学位論文

A serine protease inhibitor attenuates aldosterone-induced kidney injuries
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（アルドステロン関連腎障害におけるプラスミン活性抑制を介した
セリンプロテアーゼ阻害薬の腎保護効果）

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2017年3月

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２０１７年３月

Journal of Pharmacological Sciences 132 (2016) 145–153

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

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

Full paper

A serine protease inhibitor attenuates aldosterone-induced kidney injuries via the suppression of plasmin activity

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ARTICLE INFO

Article history:
Received 13 March 2016
Received in revised form
15 September 2016
Accepted 19 September 2016
Available online 30 September 2016

Keywords:
Aldosterone
Kidney injury
Serine protease
Plasmin
Serine protease inhibitor

ABSTRACT

Emerging evidence has suggested that aldosterone has direct deleterious effects on the kidney independently of its hemodynamic effects. However, the detailed mechanisms of these direct effects remain to be elucidated. We have previously reported that camostat mesilate (CM), a synthetic serine protease inhibitor, attenuated kidney injuries in Dahl salt-sensitive rats, remnant kidney rats, and unilateral ureteral obstruction rats, suggesting that some serine proteases would be involved in the pathogenesis of kidney injuries. The current study was conducted to investigate the roles of serine proteases and the beneficial effects of CM in aldosterone-related kidney injuries. We observed a serine protease that was activated by aldosterone/salt in rat kidney lysate, and identified it as plasmin with liquid chromatography-tandem mass spectrometry. Plasmin increased pro-fibrotic and inflammatory gene expressions in rat renal fibroblast cells. CM inhibited the protease activity of plasmin and suppressed cell injury markers induced by plasmin in the fibroblast cells. Furthermore, CM ameliorated glomerulosclerosis and interstitial fibrosis in the kidney of aldosterone/salt-treated rats. Our findings indicate that plasmin has important roles in kidney injuries that are induced by aldosterone/salt, and that serine protease inhibitor could provide a new strategy for the treatment of aldosterone-associated kidney diseases in humans.

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1. Introduction

Aldosterone maintains sodium homeostasis mainly through the activation of epithelial sodium channel (ENaC) in the distal nephron. Therefore it is considered as a hormone to increase blood pressure (1). However, mineralocorticoid receptor (MR) is expressed in various cells other than renal tubular epithelial cells. Furthermore, aldosterone has direct deleterious effects on the kidneys and other organs independently of its hemodynamic effects (2). The nonselective MR antagonist spironolactone and the selective MR antagonist eplerenone have been shown to reduce proteinuria in patients with kidney diseases independently of their antihypertensive effects (3–6). Animal studies have revealed that aldosterone directly provokes glomerular injuries and interstitial fibrosis through the induction of oxidative stress, inflammatory and pro-fibrotic cytokines, and apoptosis, as well as the activation of the intrarenal renin-angiotensin system and other mechanisms (7–9). However, the precise mechanisms of these effects need to be further elucidated, and the role of serine proteases in aldosterone-induced kidney injuries has not been evaluated.

Camostat mesilate (CM), a synthetic serine protease inhibitor, is clinically applied for the treatment of chronic pancreatitis and postoperative reflux esophagitis in Japan. We previously reported

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http://dx.doi.org/10.1016/j.jphs.2016.09.005
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that the administration of CM attenuated hypertension and kidney injuries in Dahl salt-sensitive (DS) rats fed a high salt diet (10). In addition, CM also alleviated glomerulosclerosis and interstitial fibrosis without changing blood pressure in remnant kidney model and unilateral ureteral obstruction kidney model (11,12). Furthermore, we have recently reported that CM mitigated renal fibrosis in adenine-induced chronic kidney disease rats more than hydralazine even though blood pressure levels with both treatments were similar (13,14). In those studies, CM exerted its renoprotective effects through the suppression of inflammatory and pro-fibrotic cytokine expression, reactive oxygen species (ROS) production and transforming growth factor β (TGF-β) signaling. These results suggest that some serine proteases might be involved in the progression of kidney injuries and that CM could provide beneficial effects by inhibiting the relevant proteases.

In the current study, we demonstrated that serine protease plasmin was activated in an MR dependent manner within kidney tissue of aldosterone/salt-treated rats. CM suppressed the protease activity of plasmin and alleviated the kidney injuries that were induced by aldosterone/salt.

2. Materials and methods

2.1. Animal study

All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals that have been approved by Kumamoto University. Experiments were conducted in male Sprague–Dawley (SD) rats from Charles River Laboratories (Wilmington, MA, USA). The rats underwent left uninephrectomy at 7 weeks old and were divided into one of the following groups at 9 weeks old:

Protocol 1: Effects of aldosterone and salt on serine protease activities in the kidney. (1) Control (0.3% NaCl diet), (2) high salt diet (8.0% NaCl diet), (3) aldosterone (0.75 μg/h), (4) aldosterone + high salt diet (A + S).

Protocol 2: The effects of eplerenone on kidney injuries induced by aldosterone and salt. (1) Control, (2) aldosterone + high salt diet, (3) aldosterone + high salt diet + eplerenone (0.125% diet).

Protocol 3: The effects of camostat mesilate on kidney injuries induced by aldosterone and salt. (1) Control, (2) aldosterone + high salt diet, (3) aldosterone + high salt diet + CM (0.1% diet).

Aldosterone (Sigma–Aldrich Co., St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) and saline, and then infused subcutaneously at the dorsum of the neck using an osmotic minipump (model 2004; Alza Corp., Palo Alto, CA, USA). Systolic blood pressure (SBP) was measured under awake conditions using a tail cuff method (MK-2000; Muromachi Kikai Co., Ltd., Osaka, Japan). Twenty-four hour urine collections were performed in metabolic cages, and urinary protein was measured by a commercial laboratory (SRL, Tokyo, Japan). After 4 weeks, the rats were sacrificed under anesthetic conditions. After blood samples had been collected from the inferior vena cava, the right kidneys were weighed and sliced through the short axis to obtain approximately 3 mm thick sections. The sections were then treated as described below. Serum creatinine and albumin levels were measured by SRL.

2.2. Zymography

A slice of kidney was homogenized with a polytron in ice-cold Tissue Protein Extraction Reagent (T-PER, Thermo scientific, Rockford, IL, USA) without a protease inhibitor cocktail. Aliquots of proteins (90 μg) were subjected to serine protease specific zymography (Cosmo Bio Co., Ltd, Tokyo, Japan) following the manufacturer's instructions. Double-layer fluorescent zymography (DLF-Zymography) was carried out as described previously (15). Briefly, aliquots of proteins (90 μg) were subjected to SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) in non-reduced condition. After washing with 2.5% TritonX-100 for 30 min followed by immersion in 50 mM Tris–HCl (pH 8.2), the gel was incubated with a cellulose acetate membrane treated with 100 mM Tris–HCl and 0.2 mM N-t-Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin (QAR-MCA, the substrate for a serine protease such as trypsin), which had been purchased from Peptide Institute (Osaka, Japan). After incubation at 37 °C for 1 h, the 7-amino-4-methylcoumarin released by the cleavage at the C-terminal of arginine with the serine proteases were visualized on an ultraviolet transilluminator at wave length of 365 nm. To confirm the inhibitory effect of CM on the serine proteases in the kidney homogenate, we added CM into the substrate solution in final concentrations of 0.5 and 5.0 μM.

2.3. Identification of serine protease

Kidney homogenates were precipitated in ammonium sulfate solution in 0–30%, 30–50%, 50–70% or 70–100% concentrations. Each fraction was dissolved in 50 mM Tris–HCl pH 7.6 with 0.1% CHAPS (Buffer A) and desalted in dialysis. Thereafter, it was subjected to DLF-Zymography in non-reduced condition. The fraction of 30–50% ammonium sulfate for which we observed the strongest activity of the target serine protease was demonstrated into an ion exchange chromatography column (Hi trap Q; GE Healthcare Bio Sciences, Piscataway, NJ, USA), which was pre-equilibrated with Buffer A using an AKTA prime chromatography system (GE Healthcare Bio Sciences). After being washed with Buffer A, the column was eluted with a linear gradient of 0–1.0 M NaCl in Buffer A. After ion exchange chromatography, each fraction was also subjected to DLF-Zymography and silver staining (Wako Pure Chemical Industries., Ltd, Osaka, Japan). For fractions that showed relatively higher activity of target serine protease and relatively fewer contaminating proteins with silver staining, we applied two sets of SDS-PAGE followed by DLF-Zymography and Deep Purple Total Protein Stain (GE Healthcare Bio Sciences). Stained proteins corresponding to target protease activity in DLF-Zymography were analyzed with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

2.4. Real-time PCR

A piece of kidney was placed in RNA later (Sigma Chemical Co.) at 4 °C overnight. Total RNA was extracted with an ST Total RNA Isolation System (Promega, Fitchburg, WI, USA). One microgram of total RNA was first transcribed with a Prime Script RT Reagent Kit (Takara Bio Inc., Shiga, Japan). TaqMan probes for rat collagen I, collagen III, TGF-β, connective tissue growth factor (CTGF), B7-1, monocyte chemoattractant protein-1 (MCP-1), TNF-α, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1) and protease activated receptor-1 (PAR-1) and GAPDH were all purchased from Applied Biosystems (Foster City, CA, USA). Real-time PCR was performed with the Light Cycler 480 Sequence Detector System (Roche Diagnostics, Mannheim, Germany). The results were analyzed statistically based on the ΔCt value ($Ct_{gene\ of\ interest} - Ct_{GAPDH}$). Relative gene expressions were obtained using the ΔΔCt method ($Ct_{sample} - Ct_{calibrator}$).

2.5. Chromogenic assay of plasmin activity in the kidney tissue

In protocol 3, renal plasmin activities were measured using Chromozym PL (Tosyl-Gly-Pro-Lys-4-nitroanilide acetate,

Sigma–Aldrich Co.), a specific chromogenic substrate for plasmin, as described previously (16). This substance is cleaved by plasmin into a residual peptide and 4-nitroaniline that can be detected spectrophotometrically.

2.6. Kidney histopathology

In protocol 3, the kidneys were fixed with 4% paraformaldehyde and embedded in paraffin. Sections (4 μm thick) were stained with Periodic acid-Schiff (PAS) and Azan Mallory and were examined under a light microscope. The glomerulosclerosis score was calculated in a blind manner from approximately 50 to 100 glomeruli of each rat as described previously (17).

2.7. Immunoblotting

A slice of kidney was homogenized with a polytron in ice-cold T-PER with a protease inhibitor cocktail. Aliquots of 30 μg renal proteins were subjected to SDS-PAGE in reduced condition and were immunoblotted with anti-Wilms' tumor-1 (WT-1) and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

2.8. In vitro study

2.8.1. The effect of camostat on plasmin activity

QAR-MCA (final concentration: 1 mmol/L in 96-well microliter plates) was incubated with 20 μg/mL plasmin in the presence of different concentrations of CM (0–5 μM) in 50 mM Tris–HCl (pH 8.2). The degree of substrate hydrolysis was measured by using a fluorescent microplate reader (Ultra Evolution; Tecan, Zurich, Switzerland) at an excitation of 360 nm and an emission of 465 nm. The residual activity of plasmin (velocity of inhibited enzyme reaction/velocity of uninhibited enzyme reaction) was expressed versus the CM concentration.

2.8.2. Renal fibroblast cells

A renal fibroblast cell line (NRK-49F) was purchased from Human Science Research Resources Bank (HSRRB, Osaka, Japan). Cells were maintained in advanced DMEM supplemented with 5% fetal calf serum (Invitrogen, Carlsbad, CA, USA). The cells were seeded in 6-well culture plates and grown to subconfluence in complete medium. Subsequently, the culture medium was replaced with serum-free medium for 24 h to render the cells quiescent. Human plasmin (Sigma chemical Co.) was dissolved in serum free medium and added to the culture medium at a final concentration of 20 μg/mL, as described previously (18) in the presence of CM (0–5 μM). After cells were incubated for another 72 h, Total RNA was extracted and real-time PCR was performed as described above.

2.8.3. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Comparisons were made using analysis of variance (ANOVA) followed by the Newman–Keuls method or the two-tailed, unpaired Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Animal study protocol 1: effects of aldosterone and salt on the serine protease activities in the kidney

In accordance with previous reports (19), only the A + S group developed hypertension and severe urinary protein excretion (Fig. 1A and B). Correspondently, the mRNA expressions of collagen type I, collagen type III, TGF-β1, TNF-α, and B7-1 were significantly increased in the A + S group alone (Fig. 1C).

3.2. Serine protease activities in the kidney homogenate

Serine protease specific zymography using kidney lysate revealed an approximately 80-kDa band that was only activated in the A + S group (Fig. 2A). DLF-Zymography with QAR-MCA substrate provided a similar result (Fig. 2B).

3.3. Identification of serine protease

Following the fractionation of kidney homogenate by ammonium sulfate precipitation and ion-exchange chromatography, we isolated two fractions of kidney lysate with relatively stronger activity of the target serine protease and fewer contaminating proteins (data not shown). Those fractions were then applied together to two sets of SDS-PAGE, followed by DLF-Zymography and Deep Purple Total Protein Stain (Fig. 2C). The stained band corresponding to the target protease activity in DLF-Zymography was subjected to the LC-MS/MS analysis and thereby identified as plasminogen/plasmin.

3.4. mRNA expression of tPA, uPA, PAI-1 and PAR-1

The gene expressions of tPA, uPA and PAI-1 were measured in order to examine the mechanism by which plasminogen was activated into plasmin within the renal tissue. tPA mRNA was slightly but significantly increased by aldosterone/salt, suggesting that tPA plays roles in the activation of renal plasmin. PAI-1 mRNA was dramatically increased by aldosterone/salt possibly to compensate the overactivation of renal plasmin. mRNA expression of PAR-1, which is one of plasmin receptors, was increased by aldosterone/salt (Fig. 3B).

3.5. Animal study protocol 2: the effects of eplerenone on aldosterone/salt-induced kidney injuries and plasmin activity

Eplerenone attenuated the aldosterone/salt-induced hypertension, urinary protein excretion, and mRNA expressions of kidney injury markers, as has also been reported previously (Table 1 and Fig. 3A) (9). Eplerenone also significantly suppressed the aldosterone/salt-induced plasmin activity in the kidney (Fig. 3B), indicating that mineralocorticoid receptor had an important role in the activation of plasmin.

3.6. In vitro study

3.6.1. Inhibition of proteolytic activity of plasmin by camostat mesilate

CM inhibited the proteolytic activity of plasmin significantly in concentrations of 0.5 and 5 μM. Inhibitory rates of CM on plasmin activity were 70.2 ± 5.1% and 91.2 ± 4.0% for concentrations of 0.5 and 5 μM respectively (Fig. 4A). Accordingly, the administration of CM in DLF-zymography suppressed plasmin activity in the kidney lysate (Fig. 4B).

3.6.2. Camostat mesilate suppresses the plasmin-induced cytokines expression in cultured renal fibroblast cells

The mRNA expressions of pro-fibrotic and inflammatory cytokines (TGF-β1, CTGF, MCP-1, and TNF-α) were significantly increased by 20 μg/mL of plasmin, indicating that plasmin has detrimental effects on NRK-49F cells. Those effects of plasmin were significantly ablated by CM (0.5 and 5 μM) (Fig. 4C).

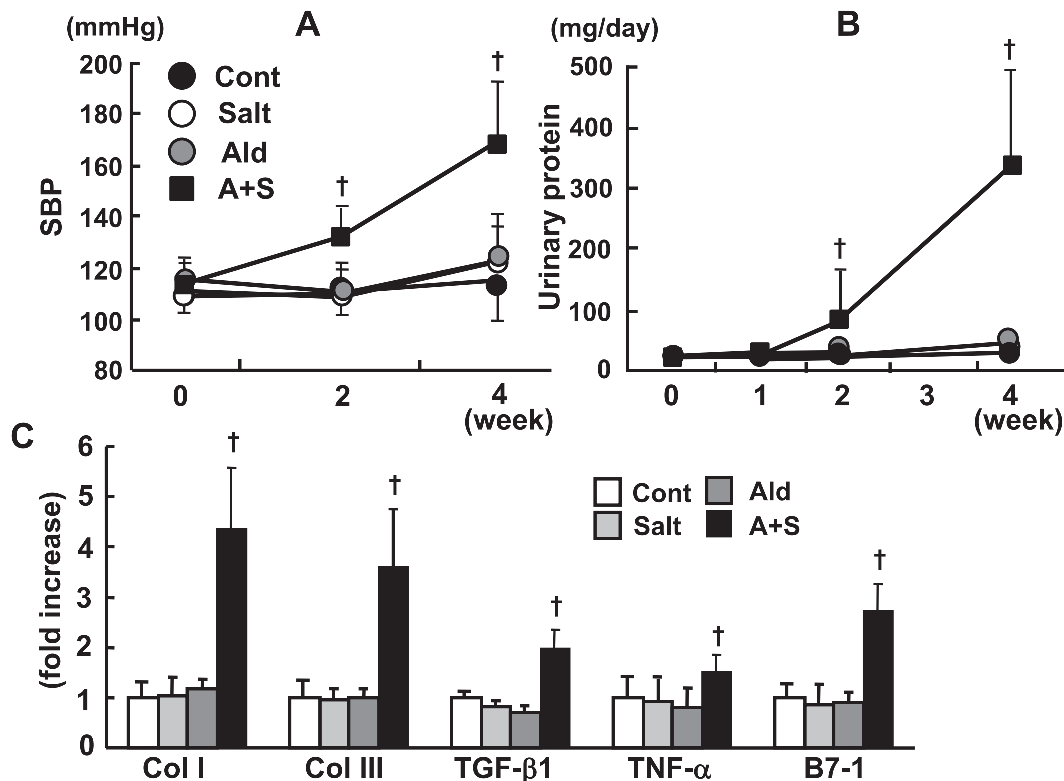

Fig. 1. Effects of aldosterone and salt on SBP (A), urinary protein (B) and mRNA expressions of kidney injury markers (C) in uninephrectised aldosterone/salt-treated rats. Aldosterone and high salt were administrated to 9-week-old uninephrectomized rats. (A) SBP was measured by tail-cuff method every second week. (B) Twenty-four hour urine collections were made in metabolic cages and urinary protein concentrations were determined at each time point. (C) mRNA expressions of kidney injury markers were determined by real-time PCR. The abundance of each mRNA was normalized by GAPDH. Values are expressed as fold increase over Control group and summarized in the bar graph. Cont, control; Salt, salt-treated group; Ald, aldosterone-treated group; A + S, aldosterone/salt-treated group; Col I and III, Collagen I and III. Data are expressed as mean ± SD (n = 5). †*P* < 0.01 vs. Control.

3.7. *Animal study protocol 3: the effects of camostat mesilate on the aldosterone/salt-induced kidney injuries*

3.7.1. *Renal plasmin activity*

Renal plasmin activity measured with a plasmin-specific substrate Chromozym PL was significantly upregulated in the A + S group and was substantially suppressed in the CM group, suggesting that renal concentration of CM was adequate to inhibit plasmin (Fig. 5A).

3.7.2. *Body weight, organ weight and blood pressure*

Body weights in the A + S and CM groups were smaller than those in the Control group (Table 2). The administration of aldosterone/salt significantly increased blood pressure immediately after 1 week, and blood pressure continued to increase until week 4. Treatment with CM significantly attenuated hypertension at week 4 (Fig. 5B). The ratio of right kidney weight to body weight was increased by aldosterone/salt, which was significantly suppressed by CM (Table 2).

3.7.3. *Blood and urine parameters*

In the A + S group, serum creatinine increased and serum albumin decreased, reflecting severe kidney injuries. However, concomitant administration of CM inhibited these changes (Table 2). Urinary protein levels dramatically increased in the A + S group, however this change was suppressed by CM as early as week 2 (Fig. 5C). CM exerted renoprotective effects earlier than its antihypertensive effect, indicating that plasmin in kidney tissue would provoke kidney injuries independently of blood pressure.

3.7.4. *Kidney histopathology*

Light microscopic examination revealed severe glomerular hypertrophy, glomerular sclerosis, and tubulointerstitial fibrosis in the A + S group; each was significantly alleviated by treatment with CM (Fig. 6A). The protein expression of WT-1 was decreased in the A + S group and was strikingly recovered in the CM group. These results indicate that CM substantially mitigated podocyte injuries caused by aldosterone/salt.

3.7.5. *mRNA expression of kidney injury markers*

mRNA levels of collagen type I, collagen type III, TGF-β1, B7-1, and MCP-1 were all significantly increased in A + S group, as compared with those in the Control group. Treatment with CM apparently suppressed all of these kidney injury markers (Fig. 6B).

4. Discussion

The current investigation was designed to identify serine protease(s) associated with kidney injuries that had been induced by aldosterone/salt. We further sought to confirm the beneficial effects of CM on these kidney injuries. We found a serine protease that is substantially activated in the kidneys of aldosterone/salt-treated rats. The column purification and LC-MS/MS analysis identified the serine protease as plasminogen/plasmin. Plasmin induced mRNA expression of pro-fibrotic and inflammatory genes in cultured renal fibroblasts, however CM suppressed these inductions. Moreover, the administration of CM significantly alleviated urinary protein excretion, glomerular sclerosis and tubulointerstitial fibrosis. These effects of CM were accompanied by

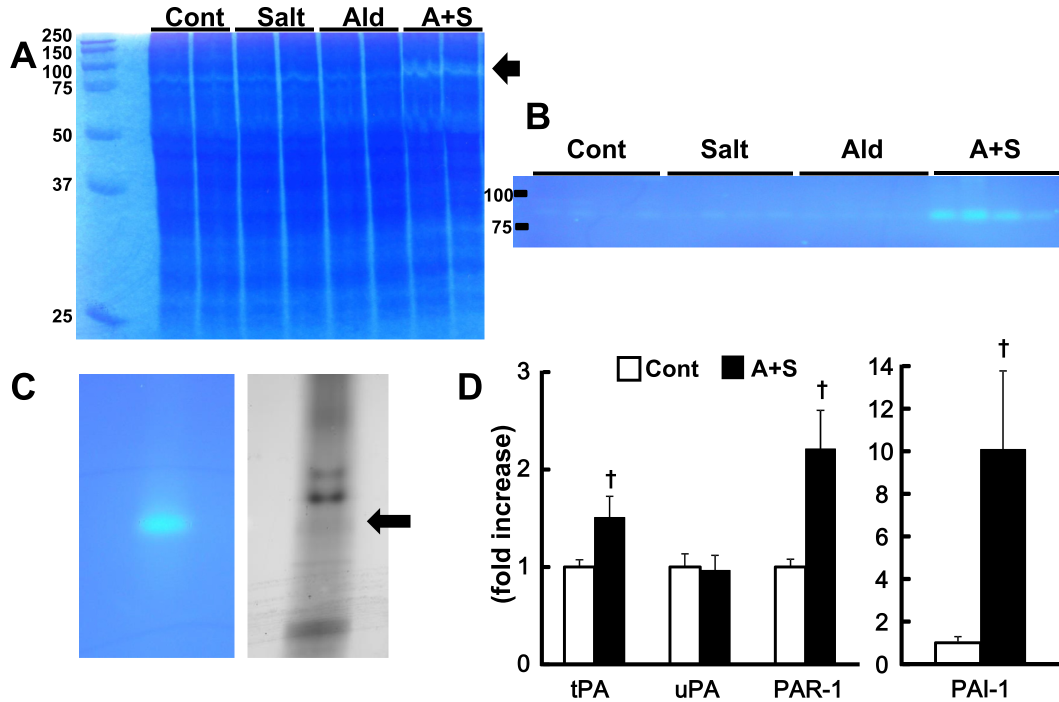

Fig. 2. Effects of aldosterone/salt on the serine protease activities in the kidney homogenate. (A) Serine protease specific zymography and (B) DLF-Zymography with QAR-MCA substrate displayed a serine protease at approximately 80 kDa that was highly activated in the kidney homogenate of A + S group. (C) Following the purification of kidney homogenate by ammonium sulfate precipitation and ion-exchange chromatography, the fractions that showed relatively higher activity of target serine protease and fewer contaminating proteins were applied to two sets of SDS-PAGE followed by DLF-Zymography (Left) and Deep Purple Total Protein Stain (Right). Ninety microgram of kidney homogenate was applied to each zymography in non-reduced condition. (D) mRNA expressions of tPA, uPA, PAI-1 and PAR-1 were determined by real-time PCR. Abundance of each mRNA was normalized for GAPDH and values are expressed as fold increase over Cont. Cont, control; Salt, salt-treated group; Ald, aldosterone-treated group; A + S, aldosterone/salt-treated group. Data are expressed as mean ± SD (n = 5). †$P < 0.01$ vs. Control.

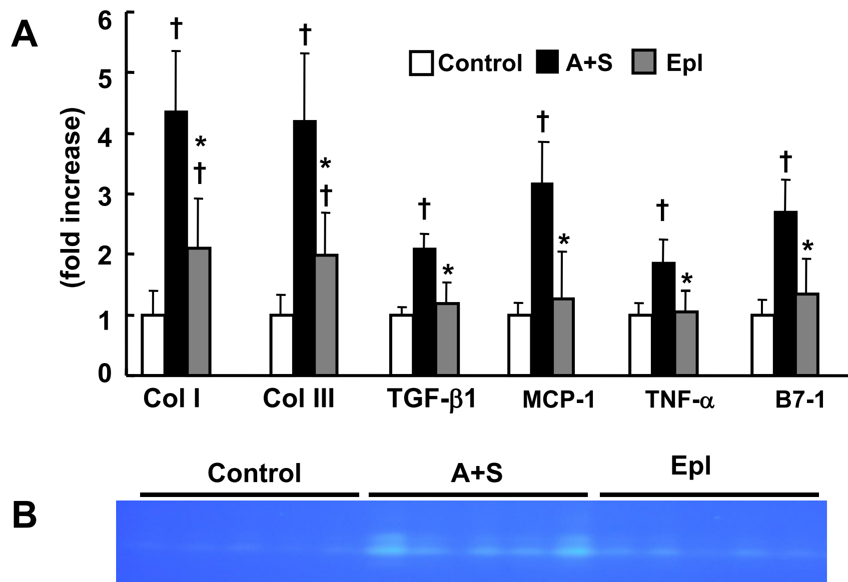

Fig. 3. Effects of eplerenone on the serine protease activity and kidney injury markers in uninephrectised aldosterone/salt rats. Eplerenone (0.125% diet) was administrated to 9-week-old uninephrectomized rats treated with aldosterone/salt. (A) mRNA expressions of kidney injury markers were determined by real-time PCR. The abundance of each mRNA was normalized by GAPDH. Values are expressed as fold increase over Control group and summarized in the bar graph. Data are expressed as mean ± SD (n = 8). †$P < 0.01$ vs. Control; *$P < 0.01$ vs. A + S. (B) The effect of eplerenone on the protease activity of plasmin in the kidney. Kidney homogenates (90 μg) of each group were applied to DLF-Zymography with QAR-MCA substrate.

suppressed mRNA expression of kidney injury markers in aldosterone/salt-treated rats.

The plasminogen-plasmin system plays pivotal roles in the fibrinolytic system, converting fibrin to fibrin degradation products in the intravascular area. The pro-enzyme plasminogen is synthesized in the liver and converted to its active form plasmin by cleavage with tPA and uPA (20,21). Both tPA and uPA are inhibited by plasminogen activator inhibitors (PAIs), and PAI-1 is their

Table 1
Effects of eplerenone on (patho)physiological parameters in uninephrectised aldosterone/salt rats.

	Cont	A + S	Eple
BW (g)	410 ± 11	393 ± 36	412 ± 27
rt Kidney wt (mg/g BW)	4.6 ± 0.2	9.0 ± 0.9†	6.0 ± 0.7†§
SBP (mmHg)	110 ± 5	174 ± 11†	140 ± 10†§
U-TP (mg/day)	15 ± 7	501 ± 86	52 ± 62*
Alb (g/dL)	3.8 ± 0.1	3.2 ± 0.2†	3.7 ± 0.3§
Cr (mg/dL)	0.41 ± 0.02	0.52 ± 0.04†	0.39 ± 0.02*

Cont, control; A + S, aldosterone/salt-treated group; Eple, aldosterone/salt and concomitant eplerenone-treated group; BW, body weight at week 4; SBP, systolic blood pressure at week 4; U-TP, urinary protein excretion at week 4; Alb, serum albumin level; Cr, serum creatinine level. Data are expressed as mean ± SD (n = 8). $^{†}P < 0.01$ vs. Cont, $^{§}P < 0.05$ vs. A + S, $^{*}P < 0.01$ vs. A + S.

dominant physiological inhibitor (22). In the kidney, tPA is the major glomerular plasminogen activator, while uPA is prominently expressed in the renal tubulointerstitium (23). Plasmin in urine has been reported to be associated with sodium retention in the nephrotic syndrome. Plasminogen filtered through damaged glomeruli is converted to plasmin by uPA within the renal tubules, and plasmin activates ENaC with the proteolytic cleavage of γ subunit leading to edema and hypertension (24). As we reported that CM inhibited the proteolytic cleavage of γENaC in vivo, CM might suppress hypertension via the inhibition of ENaC and mitigate the kidney injuries in the current experiment (25). However, CM reduced urinary protein excretion (beginning at week 2) before having an evident anti-hypertensive effect (which was observed at week 4), suggesting that plasmin activated by aldosterone/salt within the kidney tissue is highly associated with blood pressure-independent detrimental effects of aldosterone.

Although the role of plasminogen-plasmin system in kidney injury has been reported in various studies, it remains a matter of debate. A study of crescentic glomerulonephritis demonstrated that endogenous plasmin that was activated by exogenous tPA inhibited glomerular fibrin accumulation and glomerular damage (26). In contrast, it was also found that plasmin, which was attached to glomerulus through nephritis-associated plasmin receptor (NAPlr), could initiate glomerular injuries in human acute post-streptococcal glomerulonephritis (27). These conflicting reports suggest that plasmin could have biphasic effects on the progression of kidney injuries, depending on disease states. The pathogenic roles of plasmin in the development of interstitial fibrosis also remain to be elucidated. Plasmin has been shown to directly degrade extracellular matrix proteins, such as fibronectin, laminin, and proteoglycan (28). Furthermore, plasmin has been shown to activate matrix metalloproteinases, which also alleviate interstitial collagen accumulation (29). Therefore, plasmin had been considered to be anti-fibrotic protease in some organs (30–32). However, recent studies have demonstrated that plasmin exacerbates renal fibrosis in unilateral ureteral obstruction mice by activating TGF-β1 and stimulating PAR-1 and its downstream molecule ERK-1/2 (18,33). In addition to its pro-fibrotic effects, plasmin contributes to inflammation by provoking cytokine productions (such as TNF-α and MCP-1) and inflammatory cell infiltrations (34,35). Furthermore, plasmin produces reactive oxygen species (ROS) (35). In the current study, plasmin might have caused severe kidney injuries through the induction of these molecules, which were recovered by

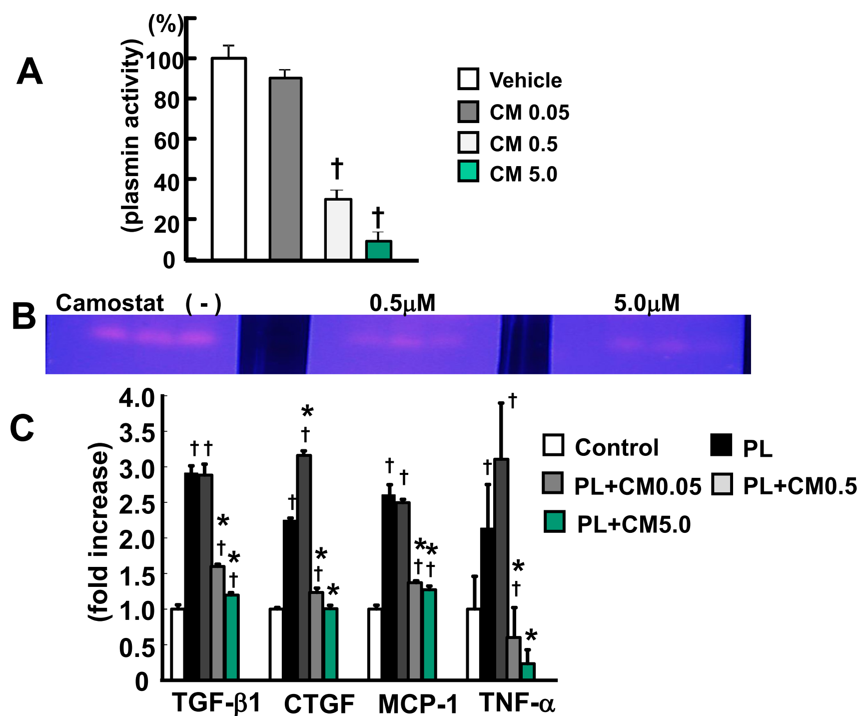

Fig. 4. Effects of camostat mesilate on the plasmin activity and the expression of kidney injury markers induced by plasmin in cultured renal fibroblast cell. (A) QAR-MCA (final concentration: 1 mmol/L) was incubated with 20 μg/mL plasmin in the presence of different concentrations of CM (0–5 μM) in 50 mM Tris–HCl (pH 8.2). The degree of substrate hydrolysis was measured by using a fluorescent microplate reader. The residual activity of plasmin (velocity of inhibited enzyme reaction/velocity of uninhibited enzyme reaction) was shown versus CM concentration. Data are expressed as mean ± SD (n = 4). $^{*}P < 0.01$ vs. Vehicle. (B) Vehicle, 0.5 μM and 5.0 μM of CM (final concentration) were into DLF-zymography substrate solution. CM suppressed plasmin activity in the dose-dependent manner. (C) After serum deprivation, the cultured renal fibroblast cells were incubated with 20 μg/mL plasmin and CM (0–5.0 μM, final concentration) for 72 h. CM suppressed the mRNA expression of kidney injury markers induced by plasmin from 0.5 μM. mRNA expressions of kidney injury markers were determined by real-time PCR. The abundance of each mRNA was normalized for GAPDH. Values are expressed as fold increase over Control group and summarized in the bar graph. Data are expressed as mean ± SD (n = 6). $^{†}P < 0.01$ vs. Control, $^{*}P < 0.01$ vs. PL.

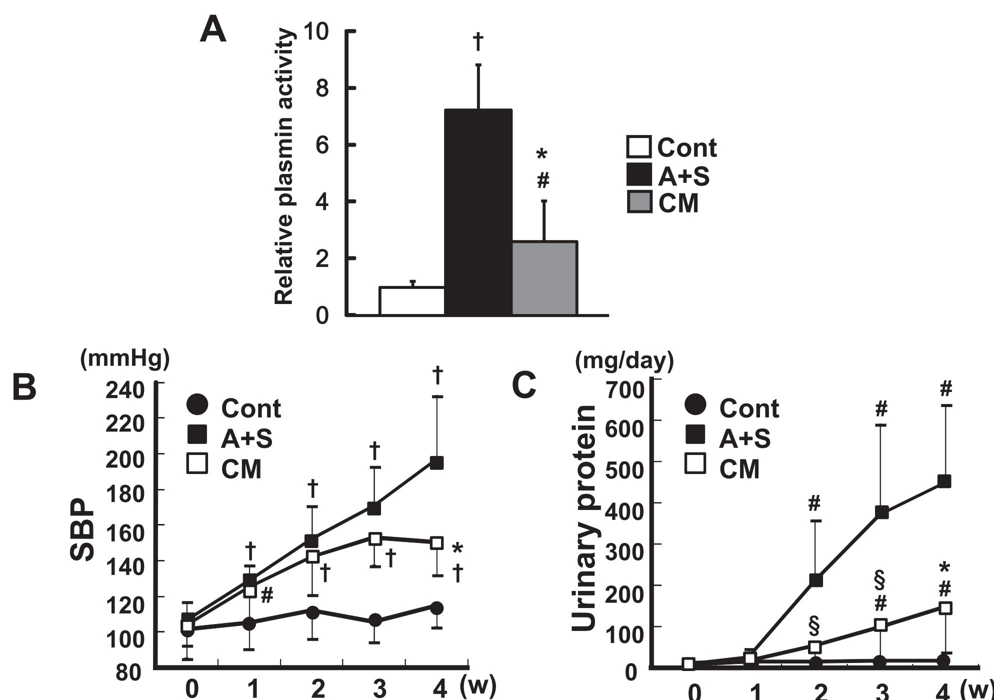

Fig. 5. Effects of camostat mesilate on renal plasmin activity (A), SBP (B) and urinary protein (C) in uninephrectised aldosterone/salt rats. CM (0.1% diet) was administrated to 9-week-old uninephrectomized rats treated with aldosterone/salt. (A) Renal plasmin activities in each group were measured using Chromozym PL that is specifically cleaved by plasmin. Relative plasmin activities were expressed as fold increase over Control group and summarized in the bar graph. (B) SBP was measured by tail-cuff method weekly. (C) Twenty-four hour urine collections were made in metabolic cages and urinary protein concentrations were determined at each time point. Cont, control; A + S, aldosterone/salt-treated group; CM, aldosterone/salt and concomitant CM-treated group. Data are expressed as mean ± SD (n = 8). $^{\#}P < 0.05$ vs. Cont, $^{\dagger}P < 0.01$ vs. Cont, $^{\S}P < 0.05$ vs. A + S, $^{*}P < 0.01$ vs. A + S.

Table 2
Effects of camostat mesilate on (patho)physiological parameters in uninephrectised aldosterone/salt rats.

	Cont	A + S	CM
BW (g)	438 ± 23	375 ± 22†	366 ± 17†
rt Kidney wt (mg/g BW)	4.8 ± 0.6	9.9 ± 1.3†	7.8 ± 0.5†§
Alb (g/dL)	3.8 ± 0.1	3.2 ± 0.4†	3.6 ± 0.2§
Cr (mg/dL)	0.38 ± 0.04	0.54 ± 0.06†	0.40 ± 0.09*

Cont, control; A + S, aldosterone/salt-treated group; CM, aldosterone/salt and concomitant camostat-treated group; BW, body weight at week 4; Alb, serum albumin level; Cr, serum creatinine level. Data are expressed as mean ± SD (n = 8). $^{\dagger}P < 0.01$ vs. Cont, $^{\S}P < 0.05$ vs. A + S, $^{*}P < 0.01$ vs. A + S.

CM treatment. Consistent with this hypothesis, we have already reported that CM prevented pro-fibrotic and inflammatory molecules and oxidative stress in other experimental rat models of kidney disease (10–14).

To the best of our knowledge, there has been no report to elucidate the relationship between aldosterone, MR and PAR-1 in the progression of kidney diseases. In this study, aldosterone/salt increased plasmin activity as well as mRNA expression of PAR-1 in the kidney, indicating that both effects synergistically exacerbated kidney damages. As CM suppressed plasmin activity in the kidney, the following inhibition of PAR-1 activation might have attenuated aldosterone-induced kidney injuries. Correspondingly, mRNA of PAR-1 was detectable in NRK-49F cells (data not shown) and CM prevented pro-fibrotic and inflammatory cytokine inductions caused by plasmin.

We were not able to clearly demonstrate the mechanism by which plasmin was activated in the kidney tissue of aldosterone/salt-treated rats. Because mRNA expression of plasminogen was not detectable in the kidney (18), and because we could never observe plasmin activity in the plasma from control or aldosterone/salt rats in DLF-Zymography (data not shown), circulating plasminogen could be trapped in the kidney tissue and activated locally via the interaction of aldosterone and MR, which was suppressed by eplerenone. Although mRNA expression of tPA in the kidney was slightly upregulated by aldosterone/salt, it is difficult to conclude that tPA was the dominant plasminogen activator in this model because upstream protease activity is more important than its gene expression in the serine protease cascade. In this study, we did not elucidate the protease activities of each plasminogen activator in the kidney. Additional studies are necessary to shed light on the detailed mechanism of renal plasmin activation.

In the present study, we used two different substrates for zymography to identify a serine protease plasmin that is activated by aldosterone/salt in the kidney. However, we cannot exclude the possibility that kidney injuries caused by aldosterone/salt involve other serine proteases that are undetectable with our methods. Other serine proteases such as elastase, cathepsin G, neutrophil serine protease and coagulation factor Xa have been known to induce kidney diseases (11,12,36,37). Although we did not evaluate the inhibitory effects of CM on these proteases, there were possibilities that CM inhibited kidney injuries via the suppression of these protease cascades in addition to plasmin. Further studies, particularly using plasminogen deficient mice, are definitely required to address the role of plasmin in kidney injuries.

The results of the current study suggest that serine protease plasmin induced by aldosterone/salt would cause kidney injuries. Serine protease inhibitors, such as CM, could provide a new strategy for the treatment of aldosterone-associated kidney injuries in humans.

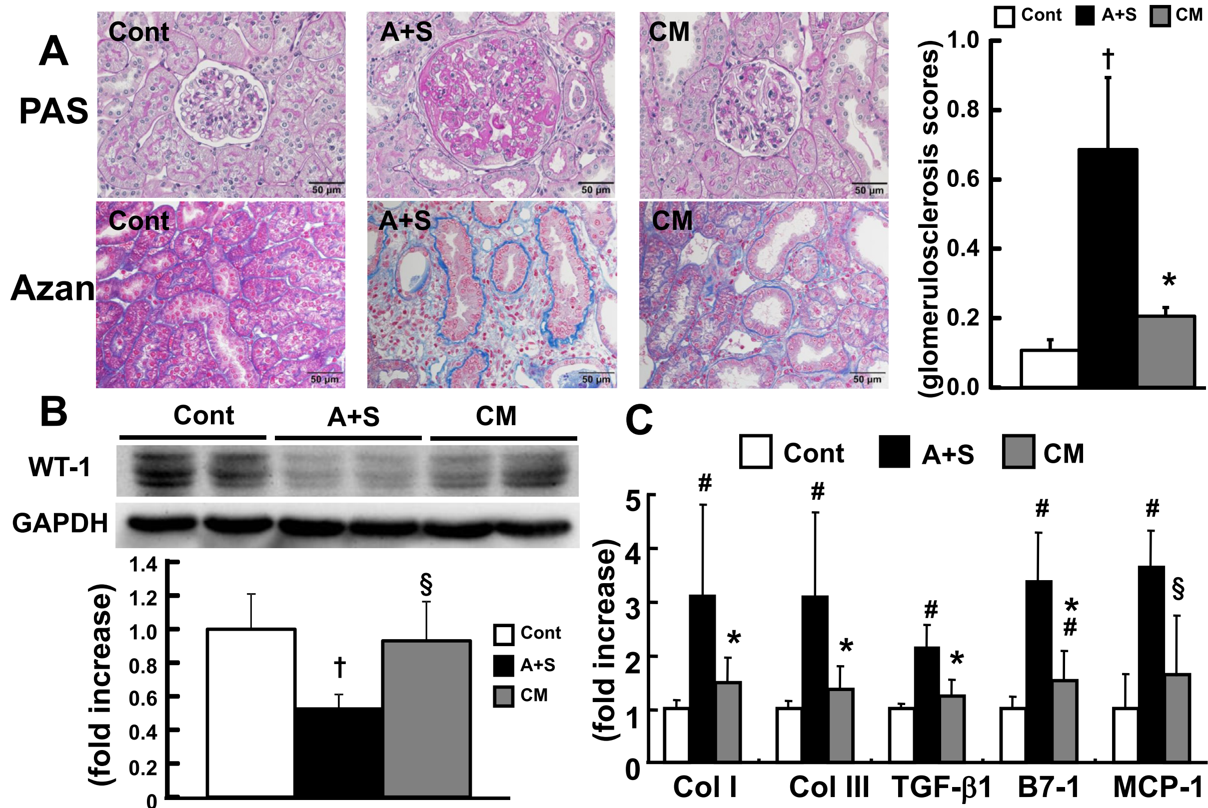

Fig. 6. Effects of camostat mesilate on kidney histopathology (A), WT-1 (B) and kidney injury markers (C) in uninephrectised aldosterone/salt rats. (A) Representative photomicrographs (×400) of periodic acid-Schiff (PAS) and Azan Mallory (Azan) stained kidney sections. The glomerulosclerosis score was calculated from approximately 50 to 100 glomeruli of each rat. (B) Protein expressions of WT-1 in the kidney were evaluated by Western blot analysis. The densitometry values were normalized for GAPDH and were expressed as fold increase over Control group. (C) mRNA expressions of kidney injury markers were determined by real-time PCR. The abundance of each mRNA was normalized by GAPDH. Values are expressed as fold increase over Control group and summarized in the bar graph. Cont, control; A + S, aldosterone/salt-treated group; CM, aldosterone/salt and concomitant CM-treated group. Data are expressed as mean ± SD (n = 8). $^{\#}P < 0.05$ vs. Cont; $^{\dagger}P < 0.01$ vs. Cont, $^{\S}P < 0.05$ vs. A + S, $^{*}P < 0.01$ vs. A + S.

Conflict of interest

The authors indicated no potential conflicts of interest.

Acknowledgments

The authors thank Ms. Noriko Nakagawa and Ms. Naoko Hirano (Graduate School of Medical Sciences, Kumamoto University) for their expertise in histopathology. This work was supported by the Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture in Japan (23790948 to Y. Kakizoe), The Salt Science Research Foundation (1532 to Y. Kakizoe).

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