Rivaroxaban, a Direct Factor Xa Inhibitor, Ameliorates Hypertensive Renal Damage Through Inhibition of the Inflammatory Response Mediated by Protease-Activated Receptor Pathway

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Background—An enhanced renin-angiotensin system causes hypertensive renal damage. Factor Xa not only functions in the coagulation cascade but also activates intracellular signaling through protease-activated receptors (PAR). We investigated the effects of rivaroxaban, a factor Xa inhibitor, on hypertensive renal damage in hypertensive mice overexpressing renin (Ren-TG).

Methods and Results—The 12- to 16-week-old Ren-TG and wild-type mice were orally administered with or without 6 or 12 mg/kg of rivaroxaban for 1 or 4 months. Plasma factor Xa was significantly increased in the Ren-TG compared with the wild-type mice and was reduced by 12 mg/kg of rivaroxaban ($P<0.05$). Urinary albumin excretion (UAE) was higher in the nontreated 8-month-old Ren-TG than in the wild-type mice (69.6±29 versus 20.1±8.2 μg/day; $P<0.01$). Treatment with 12 mg/kg of rivaroxaban for 4 months decreased the UAE to 38.1±13.2 μg/day ($P<0.01$). Moreover, rivaroxaban treatment attenuated histologic changes of glomerular hypertrophy, mesangial matrix expansion, effacement of the podocyte foot process, and thickened glomerular basement membrane in the Ren-TG. The renal expression of PAR-2 was increased in the Ren-TG, but was inhibited with rivaroxaban treatment. In vitro study using the human podocytes showed that the expressions of PAR-2 and inflammatory genes and nuclear factor—κB activation were induced by angiotensin II stimulation, but were inhibited by rivaroxaban. PAR-2 knockdown by small interfering RNA also attenuated the PAR-2-related inflammatory gene expressions.

Conclusions—These findings indicate that rivaroxaban exerts protective effects against angiotensin II—induced renal damage, partly through inhibition of the PAR-2 signaling-mediated inflammatory response. (***J Am Heart Assoc***. 2019;8:e012195. DOI: 10.1161/JAHA.119.012195.)

Key Words: albuminuria • podocytes • protease-activated receptor-2 • renin-angiotensin system • rivaroxaban

Hypertension is one of the most important risk factors for chronic kidney diseases, leading to hypertensive nephrosclerosis. Furthermore, increased activity of the renin-angiotensin system is associated with target organ damage in hypertensive patients. Recently, accumulating evidences support a potential link between hypertension and coagulation.[1–3] For instance, increased plasma levels of D-dimer, fibrinogen, and prothrombin fragment 1+2, all of which represent activated coagulation pathways, are detected in hypertensive patients. Furthermore, angiotensin II infusion increases the plasma levels of plasminogen activator inhibitor (PAI)-1 in humans.[3–5] Thus, it seems that both an enhanced renin-angiotensin system and a prothrombotic state may synergistically contribute to the development of hypertensive target organ damage.

The recently developed direct oral anticoagulants have been widely used for the prevention of thromboembolic diseases. Rivaroxaban, which is the first factor Xa (FXa) inhibitor, inhibits free FXa and prothrombinase activity, thereby effectively blocking thrombin generation in a concentration-dependent manner.[6–8] FXa not only functions in the coagulation cascade but also activates the intracellular signaling pathways through G-protein—coupled protease-activated receptors (PARs). Four PARs have been identified in mice and humans.[9–11] FXa mainly acts through PAR-2 and subsequently stimulates multiple intracellular signaling

pathways, including the nuclear factor–κB and mitogen-activated protein kinase pathways and induces inflammatory and fibrotic responses.[12–14]

In this study, we tested the hypothesis that rivaroxaban protects against renal damage in renin-angiotensin system–activated hypertension partly through the PAR-2 signaling pathway. For this purpose, we used hypertensive mice with a genetically clamped renin transgene in the liver (Ren-TG mice), which showed high blood pressure, cardiac hypertrophy, and renal damage.[15–17]

Methods

The data, analytic methods, and study materials will be available to other researchers for purposes of reproducing results or replicating procedures, as described in this article or by contacting corresponding author on reasonable request.

Animals

The Ren-TG mice, which were originally described as Ren-TG MK mice, were kindly provided by Drs Oliver Smithies and Nobuyo Maeda (University of North Carolina at Chapel Hill, Chapel Hill, NC). Briefly, a modified mouse renin transgene that was driven by a liver-specific albumin promoter/enhancer was inserted into the genome as a single copy at the liver-specific ApoA1/ApoC3 locus.[15,16] The resulting transgene expressed renin ectopically in the liver at constantly high levels, which resulted in elevated plasma levels of active renin and angiotensin II. The 12- to 16-week-old male heterozygous (1 copy of the renin transgene) Ren-TG mice were used in the present study, which were backcrossed to C57BL6/N mice for >10 generations, because of the availability of mouse strain. The wild-type (WT) and Ren-TG mice were administered with or without rivaroxaban at 6 and 12 mg/kg as a mixed chow diet for 1 or 4 months. The WT littermates were used as the control. For euthanasia and tissue harvest, mice were fully anesthetized with isoflurane or CO_2 and immediately euthanized by cervical dislocation.

All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Hirosaki University Graduate School of Medicine.

Blood Pressure Measurement

The mice were kept in a chamber warmed at 37°C for ≈10 minutes; thereafter, the systolic blood pressure and pulse rate were measured in the conscious mice by the tail-cuff method (BP-98A; Softron, Tokyo, Japan). After discarding the highest and lowest readings, at least 10 readings were averaged each day for 3 consecutive days, as previously described.[17]

Biochemical Measurements

Urinary albumin excretion was measured using the ALBUWELL M TEST Kit (Exocell, Philadelphia, PA). Anti-FXa activity was measured by the HemosIL Liquid Heparin (Instrumentation Laboratory, Lexington, MA). The plasma rivaroxaban concentration was measured by liquid chromatography–mass spectrometry. Prothrombin time was measured by the HemosIL Heparin Kit (Instrumentation Laboratory, Bedford, MA). Coagulation FXa was measured by the Mouse Coagulation FXa ELISA kit (MyBioSouse, San Diego, CA). Blood urea nitrogen and creatinine were measured using the SPOTCHEM™ II (ARKRAY Factory, Shiga, Japan).

Histologic Examination of the Renal Cortex

The kidney samples were fixed with 10% formalin. Paraffin-embedded tissues were cut into 5-μm sections, which were stained with PAS, hematoxylin and eosin, and Masson's trichrome. The stained sections were examined using an all-in-one fluorescence microscope (BZ-X710; Keyence, Osaka, Japan). Glomerular hypertrophy was quantitatively assessed by glomerular tuft surface area measured on at least 50 randomly chosen glomeruli, and mean values were obtained as a representative of an individual.

Measurement of Glomerular Basement Membrane Thickness and Number of Open Slit Pores

The samples were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer at pH 7.4. The images were observed using a transmission electron microscope (JEM-1400 Plus; JELOL, Tokyo, Japan). The glomerular basement membrane (GBM) thickness was determined as the distance between the outer limit of the endothelial cells and the base of the cell membranes of the podocyte foot processes; 20 glomeruli were measured at 10 positions of each glomerulus. The number of slit pores was counted and divided by the GBM length (per 100 μm) using ImageJ.

Cell Culture

Immortalized human podocytes, which were kindly provided by Dr Moin A Saleem (University of Bristol, Bristol, UK), were maintained in RPMI 1640 containing 10% fetal bovine serum; 1% insulin–transferrin–selenium-A supplement (Invitrogen, Carlsbad, CA); penicillin (10 U/mL); and streptomycin (100 μg/mL). The cells were grown at 33°C in 95% air and 5% CO_2, then were converted to differentiated cells by incubating at 37°C for 10 days. The cells were kept in 1% fetal bovine serum for 24 hours, followed by pretreatment with rivaroxaban (500 μg/L) for 1 hour, and then angiotensin II (1.0 μmol/L) was added and the cells were incubated for the designated hours.

Small Interfering RNA

The cultured podocytes were transfected with small interfering RNA (siRNA) (5 nmol/L) without antibiotics for 24 hours using Dharmafect transfection reagent (GE Healthcare, Little Chalfont, UK), according to the manufacturer's protocol. An siRNA that was targeted to an irrelevant mRNA served as a nonspecific control.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from the kidney cortex of the mice and from the human podocytes using RNeasy Protect Mini Kit (QIAGEN, Valencia, CA). The extracted RNA was transcribed into the first-strand cDNA using the Omniscript RT kit (QIAGEN), according to the manufacturer's protocol. Quantitative reverse transcriptase–polymerase chain reaction was performed using a CFX connect (Applied Biosystems, Foster City, CA) with TaqMan Universal PCR Master Mix (Applied Biosystems). The specific primers and probes (Applied Biosystems) were acquired to detect PAR-2 (Assay ID: Mm00433160_m1 and Hs00608346_m1); monocyte chemoattractant protein (MCP)-1 (Assay ID: Mm00441342_m1 and Hs00234140_m1); tumor necrosis factor (TNF)-α (Assay ID: Mm0043258_m1 and Hs00174128_m1); PAI-1 (Assay ID: Mm00435860_m1); interleukin-6 (Assay ID: 00985639_m1); synaptopodin (Assay ID: Hs00702468); and glyceraldehyde–3-phosphatase dehydrogenase (GAPDH) (Assay ID: Mm99999915_g1 and Hs0275 8991_g1). The results were normalized by GAPDH.

Western Blot Analysis

After the euthanasia of the mice, the kidneys were immediately excised, homogenized, and centrifuged in 3% sodium dodecyl sulfate buffer and 7.5% β-mercaptoethanol; the supernatant was collected for western blot analysis. Protein concentrations were determined using Protein Quantification Assay (MACHEREY-NAGEL GmbH & Co KG, Duren, Germany). The proteins were separated by sodium dodecyl sulfide–polyacylamide gel electrophoresis and were electrophoretically transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA). After blocking with 5% milk for 1 hour, the membranes were incubated with primary antibodies for PAR-2 (sc-13504; Santa Cruz Biotechnology, Dallas, TX) diluted to 1:3000 at 4°C overnight. Horseradish peroxidase and alkaline phosphate–conjugated antimouse antibody (sc-2005; Santa Cruz) diluted to 1:10 000 were used for PAR-2 as secondary antibodies. The primary antibody for GAPDH (sc-25778; Santa Cruz) was diluted to 1:3000 at 4°C overnight. Horseradish peroxidase and alkaline phosphate–conjugated antirabbit antibodies (sc-2004; Santa Cruz) diluted to 1:15 000 were used for GAPDH as secondary antibodies. The protein bands were detected by Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare). Densitometric analysis was performed with ChemiDoc XRS+ with Image Lab Software (Bio-Rad Laboratories, Woodinville, WA), and a relative value of the target protein to GAPDH was calculated in each sample.

Immunofluorescence Study

Podocytes were grown and differentiated on glass-bottom culture dishes (MatTec, Ashland, MA). The cells were pretreated with rivaroxaban (500 μg/L) for 1 hour, followed by treatment with or without angiotensin II (1.0 μmol/L) for 1 hour for the assessment of nuclear factor–κB and for 24 hours for assessment of F-actin staining. Then, the cells were fixed with 4% paraformaldehyde for 30 minutes and were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 10 minutes. To assess the activation of nuclear factor–κB, the cells were blocked with 5% milk in phosphate-buffered saline (blocking buffer) for 1 hour and incubated overnight with rabbit antiphospho-p65 antibody (Cell Signaling, Danvers, MA), diluted to 1:100 in a blocking buffer, then incubated with Alexa-Fluor 488 goat antirabbit

antibody for 2 hours. For F-actin staining after fixation, the cells were incubated with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Carlsbad, CA) for 1 hour. Thereafter, the samples were covered with an antifading mounting medium with or without 4′-6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). The preparations were visualized under a fluorescence microscope.

Statistical Analyses

All data were given as mean± SD. The distribution of all measurements was tested for normality using the Shapiro–Wilk test. Comparisons of parametric data were performed using 1-way ANOVA, followed by the Tukey's honest significant difference test. Comparisons of nonparametric data were performed using the Kruskal–Wallis test, followed by the

Steel–Dwass multiple comparison test. A simple linear regression analysis was performed to examine the correlation between anti–factor Xa activity and rivaroxaban concentration in the blood. The significance and interactions of the effects of genotype, treatment, and age were determined by 2-way or 3-way ANOVA. Statistical analyses were performed with a commercially available software program JMP 12.1.1 (SAS, Cary, NC). A $P<0.05$ was considered to be statistically significant.

Results

Effects of Rivaroxaban on Anticoagulant Activity

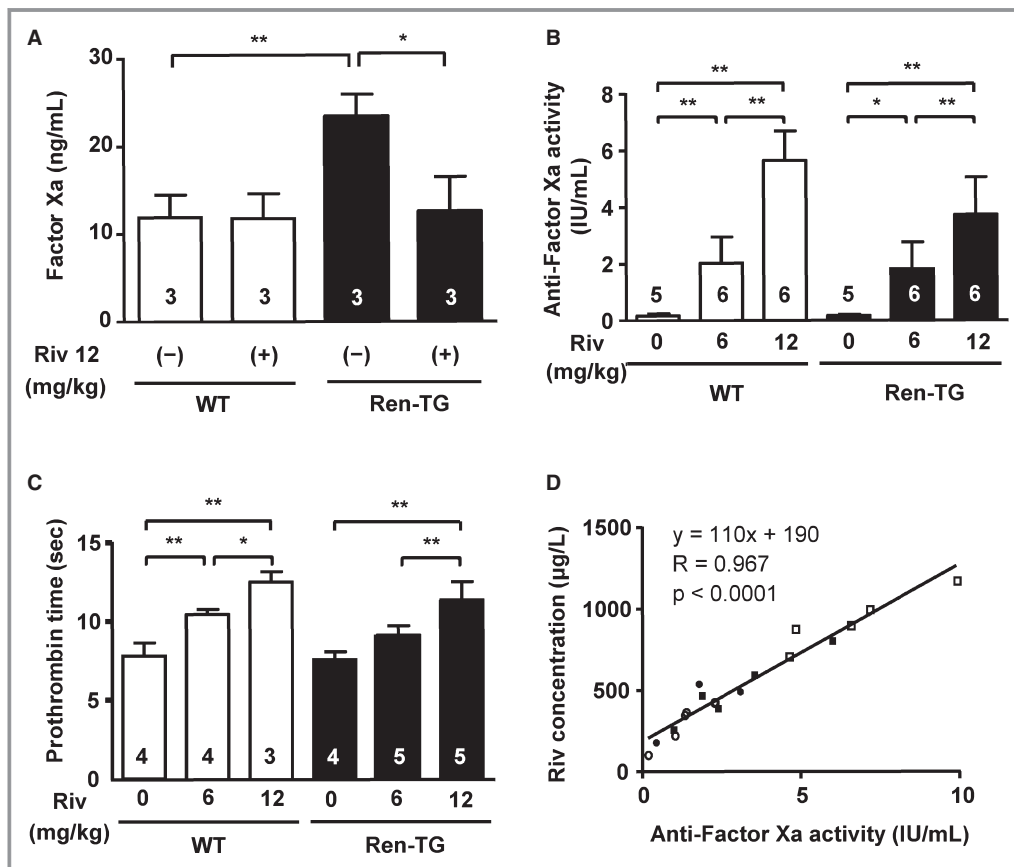The 12- to 16-week-old Ren-TG and WT mice were administered with or without 6 or 12 mg/kg of rivaroxaban as a

Figure 1. Effects of rivaroxaban on coagulation activity. The 12- to 16-week-old Ren-TG and WT mice were administered with or without 6 or 12 mg/kg of rivaroxaban for 1 month. **A**, Plasma factor Xa in the WT and Ren-TG mice administered with or without 12 mg/kg of rivaroxaban. **B** and **C**, Anti–factor Xa activity, and prothrombin time in WT and Ren-TG mice administered with or without rivaroxaban at 6 and 12 mg/kg. Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. **D**, Relationship between anti–factor Xa activity and rivaroxaban concentration in the blood (white circle; WT with rivaroxaban at 6 mg/kg, n=5, white square; WT with rivaroxaban at 12 mg/kg, n=5, solid circle; Ren-TG with rivaroxaban at 6 mg/kg, n=3, solid square; Ren−TG with rivaroxaban at 12 mg/kg, n=5). *$P<0.05$, **$P<0.01$. Sample sizes are given on each bar. Ren-TG indicates renin transgenic; WT, wild-type.

mixed chow diet for 1 month. The FXa in plasma was significantly higher in the Ren-TG mice than in the WT mice treated without rivaroxaban for 1 month (23.4±2.6 versus 11.9±2.6 ng/mL, $P<0.01$, each n=3) and was significantly reduced in the Ren-TG mice treated with 12 mg/kg of rivaroxaban for 1 month (12.7±3.9 ng/mL, $P<0.05$ versus Ren-TG without rivaroxaban for 1 month, n=3) (Figure 1A). The plasma anti-FXa activity was significantly increased in both WT and Ren-TG mice in a dose-dependent manner with rivaroxaban at 6 or 12 mg/kg for 1 month compared with no rivaroxaban for 1 month (n=5–6) (Figure 1B). Consistent with this, prothrombin time was significantly prolonged in both WT and Ren-TG mice in a dose-dependent manner (n=3–5) (Figure 1C). Anti-FXa activity and rivaroxaban concentration in the blood were strongly positively correlated, and the Pearson correlation coefficient was 0.967 (Figure 1D).

Characteristics of Mice at 8 Months of Age

The 12- to 16-week-old WT and Ren-TG mice were administered with or without 12 mg/kg of rivaroxaban for 4 months. Systolic blood pressure was significantly higher in the Ren-TG mice than in the WT mice treated without rivaroxaban for 4 months (150.5±6.6 versus 112.3±6.5 mm Hg, $P<0.01$, each n=7), and treatment with 12 mg/kg of rivaroxaban for 4 months decreased to 126.2±11.0 mm Hg in the Ren-TG mice ($P<0.01$ versus Ren-TG without rivaroxaban for 4 months, n=7), as shown in Table (genotype effect, $P<0.01$; drug effect, $P<0.01$; interaction, $P<0.05$). Although pulse rate had a significant genotype effect ($P<0.05$), there was no drug effect. There were no significant differences in body weight, blood urea nitrogen, creatinine, and kidney weight/body weight among the 4 groups.

Effects of Rivaroxaban Treatment on Urinary Albumin Excretion

The 12- to 16-week-old WT and Ren-TG mice were administered with or without 12 mg/kg of rivaroxaban as a mixed chow diet for 1 or 4 months. As shown in Figure 2A and 2B, urinary albumin excretion was significantly increased in the Ren-TG mice than in the WT mice treated without rivaroxaban for 1 month (39.7±13.3 versus 15.1±4.0 µg/day, $P<0.01$, each n=5) and for 4 months (69.6±29.0 versus 20.1±8.2 µg/day, $P<0.01$, each n=7). Although rivaroxaban treatment for 1 month did not reduce urinary albumin excretion (n=5) (Figure 2A), 4-month treatment with rivaroxaban significantly reduced it to 38.1±13.2 µg/day ($P<0.01$ versus Ren-TG without rivaroxaban for 4 months, n=7) (Figure 2B). As shown in Figure 2C, urinary albumin excretion was significantly affected by genotype ($P<0.01$), age ($P<0.01$), and drug ($P<0.05$).

Histologic Assessment

Glomerular hypertrophy was evaluated in the WT and Ren-TG mice with or without rivaroxaban at 6 or 12 mg/kg (Figure 3). The Ren-TG mice showed glomerular hypertrophy compared with the WT mice at 8 months of age (Figure 3A and 3D). Rivaroxaban treatment at 6 mg/kg (Figure 3E) and 12 mg/kg (Figure 3F) for 4 months attenuated glomerular hypertrophy in the Ren-TG mice. The quantitative assessment was summarized in Figure 3G. Rivaroxaban significantly ameliorated

Table. Characteristics of Mice at 8 Months of Age

Genotype	WT		Ren-TG		Effects (*P* Value)		
Riv 12, mg/kg	−	+	−	+	Genotype	Drug	Interaction
Number of mice	7	7	7	7			
SBP, mmHg	112.3±6.5	109.3±5.2	150.5±6.6*	126.2±11.0[†]	<0.01	<0.01	<0.05
PR, bpm	644.5±35.3	639.5±22.9	679.8±33.1	654.6±28.8	<0.05	ns	ns
BW, g	40.1±3.5	41.2±2.7	43.2±3.9	40.9±2.7	ns	ns	ns
BUN, mg/dL	19.3±4.4	20.3±2.9	21.1±4.8	21.9±2.1	ns	ns	ns
Cre, mg/dL	0.61±0.04	0.69±0.10	0.63±0.05	0.67±0.08	ns	ns	ns
Lt Kid/BW, mg/g	4.21±0.29	4.47±0.40	4.39±0.51	4.45±0.52	ns	ns	ns
Rt Kid/BW, mg/g	4.74±0.28	4.57±0.43	4.45±0.63	4.38±0.52	ns	ns	ns

The 12- to 16-week-old WT and Ren-TG mice were administered with vehicle or 12 mg/kg of rivaroxaban as a mixed chow for 4 months. Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. Effects of genotype, drug, and their interaction on each variable were evaluated by 2-way ANOVA. BUN indicates blood urea nitrogen; BW, body weight; Cre, creatinine; Kid, kidney weight; ns, not significant; PR, pulse rate; Ren-TG, renin transgenic mice; SBP, systolic blood pressure; WT, wild-type mice.

*$P<0.01$ vs WT without rivaroxaban.

[†]$P<0.01$ vs Ren-TG without rivaroxaban.

glomerular hypertrophy in a dose-dependent manner in the Ren-TG mice.

Furthermore, the Ren-TG mice exhibited mesangial matrix expansion compared with the WT mice (Figure 4). Rivaroxaban at 6 and 12 mg/kg for 4 months attenuated mesangial matrix expansion in the Ren-TG mice (Figure 4E and 4F).

Effects of Rivaroxaban on GBM Thickness and Foot Process Effacement of Podocytes

The GBM thickness was greater and more irregular in the Ren-TG mice than in the WT mice at 8 months of age (Figure 5A and 5B). These changes were ameliorated by 12 mg/kg of rivaroxaban treatment for 4 months (Figure 5C). Effacement of the podocyte foot process was evaluated based on the number of open slit pores. It was more pronounced in the Ren-TG mice than in the WT mice (Figure 5D and 5E).

Likewise, these changes were ameliorated by 12 mg/kg of rivaroxaban (Figure 5F). Quantitative assessments of GBM thickness and effacement of the podocyte foot process are summarized in Figure 5G and 5H.

Effects of Rivaroxaban on Renal PAR-2 Expression

The gene expression of PAR-2 was significantly higher in the Ren-TG mice than in the WT mice (1.67 ± 0.34-fold, $P<0.05$, n=4–6) (Figure 6A) and was significantly reduced (1.00 ± 0.64-fold, $P<0.01$, n=6) by rivaroxaban treatment. Similarly, the protein expression of PAR-2 was significantly higher in the Ren-TG mice than in the WT mice (4.81 ± 1.59-fold, $P<0.01$, each n=4) and was significantly reduced (2.23 ± 1.34-fold, $P<0.05$, n=4) in the Ren-TG mice by rivaroxaban treatment (Figure 6B and 6C). There were no significant differences in the expressions of PAR-1, PAR-3, and PAR-4 between the Ren-TG mice and the WT mice (data not shown).
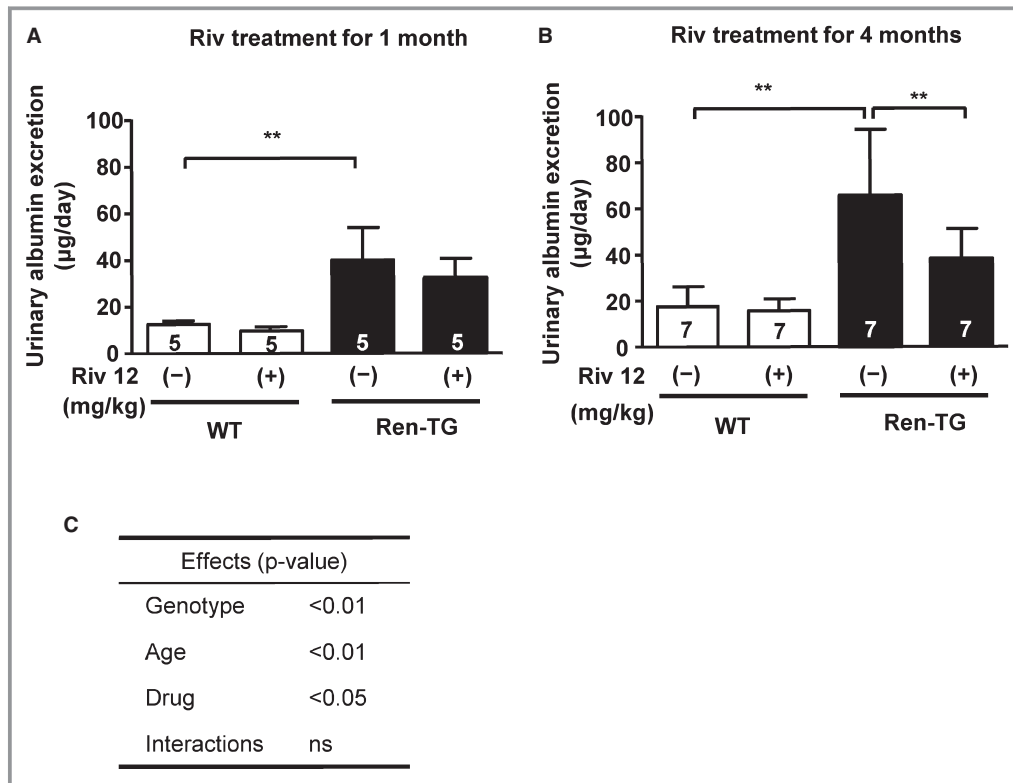

Figure 2. Effects of rivaroxaban on UAE. **A** and **B**, UAE in the WT and Ren−TG mice treated with or without 12 mg/kg of rivaroxaban for 1 month (**A**) and for 4 months (**B**). Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. **$P<0.01$. Sample sizes are given on each bar. **C**, Effects of genotype, age, and drug on UAE were evaluated by 3-way ANOVA. Their interactions were evaluated by both 2-way and 3-way ANOVA. Age was used as a categorical (5 months old for 1-month rivaroxaban treatment and 8 months old for 4-month rivaroxaban treatment). ns indicates not significant; Ren-TG, renin transgenic; UAE, urinary albumin excretion; WT, wild-type.

Effects of Rivaroxaban on Renal Proinflammatory Genes

The gene expression of TNF-α was significantly higher in the Ren-TG mice than in the WT mice (1.45±0.18-fold, $P<0.05$, n=5–6), and rivaroxaban significantly reduced the increase (0.62±0.34-fold, $P<0.01$, n=5) (Figure 6D). Similarly, the gene expressions of PAI-1 and MCP-1 were significantly higher in the Ren-TG mice than in the WT mice (1.52±0.31-fold, $P<0.05$, n=5–6, and 1.96±0.36-fold, $P<0.01$, n=5–6, respectively) (Figure 6E and 6F), and these increases were significantly reduced by rivaroxaban treatment (0.59±0.15-fold, $P<0.01$, n=6; and 1.31±0.37-fold, $P<0.05$, n=6, respectively).

Effects of Rivaroxaban on Angiotensin II–Induced Actin Reorganization in Human Podocytes

The effacement of foot processes is caused by the disruption of actin filaments in the podocytes.[18] F-actin staining by phalloidin revealed that untreated human podocytes showed normal central stress fibers (Figure 7A). The central stress fibers were disrupted by angiotensin II (1.0 μmol/L) stimulation for 24 hours, and F-actin was increased on the cell periphery, demonstrating actin reorganization (Figure 7B). It was partially prevented by 500 μg/L of rivaroxaban treatment (Figure 7C). Likewise, the gene expression of synaptopodin, which contributes to the stabilization of the glomerular filter,[19] was significantly decreased by angiotensin II (1.0 μmol/L) stimulation for 24 hours (0.75±0.12-fold, $P<0.05$ versus control, n=4–6), whereas rivaroxaban significantly ameliorated this decrease (1.21±0.12-fold, $P<0.01$, n=5) (Figure 7D).

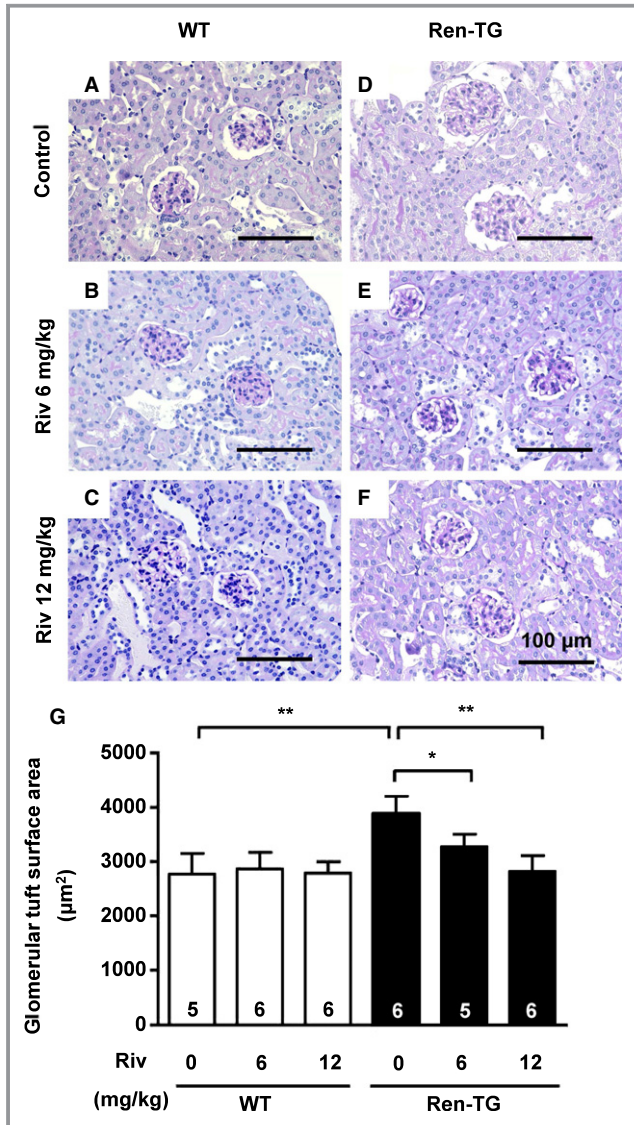**Figure 3.** Effects of rivaroxaban on glomerular hypertrophy. **A** through **F**, Representative images (×200) of the kidneys stained with PAS in WT and Ren-TG mice with or without rivaroxaban at 6 and 12 mg/kg for 4 months. **G**, Comparison of glomerular tuft surface area among the groups. Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. *$P<0.05$, **$P<0.01$. Sample sizes are given on each bar. PAS indicates periodic acid-Schiff; Ren-TG, renin transgenic; WT, wild-type.
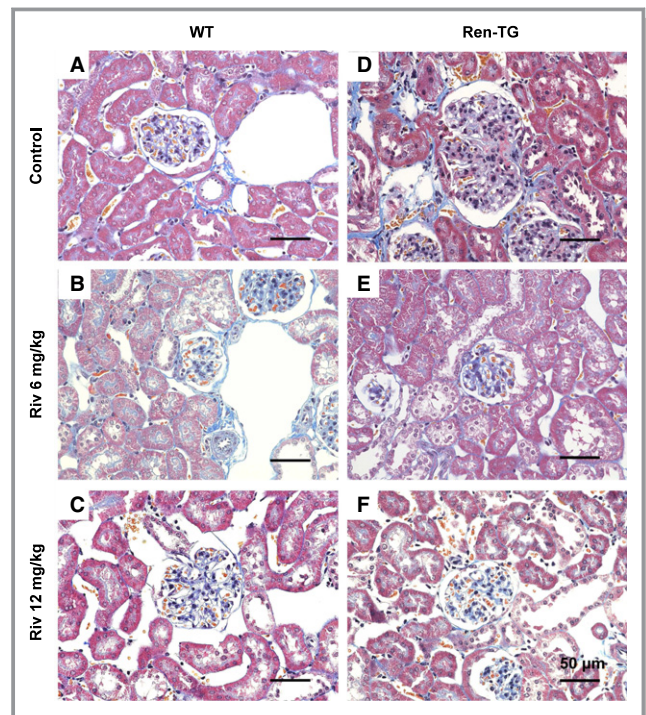

Figure 4. Histologic examination with Masson trichrome staining. **A** through **F**, Representative images (×400) of the kidneys stained with Masson trichrome in WT and Ren-TG mice with or without rivaroxaban at 6 and 12 mg/kg for 4 months. Ren-TG indicates renin transgenic; WT, wild-type.

Effects of Rivaroxaban on Angiotensin II–Induced Proinflammatory Gene Expressions

Treatment with angiotensin II (1.0 μmol/L) for 6 hours significantly increased the gene expressions of MCP-1, TNF-α, and interleukin-6 in human podocytes by 1.49±0.21-fold, 1.29±0.07-fold, and 1.29±0.11-fold, respectively ($P<0.01$, <0.05, and <0.01, each n=6) (Figure 8A through 8C). Rivaroxaban significantly ameliorated these increases (0.78±0.24-fold, n=6; 0.93±0.23-fold, n=5; and 1.01±0.16-fold, n=6, respectively).

In the clinical setting, the maximum concentration values for oral administration of 20 mg of rivaroxaban in humans is approximately in the range of 225.4 to 360.6 μg/L.[20] By using rivaroxaban at concentrations of 100, 500, and 1000 μg/L, MCP-1 gene expression levels were reduced in a dose-dependent manner (data not shown).

Effect of Rivaroxaban on Nuclear Factor–κB Activation

In the control human podocytes, p65 is present in the cytoplasm as shown in red fluorescence (Figure 8D). The translocation of p65 into the nucleus was induced by angiotensin II (1.0 μmol/L) stimulation for 1 hour (Figure 8E) and was partially blocked by 500 μg/L of rivaroxaban treatment (Figure 8F).

Relationship Between the PAR-2 Pathway and the Inflammatory Responses in Human Podocytes

We evaluated the gene expression of PAR-2 by quantitative reverse transcriptase–polymerase chain reaction in human podocytes. Angiotensin II (1.0 μmol/L) for 6 hours signifi-cantly increased its gene expression (1.19±0.10-fold, $P<0.05$
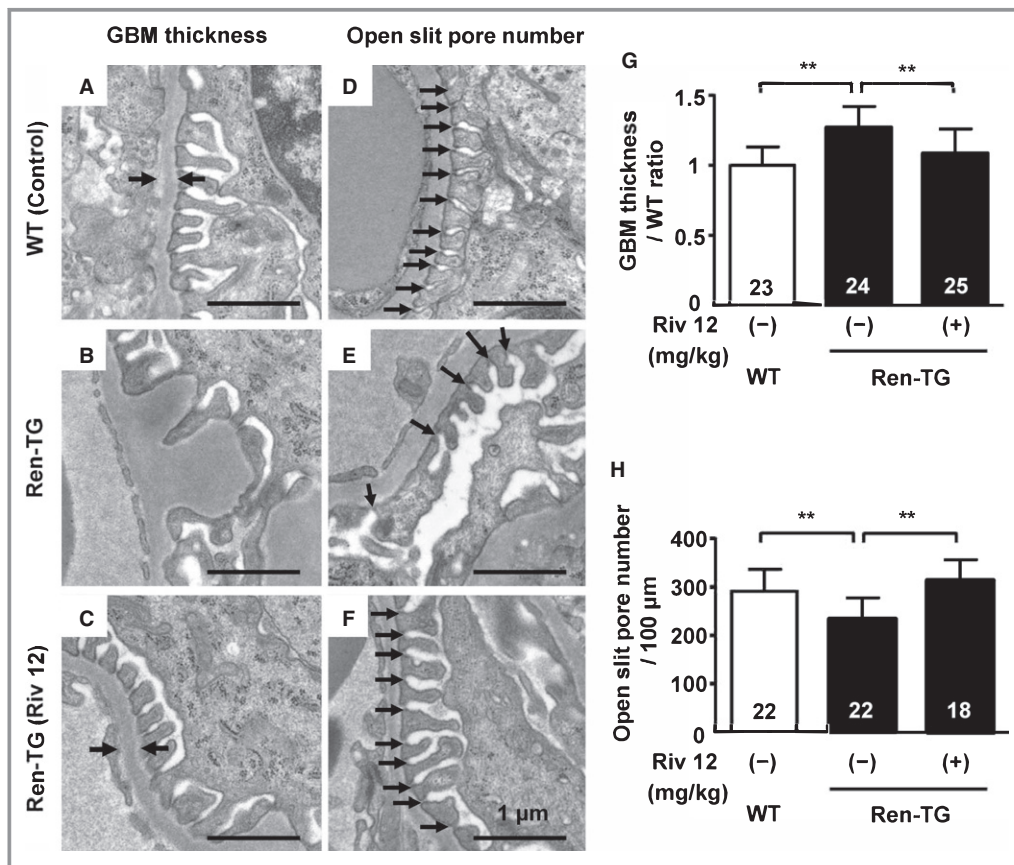

Figure 5. Effects of rivaroxaban on GBM and podocyte foot process effacement. **A** through **F**, Representative images of GBM thickness and the podocyte foot process effacement by electron microscopy (×19 000) in the WT (**A** and **D**), Ren-TG (**B** and **E**), and Ren-TG treated with 12 mg/kg of rivaroxaban for 4 months (**C** and **F**). **G** and **H**, Quantification of GBM thickness (the ratio to the WT mice) (**G**) and open slit pore number per 100 μm (**H**). Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. ******$P<0.01$. Sample sizes are given on each bar. GBM indicates glomerular basement membrane; Ren-TG, renin transgenic; WT, wild-type.

versus control, n=5–6), whereas rivaroxaban significantly attenuated this increase (0.97±0.12-fold, *P*<0.01, n=6) (Figure 9A).

To investigate the role of PAR-2, PAR-2 was temporary knocked down with siRNA. The gene expression of PAR-2 was effectively suppressed by siRNA in human podocytes (*P*<0.01, n=5) (Figure 9B). Treatment with PAR-2 siRNA ameliorated the gene expressions of MCP-1 (Figure 9C) and TNF-α (Figure 9D). The gene expression of synaptopodin was

significantly decreased by angiotensin II, and was recovered by the PAR-2 siRNA (Figure 9E).

Discussion

In the present study, we assessed the protective effects of rivaroxaban against hypertensive renal damage using a Ren-TG hypertensive mouse model with increased albuminuria, glomerular hypertrophy, mesangial matrix expansion,
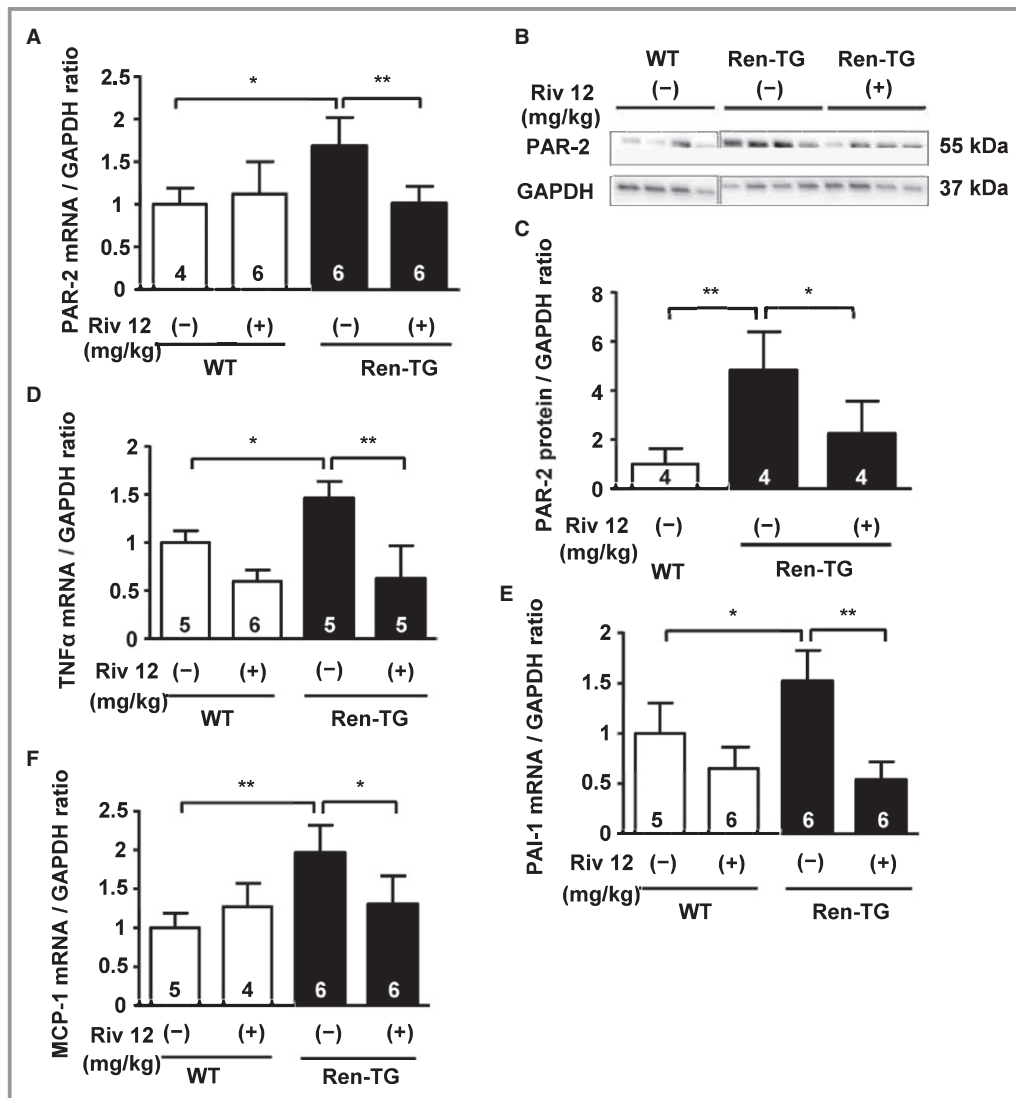

Figure 6. Effects of rivaroxaban on renal expressions of PAR-2 and proinflammatory genes. **A**, Comparison of the renal gene expression of PAR-2 among the WT and Ren-TG mice with or without 12 mg/kg of rivaroxaban for 1 month. **B** and **C**, Representative bands of PAR-2 by western blotting (molecular weight markers are shown) and relative levels of renal protein expression of PAR-2. **D** through **F**, Comparisons of the renal gene expression of TNF-α (**D**), PAI-1 (**E**), and MCP-1 (**F**). Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. *P*<0.05, **P*<0.01. All data are normalized to GAPDH and are described as a ratio to the WT mice. Sample sizes are given on each bar. GAPDH, glyceraldehyde-3-phosphatase dehydrogenase; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1; PAR-2, protease-activated receptor-2; Ren-TG, renin transgenic; TNF-α, tumor necrosis factor-α; WT, wild-type.

effacement of the podocyte foot process, and thickened GBM. Rivaroxaban treatment significantly reduced albuminuria and attenuated these histologic changes, partly through inhibition of the PAR-2 signaling-mediated inflammatory response. Although this is a mild model of the hypertensive renal damage, our results could be applicable to the millions of potential chronic kidney disease patients with hypertension and albuminuria without apparent renal dysfunction.

The role of coagulation factors has been suggested as an important factor of glomerular damage leading to glomerulosclerosis. Fibrin formation within the glomeruli and tubulointerstitium is commonly observed in renal damage, and various anticoagulant agents have been suggested to decrease proteinuria, including the FXa inhibitor danaparoid, which reduces proteinuria in high IgA mice. Nevertheless, there is a risk of bleeding, and these agents have not been accepted in clinical use for the treatment of chronic kidney disease.[21] A recent report further showed that PAR-2 expression was elevated in the kidney of diabetic Akita mice lacking endothelial nitric oxide synthase, and that another FXa inhibitor, edoxaban, suppressed the accumulation of mesangial matrix and podocyte foot process effacement and tended to reduce albuminuria.[22] All these studies strongly indicate
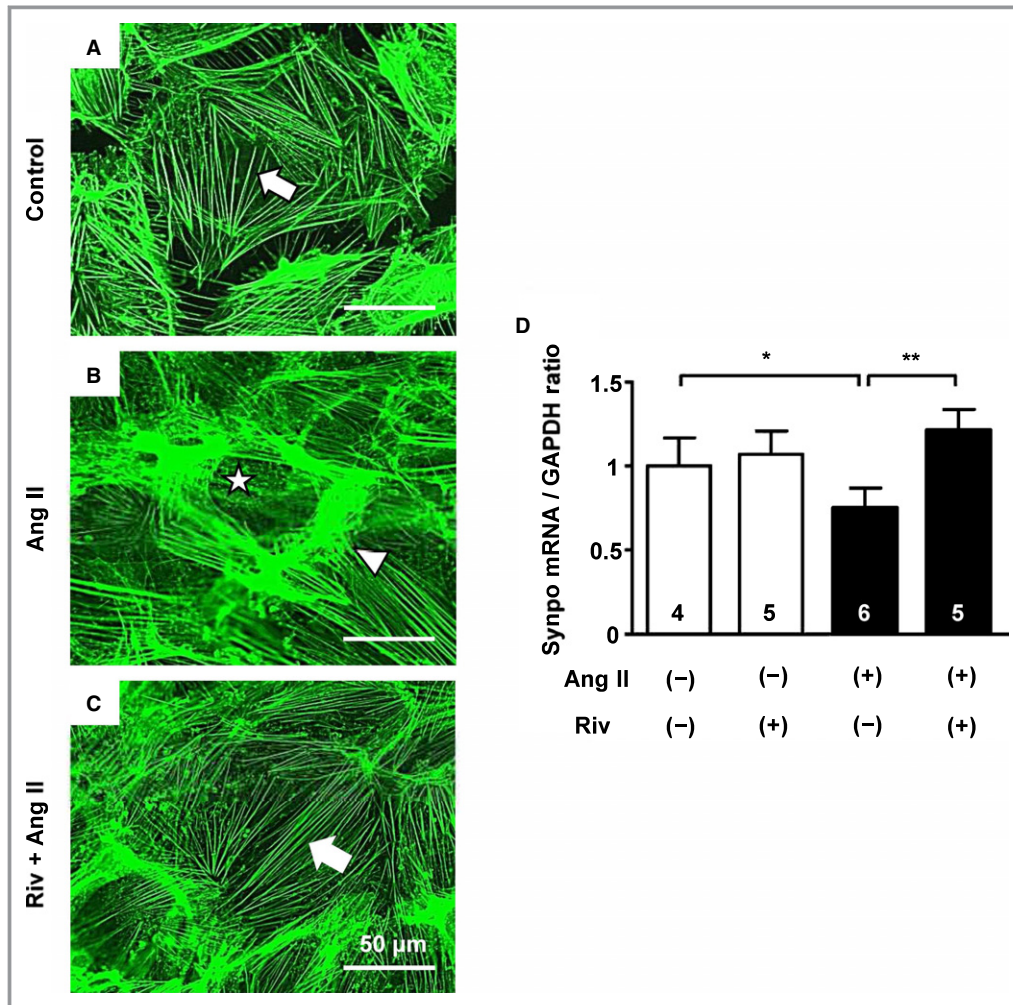

Figure 7. Effects of rivaroxaban on podocyte damage induced by angiotensin II stained by phalloidin. **A,** Arrow indicates normal actin filament structure in the control. **B,** Treatment with angiotensin II (1.0 μmol/L) for 24 hours resulted in reorganization of the actin cytoskeleton with disruption of central stress fibers (stars) and its accumulation on the cell periphery (arrowhead). **C,** Rivaroxaban (500 μg/L) partially ameliorated actin reorganization induced by angiotensin II. **D,** Comparison of the gene expression of synaptopodin in human podocytes. Data are normalized to GAPDH and are described as a ratio to the control. Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. *$P<0.05$, **$P<0.01$. Sample sizes are given on each bar. GAPDH indicates glyceraldehyde-3-phosphatase dehydrogenase.

that FXa-associated PAR-2 signaling plays a significant role in renal inflammation and suggest a beneficial effect of FXa inhibitors.

Although thickening of the GBM is a well-known feature of diabetic nephropathy, it is also associated with hypertensive nephropathy and increased renin-angiotensin system
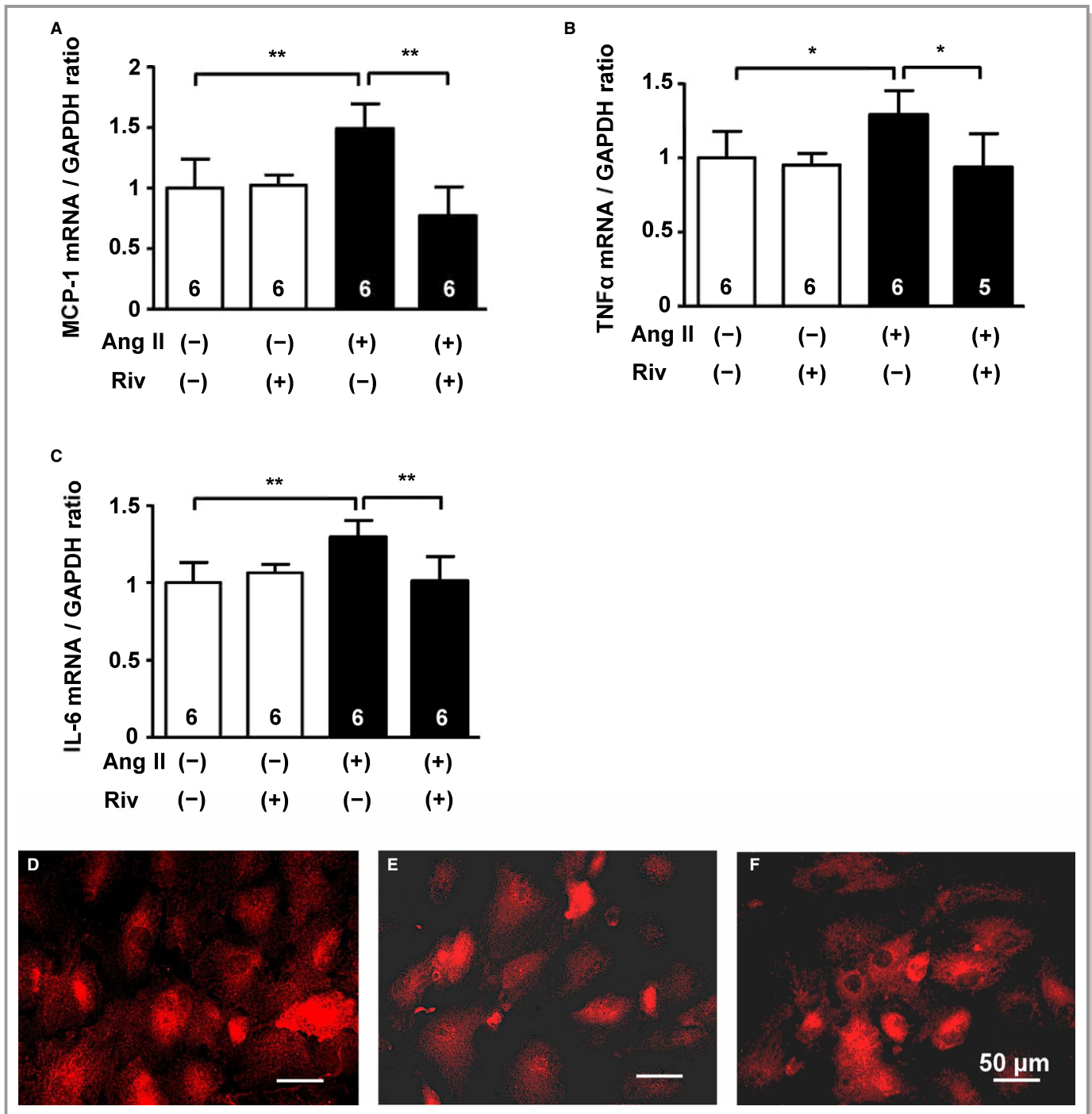

Figure 8. Effects of rivaroxaban on proinflammatory response induced by angiotensin II in human podocytes. Cells were stimulated with angiotensin II (1.0 μmol/L) for 6 hours with the presence or absence of rivaroxaban (500 μg/L). **A** through **C**, Comparisons of the gene expressions of MCP-1 (**A**), TNF-α (**B**), and interleukin-6 (**C**). Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. *$P<0.05$, **$P<0.01$. **D** through **F**, Representative images of the human podocytes stained with p65 nuclear factor–κB in the control (**D**), stimulation with angiotensin II (1.0 μmol/L) for 1 hour (**E**), and rivaroxaban+angiotensin II (**F**). Sample sizes are given on each bar. MCP-1, monocyte chemoattractant protein-I; TNF-α, tumor necrosis factor-α.

activity.[23,24] Furthermore, glomerular hypertrophy is recognized as an integral feature of hypertensive nephropathy[23] and precede renal dysfunction.[25] Accordingly, the Ren-TG mice in this study showed thickened GBM and glomerular hypertrophy, both of which were significantly inhibited by

rivaroxaban treatment. Our study on human podocytes further showed that actin reorganization induced by angiotensin II stimulation was prevented by rivaroxaban. Consistently, decreased gene expression of synaptopodin by angiotensin II was improved by rivaroxaban. Although the detailed
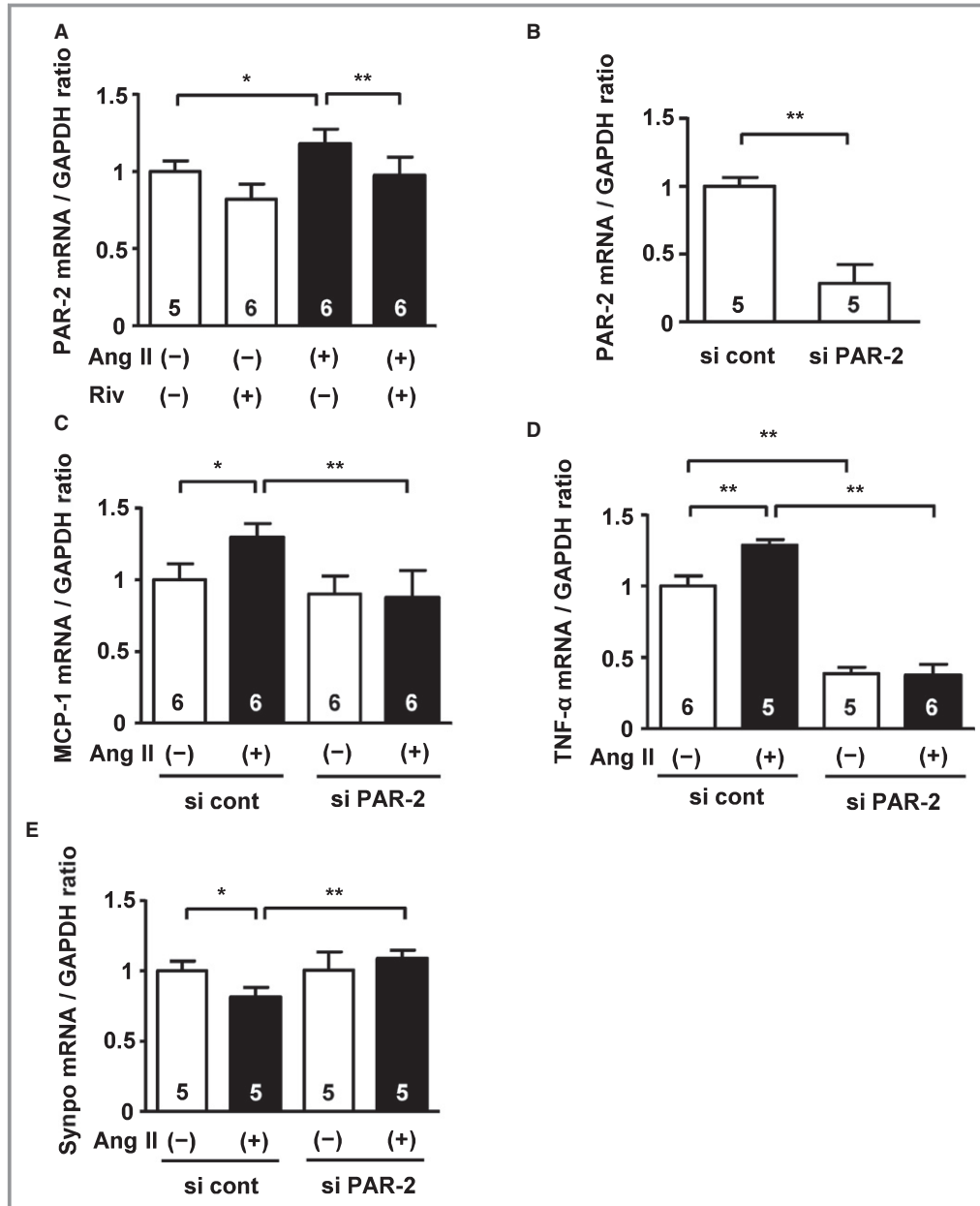

Figure 9. Role of PAR-2 signaling on proinflammatory genes in human podocytes. **A,** Effect of rivaroxaban (500 μg/L) on the gene expression of PAR-2 in human podocytes in the presence or absence of angiotensin II (1.0 μmol/L). **B,** Validation of knockout of PAR-2 by small interfering RNA (si PAR-2) in human podocytes. **C** through **E,** Human podocytes were treated with control small interfering RNA (si cont) or small interfering PAR-2 in the presence or absence of angiotensin II (1.0 μmol/L), and the gene expressions of MCP-1 (**C**), TNF-α (**D**), and synaptopodin (**E**) were quantified. Data are presented as means±SD. Statistical analysis was performed by 1-way ANOVA, followed by the Tukey's honest significant difference test. *$P<0.05$, **$P<0.01$. Sample sizes are given on each bar. MCP-1 indicates monocyte chemoattractant protein-l; PAR-2, protease-activated receptor-2; TNF-α, tumor necrosis factor-α.

mechanism remains largely uncertain, the protective effect of rivaroxaban treatment on GBM thickening, as well as podocyte injury, may contribute to the reduced albuminuria.

PAI-1 is considered as an acute-phase reactant, which is being closely influenced by inflammatory cytokines, such as interleukin-6 and TNF-α.[26,27] PAI-1 is highly expressed in several kidney diseases, both acute and chronic stages.[28] Moreover, numerous animal studies have established a significant link between the PAI-1 levels and glomerulosclerosis.[29] In the present study, rivaroxaban treatment in the Ren-TG mice attenuated mesangial matrix expansion and concomitantly decreased the expressions of PAI-1, TNF-α, and MCP-1. These findings suggest anti-inflammatory effects of rivaroxaban on hypertensive renal damage.

The point to be noted is that rivaroxaban suppressed blood pressure in the Ren-TG hypertensive mice. The relationship between albuminuria and high blood pressure was confirmed in the previous studies.[28–30] We previously showed that hydralazine, a direct vasodilator, administered to this mouse model did not improve albuminuria even though blood pressure was lowered, suggesting that albuminuria observed in the Ren-TG mice is not improved only by lowering blood pressure.[31] These findings indicate that rivaroxaban exerts a protective effect, at least in part through blood pressure–independent effect. In the previous report, edoxaban did not lower blood pressure in the diabetic mouse model. Also, there is no evidence of blood pressure–lowering effect of FXa inhibitors in humans. Further experimental studies are needed to elucidate the underlying mechanism for the blood pressure–lowering effect of rivaroxaban.

Podocytes are the final barrier in the glomerular filtration, and therefore podocyte injury has a significant role in proteinuric conditions. To elucidate further mechanisms of reduced albuminuria by rivaroxaban treatment, we performed an in vitro study on human podocytes. We showed that PAR-2 expression and nuclear factor–κB activation were increased by angiotensin II, and were inhibited by rivaroxaban treatment. In addition, angiotensin II stimulation increased the expression of MCP-1 and TNF-α, whereas temporary knockdown of PAR-2 by siRNA reversed these changes. These results indicate involvement of PAR-2 signaling in the renal inflammatory process and podocyte damage. However, we focused only on the podocyte injury in this study, and therefore experiments using tubular cells and other intrinsic renal cells are clearly required.

FXa inhibitors have been widely used for the prevention of stroke and systemic embolism in patients with nonvalvular atrial fibrillation (NVAF).[30,32,33] In the ROCKET AF (An Efficacy and Safety Study of Rivaroxaban With Warfarin for the Prevention of Stroke and Non–Central Nervous System Systemic Embolism in Patients With Non-valvular Atrial Fibrillation) study, rivaroxaban at 20 or 15 mg per day was shown to be noninferior to warfarin in preventing stroke or systemic embolism in patients with NVAF.[30] Notably, 90% of the patients in that study had hypertension as a coexisting condition. Although renal dysfunction in patients with NVAF has been shown to increase the risk of stroke and bleeding,[34,35] a subanalysis of the ROCKET AF study showed that the rate of decline in mean estimated glomerular filtration rate (eGFR) during the follow-up period was significantly slower in patients treated with rivaroxaban than in those treated with warfarin (3.5 versus 4.3 mL/min).[36] Furthermore, a recent real-world data analysis that used a large US database confirmed that compared with warfarin, rivaroxaban and dabigatran, which is a direct thrombin inhibitor, were associated with lower risks of estimated glomerular filtration rate decline, doubling of serum creatinine, and acute kidney injury.[37] Moreover, in animal studies, warfarin has been shown to induce renal damage due to glomerular hemorrhage and vascular calcification.[38,39] Taken altogether, the results of the present study might provide an important clinical implication on the underlying mechanism by which rivaroxaban exerts its beneficial effects against renal damage in hypertensive patients with NVAF. Further experimental and clinical studies on this matter are certainly warranted.

In conclusion, rivaroxaban inhibited progressive renal damage in the Ren-TG hypertensive mice. Furthermore, rivaroxaban exerted protective effects against angiotensin II–induced podocyte injury not only through the coagulation cascade but also partly through inhibition of the PAR-2 signaling-mediated inflammatory response. All these findings may provide a theoretical basis on the renal protective effect of rivaroxaban treatment in hypertensive patients with NVAF.

Disclosures

Dr Tomita received research funding from Boehringer Ingelheim, Bayer, Daiichi-Sankyo, and Pfizer, and Speakers' Bureau/Honorarium from Boehringer Ingelheim, Bayer, and Daiichi-Sankyo. Dr Okumura received Speakers' Bureau/Honorarium from Daiichi-Sankyo, Boehringer Ingelheim, and Bayer. The remaining authors have no disclosures to report.

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