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New insights regarding epithelial Na⁺ channel regulation and its role in the kidney, immune system and vasculature

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Abstract

Purpose of review: This review describes recent findings regarding the epithelial Na⁺ channel (ENaC) and its roles in physiologic and pathophysiologic states. We discuss new insights regarding ENaC's structure, its regulation by various factors, its potential role in hypertension and nephrotic syndrome, and its roles in the immune system and vasculature.

Recent findings: A recently resolved structure of ENaC provides clues regarding mechanisms of ENaC activation by proteases. The use of amiloride in nephrotic syndrome, and associated complications are discussed. ENaC is expressed in dendritic cells and contributes to immune system activation and increases in blood pressure in response to NaCl. ENaC is expressed in endothelial ENaC and has a role in regulating vascular tone.

Summary: New findings have emerged regarding ENaC and its role in the kidney, immune system and vasculature.

Keywords

ENaC; amiloride; hypertension; nephrotic syndrome; dendritic cells; endothelial cells

Introduction

The Na⁺ content of the extracellular fluids is a major determinant of the extracellular fluid volume (ECFV), intravascular volume and blood pressure (BP). Epithelial Na⁺ channels (ENaCs) are one of several key Na⁺ transporters in the aldosterone-sensitive distal nephron (ASDN) that have important roles in regulating the reabsorption of filtered Na⁺ and ECFV [1–3]. In addition to Na⁺ absorption, ENaCs have a key role in facilitating K⁺ secretion in the ASDN.

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Conflicts of interest

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ENaC/Degenerin family members have a trimeric architecture with large highly organized extracellular regions that sense extracellular factors that modulate channel activity [4–15]. For example, extracellular Na⁺ binds to ENaC at a defined site in the α subunit, driving allosteric changes that are transmitted to the channel gate, which reduces ENaC open probability [2,15]. This response, referred to as Na⁺ self-inhibition [2], provides a mechanism for cells in the distal nephron to rapidly tune the rate of Na⁺ influx according to fluctuations of urinary [Na⁺]. Numerous sites in the different domains of the extracellular regions of ENaC have been identified where amino acid substitutions alter channel activity, primarily by changing the Na⁺ self-inhibition response [9,12,13,16–21].

Do ENaC variants influence blood pressure?

ENaC gain-of-function mutations have been described in Liddle syndrome, an Mendelian disorder characterized by profound hypertension and hypokalemia [22–32]. These mutations generally result in a loss of a Pro-Tyr motif in the cytoplasmic C-terminus of the β or γ subunits of ENaC and an increase in channel surface expression. Is it possible that some individuals with Liddle syndrome have mutations in the extracellular regions of ENaC subunits that result in a gain-of-function due to a loss of Na⁺ self-inhibition? A recent report described siblings presenting with a Liddle syndrome phenotype and ENaC gain-of-function mutation in the extracellular domain of the α subunit (α C479R) [33**]. We previously found that an Ala substitution at this site increases channel activity due to a loss of Na⁺ self-inhibition [18], and it is likely that α C479R also exhibits a loss of Na⁺ self-inhibition. This report raises the possibility that humans with ENaC variants at other sites that reduce the Na⁺ self-inhibition response are at risk for hypertension. It also raises the question of whether humans with ENaC variants that enhance the Na⁺ self-inhibition response have a reduced risk of hypertension. We and other have identified a number of human ENaC nonsynonymous single nucleotide variants (nsSNVs) whose functional properties are altered when expressed in heterologous expression systems [34–42*]. However, the functional properties of most ENaC nsSNVs are unknown, as well as their effects on the risks of developing hypertension.

ENaC structure, biogenesis and regulation by proteases

Assembled $\alpha\beta\gamma$ ENaCs are processed in Golgi and post-Golgi compartments, where maturation of N-glycans and subunit cleavage by the protease furin occurs [43–46]. We recently reported that ENaCs expressed in *Xenopus* oocytes have reduced activity when a single subunit lacked N-glycans, with reductions in both whole cell and surface levels of cognate subunits [47*]. The most pronounced effect was seen when the β subunit was devoid of its N-linked glycans. A large number of studies have examined the role of proteases in cleaving and activating ENaC. Furin, a member of the proprotein convertase family of serine proteases that resides in the trans-Golgi network, has a key role in this process [2,43,46]. The α subunit is cleaved twice by furin, releasing a 26-residue imbedded inhibitory tract that transitions channels from a low open probability (or activity) state to a moderate open probability state [17,48]. Furin cleaves the γ subunit once [43]. Subsequent cleavage by a second protease releases another inhibitory tract of >40-residues, transitioning channels to a high open probability state [45]. A growing number of proteases have been

identified that can cleave the γ subunit at sites distal to its inhibitory tract, releasing the inhibitory tract. These proteases include prostatic, matriptase, cathepsin B, elastase, kallikrein, urokinase and plasmin [45,49–62].

Recent work has provided insights regarding how the imbedded inhibitory tracts reduce channel activity. The resolved structure of an acid sensing ion channel (ASIC1) provided important clues regarding the structure of the extracellular regions within ENaC subunits [4,63–66*]. The extracellular region of ASIC1 is a highly ordered structure that resembles an outstretched hand containing a ball, and has clearly defined subdomains termed finger, thumb, palm, knuckle and β -ball. The palm and β -ball are β strands and form a central core, whereas the peripheral finger, thumb and knuckle are α -helical structures. A structural model of the α subunit of ENaC, in conjunction with peptide-ENaC crosslinking studies, suggested that the α subunit inhibitory tract lies within the periphery of the subunit at an interface between the thumb domain and an α helix in the finger domain [13,67]. We suggested that the inhibitory tracts bind to and limits the relative mobility of the thumb and finger domains, favoring a low activity state [13,67,68]. Consistent with this hypothesis, chemically crosslinking the thumb and finger domains stabilized the channel in a low activity state [69]. Recent work suggests that the γ subunit inhibitory tract also lies within the periphery of the subunit at an interface between the thumb and finger domains [70*]. Furthermore, a recent study examining ASIC structures by cryo-electron microscopy suggested that movement of the thumb domain is associated with transitions between conducting and non-conducting states [66*]. A high resolution cryo-electron microscopy structure of $\alpha\beta\gamma$ ENaC was just published, confirming that ENaC is a trimer and that the organization of the extracellular region of the ENaC subunits is similar to that of ASIC1 [71**]. The authors resolved the structure of the region encompassing the inhibitory tracts, which is formed by antiparallel β -strands linked by a disulfide bond that places the protease cleavage sites in close proximity. The inhibitory tracts interface with the thumb and finger domains, in part, via aromatic amino acid residues.

While *in vitro* studies clearly show that proteases have a role in ENaC activation, there are few *in vivo* studies that directly support the *in vitro* observations. These *in vivo* studies have focused primarily on blocking expression of selected proteases, or administration of serine protease inhibitors. For example, a kallikrein knockout mouse exhibited enhanced Na^+ absorption in CCDs with no change in transepithelial voltage, suggesting activation of an electroneutral process [72]. Prostatic knockout mice have early mortality due to abnormal skin development [73]. A moderate effect on colonic potential difference (PD) was observed when prostatic was knocked out in the colon [74], and reduced fluid clearance was observed when prostatic was knocked out in alveolar epithelia [75]. To date, the phenotype of a kidney-specific prostatic knockout has not been described.

ENaC's role in nephrotic syndrome

In animal models of nephrotic syndrome, renal Na^+ retention may be due, in part, to filtered plasminogen that is converted to plasmin by urokinase within the tubular lumen, which cleaves and activates ENaC [60,61,76]. In support of this hypothesis, it was recently reported that mice with doxorubicin-induced nephrotic syndrome receiving a non-selective serine

protease inhibitor (aprotinin) exhibited a natriuresis [77**]. The question of whether renal Na^+ retention in the setting of nephrotic syndrome in humans is primarily due to ENaC activation is still unsettled. Numerous studies have shown that plasminogen and plasmin are readily detected in the urine in the setting of nephrotic syndrome, and its levels in urine correlate with levels of urinary albumin [78–80**,81*,82,83*]. In a long term observation study of type 1 diabetics, baseline urinary plasminogen/plasmin correlated with the incidence of hypertension, all-cause and cardiovascular mortality. However, these correlations were not independent of urinary albumin [83*].

The question of whether the ENaC inhibitor amiloride is efficacious in inducing natriuresis, reducing weight and/or blood pressure in humans with nephrotic syndrome is still unclear. Short term (2 days) administration of amiloride to diabetics with nephropathy and controls led to a natriuresis in both groups [84]. A recent study comparing the effects of hydrochlorothiazide and amiloride in 9 subjects with type 2 diabetes and proteinuria did not find differences between the two diuretics [80**]. The study was stopped after two individuals developed severe hyperkalemia and acute kidney injury while on amiloride. It was unclear whether the acute kidney injury reflected an exaggerated natriuretic response to amiloride. A recent case report described an individual with hyperkalemia and acute kidney injury while receiving amiloride, associated with significant weight loss [85**]. Another case report described a patient with nephrotic syndrome, hypertension, hypokalemia and edema who responded to triamterene (an ENaC inhibitor) with improvements in blood pressure, edema and serum $[\text{K}^+]$ [86*]. These studies raise the possibility that, in some instances, an ENaC inhibitor (amiloride or triamterene) may be efficacious in the management of edema and hypertension in the setting of nephrotic syndrome. While more work is needed, these studies also suggest that amiloride should be used with caution in the setting of nephrotic syndrome.

It has been assumed that ENaC subunit proteolysis in kidney correlates with channel activation *in vivo*. However, this has not been directly shown and recent work suggests that this is not necessarily the case. A clear discrepancy between ENaC subunit cleavage (early response) and increases in channel open probability (delayed response) in rat kidney in response to a low Na^+ diet has been observed [87**]. This is not surprising, as numerous factors influence channel open probability. For example, anionic phospholipids such as PIP_2 and PIP_3 enhance channel open probability, presumably by binding to cationic sequences within specific ENaC subunits [88–96*]. Cys-palmitoylation is a reversible attachment of palmitate to cytoplasmic Cys residues on proteins. We showed that post-translational modification of the β and γ subunits by cys-palmitoylation is another mechanism by which lipid molecules increase ENaC open probability [97,98]. Five of the 23 known palmitoyltransferases (referred to as DHHCs) activate ENaC when co-expressed in *Xenopus* oocytes [99*], suggesting that multiple DHHCs may have a role in regulating ENaC via palmitoylation.

ENaCs are expressed in dendritic cells and influence inflammation and blood pressure

ENaCs contribute to BP regulation through both renal and extrarenal mechanisms. In addition to the ASDN, ENaCs are expressed at numerous sites that influence blood pressure. ENaCs in lingual epithelium mediate salt taste and influence Na⁺ ingestion, while ENaCs in the distal colon serve as the final site for absorption of ingested Na⁺. Recent work suggests that ENaCs are expressed in antigen presenting dendritic cells, and may have a role in linking a high Na⁺ diet, inflammation and hypertension [100**]. Rodents fed a high Na⁺ diet accumulate Na⁺ in the interstitium, with the [Na⁺] in skin reaching 190 mM although plasma [Na⁺] is unchanged [101–103*]. A recent study reported that dendritic cells respond to increases in extracellular [Na⁺] in an ENaC dependent manner, resulting in Ca²⁺ influx and activation of a signaling pathway that includes protein kinase C and NADPH oxidase, superoxide production and formation of immunogenic isolevuglandin-protein adducts. In turn, this leads to T cell activation, release of inflammatory cytokines (IL-17 and INF- γ) and an angiotensin II-dependent increase in blood pressure [100**]. Dendritic cells express the α and γ subunits of ENaC, but not the β subunit [100**].

Endothelial ENaC

ENaCs are expressed in endothelial cells (EC), where they may have a role in the regulation of vascular reactivity [104–107]. In cultured ECs, ENaC reduces nitric oxide (NO) production via a mechanism that involves an increased density of the cortical actin cytoskeleton in association with cellular stiffening and decreased NO synthase activation by fluid shear stress [108–110]. Based on these observations, recent work has sought to elucidate the effects of endothelial ENaC on vascular parameters *in vivo*. The prediction is that ENaC activation will lead to reduced NO release and vasoconstriction, whereas ENaC inhibition should result in increased NO release and vasodilation. Not all studies have observed this paradigm.

Several studies have examined the responsiveness of arteries to the vasodilator acetylcholine when ENaC is either acutely inhibited by amiloride or the α subunit has been genetically deleted. Decreased ENaC expression and activity were observed in mesenteric arteries from Sprague-Dawley rats on a high salt diet. This decrease in expression was associated with increased acetylcholine responsiveness [111]. In contrast, Dahl salt-sensitive rats responded to high salt diet with an increase in mesenteric ENaC expression and activity, leading to a reduction in acetylcholine responsiveness that was restored with acute amiloride treatment [112*]. Taken together, these studies suggest that endothelial ENaC regulates acetylcholine responsiveness, and that its dysregulation may mediate vascular dysfunction seen in the Dahl salt-sensitive strain. These findings are consistent with observations in cultured ECs.

Other studies in murine vasculature found that acute treatment of mesenteric arteries with the ENaC inhibitor benzamil reduced dilation to acetylcholine [113*]. However, when the α subunit was genetically deleted from the endothelium, mesenteric arteries showed no difference in acetylcholine-mediated vasodilation, which may be due to enhanced eNOS expression and phosphorylation [113*]. Reduced acetylcholine response was still seen in

other vessels [114*]. These data suggest the role of endothelial ENaC in altering acetylcholine-induced dilation is more complex than previously predicted, with the response being dependent on method of ENaC inhibition and vascular bed. Furthermore, there may be other targets of amiloride in the vasculature [115*].

ENaC is a mechano-activated ion channel [2], and endothelial ENaC has been postulated to have a role in flow-mediated vascular signaling [116]. Blockade of ENaC in various vessels either by pharmacological inhibition [113*,117*,118] or genetic deletion [113*,114*] has been shown to alter the vascular response to fluid shear stress. While murine carotid arteries acutely treated with amiloride showed an increased vasodilatory response to flow, mesenteric arteries had an opposite response, constricting in response to flow [117*]. Benzamil did not invoke constriction of mesenteric arteries in response to flow; however, it significantly inhibited the flow-mediated dilatory response seen in control vessels [113*]. This response was mimicked by genetic deletion of the α subunit of ENaC in endothelium [113*,114*]. These differences in flow-mediated responses could be explained by the structural elements that ENaC regulates. In cell culture the deletion of ENaC prevented stiffening of the endothelial cells as assessed by atomic force microscopy (AFM) [119], and recent work has shown this is true *in vivo* as well. Deletion of the α subunit not only decreased the stiffness of individual aortic EC as measured by AFM in baseline knockout mice [113*], but decreased pulse wave velocity in aldosterone treated knockout females as compared to controls [120*]. More work is needed to understand if alteration of vascular stiffness extends beyond the large conductance arteries to the smaller resistance vessels. Taken together, these data suggest that endothelial ENaC alters vascular signaling, and its role is more complicated than cell culture data suggested.

Conclusions

In summary, a growing body of work continues to highlight ENaC and its roles in various organ systems. A case report describing a family with Liddle syndrome bearing an ENaC mutation in the extracellular region that likely leads to a loss in Na^+ self-inhibition raises the possibility that mutations at other sites that inhibit Na^+ self-inhibition may be associated with hypertension [33**]. The resolved structure of ENaC has provided a view of subunit finger domains and imbedded inhibitory tracts, and new insights regarding mechanisms by which proteases activate ENaC [71**]. In addition to the kidney, new work raises the possibility that ENaCs in dendritic cells may contribute to salt-sensitive hypertension [100**]. While ENaCs are expressed in vascular endothelium and contribute to vascular tone, the role ENaC in the vasculature is complex.

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Bullet points

- Numerous human ENaC nonsynonymous single nucleotide variants have been found that alter the functional channel properties, and a recent case report suggests that selected functional channel variants influence blood pressure in humans.
- A resolved structure of ENaC provides new insights regarding the mechanism of channel activation by proteases.
- While an increasing number of studies suggest that ENaC is activated in nephrotic syndrome, the efficacy of amiloride in this setting is still unclear and significant drug-related side effects have been observed.
- ENaC is expressed in dendritic cells, has a role in activation of the immune system, and may contribute to the increase in blood pressure in response to dietary salt.
- Endothelial ENaC affects vascular signaling. However, its role appears to differ among the various vascular beds that have been studied.