学位論文

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in a mouse model of nephropathy

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Journal of Pharmacological Sciences 135 (2017) 81–88

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full paper

A protease-activated receptor-1 antagonist protects against podocyte injury in a mouse model of nephropathy

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ARTICLE INFO

Article history:
Received 9 June 2017
Received in revised form
13 August 2017
Accepted 15 August 2017
Available online 14 September 2017

Keywords:
Protease-activated receptor-1
Podocyte
Transient receptor potential cation channel
Kidney injury

ABSTRACT

The kidney expresses protease-activated receptor-1 (PAR-1). PAR-1 is known as a thrombin receptor, but its role in kidney injury is not well understood. In this study, we examined the contribution of PAR-1 to kidney glomerular injury and the effects of its inhibition on development of nephropathy. Mice were divided into 3 groups: control, doxorubicin + vehicle (15 mg/kg doxorubicin and saline) and doxorubicin + Q94 (doxorubicin at 15 mg/kg and the PAR-1 antagonist Q94 at 5 mg/kg/d) groups. Where indicated, doxorubicin was administered intravenously and PAR-1 antagonist or saline vehicle by subcutaneous osmotic mini-pump. PAR-1 expression was increased in glomeruli of mice treated with doxorubicin. Q94 treatment significantly suppressed the increased albuminuria in these nephropathic mice. Pathological analysis showed that Q94 treatment significantly attenuated periodic acid–Schiff and desmin staining, indicators of podocyte injury, and also decreased glomerular levels of podocin and nephrin. Furthermore, thrombin increased intracellular calcium levels in podocytes. This increase was suppressed by Q94 and Rox4560, a transient receptor potential cation channel (TRPC)3/6 antagonist. In addition, both Q94 and Rox4560 suppressed the doxorubicin-induced increase in activities of caspase-9 and caspase-3 in podocytes. These data suggested that PAR-1 contributes to development of podocyte and glomerular injury and that PAR-1 antagonists have therapeutic potential.

1. Introduction

Chronic kidney disease is a substantial worldwide burden to patients and society.[1,2] Albuminuria is a typical characteristic of kidney disease and results from disruption of the glomerular filtration barrier. Podocytes are important cells for maintaining glomerular filtration barrier function. Thus, podocyte loss causes serious nephropathy.[3–5] For this reason, podocytes have been considered a principal therapeutic target for protecting the glomerular filtration barrier against plasma protein leakage into urine.[6,7]

The PAR-1 (protease-activated receptor 1), a Gi protein-coupled receptor,[8] is activated by thrombin-induced proteolytic cleavage. The N-terminal extracellular region of PAR-1 is cleaved by thrombin and the cleaved peptide then interacts with the PAR-1 receptor to induce transmembrane signaling.[8] Both human and rodent kidneys express PAR-1.[9] A recent review suggested that PAR-1 plays a functional role in rat glomeruli.[10] In a clinical study, urinary thrombin excretion in patients with glomerulonephritis was higher than in healthy subjects.[11] However, the contribution of PAR-1 to progression of glomerular injury has not been elucidated.

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Peer review under responsibility of Japanese Pharmacological Society.

http://dx.doi.org/10.1016/j.jphs.2017.09.002

In this study, we examined the role of PAR-1 on development of glomerular podocyte injury and its potential value as a therapeutic target. PAR-1 upregulation was confirmed in two glomerular injury models, doxorubicin-induced nephritis and anti-glomerular basement membrane (GBM) antiserum-induced nephritis model. The pharmacological inhibition of PAR-1[12] in doxorubicin model prevented development of podocyte injury. Moreover, to address biological changes in the podocyte after PAR-1 stimulation, we assessed PAR1-dependent regulation of apoptosis and intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in podocytes. These processes were reported to represent a common pathway driving loss of podocyte foot processes.[13]

2. Materials and methods

2.1. Animal

All experiments were approved by the Institutional Animal Care and Use Committee of Kagawa University. BALB/c and C57BL/6 mice were from Clea Japan (Tokyo, Japan).

2.2. Materials

Q94, a PAR-1 antagonist, was purchased from Tocris Bioscience (Bristol, UK). Chemicals were from Sigma–Aldrich (St. Louis, MO, USA) or Nacalai Tesque (Kyoto, Japan), unless otherwise specified. Rox4560, named 6228-0473 in a previous report,[14] was synthesized in the Faculty of Pharmaceutical Sciences, Kyushu University.

2.3. Mouse model for podocyte injury

Nephropathy with podocyte injury was induced by a single intravenous injection of anti-GBM antiserum[15] or by a single intravenous injection of doxorubicin, at 15 mg/kg (D1515, Sigma–Aldrich), in 0.9% saline.[16,17] Preparation of anti-GBM antiserum in rabbits was performed based on the Spiro's method[18] with some modification for mice experiment.[19] In brief, glomeruli were isolated by differential sieving from mouse renal cortex and disrupted by sonication. The GBM was collected by centrifugation, emulsified with complete Freund's adjuvant (Difco, Detroit, MI, USA) and immunized in rabbits. C57Bl/6 mice, at 8 weeks of age, were immunized by an intraperitoneal injection of 25 mg/kg normal rabbit IgG (MP Biomedicals, Santa Ana, CA, USA) emulsified with complete Freund's adjuvant (Difco), given 5 days before either vehicle (normal rabbit serum) or anti-GBM antiserum (0.3 mL, i.v.). The kidneys were removed and preserved for later processing for mRNA at week 4 after the anti-GBM antiserum injection. C57Bl/6 mice in the control group received the same volume of control antiserum. To investigate effects of PAR-1 inhibition on kidney damage, BALB/c mice were divided into the following groups: control ($n = 4$), doxorubicin + vehicle (15 mg/kg doxorubicin and saline, $n = 5$) and doxorubicin + Q94 (doxorubicin at 15 mg/kg and Q94 at 5 mg/kg/d, $n = 7$) groups. Where indicated, doxorubicin was administered intravenously and PAR-1 antagonist or saline vehicle by subcutaneous osmotic mini-pump. Albuminuria and podocyte injury were analyzed at 2 wk after injections. Urine samples were collected for 24 h in a metabolic cage before sacrifice. The kidneys were removed and preserved for later processing for mRNA, histology and immunohistochemistry (IHC) analyses.

2.4. Assay for albuminuria

Albumin levels were measured using commercially available assay kits (Mouse Albumin ELISA kit, Shibayagi, Gunma, Japan).

2.5. Assays for urinary and plasma creatinine

Commercially available assay kits were used to measure creatinine (LabAssay Creatinine kit; Wako, Osaka, Japan).

2.6. Histology

Kidneys were fixed with 15% formalin (pH 7.4), embedded in paraffin, cut into 2 μm sections and mounted on slides. The sections were then stained with periodic acid–Schiff (PAS) reagent and sirius red. Images were evaluated using light microscopy (BX-51/DP-72; Olympus, Tokyo, Japan). Glomerular sclerotic and sirius red positive areas were determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. Immunohistochemistry

Kidney tissues were fixed with 15% formalin (pH 7.4), embedded in paraffin, cut into 2 μm sections and mounted on slides. IHC staining for desmin[20] and 4-hydroxynonenal (4-HNE)[21] was performed with Histofine Simple Stain MAX-PO MULTI (Nichirei Biosciences, Tokyo, Japan). After deparaffinization with xylene, sections were incubated with 0.3% hydrogen peroxide, for 15 min for desmin or 30 min for 4-HNE, to block endogenous peroxidases. For desmin antigen retrieval, sections were incubated for 30 min in 0.01 mol/L citrate buffer (pH 6.0) at 100 °C. Proteinase K (DAKO Cytomation, Glostrup, Denmark) was used for 4-HNE antigen retrieval, with incubation for 10 min. After blocking with 10% goat serum, sections were incubated overnight at 4 °C with primary antibodies, each at 1:200 dilution (anti-human desmin mouse monoclonal antibody, D33, DAKO Cytomation; anti-hydroxynonenal antibody, ab46545, Abcam, Cambridge, UK). After washing sections and incubating them with secondary antibody for 1 h at room temperature, DAB substrate (DAKO Cytomation) was used to visualize IHC staining. Finally, counterstaining was performed with hematoxylin (DAKO Cytomation). Positively stained areas were analyzed using ImageJ software.

2.8. Laser-capture microdissection

Laser-capture microdissection (LCM) was performed as previously described.[22] Frozen tissues embedded in OCT were subsequently cryosectioned into 20 μm sections and fast-stained using Arcturus histogene frozen section staining kit (Ambion Inc., Austin, TX, USA). For each sample, 300–400 glomeruli were captured under direct visualization with CapSure HS Laser-capture microdissection tubes, using a laser microdissector pressure-catapulting device (Arcturus® LCM, Applied Biosystems, Waltham, MA, USA). Glomerular mRNA, for podocin and nephrin measurements, was extracted using an RNAqueous-Micro kit (Ambion Inc.).

2.9. Cell culture

Conditionally immortalized mouse podocytes were cultured as previously described.[20] Under growth permissive conditions, cells were seeded on type I collagen coated dishes at 33 °C. The growth medium was RPMI 1640 (Sigma–Aldrich) with 10% fetal bovine serum and 20 U/mL mouse interferon-γ (Sigma–Aldrich) to drive the expression of a thermosensitive T-antigen. To induce differentiation, cells were maintained at 37 °C without interferon-γ for 10–14 d. All experiments were performed with differentiated podocytes.

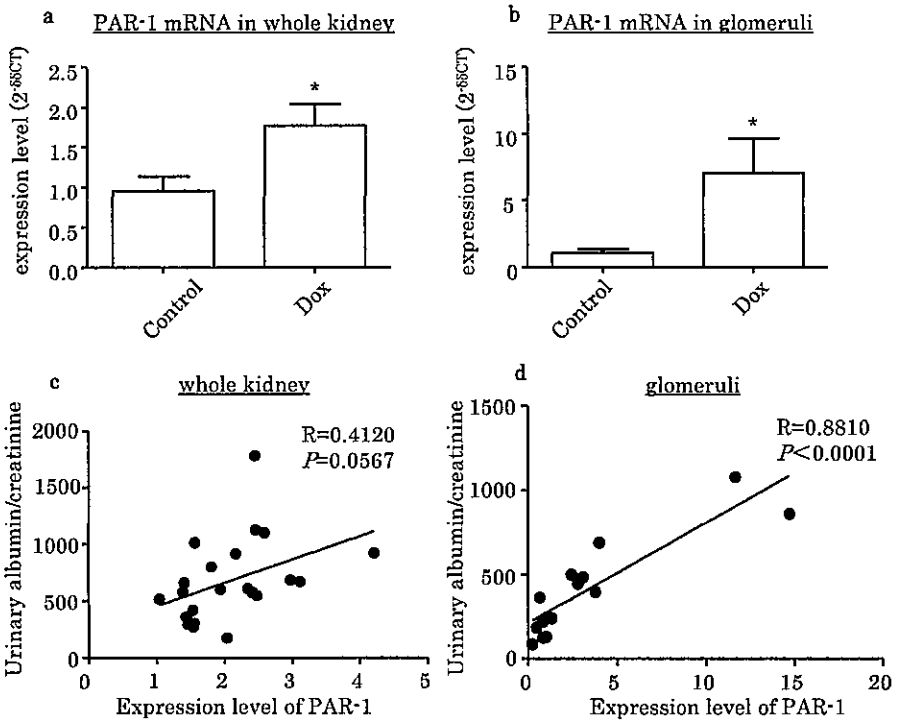

Fig. 1. PAR-1 expression in the renal glomeruli. Doxorubicin induced the increase in PAR-1 mRNA both in whole kidney tissue and glomeruli (n = 5–22) (a,b). Correlation between mRNA level of PAR-1 with urinary albumin/creatinine ratio in whole kidney tissue and glomeruli (c,d). *p < 0.05 vs control.

2.10. Fura-2 fluorometry

Fura-2 fluorometry was performed as previously described.[23] Briefly, differentiated cells were loaded with fura-2 by incubating them in medium containing 5 μmol/L fura-2 acetoxymethyl ester (Dojin Chemical, Kumamoto, Japan) at 37 °C for 1 h. Cells were then equilibrated in HEPES buffered saline (HBS) at room temperature for 15 min. After equilibration, fura-2 fluorescence was measured with a front-surface fluorometer CAM230-OF2. In the end of each measurement, the response to 75 μmol/L ionomycin was recorded as a reference, used to normalize the fluorescence data.

2.11. Caspase 3 and 9 assay

Caspase 3 and 9 activity were measured using commercially available assay kits (APOPCYTO™ Caspase Fluorometric Assay Kit, Medical Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions.

2.12. Real-time PCR

To analyze mRNA expression, mRNA was isolated from kidney cortex homogenates or glomeruli captured by LCM. Levels of 18S, TGF-β, PAR-1, podocin and nephrin mRNAs were analyzed by real-time PCR, using a 7300 Fast Real-Time PCR System (Applied Biosystems) and the Light Cycler Fast Start DNA Master SYBR Green I kit (Applied Biosystems). The mouse primer sequences (forward and reverse) were: 18S: 5′-GTAACCCGTTGAACCCCATT-3′, 5′-CCATCCAATCGGTAGTAGCG-3′; TGF-β: 5′-GCTGCTGACCCCCACT-GATA-3′, 5′-ACAAGAGCAGTGAGCGCTGAA-3′; PAR-1: 5′-GTTGA-TCGTTTCCACGGTCT-3′, 5′-GCTGCCTCTGTACCAGGACT-3′; podocin: 5′-CTTGGCACATCGATCCCTCA-3′, 5′-CGCACTTTGGCCTGTCTTTG-3′;

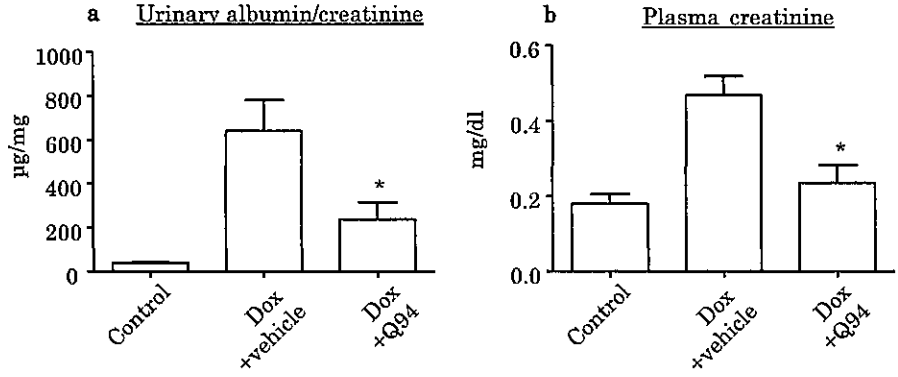

Fig. 2. PAR-1 inhibition on kidney damage. Treatment with Q94 attenuated both the development of albuminuria (a) and the increased plasma creatinine levels (b) in doxorubicin (Dox)-injected mice (n = 4–7). *p < 0.05 vs Dox + vehicle.

nephrin: 5′-GTGCCCTGAAGGACCCTACT-3′, 5′-CCTGTGGATCCC-TTTGACAT-3′. Relative mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method. The ddCt value was calculated using data from the normal control group.

2.13. Data and statistical analysis

Student's *t* test was used to compare data from two individual groups. Multiple group comparisons were made using one-way analyses of variance (ANOVA), followed by Tukey's multiple comparison test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Correlation between proteinuric kidney disease and mRNA expression of PAR-1

First, we examined PAR-1 expression in the mouse model for glomerular filtration barrier injury. Mice with doxorubicin-induced nephropathy had increased PAR-1 mRNA expression in both whole kidney tissue and isolated glomeruli (Fig. 1a and b). There was a positive correlation between albuminuria and glomerular PAR-1 mRNA expression in doxorubicin-induced nephropathy. However, there was no correlation between whole kidney PAR-1 mRNA

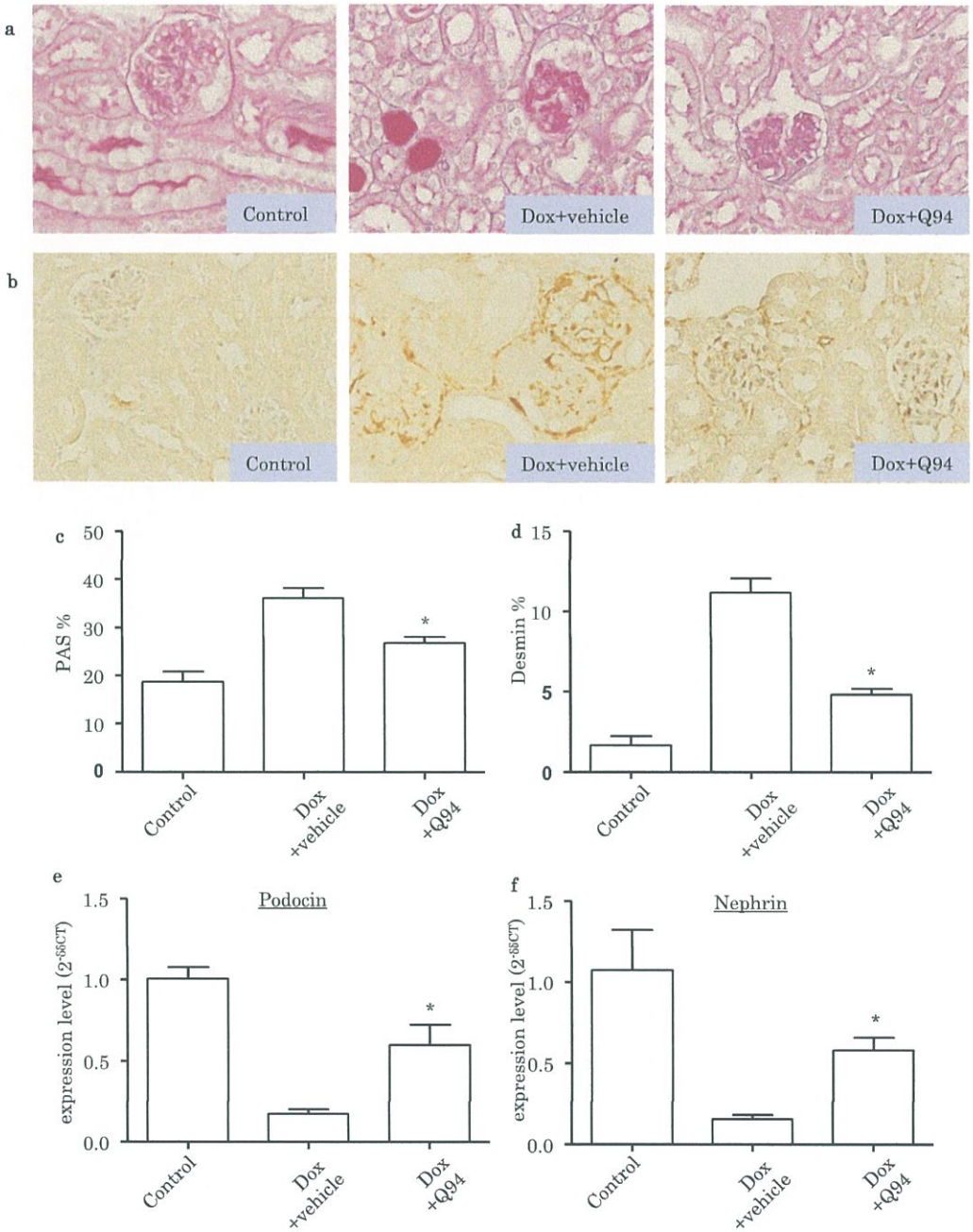

Fig. 3. PAR-1 inhibition on glomerular and podocyte damage. Q94 treatment suppressed the increased PAS-positive region in the glomeruli of doxorubicin (Dox)-injected mice (a and c) (n = 4–7). The desmin-positive area showed same trend (b and d) (n = 4–7). The loss of both nephrin and podocin mRNA levels was attenuated by Q94 (e and f). *$p < 0.05$ vs. Dox + vehicle.

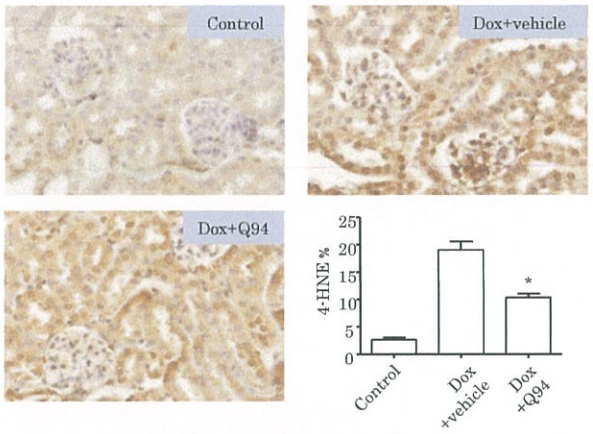

Fig. 4. PAR-1 inhibition on renal oxidative stress. Treatment with Q94 decreased oxidative stress, which evaluated by immunohistochemical staining for 4-hydroxy-2-nonenal (4-HNE) in doxorubicin (Dox)-injected mice (n = 4–7). $^{*}p < 0.05$ vs. Dox + vehicle.

expression and albuminuria (Fig. 1c and d). To confirm PAR-1 upregulation in the nephropathic kidney, we assessed PAR-1 mRNA expression in mice receiving an anti-GBM antiserum. Nephritis induced by the anti-GBM antiserum was accompanied by significantly increased PAR-1 expression both in whole kidney samples and isolated glomeruli (vehicle 1.01 ± 0.06, anti-GBM 3.19 ± 0.37, $p < 0.01$ in whole kidney; vehicle 1.06 ± 0.19, anti-GBM 10.23 ± 2.95, $p < 0.01$ in isolated glomeruli).

3.2. Effects of a PAR-1 antagonist on renal function and histopathological changes in doxorubicin-induced nephropathy

The doxorubicin treated mice had higher urinary albumin excretion and plasma creatinine levels than vehicle treated mice (Fig. 2a and b). The increased albuminuria/creatinine ratios and plasma creatinine levels in doxorubicin treated mice were significantly suppressed by treatment with Q94, a PAR-1 antagonist (Fig. 2a and b).

Glomerulosclerosis, detected by PAS staining, was induced by doxorubicin treatment, and this effect was attenuated by Q94 (Fig. 3a and c). We also examined IHC staining for desmin, a marker for podocyte injury.[24] An increase in desmin-positive areas in the glomeruli in mice treated with doxorubicin was suppressed by Q94 (Fig. 3b and d). To confirm effects of Q94 on doxorubicin-induced nephropathy, glomeruli isolated by laser capture were analyzed for podocin and nephrin mRNA expression. Podocin and nephrin mRNA levels were decreased in doxorubicin-induced nephropathy and were restored by Q94 treatment (Fig. 3e and f).

Oxidative stress was evaluated by IHC staining for 4-HNE. 4-HNE deposition was increased in both glomeruli and tubules in doxorubicin treated mice (Fig. 4). Q94 significantly decreased 4-HNE-positive areas (Fig. 4). On the other hand, renal interstitial collagen

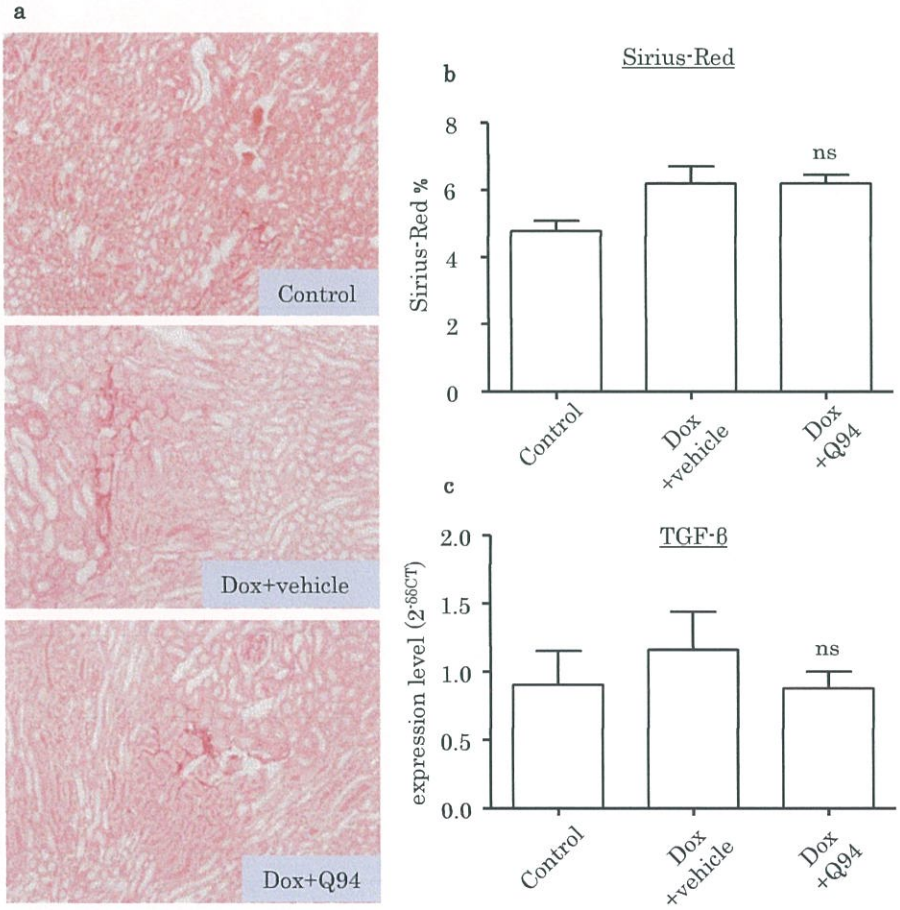

Fig. 5. PAR-1 inhibition on renal fibrosis. Neither doxorubicin nor Q94 affected the Sirius red-positive area (a and b) and TGF-β mRNA in doxorubicin (Dox)-injected mice (n = 4–7). *ns* vs Dox + vehicle.

deposition, detected by sirius red staining, was not affected by doxorubicin or Q94 (Fig. 5a and b). TGF-β mRNA levels were also unaffected during the 2 wk experimental period (Fig. 5c).

3.3. Involvement of transient receptor potential canonical channel (TRPC) 3/6 in PAR-1 mediated podocyte apoptosis

Thrombin is known to activate calcium influx via PAR-1 in endothelial cells.[25] We found that thrombin activated calcium influx in cultured mouse podocytes and that this effect was suppressed, in a dose-dependent manner, by Q94 (Fig. 6a). The increase in intracellular Ca^{2+} concentrations induced by thrombin was also suppressed by a TRPC3/6 inhibitor, Rox4560 (1.2 μmol/L) (Fig. 6b), supporting the importance of TRP channels in regulating calcium influx in podocytes.[26] Previous studies reported that TRPCs were involved in maintaining $[Ca^{2+}]i$ in podocytes and in development of proteinuric kidney disease.[27,28] Therefore, we next investigated whether PAR-1 induced apoptosis through a TRPC3/6 dependent pathway. Doxorubicin significantly increased activities of both caspase-3 and 9, which are known to induce apoptosis, in cultured podocytes (Fig. 7a and b). Q94 suppressed the doxorubicin-induced increase in activities of both caspase-3 and 9. Rox4560 also suppressed the increase in activities of both caspase-3 and 9 (Fig. 7c and d). In addition, doxorubicin treated podocytes were also administered Q94 (60 μmol/L) and Rox4560 (1.2 μmol/L) together. No further decrease in caspase-9 activity was observed, compared with in cells treated with only Q94 or Rox4560 (Fig. 7e and f). This suggested that PAR-1 and TRPC3/6 induced apoptosis via a shared mechanism.

4. Discussion

An intact kidney filtration barrier can protect essential proteins in the blood.[4] Podocytes are important components of this barrier. Once podocytes are damaged, plasma albumin can leak into the urinary space, resulting in a pathologic condition known as albuminuria.[29] Treatment options to prevent or attenuate podocyte damage or loss are, therefore, greatly needed. In our study, PAR-1 expression was increased in the glomeruli in two mouse nephropathy models. Furthermore, Q94 treatment attenuated albuminuria and podocyte injury *in vivo*. *In vitro*, Q94 suppressed caspase activity and increases in intracellular calcium levels. These data supported PAR-1 inhibition as a potential therapeutic strategy against podocyte damage. These findings were consistent with a recently published study showing increased PAR-1 expression and involvement of PAR-1 in tissue pathophysiology in a mouse model for diabetic nephropathy.[30]

Our data supporting involvement of TRPC on thrombin-induced regulation of $[Ca^{2+}]i$ in podocytes provided further insights into the pathophysiology of podocytopathy in glomerular disease. Previous studies reported that disrupted Ca^{2+} signaling, such as sustained Ca^{2+} influx, was involved in development of podocyte injury.[10,31–33] We demonstrated that thrombin-induced Ca^{2+} influx was both PAR-1- and TRPC3/6-dependent. PAR-1 is coupled with

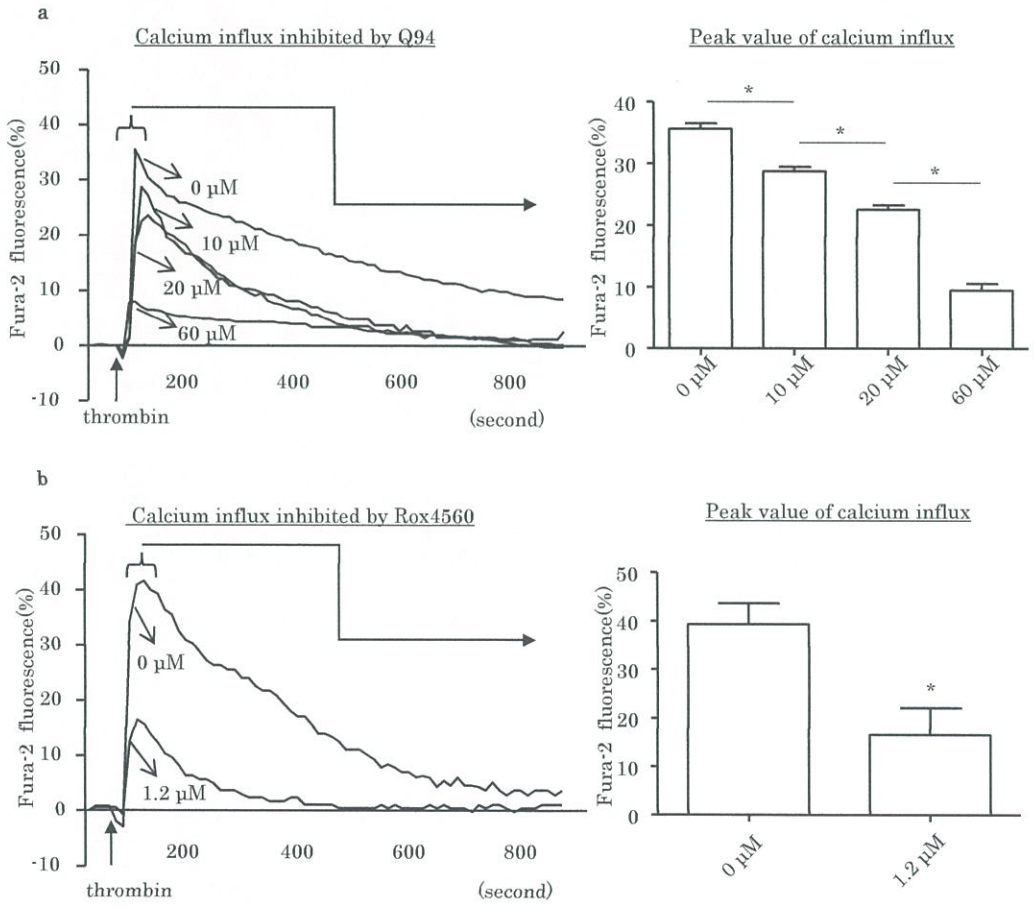

Fig. 6. PAR-1 on calcium regulation in cultured podocytes. Thrombin (1 mU/L)-induced intracellular calcium influx was dose-dependently suppressed by Q94 (n = 5) (a). A TRPC3/6 inhibitor, Rox4560 (1.2 μmol/L), also suppressed the thrombin-induced intracellular calcium influx (n = 5) (b). *: $p < 0.05$, vs. vehicle.

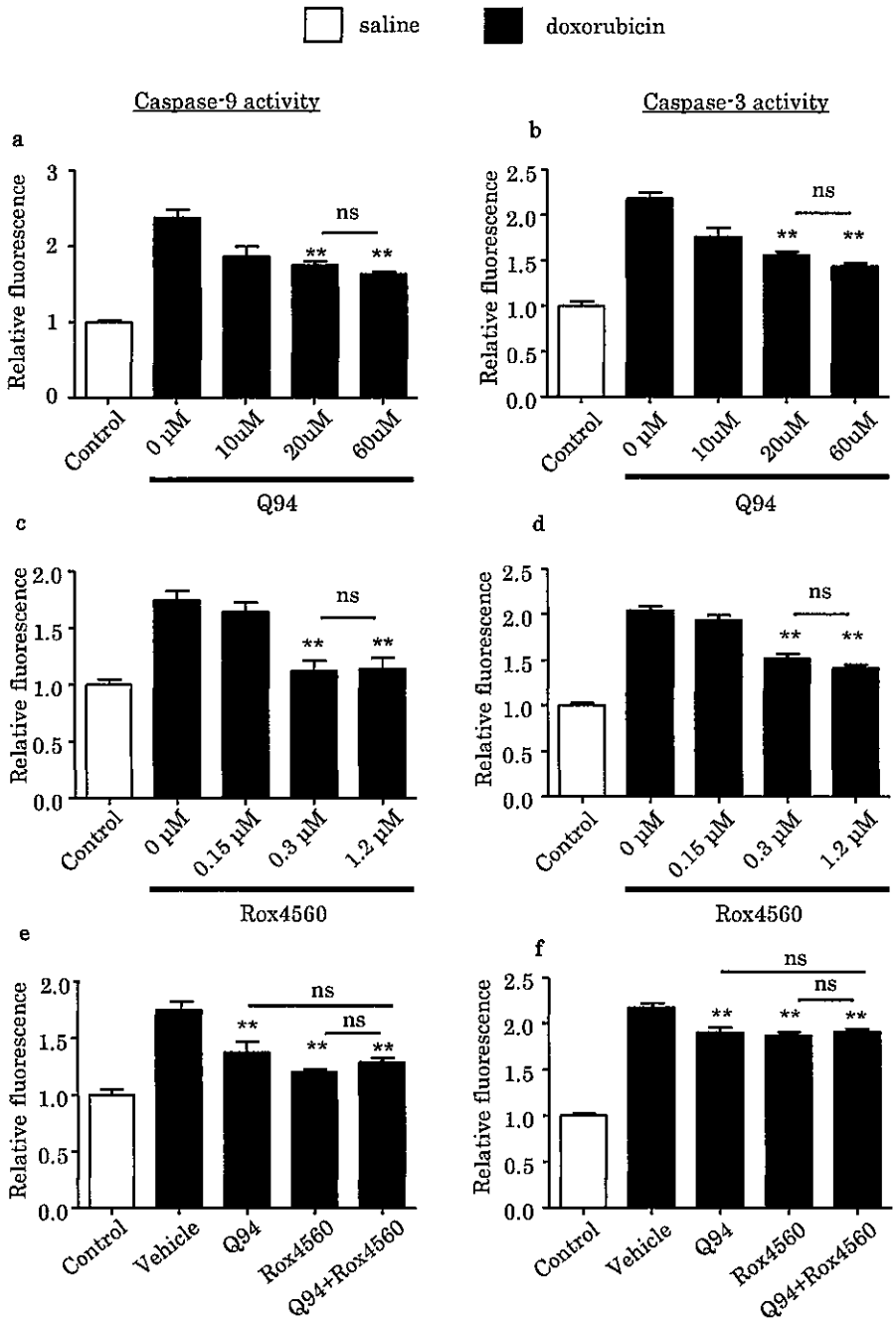

Fig. 7. PAR-1 on caspase activity in cultured podocytes. The activities of caspase 9 (a, c and e) and caspase 3 (b, d and f) were measured in the cultured podocytes. Treatment with Q94 prevented the increase in caspase-9 and 3 activity by doxorubicin (1 μmol/L) (n = 5) (a and b). The increased activities of caspase-9 and 3 were also attuned by Rox4560 (n = 5) (c and d). Co-treatment with Q94 (60 μmol/L) with Rox4560 (1.2 μmol/L) did not further suppressed the increased activities of both caspase 9 and 3 in comparison with the monotherapy (n = 5) (e and f). **: $p < 0.001$, vs. vehicle. *ns*: not significant.

Gq/11 and second messengers, such as diacylglycerol, were implicated in activating TRPCs.[34,35] In addition, podocyte apoptosis was suppressed by Rox4560, a TRPC3/6 antagonist, and co-treatment with Q94 and Rox4560 showed no additional inhibition of caspase-9 activation. This suggested that PAR-1 and TRPC shared the same pathway for regulating $[Ca^{2+}]i$ and apoptosis in podocytes.

It is well known that thrombin is a coagulation factor. Importantly, a key feature of glomerulonephritis is formation of a fibrin matrix in Bowman's capsule.[36] Thrombotic disease is a significant factor increasing morbidity in kidney diseases, especially in patients with nephrotic syndrome and undergoing hemodialysis.[37] In addition, it was reported that patients with crescentic glomerulonephritis had high thrombinuria.[38] Moreover, anticoagulants, such as soluble thrombomodulin, can decrease inflammation and prevent microalbuminuria induced by chronic endothelial activation,[39] suggesting a potential link between chronic kidney disease and hypercoagulability. Evidence suggested that PAR-1 inhibition would have long-term therapeutic effectiveness in the glomeruli, although, in our study, we did not find any thrombosis in the

kidneys of the doxorubicin treated mice. Doxorubicin was reported to induce both thrombocytopenia[40] and procoagulant activation.[41] Thus, in future research, we should also examine a chronic kidney disease model with both apparent thrombotic features and podocyte injury.

We did not observe renal interstitial fibrosis in our model, although a previous study suggested that the thrombin/PAR-1 pathway contributed to TGF-β secretion in proximal tubular epithelial cells.[42] In addition, other studies reported that continuous albumin leakage from the glomerular filtration barrier resulted in renal fibrosis.[43] These conflicting results might have been attributable to the short experimental duration of our study.

In conclusion, our study showed that PAR-1 levels in glomeruli was closely associated with damage to the glomerular filtration barrier and that PAR-1 upregulation during nephropathy might exacerbate nephropathy. PAR-1 inhibition, not only for anticoagulation but also for glomerular protection, should be considered as a new therapeutic target.

Conflict of interest

The authors have declared that no conflicts of interest exist.

Acknowledgments

This work was supported by Yokoyama Foundation for Clinical Pharmacology (grant no. YRY1504).

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