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Sodium potassium adenosine triphosphatase (Na/K-ATPase) as a therapeutic target for uremic cardiomyopathy

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Abstract

Introduction—Clinically, patients with significant reductions in renal function present with cardiovascular dysfunction typically termed, uremic cardiomyopathy. It is a progressive series of cardiac pathophysiological changes, including left ventricular diastolic dysfunction and hypertrophy (LVH) which sometimes progress to left ventricular dilation (LVD) and systolic dysfunction in the setting of chronic kidney disease (CKD). Uremic cardiomyopathy is almost ubiquitous in patients afflicted with end stage renal disease (ESRD).

Areas covered—This article reviews recent epidemiology, pathophysiology of uremic cardiomyopathy and provide a board overview of Na/K-ATPase research with detailed discussion on the mechanisms of Na/K-ATPase/Src/ROS amplification loop. We also present clinical and preclinical evidences as well as molecular mechanism of this amplification loop in the development of uremic cardiomyopathy. A potential therapeutic peptide that targets on this loop is discussed.

Expert opinion—Current clinical treatment for uremic cardiomyopathy remains disappointing. Targeting the ROS amplification loop mediated by the Na/K-ATPase signaling function may provide a novel therapeutic target for uremic cardiomyopathy and related diseases. Additional studies of Na/K-ATPase and other strategies that regulate this loop will lead to new therapeutics.

Keywords

Na/K-ATPase; ROS; cell signaling; uremic cardiomyopathy

1. Epidemiology of uremic cardiomyopathy

Based on data from the 2015 US Renal Data System (USRDS) [1], the prevalence of CKD in the general population was approximately 14%, with Stage 3 kidney disease showing the

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Declaration of interest

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largest growth over the past 20 years. This is not unexpected as older age, diabetes, hypertension, cardiovascular diseases and higher body mass index (BMI) are associated with the development of CKD, and the prevalence of these conditions are also on the rise. Although most cases of CKD do not progress to ESRD, the prevalence of ESRD has increased to approximately 2 cases per thousand people in the USA. Mortality rates are significantly increased in patients with CKD and ESRD and cardiovascular disease accounts for a substantial fraction of this increase. Despite trending decreases in mortality rates for dialysis and transplant patients from 1996 to 2013 which may be related to improvements in treatments, the adjusted mortality rate for ESRD patients are still several fold higher than in populations without CKD, and these mortality rate differences persist across the continuum of age [1].

Clear evidence supports the existence of a ‘cardio-renal’ axis in which injury or dysfunction of either the heart or the kidneys leads to pathological changes in both organ systems. For example, the presence of renal insufficiency significantly worsens outcomes in congestive heart failure (CHF) patients [2–5]. Mortality due to cardiovascular disease (CVD) is 10–30 times higher in dialysis patients than in the general population [6]. Among all major causes of death in patients with CKD or ESRD, CVD accounts for 69.6% in patients with CKD compared to 34.7% without. The 2-year survival rate of patients with both CKD and CHF is around 75%, compared with 90 and 82% for patients with CKD or CHF alone [1]. Cardiovascular comorbidities of CKD include coronary artery disease (CAD), CHF, valvular heart disease, stroke, peripheral artery disease and cardiac arrest and arrhythmias [7–10].

Although the relationship between CVD and CKD is still under investigation, some common risk factors including diabetes mellitus, hypertension, obesity, aging, metabolic syndrome and smoking, have been identified that correlate with the development of both CVD and renal disorders [9,11,12]. Moreover, CVDs and renal disorders have certain molecular changes in common, in particular, markers of inflammation and oxidant stress (e.g. elevated reactive oxygen species (ROS)) [9,11–14].

2. Pathophysiology of uremic cardiomyopathy

A number of factors are present in CKD and especially ESRD patients that can lead to cardiomyopathy (Figure 1). Hypertension is one of the main factors present which can lead to diastolic dysfunction and LVH [7,8]. Also, volume overload due to salt and water retention along with anemia can lead to LV dilation. Sustained increase in preload and afterload activates a series of intracellular signaling cascades that lead to maladaptive cardiac cell responses like apoptosis of myocytes, initiation of fibrosis via cardiac fibroblasts, and possible cardiac cell regeneration [15–19].

That said, it is very clear that there is something about the CKD milieu that facilitates the development of cardiomyopathy. In particular, neither intensive blood pressure control nor reduced preload through hemodialysis and aggressive ultra-filtration are able to ameliorate the pathological alterations found in patients who have developed uremic cardiomyopathy [13,20,21]. Over the past 20 years, other factors have been identified as important for the

development of LV hypertrophy, fibrosis, and apoptosis independent of pressure and volume overload.

Insulin resistance is known to be involved in the development of atherosclerosis, hypertension, dyslipidemia, and LV ventricle hypertrophy. Multiple laboratories have identified insulin resistance as an independent factor for the development of uremic cardiomyopathy as well as a strong predictor of cardiovascular death in chronic renal failure [22–25]. Specifically, in 227 nondiabetic renal dysfunction patients, insulin resistance, which was evaluated by the homeostasis model assessment method (HOMA-IR), appeared to be significantly elevated and had an elevated association with cardiovascular events than those observed in healthy subjects [26]. Although the exact mechanism of insulin-induced protein kinase B (AKT) signaling in the uremic cardiomyopathy is not fully understood, it is clear that insulin resistance during the CKD altered AKT signaling is involved in uremic cardiomyopathy.

Parathyroid hormone (PTH) has been known as an important indicator of cardiac dysfunction in uremia since early 1960s [27,28]. A study evaluating hemodialysis patients without hypertension showed that left ventricle hypertrophy and diastolic dysfunction were correlated with secondary hyper-parathyroidism [29]. Furthermore, administration of PTH causes more cardiac lesions in uremic rats, while parathyroidectomy could attenuate the development of cardiac lesions and fibrosis. Although uremia-related PTH increase plays an important role in the development of uremic cardiomyopathy, the mechanism is still not elucidated. It is possible that elevated PTH which promotes Ca^{2+} /PKC downstream signaling is involved in the development of left ventricle hypertrophy.

Additionally, the uremic state itself appears to result in the transactivation of downstream signaling events that induce cardiomyocyte enlargement and cardiac fibroblast proliferation which account for the process of cardiac [11,30,31].

Furthermore, increase in ROS stress has also been well documented as an important indicator for CKD in both patients and animal models. Specifically, Tepel et al., have shown that the spontaneous production of ROS was significantly increased in lymphocytes from patients with ESRD compared with healthy volunteers [32]. Another clinical study with 60 CKD patients finished by Oberg et al., also presents elevated oxidative stress biomarkers compared with healthy subjects [33]. Additionally, mitochondrial dysfunction and increased oxidative stress were reported in uremic cardiomyopathy murine model [34]. Therefore, the medical hypothesis of ROS generation/oxidative stress as a therapeutic target for CKD patients with uremic cardiomyopathy is one of our focuses in this review.

More importantly, a number of laboratories including our own have identified elevated concentrations of cardiotonic steroids (CTS) in patients with CKD and or CVDs (Table 1) [35–39]. We have demonstrated the involvement of these cardiac glycosides-specific receptor Na/K-ATPase and ROS amplification, in mediating the development of LV hypertrophy, cardiac cell apoptosis and fibrosis in the process of uremic cardiomyopathy [11,19,40,41]. For the remainder of this review, we will focus on the mechanisms by which this appears to occur.

3. Na/K-ATPase – an ion pump, a signaling receptor and a signaling scaffold

3.1. Na/K-ATPase as an ion pump

The Na/K-ATPase, first discovered by Skou [42], belongs to the family of P-type ATPases and transports Na⁺ and K⁺ across the plasma membrane in animals in an ATP-dependent process [43–46]. It consists of two noncovalently linked polypeptides, an α -subunit and β -subunit as well as other subunits which are inconsistently expressed in different tissues.

The α -subunit contains the binding sites for ATP, CTS, and other ligands, and plays the central role in the ion pumping function. The intracellular part of this transmembrane subunit is divided into three well-characterized regions according to their distinct functions (Figure 2): the actuator (A) domain is composed of the N-terminus and the second cytosolic domain (CD2) connecting the M2 and M3 transmembrane helices; the phosphorylation (P) domain and the nucleotide binding (N) domain are located in the loop between M4 and M5 transmembrane helices [47–49]. The β subunit is important for the assembly of the functional enzyme on the plasma membrane. Several α and β subunits have been identified and functionally characterized [43,44]. In addition, isoforms of α subunit are expressed in a tissue-specific manner [50]. The α 1 isoform is found in all kinds of cell types. The α 2 and α 3 isoforms are expressed in skeletal muscle, neuronal tissue, and cardiac myocytes. The α 4 isoform is expressed exclusively in spermatozooids and regulates sperm motility [51,52].

3.2. Receptor function of Na/K-ATPase

3.2.1. Endogenous CTS—CTS include plant-derived digitalis drugs such as digoxin and ouabain, and vertebrate-derived aglycones such as bufalin and MBG [53–55]. They are specific inhibitors of Na/K-ATPase with vast difference in affinity between them [56,57]. On the other hand, the affinity of Na/K-ATPase to CTS are highly dependent on the type of CTS, the type of the α subunit, the combination of α/β subunit, as well as the species studied. For instance, the IC₅₀ of the human α 1 is about 50 nM to ouabain, whereas the IC₅₀ of rat α 1 is 48,000 nM [58–61]. Depending on the methodology and experimental condition, the values of IC₅₀ may also be vastly varied. CTS have largely only been considered for their use as drugs since their discovery, recent studies have identified both ouabain and MBG as endogenous hormones whose production and secretion are regulated by multiple physiological stimuli including ACTH and angiotensin II [62,63]. Evidence for this assertion was generated by specific identification of subnanomolar concentrations of endogenous CTS in both human and experimental animals, and raised questions about their roles in human physiology as a pump inhibitor [55,58,59,64–66]. These CTS have mostly been measured using antibody based assays [67,68]. However, some studies have confirmed the presence of some CTS with mass spectroscopy and NMR and found differences in humans with diseases such as kidney failure and heart failure [68–71]. Although the exact pathophysiological role of increased endogenous CTS in these diseases continues to be debated [72–74], a study from Lingrel's laboratory using gene replacement technology demonstrated that endogenous CTS, do play a measurable role in the regulation of blood pressure, cardiac function and muscle physiology [75,76]. Under normal conditions, the rodent Na/K-ATPase α 1 isoform has a very low sensitivity to ouabain compared with human

$\alpha 1$, whereas the rodent $\alpha 2$ isoform is sensitive. By manipulations of the Na/K-ATPase genetic code, this group created mice with mutant Na/K-ATPase (ouabain-sensitive $\alpha 1$ isoform and ouabain-resistant $\alpha 2$ isoform). Using these genetically modified animals, they have shown that mutant Na/K-ATPase animal appeared to possess remarkably increased effects of low concentration of CTS (0.48 nmol/g body weight) resulting in threefold elevated cardiac contractility compared with wild-type mice [77]. Another group using these genetically modified animals has identified dramatically increased cardiac hypertrophy and fibrosis as well as cardiac dysfunction in terms of left ventricular dilatation and systolic dysfunction in $\alpha 1$ -sensitive mice than wild-type control mice after four-weeks of pressure overload induced by transverse aortic constriction (TAC) [78]. Taken together, these studies indicate that Na/K-ATPase plays a significant role in regulation of cardiac function and development of heart diseases. It is reasonable to propose the potential involvement of endogenous CTS and Na/K-ATPase signaling function (please see section 4.3.1. for further discussion). In addition, Aperia et al. and Lichtstein et al. have suggested a role for endogenous CTS in fetal growth in general and kidney development specifically [79,80]. Although we cannot ignore the effect of endogenous CTS on the inhibition of Na/K-ATPase and the subsequent elevation of intracellular Ca^{2+} levels, recent studies mentioned above have demonstrated that binding of CTS to the α -subunit of the Na/K-ATPase may also directly initiate a variety of intracellular signaling cascades, independent of changes in transport activity.

3.2.2. Na/K-ATPase as a signaling receptor—The identification of endogenous CTS along with the evidences that low concentrations of CTS, like ouabain or marinobufagenin (MBG), in concentrations which do not affect ion pumping function, can activate downstream signaling cascades through tyrosine phosphorylation, ultimately altering gene expression, suggest the possible receptor function of Na/K-ATPase rather than its canonic pumping function. Like other cytokine receptors and GPCRs, Na/K-ATPase does not have intrinsic activity. Therefore, it requires coupling with non-receptor kinase to fulfill the phosphorylation capability. Based on the previous studies from Xie's lab, Src kinase turns out to be one of the most proximal kinase associating with Na/K-ATPase. First, researchers from this lab and many other labs have reported the activation of Src by ouabain in different types of cells [81–83]. Follow-up studies suggest that the receptor function of Na/K-ATPase require Src-mediated trans-activation of EGF receptor, and subsequent recruitment and assembly of signaling proteins for the activation of protein/ lipid kinase cascades [84]. Second, using a combination of several different methods including coimmunoprecipitation, confocal imaging, FRET and GST pull-down assay [84], we have identified two potential binding sites between $\alpha 1$ Na/K-ATPase and Src. One is between A domain of Na/K-ATPase $\alpha 1$ subunit and Src homology2 (SH2) domain. The second site involved N domain of Na/K-ATPase $\alpha 1$ subunit and Src kinase domain. The latter interaction between Na/K-ATPase and Src keeps Src in an inactivate state by inhibiting Try 418 phosphorylation without affecting the phosphorylation of Tyr 529. Ouabain binding releases Src kinase domain from Na/K-ATPase/Src receptor complex and activates the Na/K-ATPase-associated Src [84]. The activation of Src results in transactivation of multiple downstream effectors, and regulates cellular activities such as clathrin-mediated endocytosis of $\alpha 1$ Na/K-ATPase [85]. Third, genetic modification of Na/K-ATPase expression in live cells show that knockdown of

endogenous Na/K-ATPase $\alpha 1$ subunit using siRNA significantly elevated Src activity as well as abolished ouabain-induced downstream signaling, whereas restoring Na/K-ATPase inhibited Src activity and receptor function of Na/K-ATPase. Although the involvement of Src and EGF receptor in the Na/K-ATPase $\alpha 1$ -mediated signal transduction and the interaction between Na/K-ATPase and Src have reaffirmed and accepted by many laboratories (Figure 2). There are some data that are opposite with our model. It has been shown that c-Src activation was not involved in ouabain induced Na/K-ATPase endocytosis in non-small cell lung cancer cells [86] and no stable interactions were detected between purified recombinant Na/K-ATPase and purified human Src kinase [87]. We would like to point out that these observations are different from what we have reported using purified pig kidney Na/K-ATPase, LLC-PK1 cell, human HK-2, human dermal fibroblasts, renal fibroblast cell line, and rat cardiac fibroblasts cell line [84,88,89]. Therefore, the model that Na/K-ATPase signal from interaction with Src is reliable and worthy to investigate. Taken together, these evidences indicate that besides its classic pumping function, Na/ K-ATPase is capable of associating with Src kinase to form a receptor complex for CTS to activate protein kinases in live cells.

3.3. Na/K-ATPase as signaling scaffold

Based on the characterization of the A and N domains of Na/K-ATPase $\alpha 1$ subunit, both are highly exposed and enable interacting with other intracellular proteins. Work in our and other labs have identified that the $\alpha 1$ subunit is capable of interacting with various proteins, which play important roles in protein trafficking and signal transduction processes. Depending of the binding site on the $\alpha 1$ subunit, they can be divided into two groups, The first group contains proteins bind to the A domain of $\alpha 1$ subunit including 14-3-3 [90], phosphatidylinositol-3-kinases (PI3 K) [91], inositol trisphosphate receptor (IP3R)[92], Src kinase[84] and caveolin-1 [93]. The second group includes those interact with N domain of $\alpha 1$ subunit such as arrestin, spinophilin, GPCR kinase, 14-3-3 epsilon [94], adapter protein-1/2 [95,96], ankyrin [97], and Src, On the other hand, depending on the function of Na/K-ATPase $\alpha 1$ -association, they can be separated into three groups. The first group contains those bind to Na/K-ATPase $\alpha 1$ subunit and modulate its trafficking. For instance, in response to dopamine-induced GPCR signals, several proteins were recruited to the $\alpha 1$ subunit including arrestin, spinophilin, 14-3-3 epsilon, and GPCR kinase, which mediated endocytosis of Na/K-ATPase [94]. Moreover, the interaction between N terminal Na/K-ATPase $\alpha 1$ subunit and caveolin-1 appears to be important for ouabain-induced endocytosis of Na/K-ATPase [85]. The second group includes those that dynamically interact with Na/K-ATPase $\alpha 1$ subunit, which affect Ca^{2+} signaling events or kinase activity. For example, binding of IP3R and phospholipase C- γ (PLC- γ) to $\alpha 1$ formed the signaling complex that regulated intracellular Ca^{2+} signal [92,98]. Additionally, as we mentioned above, interaction of $\alpha 1$ subunit with Src kinase inhibited its kinase activity. Binding of ouabain to the $\alpha 1$ subunit released Src and activated its kinase activity, which led to activation of mitogen-activated protein kinase (MAPK) pathway [84]. The third group consists of those directly enhance the Na/K-ATPase activity such as cofilin and polycystin-1 [99,100].

Further studies by Xie's laboratory have demonstrated that cells appear to contain two functionally separable pools of Na/ K-ATPase i.e. the so called ion pumping pool and the

non-pumping pool [101]. The non-pumping Na/K-ATPase apparently resides in caveolae and interacts directly with multiple proteins including Src, ion transporters, and caveolin to fulfill its signal transducer function [85,102,103]. Caveolae were first identified as flask-shaped vesicular invaginations of cellular plasma membranes enriched in cholesterol, glycosphingolipids and sphingomyelin [104,105]. Now, caveolae have been implicated in endocytosis, transcytosis, calcium signaling, and many other signaling processes [105,106]. Additionally, many signaling proteins have been discovered to be localized and concentrated within caveolae [93,107], which indicates that caveolae may serve as a micro domain for compartmentalization of signal transduction and facilitate the regulation of different signaling processes. Interestingly, previous work from our and Xie's lab have shown that Na/K-ATPase α 1 sub-unit interact with caveolin-1 and highly concentrated in caveolae [93]. The signaling Na/K-ATPase mainly resides in caveolae. Disruption of the caveolae structure by either removal of cholesterol or knockdown of caveolin-1, leads to disassembly of the Na/K-ATPase/Src signaling complex, abolishes the activation of Na/K-ATPase-mediated signal transduction by ouabain and interrupts endocytosis of Na/K-ATPase [93,102,108]. Taken together, it is clear that Na/K-ATPase, mainly localized in caveolae, could also serve as a signaling scaffold through interaction with other proteins or receptors.

4. Na/K-ATPase and ROS in uremic cardiomyopathy

4.1. Reactive oxygen species

Over the past five decades, oxygen-derived free radicals called ROS have been identified in both intracellular and extracellular locations and have been shown to play important roles in physiology and pathology. Common forms of ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{OH}\cdot$) [109]. ROS was initially thought to exclusively induce cellular damage but not physiological function [110,111]. However, evidence has accumulated linking ROS to multiple pathophysiological events, such as like diabetes, cancer, CVD, obesity, CKD, ESRD, aging, and neuronal disorder [112–117]. Furthermore, many publications have indicated that H_2O_2 induces tyrosine phosphorylation and affects cell signaling pathways [118].

4.2. Na/K-ATPase/ROS amplification loop

Interestingly, Liu et al. made the connection of Na/K-ATPase-mediated signal transduction to the generation of ROS, and provided strong evidence that this regulation occurs independent of changes in intracellular calcium and sodium concentrations [88,119]. Specifically, ouabain caused no change in calcium concentration when cardiac myocytes were incubated in calcium-free medium, whereas it did induce ROS generation under the same conditions. Moreover, ouabain-induced protein tyrosine phosphorylation was also independent of changes in intracellular calcium concentration, but can be blocked by Ras inhibitors. This finding again emphasizes that Na/K-ATPase as a signaling scaffold in cell physiology. Our early studies suggest that ouabain could stimulates ROS generation in a Ras-dependent way through Na/K-ATPase/Src signaling complex [88,119]. Further studies by Xie's lab show that disruption of Na/K-ATPase/Src signaling by either pNaktide (an antagonist of Na/K-ATPase/Src signaling) or by the expression of Src-binding sites null Na/K-ATPase (A420P) abolishes ROS-induced Src/ERK/MAPK activation and protein

carboxylation [120]. We also observed that ouabain is able to induce direct carbonylation on Pro 222 and Thr 224 of pig Na/K-ATPase α 1 subunit, and that inhibition of the carbonylation by antioxidants or mutation of Pro222 attenuates ouabain-induced activation of protein kinase cascades [121].

Conversely, oxidative stress can also induce Na/K-ATPase downstream signaling. First, increases in ROS generation could oxidize the Na/K-ATPase α and β subunits as well as its independent regulator FXYD proteins. Oxidation of Na/K-ATPase inhibits its activity and promotes its sensitivity to protein endocytosis pathway [122–126]. For instance, oxidative stress could induce glutathionylation of Na/K-ATPase β 1 subunits in pig kidney and cardiomyocytes. Peroxynitrite-mediated oxidation of Na/K-ATPase in purified pig kidney inhibits its activity by stabilizing the enzyme in an E2-P prone conformation. FXYD proteins could reverse oxidative stress induced inhibition of Na/K-ATPase by deglutathionylation of β 1 subunit [124]. On the other hand, administration of either H₂O₂ or glucose oxidase in cardiac myocytes activates Na/K-ATPase signaling in cardiomyocytes [127], whereas pretreatment with antioxidant N-acetyl cysteine (NAC) prevents ouabain-induced Na/K-ATPase downstream signaling [88,128]. Thus, we propose that Na/K-ATPase/Src signaling complex and ROS form a signal amplification loop allowing not only CTS but also ROS to generate signaling from the Na/K-ATPase.

4.3. Na/K-ATPase/ROS amplification loop in diseases

In view of the well-established role of oxidative stress in the development of many chronic disease, Chen et al. and Kennedy et al. have recently explored whether the newly appreciated Na/K-ATPase/Src/ROS amplification loop is critical for ROS signaling during diseases. Using gene modified animal, Chen et al. indicated the role of Na/K-ATPase in participating oxidative stress induced atherosclerosis [129]. Whereas Kennedy et al. suggested the involvement of this signaling loop in hyperlipidemic states induced renal inflammation and tissue damage [130]. Furthermore, our recent animal studies directly point the role for Na/K-ATPase/Src-ROS amplification in high-fat diet induced adipogenesis [131]. Specifically, it is well known that elevated oxidative stress due to high-fat diet is an important pathological mechanism in obesity and metabolic imbalance in adipocytes, which makes the redox state of adipocyte become a therapeutic target for obesity and metabolic syndrome [115,132,133]. C57/B6 mice fed high-fat chow for 12 weeks presented with obesity and metabolic imbalance due to the activation of ROS. Using pNaktide, we observed significant attenuation of obesity and development of metabolic syndrome. More importantly, our results also showed that targeting on Na/K-ATPase/Src/ROS amplification loop could have decreased visceral and subcutaneous fat mass potentially due to attenuated oxidative stress and insulin resistance with an increase in adiponectin level in adipocytes.

4.3.1. Na/k-ATPase/ROS amplification loop in uremic cardiomyopathy—Uremic cardiomyopathy is complicated by diastolic dysfunction in its earliest stages followed by significant ventricular hypertrophy and ultimately, systolic dysfunction [13]. Among many factors, cardiac hypertrophy and sequential replacement of cardiomyocytes with fibrosis seem to attribute more to cardiac dysfunction than other mechanisms [7,8,134]. Although many molecular signals induce fibrosis in uremic cardiomyopathy, oxidative stress is

certainly a significant contributory mechanism [34,41,135]. In CKD, elevated ACE and endogenous CTS target cardiac cells and upon binding induce the generation of ROS through different signaling pathways. Increased ROS generation stimulates downstream signaling that induces cell proliferation, markers for cardiac hypertrophy, as well as collagen synthesis [40,88,136]. On the other hand, many laboratories including ours have shown that elevated CTS levels or H₂O₂ can induce cardiomyocyte hypertrophy as well as cardiovascular remodeling both *in vitro* and *in vivo* [19,40,137,138]. The first clue that link Na/K-ATPase and CTS with cardiac hypertrophy based on the observation that cultured cardiomyocytes appeared to display increased cellular survival signaling instead of activation of apoptosis following CTS treatment [88]. Following studies by Xie et al. demonstrated that CTS treatment in cardiac myocytes stimulated ROS generation and NF-kappaB activation which could be blocked by pretreatment with antioxidants such as N-acetylcysteine (NAC) or vitamin E [88]. These *in vitro* studies not only reveal the signaling receptor function of Na/K-ATPase aforementioned, but also suggest that Na/K-ATPase/Src/ROS feed-forward amplification loop that activates downstream signaling cascades responsible for cardiac hypertrophy. Clinically, many lab including ours have reported an increase in endogenous CTS in CKD patients [35,36], as well as a decrease in Na/K-ATPase expression in the heart of dilated cardiomyopathy patients [139,140], and experimental animal model [40,141]. In addition, MBG infusion through mini-pump was shown to produce the same uremic cardiomyopathy phenotype as PNx surgery in rats, in terms of elevated circulating MBG, cardiac hypertrophy, impaired cardiac function, and cardiac fibrosis [136]. Subsequently, antagonizing CTS by spironolactone or targeting mTOR pathway with rapamycin or neutralization of CTS attenuates pathological remodeling characterized by cardiomyocyte hypertrophy, cardiac fibrosis and diastolic dysfunction in experimental uremic cardiomyopathy animal models [14,134].

Importantly, in our recently *in vivo* study, we not only first demonstrate the essential role of Na/K-ATPase/Src/ROS signaling amplification loop in the development of uremic cardiomyopathy *in vivo*, but also provide a potential drug candidate for uremic cardiomyopathy [41]. Specifically, using a 5/6th partial nephrectomy (PNx) mouse model, we observed increased Src/ERK phosphorylation along with elevated expression of heme oxygenase-1 (HO-1), collagen-1 and protein carbonylation in PNx mouse left ventricle. The Na/K-ATPase/Src signaling complex antagonist, pNaktide, could attenuate physiological, morphological, and biochemical alterations of uremic cardiomyopathy, which including reduced left ventricle hypertrophy and improved diastolic function, decreased cardiac fibrosis, and decreased Src/ERK phosphorylation as well as protein carbonylation. Surprisingly, pNaktide but not induction of HO-1 appeared to ameliorate anemia in renal dysfunction animal. More interestingly, in reversal study, pNaktide was found to have a dose-dependent effect in developed cardiac hypertrophy, fibrosis and anemia. Taken together, it is clear that Na/K-ATPase/Src/ROS signaling amplification loop is important for the development of uremic cardiomyopathy. Therapeutic targeting on this signaling pathway may provide a new direction for this disease.

5. Development of pNaktide

As we mentioned above, pNaktide a novel peptide that was developed in our lab, could attenuate uremic cardiomyopathy and high-fat diet induced obesity [41,131]. The idea for the development of pNaktide arose from the observation that Na/ K-ATPase allosterically interacts with Src kinase, keeping it inactive; however, when CTS-bind to the Na/K-ATPase $\alpha 1$ sub-unit, a conformational change occurs in this subunit which frees the allosteric inhibition of Src kinase allowing it to become activated [84]. The first step in this process was afforded by the studies in LLC-PK1 cells mentioned above which demonstrated that downregulation of Na/K-ATPase with siRNA lead to significantly elevated Src activity [89]. Detailed sequencing analysis showed that a 20-amino acid sequence on the N domain of Na/K-ATPase $\alpha 1$ subunit is essential for Src inhibition [142]. Based on this sequence (Figure 2), we synthesized a patented 33-amino acid peptide (pNaktide-GRKKR RQRRR PPQSA TWLAL SRIAG LCNRA VFQ) the extra 13 amino acids correspond to an HIV-TAT leading sequence which facilitates cellular permeation. This leading sequence renders pNaktide permeable to the cell membrane and leads to localization of pNaktide to the intracellular face of plasma membrane, limiting the effects of this peptide to cellular membrane-associated Src thus preventing inhibition of cytosolic Src activity a feature of its specificity [142]. pNaktide induces Src inhibition at an $IC_{50} = 70$ nM in LLC-PK1 cells and cardiomyocytes, importantly pNaktide is a specific inhibitor of Na/K-ATPase/Src complex signaling and appears to have no effect on Na/K-ATPase ion pumping function nor IGF1 induced Src activity [142]. In cultured neonatal cardiac myocytes, pNaktide has the capability to abolish CTS-induced hypertrophy in a dose-dependent manner. Furthermore, based on the observation that expression of Na/K-ATPase $\alpha 1$ is significantly reduced with accompanying elevated Src activity in human prostate carcinoma, we have shown that administration of pNaktide to NOD/SCID mice with this tumor xenograft potentially inhibit tumor growth likely due to attenuation of overactive Src and angiogenesis [143,144].

6. Conclusion

In accord with the focus of the current review, we suggest that increased endogenous CTS or oxidative stress-induced Na.K-ATPase signaling complex activation and/or the subsequent oxidant amplification loops are well known to occur in CKD patients with cardiac dysfunction. As such we propose that Na/ K-ATPase/Src/ROS amplification loop is a suitable target to regulate the development of uremic cardiomyopathy and that work in our lab and others specifically focused on inhibition of this signaling cascade, like the development of pNaktide will function to prevent or reverse uremic cardiomyopathy and act as a therapeutic agent by inhibiting Na/K-ATPase signaling and oxidant amplification (Figure 1).

7. Expert opinion

Over the past 20 years, many molecular mechanisms leading to uremic cardiomyopathy have been proposed and explored [8,13,34,40]. However, clinical treatment targeting these mechanisms remains disappointing.

We would suggest that this is probably due to two factors. First, the cardiac abnormalities are usually present when dialysis treatment is initiated, and reversal is almost always much, much harder to achieve than prevention. This indicates the importance of recognizing and correcting cardiac alterations and/or their risk factors in the patients before the cardiomyopathy becomes advanced. Second and of greater relevance to this review, oxidative stress is clearly a contributor, but our clinical approaches have been inadequate. To date, most strategies to address oxidant stress employ the use of ‘antioxidants’ or free radical ‘scavengers.’ As these do not impact the production of oxidants, we think they will eventually become overwhelmed. Whether it is through this mechanism or others, they have not been effective clinically. We believe that targeting the amplification of ROS generation will be effective clinically as we have shown for experimental animals. However, it is still very clear that we have a very long way to go before this can be proven.

As discussed, we believe that the Na/K-ATPase/Src/ROS signaling amplification loop is important in the development of uremic cardiomyopathy, and thus, may become a fertile therapeutic target for the prevention and treatment of uremic cardiomyopathy as well as other medical issues that are derived from the oxidant stress seen with CKD. Both *in vitro* and *in vivo* studies have demonstrated the effectiveness of pNaKtide in blocking this signaling loop. As such, we and others have shown that administration of pNaKtide attenuates/reverses cardiac hypertrophy and fibrosis in animal models of uremic cardiomyopathy. Although the role of Na/K-ATPase in cellular signal transduction has been well documented in the literature, it should be noted that Karlisch and his colleagues have recently questioned whether there is a direct interaction between Na/K-ATPase and Src. Needless to say, further investigation is needed to resolve this important issue. However, it is equally worth noting that the concept of direct interaction between the $\alpha 1$ Na/K-ATPase and Src has been evolved from multiple studies from several independent laboratories. Moreover, our recent success in the use of pNaKtide in several disease models has re-enforced the concept of direct protein interaction. On the other hand, the concept that increase in ROS generation can occur through the pathophysiology of CKD and uremic cardiomyopathy is well characterized, suggests that targeting on ROS amplification may serve as a newly potential therapeutic target in a number of conditions characterized by oxidative stress. For instances, elevated oxidative stress has been documented as important mechanism for many risk factors of CKD such as diabetes, hypertension, and obesity [145–147]. Based on these observation, we believe that further investigation targeting on ROS amplification would benefit not only CKD and uremic cardiomyopathy, but also its risk factors and provide better therapeutic effect. In this case, Na/K-ATPase/Src/ROS amplification loop based on this review provides a new direction and a good start point for this hypothesis. Although a novel, well-characterized anti-ROS generation agent would be a welcome addition to our clinical armamentarium, much work is necessary to fill the gap between rodents and humans. Furthermore, additional studies on pNaKtide and other strategies that target this loop focusing on the tissue-specific and time-specific manner will be necessary to test this hypothesis.

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Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

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Article highlights

- Epidemiology and pathophysiology of uremic cardiomyopathy
- Na/K-ATPase and its pumping function, receptor function and signaling scaffold
- The role of ROS and the newly discovered Na/K-ATPase/Src/ROS amplification loop in uremic cardiomyopathy
- The development of a potential therapeutic peptide targeting on this amplification loop
- Expert opinion of current study and future direction

This box summarizes key points contained in the article.

Figure 1. Schematic showing central role for Na/K-ATPase alpha1 subunit in the amplification of ROS in chronic kidney disease, ultimately leading to uremic cardiomyopathy.

Figure 2. Schematic of Na/K-ATPase alpha1 subunit A, N and P domains on the cytosolic aspect of the membrane and its interaction with Src kinase.

Table 1

Plasma level of related endogenous digitalis in different diseases.

Disease	Patients	Healthy volunteers/control	Ref.
Essential hypertension	EO: 377 ± 19 pmol/L *	EO: 253 ± 53 pmol/L	[37]
Heart failure	Median MBG: 583 pmol/L *	Median MBG: 241 pmol/L	[38]
End-stage renal disease	MBG: 3.81 ± 1.92 ng/ml *	MBG: 0.94 ± 0.28 ng/ml	[36]
First Day AMI	MBG: 1850 ± 380 pmol/L *	MBG: 500 ± 70 pmol/L	[39]
Chronic renal failure	MBG: 860 ± 70 pmol/L *	MBG: 280 ± 20 pmol/L	[35]

* Significant difference compare to healthy volunteer.

EO: endogenous ouabain-like factor; AMI: acute myocardial infraction.