls and values not significantly different from the db/m controls in 23CPPA-treated diabetic mice (Fig. 5). The difference between the nondiabetic and diabetic controls was accentuated when clearance was normalized to body weight, which was significantly greater in the diabetic animals, and values in the diabetic mice receiving the test compound were significantly higher than those in the diabetic controls, although they remained significantly lower than in the nondiabetic controls: 0.15 ± 0.01, 0.08 ± 0.01, and 0.12 ± 0.02 ml·h⁻¹·g body wt⁻¹ in db/m, db/db, and 23CPPA-treated db/db mice.

**DISCUSSION**

The foregoing results indicate that inhibiting the nonenzymatic glycation of albumin in the db/db mouse affords significant protection against the development of the renal insufficiency that occurs in the untreated state. The primary evidence for a beneficial effect of the treatment regimen is the significantly lower serum creatinine, which in the diabetic controls was double that in the nondiabetic mice and would represent an ~50% reduction in glomerular filtration. The rise in creatinine in the db/db mice compared with the db/m mice observed in the
al. (21) indicated that creatinine clearance was increased in after streptozotocin-induced diabetes (25) in contrast to the decrease although clearance increased in mice with short-term (4-wk) renal insufficiency and as a relative index between experimental groups. The creatinine clearance values reported here are in the range of values that have been reported by others (25, 42), thereby demonstrating the utility of the former as a marker for creatinine clearance and that quantified by inulin clearance, differences between the extent of reduction in filtration measured by GFR by 15 wk of age in db/db mice and excellent correspondence between the extent of reduction in filtration measured by creatinine clearance and that quantified by inulin clearance, thereby demonstrating the utility of the former as a marker for renal insufficiency and as a relative index between experimental groups. The creatinine clearance values reported here are in the range of values that have been reported by others (25, 42), although clearance increased in mice with short-term (4-wk) streptozotocin-induced diabetes (25) in contrast to the decrease after ~12 wk of diabetes in the db/db mouse (42). Flyvbjerg et al. (21) indicated that creatinine clearance was increased in db/db compared with db/m mice at an age comparable to that of animals in the present experiments but did not give actual values for creatinine in urine and serum. If we assume comparable body weight, the creatinine clearance value in the db/m mouse in the report of Flyvbjerg et al. was less than half of that found in the present study, suggesting that the serum creatinine concentrations reported by Flyvbjerg et al. were higher than those reported here or by Dunn et al. (20). Also, the very low values for albumin excretion reported by Flyvbjerg et al. (1–5 μg/24 h) raise questions concerning technical issues.

The beneficial effect of 23CPPA on the development of renal insufficiency may relate to reduction of the glycated albumin-induced increase in TGF-β1-stimulated glomerular extracellular matrix production, which operates independently of TGF-β1-mediated changes induced by high glucose. Previous studies have shown that glycated albumin in a euglycemic milieu upregulates the TGF-β system and the expression of extracellular matrix proteins in glomerular cells (4, 6, 12, 13, 15) and that inhibiting the formation of Amadori-modified albumin with 23CPPA in mice reduces the increased glomerular TGF-β1 mRNA expression and renal concentration of translated TGF-β1 protein in hyperglycemic db/db controls (9, 14). Although it has not been directly examined, glycated albumin may sustain TGF-β1 overexpression independently of the renin-angiotensin system, which influences renal TGF-β in experimental diabetes (28, 38). Of interest in this regard are the findings of Benigni et al. (3), who reported that the concomitant administration of neutralizing monoclonal anti-TGF-β antibodies and the angiotensin-converting enzyme inhibitor lisinopril to uninephrectomized streptozotocin-diabetic rats afforded greater protection than either agent alone with respect to proteinuria, collagen III deposition in the renal interstitium, and histopathological changes of glomerulosclerosis, tubular damage, interstitial volume expansion, and infiltration of lymphocytes. However, these investigators found that anti-TGF-β monoclonal antibody alone did not modulate renal TGF-β expression, although others have reported that it does (17, 37), and that serum creatinine concentrations after treatment with anti-TGF-β antibody and lisinopril, alone or in combination, were identical. Ziyadeh et al. (42) reported that absolute levels of urinary albumin did not significantly differ in anti-TGF-β

Fig. 3. Western blots of collagen IV subchain immunoreactivity. Relative ratios of collagen IV subchain to β-actin in db/m controls were assigned an arbitrary value of 1.0. The αs(IV) and α5(IV) collagen immunoblots were performed on different gels, each of which was reprobed with β-actin. *P < 0.05 vs. db/m. *P < 0.05 vs. db/db.

Fig. 4. Western blot of nephrin in renal cortex probed with affinity-purified rabbit polyclonal antibody directed against the cytoplasmic domain of mouse nephrin and developed with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences) and chemiluminescent substrate. Nephrin band was observed at ~185 kDa under reducing conditions. *P < 0.05 vs. db/m. *P < 0.05 vs. db/db.
antibody-treated vs. control db/db diabetic mice but commented that the urinary excretion of albumin factored for creatinine clearance was slightly lower in the anti-TGF-β antibody-treated group, which is consistent with results in the present study.

Upregulation of renal VEGF and VEGFRs has been found in rodents with experimental diabetes, although an early increase in VEGFR-2 ~3 wk after induction of diabetes is followed by a decline (16, 27, 31, 36), which may explain the unchanged renal VEGFR-2 protein levels in the present study, which analyzed renal cortex from db/db mice that, at 18~19 wk of age, had been diabetic for ~14 wk. VEGF signaling has been implicated in the differential glomerular expression of collagen IV subchains (7, 8, 26), which encompass the α1(IV) and α2(IV) chains predominant in the mesangial matrix and the α3(IV) and α5(IV) chains in the glomerular basement membrane produced by endothelial cells and podocytes (39, 40). Mesangial α1(IV) and α2(IV) collagen are increased in human (2, 39) and experimental (14, 22) diabetes, and the increased renal α1(IV) expression in db/db mice is significantly lessened by reduction of the elevated levels of glycated albumin (14). In glomeruli of rats with membranous nephropathy, levels of mRNA encoding α3(IV) and α5(IV) subchains are elevated (32), and increased α3(IV) collagen mRNA has been reported in renal cortex from KKAy mice (22), suggesting that expression of minor collagen IV subchains is altered in diabetes or with podocyte injury. The present observations are consistent with dysregulation of collagen IV subchain production, although the direction of changes in α3 protein was different from that reported for α3(IV) collagen mRNA in human non-insulin-dependent diabetes (39) or in diabetic KKAy mice (22), a discrepancy that may in part be due to human vs. rodent diabetes or different mouse strains or to analysis of translated protein in the present study vs. mRNA. Hyperglycemia was relatively modest in the study of KKAy mice: the mean α3(IV) collagen mRNA levels (error bars not reported) declined after 16 wk of age, and the actual duration of diabetes was comparable to a younger (~12 wk) age than the age of the animals in the present experiments at the time of renal protein analysis. Nevertheless, the amelioration of the altered renal level of α3(IV) collagen in 23CPPA-treated animals implicates glycated albumin in its genesis. The cellular source of this change remains speculative, but it is assumed that glycated albumin-induced VEGF overactivity substantially contributes to the decrease in α3(IV) collagen. VEGFR-1 is present in mesangial, endothelial, and tubular cells and in podocytes, glomerular endothelial and epithelial cells express α3(IV) and α5(IV) collagen, and, in theory, any of these renal cell types may variably contribute to altered levels of collagen IV subchains in preparations of renal cortex. In contrast, the podocyte is the exclusive source of nephrin, and changes in renal cortical levels of this protein uniquely reflect podocyte components, which express VEGF-1 but may not express VEGF-2 (7). Thus the reduced nephrin and at least part of the increased VEGFR-1 could be considered glomerular in origin, whereas nonglomerular sources likely contributed to the reduced α3(IV) collagen content. Identification of the contributions to the overall α3(IV) collagen level by potential sources in the kidney requires further localization analysis, including immunohistochemistry. Glycated albumin has been shown to decrease nephrin expression in vitro (19) and in vivo, the latter supported by the observation that administration of a glycation inhibitor prevents nephrin depletion assessed by glomerular immunofluorescence (9) and, in this study, by immunoblotting of renal cortex protein.

Anti-VEGF therapy reduces albuminuria in diabetic rodents (18, 21), and results of recent studies suggest additional roles for VEGF signaling through the VEGFR-1 tyrosine kinase in diabetic nephropathy. VEGFR-1 activation has angiogenic and antiangiogenic effects, depending on specific cells or tissues and in relation to the stage of injury (4, 5, 23, 34). Thus increased VEGFR-1, stimulated and sustained by diabetes-associated factors such as glycated albumin, may operate independently of autoregulatory constraints and exert an antiangiogenic effect that negatively influences remodeling events in the glomerular capillary network that are involved in the regression of glomerulosclerosis and repair of renal tissue (1, 35).

In conclusion, we have provided evidence that the decline in glomerular filtration function, increased renal VEGFR-1, and dysregulation of collagen IV subchain production in diabetic db/db mice are attenuated by administration of a compound that impedes the accelerated Amadori modification of albumin, which is associated with the diabetic state. The findings implicate glycated albumin in the genesis of these changes, which are likely related to alterations in TGF-β1 expression, VEGF, and VEGFR-1 production, and podocyte damage.

Diabetic nephropathy is the leading cause of end-stage renal disease in the United States (29, 33), prompting extensive research into its pathogenesis and an appreciation of the need for innovative approaches to its prevention and treatment (24). Our demonstration that administration of a compound that inhibits the nonenzymatic glycation of albumin significantly reduces the loss of filtration function in the diabetic db/db mouse holds promise in this regard.
REFERENCES


