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

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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 prostasin 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-to-K ratio. Because CM inhibits the protease activity of serine proteases such as prostasin 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

THE CONTROL OF BLOOD PRESSURE (BP) and extracellular fluid volume primarily depends on renal regulation of sodium reabsorption and excretion. Among several sodium transporters and channels in the kidney, the epithelial sodium channel (ENaC) plays an important role in the regulation of BP by modulating sodium reabsorption in the distal nephron (26). Mutations in ENaC cause disturbances of BP as observed in Liddle’s syndrome and pseudohypoaldosteronism type 1 (23), indicating the importance of this channel for the pathogenesis of hypertension.

ENaC consists of α-, β-, and γ-subunits (4). Each subunit has a long extracellular domain in the luminal space, two transmembrane domains, and small cytoplasmic domains. The activation of ENaC is mainly regulated by the renin-angiotensin-aldosterone system. In particular, aldosterone increases sodium absorption through ENaC via several mechanisms [increased expression, trafficking to cell membrane, and suppression of ubiquitination, etc. (17, 20)]. Recently proteolytic cleavage of γENaC was recognized as a physiological mechanism of aldosterone-induced activation of ENaC. In normotensive rats, aldosterone increases αENaC protein abundance and leads to the redistribution of its three subunits to the apical region in the cortical collecting duct principal cells (20). In addition, aldosterone induces a molecular weight shift of γENaC from 85 to 70 kDa (20). This shift is believed to be a result of proteolytic cleavage by serine proteases and is necessary for the activation of ENaC. In vitro experiments demonstrated the possible involvement of several serine proteases such as trypsin, furin, prostasin, plasmin, and elastase in the proteolytic activation of γENaC (10).

Prostasin is one of the trypsin-like serine proteases purified from human seminal fluid (30). Vallet et al. (28) and we previously reported that prostasin increased sodium transport through ENaC in a coexpression system using Xenopus oocytes (1, 28) and that aldosterone increased the abundance of prostasin in human and rat urine and in the cultured medium of a mouse cortical collecting duct cell line, M-1 cells (22). It was also reported that urinary prostasin excretion was correlated with plasma aldosterone concentration or urinary Na-to-K (Na/K) ratio in humans (15). Therefore, it is conceivable that prostasin is involved in the cleavage of γENaC induced by aldosterone in vivo. Furthermore, we showed that urinary prostasin excretion was paradoxically increased and the cleavage of γENaC was accordingly increased in spite of high salt loading in Dahl salt-sensitive (DS) rats (13). Oral administration of the synthetic serine protease inhibitor camostat mesilate (CM), which is clinically used for the treatment of chronic pancreatitis or postgastrectomy reflux esophagitis in Japan (14, 16), significantly lowered systolic BP concomitantly with an increase in urinary Na/K ratio in DS rats fed a high salt diet (19). Although these results emphasize the importance of serine proteases such as prostasin in the activation of γENaC, it still remains unclear whether the molecular weight shift of γENaC induced by aldosterone in vivo is indeed the result of proteolytic cleavage by serine proteases.

In this study, we investigated the effect of CM on the molecular weight shift of γENaC in the kidney of aldosterone-infused rats to demonstrate the involvement of serine proteases in the cleavage of this subunit in vivo. Furthermore, we studied the effect of CM on the protein abundance of prostasin, one candidate serine protease, for the γENaC cleavage.

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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 9% dextrose. Ten days after implantation, rats were killed under anesthesia with pentobarbital (Sigma) at 4°C overnight. Total RNA was extracted with the ST Total RNA Isolation System (Promega, Fitchburg, WI). A piece of kidney cortex was placed in RNAlater (Sigma, St. Louis, MO) and stored at −80°C until use. Total RNA was reverse transcribed with the Advanced First-Strand cDNA Synthesis System (Promega, St. Louis, MO) was dissolved in dimethyl sulfoxide and diluted with isotonic 9% dextrose. One microgram of total RNA was then 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 ∆∆Ct method (Ct sample − Ct calibrator).

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 kidneys 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 prosta
in 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 matrkapase 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, whereas CM treatment significantly decreased its urinary excretion (Fig. 1). Aldosterone treatment caused a significant increase in the abundance of the γ-subunit protein, whereas CM treatment significantly decreased it (Fig. 2). These results suggest that CM suppressed the proteolytic activity of prostasin in vivo.
increased in the Aldo group, but prostasin protein abundance in the kidney was not increased. We cannot clearly shed light on these discrepancies at this point. It is possible that prostasin induced by aldosterone cleaved γENaC and was excreted into urine thereafter. Therefore, this might be the reason why we did not observe any increase in prostasin abundance in the kidney by aldosterone. In addition, CM increased the molecular weight of prostasin in the kidney by ~3 kDa, and at the same time significantly inhibited urinary prostasin excretion, resulting in an increase in the abundance of pro-prostasin protein remaining in the kidney. These findings suggest the possibility that CM inhibited the processing of pro-prostasin as well as the shedding or secretion of prostasin from the apical membrane. We previously reported that the synthetic serine protease inhibitor nafamostat mesilate (NM) inhibited prostasin excretion/shedding into rat urine and culture medium of M-1 cells (12). While prostasin is a GPI-anchored serine protease (6), the soluble form of prostasin can be released by shedding or secretion by GPI-specific phospholipase D1 or tryptic peptidases depending on the cell type (29). Because it is unlikely that CM inhibits the activity of phospholipase D, CM would interfere with the proteolytic shedding of prostasin although the tryptic peptidase responsible for shedding of the prostasin has not been identified yet. Aprotinin has been shown to inhibit the proteolytic processing of prostasin in human airway epithelial cells (21). However, it did not affect the secretion of prostasin, suggesting that the inhibitory effect on prostasin shedding/secretion might be specific to the synthetic small-molecular-weight serine protease inhibitors such as NM and CM.

A previous study from Andreasen and colleagues (2) reported that coexpression of a mutant prostasin with substitu-
REFERENCES


