Loss of diacylglycerol kinase epsilon in mice causes endothelial distress and impairs glomerular Cox-2 and PGE\textsubscript{2} production

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genes encoding factors that participate in the complement cascade (30). Recently, our laboratory and others have described a genetic form of TMA that is caused by mutations in the gene that encodes the lipid kinase diacylglycerol kinase-ε (DGKε), and it is unrelated to the complement pathway (20, 31, 42). Recessive loss of function mutations in the DGKE gene cause TMA with histological features (increased deposition of mesangial matrix, thickening of the glomerular capillary walls, and splitting of the glomerular basement membrane) that resemble membrano-proliferative glomerulonephritis, but the mechanisms that lead to this disease are still unexplained (20, 31, 42). DGKs are intracellular lipid kinases devoted to phosphorylate diacylglycerol (DAG) to phosphatidic acid (36). At least 10 DGKs have been identified in mammals, differing mostly for the composition of the noncatalytic domains, which confer specific activity to each isoform (24). The epsilon isoform is a ubiquitous enzyme with higher level of expression in testis, cardiac and skeletal muscle, and kidney, where it has been demonstrated in podocytes and endothelial cells (20, 31). DGKε is constitutively active and has high specificity for DAG.
acetylated with arachidonic acid (AA) in sn-2 position (sn-2-arachidonoyl-DAG). The high substrate selectivity, together with the constitutive activation of this enzyme, results in enrichment of the cellular polyunsaturated lipids with AA acyl chains (25, 41). AA is the main precursor in the production of prostaglandins (PGs), and its cellular availability is the rate-limiting factor for the synthesis of prostanooids (19). PG synthesis is usually triggered by activation of membrane phospholipase A2 that releases AA from phospholipids in the cell membrane (19). Then the inducible enzyme PG endoperoxide synthase 2, also known as cyclooxygenase-2 (Cox-2), converts AA to the intermediate PG H2 (PGH2) that, in turn, metabolized by specific PG synthases into PG E2 (PGE2), PG I2 (PGI2), thromboxane A2 (TXA2), PGF2α, and PGD2 (12). PGs are short-lived ubiquitous signaling molecules that are important mediators in kidney physiology and pathology and are required for microvascular angiogenesis and function (12, 34). Inactivation of Dgke in mice has been shown to affect recovery from seizures after brain electric stimulation, but no glomerular phenotype has been described. Since mutations in humans result in TMA, we hypothesized that loss of Dgke in mice may cause subclinical glomerular lesions and may predispose to disease. Here we report that Dgke knockout mice have subclinical microscopic anomalies of the glomerular endothelium and basal membrane that worsen with age and develop extensive glomerular capillary occlusion when challenged with nephrotoxic serum (NS). We found that Dgke knockout mice fail to induce glomerular Cox-2 and the proangiogenic prostaglandin PGE2 after puromycin aminonucleoside (PAN)-mediated glomerulonephritis. Concordantly, with the reduced expression of Cox-2, Dgke knockout mice were partially protected from developing podocyte foot process effacement and proteinuria after PAN injury. Dgke knockout mice also showed reduced expression of the antithrombotic cell adhesion molecule platelet endothelial cell molecule-1 (PECAM-1) in the glomerular endothelium. Notably, PGE2 supplementation was able to rescue motility defects of Dgke knockout cells in vitro and to restore angiogenesis in an in vivo test. Our results suggest that Dgke has both endothelial and podocyte-specific functions, and that it is required for glomerular induction of Cox-2 and synthesis of vasoactive prostanooids.

**MATERIALS AND METHODS**

**Mice knockouts.** Dgke knockout mice were developed on a C57/B6 background and have been previously described (36). All of the experiments were previously approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center.

**NS and PAN-induced glomerulonephritis.** Glomerular basal membrane (GBM)-reactive NS was generated by Lampire Laboratories (Pipersville, PA), as previously described (43). Mice were challenged with intraperitoneal injections of complete Freund adjuvant, then with a first dose of NS (12.5 μg/g), followed by a second dose (5 μg/g) after 5 days. Equal amount of nonimmune isotypic serum (NIS) was used as a control. Fourteen days later, 24-h urine was collected, and kidneys were harvested for fixation or glomeruli isolation. PAN (Sigma, St. Louis, MO) was administered by intraperitoneal injection at the dose of 0.5 mg·g⁻¹·day⁻¹ for 2 days. An equal amount of volume per gram of body weight of saline was injected as a control. Mice were killed 3 or 10 days later, depending on the experiment. Kidneys were either fixed in 4% paraformaldehyde for histological analysis or snap frozen for immunofluorescence.

**Transmission electron microscopy.** Samples were fixed/perfused with a 0.05% glutaraldehyde solution, embedded in Epon-812, cut in thin sections (600–900 nm), and stained with Reynolds lead citrate. Electron microscopy images were acquired under a Philips Tecnai-12 electron microscope (Philips, Eindhoven, The Netherlands).

**Mouse glomeruli morphometric analysis.** Morphometric analysis of glomeruli of mice after NS and PAN-induced glomerulonephritis was performed using the software ImageJ [National Institutes of Health (NIH), Bethesda, MD; http://rsb.info.nih.gov/ij/]. Whole kidneys stained with hematoxylin and eosin (H&E) were imaged using Zeiss Axiocam Z1 microscope. Glomerular occlusion was determined by measuring the ratio of patent capillary area over total glomerular area examined. Electron microscopy images were used to quantify the extent of podocyte foot process effacement and the proportion of stressed endothelial cells. Podocyte foot process effacement was expressed as proportion of the length of basal membrane covered by two or more consecutive effaced foot processes over total linear length examined. Stressed endothelio-
lial cells (swollen cells with nuclei occluding more than 50% of the capillary lumen or with clear signs of endotheliosis) were manually detected in 20 consecutive capillary loops from multiple glomeruli. Results were expressed as proportion of stressed cells per number of examined capillary loops.

**Immunofluorescence microscopy.** The following antibodies were used: Alexa Fluor 488 conjugate goat anti-rat IgG (Invitrogen, A-11006), C3 (Abcam, ab11862) PECAM-1/CD31 (BD Biosciences, 553930), fibrin (Dako, a0080), and WT-1 (Santa Cruz, sc-7385). After standard preparation, tissues were incubated overnight at 4°C in primary antibody and for 1 h at room temperature with the fluorescently labeled secondary antibody and mounted. Images were acquired using a Zeiss Axiosplan 2 deconvolution microscope or a Zeiss LSM 510 confocal microscope.

**Lentiviral infection, shRNA-mediated gene silencing.** Human umbilical vein epithelial cells (HUVECs) were obtained by American Type Culture Collection (Manassas, VA). DGKE and green fluorescent protein (GFP)-targeting short hairpin RNA (shRNA) lentiviral constructs were purchased by Open Biosystems (Lafayette, CO). The experiments were performed as previously described (21).

**Wound-healing test for endothelial cell migration.** HUVECs were plated on glass-bottomed dishes for 48 h to allow them to reach full confluence. A wound in the monolayer was then created by scratching the cells with a pipette tip, and images were taken 6 h later using a Zeiss LSM 510 microscope at four different points along the wound. Migration quantification was performed using the software ImageJ (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/) and was expressed as a percentage of the distance covered by migrating cells over the initial distance between the edges of the wound. PGE2 was purchased by Cayman Chemicals (Ann Arbor, MI) and was used at a final concentration of 3 μM.

**In vivo angiogenesis assay.** The subcutaneous sponge model was used to determine the effects of prostanoids on in vivo angiogenesis (34). Sterile polyvinyl-acetal CF-50 round sponges (6-mm diameter, 50-μm pores, Medtronic Xomed) were implanted under the flank skin of wild-type and Dgke knockout mice (10 wk of age, 25 g body wt). After standard preparation, tissues were incubated overnight at 4°C in primary antibody and for 1 h at room temperature with the fluorescently labeled secondary antibody and mounted. Images were acquired using a Zeiss Axiosplan 2 deconvolution microscope or a Zeiss LSM 510 confocal microscope.

**Quantitative real-time PCR.** Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and purified with Qiagen RNeasy Mini Kit, according to the manufacturer’s protocols. First-strand reverse transcription reactions were performed using the ThermoScript RT-PCR Kit (Invitrogen). Real-time PCR were performed using iQ SYBR Green Supermix (Bio-Rad). The β-actin gene was used as a normalizer.

**Measurement of serum creatinine and blood urea nitrogen.** Serum creatinine concentrations were determined by capillary electrophoresis at the physiology core of O’Brien Center for Kidney Diseases in Dallas, TX. Blood urea nitrogen measurements were obtained using the Vitrosp 250 chemistry analyzer.

**Measurement of urinary albumin and creatinine.** Twenty-four-hour urine was collected using metabolic cages. Urinary albumin concentration was determined using the Albuewell microalbumin ELISA essay (Exocell, Philadelphia, PA). Urinary creatinine concentration was determined using a P/ACE MDQ Capillary Electrophoresis System and photodiode detector (Beckman-Coulter, Fullerton, CA) at 214 nm (44) in the physiology core of the O’Brien Center for Kidney Diseases in Dallas. Urinary albumin concentrations were normalized to the amount of excreted creatinine.

**Measurement of urinary prostanoids.** Prostanoids were extracted by solid phase chromatography using octadecyl silica cartridges, resuspended in 25 μl of 70:30 methanol/water solution, and subjected to triple-quadruple (Waters, TQ-S) electrospray tandem mass spectrometry after adding an internal standard (Cayman Biochemicals, Ann Arbor, MI) for quantification. Urinary prostanoid concentrations were normalized to milligrams of excreted creatinine.

**Statistical analysis.** Data analysis was performed using Excel (Microsoft) and Prism (version 5.03) software (GraphPad Software, San Diego, CA). Data are presented as means ± SE, unless otherwise specified. Two-tailed unpaired Student’s t-test or nonparametric Mann-Whitney test were used to compare two groups of data.

**RESULTS**

Glomeruli of Dgke knockout mice have submicroscopic signs of endothelial impairment that become more pronounced with age. Dgke knockout mice, which were previously generated to study the contribution of arachidonate signaling to epileptogenesis, are fertile and do not present any apparent phenotype (36). Dgke−/− light microscopy examination of H&E sections of Dgke knockout kidneys between 4 and 6 wk of age did not show significant differences, compared with wild-type littermates (Fig. 1A). Careful examination of transmission electron microscopy (TEM) images of Dgke knockout glomeruli, instead, evidenced modest signs of endothelial stress that were not detected in wild-type mice (Fig. 1, B and C): some endothelial cells in Dgke−/− glomeruli lost the normal fenestrated appearance and presented swelling of the endothelial rim. Segments of the GBMs were also abnormally inspissated or duplicated for the interposition of mesangial cells appendices, whereas the epithelial compartment was relatively well preserved, but no significant differences in albuminuria were detected between knockout and wild-type mice (Fig. 1D). The glomerular lesions became more severe in Dgke−/− kidneys.
Fig. 2. Dgke KO mice develop glomerular thrombotic microangiopathy when injected with nephrotoxic serum (NS). A: representative images of Dgke KO mice glomeruli (left) after injection of subtoxic doses of rabbit NS compared with WT littermates (right). Glomeruli of KO mice show increased mesangial matrix (arrowheads), diffuse congestion of the capillary loops (black arrow), and numerous entrapped erythrocytes (white arrow). Capillary obliteration is not detectable in WT littermates (right) after injection of the same dose of NS (arrows indicate patent capillary loops) and in Dgke KO mice treated with nonimmune isotypic serum (NIS; center). H&E staining, scale bars = 20 μm. Bottom: immunofluorescence microscopy images of glomeruli of the same mice probed with an antibody against rabbit IgG (rIgG), showing that the animals received comparable amount of NS or no NS in the NIS control. Mice were 6 wk of age at the date of the experiment. Scale bars = 20 μm. B: quantification of the average patent capillary area in glomeruli of WT and Dgke KO mice after injection of NS or of isotypic NIS as a control, obtained by digital image analysis. Values are means ± SE; n = 2 mice per group, with 10 optical fields per mouse. P values were calculated by Student’s t-test. C: TEM images of Dgke KO after NS or isotypic NIS injections and WT littermate glomeruli after NS injection. In kidneys of Dgke KO mice after NS injection (left) mesangial cells (m) are focally increased in number and infiltrate segments of the GBM (white asterisk). Endothelial cells (black asterisks) are swollen, and capillary rims are irregular and lack the typical fenestrations (black arrows). Lumens are partially obliterated by swollen endothelium and trapped irregular-shaped erythrocytes (stars). Notice that podocytes’ foot processes (black arrowhead and inset) are relatively well conserved. Capillary loops of Dgke KO treated with NIS and of WT littermates after NS injections (stars; middle and right) are patent, endothelial fenestrations are clearly detectable (arrows and insets), and podocytes’ foot processes appear normal (arrowhead). Scale bars = 10 μm. D and E: TEM images at higher magnification of Dgke KO glomeruli after injection of NS that show bulky endothelial cells (black asterisks) with almost completely obstructed capillary lumens and the interposition of a mesangial cell (E; white asterisk), which results in the splitting of the basal membrane (E; black arrowheads). Scale bars = 2 μm. F: quantification of the number of stressed endothelial cells per number of capillary loops in glomeruli of Dgke KO and WT mice 14 days after injection with NS. Values are means ± SE; n = 20 consecutive capillary loops form multiple glomeruli, with 2 mice per experimental group.
knockout mice at 1 yr of age: in H&E kidney sections, glomeruli had more frequently the typical “avascular” appearance, focal thickening of the capillary walls, and prominent endothelial cells nuclei (Fig. 1E). By TEM, signs of important endotheliosis, mesangial interposition, and splitting of the basal membrane were also evident (Fig. 1F and G), recapitulating the glomerular human pathology of subjects with DGKE loss of function mutations. Urinary albumin tended to be higher in Dgke knockout mice compared with wild-type controls (Fig. 1H).

A

B

C

D

E

F

H

G

I

Image A: ACR (μg/mg)

Image B: Effective renal glomerular length (μm)

Image C: WT vs Dgke−/−

Image D: WT-1 positive cells/glomerulus

Image E: Serum hemoglobin (g/dL)

Image F: Dgke−/− vs Dgke+/−

Image H: CD31

Image G: % of positive glomerular area

Image I: CD31+ area/glomerular (%)
Dgke knockout mice are more susceptible to glomerular injury when challenged with NS. To determine if Dgke knockouts were more exposed of wild-type mice to develop acute phenotype at younger age, we used an experimental model of immune-mediated glomerulonephritis, challenging the mice with NS (10, 43). To this purpose, we injected 4-wk-old Dgke knockouts and wild-type littermates with Freund’s adjuvant, followed, after 5 days, by the injection of subtoxic doses of NS or NIS as a control. The mice were killed 14 days later, and kidneys were examined by light and electron microscopy. H&E sections of kidneys of Dgke knockout mice that received NS had evident signs of microvascular congestion: capillary loops, were partially or completely obliterated, and trapped erythrocytes were frequently observed. Hypercellularity and mesangial expansion were also evident (Fig. 2, A, top left, and B). Oppositely, Dgke+/+ mice challenged with NS (Fig. 2, A, top right, and B) and Dgke knockout mice that received NIS (Fig. 2, A, top middle, and B) appeared mostly normal, showing normocellular glomeruli and patent capillary tufts. Similarly, TEM of glomeruli of Dgke−/− mice treated with NS (Fig. 2, C, left, and F) presented mesangial expansion and infiltration of the GBM. Loss of endothelial fenestrae, diffuse swelling of the endothelial cells that partially obliterated the capillary lumens, and trapped erythrocytes. Instead, capillary loops of Dgke knockout mice treated with NIS and of wild-type littermates’ glomeruli after NS (Fig. 2, C, center and right, and F) were patent with clearly detectable endothelial fenestrations. Dgke knockout mice had higher levels of urinary albumin (Fig. 3A) and more diffuse foot process effacement (Fig. 3B) 14 days after NS injection, suggesting impaired repair capacity after immune-mediated glomerular injury. No significant differences were detected in the number of glomerular podocytes (Fig. 3, C and D) and in serum hemoglobin, red blood cells, and platelet counts among all of the experimental groups (Fig. 3E). On immunofluorescence microscopy, glomeruli of Dgke knockout mice treated with NS were more often positive for complement C3 (Fig. 3, F and G) and expressed less anti-thrombotic PECAM-1 (CD31 in the figures, Fig. 3, H and I), compared with wild-type NS-treated controls. This data indicate that lack of Dgke predisposes to extensive glomerular capillary occlusion after immunemediated glomerular injury.

Dgke knockout mice are resistant to PAN-induced glomerulonephritis. The model of immune-mediated nephritis used in the previous experiment is based on complement activation that is triggered by the deposition of antibodies raised against GBM antigens. To test if Dgke knockout mice are more susceptible also to IgG/complement-independent glomerular injury, we treated mice with PAN, a drug with selective podocyte toxicity that induces self-limited damage, independent from complement activation (16). Dgke knockouts and wild-type littermates between 8 and 10 wk of age were injected with PAN (0.5 mg·g−1·day−1 for 2 days), and kidney histology was evaluated after 3 days, when the highest acute PAN toxicity has been reported (2), and 10 days, to monitor recovery from injury. By light microscopy of H&E-stained kidney sections, we observed congestion in some glomeruli of Dgke knockout and wild-type mice treated with PAN (Fig. 4A), but no statistically significant difference was detected between these two groups (Fig. 4B, 150 glomeruli examined, 50 random chosen glomeruli per mouse, 3 mice per group). In TEM images (Fig. 4C), some glomeruli of knockout and wild-type mice treated with PAN showed signs of mild endothelial stress (swollen endothelial cells with enlarged nuclei, absence of capillary fenestrations, and blebs of the endothelial rim), whereas podocytes’ foot processes were often well conserved. Conversely, podocytes’ foot processes in wild-type mice treated with PAN were extensively effaced, as shown by morphometric analysis of foot process effacement of TEM images (Fig. 4D, 10 glomeruli per mouse, n = 4 mice per group). Consistent with this finding, urinary albumin-to-creatinine ratios from 24-h urine collections were lower in Dgke knockouts after PAN injection compared with wild-type controls 3 days after treatment, and recovered faster by day 10 (Fig. 4E). No differences in the number of podocytes were detected between the experimental groups (Fig. 4F). By immunofluorescence microscopy, we detected complement C3 in a single glomerulus in one Dgke knockout (Fig. 4G, arrow) but never in wild-type mice (50 glomeruli counted per mouse, n = 3 mice per group). We conclude that, at the used doses, PAN induces mild endotheliosis in both Dgke knockout and wild-type mice, and that Dgke knockout podocytes are more resistant to PAN injury compared with wild-type controls.

Dgke is required for the expression of the inducible enzyme Cox-2 in normal glomeruli. Cox-2 is a ubiquitous enzyme required for a rate-limiting reaction in the synthesis of PGs from AA. Unlike the cognate enzyme Cox-1 that is constitutively active, Cox-2 is an inducible enzyme (33, 38). In the kidney, Cox-2 is expressed in glomeruli, thick ascending limb of Henle, and in medullary interstitial cells (14). Importantly, Cox-2 is expressed by podocytes, and it is required for correct glomerular homeostasis (3, 13): both increased and decreased Cox-2 expression in mouse podo-
cytes, in fact, result in different forms of glomerular disease, indicating that a balanced activity of this enzyme is necessary for correct glomerular development and maintenance (3). Microarray expression analysis of mouse brains after electrical shock had previously revealed that Dgke knockouts failed to induce transcription of Cox-2 compared with wild-type controls (22). We tested if Cox-2 expression after stress was also compromised in other tissues and more specifically in kidneys. Since Cox-2 expression is induced in macrophages (39) and fibroblasts (5) when stimulated with lipopolysaccharide (LPS) and with interleukin 1β (IL-1β), respectively, we measured by quantitative real-time PCR the levels of Cox-2 mRNA in Dgke−/− and wild-type bone marrow-derived macrophage precursors, 12 h after stimulation with LPS (4 ng/ml) and in skin primary fibroblasts cultured for 4 h in medium supplemented with IL-1β (2 ng/µl). We found that expression of Cox-2 was upregulated after exposure to LPS in wild-type bone marrow-derived macrophages.
macrophage precursors (Fig. 5A), but not in Dgke−/− cells, and in wild-type, but not in knockout fibroblasts, exposed to IL-1β (Fig. 5B). Western blots of fibroblasts extracts confirmed the impaired expression of Cox-2 in Dgke null cells, both in basal conditions and after IL-1β stimulation (Fig. 5C). No changes in Cox-1 expression in wild-type and knockout fibroblasts were detected by quantitative real-time PCR and Western blot (not shown). Then we asked if knockout fibroblasts were detected by quantitative real-time PCR and by Western blot (not shown). We examined by quantitative real-time PCR and by Western blot cortexes from Dgke knockout mice after PAN injection compared with wild-type littersmates (Fig. 5E). Importantly, Cox-2 expression was also impaired in Dgke−/− isolated glomeruli (Fig. 5F). The lower expression levels of Cox-2 in Dgke null glomeruli compared with wild-type is consistent with the reduced proteinuria and podocytes foot process effacement observed in Dgke knockout mice, in agreement with the detrimental effect of Cox-2 on these parameters (3, 4, 40).

**Lack of Dgke affects prostanoid synthesis in mouse kidneys.** Cox-2 and the cognate enzyme Cox-1 catalyze the formation of the cyclic endoperoxides PGG2 and PGH2, which are obligate intermediates for the synthesis of prostanoids. Although Cox-2 and Cox-1 potentially act on the same substrate, they have a different affinity for AA and are functionally coupled with different prostanoid synthases (27). As a consequence, PGE2 and PGF2α synthesis in the kidney is mostly dependent on Cox-2 activity (33). PGE2 is the most abundant prostanoid in the kidney and has multiple effects on the glomerular circulation, acting as a vasodilator of the afferent arteriole and involved in complement activation. PGE2 supplementation could also improve the migration of HUVEC cells in vitro and to sustain angiogenesis in vivo (28, 34). For this reason, we measured the levels of PGE2 and PGF2α in the urine of Dgke−/− mice and wild-type littersmates, 3 days after PAN injection, by liquid chromatography-electrospray tandem mass spectrometry (23). Concordant with Cox-2 expression, the urinary excretion of these two Cox-2 enzymatic products was reduced in Dgke−/− mice compared with wild-type controls after PAN injection (Fig. 6), indicating impaired induction of prostanoid synthesis in Dgke−/− null mice after podocyte stress. Twenty-four-hour urinary 11-dehydrothromboxane B2, the degradation product of TXA2, was also lower in knockout mice after PAN injection compared with the wild type, although the difference did not reach statistical significance.

Dgke knockout endothelial cells present defects that improve with PGE2 supplementation. Recently, loss of Dgke has been associated with impaired motility and angiogenesis, distress of endothelial cells, and increased apoptosis in vitro, suggesting a cell-autonomous endothelial function of Dgke (1). Given the important pro-angiogenic effect of PGE2 on endothelial cells (1, 34), we hypothesized that impaired PGE2 production in Dgke knockout mice may concur to the endothelial stress observed in the absence of Dgke. Activated endothelial cells express more pro-thrombotic factors, such as intercellular adhesion molecule-1 and tissue factor, and less anti-thrombotic molecules, such as PECAM-1. PECAM-1 is a cell-cell junction protein that is important for normal angiogenesis (6) and is known to inhibit thrombus formation in vivo (8). To test the expression of this protein in Dgke knockout glomeruli, we probed wild-type and Dgke knockout kidneys treated either with PAN or vehicle, with an antibody against PECAM-1. Digital analysis of immunofluorescence microscopy images showed that glomeruli of knockout mice after PAN had reduced PECAM-1 expression, compared with wild-type mice (Fig. 7, A and B). Expression of PECAM-1 was also impaired in cortexes of Dgke knockout mice in basal conditions and 3 days after PAN injection, as detected by quantitative real-time PCR (Fig. 7C). To verify if PGE2 may affect PECAM-1 expression in Dgke defective endothelial cells, we silenced Dgke in HUVEC cells by shRNA and estimated the amount of PECAM-1 by Western blot after exposing these cells to PGE2 for 4 h. We found that PECAM-1 protein levels were lower in HUVEC cells after Dgke silencing compared with the GFP-silenced controls. The amount of PECAM-1 increased, though, after exposing HUVECs to PGE2-supplemented medium, indicating that PGE2 is required to induce PECAM expression in HUVECs (Fig. 7D). We asked, then, if PGE2 supplementation could also improve the migration defects presented by the Dgke knockout HUVECs in vitro (1). To this purpose, we assayed the effect of PGE2 on migratory ability of Dgke knockout HUVEC cells in a wound-healing assay in vitro, by measuring the migration of Dgke knockout cells on a coverslip after scratching the cell monolayer with a pipette tip. The distance covered by migrating cells after wounding was lower in Dgke knockout cells compared with GFP-targeted controls, as previously described.
Cyclooxygenase-2 (Cox-2) induction is impaired in Dgke null cells. 

**A**: quantification of Cox-2 expression, measured by real-time PCR, in bone marrow-derived macrophage (Mφ) precursors, isolated from Dgke KO mice and WT littermates and exposed to LPS or Veh as a control. Cox-2 expression is induced by stimulation with LPS in Mφ isolated from WT but not from Dgke KOs (n = 6 mice per group). AU, arbitrary units. Values are means ± SE. P values were calculated by Student’s t-test.

**B**: quantification by real-time PCR of Dgke KO and WT skin fibroblasts stimulated with IL-1β. Cox-2 expression is decreased in mutant fibroblasts and fails to be induced after IL-1β stimulation. Values are means ± SE; n = 4 mice per group. P values were calculated by Student’s t-test.

**C**: Western blot of Dgke KO and WT fibroblast lysates exposed to IL-1β or Veh as a control, probed with an antibody against Cox-2. Two replicates were loaded for each experimental condition. Cox-2 was undetectable in Dgke KO fibroblasts before and after stimulation with IL-1β, but was abundantly expressed in WT cells. For two WT samples (column 3 and 4 from left), one-half of the amount of protein was loaded to show the increase of Cox-2 expression after exposure to IL-1β.

**D**: quantification by real-time PCR of the transcripts of Cox-2 in kidney cortices of Dgke KO mice and WT littermates, 3 days after the injection of PAN or Veh as a control. Cox-2 expression in Dgke KO cortices is increased in PAN-treated WT mice, but not in Dgke KOs. Values are means ± SE; n = 4 mice per group. P values were calculated by Student’s t-test.

**E**: Western blot showing the amount of Cox-2 in kidney cortices of Dgke KO mice and WT controls, 3 days after treatment with PAN or Veh as a control. Cox-2 is increased in cortices of PAN-treated WT mice, but not in Dgke KOs. Values are means ± SE; n = 4 mice per group. P values were calculated by Student’s t-test.

**F**: quantification by real-time PCR of Cox-2 expression in isolated glomeruli from Dgke KO mice and WT littermates, 3 days after the injection of PAN or Veh as a control. Cox-2 expression in Dgke KO glomeruli is induced in PAN-treated WT mice, but not in Dgke KOs. Values are means ± SE; n = 4 mice per group. P values were calculated by Student’s t-test.

Fig. 6. Lack of Dgke affects prostanooids synthesis in mouse kidneys. Urinary concentrations of prostaglandins (PG) E2 (A), PGF2α (B), and 11-dehydrothromboxane B2 (11-dh-TxB2; C) in 24-h urine of Dgke KO mice and WT controls in basal conditions and 3 days after PAN injection or Veh as a control, determined by liquid chromatography-electrospray tandem mass spectrometry. Excretion is significantly lower in Dgke KOs compared with controls after PAN treatment. Box and whiskers are 25th and 75th and minimum and maximum, respectively. Lines represent medians; dots correspond to means. Values are means ± SE; n = 4 samples per group. P values were calculated with the Mann-Whitney rank sum t-test. uCr, urinary creatinine.
(1), but improved with PGE2 supplementation (Fig. 7, E and F). Finally, to explore if PGE2 supplementation may affect endothelial angiogenesis also in vivo, we performed experiments of angiogenesis in subcutaneous sponge implants (1, 32). Sterile polyvinyl-acetal sponges were implanted under the flank skin of Dgke knockout mice and of wild-type littermates (8–12 wk of age, n = 4 mice) and were injected with PGE2 or with equal volume of PBS, on alternate days for 14 days. After this time, mice were killed, and sponges were collected, fixed with paraformaldehyde, and probed with an antibody against PECAM-1. Images were taken by immunofluorescence confocal microscopy, and the extent of PECAM-1-positive surface or the number of positive neovascularized structures per random microscopy field was quantified by digital image analysis. We found that sponges implanted in Dgke knockout mice had reduced PECAM-1-positive structures and reduced average PECAM-1-positive surface per neovascularized structure, compared with sponges implanted in wild-type littermates (Fig. 7, G–I, n = 2 sponges per mouse in each group, 4 mice per group). Importantly, expression of PECAM-1 increased in sponges injected with PGE2, suggesting that the defective angiogenic potential of Dgke null endothelial cells may be partially reversed by PGE2.

DISCUSSION

In the present work, we have characterized the glomerular phenotype of Dgke knockout mice and investigated possible mechanisms of disease promoted by lack of Dgke. Dgke−/− mice are more resistant than wild type to develop seizures after brain electrical stimulation, but no apparent kidney phenotype has been so far reported (36). Here we have shown that, although Dgke null mice do not have spontaneous clinical signs of kidney disease and have normal serum creatinine and urinary albumin, they present microscopic glomerular lesions (endotheliosis and mesangial interposition of the GBM) that worsen with age, and that they are more susceptible to develop overt glomerular congestion after NS injury. We also tested the susceptibility of Dgke knockout mice to immuno-independent glomerular injury by administering PAN, a drug with selective podocyte toxicity (16). Administration of PAN was associated with signs of endothelial stress in glomeruli of both Dgke knockout and wild-type mice, suggesting that these lesions are secondary to podocyte injury. We did not detect significant glomerular deposition of complement C3 between Dgke knockout and wild-type mice, at the used PAN dosage. Unfortunately, we were not able to perform the experiment with higher dosage or prolonged exposure to PAN, because of the high mouse mortality that we observed in these experimental conditions. However, after NS-induced nephritis, we noticed that more glomeruli were positive for complement C3 in Dgke knockout mice compared with controls. In the attempt to reconcile these evidences, we can speculate that complement activation is not the initial determinant of the phenotype in Dgke knockouts, but Dgke null endothelial cells may be more susceptible to fix the complement secondarily, because they are more susceptible to injury and death. This hypothesis is compatible with the recent report that DGKE knockout HUVEC cells have an increased tendency to apoptosis and decreased expression of two cell surface regulators of the complement activation, the complement inhibitor membrane cofactor protein and the decay accelerating factor (1). The authors of this paper, though, failed to detect complement deposition on these cells in a test in vitro, and, therefore, more targeted in vivo studies will be required to clarify this point. Another question that will need to be addressed, which will require the use of tissue-specific Dgke knockouts, is if endothelial cells, podocytes, or both contribute to determine the glomerular lesions in the absence of Dgke. The effect of DGKε small interfering RNA-mediated silencing on HUVEC cell survival and angiogenic potential indicates cell-autonomous functions of Dgke in the endothelium. On the other hand, the impaired Cox-2 expression after stress in all of the cell lines and tissues that we have examined, including kidney glomeruli, infers that Dgke is required for Cox-2 induction also in podocytes, which is the principal glomerular source of Cox-2 (18), and may affect the defective endothelium in a cell nonautonomous fashion, through impaired production of PGE2. This hypothesis is corroborated by our in vitro and in vivo experiments that show improved angiogenic potential and PECAM-1 expression of DGKε knockout HUVEC cells after PGE2 supplementation. The resistance of Dgke null podocytes to PAN injury, in fact, argues that the reduced Cox-2 expression in Dgke null glomeruli is not secondary to loss of podocytes. Finally, the mechanisms by which Dgke controls Cox-2

Fig. 7. Dgke KO mice have endothelial defects that improve with PGE2 supplementation. A and B: representative immunofluorescence microscopy images (A) and quantification by digital image analysis (B) of glomerular CD31 expression in Dgke KO kidneys and WT mice 3 days after treatment with PGE2 or with PBS as a control. CD31 signal was lower in Dgke KO compared with controls. Glomeruli are highlighted by dotted lines. Scale bars = 50 μm. Quantification data are expressed as average ± SD (n = 3 mice, 10 optical fields per mouse). C: expression of CD31 in cortices of Dgke KO and WT mice, 3 days after treatment with PGE2 or with PBS as a control, measured by quantitative real-time PCR. Values are means ± SD of triplicate samples. P values were calculated by Student’s t-test. D: Western blot of human umbilical vein epithelial cell (HUVEC) lysates after short hairpin RNA (shRNA)-mediated silencing of DGKE (sh-Dgke) compared with control cells infected with a green fluorescent protein (GFP)-targeting shRNA (sh-GFP). CD31 is lower in Dgke-silenced HUVECs compared with the controls and increases after 4-h exposure to PGE2 supplemented medium. E: representative differential interference contrast microscopy images of DGKE knockdown and GFP-targeted control HUVECs immediately after wounding the cell monolayers (0 h) and 6 h after exposure to PGE2-supplemented medium or to Veh-supplemented medium, as a control. Dashed lines were overlaid to the images to mark the border of the wound. F: quantification by digital image analysis of the distance covered by endothelial cells migrated from the border of the wound 5 h after exposure to PGE2-supplemented medium, expressed as the ratio between covered distance and total width of the wound. Data from triplicate experiments are expressed as average ± SE. P values were calculated by Student’s t-test. G: representative immunofluorescence confocal microscopy images of polyvinyl-acetal sponges implanted subcutaneously in Dgke KO mice (8–12 wk of age, n = 4 mice per mouse, 4 mice) and of WT littermates injected with PGE2 or with equal volume of Veh on alternate days for 14 days, probed with an antibody against CD31. Scale bars = 20 μm. H and I: quantification by digital image analysis of the number of positive neovascularized structures, expressed as average of CD3-immunoreactive area of CD31-positive structures (H), or as number of CD31-positive structures per random microscopy field (I). Data were obtained analyzing 10 random images per sponge and were expressed as average ± SD, n = 4 sponges per mouse, with 4 mice per each experimental group. P values were calculated by Student’s t-test.
induction will also need to be investigated. Dgkε is required to maintain the phospholipids acyl composition of the cell membranes, and thus is likely to affect multiple downstream signaling pathways. If and how any of these pathways may regulate Cox-2 expression will need to be experimentally proved. In summary, we have reported that Dgke knockout mice have glomerular pathology that reproduces the lesions observed in patients with biallelic loss of function mutations in DGKE. Our studies also show that the lipid kinase Dgkε is required for the maintenance of the glomerular endothe-

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\begin{align*}
\text{A} & \quad \text{Vehicle} \quad \text{PAN} \\
\text{KO} & \quad \text{CD31} \\
\text{WT} & \quad \text{CD31} \\
\text{B} & \quad \text{P=0.002} \\
\text{C} & \quad \text{P<0.001} \\
\text{D} & \quad \text{CD31} \\
\text{E} & \quad \text{sh-Dgke} \\
\text{sh-GFP} & \quad \text{+ PGE_2} \\
\text{F} & \quad \text{P=0.003} \\
\text{G} & \quad \text{KO} \quad \text{WT} \\
\text{H} & \quad \text{P=0.005} \\
\text{I} & \quad \text{P<0.001} \quad \text{P=0.001} 
\end{align*}
\]
lum and participates in the regulation of the expression of Cox-2 and the production of PGE$_2$ in normal glomeruli.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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