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Aberrant production of extracellular matrix proteins and dysfunction in kidney endothelial cells with a short duration of diabetes

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Grutzmacher C, Park S, Zhao Y, Morrison ME, Sheibani N, Sorenson CM. Aberrant production of extracellular matrix proteins and dysfunction in kidney endothelial cells with a short duration of diabetes. Am J Physiol Renal Physiol 304: F19–F30, 2013. First published October 17, 2012; doi:10.1152/ajprenal.00036.2012.—Diabetic nephropathy is the most common cause of end-stage renal disease and is a major risk factor for cardiovascular disease. In the United States, microvascular complications during diabetic nephropathy contribute to high morbidity and mortality rates. However, the cell-autonomous impact of diabetes on kidney endothelial cell function requires further investigation. Male Akita/+ [autosomal dominant mutation in the insulin II gene (Ins2)] mice reproducibly develop diabetes by 4 wk of age. Here, we examined the impact a short duration of diabetes had on kidney endothelial cell function. Kidney endothelial cells were prepared from nondiabetic and diabetic mice (4 wk of diabetes) to delineate the early changes in endothelial cell function. Kidney endothelial cells from Akita/+ mice following 4 wk of diabetes demonstrated aberrant expression of extracellular matrix proteins including decreased osteopontin and increased fibronectin expression which correlated with increased a5-integrin expression. These changes were associated with the attenuation of migration and capillary morphogenesis. Kidney endothelial cells from Akita/+ mice had decreased VEGF levels but increased levels of endothelial nitric oxide synthase(eNOS) and NO, suggesting uncoupling of VEGF-mediated NO production. Knocking down eNOS expression in Akita/+ kidney endothelial cells increased VEGF expression, endothelial cell migration, and capillary morphogenesis. Furthermore, attenuation of sprouting angiogenesis of aortas from Akita/+ mice with 8 wk of diabetes was restored in the presence of the antioxidant N-acetylcysteine. These studies demonstrate that aberrant endothelial cell function with a short duration of diabetes may set the stage for vascular dysfunction and rarefaction at later stages of diabetes.

angiogenesis; capillary morphogenesis; extracellular matrix proteins

DIABETIC NEPHROPATHY REPRESENTS nearly half of the new cases of end-stage renal disease (ESRD) reported in the United States. Unfortunately, the highest mortality rate in ESRD is in patients with diabetic nephropathy. Endothelial cell dysfunction precedes and predicts the onset of microalbuminuria in diabetic nephropathy (25). Since microvascular complications can contribute to the high morbidity and mortality rates in diabetes (30), understanding how early changes in endothelial cell function contribute to diabetic nephropathy is essential. This knowledge will aid with early detection of vascular dysfunction and decrease the cost of treating this disease, which has become an increasing burden in the United States (27).

The Akita spontaneous mutation (Akita/++; commonly referred to as maturity-onset diabetes of the young or MODY) is an autosomal dominant mutation in the insulin II gene (Ins2) (32). This missense mutation results in an amino acid substitution (Cys 96 to Tyr) corresponding to the seventh amino acid position of the insulin II A chain. Cysteine 96 is involved in forming one of the two disulfide bonds between the A and B chains. Cysteine 96-to-tyrosine replacement causes incorrect folding of the insulin II proteins so that insulin release is abnormal and diabetes develops. Male mice heterozygous for the Akita spontaneous mutation (Akita/+ ) reproducibly exhibit hyperglycemia, hypoinsulinemia, polydipsia, and polyuria beginning ~3–4 wk of age. The diabetic phenotype is more severe and progressive in the male. Obesity or insulitis does not accompany diabetes in these mice. These mice also respond to exogenously administered insulin, indicating that Akita/+ mice serve as an excellent substitute for mice made insulin dependent by treatment with alloxan or streptozotocin. Chronic hyperglycemia during diabetes can trigger the generation of free radicals and oxidative stress. Enzymatic and nonenzymatic sources contribute to reactive oxygen species (ROS) observed in the diabetic kidney, including advanced glycation, mitochondrial respiration chain deficiencies, xanthine oxidase activity, peroxidases, nitric oxide synthase (NOS), and NAD(P)H oxidase. Chronic hyperglycemia promotes excessive oxidative stress in the vasculature. Although glomerular changes including mesangial proliferation and extracellular matrix accumulation are well documented, hyperglycemia-induced changes in the vasculature are less clearly understood and may set the stage for diabetic nephropathy.

Here, we examined whether a short duration of diabetes was sufficient to induce kidney endothelial cell dysfunction. Kidney endothelial cells were prepared from 3–4 wk-old wild-type and Akita/+ mice (4 wk of diabetes). Akita/+ kidney endothelial cells displayed aberrant production of extracellular (ECM) matrix proteins. These changes were associated with attenuation of cell migration, VEGF expression, and capillary morphogenesis. Akita/+ kidney endothelial cells also expressed increased levels of eNOS and p-eNOS consistent with
the increased NO production and nitrative stress observed in these cells. Cell migration and capillary morphogenesis was restored when eNOS expression was knocked down in Akita/+ endothelial cells. In addition, attenuation of sprouting angiogenesis in aortas from Akita/+ mice with 8 wk of diabetes was restored by addition of the antioxidant N-acetylcysteine (NAC). Thus understanding how kidney endothelial cell function is aberrantly modulated early in diabetes will further our understanding of the course of this disease so that more effective therapies can be designed for early intervention.

**MATERIALS AND METHODS**

Experimental animals and cell cultures. The mice used for these studies were maintained and treated in accordance with our protocol approved by the University of Wisconsin Animal Care and Use Committee. Immortomice expressing a temperature-sensitive SV40 large T antigen were obtained from Charles River Laboratories (Wilmington, MA). Akita/+ mice (Jackson Laboratory, Bar Harbor, ME) were crossed with the Immortomouse and screened as previously described (7, 23). To isolate kidney endothelial cells, kidneys from three 8-wk-old wild-type and Akita/+ Immortomice were dissected out aseptically and placed in serum-free DMEM (no. 1200046, Invitrogen, Carlsbad, CA) containing penicillin/streptomycin (Sigma, St. Louis, MO). The kidneys were pooled, rinsed with DMEM, minced into small pieces in a 60-mm tissue culture dish using sterilized razor blades, and digested in 5 ml of collagenase type I (1 mg/ml in serum-free DMEM, Worthington, Lakewood, NJ) for 30–45 min at 37°C. Following digestion, DMEM with 10% FBS was added, and cells were pelleted. The cellular digests were then filtered through a double layer of sterile 40-μm nylon mesh (Sefar America, Hanover Park, IL), centrifuged at 400 g for 10 min to pellet cells, and then the cells were washed twice with DMEM containing 10% FBS. The cells were resuspended in 1.5 ml medium (DMEM with 10% FBS) and incubated with sheep anti-rat magnetic beads precoated with anti-platelet endothelial cell adhesion molecule (PECAM)-1 antibody (MEC13.3; BD Biosciences, Bedford, MA), as described previously (28). After affinity binding, magnetic beads were washed six times with DMEM with 10% FBS and bound cells in endothelial cell growth medium and plated into a single well of a 24-well plate precoated with 2 μg/ml of human fibronectin (BD Biosciences). Endothelial cells were grown in DMEM containing 10% FBS, 2 mM l-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% nonessential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, heparin at 55 U/ml (Sigma), 100 μg/ml endothelial growth supplement (Sigma), and murine recombiant interferon-γ (R&D Systems, Minneapolis, MN) at 44 U/ml. Cells were maintained at 33°C with 5% CO2. Cells were progressively passed to larger plates, maintained, and propagated in 1% gelatin-coated 60-mm dishes. Kidney endothelial cells were positive for B4-lectin (a mouse endothelial cell-specific lectin) and expressed PECAM-1 and vascular endothelial (VE)-cadherin as previously described (7, 13). The experiments described here were performed with three separate isolations of cells with similar results.

**Cell apoptosis assay.** Cell apoptosis was assessed using Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij). As an apoptotic stimulus, wild-type and Immortomice were incubated with 5 mM 5-fluoro-2′,3′-dIDEOXycytidine (FUDR) or growth medium for 48 h. Cell apoptosis was determined as the percentage of total distance for quantitative assessment as described previously (7). These experiments were repeated at least twice with two different isolations of cells.

**Proliferation assay.** The cell proliferation assays were performed by plating cells in 60-mm tissue culture dishes and counting the number of cells every other day for 2 wk. Cells (1 × 10⁴) were plated in triplicate in multiple sets on 60-mm tissue culture plates. Cells were fed every other day, and the cell number in one set of plates was determined by counting on each indicated day.

**Capillary morphogenesis in Matrigel.** Matrigel (10 mg/ml; BD Biosciences) was applied at 0.5 ml/35-mm tissue culture dish and incubated at 37°C for at least 30 min to harden. Cells were removed using trypsin-EDETA, washed with growth medium once, and resuspended at 1 × 10⁵ cells/ml in serum-free growth medium. Cells (2 ml) were gently added to the Matrigel-coated plates, incubated at 37°C, monitored for 18 h, and photographed using a Nikon microscope equipped with a digital camera. For quantitative assessment of the data, the mean number of branch points in 10 high-power fields (×100) was determined after 18 h. A longer incubation of the cells did not result in further branching morphogenesis (28).

**Aortic ring ex vivo spraying assay.** Thoracic aortas from 4-, 8-, and 12-wk-old mice were removed and immediately transferred to a 50-ml tube with 40 ml of ice-cold serum-free DMEM and then washed by shaking the tube for 15 s. The periadventitial adipose tissue was carefully removed with fine microdissecting forceps and iridectomy scissors, with special attention paid so as not to damage the aortic wall. One-mm-long aortic rings (8/aorta) were sectioned and rinsed extensively in three consecutive washes of DMEM. The mouse aorta pieces were then embedded in Matrigel (10 mg/ml)-plated wells (0.3 ml/well) in 12-well plates on ice with up to 6 aortic rings per well. The Matrigel-embedded aortic rings were incubated at 37°C for 30 min to harden. Then, 1 ml DMEM containing 10% FBS was added to each well. In some cases, 1 mM NAC was added to the cultures. The cultures were kept at 37°C in a humidified environment and fed every other day. Five-day cultures were photographed using a Nikon microscope equipped with a digital camera. For quantitative assessment of spraying, the area of spraying per millimeter of tissue was assessed using Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij).

**Cell adhesion assays.** Cell adhesion to various matrix proteins was performed as previously described (21). Briefly, varying concentrations of fibronectin, vitronectin, collagen type I, and collagen type IV (BD Biosciences) prepared in TBS with Ca²⁺ and Mg²⁺ (2 mM each; TBS with Ca/Mg) were coated on 96-well plates (50 μl/well; Nunc Maxisorbe plates, Fisher Scientific) overnight at 4°C. As a control, wells were coated with 1% BSA. Plates were rinsed four times with 200 μl of TBS with Ca/Mg and blocked with 200 μl of 1% BSA prepared in TBS with Ca/Mg for at least 1 h at room temperature. Cells were removed by dissociation solution (Sigma), washed with TBS, and resuspended at 5 × 10⁴ cells/ml in HBS (20 mM HEPES, 150 mM NaCl, pH 7.6, and 4 mg/ml BSA). After blocking, plates were rinsed with TBS with Ca/Mg once, 50 μl of cell suspension was added to each well containing 50 μl of TBS with Ca/Mg, and the cells were allowed to adhere to the plate for 1.5 h at 37°C. The nonadherent cells were removed by gently washing the plate four times with TBS with Ca/Mg, or until no cells were left in wells coated with BSA. The number of adherent cells in each well was quantified by measuring the cellular phosphatase activity as previously described (33). All samples were done in triplicate.

**Western blot analysis.** Cells were plated at 4 × 10⁴ in 60-mm dishes coated with 1% gelatin and allowed to reach nearly 90% confluence in 2 days. The cells were then rinsed once with serum-free medium and incubated with serum-free DMEM for 48 h. Then, conditioned medium (3.5 ml) was collected and clarified by centrifugation. The 40 μl of sample was mixed with appropriate volume of
6× SDS buffer and analyzed by SDS-PAGE (4–20% Tris glycine gel; Invitrogen). In some cases, total protein lysates were prepared from these cells in a modified RIPA buffer (142.5 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.4, 2 mM orthovanadate, and 2 mM sodium fluoride, 1% Nonidet P-40, and a complete protease inhibitor cocktail, Roche, Mannheim, Germany). The proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with an anti-fibronectin (Sigma), a rabbit anti-chicken tenascin C polyclonal antibody (AB91013; Millipore, Billerica, MA), anti-thrombospondin-1 (TSPI) monoclonal antibody (Clone A6.1; Neo Marker, Fremont, CA), anti-osteopontin (R&D Systems), anti-β-catenin (Sigma), anti-heat shock protein 90 (HSP90; Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti-phospho-Akt (Cell Signaling Technology), anti-β-actin (Sigma), anti-phospho-eNOS (Cell Signaling Technology), and anti-eNOS (Santa Cruz Biotechnology, Santa Cruz, CA). The blot was washed, incubated with appropriate secondary antibody, and developed using ECL (Amersham, Piscataway, NJ) (24, 26).

Fluorescence-activated cell scanner analysis. Fluorescence-activated cell scanner (FACScan) analysis was performed as previously described (13). The cells were washed once with PBS containing 0.04% EDTA and incubated with 2 ml of dissociation solution (Sigma) to remove the cells from the plate. The cells (10⁶) were washed with TBS, blocked in TBS containing 1% goat serum on ice for 20 min, and incubated with the appropriate dilution of primary antibody: anti-PECAM-1 (BD Pharmingen), anti-VE-cadherin (Alexis Biochemical, San Diego, CA), B4-lectin (Sigma), anti–β1 (Millipore), anti–α5 (MABI949; Millipore), anti–α4 (Millipore), anti–β3 (MABI957; Millipore), anti–αvβ3 (MABI976Z; Millipore), anti–VCAM-1 (CLB1300; Millipore), anti–ICAM-1 (553250; BD), or control IgG (Millipore). For antibodies that required cell permeabilization, cells were removed from the dish, washed with PBS, fixed with 2% paraformaldehyde on ice for 30 min, washed with PBS, and resuspended in PBS containing 0.1% Triton X-100 and 0.1% BSA containing an appropriate dilution of primary antibody. The cells were washed with TBS containing 1% BSA and then incubated with the appropriate secondary antibody (1:200) on ice for 30 min. After the incubation, the cells were washed twice with TBS containing 1% BSA and resuspended in 0.5 ml of TBS containing 1% BSA. FACScan analysis was performed on a FACScan caliber flow cytometer (Becton-Dickinson, Franklin Lakes, NJ).

Transwell assay. Transwell filters (Costar 3422) were coated with 1% gelatin, rinsed with PBS, and then blocked with 2% BSA in PBS. Five hundred microliters of serum-free DMEM was added to the bottom of each well, and 1 × 10⁴ cells in 100 μl of medium was added to the top of each well. Each condition was done in duplicate. Following 4 h in a 37°C tissue culture incubator, the cells and medium were aspirated, and the upper side of the membrane was wiped with a cotton swab. The cells that had migrated through the membrane were fixed with 2% paraformaldehyde and stained with hematoxylin and eosin. Ten fields of cells were counted for each condition, and the average and SD were determined.

VEGF analysis. VEGF protein levels were determined from conditioned medium prepared from kidney endothelial cells, utilizing a mouse VEGF Immunoassay kit (R&D). Briefly, kidney endothelial cells were grown for 2 days in serum-free medium. The conditioned medium (50 μl) was used in the VEGF immunoassay, which was performed in triplicate as recommended by the manufacturer and was normalized to the number of cells. The assay was repeated twice using two different isolations of endothelial cells with similar results.

NO analysis. Kidney endothelial cells were plated in black wall, clear bottom Microtest TM 96-well plates (no. 35 3948; 5 × 10⁴ cells in 100 μl; BD). The next morning, the medium was changed to endothelial cell medium containing 30 μM DAF-FM diacetate (D-23842; Invitrogen) and 5 μg/ml of Cell trackerRed (C34552; Invitrogen). Following a 40-min incubation at 33°C, fresh endothelial cell medium was placed on the cells, and the incubation continued for 20 min. The wells were washed with TBS, the cells in the wells were resuspended in 100 μl of TBS, and the absorbance was read at 495/515 nm using a fluorescence plate reader (7). These experiments were performed in triplicate and repeated twice with similar results.

Indirect immunofluorescence staining. Kidney endothelial cells were plated on fibronectin (2 μg/ml)-coated coverslips. The cells were then rinsed with PBS, fixed with 3% of paraformaldehyde (PFA) for 10 min on ice, washed two times with PBS, and incubated with anti-N-tyr (1:200; StressMarq, Victoria, BC) for 30 min at 37°C. After washing three times with TBS, cells were incubated with appropriate C3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) at 37°C for 30 min. Cells were washed three times with TBS, mounted, and photographed using a Zeiss fluorescence microscope (Axiopt, Zeiss, Germany) equipped with a digital camera. The fluorescence intensity was quantified with ImageJ software from NIH (rsweb.nih.gov/ij/).

Expression of eNOS small interfering RNAs. Multiple MISSION Lentiviral Transduction Particles were purchased from Sigma (no. SHCLNV) targeting different regions of mouse eNOS (accession no. NM_008713, Clone ID TRC0000075863, 64, 65, 66, and 67). Akita/+ kidney endothelial cells were transfected with multiple individual constructs and selected for puromycin resistance according to the manufacturer’s instructions. Briefly, cells were seeded on wells of a 12-well plate at 75% confluence before transduction. The next day, an appropriate amount of viral particles at multiplicity of infection = 2 TU/cell was added along with 8 μg/ml hexadimethrine bromide, which would enhance transduction, and was incubated with cells at 33°C overnight. MISSION pl.KO.1-puro Non-Target short hairpin (sh) RNA Control Transduction Particles (no. SHC002V; Sigma) was used as the nontargeting negative control. Cells were fed with fresh growth medium on day 3. The selection began on day 4 with medium containing 1.5 μg/ml puromycin to select for the cells expressing specific small interfering (si) RNA constructs. The medium was replaced with fresh, puromycin-containing medium every 4 days until resistant colonies were identified. Colonies were further expanded and evaluated for knockdown of mouse eNOS by Western blotting. A stable cell population expressing specific siRNA (TRCN0000075867) was used for further analysis.

Processing of kidneys for histological studies. Following surgical removal from mice, kidneys were fixed with formalin overnight and processed for paraffin sectioning. For immunohistochemical staining, paraffin sections were deparaffinized with xylene and rehydrated. Antigen unmasking was performed using antigen-unmasking solution (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. The sections were then washed in PBS and incubated for 15 min in PBS blocking buffer (PBS containing 1% bovine serum albumin, 0.3% Triton X-100, and 0.2% skim milk powder). The sections were incubated overnight with anti-PECAM-1 (1:150; R&D Systems). The sections were then incubated with indocarbocyanine (CY3)-labeled secondary antibody (Jackson ImmunoResearch) and photographed. Vascular density was determined by counting the number of capillaries and tubules or glomeruli on at least 10 high-magnification fields (×400). The data are represented as number of capillaries per tubule or capillaries per square millimeter of glomerulus.

Statistical analysis. Statistical differences between control and treated samples were evaluated with Student’s unpaired t-test (2-tailed) or two-way ANOVA with Bonferroni correction for multiple comparisons when appropriate. Mean ± SDs are shown. P values <0.05 were considered significant.

RESULTS

Akita/+ kidney endothelial cells demonstrated similar levels of apoptosis and proliferation compared with their non-diabetic wild-type counterparts. To determine the impact early stages of diabetes have on kidney endothelial cell function, we isolated...
kidney endothelial cells from 8-wk-old wild-type and Akita/+ mice (4 wk of diabetes). We first examined kidney endothelial cell morphology and expression of endothelial cell markers to confirm that these cells maintain their endothelial cell characteristics. Wild-type and Akita/+ kidney endothelial cells expressed VE-cadherin and PECAM-1 and were positive for B4-lectin (a mouse microvascular endothelial cell-specific lectin) (Fig. 1A). Figure 1B shows that the morphology of kidney endothelial cells prepared from wild-type and Akita/+ mice was similar on gelatin-coated plates.

We next assessed apoptosis and proliferation rates in kidney endothelial cells. Minimal amounts of apoptosis were observed under normal growth conditions (Fig. 1C).

Wild-type and Akita/+ kidney endothelial cells were then incubated with 5-FU for 48 h as an apoptotic challenge. A twofold decrease in viability was observed when wild-type or Akita/+ endothelial cells were incubated with 5-FU (Fig. 1C). Next, cell proliferation was examined by counting the number of cells every other day for 2 wk. Figure 1D shows that wild-type and Akita/+ kidney endothelial cells proliferated at a similar rate.

Increased fibronectin expression in Akita/+ kidney endothelial cells. The ECM composition impacts many endothelial cell functions including migration, adhesion, and ultimately the ability to undergo capillary morphogenesis. We examined ECM protein expression in serum-free conditioned medium

Figure 1. Isolation and characterization of mouse kidney endothelial cells. In A, kidney endothelial cells prepared from 8-wk-old wild-type (WT) and Akita/+ male mice were examined for expression of platelet endothelial cell adhesion molecule (PECAM)-1, vascular endothelial (VE)-cadherin, and B4-lectin by FACSscan analysis. The shaded areas show staining in the presence of control IgG. Please note similar expression of these cellular markers in all cells. In B, WT and Akita/+ kidney endothelial cells grown on gelatin-coated plates. Cells were photographed using a phase microscope in digital format at low magnification. In C, WT and Akita/+ kidney endothelial cells were incubated with solvent control or 1 mM 5-fluorouracil (5-FU) for 48 h. Apoptotic cells were determined by in situ monitoring of caspase activity. The percentage of apoptotic cells was determined as the percentage of positive cells relative to the total number of cells per 5 high-power fields (×100). *P < 0.05 (WT vs. WT + 5-FU). **P < 0.05 (Akita/+ vs. Akita/+ plus 5-FU). In D, the growth rate is shown over a 2-wk time frame for WT (○) and Akita/+ (■) kidney endothelial cells. These experiments were repeated twice with similar results.
from wild-type and Akita/+ endothelial cells. We observed a dramatic increase in fibronectin expression and decreased osteopontin expression in Akita/+ endothelial cells. TSP1 and tenascin C expression was similar in both cell types (Fig. 2). The β-catenin level in cells was used as a control for loading.

**Attenuation of Akita/+ kidney endothelial cell migration.** Changes in the ECM milieu can affect cell migration. Here, we examined endothelial cell migration using scratch wound and Transwell migration assays. Akita/+ kidney endothelial cells demonstrated a twofold decrease in the rate of migration compared with their wild-type counterparts in both the scratch wound (Fig. 3, A and B) and Transwell migration assays (Fig. 3C). Figure 3B is a quantitative assessment of the data in Fig. 3A.

**Wild-type and Akita/+ kidney endothelial cells demonstrated similar cell adhesion.** Changes in migration of Akita/+ kidney endothelial cells could be attributed to altered cell adhesion. Next, we examined the ability of wild-type and Akita/+ kidney endothelial cells to adhere to various extracellular matrix proteins including fibronectin, collagen I, collagen IV, and vitronectin (Fig. 4A). Wild-type and Akita/+ kidney endothelial cells demonstrated similar adhesion to all of these matrices. Thus changes in cell adhesion do not contribute to the aberrant migration observed in Akita/+ kidney endothelial cells.

To ensure that the changes in migration we observed were not due to expression and/or activity of integrins expressed on the surface of these cells, we determined expression of various integrins on the surface of kidney endothelial cells by FACScan analysis (Fig. 4B). Akita/+ kidney endothelial cells demonstrated increased expression of α5-integrin compared with their wild-type counterparts. However, both wild-type and Akita/+ kidney endothelial cells expressed similar levels of α4-, αβ3-, β1-, and β3-integrin on their surfaces. We also examined expression of ICAM-1 and VCAM-1 (Fig. 4C). ICAM-1 and VCAM-1 levels were similar in wild-type and Akita/+ kidney endothelial cells.

**Attenuation of Akita/+ kidney endothelial cell capillary morphogenesis and aortic sprouting.** Capillary morphogenesis plays a fundamental role in vascular development and remodeling. To assess capillary morphogenesis, wild-type and Akita/+ kidney endothelial cells were plated on Matrigel. Wild-type kidney endothelial cells formed an extensive network within 24 h, while Akita/+ kidney endothelial cells only formed rudimentary structures (Fig. 5A). Longer incubation of the cells did not result in further branch formation. The quantitative assessment of the data demonstrated a 10-fold decrease in branch points in Akita/+ endothelial cells in this assay.

Since we observed that Akita/+ kidney endothelial cells underwent minimal capillary morphogenesis (Fig. 5A), we utilized an aortic ex vivo angiogenesis assay to further investigate the impact diabetes had on vascular sprouting. We harvested aortas from Akita/+ at 4 wk of age (onset of diabetes), 8 wk of age (4 wk of diabetes), and 12 wk age (8 wk of diabetes). At 4 and 8 wk of age, the number of sprouting outgrowths in aortas from wild-type and Akita/+ mice were similar. However, at 12 wk of age, the ability of aortas from Akita/+ mice to generate sprouting outgrowths decreased significantly (Fig. 5B). The quantitative assessment of the data demonstrates a fivefold decrease in sprouting per millimeter tissue edge in aortas from 12-wk-old Akita/+ mice compared with their wild-type counterparts. Next, aortic ex vivo cultures from 12-wk-old Akita/+ mice were incubated with an antioxidant, 1 mM NAC. NAC incubation resulted in a threefold increase in sprouting outgrowths in aortas from 12-wk-old Akita/+ mice (Fig. 5C). Thus increased generation of ROS in aortas from 12-wk-old Akita/+ mice may inhibit sprouting.

**Increased phospho-eNOS expression in Akita/+ kidney endothelial cells.** VEGF promotes angiogenesis through activation of Akt1 and eNOS (2, 6, 11). Akita/+ kidney endothelial cells demonstrated increased phospho-eNOS and total eNOS expression compared with wild-type cells (Fig. 6A). NO production also increased approximately fourfold in Akita/+ kidney endothelial cells compared with their wild-type counterparts (Fig. 6B). Consistent with the inability of Akita/+ endothelial cells to undergo capillary morphogenesis, we observed a fourfold decrease in VEGF expression (Fig. 6C). We next examined the expression of Akt, phosphorylated Akt1 and HSP90 in lysates from wild-type and Akita/+ endothelial cells.
Expression of Akt1, phospho-Akt1, and HSP90 was similar in wild-type and Akita/+ kidney endothelial cells. Thus increased p-eNOS expression correlated with increased NO production in Akita/+ kidney endothelial cells and may contribute to increased oxidative stress associated with diabetes.

Increased NO production and eNOS expression in Akita/+ kidney endothelial cells (Fig. 6A) corresponded with an inability to undergo capillary morphogenesis (Fig. 5A), suggesting uncoupling of NO function resulting in enhanced production of peroxynitrate. Thus increased nitrative stress may exist in Akita/+ endothelial cells. To determine whether this was indeed the case, wild-type and Akita/+ endothelial cells were stained with anti-nitrotyrosine (Fig. 6D). Akita/+ endothelial cells stained with anti-nitrotyrosine demonstrated an increased mean fluorescence intensity compared with their wild-type counterparts, indicating increased nitrative stress.

siRNA knockdown of eNOS in Akita/+ kidney endothelial cells restores migration and capillary morphogenesis. Figure 7A demonstrates knockdown of eNOS expression in Akita/+ kidney endothelial cells utilizing targeted siRNA reduced eNOS and p-eNOS expression while the siRNA control did not. Next, we examined whether decreasing eNOS expression in Akita/+ kidney endothelial cells was sufficient to increase cell migration and restore capillary morphogenesis. The sieNOS Akita/+ kidney endothelial cells displayed increased migration to the level observed in wild-type cells (Fig. 7B). As shown in Fig. 7B, sicontrol Akita/+ kidney endothelial cells migrated at a similar rate to Akita/+ cells. A quantitation is shown in Fig. 7C. Previous studies from our laboratories indicated that optimal endothelial cell migration is essential for capillary morphogenesis in vitro (4, 7, 14, 29). Next, we determined whether decreasing eNOS expression restored capillary morphogenesis. Figure 7D demonstrates partial restoration of capillary morphogenesis in si eNOS Akita/+ kidney endothelial cells. The enhanced endothelial cell migration and capillary morphogenesis of si-eNOS kidney endothe-
lial cells may be due to restoration of VEGF expression in these cells (Fig. 7E). Thus increased eNOS expression may dampen the ability of Akita/+ kidney endothelial cells to undergo angiogenesis.

Similar kidney vascular density in wild-type and Akita/+ mice with a short duration of diabetes. To determine whether the functional changes observed in endothelial cells translated in vivo with peritubular capillary loss during the early stages of diabetes, we immunostained kidney sections from 12-wk-old wild-type and Akita/+ mice with anti-PECAM-1. Wild-type and Akita/+ mice demonstrated significant PECAM-1 staining. The number of peritubular capillaries per tubule and capillaries per square millimeter of glomeruli were similar (Fig. 8). Thus vascular rarefaction was not observed with short-duration diabetes in vivo, but rather early endothelial dysfunction may set the stage for kidney vascular rarefaction as the disease progresses.

DISCUSSION

Diabetes incidence and complications, such as diabetic nephropathy and retinopathy, are steadily rising each year. Both diabetic nephropathy and retinopathy have endothelial dysfunction contributing to disease progression in a yet undeter-
Fig. 5. Akita+/+ kidney endothelial cells fail to undergo capillary morphogenesis. In A, WT and Akita+/+ kidney endothelial cells were plated in Matrigel and photographed in digital format. The quantitative assessment of the data is shown on the right. The data are the mean number of branch points from 10 high power fields (×100) ± SD. Note Akita+/+ kidney endothelial cells fail to undergo capillary morphogenesis. In B, aortic rings were prepared from 4-, 8-, and 12-wk-old WT and Akita+/+ male mice and embedded in Matrigel for 5 days, photographed, and the mean area of outgrowths was quantitated. Note the significantly decreased vascular sprouting in aortas from 12-wk-old Akita+/+ mice (*P ≤ 0.05). In C, aortic rings were prepared from 12-wk-old mice and embedded in Matrigel in the presence or absence of 1 mM N-acetylcysteine (NAC). These experiments were repeated with aortas from 5 different mice with similar results. *P < 0.05 (WT vs. Akita+/+). **P < 0.05 (Akita+/+ vs. Akita+/+ NAC).
mained manner. Recent studies suggest that glycemic control, particularly in the early stages of diabetes, can reduce the incidence of microvascular complications (9, 20). Hyperglycemia upregulates fibronectin expression and leads to vascular basement membrane thickening and dysfunction (3, 22). Recent studies indicated that tight glycemic control downregulated fibronectin expression and prevented basement membrane thickening. Here, we examined whether a short duration of diabetes in vivo impacts the kidney endothelial cell function in vitro, when these cells are maintained in culture under ambient glucose conditions. We observed that kidney endothelial cells exposed to hyperglycemia in vivo display endothelial cell dysfunction, including upregulation of fibronectin expression, in vitro, consistent with metabolic memory contributing to renal vascular fate during diabetes (10, 16, 17).

Excessive deposition of ECM in the mesangium and tubulointerstitium during diabetes mirrors the progressive decline in renal function associated with this disease. Although enhanced production of ECM proteins is well recognized during diabetes, altered expression of these proteins in the endothelium is less defined. Here, we show that aberrant ECM expression in Akita/+ endothelial cells, particularly fibronectin, was accompanied by decreased capillary morphogenesis. Our previous studies in kidney epithelial cells showed that increased fibronectin and α5-integrin expression correlated with attenuation of tubular morphogenesis (Ref. 33 and our unpublished observations). Enhanced fibronectin production and increased oxidative stress with the progression of diabetes could result in an inability to repair damage incurred during the course of the disease. Therefore, inability to repair damage due to attenuated capillary morphogenesis could lead to destabilization of kidney vasculature and rarefaction with disease progression.

Microvascular complications can be the result of unbalanced production of angiostatic and proangiogenic factors and are implicated in high diabetic morbidity and mortality rates (30). For example, dysregulation of kidney VEGF expression is thought to play a causative role in the pathogenesis of diabetic nephropathy. VEGF enhances eNOS activity, thereby inducing NO production. NO functions in vasodilation, regulation of smooth muscle proliferation, and expression of cellular adhesion molecules such as VCAM-1. Kidney biopsies from diabetic patients demonstrated reduced levels of VEGF (1). Our studies also demonstrated decreased VEGF levels but increased levels of eNOS and NO, suggesting uncoupling of VEGF-mediated NO production. Other investigators have also noted increased eNOS expression and activity during the early stages of diabetes (12, 31). The increased NO produced via increased eNOS expression and/or activity through a VEGF-independent mechanism may explain the intrarenal vasodilation and hyperfiltration observed during the early stages of diabetes. We observed not only increased activation but also increased eNOS expression in Akita/+ endothelial cells. Protein kinases such as Akt can regulate NO production. NO functions in vasodilation, regulation of smooth muscle proliferation, and expression of cellular adhesion molecules such as VCAM-1. Kidney biopsies from diabetic patients demonstrated reduced levels of VEGF (1). Our studies also demonstrated decreased VEGF levels but increased levels of eNOS and NO, suggesting uncoupling of VEGF-mediated NO production. Other investigators have also noted increased eNOS expression and activity during the early stages of diabetes (12, 31). The increased NO produced via increased eNOS expression and/or activity through a VEGF-independent mechanism may explain the intrarenal vasodilation and hyperfiltration observed during the early stages of diabetes. We observed not only increased activation but also increased eNOS expression in Akita/+ endothelial cells. Protein kinases such as Akt can regulate NO production and eNOS activation. Here, we demonstrated that increased eNOS expression in Akita/+ kidney endothelial cells occurred in an Akt-independent manner. Thus it is tempting to speculate that activation of eNOS per se is not the limiting step but rather that it is the increased eNOS expression that lends itself to increased eNOS activation. This is consistent with the supposition that increased eNOS expression during diabetes is a
Fig. 7. Small interfering (si) RNA knockdown of eNOS in Akita/+ cells increases migration and capillary morphogenesis. Protein lysates (35 μg) from WT and Akita/+ kidney endothelial cells and Akita/+ cells infected with control (sictrl) or siNOS were analyzed by Western blot analysis for expression of phospho-eNOS and total eNOS. β-actin expression was assessed as a loading control (A). In B, cell migration was determined by scratch wounding of kidney endothelial cell monolayers, and wound closure was monitored by photography and quantitated in C. In D, kidney endothelial cells were plated in Matrigel and photographed in digital format. The quantitative assessment of the data is shown in on the bottom. The data are the mean number of branch points from 10 high-power fields (×100) ± SD. Note sieNOS Akita/+ kidney endothelial cells demonstrate increased capillary morphogenesis. *P < 0.05 (WT vs. Akita/+). **P < 0.05 (Akita/+ siCtrl vs. Akita/+ sieNOS). E: an immunoassay was used to determine VEGF levels (pg/ml) in kidney endothelial cells from WT and Akita/+ mice and those expressing siCtrl or sieNOS constructs.* P < 0.05 (WT vs. Akita/+ and Akita/+ vs. siCtrl). **P < 0.05 (Akita/+ and Akita/+ siCtrl vs. Akita/+ sieNOS).
counterregulatory mechanism to increase NO production (8). PKC activation (18) and hydrogen peroxide (5) can increase eNOS expression, both of which could be a factor during diabetes (8). The identity of the mechanisms and the proteins involved are the subject of current investigation in our laboratories.

Chronic hyperglycemia during diabetes can trigger the generation of free radicals and oxidative stress. Enzymatic and nonenzymatic sources contribute to ROS observed in the diabetic kidney, including advanced glycation, mitochondrial respiration chain deficiencies, xanthine oxidase activity, peroxidases, NOS, and NAD(P)H oxidase. Kidney vascular cells are negatively affected by oxidative stress during diabetes. Kidney endothelial cells from Akita/+ mice in the early stages of diabetes (4 wk of diabetes) demonstrate a fourfold increase in NO production and increased expression of phospho-eNOS expression compared with their wild-type counterparts. Increased generation of NO can lead to enhanced production of peroxynitrite. Knocking down eNOS expression increased VEGF expression, restoring Akita/+ endothelial cell migration and capillary morphogenesis. Chronic hyperglycemia promotes excessive oxidative stress in the vasculature, leading to vascular dysfunction. Consistent with this notion, NAC incubation of aortas from 12-wk-old Akita/+ mice facilitated increased sprouting outgrowths. Although glomerular changes including mesangial proliferation and ECM accumulation are well documented, hyperglycemia-induced changes in the vasculature are less clearly understood and may set the stage for diabetic nephropathy.

The role of angiogenesis during diabetes remains unclear. Several studies suggest that increased angiogenesis in the early stages of diabetes may correlate with glomerular hypertrophy associated with diabetes (19). Angiogenesis can be associated with imbalanced proliferation and apoptosis. Levels of apoptosis and proliferation were similar in wild-type and Akita/+ kidney endothelial cells. VEGF levels decreased in Akita/+ kidney endothelial cells similar to what is typically observed in the kidney as diabetes progresses. Akita/+ kidney endothelial cells also had increased eNOS expression and NO production, which could stimulate vasodilation. Previous studies demonstrated that lowering blood pressure in diabetic mice blocked aberrant angiogenesis and inhibited VEGF expression (15).

The studies presented here demonstrate that the angiogenic capacity of Akita/+ endothelial cells is greatly diminished following a short duration of diabetes. In addition, uncoupled eNOS/NO production leads to increased oxidative stress, as assessed by increased peroxynitrite production. In the context of the kidney, such changes could lead to damage to the vasculature which would be difficult to repair due to its inability to undergo angiogenesis. Such changes would set the stage for vascular rarefaction and decreased kidney function as a consequence of poorly controlled hyperglycemia during diabetes. Here, we noted that knocking down eNOS expression in Akita/+ kidney endothelial cells increased VEGF expression and restored angiogenic capacity. Thus it is tempting to speculate that decreased eNOS expression may prevent vascular rarefaction during diabetes, providing a future target for therapeutic intervention.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: C.G., S.P., Y.Z., and M.E.M. performed experiments; C.G., S.P., Y.Z., M.E.M., N.S., and C.M.S. analyzed data; C.G., S.P., M.E.M., and C.M.S. prepared figures; C.G., S.P., Y.Z., M.E.M., N.S., and C.M.S. approved final version of manuscript; N.S. and C.M.S. provided conception and design of research; N.S. and C.M.S. interpreted results of experiments;
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