Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Roxadustat Does Not Affect Platelet Production, Activation, and Thrombosis Formation

Jiaxin Zhao,* Yanyan Xu,* Jingyuan Xie, Junling Liu, Ruiyan Zhang, Xiaoxiang Yan

OBJECTIVE: Roxadustat is a new medication for the treatment of renal anemia. EPO (erythropoietin)—the current treatment standard—has been reported to enhance platelet activation and production. However, to date, the effect of roxadustat on platelets is unclear. To address this deficiency, herein, we have evaluated the effect of roxadustat on platelet production and function.

APPROACH AND RESULTS: We performed several mouse platelet functional assays in the presence/absence of in vitro and in vivo roxadustat treatment. Both healthy and 5/6 nephrectomized mice were utilized. The effect of roxadustat on platelet function of healthy volunteers and chronic kidney disease patients was also evaluated. For platelet production, megakaryocyte maturation and proplatelet formation were assayed in vitro. Peripheral platelet and bone marrow megakaryocyte counts were also determined. We found that roxadustat could not stimulate washed platelets directly, and platelet aggregation, spreading, clot retraction, and P-selectin/JON/A exposure were similar with or without in vitro or in vivo roxadustat treatment among both healthy and 5/6 nephrectomized mice. In vivo mouse thrombosis models were additionally performed, and no differences were detected between the vehicle and roxadustat treatment groups. EPO, which was considered a positive control in the present study, promoted platelet function and production as reported previously. Megakaryocyte maturation and proplatelet formation were also not significantly different between control mice and those treated with roxadustat. After receiving roxadustat for 14 days, no difference in the peripheral platelet count was observed in the mice.

CONCLUSIONS: Administration of roxadustat has no significant impact on platelet production and function.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: blood platelets ■ platelet aggregation ■ platelet count ■ renal insufficiency, chronic ■ thrombosis

Renal anemia is one of the most common complications of chronic kidney disease (CKD) and is characterized by decreased hemoglobin, hematocrit, and circulating erythrocytes. This type of anemia occurs due to lack of EPO (erythropoietin) synthesis by the kidneys.[1] Therefore, exogenous EPO has become an important treatment for renal anemia. Although EPO is effective in correcting anemia, there are some concerns that need to be considered. EPO has already been reported to enhance platelet activation and production,[2,3] which may increase the risk of thrombosis in CKD patients. Previous studies have revealed that platelet activity is significantly increased in patients with CKD. This hyperactivity is caused by uremic toxins and inflammatory reactions that have activating effects on platelets, placing CKD patients at a higher risk of thrombosis than people with normal kidney function.[4,5] Thus, for these patients, EPO therapy with its effect on platelet activation might not be a good choice.

HIFs (hypoxia-inducible factors)—key transcription factors for cell adaptation to hypoxia—have been reported

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For Sources of Funding and Disclosures, see page 2536.

Nonstandard Abbreviations and Acronyms

5/6 Nx	5/6 nephrectomy
CKD	chronic kidney disease
DMSO	dimethyl sulfoxide
EPO	erythropoietin
HIF	hypoxia-inducible factor
HRP	horseradish peroxidase
IL-3	interleukin-3
PAI-1	plasminogen activator inhibitor 1
PE	phycoerythrin
PGE1	prostaglandin E1
PHD	prolyl hydroxylase domain
ROS	reactive oxygen species

to participate in glucose metabolism, cell proliferation/survival, angiogenesis, and iron metabolism.[6] HIFs also upregulate the expression of EPO by binding to a consensus sequence (5′-TACGTGCT-3′) in the EPO 3′ enhancer region.[7] In normoxia, the HIF-α subunit is hydroxylated by PHD (prolyl hydroxylase domain), ubiquitinated, and then finally degraded by the proteasome. When oxygen levels decrease, hypoxia impairs the activity of PHD, thus reducing HIF degradation.[8] Roxadustat stabilizes HIF by inhibiting PHD; HIF thus accumulates intracellularly by avoiding recognition by ubiquitinating enzymes. This upregulates the expression of several HIF-responsive genes including EPO, the EPO receptor, and proteins promoting iron absorption, iron transport, and heme synthesis,[9] which makes roxadustat treatment of renal anemia possible.

As a new favorite, roxadustat is a potential alternative to replace recombinant EPO as a therapy for renal anemia on the basis of its oral administration, good efficacy, and convenience. However, whether this new substitute affects platelet activation and production remains unknown. Some previous studies have indicated that exogenous EPO enhances platelet activation and production[2] and that upregulation of platelet HIF induces a prothrombotic phenotype.[10] These all have created an urgent need to clarify whether this HIF-PHD inhibitor roxadustat has an impact on platelets by upregulating the expression of both EPO and HIF. Thus, the aim of the present study was to evaluate the effect of roxadustat on platelets using both in vitro and in vivo assays.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Experimental Design

For the in vitro experiments, platelets that had been isolated from healthy C57BL/6J mice and healthy volunteers were incubated with roxadustat or vehicle for 30 minutes, and platelet functional assays were performed to evaluate the effect of roxadustat on platelet function (Figure 1A). The healthy mice were orally administered roxadustat or vehicle to confirm the impact of the drug on platelets in vivo (Figure 2A). Mice that had received 5/6 nephrectomy (5/6 Nx) were orally administered roxadustat or vehicle or subcutaneously injected with EPO and multiple thrombosis models were performed to evaluate the effect of roxadustat on platelet activation and thrombosis formation in the state of chronic renal failure (Figure 4A). Platelet function was also measured among CKD patients prescribed roxadustat or not. Platelet production after administration of roxadustat or vehicle was also assessed both in vitro and in vivo.

Highlights

- HIF (hypoxia-inducible factor)-1α and HIF-2α are expressed in platelets and accumulate in the presence of proline hydroxylase inhibitors.
- Conventional therapeutic dose of roxadustat increases intracellular HIFs and upregulates EPO (erythropoietin) expression lightly and transiently in vivo.
- We revealed for the first time that roxadustat does not affect platelet functions both in vitro and in vivo.
- Roxadustat has no effect on platelet production.

Animals

C57BL/6J male mice were purchased from Model Organisms, Shanghai, China. All animal procedures met the standard of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23, revised 1996) and were approved by the Animal Care Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. Only male mice were studied to avoid the potential impact of hormones on platelets.[10–12]

Human Study

Platelets of healthy male volunteers and CKD patients were isolated from venous blood under informed consent. All CKD patients were recruited from inpatients in the Nephrology Department of Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China. Patients experiencing hematologic diseases, infections, thrombotic diseases, or those receiving antithrombotic therapy were excluded. Only nondialysis-dependent CKD patients were included due to the potential impact of dialysis on platelets. The patients were divided into 2 groups according to whether they received roxadustat treatment or not. The patients taking roxadustat were defined as those taking the drug (≈2 mg/kg, thrice per week) continuously for >2 weeks. Human platelets were prepared by the same method used for mouse. Platelet aggregation and P-selectin/PAC1 (procaspase-activating compound 1) exposure upon activation were assessed to evaluate the effect of roxadustat on human platelet function. The platelet aggregation assay was performed on only 5 patients in each group because the test required collecting a relatively large amount of blood, which was not recommended for patients with renal anemia. The human

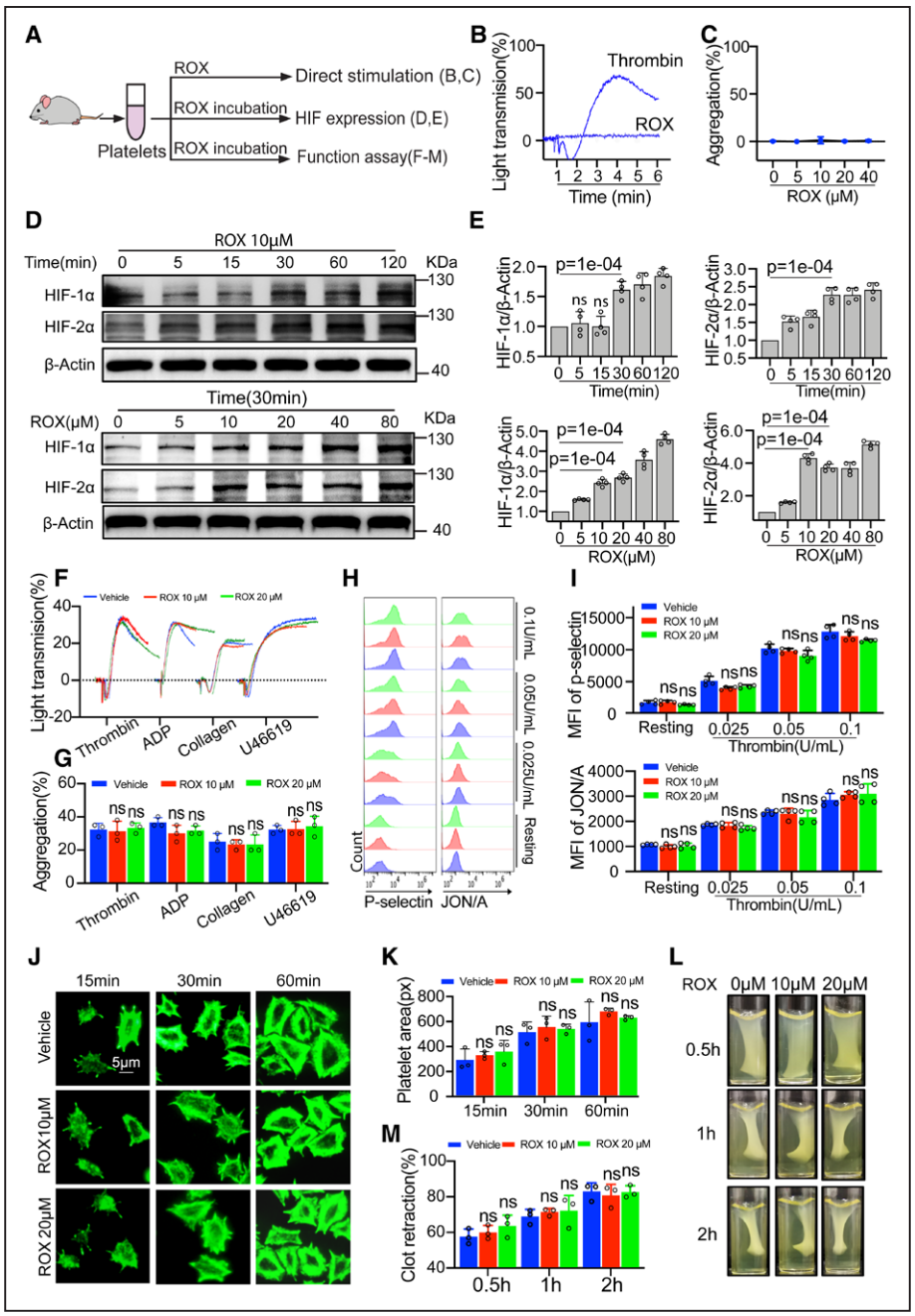

Figure 1. Roxadustat (ROX) has no effect on platelet activation in vitro.
A, Design of in vitro ROX pretreatment experiment. **B**, Platelet aggregation curves were recorded when platelets were stimulated directly with ROX (10 μM) or thrombin (0.03 U/mL). **C**, Maximum platelet aggregation of mouse washed platelets after direct stimulation with ROX at indicated concentrations. Aggregation level is presented as mean±SD of 3 different determinations. **D** and **E**, Western blotting and relative quantification of HIF (hypoxia-inducible factor)-1α and HIF-2α expression levels of mouse platelets incubated with ROX at indicated concentrations and times. Each symbol represents one experiment, and data are means±SD of 4 independent experiments. **F** and **G**, After preincubating with ROX (10 or 20 μM) or vehicle (0.1% dimethyl sulfoxide [DMSO], 0 μM ROX), aggregation of mouse washed platelets by stimulation with thrombin (0.033 U/mL), ADP (6 μM), U46619 (0.5 μg/mL), or collagen (2 μg/mL) was tested. Each symbol indicates the average aggregation level of one experiment, and statistical results are shown from 3 independent experiments. Bars represent the means±SD of the extent of aggregation. ns, no significance compared with vehicle (0.1% DMSO). **H** and **I**, Surface exposure of platelet P-selectin and JON/A in the presence of thrombin (0.025 U/mL, 0.05 U/mL, 0.1 U/mL) was evaluated by flow cytometry with PE (phycoerythrin)-conjugated anti-mouse CD62p (p-selectin) antibody and PE-conjugated anti-mouse JON/A antibody. Mean fluorescence intensity (MFI) is plotted as mean±SD of 4 independent experiments, and each symbol represents the average MFI level of one experiment. **J** and **K**, Spreading platelets preincubated with ROX (10 or 20 μM) or vehicle (0.1% DMSO) were stained with rhodamine-conjugated phalloidin at the indicated time points. Plotted bars represent means±SD of adhering platelet areas from 3 independent experiments, and each symbol represents the average area of one experiment. Scale bar = 5 μm. **L** and **M**, Platelets preincubated with ROX (10 or 20 μM) or vehicle (0.1% DMSO) were added to human plasma, and clot retraction is displayed at the indicated time point. Bar plots show means±SD of clot retraction levels from 3 independent experiments. Each symbol represents the average extent of clot retraction from one experiment. ns indicates no significance compared with vehicle.

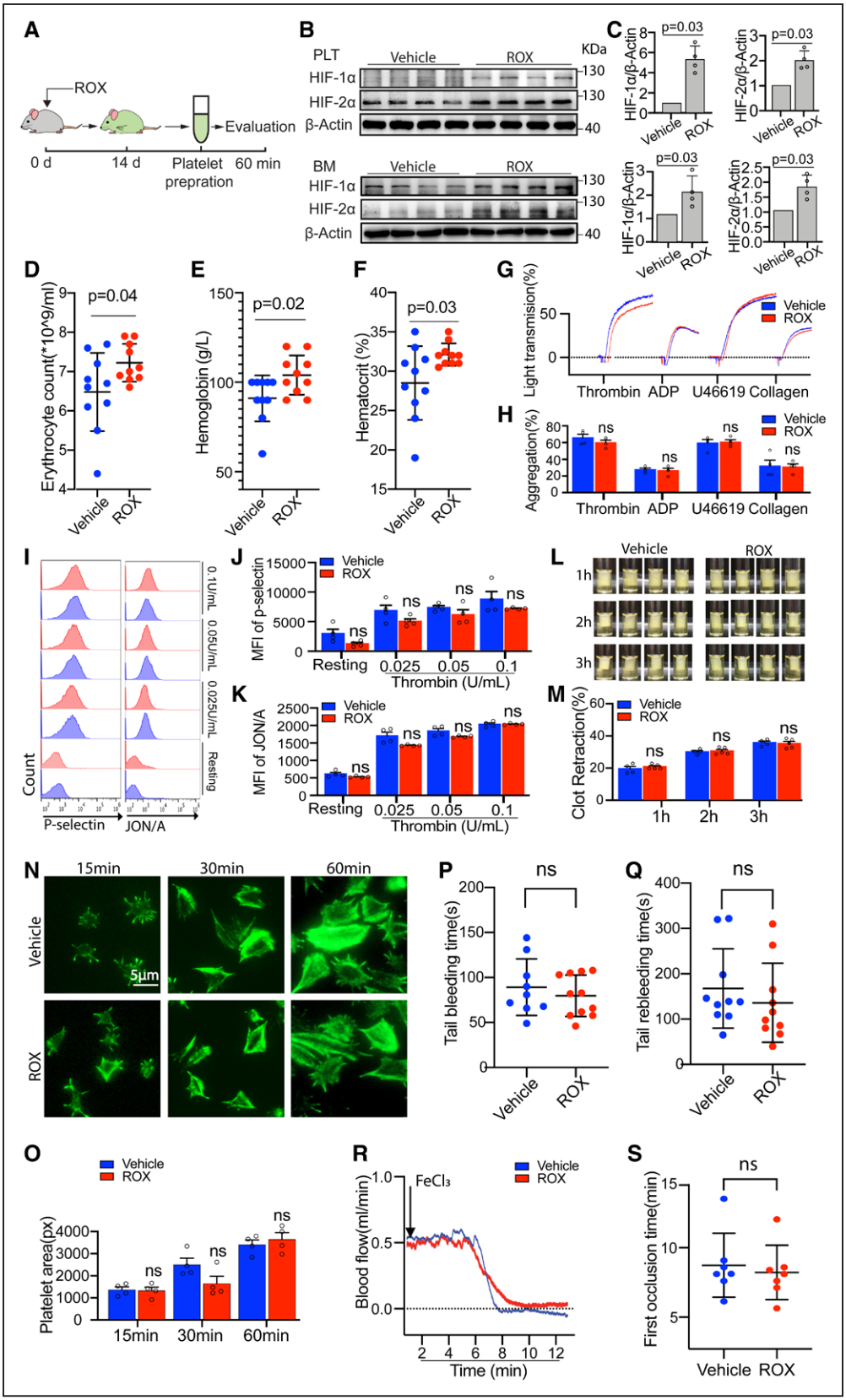

Figure 2. Roxadustat (ROX) has no effect on platelet (PLT) activation in healthy mice in vivo.
A, Design of in vivo ROX administration experiment examining PLT function. **B** and **C**, Western blotting and relative quantification of HIF (hypoxia-inducible factor)-1α and HIF-2α expression levels in PLTs and bone marrow of mouse administered ROX (20 mg/kg) or vehicle (dimethyl sulfoxide [DMSO] 1 μL/g body weight). Each lane or symbol represents one animal, and plotted data are means±SD from 4 mice. (*Continued*)

study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine.

Drug Administration

For drug preparation, 10 mg roxadustat (catalog No. S1007; Selleck Chemicals, Co, Ltd, China) was dissolved in 500 µL dimethyl sulfoxide (DMSO) and stored at −80 °C for no more than 3 months. This stock solution was further diluted with PBS to 1 mg/mL before use and orally administered within 20 minutes. The healthy male mice (8–10 weeks old) received oral roxadustat at 20 mg/kg or an equal volume of vehicle (DMSO, 1 µL/g body weight) daily for 2 weeks before experimental evaluation. The mice that had received 5/6 Nx were orally administered roxadustat (20 mg/kg) or vehicle (DMSO, 1 µL/g body weight) or subcutaneously injected EPO (500 U/kg) thrice per week for 2 weeks before platelet preparation. The selected roxadustat dose was equivalent to the upper bounds in phase II trials of roxadustat treatment for renal anemia,[13] and the treatment duration was selected because it is the physiological life span of platelets.

Induction of Megakaryocyte Differentiation, Polyploidy Assay, and Proplatelet Formation Assay

A fetal liver single-cell suspension of 13.5-day-old mouse embryos was prepared and cultured in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin/streptomycin, 10 ng/mL IL-3 (interleukin-3; catalog No. SRP4134; Sigma-Aldrich), and 50 ng/mL recombinant human thrombopoietin (catalog No. SRP3178; Sigma-Aldrich) at 37 °C in 5% CO_2 for 4 days. For the polyploidy test, cultured megakaryocytes on day 4 were harvested and labeled with fluorescein isothiocyanate–conjugated anti-mouse CD41 (integrin alpha-IIb) antibody. The labeled cells were then fixed with 70% ethanol at 4 °C overnight. After washing twice with PBS, the cells were incubated with DNA staining buffer containing 50 µg/mL propidium iodide, 100 µg/mL RNase A, and 0.2% Triton X-100 at 37 °C for 30 minutes. Polyploidy was then analyzed by flow cytometry. To assess proplatelet formation, cultured megakaryocytes were purified by precipitating in gradient 3%/1.5% BSA solution for 1 hour at 37 °C and then plated on glass slides coated with 50 µg/mL fibrinogen (catalog No. F3879; Sigma-Aldrich) at 4 °C overnight. The proplatelets were then fixed with 4% paraformaldehyde at 4 °C overnight and stained with an α-tubulin primary antibody (catalog No. ab7291; Abcam, United Kingdom) and Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories). Images were acquired on a BX53MTRF-S fluorescence microscope (Olympus, Japan).

Platelet Life Span Assay

Endogenous platelet survival was measured after tail intravenous injection of Sulfo-NHS-LC-Biotin (30 µg/g; catalog No. PG82075; Invitrogen). The platelets were purified from orbital blood collected once daily for 6× in total, and the biotin-labeled platelets were detected by flow cytometry with APC (allophycocyanin)-streptavidin (10 µg/mL; catalog No. 405207; BioLegend).

Complete Blood and Washed Platelet Counts

Orbital mouse blood samples (100 µL) were collected into heparinized tubes (BD Microtainer) and stored in a thermostatic water bath at 37 °C. Before testing, the blood samples were diluted with an equal volume of normal saline preheated to 37 °C. Platelet and red blood cell counts, hemoglobin level, and hematocrit were analyzed on an automated hematology analyzer (Sysmex XT-2000i). The results are presented as the mean±SD for n=5 to 10 mice. For washed platelet counts, one volume of platelets was diluted with 9 volumes of modified Tyrode solution, and the final measurement was the average of 3 repeated tests.

Platelet Preparation

Washed platelets were prepared as described previously.[14] Briefly, blood was collected from the abdominal aorta of isoflurane-anesthetized mice into syringes containing 100 µL/mL White anticoagulant (2.94% sodium citrate and 136 mmol/L glucose [pH 6.4]), 0.1 µg/mL PGE1 (prostaglandin E1; catalog No. 745-65-3; Sigma-Aldrich), and 1 U/mL apyrase (catalog No. 9000-95-7; Sigma-Aldrich). The samples were diluted with equal volumes of normal saline preheated to 37 °C and then centrifuged at 250*g* for 10 minutes. The platelet-rich plasma was transferred to a new centrifuge tube and centrifuged at 850*g* for 10 minutes. The supernatant was discarded and the platelets were resuspended in modified Tyrode solution (12 mmol/L $NaHCO_3$, 138 mmol/L NaCl, 5.5 mmol/L glucose, 2.9 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.42 mmol/L NaH_2PO_4, 10 mmol/L HEPES, pH 7.4).[14] The platelets were then counted and adjusted to 3×10^8 platelets/mL. For the in vitro experiment, the

Figure 2 Continued. **D**–**F**, Peripheral erythrocyte counts, hemoglobin levels, and hematocrits of healthy mice administered oral ROX (20 mg/kg) or vehicle. Each symbol represents one animal; plotted lines show means±SD, n=10. **G** and **H**, Aggregation curves of PLTs from healthy mice that received ROX (20 mg/kg) therapy or vehicle for 14 d. PLTs were stimulated with thrombin (0.1 U/mL), ADP (6 µM), U46619 (0.5 µg/mL), or collagen (2 µg/mL). Each symbol indicates the average aggregation level of one experiment, and plotted data are means±SD of 4 independent experiments; ns, no significance compared with vehicle. **I**–**K**, Surface exposure of PLT P-selectin and JON/A in the presence of thrombin (0.025, 0.05, and 0.1 U/mL) was evaluated by flow cytometry between mice receiving ROX administration or not. Mean fluorescence intensity (MFI) is plotted as the mean±SD of 4 independent experiments, and each symbol represents the average MFI from one experiment; ns, no significance compared with vehicle. **L** and **M**, PLTs from mice administered ROX (20 mg/kg) or vehicle (DMSO 1 µL/g body weight) were added to human plasma, and clot retraction is displayed at the indicated time point. Bar plots show the means±SD of clot retraction levels from 4 independent experiments. Each symbol represents the average extent of clot retraction in one experiment; ns, no significance compared with vehicle. **N** and **O**, Spreading PLTs from mice administered ROX (20 mg/kg) or vehicle (DMSO 1 µL/g) were stained with rhodamine-conjugated phalloidin at the indicated time points. Data are means±SD of adhering PLT areas from 4 independent experiments, and each symbol represents the average area of one experiment. Scale bar as indicated; ns, no significance compared with vehicle. **P** and **Q**, Tail-bleeding and rebleeding time of mice receiving roxadustat ROX (20 mg/kg) or vehicle (DMSO 1 µL/g). Each symbol represents one animal, and data are means±SD of time (s); n=10; ns, no significance. **R** and **S**, Blood flow records of FeCl3-induced carotid artery thrombosis formation in mice receiving ROX (20 mg/kg) or vehicle (DMSO 1 µL/g). Lines on dot plots show means±SD of the first occlusion time of blood flow. Each symbol indicates one animal; n=7; ns, no significance.

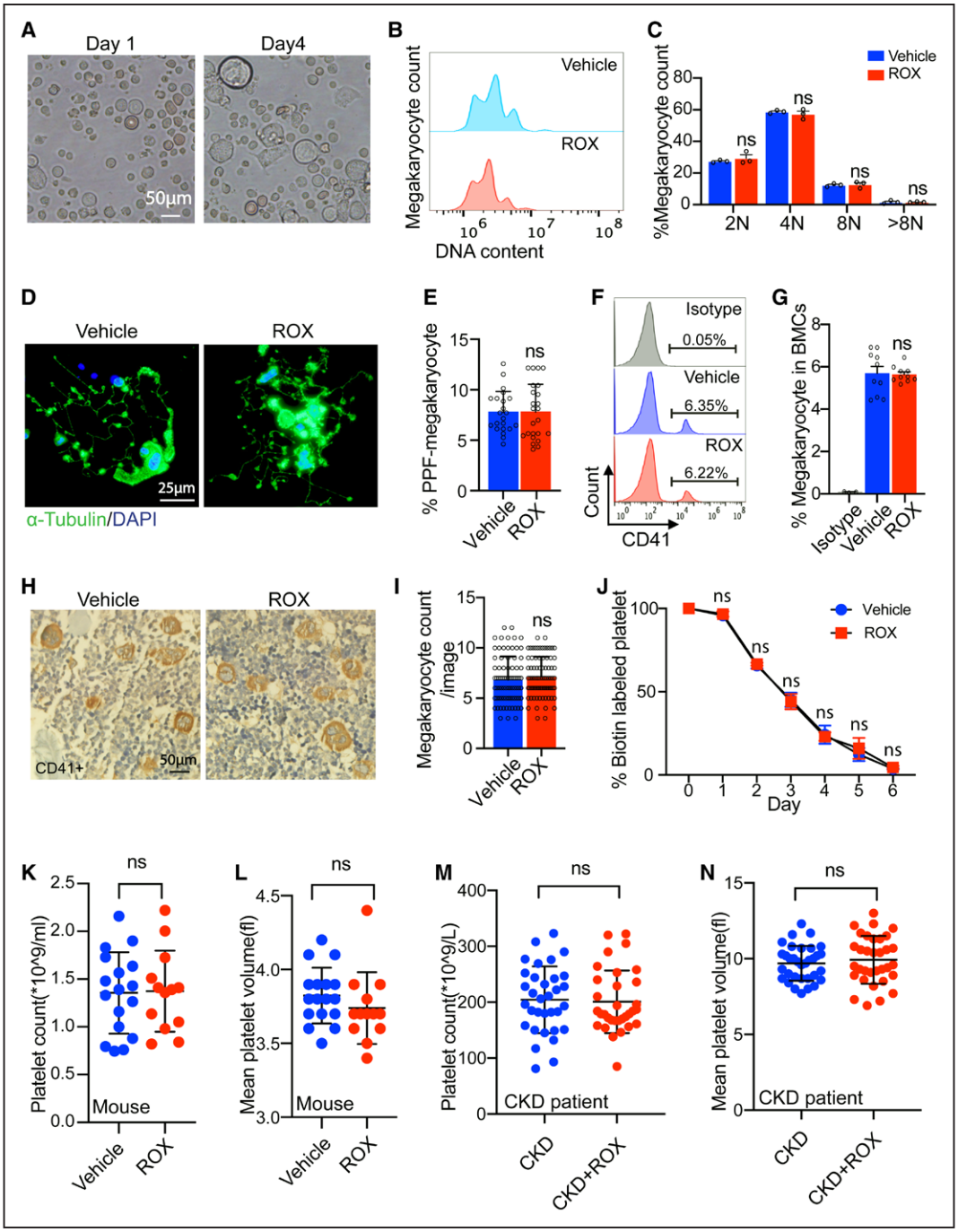

Figure 3. Roxadustat (ROX) does not affect platelet production.

A, Images of fetal liver–induced megakaryocytes are shown at indicated time points. **B** and **C**, Polyploidy assay of cultured megakaryocytes was performed by flow cytometry. The megakaryocytes were first labeled with fluorescein isothiocyanate–anti-mouse CD41 (integrin alpha-IIb) antibody and then stained with PI. Polyploidy percentages are shown as means±SD from 3 independent experiments. Each symbol indicates the average percentage in one experiment. **D** and **E**, Megakaryocyte proplatelet formation was assessed by adding megakaryocytes on glass slides coated with fibrinogen. Images show proplatelet-forming megakaryocytes (PPF-megakaryocytes) in the presence of ROX (10 μM) or vehicle (0.1% dimethyl sulfoxide [DMSO]). Percentages of PPF-megakaryocyte are presented as means±SD of 22 to 24 counts in each group from 3 independent experiments. **F** and **G**, Flow cytometry analysis of CD41+ megakaryocytes from bone marrow of mice administered ROX (20 mg/[kg·day]) or vehicle (1 μL/g body weight). Percentages of megakaryocytes in the total bone marrow cells between the two treatment conditions are presented as means±SD; n=10. **H** and **I**, Immunohistochemistry of sternum bone marrow sections from ROX-treated (20 mg/[kg·day]) or vehicle-treated (1 μL/g body weight) mice. The number of megakaryocytes per image was calculated and plotted as mean±SD of 80 counts from 5 mice in each group. **J**, Platelet life span of healthy mice receiving ROX (20 mg/kg) or vehicle (1 μL/g body weight) was evaluated by flow cytometry. Date are means±SD; n=6. **K** and **L**, Mean platelet volumes and platelet counts of mice receiving ROX (20 mg/[kg·day]) or vehicle (1 μL/g body weight) are shown as means±SD. Each symbol represents 1 animal; n=13 to 17. **M** and **N**, Mean platelet volumes and platelet counts of chronic kidney disease (CKD) patients prescribed ROX (≈2 mg/kg, thrice per week) or not are plotted as means±SD; n=32. BMC indicates bone marrow cell; DAPI, 4′,6-diamidino-2-phenylindole; and ns, no significance.

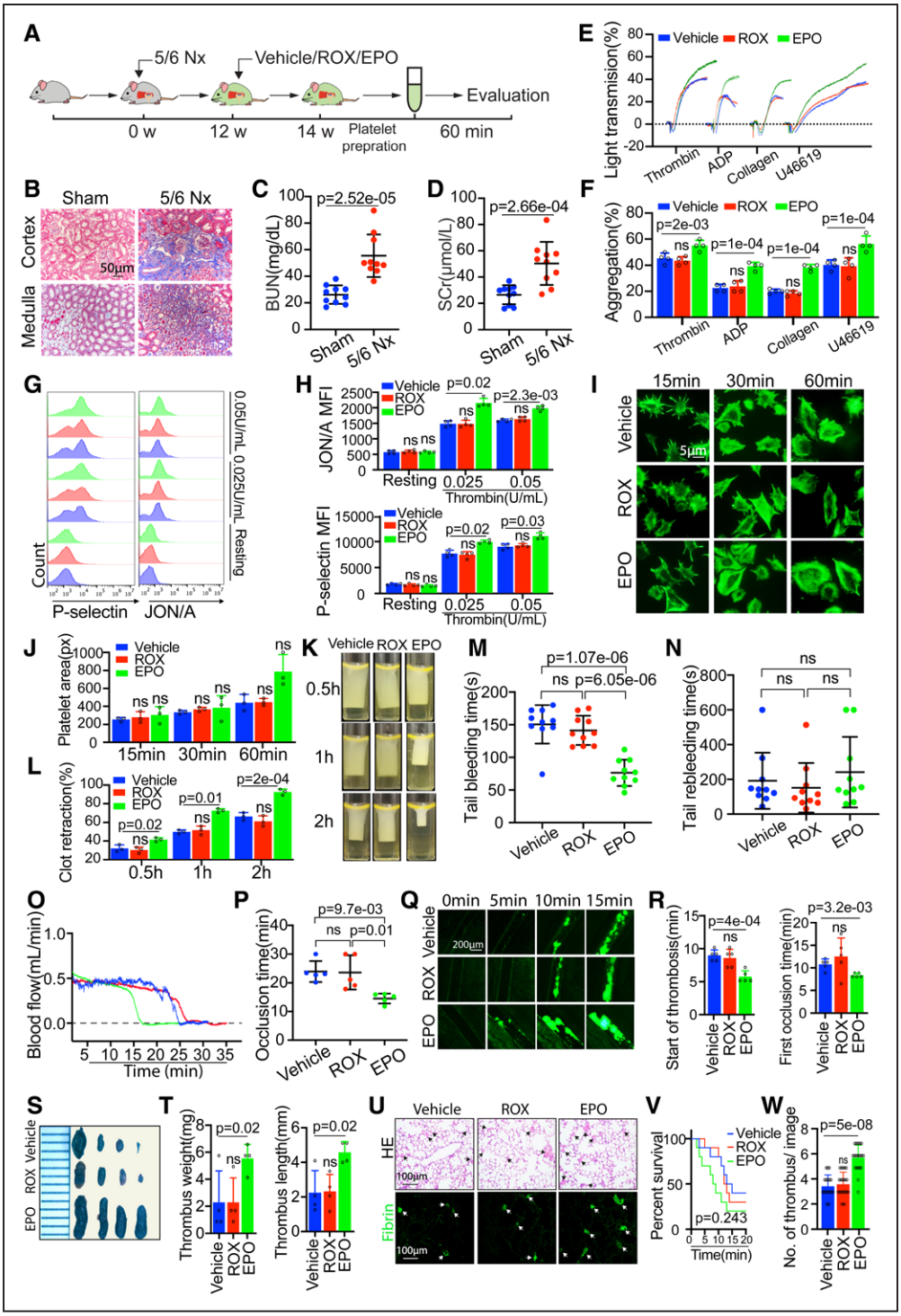

Figure 4. Roxadustat (ROX) has no effect on platelet activation in 5/6 nephrectomized (5/6 Nx) mice.
A, Design of in vivo ROX administration experiment examining platelet functions in 5/6 nephrectomized mice. **B**, Extent of renal fibrosis is shown by Masson trichrome staining of cortex and medulla tissue sections from mice that underwent 5/6 nephrectomy or sham surgery. **C** and **D**, Blood levels of urea nitrogen and serum creatinine in 5/6 nephrectomized and sham-operated mice are plotted as means±SD; n=10. **E** and **F**, Aggregation curves of platelets from chronic kidney disease (CKD) mice administered ROX (20 mg/kg, thrice per week) or EPO (erythropoietin; 500 U/kg, thrice per week) or vehicle (1 μL/g body weight) for 2 wk. Platelets were stimulated with thrombin (0.1 U/mL), ADP (6 μM), U46619 (0.5 μg/mL), or collagen (2 μg/mL). Each symbol indicates the average aggregation level of one experiment, and data are means±SD of 4 independent experiments; ns, no significance compared with vehicle. **G** and **H**, Surface exposure of platelet P-selectin and JON/A in the presence of thrombin (0.025 and 0.05 U/mL) was evaluated by flow cytometry between ROX-, EPO-, or vehicle-treated CKD mice. Mean fluorescence intensity (MFI) is plotted as mean±SD of 4 independent experiments, and each symbol represents the average MFI level of one experiment; ns, no significance compared with vehicle. **I** and **J**, Spreading platelets from CKD mice administered ROX (20 mg/kg), EPO (500 U/kg), or vehicle (dimethyl sulfoxide [DMSO] 1 μL/g) were stained with rhodamine-conjugated phalloidin (*Continued*)

washed platelets were allowed to rest for 30 minutes and then incubated with roxadustat or DMSO for another 30 minutes before the aggregation and spreading tests were performed as described below.

Platelet Aggregation and Spreading

The aggregation of washed platelets was performed as described previously[15] on a whole-blood lumiaggregometer (Chrono-Log, Havertown, PA) upon stimulation with the indicated agonists including thrombin, ADP, U46619, and collagen. Platelet spreading was performed as before.[16] Briefly, glass slides (Lab-Tek II) were precoated with fibrinogen (40 μg/mL in 0.1 M $NaHCO_3$, pH 8.1) overnight at 4 °C and blocked with 1% BSA for 1 hour at room temperature. The mouse washed platelets were diluted with Ca^{2+} free modified Tyrode solution containing 2 mmol/L Mg^{2+} and allowed to spread on the immobilized fibrinogen on a chamber slide for different time intervals at 37 °C. The adhesion was stopped by fixation with 4% paraformaldehyde, and the platelets were permeabilized with Labeling solution (0.2% Triton X-100, 0.5% BSA). Rhodamine-conjugated phalloidin was used to stain the adherent platelets, and images were captured under fluorescence microscopy (×100 objective). The areas of the spreading platelets were quantified by Image J software (version 1.47).

Clot Retraction

For clot retraction, 100 μL of washed platelets was mixed with 300 μL of human platelet-depleted plasma, and 0.4 U thrombin was added to the final concentration of 1 U/mL. The mixture was incubated at 37 °C for different time intervals and then photographed. The degree of clot retraction was analyzed using the Image J software (version 1.47).

Flow Cytometry

To evaluate platelet P-selectin and αIIbβ3 exposure upon activation, washed platelets were diluted with Tyrode solution and adjusted to 3×10^7 platelets/mL. The diluted washed platelets were stained with 10 μg/mL PE (phycoerythrin)-conjugated anti-mouse CD62P or 50 μL/mL PE-conjugated anti-mouse JON/A (10 μg/mL fluorescein isothiocyanate–conjugated anti-human CD62P (p-selectin) or 5 μg/mL fluorescein isothiocyanate–conjugated PAC1 for human platelets). Thrombin or collagen was then added to the final indicated concentration. The mixture was incubated at 37 °C for 30 minutes, and the platelets were then fixed with 200 μL 4% paraformaldehyde. P-selectin and αIIbβ3 expression was quantified on a CytoFLEX S Flow Cytometer (Beckman Coulter Life Sciences) to assess the effect of roxadustat on platelet activation.

Annexin V Binding Assay

Washed mouse platelets were resuspended in modified Tyrode buffer containing 1 mmol/L Ca^{2+} and adjusted to 1×10^7 platelets/mL. The platelets were then stimulated with different concentrations of thrombin in the presence of fluorescein isothiocyanate–annexin V (50 μL/mL; catalog No. 640905; BioLegend) at 37 °C for 40 minutes. Samples were further diluted with Tyrode buffer, and the proportion of annexin V+ platelets was assessed using flow cytometry.

Reactive Oxygen Species Measurement

Platelet reactive oxygen species (ROS) were tested in the platelets pretreated with 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (catalog No. C2938; Thermo Fisher) as before.[17] Briefly, washed platelets were incubated with 10 mmol/L dichlorodihydrofluorescein diacetate at 37 °C for 15 minutes and washed with Tyrode buffer 3×. Platelets were then treated with vehicle (0.1% DMSO) or roxadustat (20 μM) for 30 minutes and stimulated by collagen to promote intracellular ROS production. The dichlorodihydrofluorescein fluorescence intensity of the platelets was detected by flow cytometry.

Tail-Bleeding Assay

Tail-bleeding was performed as described previously.[18] Briefly, the mice were anesthetized with isoflurane, and their tails were prewarmed in 15 mL sterile, normal saline (37 °C) for 5 minutes. A short section of the tail tip (≈3 mm) was removed, and the tail was immediately returned to the warm saline. The bleeding time was calculated from the start of bleeding to the first cessation of bleeding. The maximum recorded bleeding time was 10 minutes; tail-rebleeding time was also recorded from the time the bleeding stopped to the time it started again. The wounded tail was cauterized if bleeding persisted beyond 10 minutes or rebleeding occurred. For quantitative analysis of bleeding, blood mixed with normal saline (15 mL) was centrifuged at 1000*g* for 10 minutes. The supernatant was discarded, and the

Figure 4 Continued. at the indicated time points. Data are means±SD of adhering platelet areas from 3 independent experiments, and each symbol represents the average area of one experiment. Scale bar as indicated; ns, no significance compared with vehicle. **K** and **L**, Platelet clot retraction from CKD mice administered ROX (20 mg/kg), EPO (500 U/kg), or vehicle (DMSO 1 μL/g) is displayed at the indicated time points. Bar plots show the means±SD of clot retraction levels from 3 independent experiments. Each symbol represents the average extent of clot retraction in one experiment; ns, no significance compared with vehicle. **M** and **N**, Tail-bleeding and rebleeding time of CKD mice receiving ROX (20 mg/kg), EPO (500 U/kg), or vehicle (DMSO 1 μL/g). Each symbol represents one animal, and data are means±SD of time (s); n=10; ns, no significance. **O** and **P**, Blood flow records of FeCl3-induced carotid artery thrombosis formation in CKD mice receiving ROX (20 mg/kg), EPO (500 U/kg), or vehicle (DMSO 1 μL/g). Data show means±SD of the first occlusion time of blood flow; n=5; ns, no significance. **Q** and **R**, Images of FeCl3-induced mesenteric artery thrombosis formation in CKD mice receiving ROX (20 mg/kg), EPO (500 U/kg), or vehicle (DMSO 1 μL/g) in vivo are shown at different time points. Data are means±SD of the start time of thrombosis and first occlusion time of blood flow. n=5; ns, no significance compared with vehicle. **S** and **T**, Experimental deep vein thrombosis of CKD mice administered the indicated drugs or vehicle. Data are shown as means±SD of weight and length of thrombosis from 4 mice that underwent inferior vena cava stenosis; ns, no significance compared with vehicle. **U**–**W**, Lung sections of CKD mice that received ROX, EPO, or vehicle before induction of pulmonary thromboembolism were stained with hematoxylin-eosin (HE) and subjected to immunofluorescence using anti-mouse fibrin antibody. Survival curves (n=10) are also shown. Plotted data are means±SD of 25 counts from 5 mice in each group; ns, no significance compared with vehicle. BUN indicates blood urea nitrogen; and SCr, serum creatinine.

blood cells were resuspended in 1 mL warmed normal saline before hematology testing.

5/6 Nephrectomy

Mouse CKD was simulated with a 5/6 Nx model. Briefly, mice were anesthetized with isoflurane, shaved, and disinfected with 70% ethanol. An incision near the left waist was performed to expose the left kidney completely. One-third of each of the upper and lower poles of the left kidney were removed with sharp scissors, and the remaining tissue was quickly compressed with a pair of hemostatic sponges for 1 minute. Hemostasis was confirmed, and the remaining 1/3 of the kidney was returned to the abdominal cavity. Antibiotic-containing saline was applied before the abdominal cavity was closed carefully. After recovery from anesthesia, the mice were placed in a cage with adequate water and food. One week later, the entire right kidney was removed following a similar procedure on the other side of the abdomen. Sham surgery involved all procedures except nephrectomy. The mice that underwent 5/6 Nx were housed for 12 weeks before further experimentation.

$FeCl_3$-Induced Thrombosis Model

Assays using carotid artery[19] and mesenteric artery[20] thrombosis were performed as described previously. To evaluate thrombosis formation in elastic arteries, the mice were anesthetized, and the common carotid arteries were exposed and detached from the surrounding tissue. A microvascular flow probe attached to a transit-time perivascular flowmeter (Transonic Systems, Inc, New York) was positioned on the common carotid artery to monitor blood flow. Vascular injury was induced by applying a 2-mm piece of filter paper saturated with 10% $FeCl_3$ to the top of the vessel for 3 minutes. Blood flow was recorded immediately after placing the filter paper, and the first occlusion was defined as blood flow <0.05 mL/min. For the muscular artery thrombosis model, the mice were intravenously injected with DyLight 488-conjugated anti-mouse GPIbβ antibody (0.1 μg/g body weight; catalog No. X488; Emfret Analytics) 10 minutes before electrolyte injury. The mesenteric artery was gently exteriorized and moistened with warm saline in a 10-cm sterile cell culture dish. An unbranched mesenteric artery (35–60 μm diameter) was selected and visualized with an inverted fluorescence microscope (×10 objective) equipped with a 100-W HBO fluorescent lamp source and a digital camera connected to a video recorder. Vascular injury was induced by topical application of a 2-mm piece of filter paper saturated with 20% $FeCl_3$ for 10 s. Upon removal of the filter paper, fluorescence photos were acquired every minute until 30 minutes had elapsed or the vessel lumen was completely blocked by thrombus for at least 2 minutes.

Experimental DVT Model

The mouse DVT model was generated as described previously.[21] Briefly, the mice were anesthetized with isoflurane; their intestines were exteriorized and protected with sterile gauze moistened with warm normal saline. The inferior vena cava was carefully separated from the aorta and ligated with a 7-0 suture passing under the inferior vena cava and a 30*g* needle (diameter of 0.31 mm) just below the renal veins. The needle was then gently removed, and all visible inferior vena cava side branches were also sutured. Thereafter, the intestines were returned to the abdomen and the abdominal cavity was closed completely. The mice were euthanized 2 days later, and thrombi were harvested.

Pulmonary Thromboembolism Model

Pulmonary thromboembolism was assayed as reported previously.[22] Briefly, mice were anesthetized with isoflurane, and pulmonary thromboembolism was induced by intravenously injecting a mixture of collagen (170 μg/kg) and epinephrine (60 μg/kg). For survival analysis, death was defined as cessation of breathing for >2 minutes, and time to death was recorded for no more than 20 minutes. For histological studies, the mice were euthanized 2 minutes post-injection, and the lungs were perfused with 4% formaldehyde solution and collected for further hematoxylin/eosin and immunofluorescence staining.

Western Blotting

To confirm the activity of roxadustat treatment, HIF-1α and HIF-2α were analyzed by Western blotting. Tissue from different organs was collected after 14 days of drug administration. HIFs in total protein extracts were detected using primary rabbit anti-mouse HIF-1α antibody (catalog No. ab216842; Abcam), rabbit anti-mouse HIF-2α antibody (catalog No. ab199; Abcam), and secondary HRP (horseradish peroxidase)-linked mouse anti-rabbit IgG antibody (catalog No. ab205718; Abcam). Protein expression levels were quantified through band intensity analysis by Image J software (version 1.47).

Immunohistochemistry

To assess the effect of roxadustat on platelet production in vivo, megakaryocytes in bone marrow sections from mice treated with the drug (20 mg/kg) or vehicle were subjected to immunohistochemistry. Briefly, antigens were unmasked by microwaving the bone marrow sections in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes. After cooling, goat serum was used to block for 1 hour. Immunostaining was undertaken by incubating at 4 °C overnight with antibodies against mouse CD41 (catalog No. sc-365938; Santa Cruz Biotechnology) at 2 μg/mL or equivalent concentrations of polyclonal nonimmune IgG (catalog No. sc-2025; Santa Cruz Biotechnology) as a control. The sections were washed 3× and incubated with the appropriate biotin-conjugated secondary antibody and subsequently with streptavidin solution. Color development was performed with 3,3′-diaminobenzidine tetrahydrochloride. Finally, the sections were dehydrated using increasing concentrations of ethanol and then xylene.

ELISA

Mouse blood EPO levels were measured with a mouse EPO ELISA kit (catalog No. 148305; BioLegend) following the supplied instructions. Briefly, mouse serum samples were diluted 1:2 with sterile PBS, and 100 μL of each diluted sample was added into a 96-well plate precoated with mouse EPO antibody (capture antibody). The plate was incubated at room temperature for 2 hours with shaking (220 rpm). After washing, 100 μL of the detecting antibody solution was added and incubated at room temperature for 1 hour with shaking (220 rpm).

Avidin-HRP solution and substrate solution F were added and incubated for 30 and 10 minutes, respectively. Finally, 100 µL stop solution was added to stop the reaction. The absorbance was read at 450 nm within 20 minutes, and the EPO level was calculated using a standard curve.

Statistical Analysis

Continuous variables were presented as means±SD and tested for normality and equal variance before parametric analysis. To evaluate the influence of a single factor, Mann-Whitney *U* tests (n<6) or unpaired Student *t* tests (n≥6) were used for comparisons between two groups, and 1-way ANOVA followed by Tukey multiple comparison tests was used for comparisons of ≥3 groups. Two-way ANOVA followed by Tukey multiple comparison tests was performed for estimating the influence of 2 factors. A log-rank (Mantel-Cox) test was used to compare the survival curves of the experimentally induced pulmonary thromboembolism mice. The parametric analysis represented relatively low statistical power when the sample sizes were small (n<6). All statistical analyses were performed using GraphPad Prism (version 8.2.1 for MacOS; GraphPad Software, Inc, La Jolla, CA). Statistical significance was defined as $P<0.05$.

RESULTS

Roxadustat Has No Direct Stimulating Effect on Mouse Platelets

We initially explored whether roxadustat can stimulate mouse platelets directly. We measured platelet aggregation using roxadustat as the platelet agonist. As indicated in Figure 1B and 1C, no aggregation was apparent after stimulation with roxadustat at different concentrations.

Pretreatment With Roxadustat Has No Effect on Platelet Activation Induced by Conventional Agonists In Vitro

To confirm whether roxadustat enhances or suppresses platelet activation in vitro, the functions of washed platelets pretreated with roxadustat or vehicle were tested. We analyzed both the time and concentration dependency of the effect of roxadustat on washed platelets. The protein levels of platelet HIF-1α and HIF-2α were upregulated significantly in a time- and concentration-dependent manner (Figure 1D and 1E). Based on this result, washed mouse platelets were incubated with roxadustat (10 or 20 µM) or vehicle (0.1% DMSO) for 30 minutes before functional evaluation in vitro. First, the aggregation of platelets pretreated with different concentration of roxadustat or vehicle was measured, and there was no remarkable effect on the aggregation induced by thrombin (0.033 U/mL), ADP (6 µM), U46619 (0.5 µg/mL), and collagen (2 µg/mL; Figure 1F and 1G). Exposure of P-selectin and JON/A, markers of platelet activation, in the presence of thrombin (0.025, 0.05, or 0.1 U/mL) was also unaffected (Figure 1H and 1I). Platelet spreading on immobilized fibrinogen, which was performed in $Ca2^{+}$ free modified Tyrode buffer containing 2 mmol/L Mg^{2+}, also revealed no difference between the three pretreatment conditions (Figure 1J and 1K). Finally, platelets incubated with 2 different concentrations of roxadustat or vehicle were added to human plasma to perform a clot retraction test. Similar to the above results, no remarkable differences were observed (Figure 1L and 1M).

Roxadustat Administration Has No Effect on Platelet Activation and Thrombosis Formation in Healthy Mice In Vivo

To test whether roxadustat affects platelet function in vivo, healthy male mice were treated with roxadustat (20 mg/kg) or vehicle (DMSO, 1 µL/g) for 14 days—the time required for complete platelet turnover. The last drug dose was administered the night before euthanasia, and platelet function was measured the following morning. Consistent with the results of HIF expression in vitro, the HIF-1α and HIF-2α protein levels in platelets and bone marrow (Figure 2B and 2C), as well as other tested organs (Figure IA through IE in the Data Supplement), were remarkably elevated. The antianemia effect of roxadustat depends on the upregulation of EPO and its receptor, as well as the optimization of iron metabolism, which is more efficient and faster than that of EPO.[9] Thus, we compared the peripheral erythrocyte counts, hemoglobin content, and hematocrit between mice receiving roxadustat and those receiving vehicle. As expected, all of these hematologic indexes were increased with roxadustat treatment (Figure 2D through 2F). These above in vivo results demonstrated that the administered roxadustat was successfully absorbed and functional. On this basis, we then performed platelet function and thrombosis formation assays. Platelet aggregation and surface exposure of P-selectin and JON/A with thrombin stimulation were not significantly different between the tested treatment groups (Figure 2G through 2K). There was also no difference in the average spreading area (performed in modified Tyrode buffer containing 2 mmol/L Mg^{2+} without Ca^{2+}) and clot retraction extension (Figure 2L through 2O). Tail-bleeding and tail-rebleeding time assays, as well as an $FeCl_3$-induced carotid artery thrombosis model, were also utilized to verify whether roxadustat impacted hemostasis and thrombosis formation. Unsurprisingly, both tail-bleeding time, tail-rebleeding time, and first occlusion time of $FeCl_3$-induced carotid artery thrombosis formation were not significantly different between the monitored groups (Figure 2P through 2S).

Oral Administration of Roxadustat Has No Significant Effect on Platelet Production

Platelets are anuclear fragments of megakaryocytes. Thus, we next explored the effect of roxadustat on

platelet production by examining megakaryocyte maturation and proplatelet formation assays in vitro. Cultured fetal liver cells were stimulated with recombinant human thrombopoietin (50 ng/mL) and IL-3 (10 ng/mL) for 4 days to form megakaryocytes (Figure 3A) in the presence of roxadustat (10 μM) or vehicle (0.1% DMSO). We did not observe significant differences in megakaryocyte polyploidy between the two treatment conditions (Figure 3B and 3C). The proplatelet formation assay also revealed no effect of roxadustat treatment (Figure 3D and 3E). To assess the effect of roxadustat on platelet production in vivo, we first compared the number of megakaryocytes (CD41[+]) in immunohistochemically stained bone marrow sections from mice treated with the drug (20 mg/kg) or vehicle (DMSO 1 μL/g body weight) for 14 days. Megakaryocyte counts were not significantly different between the treatments (Figure 3H and 3I). To verify this result, bone marrow–derived CD41(+) megakaryocyte counting was performed by flow cytometry. Predictably, the proportion of CD41(+) megakaryocytes of all bone marrow cells from mice receiving roxadustat was identical to that from mice receiving vehicle (Figure 3F and 3G). Platelet life span was also tested and remained unchanged in the presence/absence of roxadustat (Figure 3J). Finally, we compared peripheral platelet counts and mean platelet volumes with and without roxadustat treatment among healthy mice and CKD patients. The data in Figure 2K through 2N and Figure IIE and IIF in the Data Supplement show that there was no significant effect of roxadustat administration.

Roxadustat Administration Has No Effect on Platelet Activation and Thrombosis Formation in 5/6 Nx Mice

To further evaluate the effect of roxadustat on platelet function in the pathological state of renal failure, roxadustat (20 mg/kg, thrice per week) or vehicle (DMSO, 1 μL/g body weight, thrice per week) was administrated for 2 weeks to mice that had underwent 5/6 Nx. Mice receiving EPO therapy (500 U/kg, thrice per week) were analyzed simultaneously as a positive control. We first compared the trichrome staining of kidney tissue and serum markers of renal failure between the 5/6 Nx and sham-operated mice. As expected, the renal medullary and cortical fibrosis of the nephrectomized mice increased significantly compared with the sham-operated mice (Figure 4B). The serum levels of urea nitrogen and creatinine were also remarkably elevated in the former mice (Figure 4C and 4D). These results confirmed the state of pathological renal failure in the mice that received 5/6 Nx. Peripheral erythrocyte counts, hemoglobin content, and hematocrit were all increased with roxadustat or EPO treatment, as expected (Figure IIA through IID in the Data Supplement). Thereafter, the platelet function assay was performed. Consistent with previous studies that EPO promotes platelet activation,[2,3,23] we confirmed the functionally enhanced effect of EPO on mouse platelets. As shown in Figure 4E through 4H, EPO remarkably enhanced platelet aggregation and P-selectin/JON/A expression, as well as the extent of spreading (achieved in Ca^{2+} free modified Tyrode buffer containing 2 mmol/L Mg^{2+}) and clot retraction (Figure 4I through 4L). In all of these platelet functional assays, roxadustat did not promote platelet activation as EPO did. The tail-bleeding and rebleeding time, as well as the amount of hemoglobin lost, were almost the same between the mice receiving roxadustat and vehicle (Figure 4M and 4N; Figure IIIB and IIIC in the Data Supplement). To evaluate the effect of roxadustat on thrombosis formation in elastic and muscular arteries, $FeCl_3$-induced carotid artery and mesenteric artery thrombosis models were tested, respectively. Compared with the effect of the positive control (EPO), neither $FeCl_3$-induced elastic artery thrombosis (Figure 4O and 4P) nor that of muscular artery thrombosis (Figure 4Q and 4R) was affected by roxadustat or vehicle administration in vivo. Considering that DVT and pulmonary embolism are common complications of CKD, the effects of roxadustat on experimental DVT and pulmonary embolism formation were also monitored. Similar to the results above, roxadustat had no impact on DVT formation (Figure 4S and 4T) and did not affect the survival of 5/6 Nx mice with experimentally induced pulmonary embolism (Figure V in the Data Supplement). Roxadustat did not affect the number of thromboses in the pulmonary embolism model, unlike high-dose EPO that significantly increased the number (Figure 4U and 4W).

Roxadustat Does Not Activate Platelets From Healthy Volunteers and CKD Patients

Washed platelets of healthy volunteers were pretreated with roxadustat (10 or 20 μM) or vehicle (0.1% DMSO) for 30 minutes. Aggregation and P-selectin/PAC1 surface exposure upon activation were measured. Consistent with the results of the animal experiments, roxadustat also had a negative impact on human platelet activation (Figure 5A through 5D) in vitro. To perform human studies in vivo, age- and sex-matched CKD patients were divided into 2 groups according to whether they received roxadustat (≈2 mg/kg, thrice per week) therapy. Platelet function was further compared between the two groups. As predicted, platelet aggregation was not significantly different between the groups regardless of activating agent (0.1 U/mL thrombin or 3 μg/mL collagen; Figure 5E and 5F). In addition, no difference in P-selectin/PAC1 expression in the presence/absence of thrombin between the two groups was detected (Figure 5G and 5H).

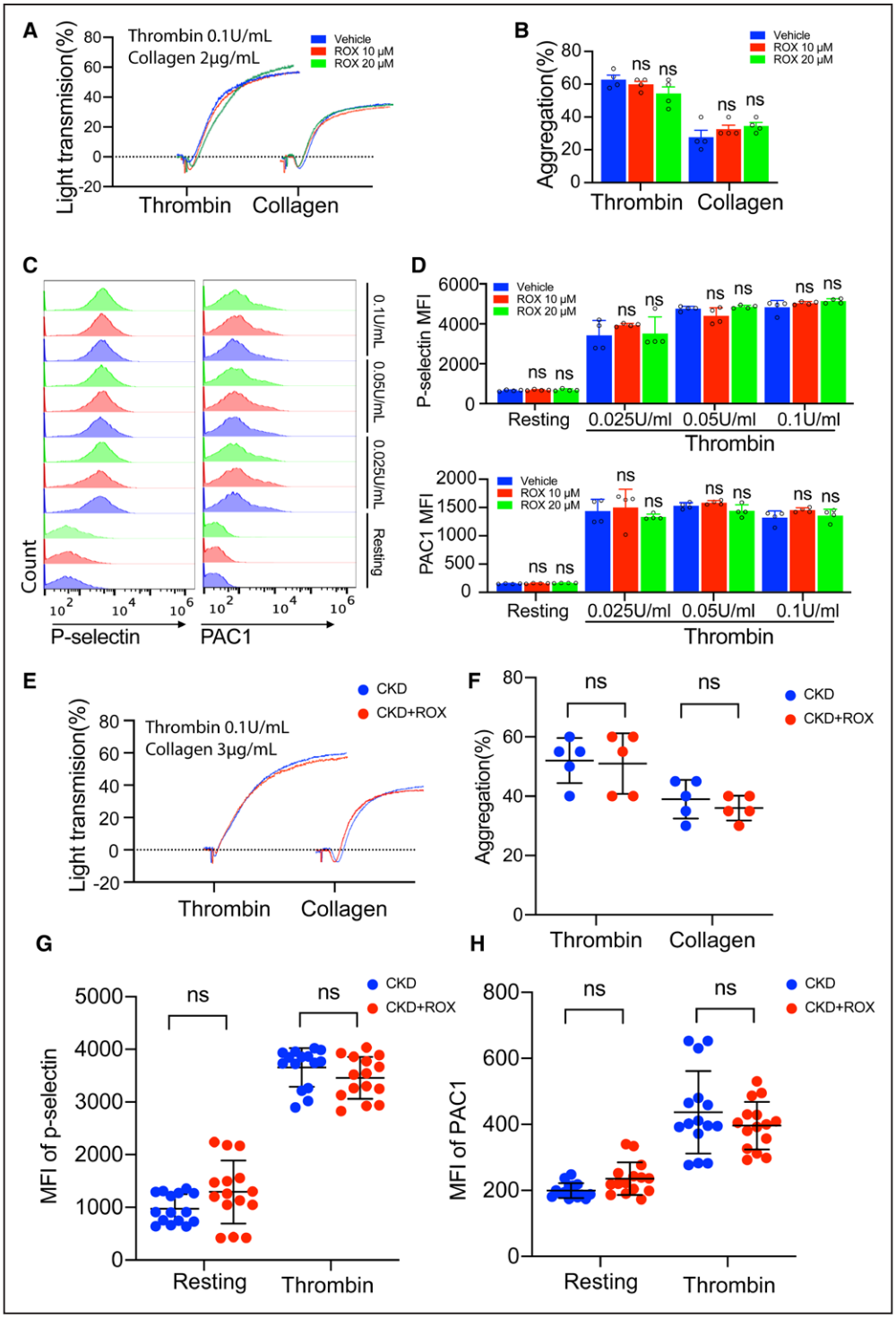

Figure 5. Roxadustat (ROX) does not activate platelets from healthy volunteers and chronic kidney disease (CKD) patients. **A** and **B**, Aggregation of healthy human washed platelets pretreated with ROX (10 and 20 μM) or vehicle (0.1% dimethyl sulfoxide [DMSO]) was performed by stimulation with thrombin (0.1 U/mL) or collagen (2 μg/mL). Each symbol indicates the average aggregation level of one person. Bars represent the mean±SD of aggregation from 4 volunteers. **C** and **D**, Human platelets were pretreated with ROX (10 and 20 μM) or vehicle (0.1% DMSO) for 30 min in vitro, and surface expression of platelet P-selectin and PAC1 (procaspase-activating compound 1) in the presence of thrombin (0.025, 0.05, and 0.1 U/mL) was evaluated by flow cytometry. Mean fluorescence intensity (MFI) is plotted as mean±SD of 4 volunteers. **E** and **F**, Aggregation of washed platelets from CKD patients receiving ROX (≈2 mg/kg, thrice per week) therapy or not was assessed after stimulation with thrombin (0.1 U/mL) or collagen (3 μg/mL). Each symbol indicates the average aggregation level of one patient. Bars represent the mean±SD of aggregation from 5 patients. **G** and **H**, Surface expression of platelet P-selectin and PAC1 in the presence of thrombin (0.05 U/mL) was evaluated by flow cytometry among CKD patients administered ROX or not. Plotted data are mean±SD of 15 patients. ns indicates no significance compared with vehicle.

DISCUSSION

In the present study, we focused on the effects of roxadustat on platelet function and production both in vitro and in vivo. For the in vitro testing, we first evaluated whether roxadustat directly activated platelets. The results were negative, which excluded any direct and unexpected effects of roxadustat as a small-molecule activator of platelets. Since platelets are anuclear blood cell fragments and have no mRNA transcriptional function, we inferred that HIF, as a transcription factor, would not have the ability to act on platelets directly. We then pretreated washed platelets with different concentrations of roxadustat for 30 minutes before conventional evaluation of platelet functions, and the results showed that roxadustat could not enhance platelet activation, which verified our supposition. The in vivo environment is more complicated than the in vitro situation. As platelet producers, megakaryocytes may be affected by roxadustat, which would subsequently alter platelet function during platelet production. In addition, roxadustat may indirectly affect platelets through other nonplatelet substances. To address whether roxadustat exerts an effect on platelet activation in vivo, we conducted experiments in healthy mice. After 14 days of oral administration of roxadustat, we first confirmed roxadustat activity in the mice by evaluating both HIF accumulation in different organs and the increase in hemoglobin. Then, platelet function was assayed, and mouse tail-bleeding time and $FeCl_3$-induced carotid artery thrombosis formation time were tested. We concluded that roxadustat does not affect platelet function in healthy mice in vivo. Next, the effect of this drug on platelet production was evaluated, and the numbers of peripheral platelets and bone marrow megakaryocytes in mice receiving roxadustat versus vehicle were compared. It was revealed that the drug had no effect on platelet production. To verify this, we performed megakaryocyte maturation and proplatelet formation assays in vitro and achieved the same result. After that, whether roxadustat affects platelet function in the pathological state of renal failure was further explored. Multiple thrombosis models were utilized, and the drug was shown to have no effect on platelets in these settings. Finally, the functions of platelets from healthy volunteers and CKD patients were tested in the presence/absence of roxadustat, and no effect of roxadustat on platelets was confirmed in human patients.

Although EPO is a downstream gene of HIF activation and roxadustat can upregulate EPO expression, we did not find any positive effect of roxadustat on platelet activation in our study. This negative result may be explained by roxadustat transiently increasing endogenous EPO levels within or near physiological range,[24,25] thus avoiding the platelet activation caused by injection of EPO, which increases blood EPO levels to a greater magnitude and duration (Figure IVA and IVB in the Data Supplement).

Platelets exposing phosphatidylserine have been found to own more ability to promote thrombin generation and act as an emerging component of the procoagulant response.[26] To evaluate the effect of roxadustat on procoagulant activity of platelets, we measured platelet phosphatidylserine exposure with annexin V–binding assay. No significant difference was observed between ROX- and vehicle-treated groups, and an elevation in the EPO-treated group was observed when platelets were stimulated with 0.1 U/mL thrombin (Figure IIIA in the Data Supplement). This kind of elevation occurred probably due to the promoting thrombocytosis effect of EPO, which induced more sensitive newly released reticulated platelets. Reticulated platelets have already been reported to convert to coated platelet more easily.[27] We found that platelet phosphatidylserine exposure was low in response to thrombin. This blunted response might be attributed to stimulating the platelets without using collagen simultaneously, and the lack of shear is also nonnegligible because platelet phosphatidylserine exposure is shear dependent.[28,29] This test could not be completed satisfactorily because we possessed no corresponding shear force instrument.

To date, few studies have focused on the relationship between HIF and platelet function. Some proline hydroxylase inhibitors have been reported to promote platelet activation by upregulating HIF-2α expression in vitro.[30] However, the in vitro result needs to be confirmed further because of lack of reverse proof experimental design. As intracellular level of ROS has been demonstrated to affect platelet activation[31] and upregulation of HIF expression has been elaborated to decrease ROS,[32] we explored whether roxadustat decreases ROS in platelets in vitro. Contrary to previous results, no significant variation of the ROS level was observed in the presence/absence of roxadustat (Figure VIA in the Data Supplement). In fact, the biological effects of HIFs mostly rely on regulating its downstream target genes as transcriptional factors. However, HIFs may not be able to play the role of transcription factor in platelets because of the absence of nuclear transcription. If proline hydroxylase inhibitors really affect platelet function in vitro, this may be achieved in an HIF-independent manner, which needs to be explored further.

It should be noted as the limitations of the present study that for patients with CKD, the risk of thrombosis is not completely the result of platelet activation, and the function of the coagulation and fibrinolytic systems should also be considered. Although roxadustat cannot activate platelets and cannot affect PT or APTT in healthy mice (Figure VA through VC in the Data Supplement), it may still be able to alter the risk of thrombosis in CKD patients through the coagulation or fibrinolytic systems. Existing studies have shown that HIF can

upregulate the expression of PAI-1 (plasminogen activator inhibitor 1) and downregulate the synthesis of protein S,[33] thereby inhibiting the function of the fibrinolytic system.[34] HIF can also increase the hepatic expression of transferrin, which can interact with clotting factors to increase the risk of thrombosis.[35] The risk of thrombosis among patients experiencing chronic obstructive pulmonary disease has been demonstrated to rely on upregulation of HIF.[30,36] In this study, we did not detect evidence that roxadustat administration is prothrombotic. This likely occurred because routine roxadustat treatment only upregulates prothrombotic HIF downstream genes transitorily and weakly, similar to what it does to EPO (Figure IVA and IVB in the Data Supplement), and the limited dosing time in the present study should also be considered. All of these findings suggest that a follow-up study on the thrombosis risk of roxadustat needs to evaluate the coagulation and fibrinolytic systems including the magnitude, impact, and duration.

In conclusion, despite this study's limitations, we have shown for the first time that roxadustat has no significant effect on platelet function and reconfirmed that platelet production is not affected. Therefore, patients with renal anemia who receive oral roxadustat will not have additionally increased risk of thrombosis due to roxadustat-induced abnormal production and activation of platelets.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Materials

Data Supplement Figures I–VI
Major Resources Table

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