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Association between endothelin-1 and collagen deposition in *db*/*db* diabetic mouse kidneys

Rangnath Mishra [a], Steven N. Emancipator [b], Timothy S. Kern [c], Michael S. Simonson [a,*]

[a] *Department of Medicine, Division of Nephrology and Hypertension, School of Medicine, Case Western Reserve University, University Hospitals of Cleveland, Cleveland, OH 44106, USA*

[b] *Department of Medicine, Division of Pathology, School of Medicine, Case Western Reserve University, University Hospitals of Cleveland, Cleveland, OH 44106, USA*

[c] *Department of Medicine, Division of Endocrinology, School of Medicine, Case Western Reserve University, University Hospitals of Cleveland, Cleveland, OH 44106, USA*

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Abstract

Endothelin-1 has been implicated in diabetic kidney injury, but there are few firm data establishing the temporal and spatial expression of kidney endothelin-1 in diabetes. We performed an immunohistochemical and histopathological analysis to determine endothelin-1 peptide expression in the kidneys of diabetic *db*/*db* mice and non-diabetic *db*/*m* controls. Diabetic mice were studied at 8 weeks, before histological damage is evident, and again at 16 weeks, when significant glomerular injury has occurred. Urinary endothelin-1 was 6.2- and 3.6-fold higher in 8- and 16-week diabetic mice compared to age-matched controls ($P < 0.01$ *db*/*db* vs. *db*/*m*). Compared to non-diabetic kidneys, immunoreactive endothelin-1 was first elevated 2.5-fold ($P = 0.02$) in the tubulointerstitial compartment at 8-week and remained high (3.8-fold, $P < 0.01$) at 16 weeks. In contrast, glomerular endothelin-1 was elevated 3.2-fold ($P = 0.03$) only in 16-week diabetic mice. Glomerular and tubulointerstitial endothelin-1 were unchanged in 8- and 16-week non-diabetic mice. Elevated endothelin-1 in diabetic mice associated temporally and spatially with collagen deposition, especially in the tubulointerstitial compartment. The localization of kidney endothelin-1 is consistent with a role for this peptide in renal fibrogenesis. These results also highlight the potential role of ET-1 in the pathogenesis of early tubulointerstitial changes in diabetes.

Keywords: Diabetes; Endothelin-1; Fibrosis; Kidney; Diabetic nephropathy

Endothelin-1 (ET-1) might contribute to kidney injury in diabetes by raising blood pressure, intra-glomerular pressure, or tubular Na^+ reabsorption. Patients with type 1 and type 2 diabetes have higher plasma ET-1 than non-diabetics [1–5]. A robust increase in urinary ET-1 excretion, which likely reflects renal ET-1 synthesis, has been observed in streptozotocin-diabetic rats [6]. Renal ET-1 is elevated in experimental models of diabetes [6–10]. ET-1 receptor antagonists slow disease progression in rodent models of diabetic kidney injury [11–18], although one study in streptozotocin-injected (mRen-2)27 rats did not support a role for ET-1 [19]. Taken together, these studies suggest that elevated ET-1 in diabetes is detrimental to renal function.

Previous studies have not established the temporal and spatial expression of ET-1 in diabetic kidney injury. The lack of data about ET-1 expression in specific nephron segments precludes a detailed model for ET-1 in the pathogenesis of diabetic kidney injury. To address mechanisms by which ET-1 might contribute to the pathogenesis of renal injury in diabetes, we localized ET-1 expression in the early stages of renal injury in the *db*/*db* mouse model of type 2 diabetes [20,21]. And, we correlated ET-1 localization with renal histopathology. Our results support a model in which

* Corresponding author. Fax: +1 216 368 1249.
E-mail address: mss5@po.cwru.edu (M.S. Simonson).

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glomerular and tubular expression of ET-1 contributes to mesangial matrix expansion and to glomerular and peritubular fibrosis.

Methods

Metabolic, renal, and histopathological parameters in db/db mice. Male *db/db* (BKS.Cg-*m*+/+*Lepr*^{db}) and *db/m* mice were used for all studies (Jackson Laboratories, Bar Harbor, ME) as previously described by our laboratory [10]. Manipulation and experiments were done in a barrier facility with gowning. To measure 24-h albumin and ET-1 excretion, mice were placed in individual diuresis cages (Nalgene, Rochester, NY) with access to water but not food for 24 h. Urinary albumin and ET-1 concentrations were measured with an ELISA specific for mouse albumin (Exocell, Philadelphia, PA) and for ET-1 (R&D Systems, Minneapolis, MN). Following 24-h ad libitum access to food and water, the mice were anesthetized and serum was collected and frozen at −40 °C. Blood glucose was monitored using an Accu-Chek meter (Roche Diagnostics, Indianapolis, IN), and the percent GHb was measured after separation on a cation exchange resin (Sigma, St. Louis, MO). Plasma creatinine was measured using a kinetic micro-Jaffe reaction (10 μl sample) to reduce interference by glucose [21]. Serum cholesterol, triglycerides, and free fatty acids were measured enzymatically in a microplate format using kits from Wako Chemicals (Richmond, VA). Morphometric analysis of renal structure used established techniques [22,23] as previously described [10]. The mesangial matrix fraction, which is highly correlated with renal function in humans with diabetic nephropathy [22], was analyzed blindly in coded sections stained with periodic acid/Schiff (PAS) reagent [24]. Forty glomeruli from each of three kidney sections for each mouse were imaged. Fibrosis in glomeruli and in the peritubular compartment was assessed using Masson's method for trichrome staining. Trichrome-stainable material was analyzed by image analysis as described below for ET-1. All animal procedures were performed according to the guidelines of the Animal Review Committee of Case Western Reserve University.

Immunohistochemical analysis of ET-1 in db/db kidneys. Two mm thin sections of kidney were immediately fixed in 10% buffered formalin at 4 °C for 24h before embedding in paraffin. Five micrometer thick sections were deparaffinized, washed with TBS-T (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), and incubated with 1.5% H_2O_2 in methanol. Sections were incubated overnight with a rabbit anti-ET-1 antibody (1:100, Chemicon, Temecula, CA) in a humidified chamber at 4 °C. For preabsorption studies the antiserum was incubated with 10 μg/ml ET-1 competitor peptide. Non-immune antibodies at the same concentration of IgG were also used as controls for specificity. Antibodies were localized with the ABC technique (Vector Labs, Burlingame, CA) and 3,3′-diaminobenzidine substrate solution. Sections were then dehydrated in ethanol, cleared in xylene, and mounted without counterstaining. Images were acquired with a SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI) using the same exposure to facilitate quantitative, blinded analysis of coded sections in NIH Image. The image was segmented for positive immunoreactivity in NIH Image by thresholding pixels below a background value. NIH Image then measured the remaining black pixels as a percentage of the total image area. For measurements of glomerular immunoreactivity, only the area in the glomerular tuft was analyzed. Twenty glomeruli from each mouse was analyzed, and positive pixels were divided by the total pixels in each glomerulus (i.e. (positive pixels/total pixels in glomerulus) × 100). The percentage of positive pixels in 10 different cortical or medullary areas (chosen at random and excluding glomeruli) for each mouse was analyzed and expressed as a percentage of pixels in that area. Details of the method can be found on the NIH Image tutorial website, http://rsb.info.nih.gov/nih-image/.

Statistical analysis. Data are means ± SD from at least $n = 4$ independent mice per group. Statistical significance was calculated by unpaired Student's t test or by ANOVA with Bonferroni multiple correction as appropriate.

Results

Diabetes and glomerular injury in db/db mice

We measured ET-1 in 8- and 16-week *db/db* mice because 8-week-old mice have proteinuria but few histological changes, but 16-week-old mice show renal histopathological features of diabetic nephropathy including mesangial matrix expansion, thickening of the basement membrane, progressive proteinuria, and a decline in GFR [10,21,23–27]. As expected, diabetic mice in our study had higher blood glucose, glycosylated hemoglobin (GHb), and albuminuria (Table 1). Typical of type 2 diabetes, *db/db* mice had markedly elevated serum cholesterol, triglycerides, and free fatty acids compared to age-matched *db/m* controls (Table 1). In 8- week-old glomeruli, the capillary tuft appeared expanded with patent capillary loops. The glomerular basement membranes were thin and delicate in 8-week *db/m* and *db/db* glomeruli (Figs. 1A and B). In contrast,16-week-old glomeruli evinced mesangial matrix expansion and collapse of capillary loops (Figs. 1C and D). Although most tubules appeared normal, at irregular intervals within the cortex of 16-week *db/db* mice, all cells in some individual tubular cross-sections showed extensive vacuolization (Fig. 1D). These highly vacuolated tubules varied from mouse to mouse, but these changes

Table 1
Metabolic and renal parameters in 8- and 16-week *db/db* mice[a,b]

Parameter	Mice			
	8-week *db/m*	16-week *db/m*	8-week *db/db*	16-week *db/db*
Body wt (g)	21.3 ± 0.6	22.9 ± 0.8	22.6 ± 0.5	43.0 ± 2.4*,**
Blood glucose (mg/dl)	69.5 ± 7.1	73.4 ± 4.8	375.2 ± 6.8*	349.9 ± 13.7*
GHb (%)	3.2 ± 0.2	3.9 ± 0.7	5.8 ± 0.7*	11.3 ± 0.6*,**
Plasma creatinine (mg/dl)	0.2 ± 0.2	0.3 ± 0.3	0.4 ± 0.3	0.8 ± 0.3*,**
Albuminuria (ug/24 h)	2.9 ± 1.3	6.8 ± 2.4	33.5 ± 17.0*	81.2 ± 32.6*,**
Mesangial matrix fraction (%)	3.6 ± 0.8	4.8 ± 0.5	4.3 ± 0.9	15.2 ± 2.5*,**
Cholesterol (mg/dl)	108 ± 12	106 ± 1	151 ± 13*	159 ± 20*
Triglycerides (mg/dl)	113 ± 14	108 ± 11	234 ± 17*	338 ± 45*,**
Free fatty acids (μmol/L)	1304 ± 185	1242 ± 76	3544 ± 649*	3193 ± 351*

[a] Data are means ± SD for 4 *db/m* and 4 *db/db* mice at each time.
[b] $^{*}P <$ at least 0.05 vs. corresponding *db/m*; $^{**}P <$ at least 0.05 vs. 8 week *db/db*.

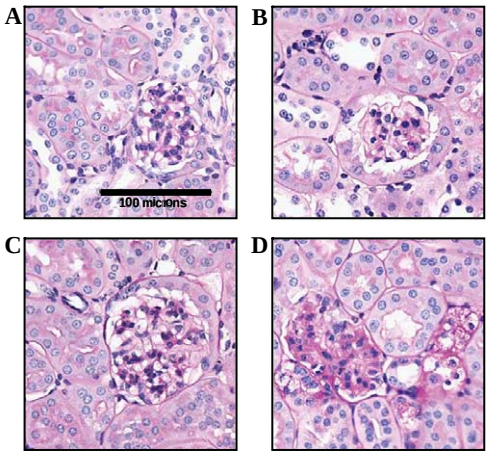

Fig. 1. Sixteen week *db*/*db* kidneys elaborate mesangial matrix expansion and tubular vacuolization. Kidney sections from (A) 8-week *db*/*m*, (B) 8-week *db*/*db*, (C) 16-week *db*/*m*, and (D) 16-week *db*/*db* mice were stained by PAS. Representative photomicrographs from four different mice per group are presented. The histopathological changes in (D) were observed in all four 16-week *db*/*db* mice studied.

were never observed in age-matched controls or in younger 8-week *db*/*db* mice. There was conspicuous PAS metachromasia in the cytoplasm of these tubular cells, and many nuclei appeared pyknotic. These tubules, by their distribution and lack of brush border, appear to be distal tubules. Collectively, the tubular changes are highly suggestive of severe cell stress. To our knowledge these tubular changes have not been previously reported.

Urinary ET-1 excretion and localization of ET-1 peptide in db/db kidneys

Urinary ET-1 excretion was 6.2- and 3.6-fold higher in 8- and 16-week diabetic mice compared to the corresponding non-diabetic controls (Fig. 2). Urinary excretion of ET-1 precedes significant renal histopathology (Fig. 1 and Table 1). We previously reported that kidney preproET-1 mRNA is elevated in 8- and 16-week *db*/*db* mice [10], but the specific nephron segments expressing ET-1 were not determined. Compared to non-diabetic kidneys, immunoreactive ET-1 in diabetic kidneys was first elevated 2.5-fold in the tubulointerstitial compartment at 8 weeks and remained high (3.8-fold) at 16 weeks (Figs. 3A, 4A and B). In contrast, glomerular ET-1-1 was elevated 3.2-fold only in 16-week diabetic mice (Figs. 3, 4B). Antigen unmasking at pH 6.0 or 8.0 did not make better ET-1 localization in non-diabetic or diabetic kidneys (data not shown) Preabsorption of the antibody with exogenous ET-1 confirmed that the immunoreactivity was specific for ET-1 (Fig. 4C). Higher magnification illustrates the tubular localization in 16-week diabetic kidneys (Fig. 4D). Glomerular and tubulointerstitial ET-1 were unchanged in 8- and 16-week non-diabetic mice (Figs. 3, 4A). The renal medulla is a rich source of ET-1 peptide [28,29], and we confirmed that the medulla of 16-week *db*/*m* kidneys had abundant ET-1 immunoreactivity (Fig. 4E). But, medullary tubular ET-1 staining was unaffected by diabetes (Fig. 4F).

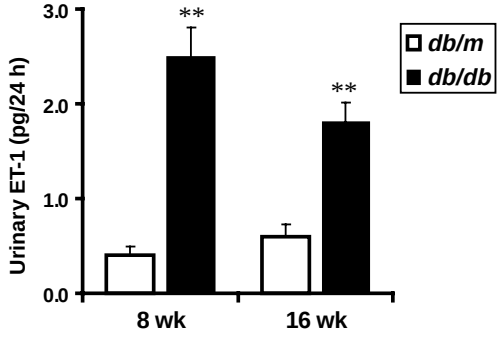

Fig. 2. Urinary ET-1 excretion is elevated in *db*/*db* mice compared with age-matched *db*/*m* controls. Urine collections (24 h) from four different mice for each group were analyzed for ET-1 excretion by ELISA. $^{**}P < 0.01$ vs. age-matched *db*/*m* mice.

Association of ET-1 immunoreactivity with collagen deposition in db/db kidneys

ET-1 immunoreactivity was associated with glomerular and tubulointerstitial histopathology in *db*/*db* kidneys. In diabetic mice, mesangial matrix expansion was temporally and spatially associated with glomerular immunoreactivity for ET-1 (Fig. 5A). ET-1 has been postulated to cause renal injury in diabetes and in other diseases by raising interstitial collagen and fibrosis [30–36]. We therefore used trichrome staining to localize fibrosis in *db*/*db* mice and to correlate

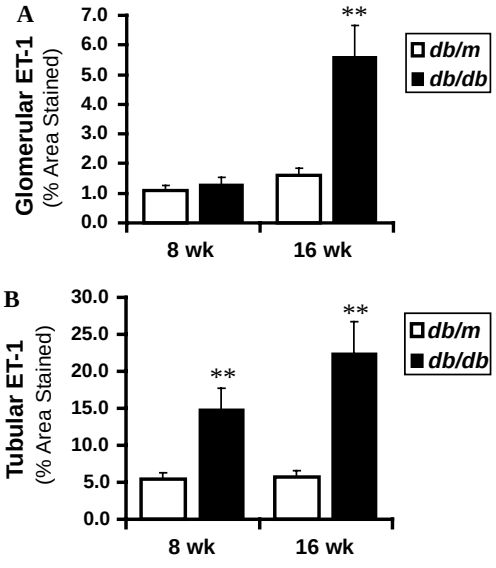

Fig. 3. Glomerular ET-1 peptide is elevated at 16 week but tubular ET-1 is increased at 8 and 16 weeks in *db*/*db* mice (A) Quantitative analysis of ET-1 immunohistochemical staining in glomeruli of 8- and 16-week *db*/*m* and *db*/*db* kidneys. $^{**}P < 0.01$ vs. age-matched controls. (B) ET-1 staining in tubules in the cortex of 8- and 16-week *db*/*m* and *db*/*db* kidneys. Results are expressed as a percentage of the area stained as described in Materials and methods.

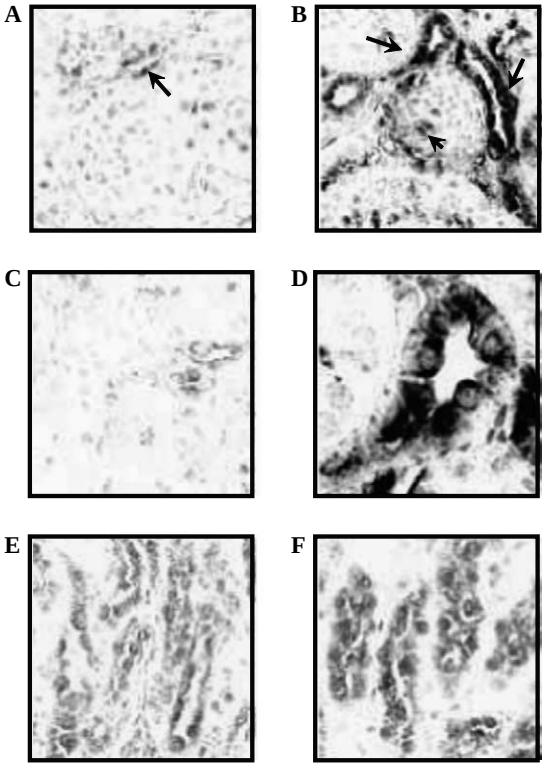

Fig. 4. ET-1 peptide is elevated in glomeruli and tubules in the cortex of *db*/*db* mice compared to non-diabetic *db*/*m* controls. ET-1 was localized by immunohistochemistry in sections from 16-week *db*/*m* or *db*/*db* mice as follows: (A) 16-week *db*/*m* cortex, (B) 16-week *db*/*db* cortex, (C) 16-week *db*/*db* cortex using antibody preadsorbed with exogenous ET-1 peptide, (D) enlargement of tubular ET-1 immunoreactivity from 16-week *db*/*db* kidney, (E) ET-1 in 16-week *db*/*m* tubules in medulla, and (F) ET-1 in 16-week *db*/*db* tubules in medulla. ET-1 immunoreactivity is representative of sections from four different mice per group. Original magnification is 400× in all panels except (D), which is 700×.

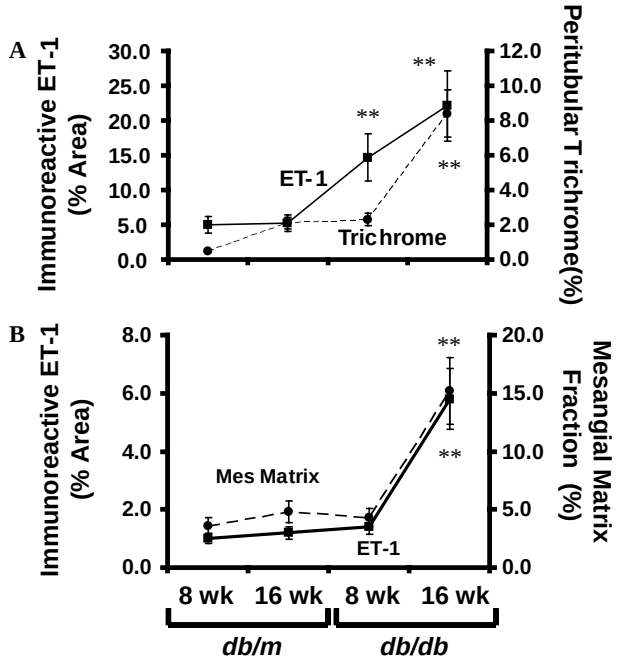

Fig. 5. Immunoreactive ET-1 associated with mesangial expansion and peritubular trichrome-staining. (A) Immunoreactive ET-1 in tubules in the kidney cortex are plotted with peritubular trichrome staining (i.e., collagen deposition) from the same sections in all four groups of mice. (B) The mesangial matrix fraction was calculated in 8- and 16-week *db*/*m* and *db*/*db* mice and is plotted with the corresponding immunoreactive ET-1 in glomeruli. $^{**}P < 0.01$ by ANOVA.

collagen deposition with renal ET-1 production. Trichrome-stainable material was not evident in 8-week *db*/*m* or *db*/*db* kidneys or in 16-week *db*/*m* kidneys (Figs. 5A, 6A). In contrast, abundant trichrome-stainable material was present in the peritubular regions of 16-week *db*/*db* kidneys in the cortex (Figs. 5A, 6B). Although interstitial fibrosis, as recognized by tracts of collagen that widen the intertubular distance, was uniformly absent in diabetic and control mice at 16 weeks, staining of tubular basement membrane with Masson's trichrome technique was prominent (Fig. 6B). Peritubular collagen deposition associated closely with immunoreactive ET-1 (Fig. 5A). We also observed significant trichrome staining in 16-week *db*/*db* glomeruli that was not observed in age-matched non-diabetic controls or in 8-week *db*/*m* or *db*/*db* kidneys (Fig. 6C). Thus, collagen deposition in glomeruli, which indicates significant glomerular injury, associates with glomerular production of ET-1.

Discussion

ET-1 immunoreactivity was temporally and spatially associated with mesangial matrix expansion and glomerular collagen deposition in *db*/*db* mice. Moreover, renal ET-1 associated with collagen deposition in peritubular locations in the cortex. These results support a model in which ET-1 contributes to glomerular and peritubular changes in diabetic kidney injury.

Consistent with our study, a previous report [9] documented elevated ET-1 in glomeruli and in tubulointerstitial cells of the SHR/N-cp rat, another rodent model of type 2 diabetes. There are at least two non-mutually exclusive mechanisms by which ET-1 could mediate glomerular and peritubular pathobiology. First, elevated glomerular ET-1 could increase capillary hydraulic pressure and reduce K_f (see [37] for review). Hemodynamically mediated glomerular injury is well documented in the kidney. Second, accumulating evidence suggests that ET-1 is an important mediator of fibroproliferative responses. Glomerular ET-1 is linked to a fibroproliferative response in the mesangium leading to cell growth and expansion of the mesangial matrix [35]. High intra-glomerular ET-1 has been implicated in structural damage to glomerular podocytes, leading to glomerulosclerosis and proteinuria [38]. Overexpression of ET-1 in transgenic mice causes mesangial remodeling and glomerulosclerosis [33,34]. Although ET-1 can act directly to raise collagen type I production in mesangial cells [39], it is also possible that ET-1 stimulates fibrosis by elevating expression of transforming growth factor β. In the studies cited above, the effects of ET-1 on glomerular cell phenotype occurred in the absence of hypertension [33] or well before hypertension or significant induction of

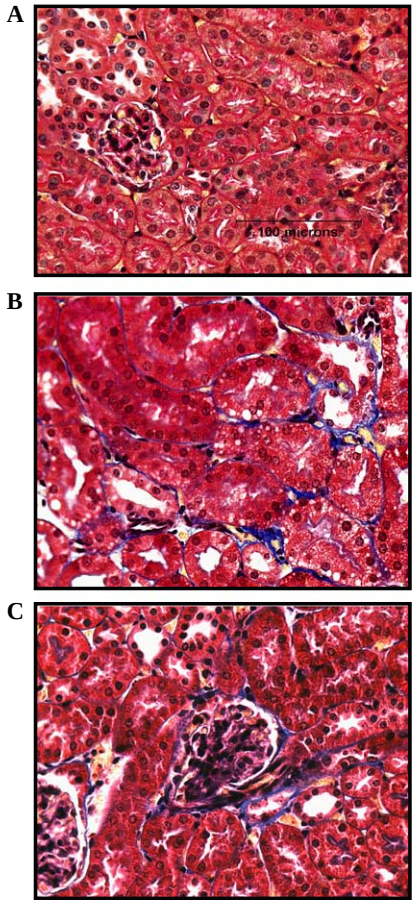

Fig. 6. Trichrome staining in 16-week *db*/*db* mice is elevated in the peritubular regions and in glomeruli. (A) Trichrome-stainable material was not apparent in the peritubular regions or in glomeruli from 16-week *db*/*m* kidneys. In contrast, trichrome staining was prominent in the peritubular compartment (B) and less so in glomeruli (C) from 16-week *db*/*db* kidneys. Photomicrographs are representative of trichrome staining in four mice per group, which is expressed quantitatively in Fig. 5. Original magnification is 400×.

fibrosis in the aorta or heart [34,36]. These results suggest that ET-1 could affect glomerular cell phenotype independent of its ability to increase blood pressure.

A noteworthy finding of our study is elevated ET-1 in the renal tubules of *db*/*db* mice. We found a rise in type I or type III (i.e., trichrome-stainable) collagen basement membrane collagen compared to the type IV collagen that is present only in normal basement membrane but does not stain with Masson's method. So there is a shift in the balance of collagen subtypes in the tubular basement membrane of *db*/*db* mice at 16 weeks that is not evident in age-matched controls. This finding is potentially important because it has been proposed that proteinuria leads to autocrine activation of tubular cells involving secretion of ET-1 toward the basolateral compartment [30,31,40–42]. This elevation in secreted ET-1 might then lead to peritubular fibrosis by several mechanisms and contribute to renal scarring [41]. In support of this hypothesis, ET-1 transgenic mice have tubulointerstitial fibrosis reminiscent of the histopathology of chronic kidney disease in humans [33,34].

In summary, ET-1 is spatially and temporally associated with expansion of the mesangial matrix and with collagen deposition in the glomerulus. ET-1 expression is also associated with peritubular deposition of collagen. Taken together, these results suggest a model in which elevated ET-1 in the diabetic kidney might drive glomerular and tubular remodeling.

Acknowledgments

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