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Targeting Na/K-ATPase Signaling: A New Approach to Control Oxidative Stress

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Abstract

Renal and cardiac function are greatly affected by chronic oxidative stress which can cause many pathophysiological states. The Na/K-ATPase is well-described as an ion pumping enzyme involved in maintaining cellular ion homeostasis; however, in the past two decades, extensive research has been done to understand the signaling function of the Na/K-ATPase and determine its role in physiological and pathophysiological states. Our lab has shown that the Na/K-ATPase signaling cascade can function as an amplifier of reactive oxygen species (ROS) which can be initiated by cardiotonic steroids or increases in ROS. Regulation of systemic oxidative stress by targeting Na/K-ATPase signaling mediated oxidant amplification improves 5/6th partial nephrectomy (PNx) mediated uremic cardiomyopathy, renal sodium handling, as well as ameliorates adipogenesis. This review will present this new concept of Na/K-ATPase signaling mediated oxidant amplification loop and its clinic implication.

Keywords

Na/K-ATPase; signaling; ROS; oxidative stress

1. THE Na/K-ATPase: ION PUMPING AND SIGNALING FUNCTION

The P-type ATP-hydrolyzing enzyme Na/K-ATPase (EC 3.6.3.9), an integral membrane protein, was first discovered by Jens Christian Skou in 1957 [1], who was awarded the Nobel Prize in 1997. The Na/K-ATPase functions as a primary ion pump. The physiological function of the Na/K-ATPase is to use ATP to maintain the electrochemical sodium gradient across the cell membrane and also to control cellular sodium homeostasis. This process balances intracellular sodium concentration by transporting Na⁺ out of cells and K⁺ into cells using an ATP/ADP-dependent phosphorylation/dephosphorylation process which

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CONFLICT OF INTEREST

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causes conformational changes in 2 states of the enzyme, E1 and E2. The Na/K-ATPase consists of two noncovalently linked subunits, α ($\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 4$) and β ($\beta 1$, $\beta 2$, and $\beta 3$) subunits [2–6]. The “catalytic” $\alpha 1$ subunit, along with $\beta 1$, is the predominant “housekeeping” enzyme of most cells. The expression of other α and β subunits ($\alpha 2$, $\alpha 3$, $\alpha 4$, $\beta 2$, and $\beta 3$) is tissue-specific. The α and β subunits are essential for different processes. The α subunits contain binding sites for Na^+ , K^+ , cardiotonic steroids (CTS, a group of specific inhibitors and ligands of the Na/K-ATPase, also known as digitalis-like substances) and ATP. The β subunits, on the other hand, are necessary for the formation of the α/β complex and its insertion into the plasma membrane. The FXYD proteins (also known as γ subunits), which are not an integral part of the Na/K-ATPase, can modulate the enzymatic activity in a tissue- and isoform-specific manner [7, 8]. The best characterized functional differences between the α subunits are in their relative sensitivities to ouabain [3, 4]. The most recognized differences are: rodent $\alpha 1$ is far less sensitive to ouabain (about 2–3 orders of magnitude) than pig, dog, or human $\alpha 1$; rodent $\alpha 2$ and $\alpha 3$ are about 2–3 orders of magnitude more sensitive than rodent $\alpha 1$.

CTS are a group of specific inhibitors and ligands of Na/K-ATPase, including plant-derived glycosides and vertebrate-derived aglycones that were classified as a new class of steroid hormones [9–11]. CTS are incredibly structurally diverse, but all contain a steroidal nucleus with a lactone moiety at position 17. They can be further categorized as cardenolides (digoxin and ouabain which have an unsaturated butyrolactone ring) and bufadienolides (bufalin and marinobufagin which have an α -pyrone ring) [12, 13]. The positive inotropic effect of CTS (such as digoxin and digitonin) have been widely used clinically to treat congestive heart failure for over 200 years. This effect involves coupling the inhibition of the Na/K-ATPase enzymatic (ion-pumping) activity to the stimulation of the $\text{Na}^+/\text{Ca}^{++}$ exchanger which leads to intracellular Ca^{++} overload.

The Na/K-ATPase functions physiologically in more ways than simply as an ion pump. It also functions as a receptor, signal transducer, and scaffold through multiple protein-protein interactions [14–24]. Binding of ouabain to the $\alpha 1$ subunit initiates different signal pathways, including c-Src kinases, EGF receptor, PI3K/Akt, Ras/Raf/ERKs, PLC/PKC, assembly of multiple protein kinase cascades, and increases in ROS generation and intracellular calcium concentration (reviewed in [10, 18, 19, 23, 25]). These pathways have been confirmed in different cells and whole animal models [9, 14, 15, 26–30]. It is important to note that ouabain-stimulated Na/K-ATPase signaling events as well as Na/K-ATPase endocytosis are largely independent of both changes in intracellular Na^+ and K^+ concentrations and significant acute inhibition of Na/K-ATPase enzymatic activity [31–33].

Receptors and signaling molecules such as Src, PLC, PI3K, IP3Rs, ankyrin, adducin, and caveolin-1 along with cytosolic proteins and membrane structural proteins interact with the multiple structural binding motifs found in the $\alpha 1$ subunit of the Na/K-ATPase [15, 34]. Like other receptors, activation of Na/K-ATPase signaling by ouabain induces endocytosis of this enzyme as well as EGFR and c-Src [35, 36]. The consequence of the signaling of the Na/K-ATPase during endocytosis still needs more research to determine whether signaling in the endocytic pathway would be terminated or extended. Because endocytosis has an integral role in the activation and propagation as well as termination of signaling pathways

on the endocytic pathways, particularly from endosomes [37–40]. Furthermore, signal transduction can also regulate endocytosis [41, 42]. Thus, it is uncertain whether the traditionally accepted receptor-mediated endocytosis would be an effective mechanism of termination as it is to other ligand-activated responses. The trafficking of receptor tyrosine kinase (RTK) receptors could regulate receptor signaling in different ways, depending on the specific cell and tissue type. Endocytic (endosomal) receptors could control the magnitude of the response via the same signaling cascades as surface-localized receptors, or initiate distinct signaling cascades from those generated at the cell surface. In this latter case endosomal signaling could be qualitatively different from that generated at the cell surface (reviewed in [43]). In this regard, the availability of substrates of RTKs is also an important variable affecting signaling responses. Indeed, other variables that affect the magnitude of endosomal signaling include both the receptor itself, and the specific cell type [44].

There are different proposed working models which explain the mechanisms underlying the activation of the Na/K-ATPase signaling function. One such model is the direct interaction of the Na/K-ATPase α 1 subunit with c-Src kinase which forms a functional Na/K-ATPase/c-Src signaling receptor complex [19, 20, 22–24]. In this model, the Na/K-ATPase α 1 subunit provides the ligand binding site, and associated c-Src provides the kinase moiety. A second model is that c-Src activation is primarily a consequence of an ATP-sparing effect (observed in a cell-free system) [45, 46]. A third model proposes that c-Src transiently interacts with a complex formed between the Na/K-ATPase α 1 subunit and caveolin-1 [47]. A common characteristic in these models is that the E2-P conformational state of the Na/K-ATPase is favored, and stabilized by Na/K-ATPase inhibitors (ouabain, vanadate, oligomycin) and energy status (ATP/ADP ratio). Though the dynamic conformational changes can affect the formation of the signaling complex, it is quite clear that c-Src activation is a proximal step in the Na/K-ATPase signaling (as described in the following section). The interaction of the Na/K-ATPase α 1 subunit and c-Src might also require other protein(s) that are not included in experiments conducted in cell-free systems.

2. THE INTERPLAY OF Na/K-ATPase AND ROS - A POSITIVE-FEEDBACK OXIDANT AMPLIFICATION LOOP

The effects of ROS on the Na/K-ATPase enzymatic activity have been well-studied [48]. Increases in ROS and/or RNS (reactive nitrogen species) cause oxidative modification of the Na/K-ATPase α and β subunits along with FXRD proteins. S-glutathionylation, S-nitrosylation, and primary (direct) protein carbonylation are some of the many modifications which can be caused by oxidative stress [49–56]. These oxidative modifications may not only regulate (inhibit) the Na/K-ATPase enzymatic activity by blocking ouabain binding site, (thereby stabilizing the enzyme in E2 state due to conformational change), but may also promote the degradation of the enzyme. Interestingly, the modification of β 1 subunit by S-glutathionylation (and the related inhibition of the enzyme activity) can be reversed by FXRD proteins in a dynamic manner [55].

Almost 17 years ago, we reported that ROS is an important component of the Na/K-ATPase signaling pathway [31, 32]. A Ras-dependent cascade is largely responsible for the ouabain-

stimulated ROS generation observed in various types of cells through Na/K-ATPase signaling. The mechanism(s) are still unclear. However, pre-treatment with different antioxidants attenuated the ouabain-induced activation of Na/K-ATPase signaling pathways and other related effects [31, 32, 57, 58]. Our studies further demonstrated a positive feedback loop of the Na/K-ATPase signaling and ROS (Fig. 1). Our proposed Na/K-ATPase signaling mediated oxidant amplification loop is based on our previous model [59], as well as 4 major observations described below [31, 32, 56–58, 60]. First, exogenous CTS stimulates Na/K-ATPase/c-Src signaling (and as a consequence, ROS generation), but in addition, Na/K-ATPase/c-Src signaling is also stimulated by increases in ROS alone (caused by a) exogenously added H₂O₂, or b) exogenous, extracellular glucose oxidase, which produces H₂O₂ at the expense of glucose in the culture medium). Neutralization of such increases in ROS by antioxidants, such as NAC, was observed to prevent CTS- and ROS-stimulated activation of the Na/K-ATPase/c-Src signaling. Secondly, deletion of Src kinase family (SYF cells), or knock-down Na/K-ATPase α 1 subunit (PY-17 cells, which was originated from pig renal proximal tubular LLC-PK1 cells), the ouabain-induced Na/K-ATPase/c-Src signaling is blocked, as well as protein carbonylation and Na/K-ATPase endocytosis. In contrast, a knock-in of c-Src in SYF cells (named SYF+c-Src cells), or a knock-in of α 1 subunit in PY-17 cells will restore the capability of ouabain-induced Na/K-ATPase signaling, protein carbonylation, and Na/K-ATPase endocytosis. These observations indicate a central role of the Na/K-ATPase/c-Src signaling activation in the process. Thirdly, the observation that the H₂O₂ mediated protein carbonylation induced by glucose oxidase in SYF cells and PY-17 cells is significantly reduced in comparison with SYF+c-Src cells and LLC-PK1 cells (the parent wild-type cells of PY-17 cells), as well as AAC-19 cells (PY-17 cells rescued with rat α 1 subunit), further suggesting a central role of the Na/K-ATPase/c-Src signaling in ROS generation, and protein carbonylation. The fourth observation was made regarding the consequences of the carbonylation of Pro222 and Thr224 amino acid residues in the actuator (A) domain of the α 1 subunit. In LLC-PK1 cells, both ouabain and glucose oxidase-induced H₂O₂ stimulate direct protein carbonylation of Pro222 and Thr224, and endocytosis of the α 1 subunit [56, 60]. When the PY-17 cells were rescued by expressing wild-type rat α 1 (AAC-19 cells as control), or the rat α 1 with a single mutation of Pro224 (corresponding to pig Pro222) to Ala, the Pro222Ala mutation did not affect either the ouabain-induced inhibition of Na/K-ATPase enzymatic activity, or the ion-exchange activity. However, it prevented the effects of ouabain on activation of Na/K-ATPase/c-Src signaling, tyrosine phosphorylation of multiple proteins, protein carbonylation, Na/K-ATPase endocytosis, and active transepithelial ²²Na⁺ transport. In contrast, control cells with an Ala416Pro mutation were observed to act like AAC-19 cells. Thus, the data indicate modification of Pro224 by direct carbonylation of the rat α 1 subunit determines ouabain-mediated Na/K-ATPase signal transduction and subsequent regulation of renal proximal tubule sodium transport [56, 60].

A bioinformatics analysis [60] indicates that Pro222 carbonylation of pig α 1 and a Pro222 to Ala mutation does not affect tertiary structure. This analysis employed a model of Na/K-ATPase/c-Src binding in which the α 1 subunit CD2 segment (amino acid residues 152–288) binds to c-Src SH2 domain (amino acid residues 161251) while α 1 subunit ND1 segment (amino acid residues 379–435) binds to c-Src tyrosine kinase domain (amino acid residues

282–531) [19, 20]. The analysis indicated that, while both native Pro222 and Ala222 are able to bind to Tyr244 of c-Src SH2 domain in E1P state, carbonylated Pro222 binds more strongly with c-Src SH2 domain by binding to Tyr244 and other amino acid residues of c-Src SH2 domain (including Asn208, Asn236, and His248). As a consequence, the interaction between the $\alpha 1$ subunit CD2 segment and c-Src SH2 domain becomes more favorable. The analysis also predicted that, carbonylated Pro222 enhances the binding of $\alpha 1$ CD2 to the c-Src SH2 domain in both the E1P and E2P state, and that the $\alpha 1$ ND1 binds to c-Src tyrosine kinase domain with more possibilities in E1P state (vs. the E2P state), thereby favoring binding in E1P state. However, these predictions need to be examined experimentally.

3. THE POSITIVE-FEEDBACK AMPLIFICATION LOOP AND EXPERIMENTAL DISEASES IN ANIMALS

In the recent years, the Na/K-ATPase has emerged as a therapeutic target for different pathological states based largely on the CTS-Na/K-ATPase signaling axis [reviewed in [9–13, 16, 17, 22, 23, 61, 62]]. However, the interplay of ROS and Na/K-ATPase signaling is still not clear. Oxidative stress has been implicated and subsequently investigated as it pertains to many pathophysiological conditions such as ischemia-reperfusion and hyperoxia as well as various disease states, including chronic cardiac disease, chronic lung disease, chronic renal failure, obesity, and diabetes [63–66]. The oxidative modification of protein can dynamically regulate protein structure, function, trafficking, and degradation, as well as cellular signaling and function [67]. Our recent findings address this issue, and have demonstrated that the positive-feedback oxidant amplification loop causes carbonylation modification of the Na/K-ATPase that contributes to the development of some experimental diseases we have studied. Direct carbonylation is one of the most commonly occurring oxidative modifications of proteins. This is likely because the Fenton reaction involves H_2O_2 which is one of the most common end products of most ROS generating biological systems.

3.1. Salt Sensitivity and Salt-Sensitive Hypertension in Dahl Rats

We have demonstrated that activation of the Na/K-ATPase/c-Src signaling induces the redistribution of the Na/K-ATPase and NHE3 (Na^+/H^+ exchanger isoform 3) in the renal proximal tubule, leading to inhibition of Na^+ reabsorption. Impairment of the Na/K-ATPase/c-Src signaling, and/or carbonylation of the Na/K-ATPase abolishes this regulation and contributes to Dahl salt-sensitive hypertension [33, 48, 56, 60, 68, 69]. Functionally, carbonylation modification of the Na/K-ATPase is implicated in renal proximal tubular sodium handling and experimental salt sensitivity [reviewed in [18, 69]].

3.2. Uremic Cardiomyopathy Induced by Mouse 5/6th Partial Nephrectomy (PNx) Model

Patients with chronic kidney disease are at great risk for cardiovascular disease events and mortality as well as progressively developing the clinical phenotype called “uremic cardiomyopathy” [70, 71] in which oxidant stress plays an important role [72–75]. The 5/6th partial nephrectomy (PNx) animal models developed uremic cardiomyopathy phenotypes that were significantly attenuated by antagonizing CTS and Na/K-ATPase signaling [29, 76–79]. When examining the possible role of the oxidant amplification loop in PNx-induced

uremic cardiomyopathy, we observed that when the oxidant amplification loop was blocked with the synthetic peptide pNaKtide, and the development of phenotypical features of uremic cardiomyopathy was significantly attenuated [80]. The pNaKtide (which is membrane permeable by a TAT leading sequence) is derived from the ND1 segment (amino acid residues 379–435) of pig Na/K-ATPase $\alpha 1$ subunit N domain [50, 81]. In the Na/K-ATPase/c-Src signaling complex model, it was proposed that the $\alpha 1$ ND1 domain binds to the c-Src tyrosine kinase domain and the $\alpha 1$ CD2 domain binds to the c-Src SH2 domain in the “resting” state. Following the E1 to E2 conformational change (which occurs upon ouabain stimulation), the binding between the $\alpha 1$ CD2 domain and the c-Src SH2 domain remains intact, while the binding between the $\alpha 1$ ND1 domain and the c-Src tyrosine kinase domain is disrupted, and the c-Src is phosphorylated (activated). The pNaKtide, mimicking part of the $\alpha 1$ ND1 domain binds to the released c-Src tyrosine kinase domain, keeping it inactive. Thus, the effect of pNaKtide fits the model very well, even though many aspects are still not totally clear.

In C57BL/6 mouse primary cardiac fibroblast cells, telecino- bufagin (i.e. TCB, a CTS) stimulated Na/K-ATPase/c-Src signaling significantly increases in protein carbonylation and type I procollagen expression (and these affects were significantly attenuated by pretreatment with pNaKtide). Compared to C57BL/6 mice with sham surgery (4 weeks post-surgery), PNx induced consistent development of cardiac hypertrophy and diastolic dysfunction (assessed by echocardiographic methods), anemia, and increase in heart weight/body weight ratio. In left ventricle homogenates, PNx stimulated Na/K-ATPase/c-Src signaling, protein carbonylation, and type I collagen expression. Notably, administration of pNaK- tide attenuated these changes. In a separate reversal study, PNx was performed, and mice were allowed to develop uremic cardiomyopathy for 4 weeks. Administration of pNaKtide (7 days) at higher doses was able to reverse PNx-induced anemia and cardiac hypertrophy, as well as some echocardiographic features of uremic cardiomyopathy. In left ventricle homogenates, administration of pNaKtide at higher doses attenuated PNx stimulated Na/K- ATPase/c-Src signaling, protein carbonylation, and type I collagen expression [80].

3.3. Adipogenesis Induced by a High Fat Diet

Oxidative stress plays an important role in the development and maintenance of the obesity phenotype. In murine preadipocytes, exposure to adipogenic differentiation medium or ROS inducers causes dysfunctional adipogenesis. As stated above, the ability of pNaKtide to attenuate oxidative stress mediated Na/K-ATPase signaling activation and protein carbonylation has been studied in a mouse model of obesity. In this model, C57BL/6 mice are fed a high fat diet to induce obesity, which causes increases in visceral and subcutaneous fat content, as well as metabolic syndrome (with its related insulin resistance and hyperglycemia). Treatment with pNaKtide was observed to effectively reduce high fat diet induced adiposity and obesity, as well as restores metabolic homeostasis by antagonizing Na/K-ATPase signaling-mediated oxidant amplification (c-Src activation and protein carbonylation) [82].

4. IMPLICATION AND PERSPECTIVE

It has been estimated that Na/K-ATPase related ATP hydrolysis uses approximately 30% of the total energy consumption in human [17]. In some cell types (such as muscle cells and neurons), Na/K-ATPase related ATP hydrolysis may use up to 60% of the cellular energy. It is reasonable to propose that ROS generated from Na/K-ATPase contributes a significant part of the total redox state both at cell, organ, and systemic levels. Oxidative modification of the Na/K-ATPase regulates both enzymatic activity and signaling function of the enzyme. We have demonstrated the proposed oxidant amplification loop both in vitro and in vivo. Surprisingly, we have noticed that the use of pNaKtide to block the oxidant amplification loop effectively restores the basal redox state in CTS-induced and adipogenic medium-induced increases in oxidative stress in vitro, as well as PNx-induced uremic cardiomyopathy and high fat diet-induced adipogenesis in vivo. These findings suggest that targeting the Na/K-ATPase signaling mediated oxidant amplification loop could be an effective way to control systemic oxidative stress, and thus is a potential therapeutic approach for chronic oxidative stress related pathological states. It is worthy to note that the oxidant amplification loop has limit(s). For example, we found that excessive chronic oxidative stress might over-stimulate the loop, leading to a weakened or disrupted Na/K-ATPase signaling function, and amplification loop in renal proximal tubular cells, and to experimental salt-sensitive hypertension (unpublished data). More work is needed to delineate the underlying mechanism(s).

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Fig. (1).

Schematic illustration of the Na/K-ATPase/c-Src signaling mediated oxidant amplification loop. CTS induced intracellular ROS generation (through Na/K-ATPase/c-Src signaling) and/or extracellularly generated ROS (such as added H₂O₂ or glucose oxidase produced H₂O₂) stimulates Na/K-ATPase/c-Src signaling cascades, ROS generation, and α1 protein carbonylation. Increases in intracellular ROS generated through Na/K-ATPase signaling or glucose oxidase might (1) further activate the same Na/K-ATPase/c-Src (α1/c-Src) signaling complex (black dot line), (2) activate other not-yet-activated α1/c-Src signaling complexes (red dot lines), and (3) increase extracellular ROS (blue dot line). This amplification loop could be blocked (stimulated by CTS) or significantly weakened (stimulated by ROS) by pretreatment with NAC (N-acetyl-L-cysteine) or pNaKtide, rat α1 Pro224Ala mutation (P/A mutation), or deletion of Src kinases. The effect of pNaKtide was also demonstrated in animal models.