Coagulation Factor Xa and Protease-Activated Receptor 2 as Novel Therapeutic Targets for Diabetic Nephropathy

Yuji Oe, Sakiko Hayashi, Tomofumi Fushima, Emiko Sato, Kiyomi Kisu, Hiroshi Sato, Sadayoshi Ito, Nobuyuki Takahashi

Objective—The role of hypercoagulability in the pathogenesis of diabetic nephropathy (DN) remains elusive. We recently reported the increased infiltration of macrophages expressing tissue factor in diabetic kidney glomeruli; tissue factor activates coagulation factor X (FXa) to FXa, which in turn stimulates protease-activated receptor 2 (PAR2) and causes inflammation.

Approach and Results—Here, we demonstrated that diabetes mellitus increased renal FX mRNA, urinary FXa activity, and FX expression in glomerular macrophages. Administration of an oral FXa inhibitor, edoxaban, ameliorated DN with concomitant reductions in the expression of PARs (Par1 and Par2) and of proinflammatory and profibrotic genes. Diabetes mellitus induced PAR2, and lack of Par2 ameliorated DN. FXa or PAR2 agonist increased inflammatory cytokines in endothelial cells and podocytes in vitro.

Conclusions—We conclude that enhanced FXa and PAR2 exacerbate DN and that both are promising targets for preventing DN. Alleviating inflammation is probably more important than inhibiting coagulation per se when treating kidney diseases using anticoagulants. (Arterioscler Thromb Vasc Biol. 2016;36:1525-1533. DOI: 10.1161/ATVBAHA.116.307883.)

Key Words: cardiovascular diseases ■ diabetic nephropathy ■ factor Xa ■ inflammation ■ prognosis

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease worldwide and is a major life-threatening problem in diabetic patients.1,2 Although pharmacological inhibition of the renin-angiotensin system slows DN progression,3,4 patient prognosis remains poor. Recent studies,5 including ours,6,7 demonstrated that lack or reduced expression of endothelial NO synthase (eNOS, Nos3) exacerbates DN, which is associated with increased expression and activity of renal tissue factor (TF), an initiator of the coagulation cascade.5,6 Anti-TF neutralizing antibody reduced inflammation in DN.7 However, the role of hypercoagulability in the pathogenesis of DN is still unclear.

Coagulation proteases contribute to tissue injury in various diseases, including cancer progression, cardiovascular diseases, and metabolic diseases; these injuries are mediated by protease-activated receptors (PARs).8–11 This family of G protein–coupled receptors consists of 4 members (PAR1–PAR4). PAR2 is activated by TF–coagulation factor VIIa complex and coagulation factor Xa (FXa) and upregulates inflammation and fibrosis through nuclear factor-kappa B (NF-xB) or mitogen-activated protein kinase (MAPK) signaling.12–14 Although FXa and PAR2 are suggested to be responsible for the progression of kidney injury,15–18 their role in DN is poorly understood.

Here, we show that FXa and PAR2 exacerbate DN in mice and that the development of inhibitors of FXa and PAR2 will be useful for combating DN.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Kidney FX in DN

We recently demonstrated the increased expression of renal TF in mice with diabetes mellitus (DM) that lacked eNOS.6,7 Because TF activates coagulation factor VIIa, which results in activation of FXa, we quantified FX expression in the liver and its activity in the plasma of mice lacking eNOS that were made diabetic by introducing the Akita mutation in the insulin 2 (Ins2) gene, a model that recapitulates human DN.6,7 The results show that plasma FXa activity was elevated in the eNOS−/− mice regardless of DM status relative to the mice with wild-type eNOS, without changes in FX mRNA expression in the liver (Figure 1A and 1B). Interestingly, the expression of FX in the kidney was elevated in DM kidneys by 2.6-fold in the eNOS−/− DM mice and by 4-fold in the eNOS−/− DM mice.
compared with the $eNOS^{-/-}$ non-DM mice, and FXa activity was elevated in the urine collected for 24 hours using metabolic cages from these mice (Figure 1C and 1D). Consistent with the report of FX expression in macrophages,\textsuperscript{19} vast majority of immunoreactive FX/FXa colocalized with MOMA-2, a macrophage marker, in the glomeruli of the $eNOS^{-/-}$ DM mice (Figure 1E). Carbonyl stress with methylglyoxal, which is present in DM, elevated FX expression in peritoneal macrophages (Figure 1F). We conclude that macrophage FX in the DM kidney likely contributes to the pathogenesis of DN.

**Inhibition of FXa Alleviates DN**

Because increased FX and TF likely accelerate DN, we tested whether inhibiting FXa via oral administration of a prescription FXa inhibitor, edoxaban, ameliorates and prevents DN. Edoxaban did not significantly affect blood pressure, kidney weight, and blood glucose levels in the $eNOS^{-/-}$ DM mice (Table I in the online-only Data Supplement; Figure 2A and 2B). Edoxaban treatment of the $eNOS^{-/-}$ DM mice resulted in a nonsignificant reduction in urinary albumin excretion (215 $\mu$g/mg creatinine versus 413 $\mu$g/mg in untreated mice; $P=0.086$; Figure 2C). Plasma cystatin C concentration in DM mice was lower than that in non-DM mice, probably because of hyperfiltration (Table I in the online-only Data Supplement). There was no interaction between $eNOS$ genotype and edoxaban treatment (Figure 2D). In contrast,

**Nonstandard Abbreviations and Acronyms**

- **DM**: diabetes mellitus
- **DN**: diabetic nephropathy
- **eNOS**: endothelial NO synthase
- **FXa**: coagulation factor Xa
- **PAR**: protease-activated receptor
- **TF**: tissue factor

**Figure 1.** Elevated kidney factor X (FX) expression in diabetic nephropathy (DN). A, Increased plasma FXa activity in mice lacking endothelial NO synthase ($eNOS$). B, FX expression in the liver. C, Increased FX expression in the diabetic kidneys. D, Increased urinary FXa activity of diabetes mellitus (DM) mice. E, Immunoreactive FX/FXa colocalized with MOMA-2 in a glomerulus from an $eNOS^{-/-}$ DM mouse. F, Stimulation of peritoneal macrophages with methylglyoxal (MG) increased FX expression. Data are mean±SEM. A–D, n≥5, samples were obtained from 6-month-old mice. E, Experiments were repeated 4 times. ANOVA followed by the Tukey–Kramer test was performed. A.U indicates arbitrary unit; HG, high glucose (33 mmol/L); Man, mannitol (33 mmol/L); and N.S., not significant.
edoxaban corrected mesangial matrix score of the eNOS−/− DM mice (28.6% in treated mice versus 32.8% in untreated mice; *P*<0.001; Figure 2E and 2F), indicating resolution of diabetic glomerulosclerosis.

### Inhibition of FXa Corrects Expression of Inflammatory/Fibrotic Genes and PARs

Because FXa induces inflammation,12,13 we tested whether FXa inhibitor changes the expression levels of proinflammatory and profibrotic genes (Tgfb, Pail, Coll1, Coll4, and Tnfa). As expected, the expression of these genes was upregulated in the kidneys of the eNOS−/− DM mice (Figure 3A; Table II in the online-only Data Supplement). Edoxaban significantly reduced Pail, Coll1, Coll4, and Tnfa mRNA levels (Figure 3A), supporting the hypothesis that inhibiting FXa alleviates DN in the eNOS−/− DM mice. Therapeutic effects of edoxaban, such as attenuation of urinary albumin excretion, mesangial proliferation, and profibrotic gene expression, were also observed in the heterozygous eNOS−/+ DM mice (Figure 1A–ID in the online-only Data Supplement).

Because FXa activates PAR1, and PAR2 and the PARs induce inflammation,14,20 we next quantified the expression of the genes encoding PARs in the kidneys. Expression level of Par1 mRNA was significantly higher in the eNOS−/− DM mice than in the eNOS−/+ DM and non-DM mice (Figure 3B; Table II in the online-only Data Supplement). That of Par2 was significantly higher in the eNOS−/− DM mice than in the non-DM mice (Figure 3B in the online-only Data Supplement). Edoxaban reduced Par1 and Par2 expression in the kidneys of the eNOS−/− DM mice (Figure 3B and 3C). Par4 mRNA levels did not differ between genotypes or in response to edoxaban treatment (Figure 3D).

### Lack of Par2 Alleviates DN

Edoxaban alleviated kidney injury in both eNOS−/+ and eNOS−/− DM mice. FXa directly activates PAR212,13; thus, if inhibiting PAR2 is beneficial for combating DN, it is worth developing PAR2 antagonists. We therefore investigated whether lack of Par2 ameliorates DN using DM mice lacking Par2 and with reduced eNOS expression.
The basic characteristics of mice at 7 months of age appear in Table III in the online-only Data Supplement. Lack of Par2 significantly reduced the urinary albumin/creatinine ratio in DM eNOS+/− mice (32.1 versus 66.5 μg/mg; P = 0.01) without affecting blood glucose or blood pressure (Figure 4A–4C; Table III in the online-only Data Supplement). DM decreased plasma cystatin C levels, but the absence of Par2 did not affect these levels (Figure 4D). Lack of Par2 inhibited glomerular mesangial matrix expansion (26.5% versus 28.9%, P = 0.007; Figure 4E and 4F). Lack of Par2 resulted in a nonsignificant reduction in glomerular basement membrane thickness (235 versus 272 nm; P = 0.06) and a significant reduction in foot process width (500 versus 573 nm; P = 0.02; Figure 4G–4I). Taken together, these data suggest that lack of Par2 alleviates DN.

Lack of Par2 and Gene Expression in the Kidneys

Expression levels of proinflammatory and profibrotic genes (Tgfβ, Pai1, Col1, Col4, Tnfa, and Ptgs2) were higher in the eNOS+/− DM kidneys than in the eNOS−/− non-DM kidneys (Figure 5A). Tgfβ, Pai1, Col1, Col4, and Ptgs2 mRNA levels were significantly lower when Par2 was absent (Figure 5A). Consistent with results in the eNOS+/− and eNOS−/− mice (Figure 1A and 1B), DM in the eNOS+/− mice did not affect plasma FXa activity or liver FX mRNA expression, and Par2 absence also did not affect these values (Figure 5B and 5C). DM increased kidney FX expression and urinary FXA activity; lack of Par2 did not affect them (Figure 5D and 5E). DM increased Par1 and Par2 mRNA levels in the kidney, whereas lack of Par2 significantly reduced renal expression of Par1 without affecting that of Par4 (Figure 5F–5H). We conclude that lack of Par2, similar to inhibition of FXA, attenuates inflammation and fibrosis in DN. Furthermore, edoxaban and lack of Par2 both decreased expression levels of Tgfβ in the kidneys, but they did not have additive effect. Lack of Par2 decreased expression levels of MCP1 in the kidneys, but edoxaban has no further benefit to diabetic Par2−/− mice, supporting the linkage of FXa and PAR2 in the pathogenesis of DN (Figure II A–II C in the online-only Data Supplement).

FXa and PAR2 Agonist Exacerbate Inflammation in Endothelial Cells and Podocytes

Ample of studies demonstrated that coagulation factor VIIa/FXa-PAR2 signaling activates NF-κB and MAPK, major mediators of inflammation.12–14 Inflammation plays a pivotal role in the progression of DN.21–24 We therefore investigated whether FXa and PAR2 activation directly cause inflammation in endothelial cells and podocytes in vitro. Stimulation of a human endothelial cell line EA.hy926 with FXa (50 nmol/L) or with the PAR2 agonist peptide SLIGKV (100 μmol/L) increased production of interleukin-8 protein and expression of MCP1 and PAI1 (Figure 6A–6D). FXa from macrophages that have infiltrated the kidney likely increases PAR2 expression in podocytes (Figure III in the online-only Data Supplement) and activates PAR2. In conditioned immortalized murine podocytes, we found that FXa increased the expression of the inflammatory mediators MCP1 and PTGS2 that latter of which encodes cyclooxygenase-2), an effect that was reversed by FSLRKY-NH2, a PAR2 antagonist (Figure 6E and 6F). Another PAR2 agonist, 2F-LIGRLO (20 μmol/L), also increased the expression of MCP1 and PTGS2 in podocytes (Figure 6G). We conclude that FXa and PAR2 activation directly increase the inflammatory response in endothelial cells and podocytes.

Discussion

Here, we demonstrated that FXa-PAR2 activation exacerbates DN, likely through an enhanced inflammatory response. This conclusion is based on the following major findings: macrophages expressing FX infiltrate the DN kidney without concomitant changes in plasma FXa activity and liver FX expression (Figure 1); inhibition of FXa with edoxaban ameliorates DN (urinary albumin excretion and mesangial matrix expansion; Figure 2; Figure I in the online-only Data Supplement) with concomitant reductions in the expression of profibrotic and proinflammatory genes, Par1 and Par2 (Figure 3A–3C; Figure I in the online-only Data Supplement); Par2 expression is higher in DN; FXa remarkably elevates Par2 expression in podocytes (Figure 3C; Figure III in the
lack of Par2 ameliorates DN and reduces the expression of profibrotic and proinflammatory genes (Figures 4 and 5A); FXa inhibition and lack of Par2 have no additive effect on reduction of inflammatory response (Figure II in the online-only Data Supplement); and FXa and PAR2 agonist elevate, whereas PAR2 antagonist decreases, levels of inflammatory cytokines in endothelial cells and podocytes (Figure 6).

FX is mainly synthesized in the liver.\textsuperscript{12,13} However, increased extrahepatic expression of FX in bronchial/alveolar epithelia and macrophages contributes to bleomycin-induced lung fibrosis and asthma, respectively.\textsuperscript{25,26} The anti-FX antibody we used recognizes both FX and FXa. Because FX is produced in macrophages, it is likely activated on secretion and works on macrophages in an autocrine manner or on other cells in the kidney in a paracrine fashion (Figure 1E). Carbonyl stress that is elevated in DM directly increased FX expression in macrophages (Figure 1F). Macrophage infiltration reportedly increased in DN lacking eNOS.\textsuperscript{5,6} The oral FXa inhibitor edoxaban alleviated DN (Figure 2). Accordingly, local FX synthesis in macrophages that have infiltrated the kidney likely contributes to the progression of DN.

Increased plasma FXa activity in eNOS\textsuperscript{−/−} mice could be because of increased TF expression on circulating monocytes, which infiltrate in the glomeruli of diabetic mice lacking eNOS as we previously reported.\textsuperscript{7} Because macrophages synthesize coagulation factor VII and factor VII-activating protease (FSAP)\textsuperscript{27,28} together with TF and FX, it is highly likely that macrophages in glomeruli can secrete and activate FX.

Figure 4. Lack of Par2 alleviates diabetic nephropathy (DN) in diabetes mellitus (DM) mice with reduced endothelial NO synthase (eNOS) expression (eNOS\textsuperscript{−/−}). A–D, Blood glucose levels (A), systolic blood pressure (BP; B), urinary albumin excretion (U-Alb; C), and levels of plasma cystatin C (P-Cystatin C; D) are shown. E, Representative histological images of kidney stained with Periodic acid–Schiff (PAS) stain. F, Mesangial matrix score from at least 150 glomeruli from 5 mice in each group. G–I, Comparison of glomerular basement membrane (GBM) thickness and foot process (FP) width on electron microscopy (EM). Data are means±SEM. Samples were obtained from 7-month-old mice. A–D, n=6 mice per group. ANOVA followed by the Tukey–Kramer test except for panel I, in which Mann–Whitney U test was used. N.S indicates not significant.
FXa activates PAR1 and PAR2 and stimulates cytokine production. FXa strongly increased the expression levels of Par2 in podocytes in vitro (Figure III in the online-only Data Supplement). Lack of Par2 significantly reduced Tgfb, Pai1, Col1, Col4, and Ptgs2 mRNA levels in diabetes mellitus (DM) kidneys. Interestingly, immunoreactive PAR2, but not PAR1, was higher in biopsy specimens from patients with DN than in specimens from patients without DN. These results support the importance of PAR2 in the pathogenesis of DN. Fondaparinux, a FXa inhibitor, is suggested to reduce DN in db/db mice.29 We extended this work using another DN model and identified connection between FXa and PAR2.

Inflammation plays a pivotal role in the pathogenesis of DN. Thus, activation of the tumor necrosis factor α pathway predicts DN in humans and deletion or inhibition of MCP1 is protective against DN. Podocyte-derived MCP1 induces podocyte death and increases permeability to albumin, which may underlie the pathogenesis of DN. Cyclooxygenase-2 expression in podocytes is upregulated in DM kidney versus non-DM kidney, and overexpression of cyclooxygenase-2 in podocytes exacerbates DN. PAR2 expression was relatively lower than that of PAR1 in murine podocyte. However, we confirmed that stimulation by FXa increased Par2 mRNA, and FXa was increased under diabetic condition (Figure 1; Figure III in the online-only Data Supplement). FXa and PAR2 agonist both increased the expression of Mcp1 and Ptgs2 (Figure 6E–6G), consistent with the hypothesis that inflammation mediates the exacerbation of DN by FXa and PAR2.

Endothelial cells also highly express PAR2. PAR2 stimulates interleukin-8 release in human umbilical vein endothelial cells and human dermal microvascular endothelial cells. Here, both FXa and the PAR2 agonist SLIGKV increased the levels of interleukin-8 protein as well as the expression of the genes encoding MCP1 and PAI1 in the kidney. Data are means±SEM. n≥5. Samples were obtained from 7-month-old mice. ANOVA followed by the Tukey–Kramer test was performed. A.U indicates arbitrary unit; eNOS, endothelial NO synthase; and N.S., not significant.
Factor Xa and PAR2 in Diabetic Nephropathy

In conclusion, our data suggest that FXa and PAR2 exacerbate DN via inflammation. Several FXa antagonists, including edoxaban, which are prescribed to prevent thromboembolic complications in atrial fibrillation,46,47 could be useful as a new therapeutic option for DN, and clinical studies to test their safety and effectiveness will be required. It is worth developing PAR2 inhibitors for preventing DN, and possibly other kidney diseases, because PAR2 inhibition does not cause bleeding complications. PAR2 inhibitors would be useful in combination with angiotensin inhibition, because the effect of the latter is limited because of angiotensin breakthrough.48 Because no teratogenic effect of Par2 deletion is reported,49 PAR2 inhibition is expected to be harmless for pregnant woman.

Acknowledgments

We thank Drs Oliver Smithies and Nobuyo Maeda (The University of North Carolina at Chapel Hill), and members of Tohoku University.

Figure 6. Factor Xa (FXa) and protease-activated receptor 2 (PAR2) agonists increase the inflammatory response of endothelial cells and podocytes. A and B, Stimulation of human endothelial cells (EA.hy926) with FXa (50 nmol/L) for 24 h increased interleukin-8 (IL-8) protein in cultured supernatant (A) and expression of MCP1 and PAI1 (B). C and D, Stimulation of EA.hy926 with the specific PAR2 agonist SLIGKV (100 µmol/L) also increased IL-8 protein after 24 h of incubation (C) and MCP1 and PAI1 mRNA levels after 4 h of incubation (D). E and F, FXa (50 nmol/L) increased Mcp1 and Ptgs2 mRNA levels in murine podocytes (E), which was corrected with FSLLRY (FSL; 50 µmol/L), a PAR2 antagonist, after 24 h of incubation (F). G, Four hours of incubation of murine podocytes with another PAR2 agonist, 2f-LIGRLO (2f-LI; 20 µmol/L), also increased Mcp1 and Ptgs2 mRNA levels. All data are shown as fold change vs control. All experiments were performed under high-glucose conditions (25 mmol/L for endothelial cells and 22 mmol/L for podocytes). Data are mean±SEM. n≥4. P values were determined by Student t test except for the analyses in A, which used the Mann–Whitney U test, and that in E, which used ANOVA followed by the Tukey–Kramer test. A.U indicates arbitrary units; Con, control; and N.S., not significant.

groups (Figure IV in the online-only Data Supplement), which is consistent with our previous finding that exacerbation of DN in the absence of eNOS is not associated with oxidative stress marker (reduced and oxidized glutathiones).6 NADPH oxidases or oxidative stress dose not cause injurious effects in our model.

FXa inhibition is known to reduce PAR1 activation and thrombin generation. Our results showed that the levels of Par1 expression increased in the kidney with DN, which was corrected by FXa inhibition and the absence of PAR2 (Figures 3B and 5F). PAR1 and thrombin are reportedly proinflammatory and pathogenic in a rodent model of glomerulonephritis and acute kidney injury.41,42 However, thrombin is protective against DN at low concentrations of ≈40 nmol/L. Antiatopotic effect on murine podocytes of activated protein C–PAR1/PAR3 pathway is also shown.35,43 Furthermore, PAR2 is also activated by other proteases, DPP4 or Cathepsin S, which could be involved in diabetic complications.44,45 We do not exclude the contribution of PAR1, thrombin, or other PAR2 activators in our model. Although FXa inhibition or lack of PAR2 reduced severity of DN, it was not corrected to the levels of non-DM in some parameters, suggesting that other injurious mechanism may exist.

In conclusion, our data suggest that FXa and PAR2 exacerbate DN via inflammation. Several FXa antagonists, including edoxaban, which are prescribed to prevent thromboembolic complications in atrial fibrillation,46,47 could be useful as a new therapeutic option for DN, and clinical studies to test their safety and effectiveness will be required. It is worth developing PAR2 inhibitors for preventing DN, and possibly other kidney diseases, because PAR2 inhibition does not cause bleeding complications. PAR2 inhibitors would be useful in combination with angiotensin inhibition, because the effect of the latter is limited because of angiotensin breakthrough.48 Because no teratogenic effect of Par2 deletion is reported,49 PAR2 inhibition is expected to be harmless for pregnant woman.
Disclosures

References

• Although accelerated coagulation is widely known in diabetes, the role of hypercoagulability in the pathogenesis of diabetic nephropathy remains unclear. Coagulation factor Xa activates protease-activated receptor 2 and causes inflammation. Here, we demonstrate that an oral coagulation factor Xa inhibitor, edoxaban, ameliorates diabetic nephropathy with concomitant reductions of proinflammatory and profibrotic gene expressions. Lack of Par2 also ameliorates diabetic nephropathy. These findings identified that coagulation factor Xa–protease-activated receptor 2 pathway is a novel therapeutic target for diabetic nephropathy.
Coagulation Factor Xa and Protease-Activated Receptor 2 as Novel Therapeutic Targets for Diabetic Nephropathy

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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 Ins2\textsuperscript{Akita/+}, eNOS\textsuperscript{+/+}, Ins2\textsuperscript{Akita/+}, eNOS\textsuperscript{+/-}, and Ins2\textsuperscript{Akita/+}, eNOS\textsuperscript{-/-} were used (abbreviated as eNOS\textsuperscript{+/+} DM, eNOS\textsuperscript{+/-} DM, and eNOS\textsuperscript{-/-} DM, respectively).\textsuperscript{1} 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 \textasciitilde30\% in mice and rats.\textsuperscript{2,3} To test whether lack of Par2 ameliorates DN, we used DM mice with reduced expression of eNOS\textsuperscript{1} that lacked Par2\textsuperscript{4} (F2rl1\textsuperscript{-/-}; Ins2\textsuperscript{Akita/+}; eNOS\textsuperscript{+/-}) and maintained them on a regular diet until 7 months of age. These mice were compared with mice with wild-type Par2 expression (F2rl1\textsuperscript{+/+}; Ins2\textsuperscript{Akita/+}; eNOS\textsuperscript{+/-}).

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.

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. 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. 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. Immortalized murine podocytes were maintained and differentiated in RPMI1640 containing 10% fetal bovine serum, as previously described. 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. 


Supplemental Figure I. Edoxaban alleviates DN in diabetic mice with reduced eNOS expression (eNOS−/−). A, Urinary albumin excretion (U-Alb). B, Mesangial matrix score of at least 120 glomeruli from more than four mice. C, Representative photos of kidneys with Periodic acid-Schiff (PAS) stain. D, Expression of Tgfb, Col1, Col4, Tnfa, Par1, and Par2 mRNA. Data are mean ± s.e.m. A and D; n≥6 mice per group. Panels A and D; Student’s t test, B; Mann-Whitney U test.
Supplemental Figure II. Edoxaban and lack of Par2 has no additive effect on inflammatory gene expression in the kidney. *Tnfa* (A), *Tgfb* (B), and *Mcp1* mRNA (C). DM was induced in male *Par2*−/− and wild-type mice by intraperitoneal injection of streptozotocin (50 mg/kg/day for five connective days). Two months later, L-NAME (50 mg/kg/day) was administered s.c. with or without edoxaban (50 mg/kg/day, orally) for a week. Data are mean ± s.e.m. n≥5. ANOVA with Tukey-Kramer test.
Supplemental Figure III. Coagulation FXa increases Pars mRNA in murine podocyte. A and B, FXa (50 nM) significantly increased Par1 and Par2 mRNA levels in cultured podocytes. Con, control; HG, high glucose (33 mM); Man, mannitol (33 mM); N.S., not significant. Data are mean ± s.e.m, n≥4. ANOVA with Tukey-Kramer test.
Supplemental Figure IV. Gene expression of Nox2 (A) and Nox4 mRNA (B) in the kidney. N.S., not significant. Data are mean ± s.e.m, n ≥ 6.
Supplemental Table I. Characteristics of mice at 6 months of age.

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<td>585 ± 66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>460 ± 45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>494 ± 49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P=0.07</td>
<td>P=0.74</td>
<td></td>
</tr>
<tr>
<td>Kidney Wt/BW (mg g&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>5.7 ± 0.2</td>
<td>5.1 ± 0.2</td>
<td>8.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.0 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.3 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.0 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P=0.73</td>
<td>P=0.23</td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>103.7 ± 2.0</td>
<td>123.9 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100.7 ± 2.8&lt;sup&gt;f&lt;/sup&gt;</td>
<td>97.8 ± 2.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>124.0 ± 3.8&lt;sup&gt;f,h&lt;/sup&gt;</td>
<td>119.1 ± 3.1&lt;sup&gt;f,h&lt;/sup&gt;</td>
<td>P&lt;0.001</td>
<td>P=0.22</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81.5 ± 1.5</td>
<td>96.1 ± 2.6</td>
<td>77.7 ± 2.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>78.9 ± 3.4</td>
<td>99.6 ± 4.1&lt;sup&gt;f,h&lt;/sup&gt;</td>
<td>91.7 ± 5.9</td>
<td>P&lt;0.001</td>
<td>P=0.41</td>
<td></td>
</tr>
<tr>
<td>Water intake (mL per 24 h)</td>
<td>1.7 ± 0.3</td>
<td>2.6 ± 0.1</td>
<td>4.6 ± 0.6</td>
<td>7.5 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.1 ± 1.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.7 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P=0.08</td>
<td>P&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Urinary volume (mL per 24 h)</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>4.0 ± 0.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.1 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.2 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.1 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P=0.48</td>
<td>P=0.09</td>
<td></td>
</tr>
<tr>
<td>Plasma cystatin C (ng mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>380 ± 13</td>
<td>434 ± 35</td>
<td>253 ± 21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>198 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>280 ± 18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>270 ± 18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P&lt;0.05</td>
<td>P&lt;0.08</td>
<td></td>
</tr>
<tr>
<td>Plasma Cre (µg dL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>75.0 ± 10.3</td>
<td>76.7 ± 6.6</td>
<td>94.2 ± 6.1</td>
<td>86.2 ± 9.6</td>
<td>P&lt;0.05</td>
<td>P=0.71</td>
<td></td>
</tr>
<tr>
<td>Cre clearance (µL min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>563 ± 82</td>
<td>612 ± 96</td>
<td>317 ± 38&lt;sup&gt;d&lt;/sup&gt;</td>
<td>364 ± 44&lt;sup&gt;d&lt;/sup&gt;</td>
<td>P&lt;0.001</td>
<td>P=0.48</td>
<td></td>
</tr>
<tr>
<td>Urinary albumin/Cr (µg mg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>20 ± 2</td>
<td>36 ± 4</td>
<td>50 ± 27</td>
<td>62 ± 26</td>
<td>413 ± 107&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;f,h&lt;/sup&gt;</td>
<td>215 ± 33</td>
<td>P&lt;0.001</td>
<td>P=0.14</td>
<td></td>
</tr>
<tr>
<td>Plasma TAT (ng mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>10.4 ± 1.7</td>
<td>9.0 ± 2.4</td>
<td>8.2 ± 0.5</td>
<td>12.7 ± 2.5</td>
<td>8.2 ± 1.2</td>
<td>5.2 ± 0.8</td>
<td>P=0.10</td>
<td>P=0.56</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± s.e.m (n≥5 in each group). Abbreviations: BW, body weight; Wt, weight; BP, blood pressure; TAT, thrombin-antithrombin complex; Cre, creatinine; N.A. not available.

There was no interaction between treatment and genotyping (p values not shown).

<sup>a</sup>P<0.05 versus eNOS<sup>+/+</sup> mice. <sup>b</sup>P<0.01 versus eNOS<sup>+/+</sup> mice. <sup>c</sup>P<0.05 versus eNOS<sup>-/-</sup> mice. <sup>d</sup>P<0.01 versus eNOS<sup>-/-</sup> mice.

<sup>e</sup>P<0.05 versus Ins2<sup>+/+</sup>; eNOS<sup>+/+</sup> mice. <sup>f</sup>P<0.01 versus Ins2<sup>+/+</sup>; eNOS<sup>-/-</sup> mice. <sup>g</sup>P<0.05 versus Ins2<sup>-/-</sup>; eNOS<sup>-/-</sup> mice with edoxaban treatment.

<sup>h</sup>P<0.01 versus Ins2<sup>-/-</sup>; eNOS<sup>-/-</sup> mice with edoxaban treatment.
### Supplemental Table II. Profiles of gene expression including non-DM groups

<table>
<thead>
<tr>
<th>Genes</th>
<th>eNOS&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>eNOS&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Ins2&lt;sup&gt;Δko/eNOS&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Ins2&lt;sup&gt;Δko/eNOS&lt;sup&gt;−/−&lt;/sup&gt; with Edoxaban</th>
<th>Ins2&lt;sup&gt;Δko/eNOS&lt;sup&gt;+/+&lt;/sup&gt; with Edoxaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfβ</td>
<td>1.00±0.11</td>
<td>1.32±0.12</td>
<td>0.70±0.06</td>
<td>0.89±0.07</td>
<td>1.93±0.48&lt;sup&gt;±c,f,g&lt;/sup&gt; 1.07±0.15</td>
</tr>
<tr>
<td>Pai1</td>
<td>1.00±0.16</td>
<td>3.26±0.99</td>
<td>2.18±0.62</td>
<td>4.33±0.61</td>
<td>6.52±1.41&lt;sup&gt;±b,i&lt;/sup&gt; 2.62±0.61&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Col1</td>
<td>1.00±0.15</td>
<td>2.37±0.62</td>
<td>1.99±0.49</td>
<td>2.27±0.42</td>
<td>8.87±1.66&lt;sup&gt;±d,i,h&lt;/sup&gt; 3.96±0.50</td>
</tr>
<tr>
<td>Col4</td>
<td>1.00±0.13</td>
<td>1.70±0.16</td>
<td>1.36±0.12</td>
<td>1.73±0.21</td>
<td>3.05±0.48&lt;sup&gt;±d,f,g&lt;/sup&gt; 1.76±0.23&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tnfa</td>
<td>1.01±0.22</td>
<td>1.77±0.66</td>
<td>1.40±0.15</td>
<td>1.54±0.35</td>
<td>3.06±0.54&lt;sup&gt;±c,f,g&lt;/sup&gt; 1.41±0.13</td>
</tr>
<tr>
<td>Par1</td>
<td>1.00±0.15</td>
<td>1.61±0.12</td>
<td>1.54±0.14</td>
<td>2.02±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.01±0.32&lt;sup&gt;±d,i,h&lt;/sup&gt; 1.34±0.20&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Par2</td>
<td>1.00±0.13</td>
<td>1.72±0.19</td>
<td>1.74±0.15</td>
<td>2.24±0.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.73±0.40&lt;sup&gt;±d,i,j&lt;/sup&gt; 1.24±0.16&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Par4</td>
<td>1.00±0.35</td>
<td>0.68±0.04</td>
<td>0.64±0.14</td>
<td>0.70±0.12</td>
<td>0.64±0.31                           0.74±0.12</td>
</tr>
</tbody>
</table>

Data are mean ± s.e.m (n=5 in each group).

<sup>a</sup>P<0.05 versus eNOS<sup>+/+</sup> mice.  <sup>b</sup>P<0.01 versus eNOS<sup>+/+</sup> mice.  <sup>c</sup>P<0.05 versus eNOS<sup>−/−</sup> mice.  <sup>d</sup>P<0.01 versus eNOS<sup>−/−</sup> mice.

<sup>e</sup>P<0.05 versus Ins2<sup>Δko/eNOS<sup>+/+</sup> mice.  <sup>f</sup>P<0.01 versus Ins2<sup>Δko/eNOS<sup>+/+</sup> mice.  <sup>g</sup>P<0.05 versus Ins2<sup>Δko/eNOS<sup>−/−</sup> mice with edoxaban treatment.

<sup>h</sup>P<0.01 versus Ins2<sup>Δko/eNOS<sup>−/−</sup> mice with edoxaban treatment.  <sup>i</sup>P<0.05 versus Ins2<sup>Δko/eNOS<sup>+/+</sup> mice.  <sup>j</sup>P<0.01 versus Ins2<sup>Δko/eNOS<sup>−/−</sup> mice.
Supplemental Table III. Characteristics of Akita DM eNOS<sup>+/−</sup> mice with or without PAR2 at 7 months of age.

<table>
<thead>
<tr>
<th></th>
<th>Ins2&lt;sup&gt;+/−&lt;/sup&gt; Akita</th>
<th>Two-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Par2&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Par2&lt;sup&gt;+/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body Wt (g)</td>
<td>29.1 ± 0.4</td>
<td>30.4 ± 0.5</td>
</tr>
<tr>
<td>Blood glucose (mg dL&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>143 ± 13</td>
<td>136 ± 8</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>100.2 ± 4.4</td>
<td>109.3 ± 2.8</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>77.1 ± 4.5</td>
<td>81.1 ± 5.2</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>84.5 ± 4.4</td>
<td>90.1 ± 4.2</td>
</tr>
<tr>
<td>Water intake (mL/24 h)</td>
<td>4.8 ± 0.7</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>Urinary volume (mL/24 h)</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Kidney Wt/Body Wt (mg g&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>5.1 ± 0.3</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Plasma cystatin C (ng mL&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>458 ± 30</td>
<td>464 ± 22</td>
</tr>
<tr>
<td>Urinary Alb/Creatinine (µg mg&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>17.1 ± 3.4</td>
<td>15.4 ± 2.1</td>
</tr>
<tr>
<td>Plasma TAT (ng mL&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>6.3 ± 1.2</td>
<td>6.8 ± 0.5</td>
</tr>
</tbody>
</table>

Data are mean ± s.e.m. (n=6 per group). Abbreviations: Wt, weight; BP, blood pressure; TAT, thrombin-antithrombin complex; Alb, albumin; Cre, creatinine. All mice are heterozygous for eNOS (Nos3<sup>+/−</sup>).

<sup>a</sup>P<0.01 versus Par2<sup>+/+</sup> mice.  <sup>b</sup>P<0.01 versus Par2<sup>+/−</sup> mice.  <sup>c</sup>P<0.05 versus Ins2<sup>+/−</sup> Akita; Par2<sup>+/−</sup> mice.
### Supplemental Table IV. The list of primers and probes

<table>
<thead>
<tr>
<th>Taqman Probe</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
<th>Probe (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>mFX</td>
<td>CTGCTGCCACTGTCTCCAT</td>
<td>GTCCACCTCGTGCAACCATCT</td>
<td>TCAAGGTGAGGTTAGGTGATCGGAACA</td>
</tr>
<tr>
<td>mPar1</td>
<td>TGCTCTGCCCGGCTTCT</td>
<td>AGTGCAACAATCAGGAGGACTT</td>
<td>CATCTTCATCGTCTGCTTTGGGCC</td>
</tr>
<tr>
<td>mPar2</td>
<td>AGCCGAGCCGAGAATCCTT</td>
<td>GGAACCCTTTCCCCACGTATT</td>
<td>CTTACTTTAGTGTGTTGGCCTCAGGGT</td>
</tr>
<tr>
<td>mPar4</td>
<td>CCTCTGGTGCTCCACAGTAGAAC</td>
<td>CCGGCTACGCTCGTGGAT</td>
<td>AGGAGGCGAGATCCCTCAGACAAGCCT</td>
</tr>
<tr>
<td>mTgfb</td>
<td>TGCTTCAGCTCCTCACAGAGAA</td>
<td>GTGATCCACTTCCACACCC</td>
<td>CCTTCCTAAAGTCAATGGAATCATTCGG</td>
</tr>
<tr>
<td>mTnfa</td>
<td>CATCTTTCTAAAAATTTGAGTGACA</td>
<td>TGGGACTAGCAAGGTACAGM</td>
<td>CAGGTCGATAGCAAACCAACACAGTG</td>
</tr>
<tr>
<td>mCol4</td>
<td>GGCTATTCTTTTCTGTGAT</td>
<td>CTAACCTTTCCAGACACAC</td>
<td>AGGTTCCCGGCAAGCCC</td>
</tr>
<tr>
<td>mMcp1</td>
<td>CTGGAGCATCACAGCTTGGT</td>
<td>TGGGATCATCTTTGCTGTTG</td>
<td>AGGCACTAGCAGTTAAGCAGCCAC</td>
</tr>
<tr>
<td>hMCP1</td>
<td>ATAGCAGCACCCTTCATTCC</td>
<td>ATCCCTGAACCCACTTCTGCT</td>
<td>CAGGACACAGCAGTTAAGCAGCCAC</td>
</tr>
<tr>
<td>hPAI1</td>
<td>TGAGATCAGCACCACAGAC</td>
<td>ATGGATAGTAATGCTGCTCTC</td>
<td>CAGGACACAGCAGTTAAGCAGCCAC</td>
</tr>
<tr>
<td>hHPRT</td>
<td>GCTGAGGATTGGAAAGGTT</td>
<td>CCTTCATCTCCTTACAC</td>
<td>CAGGACACAGCAGTTAAGCAGCCAC</td>
</tr>
</tbody>
</table>

### SYBR green

| mPai1       | TTCAGTGGCCAAATGGAAGACTCCT | AGGCGAGTCCACAACGCCTATACT | |
| mCol1       | GACTTGAAGACGAGGAGTACTG | CTTTGAGTGCCGTCAGGTT | |
| mPtgs2      | CAAGACAGATCATAAGGAGGGA | GGGCGAGTATGTTGTTCTGT | |
| hMCP1       | ATAGCAGCACCCTTCATTCC | ATCCCTGAACCCACTTCTGCT | |
| hPAI1       | TGAGATCAGCACCACAGAC | ATGGATAGTAATGCTGCTCTC | |
| hHPRT       | GCTGAGGATTGGAAAGGTT | CCTTCATCTCCTTACAC | |
Impaired eNOS

Hyperglycemia

Up-regulation of Coagulation

Tissue factor/FVIIa

FXa

PAR2

Inflammation

Diabetic nephropathy

Coagulation FXa – PAR2 pathway exacerbates diabetic nephropathy