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New role for plasmin in sodium homeostasis

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Abstract

Purpose of review—Hypertension and edema are clinical manifestations of the extracellular volume expansion generated by abnormal renal sodium handling. Perturbations in epithelial sodium channel (ENaC) activity disrupt volume homeostasis. ENaC activity can be enhanced by proteases that cleave its long extracellular domains. Recent evidence suggests that this mechanism may be involved in individuals with volume overload and proteinuria.

Recent findings—Several observations indicate a link between proteinuria and hypertension, with proteinuria preceding and predicting the onset of incident hypertension in some individuals. Recently, enhanced cleavage of ENaC's extracellular loops was identified in kidney tissue of proteinuric mice. Plasmin, a serine protease known for its role in fibrinolysis, has been implicated as an activator of ENaC in proteinuric states as (i) nephrotic urine activates ENaC expressed in a mouse collecting duct cell line, (ii) aprotinin-affinity precipitation of nephrotic urine abolishes its ability to activate ENaC, (iii) plasmin is a major component within aprotinin-affinity purified nephrotic urine and is absent in non-proteinuric urine, and (iv) plasmin activates ENaC by cleaving the extracellular loop of its γ subunit.

Summary—Enhancement of ENaC activity by proteases represents a likely mechanism for extracellular volume overload relevant to some individuals with proteinuria. Proteases not normally found in the urine can enter the urinary space across damaged glomeruli and activate ENaC. Further understanding of this mechanism may guide targeted therapeutics in individuals with proteinuria, edema, and hypertension.

Keywords

epithelial sodium channel; proteinuria; plasmin; plasminogen; kidney tubules

Introduction

Expansion of extracellular volume develops in proteinuric diseases, such as the nephrotic syndrome, and can manifest clinically as edema and hypertension. In the aldosterone-sensitive distal nephron, the epithelial sodium channel (ENaC) provides fine control of salt and fluid reabsorption. Derangements in ENaC activity affect blood pressure and extracellular volume (1-4). It is now clear that extracellular proteases enhance ENaC activity (5), a mechanism that is likely relevant to proteinuric states where proteases not normally found in urine can enter

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the urinary space across damaged glomeruli. In this review, we examine how the protease plasmin contributes to volume expansion in proteinuric states through aberrant proteolytic activation of ENaC.

Proteinuria and volume expansion

Proteinuria appears to be more than just a marker of target end organ damage. Proteinuria is generally considered abnormal if urinary losses are greater than 150-200 mg of protein per day (6). Albuminuria, a surrogate marker for proteinuria, is considered abnormal in quantities >30mg per day (7). Proteinuria not only reflects glomerular damage, but also functions as a risk factor for cardiovascular disease, stroke, and end stage renal disease (8-10). Proteinuria has been associated with extracellular volume expansion and high blood pressure. Proteinuria correlates with elevation in blood pressure in multiple disparate populations such as male US army veterans with chronic kidney disease and pre-hypertensive men and women from Korea (11,12). The relationship between proteinuria and blood pressure is complicated as hypertension can cause renal damage resulting in worsened proteinuria, and the development of essential hypertension does not require pre-existing proteinuria (13-15). However, multiple recent studies have examined the role of proteinuria as a risk factor for the development of elevated blood pressure.

A study involving normotensive adult men and women from Okinawa found the annual frequency for development of hypertension to be 2.4 fold higher if the patient had non-nephrotic proteinuria at baseline (16). Examination of nine potential biomarkers for hypertension risk in the normotensive, healthy male and female offspring of the Framingham Heart study participants found that urinary albumin-creatinine ratio, a marker of proteinuria, determined from a single void morning urine sample predicted the development of hypertension with an odds ratio of 1.21 (17). A type 1 diabetic population was also found to have increased risk of incident hypertension associated with albumin excretion rate (18). Another study found that higher levels of urinary albumin, despite being considered within the normal range, predicted incident hypertension in a population of healthy non-diabetic female nurses (7). Thus, proteinuria can predict the onset of incident hypertension in multiple different populations of normotensive individuals. How does proteinuria contribute to the pathogenesis of extracellular volume expansion and hypertension? Recent studies suggest that in some individuals with glomerular damage, proteases not normally found in urine enter the urinary space and aberrantly cleave ENaC (19-21). The proteolytic activation of ENaC would generate a primary defect in renal sodium handling, a mechanism that may be especially important in the development of the volume overload that accompanies the nephrotic syndrome.

Nephrotic syndrome is characterized by edema as well as hyperlipidemia, hypoalbuminemia, and massive proteinuria (22,23). Hypertension often accompanies this disorder. The pathogenesis of volume expansion and edema formation in the setting of massive proteinuria remains undefined, but one leading theory often described as the “overfill hypothesis” suggests that volume overload is generated by a primary defect in renal sodium handling (3,24). Evidence that supports this theory includes (i) the lack of plasma volume depletion in many nephrotic patients, (ii) suppression of components of the renin-angiotensin-aldosterone system during times of avid sodium retention in many nephrotic individuals, and (iii) the observed sodium retention only on the affected side of a unilateral nephrotic rat model (25-27). Enhanced ENaC activity by extracellular proteases may be a key event that creates the primary defect in sodium handling. However, not all patients with nephrotic syndrome fit this “overfill hypothesis” model. Up to 33% of nephrotic patients exhibit reduced plasma volume (28). Also, serum catecholamines, renin, and arginine vasopressin are increased in some nephrotic individuals (28). Enhanced ENaC activity by extracellular proteases may be one mechanism among many that generates extracellular volume overload. The ability to identify individuals

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with proteinuria, volume expansion, and enhanced ENaC activation would provide a population that might respond to targeted therapy with ENaC-specific inhibitors, like amiloride.

The kidney plays an important role in the generation of extracellular volume expansion and hypertension as sodium balance has a profound impact on extracellular fluid volume and blood pressure. Several hypertensive disease states involving ENaC implicate this channel as a vital part of renal sodium handling. Liddle's syndrome, characterized by hypertension, hypokalemia, and metabolic alkalosis, involves a mutation in ENaC that prevents retrieval of the channel from the apical membrane (29-32). In hyperaldosteronism, overexpression of the volume regulatory hormone aldosterone leads to increased ENaC activity in principal cells and subsequently hypertension (33-35). ENaC activity can also be enhanced by extracellular proteases that cleave its large extracellular loops (5). Aberrant proteolytic activation of ENaC is a likely mechanism responsible for the volume expansion seen in some proteinuric disease states.

Proteolytic activation of ENaC

ENaC is composed of three structurally related subunits (α, β, and γ) that contain intracellular N- and C- termini, two transmembrane spanning domains, and a large extracellular loop (36, 37). Enhanced ENaC activation by proteases was first observed by Chraibi et al. when they applied the serine proteases trypsin and chymotrypsin to solutions bathing cells expressing ENaC (38). Vuagniaux et al. subsequently identified a family of serine proteases that can stimulate ENaC currents when co-expressed in *Xenopus* oocytes, and designated them channel activating proteases (CAPs) (39). More recently, several proteases, including furin, prostasin (CAP1), TMPRSS4 (CAP2), plasmin, neutrophil elastase, pancreatic elastase, and kallikrein, have been implicated in cleavage and activation of ENaC (20,39-45).

Proteolytic activation of ENaC involves double cleavage events in the long extracellular loops of the channel's α and γ subunits to liberate inhibitory domains (Figure 1). Furin, a proprotein convertase residing primarily in the *trans*-Golgi network, cleaves the channel during channel maturation in the biosynthetic pathway (51). Furin cleaves the α subunit twice flanking a 26 residue inhibitory region (44,52). Electrophysiologic experiments using the *Xenopus* oocyte expression system confirmed this phenomenon. Channels with α subunits that contained mutated furin-consensus cleavage sites were not cleaved by furin and had significantly decreased activity compared to wild type channels (44,52). Furthermore, channels with α subunits containing furin-consensus cleavage site mutations and a simultaneous deletion of the intervening 26 amino acid tract were not cleaved, but exhibited activity similar to processed/cleaved wild type channels (52). A synthetic peptide corresponding to the 26-mer inhibitory domain of α, when applied externally to wild type channels in mouse cortical collecting ducts and human airway epithelial cells, inhibited these channels (52).

The processing of the γ subunit involves two important differences compared to α processing: (i) the γ subunit is only cleaved once by furin and thus requires a second protease to cleave and release an inhibitory peptide and (ii) cleavage of the γ subunit is dominant over α cleavage in activating the channels such that channels that have only γ doubly cleaved are nearly fully active (51,54). Prostasin, designated by Vuagniaux et al. as CAP1, a GPI anchored serine protease found in renal epithelia, can provide the second cleavage event distal to the furin site in the γ subunit (39,50). Wild type channels co-expressed with prostasin in *Xenopus* oocytes were fully activated. Prostasin was found to cleave at a site 43 residues distal to the furin site in γ, and mutation of this site prevented channels from being cleaved and activated by prostasin (50). A synthetic peptide corresponding to the 43-mer inhibitory domain, when applied externally to wild type channels in mouse cortical collecting ducts and human airway epithelial

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cells, inhibits these channels with an IC50 of 2-3 μM (50). Channels that possess a mutation at the γ furin consensus cleavage site and deletion of the intervening amino acid tract including the prostasin-dependent cleavage site, were not cleaved, but were fully active (50). When these mutant γ subunits were expressed along with the mutant α subunits that cannot be cleaved by furin, they were still near fully active showing the dominance of γ processing (54). Other proteases besides prostasin that have been shown to cleave the γ subunit near the prostasin-dependent cleavage site, liberate the intervening inhibitory domain, and activate the channel, include pancreatic elastase. neutrophil elastase and plasmin (20,40,41,43).

Proteolytic processing of ENaC enhances activity by increasing the channel's open probability (Po) (Figure 1) (46,49,50). ENaCs expressed in *Xenopus* oocytes undergo furin cleavage of the α subunit, liberating the α inhibitory domain, but retain their γ inhibitory domain and have a Po of ~0.3-0.4 (although ENaC Po is known to be highly variable) (50,55). Near silent channels have a Po of less than 0.1, similar to ENaCs, expressed in oocytes, that contain furin-dependent cleavage site mutations in the α subunit (i.e., these channels retain their inhibitory domains) (47,49). Application of extracellular trypsin has been shown to enhance the Po of near silent channels expressed in fibroblasts to about 0.6-0.7 (46). Co-expression of prostasin and ENaC in *Xenopus* oocytes results in channels with a Po of 0.8-0.9 (50). ENaCs that have the γ inhibitory region deleted and furin processed α subunits have a Po >0.9 (50).

Not all ENaCs undergo furin-dependent maturation. There exists a pool of ENaC that reaches the cell surface that has escaped proteolytic processing (48). This pool of channels may also be the target of extracellular proteases in disease states. Single channel recordings of near silent channels exposed to trypsin showed a significant increase in activity and Po demonstrating that near silent channels can be activated by extracellular proteases (46,53). This trypsin-dependent activation was not observed when α and β subunits were expressed without γ, whereas trypsin-dependent activation was observed in αγ channels, suggesting that the γ subunit plays a key role in channel activation by extracellular proteases (53). ENaCs in the aldosterone-sensitive distal nephron that have not been fully processed may be substrates for plasmin in proteinuric states.

Plasmin, proteinuria and processing of ENaC

In proteinuric states, proteases not normally found in the urinary space cross the glomerular barrier and have the potential to cleave ENaC (Figure 2) (61). Recently, enhanced cleavage of the γ subunit of ENaC was demonstrated by Kastner et al. in kidney tissue of proteinuric mice (injected with an anti-glomerular basement membrane (GBM) antibody) (19). Kidney homogenates blotted with antibody prepared against the C-terminus of the γ subunit showed an approximate two-fold increase in a 70kDa fragment consistent with cleavage near the known prostasin-dependent cleavage site (i.e., a cleavage event that liberates the γ inhibitory domain) (19). The enzyme responsible for ENaC activation is plasmin, a serine protease known for its role in fibrinolysis, derived from processing of its inactive precursor plasminogen by the activating enzymes tissue-type (tpa) and urokinase-type plasminogen activator (urokinase). Plasminogen is not normally found in the urine of humans that are nonproteinuric, but has been shown to be present under nephrotic conditions (56,57). Cells lining the proximal and distal nephron release urokinase to convert plasminogen, which has crossed damaged glomeruli, to plasmin in the urinary space (58-60). Furthermore, immunohistochemical staining for urokinase in human and rat nephrectomy specimens demonstrated the presence of urokinase on the apical surface of cortical collecting ducts (21).

Plasmin is identifiable in the urine of animals and humans with proteinuria. We examined urine from male obese ZSF-1 rats (diabetic and hypertensive) and their lean littermates for the presence of plasminogen and plasmin (20). Obese ZSF-1 rats were proteinuric compared to

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lean littermate controls and Western blotting with an anti-plasminogen antibody revealed plasminogen and plasmin to be readily detectable in the urine of obese ZSF1 rats and largely absent in urine from lean littermate controls (20). Svenningsen et al. found that urine from nephrotic humans and rats can activate ENaC expressed in mouse collecting duct cells, an observation that was abolished by aprotinin, a serine protease inhibitor, or heat inactivation of the urine (21). Control urine had no effect. Aprotinin-affinity precipitation of the nephrotic urine abolished the ability of the urine to activate ENaC despite not changing its total protein content, but the precipitate retained the ability to activate ENaC in collecting duct cells (21). By subjecting the precipitate to several purification steps followed by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (21), plasmin was identified as the channel activating protease. Western blotting of urine from nephrotic and control humans with an antibody against plasminogen revealed plasminogen and plasmin in only the nephrotic urine and not in controls (21).

Plasmin has the ability to activate ENaC by cleaving its γ subunit (20,21). We showed that plasmin (at 10μM) enhanced ENaC currents ~2 fold when applied externally to *Xenopus laevis* oocytes expressing wild type ENaC after four minutes of exposure to the enzyme (20). Plasmin exhibited a time dependent increase in whole cell amiloride-sensitive currents, similar to the time course for activation of ENaC by extracellular trypsin, and reached a maximal effect by 10 minutes (20,38,62). Western blotting of surface channels expressed in oocytes exposed to 10 μM plasmin for four minutes, identified a new C-terminal 70kDa cleavage product from the γ subunit that was absent in controls not exposed to plasmin (20). This 70 kDa fragment is similar in length to the fragment generated from prostasin cleavage of ENaC as well as the fragment identified in blots of kidney lysates from proteinuric mice (exposed to anti-GBM antibody) (19,50). A similar cleavage product was also demonstrated in western blots, probed for the C-terminus of γ, of surface channels exposed to extracellular trypsin, chymotrypsin, or a combination of plasminogen and urokinase (21). Plasmin's ability to cleave and activate ENaC was abolished by a specific mutation of a residue within a predicted plasmin cleavage site that is near the prostasin-dependent cleavage site, suggesting the enzyme activates the channel by liberating the intervening inhibitory tract (similar to other enzymes like prostasin and elastase that activate the channel) (20). Plasmin can cleave and activate furin processed channels. However, the ability of plasmin to activate fully non-cleaved channels, by cleaving near the furin cleavage site in the γ subunit, was not examined. Svenningsen et al. introduced a hexahistadine tag between the furin and prostasin cleavage sites in γ. Mouse collecting duct cells that expressed this tagged ENaC in the presence of NTA-Atto550 fluorophore exhibited a fluorescent signal. The signal is abolished when the cells were preincubated with plasmin or urine from nephrotic patients suggesting that plasmin cleavage liberates the inhibitory tract (21). Presumably the mouse collecting duct cells produced channels that were not fully processed. Plasmin, therefore, not only activates ENaC by cleaving the γ subunit, but appears to be the predominant aprotinin-sensitive serine protease identified in nephrotic urine from rats and humans.

Conclusions

Activation of ENaC by the serine protease plasmin appears to play a role in the development of extracellular volume expansion seen in some proteinuric states. Enhanced proteolytic cleavage of ENaC has been identified in a proteinuric mouse model. Plasminogen can cross damaged glomeruli and is activated by urokinase in the urinary space as evidenced by the detection of plasminogen and plasmin in proteinuric urine of animals and humans and their absence in control urine. Plasmin possesses the ability to activate ENaC by cleaving its γ subunit and liberates the intervening inhibitory tract when channels have also been processed by furin. Further understanding of this mechanism may help elucidate the pathogenesis of

extracellular volume overload and guide therapeutics in individuals with proteinuria, edema, and hypertension.

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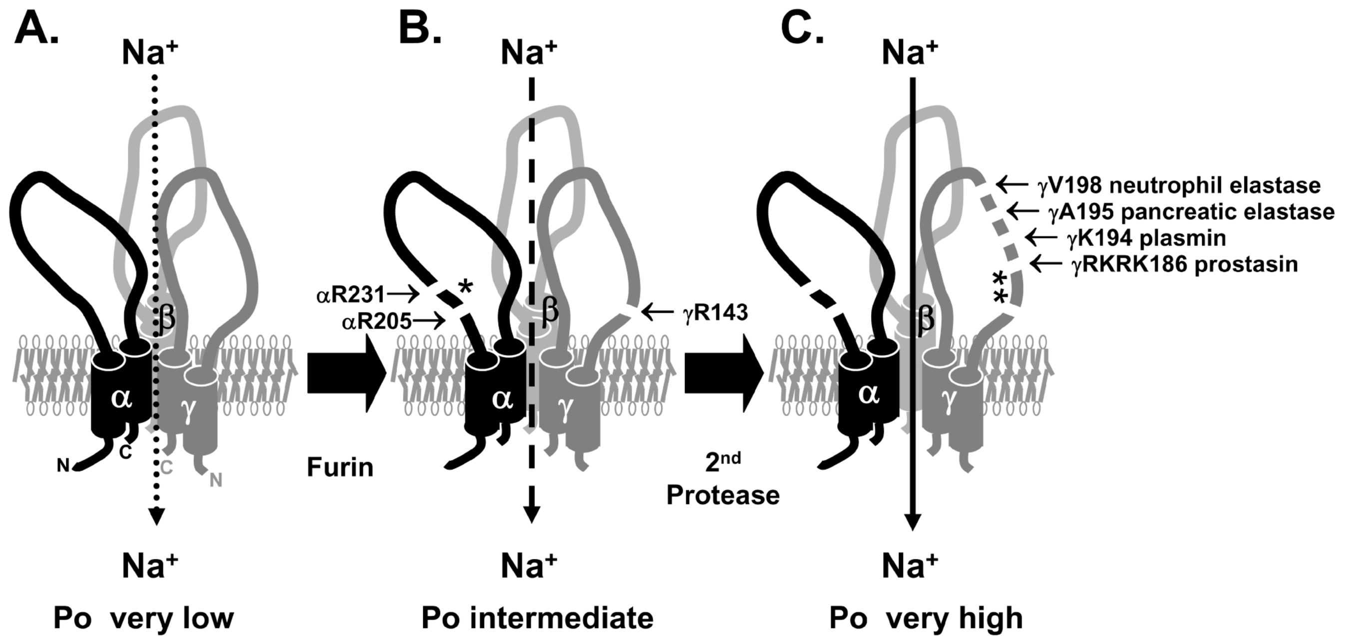

Figure 1. ENaC is activated by proteolytic cleavage and release of inhibitory peptides

(A) Non-cleaved trimers of αβγENaC likely represent near-silent channels found on the cell surface with Po<0.1 (46-49). (B) αβγENaC is normally cleaved by furin as it transits the biosynthetic pathway and exhibits an intermediate Po (50). Furin cleaves α twice to release a 26-mer inhibitory peptide (*) and γ is cleaved once (51,52). (C) Subsequent cleavage of the γ subunit at a site distal to the furin cleavage site, releases a second inhibitory peptide (**) and increases the Po to near 1 (46,50,53). Several proteases cleave γ at this distal site (20,40,50). For review, see (1,5).

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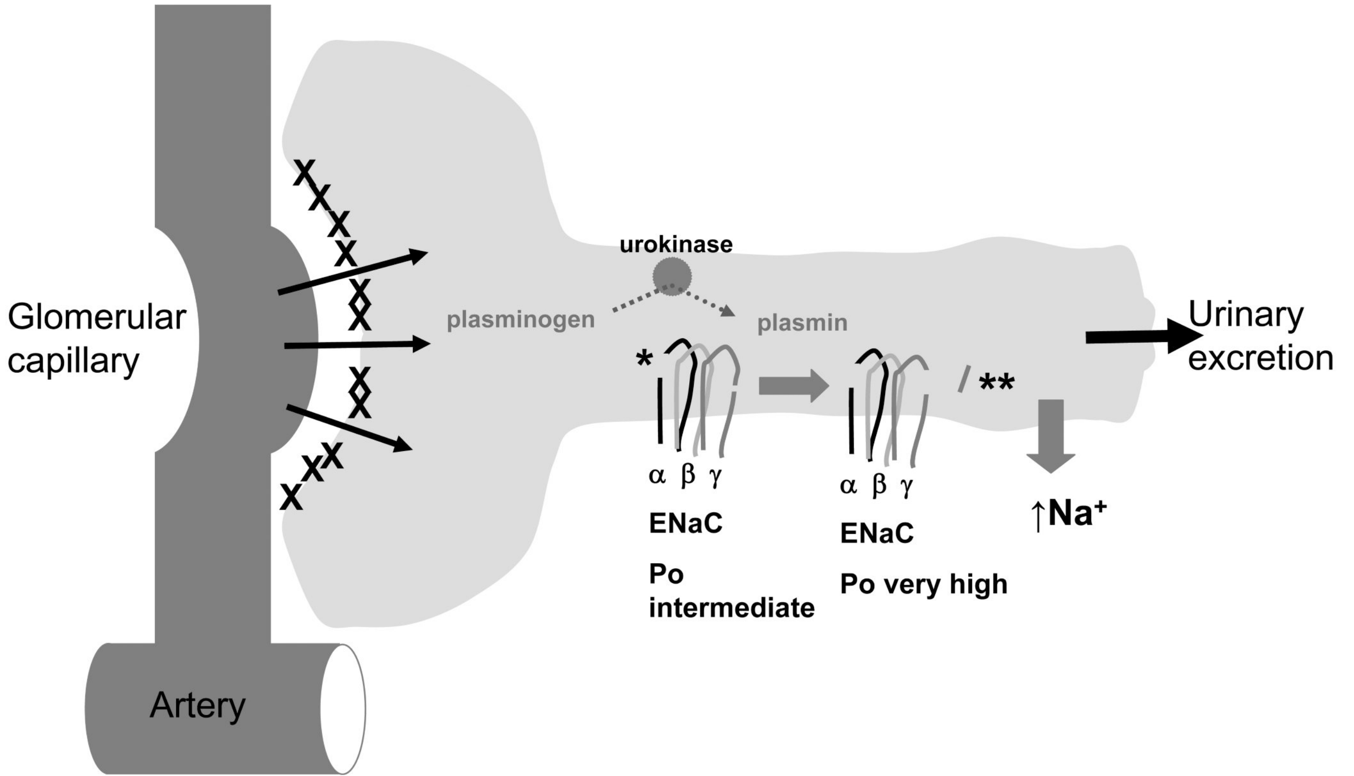

Figure 2. Plasmin in the renal tubule can aberrantly cleave and activate ENaC

Plasminogen is absent in the urine of normal rats or humans, but is present in the setting of nephrotic syndrome (20,21,56,57). It is likely that secreted tubular urokinase generates active plasmin from plasminogen, and that plasmin cleaves the γ subunit distal to the furin cleavage site to release the γ inhibitory peptide (**) (20,21,58-60). The α inhibitory peptide (*) is already released by furin cleavage at two sites (44,51). This figure is adapted from Eaton, DC and Pooler, JP (61).