Urinary Proteolytic Activation of Renal Epithelial Na\(^+\) Channels in Chronic Heart Failure

Hong Zheng, Xuefei Liu, Neeru M. Sharma, Yulong Li, Rainer U. Pliquett, Kaushik P. Patel

Abstract—One of the key mechanisms involved in renal Na\(^+\) retention in chronic heart failure (CHF) is activation of epithelial Na\(^+\) channels (ENaC) in collecting tubules. Proteolytic cleavage has an important role in activating ENaC. We hypothesized that enhanced levels of proteases in renal tubular fluid activate ENaC, resulting in renal Na\(^+\) retention in rats with CHF. CHF was produced by left coronary artery ligation in rats. By immunoblotting, we found that several urinary serine proteases were significantly increased in CHF rats compared with sham rats (fold increases: furin 6.7, prostasin 23.6, plasminogen 2.06, and plasmin 3.57 versus sham). Similar increases were observed in urinary samples from patients with CHF. Whole-cell patch clamp was conducted in cultured renal collecting duct M-1 cells to record Na\(^+\) currents. Protease-rich urine (from rats and patients with CHF) significantly increased the Na\(^+\) inward current in M-1 cells. Two weeks of protease inhibitor treatment significantly abrogated the enhanced diuretic and natriuretic responses to ENaC inhibitor benzamil in rats with CHF. Increased podocyte lesions were observed in the kidneys of rats with CHF by transmission electron microscopy. Consistent with these results, podocyte damage markers desmin and podocin expressions were also increased in rats with CHF (increased ≈2-folds). These findings suggest that podocyte damage may lead to increased proteases in the tubular fluid, which in turn contributes to the enhanced renal ENaC activity, providing a novel mechanistic insight for Na\(^+\) retention commonly observed in CHF. (Hypertension. 2016;67:197-205. DOI: 10.1161/HYPERTENSIONAHA.115.05838.) • Online Data Supplement

Key Words: heart failure • Na\(^+\) and water retention • podocyte • protease • renal function

An impaired ability to excrete Na\(^+\) load is commonly seen in patients with chronic heart failure (CHF).1,2 CHF and chronic kidney disease often coexist.3,4 This coexistence of CHF and chronic kidney disease is commonly referred to the cardio-renal syndrome. However, elucidating the contribution of specific molecular mechanism/s for the inappropriate Na\(^+\) and water retention commonly seen in CHF remains largely undefined.

Renal epithelial Na\(^+\) channels (ENaC) represent the fine-tuning, rate-limiting step in Na\(^+\) reabsorption in the distal nephron. ENaC are composed of 3 subunits: α-, β-, and γ-ENaC localized in the distal convoluted tubules, cortical collecting ducts (CCD), and medullary collecting ducts of the distal nephron.5,6 The activity of ENaC is regulated by angiotensin (ANG) II and aldosterone, which markedly increases the apical permeability of the collecting duct to Na\(^+\).7-9 Interventions studies in patients have shown that systemic ENaC inhibition by ANG II receptor blockade or aldosterone mineralocorticoid receptor blockade is an important treatment option in arterial hypertension and CHF.10 Our previous study has shown that increased renal ENaC subunits expression and activity may contribute to the renal Na\(^+\) and water retention observed in rats with CHF11. 

Recently, proteolytic cleavage of ENaC has been recognized as one of the important and major mechanisms related to ENaC activation12,13 and subsequent Na\(^+\) retention. These studies suggest that proteolytic activation of ENaC results from proteolytic release of inhibitory tracts within the α- and γ- subunits. Among the several proteases in urine, previous studies indicate a critical role for cleavage of ENaC subunits by prostasin, plasmin, and furin for the activation of ENaC.14,15 Prostasin and plasmin are extracellular proteases, whereas furin is intracellular protease. Furin is a member of a family of proprotein convertases that reside primarily in the trans-Golgi network. Prostasin was identified initially as a secreted prostate gland product with trypsin-like activity, although it was eventually realized that it was the shed version of the glycoprophatidylinositol-anchored protease. Plasmin is not present in the nephron lumen under normal conditions. Recent studies suggest that in the setting of glomerular diseases associated with podocyte injury, plasminogen is filtered by the glomerulus and is converted to plasmin by urokinase that is present within the tubular lumen.16,17 Under such circumstances, protease inhibitors could represent potential anti-hypertensive agents with renoprotective effects. For example,
camostat mesilate, an orally active synthetic serine protease inhibitor, has been shown to reduce blood pressure and renal injury in salt-sensitive hypertension.18

The protease mechanism related to ANG II–aldosterone axis has also been shown to stimulate ENaC activity in the renal tubule.19,20 Thus, we hypothesized that tubular proteases contribute to the activating ENaC in rats with CHF. In the present study, we attempted to address the following questions: (1) are the levels of proteases increased in the urine of rats with CHF and patients with CHF? (2) do urinary proteases increase ENaC activity of renal tubules and Na+ retention in rats with CHF? (3) is there increased podocyte injury which would result in increased leakage of proteases into renal tubular fluid in rats with CHF?

Methods

Study Approval
All the procedures on animals in this study were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. The experiments were conducted according to the American Physiological Society Guiding Principles for Research Involving Animals and Human Beings and the National Institutes of Health guide for the care and use of laboratory animals.

The human study was approved by the local institutional review committee (Ethik-Kommission) of the Martin-Luther University Halle-Wittenberg (Study number 2011–64). All subjects gave informed consent before study participation.

Statistics
Data were subjected to a 2-way ANOVA followed by a Multiple Range (for multiple comparisons) or Student Newman–Keuls test. *P<0.05 vs respective sham-operated rats. Detailed description of procedures is available in Methods in the online-only Data Supplement.

Results

Basal Hemodynamic Characteristics
The myocardial infarct model in the rat mimics the most common cause of CHF in humans and is used in numerous laboratories.11,21–24 Table 1 summarizes the salient characteristics of sham and CHF rats used in the present study. Heart weight, body weight, and wet lung weight were significantly higher in CHF rats compared with sham rats (P<0.05). The CHF group displayed an average myocardial infarct of >30% of the left ventricle. In contrast, sham rats had no observable damage to the myocardium. Left ventricular end-diastolic pressure was significantly elevated in CHF rats compared with sham rats (P<0.05). +dP/dt max was significantly decreased in CHF rats, indicating decreased contractility of the left ventricles. This contractile dysfunction was likely the cause for the increase in left ventricular end-diastolic pressure. −dP/dt max had a similar trend in rats with CHF. These data suggest that rats in the CHF group had decreased cardiac contractility and were experiencing diastolic dysfunction. Overall, these characteristics indicate that CHF rats were in heart failure and possibly retaining fluid.

Table 2 summarizes the renal and hemodynamic characteristics of patients with CHF and control subjects recruited in the present study. Patients with CHF (New York Heart Association III-IV) had significantly increased levels of plasma brain natriuretic peptide and creatinine compared with the control subjects without CHF. Further, left ventricular ejection fraction was significantly decreased (32±6% versus 68±1%, P<0.05) in the patients with CHF compared with the control subjects.

Urinary Serine Proteases Were Increased in CHF
We examined several urinary serine proteases in sham and CHF rats (Figure 1A). Four weeks after coronary ligation or sham surgery, 24 hours urine samples were collected from metabolic cages and processed for protease measurement by immunoblotting. We found that several urinary serine proteases were significantly increased in CHF compared with the sham rats (fold increases: furin 6.7, prostasin 23.6, plasminogen 2.06, and plasmin 3.57 versus sham, P<0.05). Urinary protease activity measured using zymogram also demonstrated increases in CHF rats (∼5-folds versus sham; Figure 1B). Similarly, we found that these 3 proteases in the urine were also increased in the patients with CHF (fold increases: furin 7.0, prostasin 7.7, plasminogen 5.4, and plasmin 3.9 versus control, P<0.05; Figure 2). This is the first evidence showing increased urinary serine proteases in the CHF condition to our knowledge.

Protease-Rich Urine Activated ENaC in Cultured Renal Cortical Collecting Duct M-1 Cells
Because we found significantly increased urinary proteases in rats with CHF, this study was designed to investigate the functional effects of protease-rich urine (from rats and patients with CHF) on ENaC activity in cultured renal CCD M-1 cells. M-1 mouse CCD cell line (purchased from ATCC) was established from normal renal tissue. These cells retain many characteristics of CCD cells, including the morphology and CCD antigens. Whole-cell patch clamp was conducted on these M-1 cells to record Na+ currents before and after exposure to the urine (collected from sham and CHF rat, or control and CHF patient). The results show that protease-rich urine, from rats or patients with CHF, increases the Na+ inward current in M-1 cells compared with the sham rats or control patients urine exposure, respectively (Figure 3A and 3C). The stimulatory effect of urine on the Na+ inward current was reduced by ENaC inhibitor amiloride and pretreatment with protease inhibitor aprotinin in the urine (Figure 3A and 3B). On the contrary, the urine collected from sham rats or control patients

Table 1. Basal Hemodynamic Characteristics of Sham-Operated and Heart Failure Rats

<table>
<thead>
<tr>
<th>Measures</th>
<th>Sham (n=12)</th>
<th>CHF (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>395±21</td>
<td>444±22*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.2±0.2</td>
<td>2.1±0.4*</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>1.7±0.2</td>
<td>2.3±0.1*</td>
</tr>
<tr>
<td>Infarct size (% of epicardial LV)</td>
<td>0</td>
<td>37±6*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>1±1</td>
<td>24±6*</td>
</tr>
<tr>
<td>+dP/dt max, mm Hg/s</td>
<td>6945±299</td>
<td>5428±154*</td>
</tr>
<tr>
<td>−dP/dt max, mm Hg/s</td>
<td>−5365±320</td>
<td>−3765±293*</td>
</tr>
</tbody>
</table>

CHF indicates chronic heart failure; LV, left ventricle; and LVEDP, left ventricular end diastolic pressure.

*P<0.05 vs respective sham-operated rats.
did not increase the ENaC activity in M-1 cells. Addition of aprotinin to the urine collected from sham rats did not affect the inward Na⁺ current. This study supports the notion that proteases in the renal tubule have a potentially important role in activating ENaC of renal tubular cells in rats with CHF.

### Diuretic and Natriuretic Responses to ENaC Inhibitor

After 2 weeks of protease inhibitor, aprotinin treatment, there were no significant differences in kidney weights between the groups of rats with or without aprotinin (Table 3). The basal urine flow before benzamil injection was not significantly different between the 2 sham groups. The basal Na⁺ excretion before benzamil injection was significantly increased in CHF group with aprotinin treatment.

Benzamil injection produced diuresis and natriuresis in both groups of rats (Figure 4). Both diuresis and the natriuresis responses were significantly increased in CHF group compared with the corresponding sham rats after benzamil injection (P<0.05), consistent with our previous observations.11 Aprotinin significantly reduced the diuretic and natriuretic responses to benzamil in CHF (Figure 4A and 4B). Aprotinin did not significantly change the diuretic and natriuretic responses to benzamil in the sham group. This study provides additional evidence to support the idea that proteases in the renal tubule have an important role to activate ENaC. Further, protease inhibitors may reduce Na⁺ retention in CHF by attenuation of ENaC activity in the kidney.

In the patch clamp study, protease-rich urine from rats with CHF increased the Na⁺ inward current in M-1 cells compared with the sham urine exposure as shown above. However, the stimulatory effect of urine on the Na⁺ inward current was reduced by the urine from CHF rats with aprotinin treatment (Figure 4C), suggesting that long-term protease treatment is effective in preventing the enhanced activation of ENaC observed in CHF.

#### Podocyte Integrity; Desmin and Podocin Expression in Rats With CHF

Podocyte lesions were found in the kidneys of rats with CHF. The number of podocytes and foot process width were detected by transmission electron microscopy (n=3; Figure 5A). Generally, there was a fairly large amount of damage in the kidneys of rats with CHF compared with sham rats. In particular, podocyte foot processes lost their normal shape and displayed numerous areas of effacement. The glomerular basement membrane also displayed abnormal thickness in rats with CHF. Further, we found that desmin immunosignal, a marker for podocyte injury,25 was enhanced in podocytes of CHF rats demonstrated by immunostaining (n = 6; Figure 5B). Finally, both desmin and podocin protein expressions (another podocyte damage marker) were significantly increased in the cortex of rats with CHF (P<0.05; Figure 5C and 5D).

## Discussion

The present study shows that several urinary serine proteases are significantly increased in patients, as well as in rats, with CHF. This protease-rich urine (from rats with CHF versus Sham-operated rats) significantly increased Na⁺ inward current in M-1 cells. Conversely, treatment with a protease inhibitor for 2 weeks significantly abrogated the enhanced diuretic and natriuretic responses to ENaC inhibitor, benzamil, in rats.
with CHF. At this phase of the disease, we observed that there was dramatic damage to the podocytes in the kidneys from rats with CHF. Taken together, these data suggest a robust increase in ENaC activation because of tubular proteases, possibly owing to podocyte damage, which leads to the enhanced Na+ reabsorption in CHF.

Na+ and fluid retention are commonly seen in patients with CHF. Recently, the role of proteases in activating ENaC in rats with CHF. Taken together, these data suggest a robust increase in ENaC activation because of tubular proteases, possibly owing to podocyte damage, which leads to the enhanced Na+ reabsorption in CHF.

Figure 2. Protein expression of urinary proteases (furin, prostasin, plasminogen, and plasmin) in the control and chronic heart failure (CHF) patients measured by immunoblotting. *P<0.05 different from respective control group.

Our previous work has shown increased ENaC abundance and enhanced ENaC functional activity in rats with CHF. This may also contribute to the Na+ retention observed in CHF. Interestingly, as mentioned above, our data show that urinary serine proteases (furin, prostasin, plasminogen, and plasmin) are all dramatically increased in CHF: Aprotinin, a broad-spectrum serine protease inhibitor, has displayed inhibitory effects on Na+ channel activity in the kidney. Based on these studies, we examined the contribution of proteases causing increased ENaC activity and Na+ retention in rats with CHF. In vitro studies demonstrated that addition of aprotinin to the urine of rats with CHF was able to abrogate the enhanced activation of ENaC, suggesting that excess proteases in the urine of CHF rats were responsible for the enhanced functionality of the ENaC in the CHF condition. Further, our results showed that in rats with CHF, treatment with aprotinin to block endogenous proteases for 2 weeks abrogated the enhanced ENaC activity responses to benzamil (increase urine volume and Na+ excretion). These results demonstrate that there is an enhanced functional activation of ENaC, which is dependent on tubular proteases in rats with CHF.

Aprotinin is an important member of a family of related protease inhibitors and has many clinically beneficial activities. In the clinic, aprotinin has been widely used as pre- and intraoperative agents with antifibrinolytic activity. It also has complex interactions with other drug therapies, including ANG-converting enzyme inhibitors. It may also affect hemodynamics in this regard. Our in vivo and in vitro studies provide evidence showing the direct effect of protease on the ENaC activity and Na+ dysregulation in CHF rats. A potential concern for this protocol is the efficacy of the protease inhibitors on renal ENaC activity. As a potential protease inhibitor, aprotinin has been shown to prolong the attenuation of ENaC function in the lung, airway, and kidney. Aprotinin has higher potency than other protease inhibitors, such as soybean trypsin inhibitors, to attenuate ENaC function and Na+ retention.

Furin is an intracellular convertase-type protease that resides primarily in the trans-Golgi network. Urinary furin is more likely from epithelial cells of the kidney rather than from circulation or glomerular podocyte leakage. The source of urinary furin may be different from extracellular proteases plasmin and prostasin. Aprotinin can block the activity of proteases, such as prostasin and plasmin; however, it has been reported that aprotinin at a dose of 0.1 mg/mL does not block furin. Administration of the synthetic serine protease inhibitor camostat mesilate inhibits the protease activity of serine proteases, such as prostasin and plasmin, but not furin. This protease inhibitor may inhibit the second cleavage of \( \gamma \)-ENaC subunits. It retains many antigenic and differentiated transport properties of the CCD. It has been used extensively to study amiloride-sensitive Na+ transport through ENaC. In our patch clamp study, the amiloride-sensitive, protease-activated inward Na+ current identified here in M-1 cells is consistent with ENaC as the target but not definitive. The contribution from other amiloride-sensitive Na+ conducting channels, such as ENaC-like channels and a nonselective cation channels in M-1 cells, cannot be conclusively excluded by these particular experiments.

Our previous work has shown increased ENaC subunit abundance and enhanced ENaC functional activity in rats with CHF. This may also contribute to the Na+ retention observed in CHF. Interestingly, as mentioned above, our data show that urinary serine proteases (furin, prostasin, plasminogen, and plasmin) are all dramatically increased in CHF: Aprotinin, a broad-spectrum serine protease inhibitor, has displayed inhibitory effects on Na+ channel activity in the kidney. Based on these studies, we examined the contribution of proteases causing increased ENaC activity and Na+ retention in rats with CHF. In vitro studies demonstrated that addition of aprotinin to the urine of rats with CHF was able to abrogate the enhanced activation of ENaC, suggesting that excess proteases in the urine of CHF rats were responsible for the enhanced functionality of the ENaC in the CHF condition. Further, our results showed that in rats with CHF, treatment with aprotinin to block endogenous proteases for 2 weeks abrogated the enhanced ENaC activity responses to benzamil (increase urine volume and Na+ excretion). These results demonstrate that there is an enhanced functional activation of ENaC, which is dependent on tubular proteases in rats with CHF.
and subsequently suppress ENaC activity on the plasma membrane.38

The sympathetic nervous system and the renin–ANG II–
aldosterone system have been suggested as possible cardio-
renal mediators.39 In CHF patients and rats with CHF, increased
sympathetic nerve activity has been well documented.40,41 The
plasma levels of norepinephrine, ANG II, and aldosterone are
all elevated in patients with CHF, as well in rats with CHF.42–44
ANG II and aldosterone are well recognized for their actions
on ENaC in increasing Na+ reabsorption.7–9 Interventional

Table 3. Basal Renal Characteristics of Sham-Operated and Heart Failure Rats

<table>
<thead>
<tr>
<th>Measures</th>
<th>Sham (n=5)</th>
<th>CHF (n=5)</th>
<th>Sham-Aprotinin (n=5)</th>
<th>CHF-Aprotinin (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney weight, g</td>
<td>1.3±0.2</td>
<td>1.9±0.2*</td>
<td>1.3±0.1</td>
<td>1.9±0.3*</td>
</tr>
<tr>
<td>Urine flow, μL/min/gkw</td>
<td>2.2±0.3</td>
<td>2.3±0.2</td>
<td>2.5±0.4</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>Na+ excretion, μEq/min/gkw</td>
<td>0.14±0.04</td>
<td>0.14±0.01</td>
<td>0.17±0.03</td>
<td>0.21±0.03†</td>
</tr>
</tbody>
</table>

CHF indicates chronic heart failure.
*P<0.05 vs respective sham-operated rats.
†P<0.05 vs respective group without aprotinin treatment.
studies in patients have shown that systemic ENaC inhibition by aldosterone mineralocorticoid receptor blocker may be an alternative to the treatment of hypertension and CHF.10 Beyond this well-known action, a recent study suggests an additional mechanism by which ANG II and aldosterone could stimulate Na+ transport through ENaC by increasing the expression of proteases in the kidney.45

Activated norepinephrine and ANG II–aldosterone systems are involved in several renal injury processes, including glomerular podocyte injury.10,46 Aldosterone is implicated in renal inflammatory and fibrotic processes, as well as in podocyte injury and mesangial cell proliferation.10 Activation of the sympathetic nervous system augments kidney renin–ANG system and oxidative stress. Intrarenal ANG II–induced increases in reactive oxygen species may contribute to the pathogenesis of glomerular podocyte injury and albuminuria.46 Severe proteinuria and renal injury are also found in patients with CHF.47,48 The defective glomerular filtration barrier allows the filtration of ENaC-activating proteases, such as plasmin converted from plasminogen into the tubular fluid. In our study, we found that podocyte foot processes lost their normal shape and displayed numerous areas of effacement in CHF rats. We also found that desmin and podocin protein expression, as markers for podocyte injury, were enhanced in the podocytes of kidneys from rats with CHF. Thus, we propose that in CHF, there is an increased damage to the podocytes that leads to leakage of proteases from the glomerulus into the tubular fluid which in turn causes an enhanced activation of ENaC, leading to enhanced Na⁺ retention (Figure 6).

Cardio-renal syndrome describes the reciprocally detrimental interaction between chronic cardiac and renal dysfunction. The syndrome is highly prevalent and carries a high risk of mortality. The main therapy of cardio-renal syndrome is loop diuretics and ANG-converting enzyme inhibition. One emergent feature of this study is that CHF presents itself as exhibiting enhanced levels of urinary proteases, and perhaps, they represent urinary biomarkers of renal injury in patients with CHF. These data lead us to speculate that urinary proteases may perhaps be used as a biomarker of cardio-renal disease. Further studies need to evaluate a therapeutic and preventive strategy to delay the onset and progression of CHF and cardio-renal syndrome by using protease inhibitor administration.

In conclusion, these findings demonstrate that increased proteases in the tubular fluid contribute to the enhanced ENaC activity and, thus, Na⁺ retention commonly seen in patients with CHF. The significance of these studies is to provide insight into the molecular and cellular mechanisms that may contribute to activation of ENaC and, consequently, lead to dysfunction in Na⁺ balance, commonly observed in CHF.
Perspectives

The significance of these studies is to provide insight into the molecular and cellular mechanisms that contribute to ENaC activation associated with dysfunction in sodium balance in CHF. Additionally, the present studies provide novel information on the beneficial effects of protease inhibition on renal sodium regulation in CHF, which may allow us to develop new treatments and management programs for the complications and consequences of CHF. This is the first study that determines the role of proteases in affecting the activity of ENaC.

Figure 5. A. Transmission electron microscopy showing glomerular podocyte in sham and chronic heart failure (CHF) rats. Magnification; 59K×. B. Immunostaining for desmin (a marker of podocyte damage, red). Scale bar: 100 μm. C, Desmin protein expression in the cortex of the kidneys from sham and CHF rats. D, Podocin protein expression in the cortex of the kidneys from sham and CHF rats. *P<0.05 vs sham.

Figure 6. Schematic of the excess protease activation of epithelial sodium channels (ENaC) in the kidney (red arrows indicate the changes in chronic heart failure [CHF]). ENaC activated by protease in the principle cell of renal tubule. ALDO indicates aldosterone; ANG, angiotensin; PN-1, protease nexin 1; and uPA, urokinase-type plasminogen activator.
of ENaC and its contribution to sodium balance dysfunction in CHF. These studies also examine the underlying possible mechanisms of enhanced protease activation of ENaC in CHF. The amalgamation of the observations in this fashion also represents a novel and an innovative approach to the study of renal handling of sodium and water in CHF.

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None.

**Disclosures**

**References**

What Is New?

- The present study shows that several urinary serine proteases are significantly increased in the renal tubular fluid during chronic heart failure. This protease-rich urine significantly increased the epithelial sodium channels–mediated Na\(^+\) inward current.
- Treatment with a protease inhibitor significantly abrogated the enhanced activation of epithelial sodium channels in rats with chronic heart failure.
- There was dramatic damage to the podocytes in the glomeruli from rats with chronic heart failure.

What Is Relevant?

- The clinical picture of advanced stages of chronic heart failure is often accompanied by the presence of edema and congestion, causing symptoms of dyspnea, fatigue, nausea, and discomfort. Avid sodium retention has been suggested to contribute further to aggravate the progression of the disease.
- The present studies provide significant new information and insight regarding the activation of epithelial sodium channels influencing sodium and water balance and the possibility of therapeutic intervention using protease inhibition on sodium retention which is endemic to chronic heart failure.

Summary

The data suggest a robust increase in epithelial sodium channel activation due in part to proteases that potentially leak into the tubular fluid because of glomerular damage, leading to the enhanced sodium retention in chronic heart failure.
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Urinary proteolytic activation of renal epithelial Na⁺ channel in chronic heart failure
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Methods

Study approval

All the procedures on animals in this study were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. The experiments were conducted according to the APS Guiding Principles for Research Involving Animals and Human Beings and the “NIH guide for the care and use of laboratory animals”.

The human study was approved by the local institutional review committee (“Ethik-Kommission”) of the Martin-Luther University Halle-Wittenberg (Study number 2011-64). All subjects gave informed consent prior to study participation.

Induction of heart failure

Male Sprague-Dawley rats weighing 200-220 g were obtained from SASCO Breeding Laboratories (Omaha, NE) and were randomly assigned to a sham-operated group and a CHF group. CHF was produced by coronary artery ligation, as previously described. Each rat was caged individually in an environment with ambient temperature maintained at 22°C and humidity at 30-40%. Laboratory chow and tap water were available ad libitum.

The degree of left ventricular dysfunction and heart failure were determined using both hemodynamic and anatomic criteria. Left ventricular end-diastolic pressure (LVEDP) was measured by using a Mikro-Tip catheter (Millar Instruments, Houston, TX) at the time of the terminal experiment. To measure infarct size, the heart was dissected and the atria and right ventricle were removed. A digital image of the left ventricle was captured using a digital camera. The percentage of infarct area to total left ventricle area was quantified using SigmaScan Pro (Aspire Software International, Ashburn, VA). Rats with both LVEDP > 15 mmHg and infarct size > 30% of total left ventricular wall were considered to be in CHF. Sham rats were treated the same as the CHF rats except their coronary arteries were not ligated.

24 hours urine collection and protein sample preparation

After 4 weeks of surgery or sham operation, rats were kept in metabolic cages, and 24 hrs urine samples were collected and concentrated by using Amicon Ultra-15 (Millipore, Billerica, MA). The total protein concentration was measured with a bicinchoninic acid assay kit (Pierce, Rockford, IL). Samples were adjusted to contain the same concentration of total protein, and then equal volumes of 2 X 4% Na+ dodecyl sulfate (SDS) sample buffer were added.

Chronic heart failure patients

Patients were hospitalized for dyspnea (NYHA III-IV, heart failure patients) or for uncontrolled arterial hypertension (control patients). Relevant comorbidities were excluded. After obtaining informed consent, both blood and urine samples were obtained. Blood samples were shipped at room temperature for immediate analysis (creatinine: Jaffé method, BNP: immunoassay provided by Beckmann-Coulter, Germany; glomerular filtration rate was calculated by MDRD equation in the hospital-attached laboratory facility). Urine samples were frozen at -20°C and shipped on dry ice for further experiments to University of Nebraska Medical Center, Omaha, NE.

Urinary proteases were studied in 6 patients (3 females and 3 males; 61 to 84 years of age) with CHF (NYHA III-IV) and 3 patients without CHF (1 female and 2 males; 61 to 77 years of age). 24 hrs urine samples from the patients were collected and processed as described above to measure proteases.
Urinary protease measured by immunoblotting and zymography

The concentrated urine samples from sham and CHF (n = 6~7/group) were used for immunoblotting analysis. The samples were mixed with an equal volume of 2 x 4% SDS sample buffer. The sample was then loaded onto the 7.5% SDS-PAGE gel for electrophoresis at 40 mA/each gel for 60 min. The fractionated proteins on the gel were electrophoretically transferred onto the polyvinylidene difluoride (PVDF) membrane at 300 mA for 90 min. The membrane was incubated with 5% milk-Tris-Buffered Saline and Tween (TBST) solution for 30 min at room temperature. The membrane was incubated with primary antibody (anti-rabbit furin, 1:2000, Santa Cruz Biotechnology, Santa Cruz, CA; anti-rabbit prostacin, 1:5000, Abnova, Walnut, CA; anti-rabbit plasmin, 1:1000, Molecular Innovations, Novi, MI) at 4°C overnight. After washing, the membrane was incubated with secondary antibody (goat anti-rabbit IgG, peroxidase conjugated, 1:5000, PIERCE, IL) for 40 min at room temperature. The signals were visualized using an enhanced chemiluminescence substrate (PIERCE) and detected by UVP digital image system (UVP LLC, Upland, CA).

Urinary protease activities were examined by zymography using Novex zymogram gels (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol.

The effects of protease-rich urine on ENaC activity in cultured renal cortical collecting duct cells

M-1 mouse CCD cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium: F12 supplemented with 5% fetal bovine serum at 37°C/5% CO2. Whole-cell patch-clamp was conducted on single M-1 cell after 24 hrs seeding the cells onto cover slips and 24 hrs serum free culture using Axopatch 200B patch-clamp amplifier (Molecular Devices LLC, Sunnyvale, CA). The holding potential was -40 mV, and current-voltage (I-V) relationship was performed using voltage ramp from -100 mV to -20 mV over 200 ms. Current traces were sampled at 10 kHz and filtered at 5 kHz. Na+ current was recorded before and after exposure to the urine collected and concentrated from sham and CHF rats with or without amiloride, sham and CHF rats with or without aprotinin (in vitro, 1 mg/ml), control and CHF patient, sham and CHF rats in vivo treated with or without aprotinin.

Renal function studies after protease inhibitor treatment

Four weeks after coronary ligation or sham surgery, rats were infused with the protease inhibitor, aprotinin (10000 KIU/kg/24hrs sc, Sigma, St. Louis, MO) via osmotic minipump (n = 5~7/group). After 14 days of treatment, we performed renal function studies in anesthetized rats. Aprotinin treatment did not significantly change the levels of proteases in the urines from both the sham and CHF groups (Supplement figure S1). On the day of the experiment, rats were anesthetized with inactin (100 mg/kg, i.p). Body temperature was maintained 36-38°C by a heated stage. After tracheal intubation, the animals were allowed to breathe independently. The left femoral artery was cannulated with PE-50 polyethylene tubing and connected to a pressure transducer for the continuous recording of arterial pressure. The left femoral vein was cannulated with PE-50 tubing for administration of supplemental anesthesia and drug.

The kidneys were exposed through a retroperitoneal flank incision. Subsequently, both ureters were cannulated with PE-10 tubing. Surgery was completed within 30 min, and an additional 30 min stabilization period was allowed before the start of the first urine collection. Urine was collected in preweighed tubes from both left and right kidney via ureteral catheters,
and urine volume was measured gravimetrically. Two urine collections (10 min each) were obtained before a bolus dose of ENaC inhibitor benzamil (0.7 mg/kg, iv, Sigma, MO) in 0.5 ml 0.9% saline. After injection of benzamil, urine was collected at 5, 10, 15, 20, 30 and 40 min. Na⁺ concentration (ion-selective electrode; Beckman ion analyzer, Brea, CA) of each of the urine samples was also analyzed.

Transmission electron microscopy

After fixation (5% glutaraldehyde) and dehydration, kidney samples were embedded in araldite. Thin sections (70-80 nm) were stained with 2% uranyl acetate and Reynolds lead citrate and examined with a Phillips 410LS TEM operated at 60 Kv. Glomeruli were photographed and printed at 59,000X magnification.

Immunohistochemistry and immunoblotting for desmin expression

For histological analyses, the rats were sacrificed; the complete kidneys were rapidly removed, and immediately immersed in 10% neutral buffered formalin and embedded in paraffin. Sections 5 μM thick were mounted on glass slide and used for immunohistochemical analysis. Briefly, sections were deparaffinized, rehydrated and boiled in 10 mM citrate buffer (pH 6.0) for 20 min for antigen retrieval. Sections were incubated with 10% goat serum to prevent nonspecific binding. The sections were then incubated with rabbit anti-desmin antibody (Santa Cruz Biotechnology) overnight at 4°C. After rinse, the sections were incubated with Alexa Fluor 596 Donkey anti-goat antibody (Molecular Probe, CA) for 2 hrs at room temperature. Sections were rinsed and then evaluated under fluorescence microscopy.

Kidney cortex lysates were prepared and processed to measure desmin and podocin protein levels in sham and CHF rats as described above.

Statistics

Data were subjected to a two-way ANOVA followed by a Multiple Range (for multiple comparisons) or Student-Newman Keuls test. P < 0.05 were considered to indicate statistical significance.

REFERENCES

**S1:** Protein expression of urinary proteases (furin, prostasin, plasminogen and plasmin) in sham, CHF rats with/without aprotinin treatment measured by immunoblotting. *P < 0.05* different from respective sham group. A: aprotinin.