Materials and Methods

Animals

All animal experiments were conducted in accordance with the guidelines of Tohoku University. To test whether inhibition of FXa attenuates DN, male diabetic mice of genotypes \( \text{Ins}^2 \text{Akita}^{+/+} \), \( \text{eNOS}^{+/+} \), \( \text{Ins}^2 \text{Akita}^{-/-} \), and \( \text{Ins}^2 \text{Akita}^{-/-} \), \( \text{eNOS}^{-/-} \) were used (abbreviated as \( \text{eNOS}^{+/+} \) DM, \( \text{eNOS}^{-/-} \) DM, and \( \text{eNOS}^{-/-} \) DM, respectively). Mice fed regular chow were divided into two groups at 3 months of age. The intervention group received 50 mg/kg/day of coagulation FXa inhibitor (edoxaban, Daiichi Sankyo CO., Ltd., Tokyo, Japan) for 3 months. Previous reports revealed that this dose was sufficient to prevent thrombus formation and to inhibit FXa activity to ~30% in mice and rats. To test whether lack of \( \text{Par}2 \) ameliorates DN, we used DM mice with reduced expression of \( \text{eNOS}^{+/-} \) that lacked \( \text{Par}2 \)\(^{+/+} \), \( \text{Ins}^2 \text{Akita}^{-/-} \), \( \text{eNOS}^{-/-} \) and maintained them on a regular diet until 7 months of age. These mice were compared with mice with wild-type \( \text{Par}2 \) expression (\( \text{F2rl}1^{+/+} \), \( \text{Ins}^2 \text{Akita}^{-/-} \), \( \text{eNOS}^{+/-} \)).

Biochemical measurements

Urinary albumin levels were determined using the Albuwell-M kit (Exocell Inc., Philadelphia, PA). Plasma thrombin-antithrombin complex (TAT) was measured using ELISA kit (Assaypro, St. Charles, MO). Plasma coagulation factor Xa activity was determined using substrate...
S-2765 (Chromogenix, Milano, Italy) as previously described. Briefly, 100 μl of diluted murine plasma or urine with Tris buffer were added to 100 μl of S-2765 (1.25 mg ml⁻¹). The reaction velocity (OD/min), defined as factor Xa activity, was measured at 405 nm for 30 min at 37 °C. Human IL-8 protein was measured using an ELISA kit (eBioscience, San Diego, CA). Urinary creatinine was measured using LC-MS/MS as previously described.

**Measurement of blood pressure**

BP was measured by the computerized tail-cuff method using the CODA system (Kent Scientific Corporation, Torrington, CT), as previously described.¹, ⁷

**Quantitative RT-PCR**

RNA was extracted from kidney cortex or cultured cells using Trizol (Invitrogen, Carlsbad, CA, USA). Gene expressions were quantified with TaqMan real-time quantitative RT-PCR (BioRad, Hercules, CA, USA) with hypoxanthine-guanine phosphoribosyltransferase (Hprt) as a reference gene, as we previously described.¹ The list of primer is shown in the Table IV in the online-only Data Supplement.

**Kidney morphometry and immunohistochemistry**

Fixed kidney samples were embedded in paraffin, and sections 2 μm in thickness were
stained with Periodic acid-Schiff (PAS) stain. The mesangial matrix score was defined as the ratio of glomerular PAS positive area to glomerular tuft area. All examinations were quantified using ImageJ (NIH). In electron microscopy, twenty to thirty capillary walls from three mice in each group were compared as previously shown.\textsuperscript{8, 9} GBM thickness or foot process width was determined using ImageJ software.

**Immunofluorescence**

Harvested kidney tissue was fixed in 2% paraformaldehyde for 24 h and treated with 20% sucrose for additional 24 h. Cryosections (5 μm) were incubated with rat anti-mouse MOMA2 antibody (1:25; AbD Serotec, Raleigh, NC) and goat anti-human factor X antibody (1:50; Santa Cruz Biotechnology). Sections were analyzed with confocal laser scanning microscopy (LSM5PASCAL, Carl Zeiss, Obercoken, Germany).

**Cell cultures**

Human endothelial cells (EA.hy926) were cultured in DMEM-H containing 10% fetal bovine serum.\textsuperscript{10} Immortalized murine podocytes were maintained and differentiated in RPMI1640 containing 10% fetal bovine serum, as previously described.\textsuperscript{11} All experiments were performed after serum starvation for 24 h. Human FXa was purchased from Haematologic Technologies Inc. (Essex Junction, VT). PAR2 agonists (SLIGKV and 2f-LIGRLO) were
purchased from Tocris Bioscience (Bristol, United Kingdom). Methylglyoxal and FSLLRY-NH2 were obtained from Sigma (St. Louis, MO).

Statistical analyses

Between-group analyses were performed with Student’s t test for normally distributed variables or the Mann-Whitney U test for non-normally distributed variables. One-way or two-way ANOVA followed by Tukey-Kramer test was used for comparing three or more groups. All analyses were performed using JMP 11.0.0 (SAS Institute Inc., Cary, NC). Values are presented as mean ± s.e.m. P < 0.05 was considered statistically significant.

References

expression of coagulation factor x contributes to the fibrotic response in human and murine lung injury.


