

Blockade of Vascular Endothelial Growth Factor Signaling Ameliorates Diabetic Albuminuria in Mice

Sun Hee Sung,^{*†} Fuad N. Ziyadeh,^{*‡} Amy Wang,[§] Petr E. Pygay,[§] Yashpal S. Kanwar,^{||} and Sheldon Chen[§]

**Renal-Electrolyte and Hypertension Division, University of Pennsylvania, Philadelphia, Pennsylvania; †Department of Pathology, Ewha Women's University, Mok Dong Hospital, Seoul, Korea; ‡Faculty of Medicine, American University of Beirut, Beirut, Lebanon; and §Division of Nephrology/Hypertension and ||Department of Pathology, Northwestern University, Chicago, Illinois*

For investigation of how the vascular endothelial growth factor (VEGF) system participates in the pathogenesis of diabetic kidney disease, type 2 diabetic *db/db* and control *db/m* mice were treated intraperitoneally with vehicle or 2 mg/kg of a pan-VEGF receptor tyrosine kinase inhibitor, SU5416, twice a week for 8 wk. Efficacy of SU5416 treatment in the kidney was verified by the inhibition of VEGF receptor-1 phosphorylation. Glomerular VEGF immunostaining, normally increased in diabetes, was unaffected by SU5416. Plasma creatinine did not change with diabetes or SU5416 treatment. The primary end point of albuminuria increased approximately four-fold in the diabetic *db/db* mice but was significantly ameliorated by SU5416. Correlates of albuminuria were investigated. Diabetic glomerular basement membrane thickening was prevented in the SU5416-treated *db/db* mice, whereas mesangial matrix expansion remained unchanged by treatment. The density of open slit pores between podocyte foot processes was decreased in *db/db* diabetes but was partly increased toward normal by SU5416. Finally, nephrin protein by immunofluorescence was decreased in the *db/db* mice but was significantly restored by SU5416. Paradoxically, total nephrin protein by immunoblotting was increased in diabetes, pointing toward a possible dysregulation of nephrin trafficking. Diabetic albuminuria is partially a function of VEGF receptor signaling overactivity. VEGF signaling was found to affect a number of podocyte-driven manifestations such as GBM thickening, slit pore density, and nephrin quantity, all of which are associated with the extent of diabetic albuminuria. By impeding these pathophysiologic processes, VEGF receptor inhibition by SU5416 might become a useful adjunct to anti-albuminuria therapy in diabetic nephropathy.

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Microalbuminuria is considered the earliest clinical indicator of incipient diabetic nephropathy. However, albuminuria is more than just a marker; it is believed to be a promoter of progressive kidney disease as well (1). Albuminuria is exacerbated by many factors, including hypertension, hyperglycemia, angiotensin II, glycosylated proteins, reactive oxygen species, signaling pathways such as protein kinase C- β , and profibrotic growth factors. Regarding the last point, we previously did not find a salient effect of TGF- β antagonism on diabetic albuminuria (2), but a deeper understanding of the underlying pathogenesis will permit novel and effective therapies to be developed.

Vascular endothelial growth factor (VEGF) likely plays a role in diabetic albuminuria. VEGF was initially implicated in proteinuria by virtue of its property as a vascular permeability factor (3). In addition, VEGF is elevated in the diabetic kidney

This paper describing the potential role of VEGF in mediating diabetic albuminuria in mice is relevant to the review of the relationship between diabetic albuminuria and left ventricular hypertrophy in diabetes in man by Nobakhtbaghi et al. in this month's issue of CJASN (pp. 1187–1190).

(4), its urinary excretion is increased (5), many features of diabetes stimulate VEGF expression in cultured renal cells (6), and blockade of VEGF ligand partly corrects diabetic albuminuria (7,8). Several questions remain. Is albuminuria mediated by signaling through the VEGF receptors? What is the target cell(s) of VEGF action? Of the many renal parameters associated with albuminuria, which ones are potentially causative? A small-molecule inhibitor, SU5416 (9), that blocks all of the VEGF receptors -1, -2, and -3 at the level of the tyrosine kinase was administered to obese, spontaneously type 2 diabetic *db/db* mice and their nondiabetic *db/m* littermates.

Although the effect on albuminuria was the primary end point, several structural and functional correlates of albuminuria were also examined. Such factors included glomerular basement membrane (GBM) thickening, mesangial matrix expansion, slit pore density, and nephrin alterations. In terms of giving rise to these parameters, the role of VEGF signaling can be ascertained with the use of SU5416. Furthermore, if SU5416 does influence albuminuria, then the amelioration or worsen-

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F.N.Z. and A.W. contributed equally to this work.

Address correspondence to: Dr. Sheldon Chen, Northwestern University, Searle 10-475, 320 E. Superior Street, Chicago, IL 60611. Phone: 312-503-6880; Fax: 312-503-0622; E-mail: sheldon-chen@northwestern.edu

ing would provide a more stringent test of the association between albuminuria and the parameter. Not only would GBM thickness, for example, have to increase with albuminuria in untreated diabetes, but it should also decrease with successful amelioration by SU5416. Conversely, a “dissociation” may militate against that parameter’s being a causative factor in albuminuria.

In our study, GBM thickening and mesangial matrix expansion, known to be correlated with diabetic albuminuria (10,11), were affected in divergent ways by SU5416. Another structural association with albuminuria is a decrease in the linear density of open slit pores between podocytes, which probably arises when the foot processes broaden out in diabetes (12,13). Slit pore density was found to revert partly in response to SU5416. Besides the histomorphologic changes coincident with albuminuria, a recently discovered functional association was studied. Nephtrin is said to decrease in diabetes and to correlate inversely with the rise in albuminuria (14,15). This was confirmed in our study by nephtrin immunofluorescence, and the decrease in nephtrin was salvaged partly by SU5416, but immunoblotting revealed an unexpected increase of nephtrin in diabetes. The seemingly contradictory result hints at further complexities in the regulation of nephtrin in diabetic kidney disease.

Materials and Methods

Experimental Animals

All protocols that used rodents were approved by the Institutional Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male diabetic *db/db* mice (C57BLKS/J-*lepr^{db}/lepr^{db}*) and male nondiabetic *db/m* mice (C57BLKS/J-*lepr^{db}/+*) were purchased at 6 to 7 wk of age from Jackson Laboratory (Bar Harbor, ME). By 8 wk of age, all *db/db* mice were hyperglycemic. Diabetic mice were randomly divided into two groups of 10: One that received SU5416 and one that received vehicle (DMSO). Similarly, the nondiabetic mice were divided into two groups of 12 and treated with either SU5416 or DMSO.

Treatment Protocol

All animals were provided food and water *ad libitum*. Preliminary studies included an 18-h urine collection in a metabolic cage. At 8 wk of age, all groups of mice had similar urine albumin excretion rates (data not shown). For the *db/m* and *db/db* mice that were randomized to receive anti-VEGF receptor treatment, 2 mg/kg SU5416 dissolved in DMSO was injected intraperitoneally twice per week. This dosing regimen was based on the IC_{50} of SU5416 (16) and the volume of distribution of SU5416 in a mouse (17). Control animals received the equivalent volume of DMSO twice per week, and the experiment was continued for 8 wk. The *db/db* mice did not require supplemental insulin.

At the end of the experimental period, the mice underwent a final 18-h urine collection. Blood was obtained from the retro-orbital sinus during terminal anesthesia with isoflurane. The mice were killed by cervical dislocation, followed by the harvesting of the heart and two kidneys.

Analytical and Immunoassay Procedures

Plasma was submitted to Thomas Jefferson University (Philadelphia, PA) for an accurate measurement of creatinine concentration by HPLC (18). The plasma concentration of glucose was measured by the glucose oxidase method using an automated analyzer, the YSI 2300 STAT Plus (YSI Inc., Yellow Springs, OH). Urinary albumin concentrations were

determined by the Albuwell M kit (Exocell, Philadelphia, PA), an indirect ELISA. Urinary creatinine concentrations were assayed by the Creatinine Companion kit (Exocell). These urinary data were used to calculate the albumin excretion rate (AER) per day and per urinary creatinine.

Immunofluorescence and Immunohistochemistry

Kidneys that were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) were frozen gently in liquid nitrogen and cut on a cryostat microtome into 4- μ m sections that were affixed to microscope slides. For nephtrin immunofluorescence, sections were incubated overnight with affinity-purified rabbit polyclonal antibody directed against the cytoplasmic domain of nephtrin (gift of Dr. L. Holzman, University of Michigan, Ann Arbor, MI) at a 1:100 dilution. Secondary antibody, a Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA), was applied at a 1:1000 dilution, and immunofluorescence photomicrographs were obtained at a $\times 600$ magnification for a 100-ms exposure time.

VEGF immunohistochemistry was performed on frozen kidney sections. Endogenous peroxidases were quenched with 2.25% H_2O_2 , and sections were blocked with avidin/biotin (Vector Laboratory, Burlingame, CA). The primary antibody was rabbit anti-VEGF (Ab-1; Lab Vision, Fremont, CA) at a 1:100 dilution, followed by biotinylated anti-rabbit secondary antibody (1:1000 dilution) and avidin-conjugated horseradish peroxidase. Signal was developed with diaminobenzidine, and the sections were counterstained with Gill’s #2 hematoxylin.

For both nephtrin immunofluorescence and VEGF immunohistochemistry, nonspecific staining was assessed by omission of the primary antibody. Photomicrographs were read by a pathologist who was unaware of the experimental group from which they were derived. Intensity of fluorescence or peroxidase staining was quantified with the IPLab software (Scanalytics, Fairfax, VA).

Renal Morphometrics

Kidneys were fixed in neutral buffered formalin (10%), embedded in paraffin, sectioned at 5 μ m, and stained with periodic acid-Schiff (PAS). Twenty glomeruli were randomly selected from each mouse, and the extent of mesangial extracellular matrix was identified by PAS-positive material in the mesangium. Glomeruli from the outer and middle thirds of the renal cortex were selected for area measurements, done with the aid of Image-Pro Plus 3.0 (Media Cybernetics, Silver Spring, MD). Care was taken to exclude juxtamedullary glomeruli.

For ultrastructural evaluation, kidney tissue was fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, imbued with uranyl acetate, and embedded in epoxy resin (Epon). The specimen was thin-sectioned and examined under a JEOL transmission electron microscope. Electron micrographs of five to 10 glomeruli per kidney were randomly taken at both $\times 1500$ and $\times 30,000$ for each mouse. At the lower magnification, mesangial matrix was readily discernible, and its extent was measured as a percentage of the glomerular tuft area, with the aid of Image-Pro Plus software. At the higher magnification, GBM thicknesses were obtained from measurements at three different sites of cross-sectioning, with the aid of Image-Pro Plus. Tangentially sectioned GBM was excluded from the analysis. Photomicrographs of the GBM were also analyzed for the density of open and “tight” slit pores between the podocyte foot processes, according to published methods (19). The numbers of each type of slit pore were counted and divided by the GBM length (mm) to arrive at the linear density.

Western Immunoblotting

The kidney cortex was homogenized in RIPA lysis buffer, supplemented with protease inhibitors and sodium orthovanadate, and spun

Table 1. Baseline characteristics of the *db/m* and *db/db* mice after 8 wk of treatment with SU5416 or vehicle

Mice	Body Weight (g)	Total Kidney Weight (g)	Heart Weight (g)	Plasma Glucose (mg/dl)
<i>db/m</i> control (<i>n</i> = 12)	29.7 ± 0.3	0.41 ± 0.01	0.15 ± 0.00	143 ± 13
<i>db/m</i> SU5416 (<i>n</i> = 12)	27.9 ± 0.4	0.40 ± 0.00	0.15 ± 0.01	152 ± 9
<i>db/db</i> control (<i>n</i> = 10)	46.3 ± 1.7 ^a	0.43 ± 0.01 ^a	0.13 ± 0.00	506 ± 39 ^a
<i>db/db</i> SU5416 (<i>n</i> = 10)	48.4 ± 8.0 ^a	0.40 ± 0.03 ^b	0.15 ± 0.03	416 ± 41 ^a

^a*P* < 0.05 versus nondiabetic *db/m* control.

^b*P* < 0.05 versus diabetic *db/db* control.

at 14,000 × *g* to pellet the nuclei and large cellular fragments. The supernatant protein concentrations were measured by the Lowry assay (Bio-Rad, Hercules, CA) and equalized with the addition of Laemmli buffer, before SDS-based electrophoresis through a 3 to 8% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA). After electrical wet transfer of the proteins to a nitrocellulose membrane, phospho-VEGF receptor-1 (phospho-VEGFR-1) or total VEGFR-1 or nephrin or β-actin was probed with the appropriate primary antibody: Rabbit anti-phospho-specific VEGFR-1 (Calbiochem), rabbit anti-VEGFR-1 (Lab Vision), rabbit anti-nephrin (gift of Dr. Holzman), and mouse anti-β-actin (Sigma, St. Louis, MO). After incubation with horseradish peroxidase-conjugated secondary antibody anti-rabbit or anti-mouse IgG (both GE Healthcare, Piscataway, NJ), the chemiluminescent reaction was developed with SuperSignal West Pico (Pierce, Rockford, IL). Computer-assisted densitometry (ImageJ) was used to quantify the bands that were captured on radiographic film, and after “normalization,” the result for each protein among the four groups of mice was graphed as a percentage of the control.

Statistical Analyses

Table and graphical data are displayed as the mean ± SEM for the number of animals indicated in each table or figure. When two independent sets of data were compared (*e.g.*, control versus diabetes), the unpaired *t* test was used. *P* < 0.05 was considered statistically significant.

Results

Characteristics of the *db/m* and *db/db* Mice Treated with SU5416

After the 8-wk experimental period, the type 2 diabetic *db/db* mice were more obese than the *db/m* mice, regardless of whether they were treated with SU5416. Treatment with SU5416 did not affect body weight (Table 1). Total kidney weight, conversely, was significantly increased by diabetes, perhaps indicative of renal hypertrophy, and this effect was prevented by SU5416 (Table 1). Heart weights were similar between nondiabetic *db/m* and diabetic *db/db* mice and between vehicle-treated and SU5416-treated mice (Table 1). Glycemic control was poor in the diabetic *db/db* mice, as expected, and plasma glucose was not affected by SU5416 (Table 1).

Renal Function by Plasma Creatinine

Plasma creatinine concentrations were measured by HPLC to avoid the error that is introduced when mouse plasma creatinines are measured by the Jaffé method (18). Control *db/m* mice had a mean plasma creatinine of 0.093 ± 0.007 mg/dl, which was not significantly different from the mean creatinine value

of 0.082 ± 0.004 mg/dl in the *db/m* mice that were treated with SU5416. Diabetes in the *db/db* mice resulted in a slightly lower mean plasma creatinine of 0.085 ± 0.004 mg/dl, perhaps indicative of hyperfiltration (8), although the difference was not

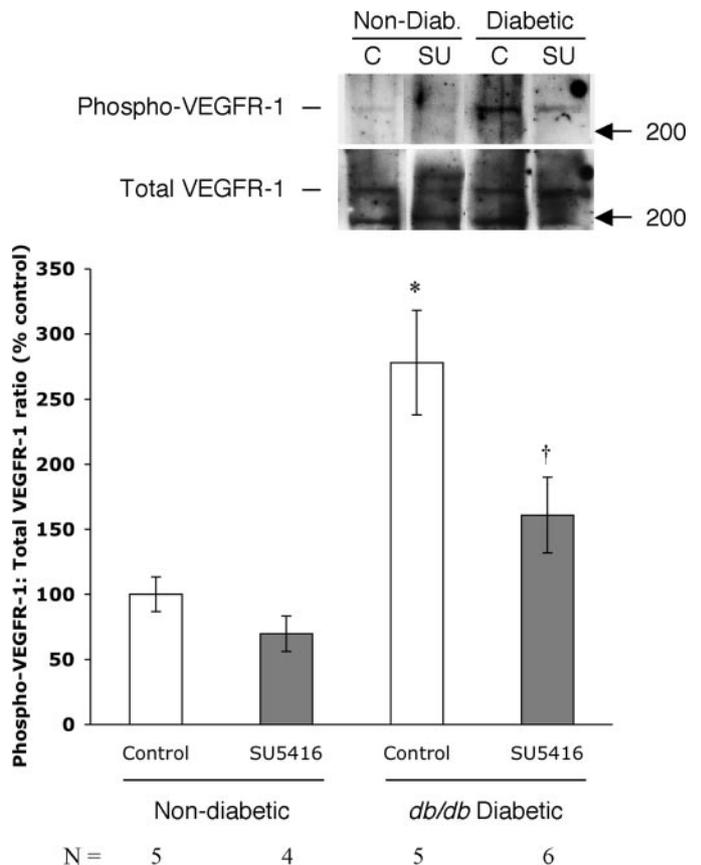


Figure 1. Vascular endothelial growth factor receptor (VEGFR) signaling is activated in *db/db* mice and inhibited by SU5416. Immunoblots of kidney lysates were probed with an anti-phospho-VEGFR-1 antibody (EMD Biosciences) and then with an anti-VEGFR-1 antibody (Lab Vision). The densitometric ratio of phosphorylated VEGFR-1 to total VEGFR-1 is one indicator of VEGF signaling activity, which was increased in diabetic *db/db* mice versus nondiabetic control (C) mice (**P* < 0.01). The autophosphorylation of VEGFR-1 was inhibited successfully by SU5416 (SU) treatment (†*P* = 0.02 versus diabetic control). Representative Western blots of phospho-VEGFR-1 and total VEGFR-1 are shown (bands juxtaposed from the same chemiluminescence film).

statistically significant. SU5416 treatment in the *db/db* mice did not affect the mean creatinine of 0.097 ± 0.009 mg/dl.

VEGF Signaling Activity

To confirm that the dosage schedule of SU5416 was effective in the treated mice, we checked for the successful inhibition of VEGFR-1 activation in the kidney. On the basis of Western blotting, VEGFR-1 was activated in the *db/db* mouse, evidenced by an increase in the ratio of phosphorylated VEGFR-1 to total VEGFR-1, compared with control (Figure 1). However, the elevated ratio of phospho-VEGFR-1:total VEGFR-1 significantly dropped in the *db/db* mice that were administered SU5416 (Figure 1). In contrast, SU5416 did not significantly alter the baseline phospho-VEGFR-1:total VEGFR-1 ratio in the *db/m* mice.

VEGF Immunostaining

The amount of immunodetectable VEGF was quantified by histochemistry. Spontaneous diabetes in the *db/db* mice increased the level of VEGF in the glomeruli. When measured by its intensity of staining, VEGF was elevated by approximately 60 to 90% in the diabetic state *versus* control (Figure 2). It is interesting that in both nondiabetic and diabetic animals, the rims of the glomerular tufts seemed to stain more intensely for VEGF, compatible with constitutive VEGF expression in the podocytes (20) and their augmented VEGF expression under diabetic conditions (4,6) (Figure 2B). Administration of SU5416 did not influence the ambient level of glomerular VEGF in either control or diabetic mice.

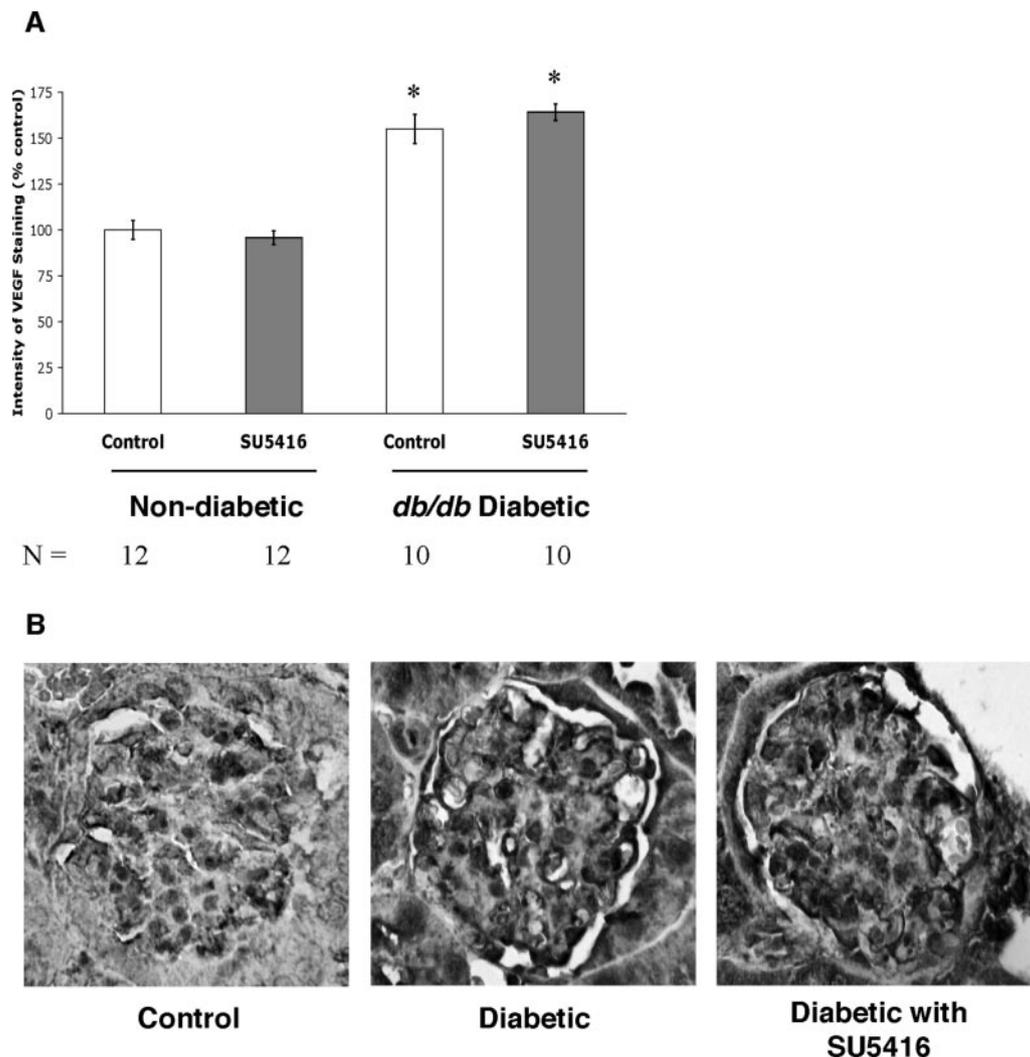


Figure 2. Extent of VEGF protein by immunohistochemistry. (A) The intensity of VEGF staining was increased in the glomeruli of diabetic *db/db* mice ($*P < 0.01$ *versus* nondiabetic control mice) but was not significantly affected by SU5416 treatment (NS *versus* diabetic control). (B) The pattern of VEGF increase with *db/db* diabetes and persistent VEGF elevation despite SU5416 treatment is evident in the representative photomicrographs. The omission of anti-VEGF antibody to evaluate for nonspecific immunoperoxidase staining showed only minimal background. Magnification, $\times 600$.

Diabetic Albuminuria

The AER per day increased with *db/db* diabetes, roughly quadrupling the AER of the nondiabetic control group (Figure 3). Albuminuria in the *db/db* mice was ameliorated by treatment with SU5416, which almost completely prevented the diabetes-induced increase in AER (Figure 3). Of note, SU5416 did not affect the mean AER of the nondiabetic *db/m* mice (Figure 3). Albuminuria also was normalized for the amount of urinary creatinine excreted to allow for an effect, if any, from the differences in muscle mass between *db/m* and *db/db* mice. This effect probably was negligible, although the trend in the urine albumin:creatinine ratio matched fairly well with the albuminuria data when expressed as the quantity excreted per day (Figure 3, B versus A).

GBM Thickening

The GBM thickness, whether measured across the lamina densa or across its full thickness (lamina densa plus lamina rara interna and rara externa), was increased by the diabetic state (Figure 4). However, in the *db/db* mice that were treated with SU5416, diabetic GBM thickening was prevented (Figure 4). In the nondiabetic *db/m* mice, the GBM thickness was not affected by SU5416. Representative electron photomicrographs of the GBM in the control, diabetic, and diabetic plus SU5416 mice are shown in Figure 4B.

Mesangial Matrix Expansion

The expansion of the mesangium with extracellular matrix was quantified by electron microscopy and shown by PAS staining. The mesangial matrix area as a percentage of the glomerular tuft area was enlarged in the *db/db* diabetic state (Figure 5). Mesangial

matrix expansion in the *db/db* mice was not blunted by treatment with SU5416, as seen in the representative photomicrographs of Figure 5B. In nondiabetic mice, SU5416 treatment did not affect the quantity of mesangial matrix (Figure 5).

Slit Pore Density

Along with podocyte foot process broadening, the density of interpodocyte slit pores along the GBM has been reported to be decreased in type 2 diabetes (12,21). In our study, the slit pore density was diminished in the *db/db* mice (Figure 6), a finding that can be seen in the representative electron photomicrographs (Figure 6B). At the same time, the density of “tight” pores, in which the space between adjacent foot processes seems to be obliterated, was found to be increased in the *db/db* mice (Figure 6). The effect of SU5416 in *db/db* diabetes was to significantly increase the open slit pore density and reduce the tight pore density toward nondiabetic control values (Figure 6). No effect of SU5416, however, was seen in the nondiabetic *db/m* mice (Figure 6). Changes in the open slit pore and tight pore densities were reciprocal to each other.

Nephrin Immunofluorescence

Nephrin protein was quantified by its immunofluorescent intensity. Type 2 diabetes in the *db/db* mouse significantly lowered the amount of nephrin in the glomerulus (Figure 7). However, the quantity of glomerular nephrin in the *db/db* mouse was partially restored by SU5416 treatment ($P < 0.05$, *db/db*-SU5416 versus *db/db*; Figure 7). These changes in nephrin correlated inversely with the degree of albuminuria (Figure 3).

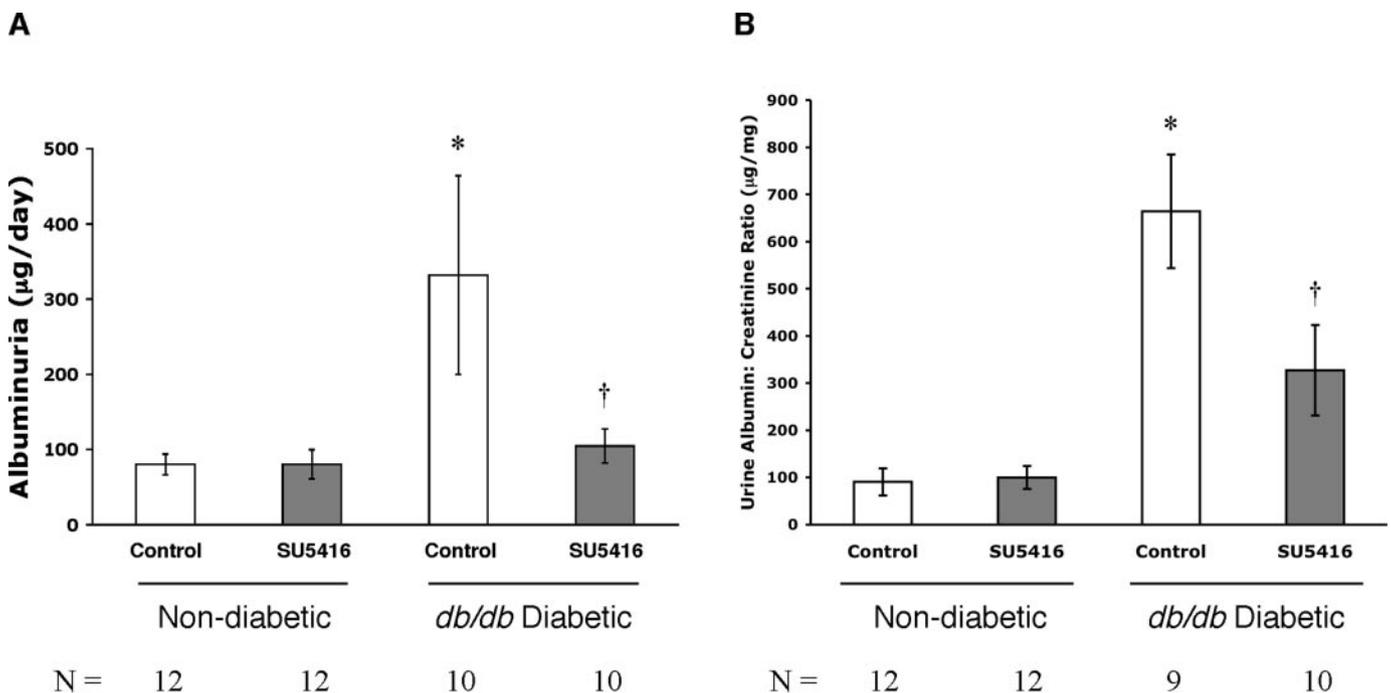


Figure 3. Albuminuria in diabetes. The albumin excretion rate (AER), measured by ELISA (Exocell) on urine samples, was increased in the *db/db* diabetic mice to at least four times the value of the nondiabetic mice, whether the AER was expressed per day (A) or corrected for the urinary creatinine (B; * $P < 0.05$ versus nondiabetic control mice). However, albuminuria in the *db/db* mice was significantly ameliorated by SU5416 ($\dagger P < 0.05$ versus diabetic control mice).

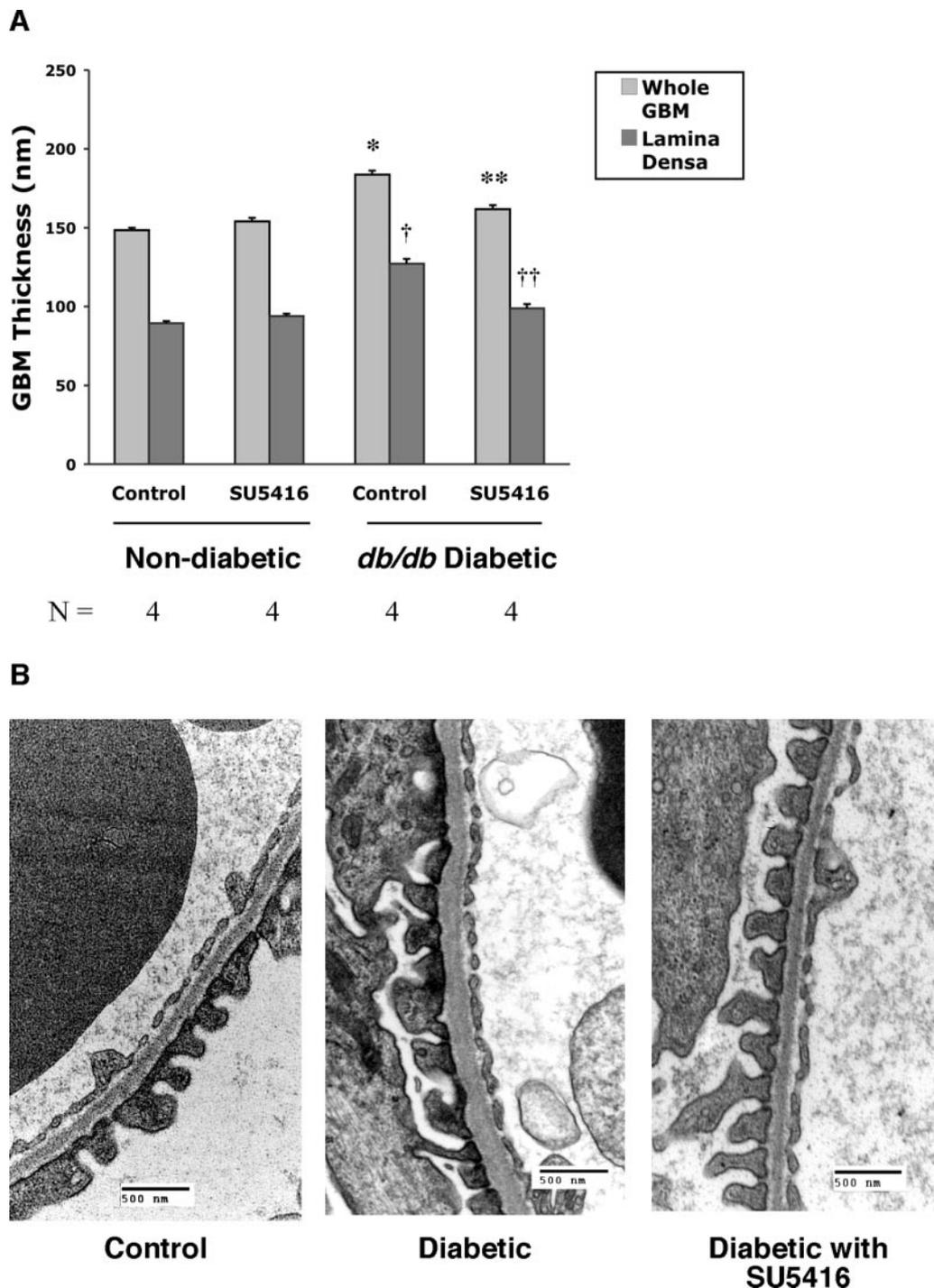


Figure 4. Glomerular basement membrane (GBM) thickening in diabetes. GBM thicknesses were measured across the whole GBM and across the lamina densa. The data are displayed as the mean \pm SEM of the GBM widths that were evaluated in at least 10 glomeruli from each of the number of mice indicated (A). Control diabetic *db/db* mice developed GBM thickening (* or $^{\dagger}P < 0.01$ versus nondiabetic control). Thickening was prevented in the *db/db* mice that were treated with SU5416 (** or $^{\dagger\dagger}P < 0.01$ versus diabetic control). Representative electron photomicrographs are shown where diabetic GBM thickening and its prevention in the *db/db* mouse by SU5416 treatment are evident by visual inspection (B). Magnification, $\times 30,000$.

Nephrin Immunoblotting

Nephrin was also measured by immunoblotting, but instead of corroborating the immunofluorescence data, it showed different results. Nephrin protein, seen as a double band at 185 kD (22) on

the Western film (Figure 8), actually increased with diabetes ($P < 0.01$ versus control; Figure 8), the opposite of what was observed by immunofluorescence. The increased nephrin in the *db/db* mice did not significantly change with SU5416 (Figure 8).

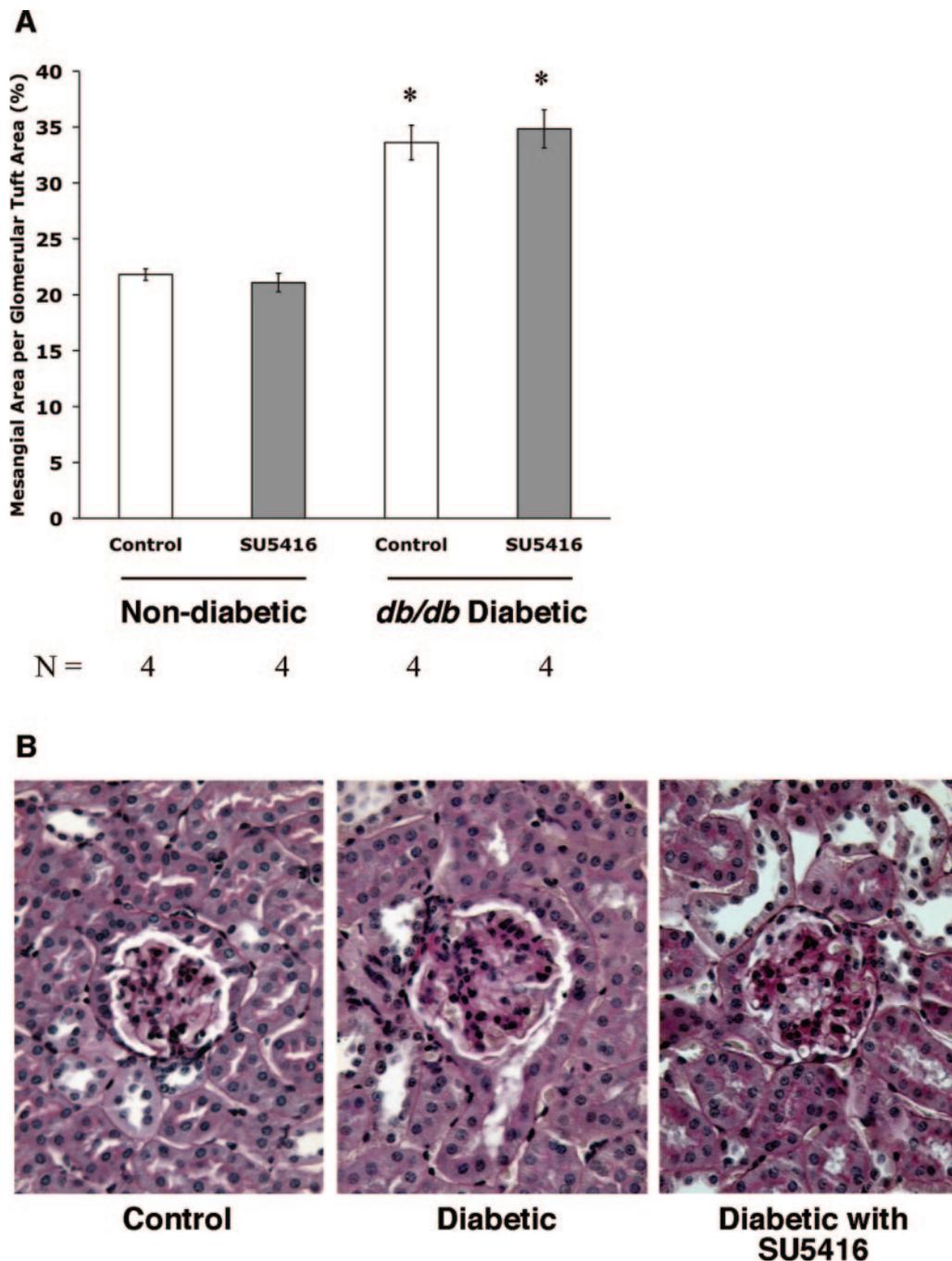


Figure 5. Mesangial matrix measurement. As assessed by electron microscopy, the area of the glomerular tuft occupied by mesangial matrix was expressed as a percentage, and the mean \pm SEM of the area percentages is shown for the number of mice evaluated (A). Mesangial matrix expansion occurred in the diabetic *db/db* mice, regardless of treatment with SU5416 ($*P < 0.01$ versus nondiabetic control mice). Increased mesangial matrix is evident by periodic acid-Schiff staining (deep purple color) in the diabetic mice but was not diminished by SU5416 treatment, as demonstrated in the representative photomicrographs (B). Magnification, $\times 400$.

Discussion

The prevention or reversal of albuminuria has become a benchmark in the therapy of diabetic nephropathy, because proteinuria seems to play a crucial role as a promoter of progressive kidney disease (23). We found that albuminuria was decreased in the diabetic *db/db* mice that were treated with the

VEGF receptor inhibitor SU5416. This positive result expands on the findings that antibody neutralization of VEGF ligand ameliorates albuminuria in both type 1 (streptozotocin-induced diabetic rats) and type 2 (*db/db* mice) models of diabetes (7,8). The mechanism of VEGF-induced albuminuria likely is related to its signaling through the VEGF receptor tyrosine kinases,

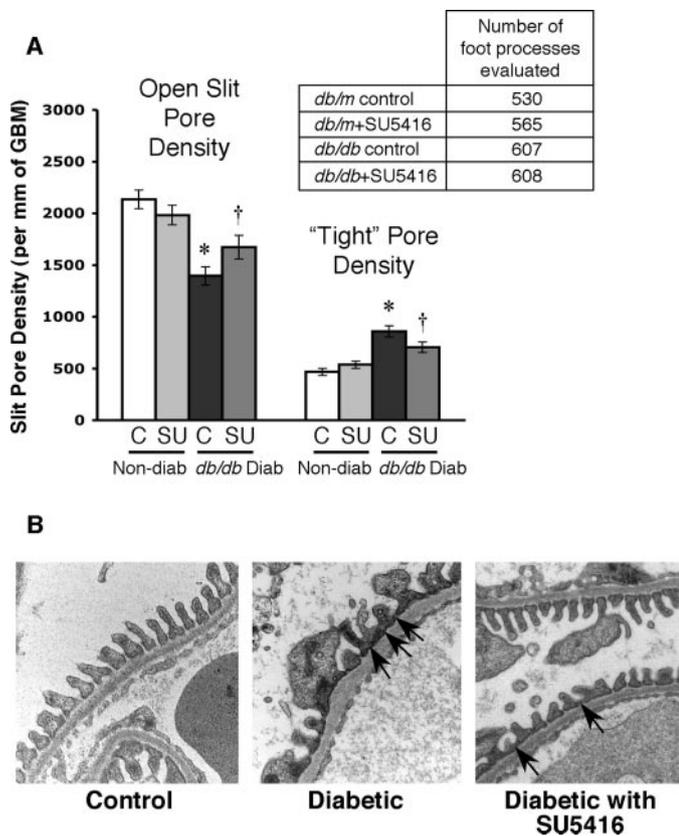


Figure 6. Podocyte morphometry for slit pore density. As assessed by electron microscopy, the number of open slit pores between podocyte foot processes and the number of “tight” pores (*i.e.*, foot processes in close apposition) were expressed per millimeter length of GBM to arrive at the mean numerical “density” (\pm SE) for each of the four groups of mice (A). Diabetic *db/db* mice had decreased open slit pore density and increased tight pore density ($*P < 0.001$ versus nondiabetic control [C] mice), but these changes were partially and significantly prevented by treatment with SU5416 ($^{\dagger}P < 0.05$ versus diabetic C mice), associated with amelioration of albuminuria. Representative electron photomicrographs show examples of tight slit pores (arrows), which are more prevalent in the diabetic *db/db* mouse (B).

because SU5416 significantly attenuated VEGF receptor activation in the diabetic state when assayed by the extent of VEGFR-1 autophosphorylation. The inhibition of VEGFR-1 activity is consistent with the known mechanism of action of SU5416 (9). It also is possible that the benefit of SU5416 in diabetes may derive partly from its modest effect on glycemic control, because the *db/db* mice that received treatment had an approximately 18% lower plasma glucose than the control *db/db* mice, although the difference was not statistically significant. In blocking the VEGFR kinases, SU5416 did not reduce glomerular VEGF expression in diabetes. SU5416 merely blocks the signaling by VEGF but does not affect its production. What could sustain VEGF overexpression in the diabetic mice are myriad stimuli, such as hyperglycemia, protein kinase C activation, angiotensin II, glycosylated proteins, cellular stretch, reactive oxygen species, and TGF- β (24).

The associations between diabetic albuminuria and renal morphometric measurements also were examined. Albuminuria had hitherto been correlated with GBM thickening (11,25) and mesangial matrix expansion (10,25), but according to our study, albuminuria can be dissociated from mesangial expansion. It suggests that the correlation between these two diabetic parameters is merely an association and not a direct cause-and-effect relationship. Of course, it does not rule out that occurrences in the mesangium may influence the albumin permeability of the filtration barrier. Furthermore, the VEGF signaling system does not seem to have an impact on the development of mesangial extracellular matrix accumulation.

What is intriguing is that the association between albuminuria and GBM thickening was upheld. The merits of the GBM as a barrier to albumin passage are still being debated, but it has been paradoxical that a thickened GBM should be more porous to protein. The quantity of GBM may be increased in diabetes, but perhaps the “quality” suffers. For reconciling the paradox, improper assembly of the GBM has been invoked, and there is some evidence now that VEGF signaling may play a role. We previously discovered that VEGF functions in an autocrine loop in the podocyte to stimulate the production of a GBM component, the $\alpha 3$ chain of collagen IV (26). Overproduction of $\alpha 3(IV)$ collagen may contribute to GBM thickening but at the same time might perturb the assembly of the GBM such that it becomes more leaky to albumin (26,27). By interrupting the pathophysiology of the VEGF autocrine loop in this study, SU5416 may have prevented GBM thickening and ameliorated diabetic albuminuria.

Podocyte-specific manifestations of diabetic nephropathy were also affected by the VEGF signaling system. The open slit pore density was noticeably reduced in the diabetic mice and was partly “rescued” by the inhibition of VEGF signaling. The reduction in slit pore density may have a direct basis in the foot process broadening that is thought to occur to compensate for the dearth of podocytes in diabetes (12). As a result of the foot process broadening, the density of open slit pores along the GBM decreases, and, concomitantly, the number of tight slit pores increases. That VEGF signaling should contribute to this morphologic chain of events is a novel discovery that warrants further investigation. Whether VEGF causes podocyte loss or cytoskeletal rearrangement as a mechanism of slit pore density changes remains to be seen. Alterations to the podocyte also could be an indirect effect of VEGF through its tendency to exacerbate diabetic proteinuria (21).

Nephrin is widely believed to decrease in diabetes, in association with an increase in albuminuria, according to the preponderance of evidence. It is interesting that most of the evidence in the literature is based on histologic assessments of nephrin (28–31), and our initial assessment, based on immunofluorescence data, was that nephrin also declines in the *db/db* model of diabetes. However, in seeming contradiction, nephrin protein seemed to be increased in the same set of *db/db* mice when measured by Western blot. The reasons for the discrepancy are unknown, but an explanation might be found in the procedural differences that handicap immunofluorescence more than Western. Perhaps the nephrin primary antibody

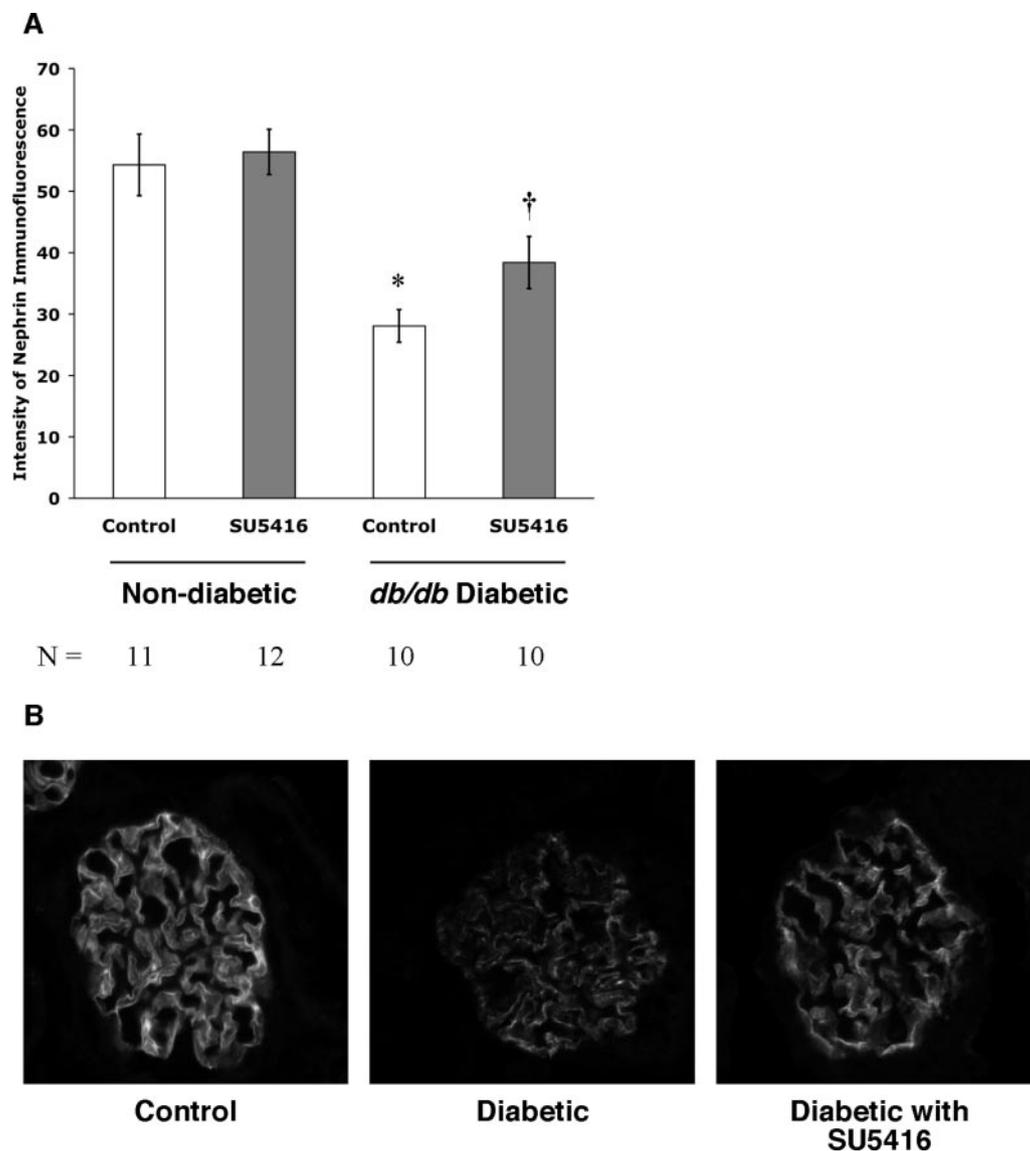


Figure 7. Nephrin immunofluorescence. (A) The intensity of immunofluorescent staining for nephrin, determined with the aid of imaging software, was markedly reduced by the *db/db* diabetic state ($*P < 0.01$ versus nondiabetic control mice). However, the staining intensity and the quantity of nephrin protein were restored partially in the diabetic mice by SU5416 therapy ($^{\dagger}P < 0.05$ versus diabetic control mice). (B) The decrease in nephrin expression and its partial restoration by SU5416 treatment can be seen in the representative fluorescence photomicrographs. Magnification, $\times 600$.

could not penetrate to its target in immunofluorescence but could freely probe for nephrin on the immunoblot. Alternatively, the wash steps might have flushed away more nephrin in the immunofluorescence procedure than in the Western, with the net result that immunofluorescence would show a decrease in nephrin at the same time that Western demonstrates an increase. The nephrin that is most vulnerable to being washed away is either extracellular and contributing to nephrinuria (32) or intracellular and located within the endoplasmic reticulum (33). Besides the procedural differences between immunofluorescence and Western, a genuine effect of diabetes on nephrin handling might help to explain the observed discrepancy. Diabetes could induce a change in the trafficking of nephrin such that an increased production is shunted toward a

soluble form of nephrin (34,35) that is susceptible to loss during the immunofluorescence procedure. The diabetic state is also known to increase nephrinuria (36), which by virtue of liberating nephrin into the extracellular space would compromise the ability of immunofluorescence but not Western to assay nephrin. Immunofluorescence may be criticized for being artifactual, but it would preferentially assay the nephrin that is membrane bound, which is arguably the more meaningful fraction of total nephrin.

The VEGF system seems to exert a salient effect on nephrin in the setting of diabetic kidney disease. The observation from immunofluorescence that SU5416 significantly restores nephrin levels would implicate overactive VEGF signaling as playing a major role in the pathogenesis of diabetes-induced

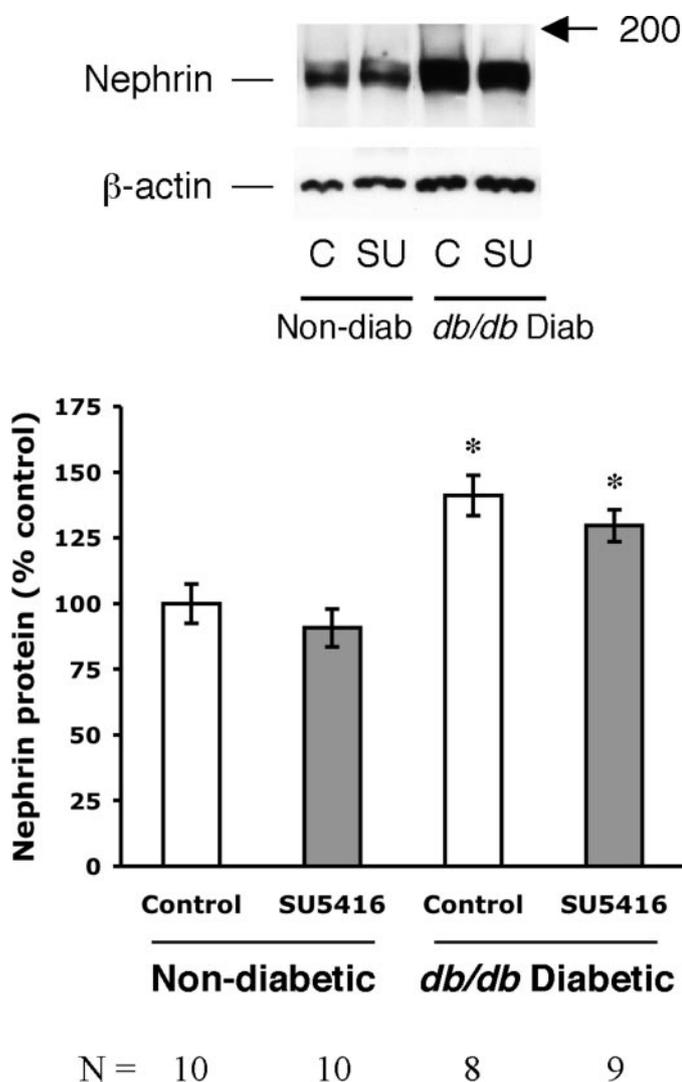


Figure 8. Nephrin protein by Western immunoblotting. In contrast to immunofluorescence, the Western blot technique revealed that nephrin, corrected for β -actin levels, was increased in the diabetic *db/db* mouse ($*P < 0.01$ versus nondiabetic control). This change in nephrin with diabetes was not significantly affected by SU5416. Chemiluminescent bands that are representative of the four groups of mice also are shown.

alterations of nephrin. Then again, a comparable effect of SU5416 to reverse an increase in nephrin was not seen by Western blotting. VEGF signaling, it seems, has a selective impact on the fraction of nephrin that is detectable by immunofluorescence but not on the total pool of nephrin that is assayable by Western. Supposing that the immunofluorescence-detectable fraction represents membrane-bound nephrin, the effect of VEGF activation would be to reduce the presence of nephrin in the slit diaphragm, possibly leading to a decrease in the filtration slit length, which itself is inversely correlated with diabetic albuminuria (13).

In the end, the amelioration of diabetic albuminuria remains a worthy goal, because proteinuria was the most potent predictor of progression in the Reduction of End-

points in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) trial of patients with type 2 diabetes (37). More effective and targeted therapies against albuminuria hold the promise of forestalling diabetic ESRD as investigations advance our knowledge of the pathogenesis. Our study disclosed that one of the crucial determinants of diabetic albuminuria is overactivity of the VEGF signaling system. It is interesting that many of the diabetic renal manifestations that were found to hinge on VEGF signaling seem to revolve around the podocyte as a target cell. GBM thickening, slit pore density, and nephrin alterations are intimately connected to podocyte structure and function, and all of these parameters are closely correlated with albuminuria. Furthermore, the regulation of nephrin in diabetes has taken on an additional layer of complexity as revealed by the incongruity between immunofluorescence and Western. Finally, among the available VEGF receptor inhibitors, SU5416 (semaxinib) already has been used safely in clinical trials (38,39). Although developed and tested as an anticancer agent, SU5416 has not yet been tried in human diabetic nephropathy. Translational research may soon follow, if not with SU5416 then with related anti-VEGF signaling compounds.

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