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Full paper

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

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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 identify 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.

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 that the administration of CM attenuated hypertension and kidney injuries in Dahl salt-sensitive (DS) rats fed a high salt diet (10). In

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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 using pentobarbital sodium 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,

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