In vivo contribution of serine proteases to the proteolytic activation of γENaC in aldosterone-infused rats

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Submitted 3 January 2012; accepted in final form 22 July 2012

Uchimura K, Kakizoe Y, Onoue T, Hayata M, Morinaga J, Yamazoe R, Ueda M, Mizumoto T, Adachi M, Miyoshi T, Shiraishi N, Sakai Y, Tomita K, Kitamura K. In vivo contribution of serine proteases to the proteolytic activation of γENaC in aldosterone-infused rats. Am J Physiol Renal Physiol 303: F939–F943, 2012. First published July 25, 2012; doi:10.1152/ajprenal.00705.2011.—Aldosterone plays an important role in the regulation of blood pressure by modulating the activity of the epithelial sodium channel (ENaC) that consists of α-, β-, and γ-subunits. Aldosterone induces a molecular weight shift of γENaC from 85 to 70 kDa that is necessary for the channel activation. In vitro experiments demonstrated that a dual cleavage mechanism is responsible for this shift. It has been postulated that furin executes the primary cleavage in the Golgi and that the second cleavage is provided by other serine proteases such as pros- tasin or plasmin at the plasma membrane. However, the in vivo contribution of serine proteases to this cleavage remains unclear. To address this issue, we administered the synthetic serine protease inhibitor camostat mesilate (CM) to aldosterone-infused rats. CM decreased the abundance of the 70-kDa form of ENaC and led to a new 75-kDa form with a concomitant increase in the urinary Na/K ratio. Because CM inhibits the protease activity of serine proteases such as pros- tasin and plasmin, but not furin, our findings strongly indicate that CM inhibited the second cleavage of γENaC and subsequently suppressed ENaC activity. The results of our current studies also suggest the possibility that the synthetic serine protease inhibitor CM might represent a new strategy for the treatment of salt-sensitive hypertension in humans.

prostasin; furin; sodium; serine protease inhibitor

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MATERIALS AND METHODS

Animals. All animal procedures were in accordance with the guidelines for care and use of laboratory animals and were approved by Kumamoto University. Experiments were conducted in male Sprague-Dawley (SD) rats (160–170 g) from Charles River Laboratories (Wilmington, MA). The SD rats (n = 6) were kept for 10 days under the following conditions: 1) control, 2) aldosterone infusion (Aldo), and 3) aldosterone infusion + CM treatment (CM) with free access to water and chow. Rats were anesthetized with pentobarbital sodium (50 mg/kg body wt) before subcutaneous implantation of osmotic minipumps (model 2002; Alzet, Palo Alto, CA) that deliver 200 μg of aldosterone/day. Aldosterone (Sigma Chemical, St. Louis, MO) was dissolved in dimethyl sulfoxide and diluted with isotonic saline. In addition, CM group rats were subcutaneously implanted with sustained-release pellets of CM (14 mg/day). Aldo group rats were implanted with vehicle pellets. Control rats received vehicle pumps and pellets. All rats were housed in a room maintained at constant temperature, humidity, and light cycle (12:12-h light-dark cycle). Aldo group rats were subcutaneously implanted with sustained-release pellets of CM (14 mg/day). Aldo group rats were implanted with vehicle pellets. Control rats received vehicle pumps and pellets. All rats were housed in a room maintained at constant temperature, humidity, and light cycle (12:12-h light-dark cycle) and 24-h urine collections were made at day 7. Ten days after implantation, rats were killed under anesthetic conditions with pentobarbital sodium. The kidneys were sliced into ~3-mm-thick sections. The cortex was separated from the medulla by sharp dissection and used as described below. Blood samples were collected from the inferior vena cava, and electrolytes were measured by a commercial laboratory (SRL, Tokyo, Japan).

Immunoblotting. Pieces of kidney cortex were homogenized with a Polytron in ice-cold solution containing 250 mM sucrose/10 mM triethanolamine with 1 μg/ml leupeptin and 0.1 mg/ml phenylmethylsulfonyl fluoride (Sigma). Centrifugal filtrations were carried out to yield membrane fractions (17,000 and 200,000 g) as described previously (20). The 200,000-g pellets were dissolved, and protein concentration was determined by a bicinchoninic acid reaction (Thermo Scientific, Rockford, IL). Aliquots of proteins (40 μg) were subjected to SDS-PAGE and immunoblotted with anti-ENaC (13), anti-prostasin (BD Biosciences, San Jose, CA), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The αENaC antibody was raised against the NH2-terminal peptide from rat ENaC (amino acids 46–68), and the βENaC and γENaC antibodies were raised against the COOH-terminal peptides (βENaC: amino acids 617–638, γENaC: amino acids 629–650). Urine samples (two-thousandth part of 24-h urine volume) from each rat were directly subjected to SDS-PAGE under reducing conditions and analyzed by immunoblotting with an anti-prostasin antibody.

Real-time RT-PCR. A piece of kidney cortex was placed in RNAlater (Sigma) at 4°C overnight. Total RNA was extracted with the ST Total RNA Isolation System (Promega, Fitchburg, WI). One microgram of total RNA was first transcribed with a PrimeScript RT Reagent Kit (TAKARA BIO, Shiga, Japan). TaqMan probes for rat ENaC α-, β-, and γ-subunits and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were all purchased from Applied Biosystems (Foster City, CA). Real-time PCR was performed with the Light Cycler 480 Sequence Detector System (Roche, Mannheim, Germany). Statistical analysis of results was performed with the ΔCt value (Ct gene of interest - Ct GAPDH). Relative gene expression was obtained by the ΔΔCt method (Ct sample - Ct calibration).

Statistical analysis. Data are expressed as means ± SD. Comparisons were made using ANOVA followed by the Newman-Keuls method. P < 0.05 was considered as statistically significant.

RESULTS

Blood and urine parameters. Serum sodium was increased and serum levels of potassium and chloride were decreased in the aldosterone-infused rats compared with control rats, but the treatment with CM significantly suppressed these changes (Table 1). Urinary Na/K ratio, which is thought to be a surrogate marker of the activation of ENaC (24), was decreased by aldosterone, but CM increased this ratio (Table 1). These results strongly suggest the possibility that CM blocks the activation of ENaC by aldosterone.

Effect of CM on mRNA expression and protein abundance of ENaC in the kidney. Aldosterone induced a significant increase in αENaC mRNA expression. The expression of α-subunit mRNA in the Aldo group was almost equal to that in the CM group (2.58 ± 0.21- and 2.61 ± 0.33-fold increase over control group, respectively), suggesting that CM did not affect the aldosterone-mediated signaling pathway through the mineralocorticoid receptor (Fig. 1). The expression of β- and γ-subunit mRNA was almost identical among the three experimental groups (Fig. 1). Protein abundance of αENaC in the kidney was strikingly increased by aldosterone, but βENaC was not affected as previously reported (20). CM had no effect on the aldosterone-induced increase in αENaC protein, suggesting that CM did not affect protein synthesis (Fig. 2). The molecular weight shift of γENaC from 85 to 70 kDa was clearly observed in the kidney of aldosterone-infused rats. In the CM group, the abundance of the 70-kDa form was significantly decreased, and instead a new 75-kDa form appeared (Fig. 2).

Effect of CM on urinary prostasin. As we reported previously (22), aldosterone significantly increased urinary prostasin excretion. Concomitant administration of CM almost completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight, which is completely inhibited urinary prostasin excretion. As we reported previously (22), aldosterone significantly increased urinary prostasin excretion. Concomitant administration of CM almost completely inhibited urinary prostasin excretion, but we observed faint expression of a form with approximately a 3-kDa higher molecular weight in the CM group (Fig. 3). Because prostasin is known to be activated when other serine proteases such as matriptase and hepsin cleave the 3-kDa light chain (21) from pro-prostasin (7, 18), we considered the possibility that this band could be pro-prostasin.

Effect of CM on the protein abundance of prostasin in the kidney. Contrary to our expectation, protein expression of prostasin in the kidneys of the Aldo group was almost equal to that of the control group. On the other hand, CM increased the protein abundance of pro-prostasin in the kidney as suggested by the 3-kDa increase in molecular weight (Fig. 4). CM inhibited urinary excretion of prostasin, and consequently the protein expression of prostasin in the kidney of the CM group was increased compared with the other two groups.

DISCUSSION

In vitro experiments demonstrated that dual cleavage of γENaC is an important step for maturation of the channel.
Furin, a proprotein convertase that resides in the Golgi apparatus, cleaves γENaC only at one site, presumably during late maturation of the channel in the Golgi (5). Other serine proteases such as prostasin and plasmin execute the second cleavage at sites distal (COOH-terminal side) to the furin site, releasing an inhibitory peptide (~5 kDa) located in the extracellular loop of γENaC and increasing the open probability of the channel (3, 25). In the current studies, aldosterone clearly induced a molecular weight shift of γENaC from 85 to 70 kDa in the kidney, and CM treatment decreased the abundance of the 70-kDa form and produced a new 75-kDa form without affecting the mRNA expression of the three ENaC subunits or the protein abundance of α- and β-ENaC. Because CM suppresses the protease activity of prostasin and plasmin (19, 27) but not that of furin (8) in vitro, it is reasonable to speculate that CM blocked only the second cleavage, resulting in the 5-kDa shift in the molecular weight of γENaC in the CM group. To our knowledge, this is the first report demonstrating the possible contribution of dual cleavage in the activation of γENaC by aldosterone in vivo. Furin is also known to cleave αENaC at two sites in the NH2-terminal side of its extracellular loop (11) and activates the channel in the same manner as the γ-subunit. Because our αENaC antibody was unable to detect the cleaved form of αENaC, we could not investigate the effect of CM on the molecular weight shift of αENaC. However, it is unlikely that CM suppresses the cleavage of αENaC because CM does not inhibit furin activity.

In this study, we focused on prostasin as a candidate serine protease involved in the second cleavage in aldosterone-infused rats because prostasin is known to be induced by aldosterone (9, 22). Urinary prostasin excretion was significantly increased in aldosterone-infused rats compared to control rats (Fig. 1). CM treatment decreased the urinary prostasin excretion to almost the same level as the control group, suggesting that CM blocked the second cleavage of γENaC.

![Fig. 1. Effects of aldosterone infusion and concomitant camostat mesilate (CM) treatment on expression of epithelial sodium channel (ENaC) mRNA in kidneys from Sprague-Dawley rats. Expression of ENaC subunit mRNA was determined by real-time PCR. The abundance of each mRNA was normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the data are expressed as fold increase over the control group. Aldo, aldosterone infusion group; CM, aldosterone infusion and concomitant CM treatment group. Data are expressed as means ± SD (n = 6 rats). †P < 0.01 vs. control.](http://ajprenal.physiology.org/)

![Fig. 2. Effects of aldosterone infusion and concomitant CM treatment on ENaC protein abundance in kidneys from Sprague-Dawley rats. Top: abundance of ENaC α-, β-, and γ-subunit protein and β-actin protein were evaluated by immunoblotting analysis. Bottom: the densitometry values for ENaC subunits were normalized for β-actin. Values are expressed as fold increase over control and summarized in the bar graph. Data are expressed as means ± SD (n = 4). #P < 0.05 and †P < 0.01 vs. control.](http://ajprenal.physiology.org/)
A previous study from Andreasen and colleagues (2) reported that coexpression of a mutant prostasin with substitu-

tions in the catalytic triad increases ENaC activity to a similar extent as wild-type prostasin. This finding raises a question whether prostasin is indeed a serine protease responsible for the activation of ENaC promoted by aldosterone. Because the removal of the inhibitory fragment from γENaC is essential for channel activation, other serine proteases such as plasmin, neutrophil elastase, and kallikrein that cleave the distal site of the inhibitory segment have been shown to activate ENaC following the first cleavage by furin. It is also likely that other unknown serine proteases can participate in the proteolytic activation of ENaC induced by aldosterone. In many cases, activation of a serine protease occurs through an activation of a cascade of serine proteases. Therefore, the inhibition of γENaC cleavage may not represent suppression of the serine protease that directly cleaves γENaC. There is a possibility that CM blocks an upstream serine protease of a protease cascade that leads to the proteolytic activation of ENaC. Because CM has a relatively broad range of inhibitory properties against serine proteases, we were unable to identify a specific target of CM in the activation of ENaC at this point. Nevertheless, CM indeed suppressed the proteolytic cleavage of γENaC in aldosterone-infused rats, and our current data suggest the possibility that the dual cleavage mechanism exists in the activation of ENaC in vivo.

In conclusion, we found that CM inhibited the proteolytic cleavage of γENaC induced by aldosterone. The effect of CM included the suppression of pro-prostasin processing and inhibition of the excretion/shedding/secretion of prostasin. These results suggest the possibility that the synthetic serine protease inhibitor CM, which is orally active and already approved for clinical use in Japan without severe side effects, could be a new strategy for the treatment of salt-sensitive hypertension in humans.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS


REFERENCES