Inhibition of Prostasin Secretion by Serine Protease Inhibitors in the Kidney

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Abstract. A serine protease, prostasin, has been shown to stimulate the activity of amiloride-sensitive sodium channels (ENaC). Prostasin is a glycosylphosphatidylinositol-anchored protein that is found free in physiologic fluids and tissue culture medium, but the mechanism by which prostasin is secreted from the cells has not been elucidated. The current studies found that serine protease inhibitor aprotinin blocked the secretion of prostasin in a mouse cortical collecting duct (CCD) cell line (M-1 cells). A synthetic serine protease inhibitor, nafamostat mesilate (NM), which is commonly used for the treatment of pancreatitis and disseminated intravascular coagulation in Japan, also inhibited the secretion of prostasin in M-1 cells. Continuous infusion of NM into rats resulted in a substantial decrease in urinary prostasin and urinary sodium excretion. p-guanidinobenzoic acid and 6-amidino-2-naphtol, catalytically inactive metabolites of NM, had no effect on prostasin secretion both in M-1 cells and in rats. These findings suggest that a serine protease-sensitive mechanism is involved in the secretion of prostasin in vitro as well as in vivo. Potassium secretion in the CCD is tightly linked to sodium reabsorption through ENaC; therefore, NM-induced decrease in prostasin secretion and subsequent inhibition of ENaC activity could account for the side effects of hyponatremia and/or hyperkalemia that are found sometimes in patients treated with NM. The results indicate an important role for prostasin in sodium reabsorption in the kidney under pathophysiologic conditions. ken@gpo.kumamoto-u.ac.jp

Proteases are involved in many biologic and physiologic processes such as digestion of proteins in the gastrointestinal tract, clotting of blood, processing of precursors of peptide hormones and growth factors, activation of proteases, and fertilization (1). Prostasin is a serine protease purified from human seminal fluid in 1994 (2). Purified prostasin displays a trypsin-like enzymatic activity and cleaves synthetic substrates in vitro, preferentially at the carboxy-terminal side of Arg residue. Prostasin is found in the kidney, prostate gland, bronchi, colon, liver, lung, pancreas, and the salivary glands. At the time prostasin was purified and the cDNA was cloned, the role of prostasin in mammalian physiology was not known (2–4). Recently a couple of laboratories including our laboratory showed that co-expression of prostasin and the epithelial sodium channel (ENaC) in Xenopus oocytes substantially increased the amiloride-sensitive sodium currents (5–9). In a mouse cortical collecting duct (CCD) cell line (M-1 cells), Nakhouli et al. and Liu et al. demonstrated that treatment of M-1 cells with serine protease inhibitors reduced sodium currents (10,11). In addition, we found that the treatment of M-1 cells with aldosterone increased the secretion of prostasin into culture media, and that prostasin then stimulated $^{22}$Na uptake (12). We also found that elevated aldosterone levels in rats markedly increased the urinary secretion of prostasin (12). Furthermore, we demonstrated that the urinary secretion of prostasin was substantially increased in patients with primary aldosteronism, and that adrenalectomy significantly reduced urinary prostasin secretion (12). These results indicate that prostasin is an important physiologic regulator of sodium reabsorption in the kidney through ENaC and suggest the possibility that prostasin is involved in the development of salt-sensitive hypertension in humans.

Molecular cloning of a full-length human prostasin cDNA revealed that its predicted amino acid residue sequence contains a carboxy-terminal hydrophobic region that is a consensus sequence of glycosylphosphatidylinositol (GPI)-anchored membrane protein (13). Although prostasin is synthesized as a GPI-anchored membrane protein, a free-form prostasin is secreted into the medium of cultured prostate cells (13). In M-1 cells, we previously demonstrated that prostasin is found only in the culture medium, but not in the either cytosolic or membrane fraction (12). We were also unable to detect prostasin in either cytosolic or membrane fractions of rat kidneys, but we easily found prostasin in the urine of rats and humans (12). Therefore, we believe that prostasin is expressed as a secretory protein both in M-1 cells and in the kidneys rather than a membrane-bound protein. However, the precise mechanism by which prostasin is expressed in the kidney remains
unknown. Understanding this mechanism could provide the new insight into the treatment of hypertension and other sodium-retaining states.

In the present studies, we found that treatment of M-1 cells with serine protease inhibitor aprotinin blocked the secretion of prostasin into the medium of cultured cells. We demonstrated that treatment with nafamostat mesilate (NM), a synthetic serine protease inhibitor that is commonly used for the treatment of pancreatitis and disseminated intravascular coagulation (DIC) in Japan, also inhibited prostasin secretion in M-1 cells as well as in rat kidneys. Furthermore, we showed that urinary sodium excretion was substantially increased in NM-treated rats. NM has been shown to induce hyponatremia and/or hyperkalemia in some cases when used for the treatment of pancreatitis or DIC (14); therefore, our results suggest the possibility that the inhibition of prostasin secretion into urine and the subsequent inhibition of prostasin-induced ENaC activity in the kidney may be responsible for the hyponatremia and/or hyperkalemia associated with NM treatment. Taken together, our results indicate that the secretion of prostasin is mediated by a mechanism that is sensitive to the serine protease inhibitors and suggest the involvement of prostasin action in pathophysiologic conditions.

Materials and Methods

Materials

Nafamostat mesilate, p-guanidinobenzoic acid (PGBA), and 6-amidino-2-naphthol (AN) are kind gifts from Torii Pharmaceutical Co., Ltd. (Nihonbashi, Tokyo, Japan). Other chemicals are obtained from Sigma (St. Louis, MI, USA).

Cell Culture

M-1 cell, an SV40-transformed mouse CCD cell line, was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco modified Eagle medium/Ham’s F-12 (1:1) mixture (Life Technologies-BRL, Rockville, MD) supplemented with 5% FBS and 100 nM dexamethasone in a humidified incubator at 37°C and 5% CO₂ as described previously (12). Experiments were performed when cells were confluent. Serum and dexamethasone were removed 48 h before experiments. All studies described in this paper were performed on cells between the fifth and twentieth passages.

Northern Blot Analyses

Total RNA from M-1 cells grown in 10-cm plastic dishes under experimental conditions was isolated by using an RNeasy kit (QIAGEN, Hilden, Germany). Total RNA (20 μg) of each sample was resolved on agarose-formaldehyde gels and transferred onto nylon membranes. A full-length cDNA of mouse prostasin and rat β-actin was individually labeled with [α-32P] dCTP, and probes were hybridized with the membranes as described previously (5).

Preparation of Proteins and TCA Precipitation

After incubation under each experimental condition, culture medium (10 ml/10 cm dish) was collected, and centrifuged at 1200 × g to pellet cell debris. Total protein in the culture medium was precipitated with TCA (final concentration, 15%). The samples were centrifuged at 12,000 × g, and the pellets were washed three times with ice-cold 80% acetone. The precipitated proteins were dried and solubilized at 100°C for 5 min in 1 × TCA buffer (200 mM unbuffered Tris, 1% SDS, 10% Glycerol, 1% β-mercaptoethanol).

Immunoblotting

Samples were size fractionated using SDS-PAGE with 12% gels and transferred electrophoretically onto nitrocellulose filters. After blocking with 5 g/dl nonfat dry milk overnight at 4°C, the blots were probed with a polyclonal antibody against prostasin peptide in Tris-buffered saline with 0.05% Tween-20 for 1 h at room temperature, followed by a secondary antibody (goat anti-rabbit IgG-conjugated with horseradish peroxidase, diluted to 1:10,000) for 1 h at room temperature. Bands were visualized using chemiluminescence substrate (ECL; Amersham Pharmacia Biotech, Buckinghamshire, England) before exposure to x-ray film. The band densities were quantitated by densitometry (Densitograph 4.0; ATTO, Tokyo, Japan).

Serine Protease Inhibitor Infusion Studies

Experiments were conducted in male Sprague-Dawley rats (180 to 200 g) from Charles River Japan, Inc. (Yokohama, Japan). Rats were anesthetized with 2% isoflurane before the implantation of catheters. The external jugular vein of rats was exposed as it crossed the clavicle via a ventral skin incision on the neck. After phlebotomy, a fine silicone catheter (Silascon, Kaneka Medics, Osaka, Japan) was introduced into the vein until the intravascular tip was located at the junction of the cranial vena cava and right atrium. The catheter was tied onto the vein by two 4-0 silk ligatures just proximal and distal of the phlebotomy. A trocar was inserted subcutaneously from the dorsal aspect of the mid-cervical region exiting at the ventral skin incision on the neck. The external end of the catheter was then threaded through the trocar to exit at the scapula, and the trocar was withdrawn. Next, the external end of the catheter was tightly attached to the swivel (Instech 375/22; Lomir Biomedical Inc., Malone, NY) through a vinyl tube, and an infusion pump was connected to the swivel. The swivel was kept over the middle of the cage by a metal hook. To keep daily sodium, potassium, and water intake constant, total parenteral nutrition was administered with this swivel system. The rats were received 260 mld/kg of body weight per day of a formulated solution composed of 100 ml of 50% glucose, 40 ml of essential amino acids (Aminpren; Ohtsuka Pharmaceutical Co., Ltd., Tokushima, Japan), 2.8 ml of 1 M NaCl, 1.5 ml of 1 M K₂HPO₄, 1.0 ml of 1 M MgSO₄, 1.5 ml of 1 M potassium aspartate, 2.5 ml of 0.2 M CaCl₂, 110 ml of sterile H₂O, and vitamins. The solutions were continuously infused with the syringe pump. Continuous infusion of a serine protease inhibitor or its stable metabolites was started 24 h after the initiation of parenteral nutrition. The rats were continuously infused with 2 mg/kg per hour of NM, 1 mg/kg per hour of PGBA, 1 mg/kg per hour of AN, or vehicle. All rats were kept in metabolic cages, and urine samples were collected and stored at 4°C. The urine electrolytes and creatinine concentrations were measured in the 24-h urine samples. Urine samples, corrected for creatinine excretion, were concentrated by TCA precipitation, and the amount of prostasin was determined by immunoblotting as described above.

Results

Effect of Protease Inhibitors on Prostasin Secretion in M-1 Cells

Since an intracellular proteolytic mechanism has been implicated in the secretion of soluble forms of GPI-anchored proteins (15), we hypothesized that protease(s) is/are involved
in the secretion of prostasin in M-1 cells. To test our hypothesis, we measured prostasin protein abundance in M-1 cells after the protease inhibitor treatment. M-1 cells (serum-deprived for 48 h) were treated with aprotinin (28 μg/ml), phosphoramidon (1.1 μg/ml), E-64 (7.2 μg/ml), leupeptin (2.0 μg/ml), antipain (2.5 μg/ml), or pepstatin A (1.0 μg/ml) in serum-free media for 24 h, and the amount of prostasin protein in the culture media was determined by the immunoblotting with anti-prostasin our peptide antibody. As shown in Figure 1A, treatment with serine protease inhibitor aprotinin almost completely inhibited the secretion of prostasin, whereas other types of protease inhibitors (cysteine, aspartic, or metalloprotease inhibitors) had no effect on the amount of prostasin protein in the medium. The prostasin protein was not found in membrane or cytosol fraction of M-1 cells under basal condition; even in the presence of aprotinin, we were unable to detect the prostasin protein in both membrane and cytosol fraction (data not shown). None of these protease inhibitors used for the experiments had significant effects on the mRNA levels of prostasin (Figure 1B). These findings suggest that a serine protease inhibitor aprotinin inhibited the translation of prostasin mRNA or the protranslational processing of prostasin protein in M-1 cells.

Effect of NM, PGBA, and AN on Prostasin Secretion in M-1 Cells

NM, a synthetic serine protease inhibitor, inhibits various serine proteases, including trypsin, thrombin, activated factor X, kallikrein, activated complements, and neutrophil elastase. Although NM has been shown to be effective in the treatment of pancreatitis and DIC (16), it sometimes causes side effects of hyponatremia and/or hyperkalemia (14). Muto et al. (17–19) reported that perfusion of rabbit CCD with NM inhibited the amiloride-sensitive sodium current, resulting in the inhibition of potassium secretion. They suggested that hyponatremia and/or hyperkalemia associated with NM treatment could be caused in part by the inhibition of amiloride-sensitive sodium conductance. We demonstrated that a serine protease inhibitor aprotinin inhibits the secretion of prostasin into culture media, and we showed that prostasin increases sodium reabsorption through the activation of ENaC; we therefore hypothesized that hyponatremia and/or hyperkalemia associated with NM treatment is caused by the inhibition of prostasin secretion and the subsequent inhibition of prostasin-induced activation of ENaC. To test this hypothesis, we treated M-1 cells with NM or its catalytically inactive metabolites, PGBA or AN, and measured the level of prostasin protein in the culture media. The stable serum concentration of NM is 10⁻⁸ to 10⁻⁷ M in patients receiving continuous infusion of NM at a dose of 0.06 to 0.20 mg/kg per hour (commonly used dosage for the DIC treatment); therefore, M-1 cells were incubated with 10⁻⁸ M of NM, PGBA, or AN for 24 h. As shown in Figure 2A, treatment with NM completely blocked the secretion of prostasin into M-1 culture media, whereas PGBA and AN had no effect on prostasin secretion. The expression of prostasin was not demonstrated in either membrane or cytosol fraction in M-1 cells treated with NM as observed in M-1 cells treated with aprotinin (data not shown). None of these compounds had significant effect on mRNA expression of prostasin in M-1 cells (Figure 2B). Next, we examined the dose-response relationship of the inhibition of prostasin secretion by NM. NM blocked prostasin secretion, even at a dose of 10⁻⁹ M, a dose that is ten times lower than the serum concentration of NM used for the treatment of DIC (Figure 3). To address the question whether aprotinin or NM binds to prostasin and reduces the ability of the antibody to recognize the epitope sequence, we added aprotinin or NM in graded concentrations to the culture medium of M-1 cells produced under the condition of no aprotinin or NM and performed immunoblottings. As shown in Figure 4, aprotinin and NM had no effect on the detection of prostasin protein by the prostasin antibody. These data suggest that serine protease activity is indeed involved in the prostasin secretion.

NM Infusion Studies

To determine the effect of NM on prostasin secretion in vivo, we continuously infused NM, PGBA, or AN into rats receiving total parenteral nutrition as described in the Materials and
Methods section. Urinary secretion of prostasin was measured before and 7 d after the initiation of continuous infusion. As shown in Figure 5, continuous infusion of NM almost completely inhibited the secretion of prostasin into urine, whereas PGBA and AN had no effect on prostasin secretion. Next, we examined the time course of urinary prostasin secretion and sodium excretion in rats receiving parenteral nutrition with or without NM. Interestingly, the inhibitory effect of NM on urinary prostasin secretion could be observed on day 1, and the effect lasted throughout the experimental period (Figure 6A). In addition, urinary sodium excretion was significantly increased in rats receiving NM when compared with rats receiving vehicle. The NM-induced increase in urinary sodium excretion was observed during the whole experimental period (Figure 6B). We also measured urinary potassium excretion in these rats. Urinary potassium excretion was as follows: at day 0 (vehicle, 1.0 ± 0.05 mEq/d; NM, 0.9 ± 0.04 mEq/d), at day 1 (vehicle, 0.8 ± 0.06 mEq/d; NM, 0.6 ± 0.04 mEq/d), at day 4 (vehicle, 1.6 ± 0.24 mEq/d; NM, 1.2 ± 0.07 mEq/d), and at day 7 (vehicle, 0.9 ± 0.12 mEq/d; NM, 0.9 ± 0.12 mEq/d). Although the difference in urinary potassium excretion between vehicle and NM was not statistically significant at each time point, we found a tendency that rats receiving NM had lower urinary potassium excretion level. These results demonstrate that a serine protease inhibitor NM almost completely blocks the secretion of prostasin in a mouse CCD cell line as well as in rats receiving parenteral nutrition.
Kidneys. Prostasin is detected only in the culture media under basal conditions. When treated with serine protease inhibitors, prostasin was not found in the culture media, membrane fraction, or cytosol fraction of M-1 cells, but mRNA expression of prostasin was not changed. These findings suggest that serine proteases are involved in the translation of prostasin mRNA or in the posttranslational processing of prostasin protein. However, in the current studies we have not addressed these possibilities. In the native tissue such as prostate gland, prostasin has been shown to be expressed as a GPI-anchored protein (13), but we were not able to demonstrate the expression of prostasin in either membrane fraction or cytosol fraction of the kidneys. We speculated that processing mechanisms of prostasin might be different between in the kidney and in the prostate gland. Human folate receptors are demonstrated to be produced as either GPI-anchored proteins or secreted proteins depending on the cell or tissue type. An intracellular proteolytic mechanism that could result in the selective and efficient secretion of soluble forms of folate receptors has been suggested (15). A same kind of mechanisms might play a role in the secretion of prostasin in the kidneys. Although renal handling mechanisms of aprotinin is not completely elucidated, it is demonstrated that aprotinin binds to the plasma membrane of renal tubules and that radio-labeled aprotinin is found in the cytoplasm of renal tubules (20,21). Recently, NM has been shown to be transported inside the renal tubular cells via organic cation transporter 2 (OCT2) (22). Therefore we speculated that these serine protease inhibitors (at least NM) can gain access to the intracellular compartments and then inhibit the intracellular proteolytic mechanism of prostasin processing. Further investigation is needed to elucidate the precise mechanism by which a serine protease regulates the secretion of prostasin.

Implications for NM-Induced Hyponatremia and/or Hyperkalemia

Previously Muto et al. (17–19) reported that perfusion of rabbit CCD with NM, PGBA, or AN inhibited the amiloride-sensitive sodium conductance in the apical membrane. They speculated that hyponatremia and/or hyperkalemia that sometimes occur in patients treated with NM are induced by inhibition of the amiloride-sensitive sodium conductance and resultant inhibition of potassium secretion. In the current studies, we also demonstrated that continuous infusion of NM into rats resulted in a substantial decrease in urinary sodium excretion and prostasin secretion. However, our data indicated that PGBA and AN had little effect on urinary prostasin secretion as well as urinary sodium excretion. The discrepancy could be explained by the experimental systems used for the studies. Muto et al. used ex vivo renal tubule perfusion system and studied the acute effect of NM on sodium conductance, whereas we performed in vitro and in vivo experiments and observed relatively long-term effects of NM.

Although Muto et al. speculated that NM, PGBA, and AN directly inhibit the amiloride-sensitive sodium conductance in rabbit CCD, the precise mechanisms by which these protease inhibitors block the sodium conductance are not defined. In the present studies, we found that the secretion of prostasin is

Discussion

In the present studies, we found that treatment of M-1 cells with a serine protease inhibitor aprotinin almost completely blocked the secretion of prostasin. We also demonstrated that treatment with NM inhibited prostasin secretion in M-1 cell as well as urinary prostasin secretion in rats. Furthermore, we showed that urinary sodium excretion was substantially increased in the NM-treated rats.

Serine Protease-Sensitive Mechanism of Prostasin Secretion

Prostasin is synthesized as a GPI-anchored membrane protein, whereas a free-form of prostasin is secreted into the culture medium of the prostate cells (13). In the studies presented here, we demonstrated that serine proteases are involved in the secretion of prostasin in M-1 cell as well as in rat kidneys. Prostasin is detected only in the culture media under...
almost completely inhibited by NM treatment, but not by AN or PGBA treatment. We previously demonstrated that prostasin is a potent ENaC activator; therefore, it is reasonable to speculate that NM blocks the secretion of prostasin into the lumen of the tubules and consequently inhibits the prostasin-induced sodium uptake through ENaC. Thus, the NM-induced inhibition of sodium reabsorption leads to a decrease in potassium secretion in the CCD. Here we propose that hyponatremia and/or hyperkalemia associated with NM treatment in the clinical situation is probably caused by the inhibition of prostasin secretion in the kidney.

Our findings would be useful to develop a strategy for the prevention of side effects of NM in the future, and they also indicate that an analogue of NM could be a candidate for the therapeutic drug for disorders of sodium handling in the kidney such as hypertension and nephrogenic edema.

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