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Reducing Cardiac Fibrosis: Na/K-ATPase Signaling Complex as a Novel Target

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Abstract

Cardiac fibrosis is a common pathological process in cardiac disease and may lead to heart failure. It can also cause sudden death even in those without cardiac symptoms. Tissue fibrosis can be categorized into two categories: replacement fibrosis (also called reparative fibrosis) and reactive fibrosis. In replacement fibrosis, infiltration of inflammatory cells and accumulation of Extracellular Matrix (ECM) proteins are the initial steps in forming scar-like fibrotic tissue after acute cardiac injury and cardiac cell necrosis. Reactive fibrosis can be formed in response to hormonal change and pressure or volume overload. Experimental studies in animals have identified important pathways such as the Renin-Angiotensin-Aldosterone System (RAAS) and the endothelin pathway that contribute to fibrosis formation. Despite the fact that clinical trials using RAAS inhibitors as therapies for reducing cardiac fibrosis and improving cardiac function have been promising, heart failure is still the leading cause of deaths in the United States. Intensive efforts have been made to find novel targets and to develop new treatments for cardiac fibrosis and heart failure in the past few decades. The Na/K-ATPase, a canonical ion transporter, has been shown to also function as a signal transducer and prolonged activation of Na/K-ATPase signaling has been found to promote the formation of cardiac fibrosis. Novel tools that block the activation of Na/K-ATPase signaling have been developed and have shown promise in reducing cardiac fibrosis. This review will discuss the recent development of novel molecular targets, focusing on the Na/K-ATPase signaling complex as a therapeutic target in treatment of cardiac fibrosis.

Keywords: Cardia fibrosis; Na/K-ATPase; Signaling; Therapeutic target

Introduction

Cardiac fibrosis is a significant health issue and a common pathological process in cardiac disease that eventually leads to heart failure [1]. Both experimental and clinical data have shown that formation of fibrotic scar tissue increases cardiac stiffness, whereas regression of fibrosis improves cardiac function [2,3]. Several categories of drugs have been developed to treat cardiac fibrosis and cardiac failure in clinics. However, there remains a major gap in elucidating the mechanisms of cardiac fibrosis and its association with heart failure, which is still the leading cause of deaths in the United States [4]. Therefore, the discovery of novel molecular targets is essential for improving patient outcomes. The Na/K-ATPase is an important transmembrane protein and is critical for maintaining ion homeostasis across the cell membrane. In the past 20 years, studies have revealed that the Na/K-ATPase can also complex with neighboring proteins and function as a signaling transducer that regulates a variety of signaling events such as the activation of Src kinase and NFκB, and the generation of reactive oxygen species [5]. Recent studies have further demonstrated that prolonged activation of Na/K-ATPase signaling promotes cardiac fibroblast proliferation, increased collagen synthesis, and contributes to the pathogenesis of cardiac fibrosis in different animal models. Therefore, components of the Na/K-ATPase signaling pathway can be novel therapeutic targets in the treatment of cardiac fibrosis and related cardiac diseases. This review will discuss the recent developments in the treatment of cardiac fibrosis with a focus on the findings that Na/K-ATPase signaling complex is a novel target for drug development.

Mechanisms of Cardiac Fibrosis

Cardiac fibrosis can be categorized into two types: reactive fibrosis and replacement fibrosis (also called reparative fibrosis) [6]. Replacement fibrosis often occurs after Myocardial Infarction (MI) when large numbers of cardiac myocytes undergo necrosis. Myocyte necrosis triggers a series of events including immune cell infiltration, inflammation, new vessel formation, removal of necrotic tissue, and eventually the replacement of damaged tissue with collagen-dominated fibrotic tissue that prevents cardiac muscle from rupture [7]. In addition to scar formation at the infarcted area, the remote non-infarcted regions can develop fibrosis in the interstitial spaces, which is referred to as reactive fibrosis or interstitial fibrosis [6,8-10]. Interstitial fibrosis can also occur in disease conditions that often involve the activation of Renin-Angiotensin-Aldosterone System (RAAS) [11-13], endothelin-1 [14-16], TGF-β [17-19], TNF-α [20,21], NFκB [22,23], or other profibrotic signaling pathways [24,25].

Cardiac fibroblast proliferation and differentiation into myofibroblasts are important processes in both types of cardiac fibrosis [1,26,27]. Myofibroblasts, the activated cardiac fibroblasts, are considered as the major source that secret collagen and other ECM proteins during the formation of fibrosis [1,28,29]. Molecular markers such as α-smooth muscle actin fibroblast-specific protein 1 and periostin were used to label myofibroblasts [1,30-32]. However, these molecular markers may also be expressed in epicardium, vascular smooth muscle, pericytes, endothelial cells, and cardiac muscle cells

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[1]. Additionally, the origin of myofibroblasts that accumulate during fibrosis formation remains elusive. Resident cardiac fibroblasts are considered the major source but other cell types such as vascular endothelial cells, perivascular cells, and progenitor cells have been shown to differentiate into myofibroblasts [1].

The RAAS and TGF-β signaling pathways are major factors that are involved in the activation of cardiac fibroblasts and formation of tissue fibrosis. Administration of angiotensin II or aldosterone has been shown to stimulate collagen and other ECM protein expression and secretion from cardiac fibroblasts [33], while inhibition of RAAS by Angiotensin Converting Enzyme (ACE) inhibitors or angiotensin type I receptor blockers (ARBs) attenuates cardiac fibrosis and improves cardiac function [34-36]. Spironolactone, an antagonist of aldosterone is also a potent drug in the treatment of cardiac remodeling that is related with cardiac fibrosis [37]. In the event of an MI, activation of NFκB was observed in different cells and subsequently drives the expression of a large panel of genes [22,23]. These genes produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin proteins that subsequently help recruit leukocytes and initiate the inflammation responses [7]. Once the necrotic tissue is cleared by leukocytes, neutrophils, and monocytes, inflammation become suppressed and fibrotic tissue formation follows. Several members of the TGF-β family are involved in the regulation of inflammation and fibrosis [18,38]. The TGF-β1 signaling pathway is an important component in the switch from inflammation to fibrosis [39]. The initial reparative fibrosis is beneficial to prevent heart wall rupture, but if this persists, it will eventually cause cardiac remodeling and reduction in cardiac function. In chronic conditions, activation of RAAS, TGF-β, and other pro-fibrotic signaling pathways can induce interstitial fibrosis in the heart and other organs [13,18,24,40,41]. Cell proliferation promoters such as non-receptor tyrosine kinases were also reported to increase organ fibrosis [42-44]. In addition to these traditional signaling pathways, epigenetic regulation has drawn attention for their regulating role in cardiac fibrosis. Numerous microRNAs were found to be either pro- or anti-fibrotic [45]. Some anti-fibrotic microRNAs such as the miR-29 mciroRNA family directly target the mRNA of several collagen isoforms, fibrilin 1, elastin, and matrix metalloprotease 2 (MMP2) and thus prevent overexpression of these ECM proteins [46-51]. When the anti-fibrotic microRNAs are reduced in disease state, syntheses of collagen and other ECM protein increase and promoting the development of fibrosis. The understanding of microRNA biosynthesis has been greatly advanced in the past decade and represents a promising area for the development of reagents that regulate specific microRNA expression and prevent organ fibrosis.

Treatment Strategies for Cardiac Fibrosis

Extensive experimental studies using animal models have revealed important pathways and identified specific molecular markers as targets for treatment of cardiac fibrosis. However, the clinical translation of these findings are limited attributing to the lack of validated non-invasive measurement of cardiac fibrosis in patients. Serum levels of procollagen type I C-terminal peptide (PCIP) and procollagen type III N-terminal peptide (PIIINP) were often used as secondary surrogates for assessment of tissue fibrosis [52], but their correlation to cardiac fibrosis and cardiac function has not been validated in the clinical studies. Echocardiography has also been used for assessment of cardiac fibrosis with diastolic dysfunction [53-55]. More accurate methods using cardiac resonance imaging (CMR) and T1 mapping have been recently used clinically to evaluate cardiac fibrosis [56,57]. These new technologies allow for a more direct measurement of cardiac fibrosis and assessment of the drug effects in clinical studies. Several categories of drugs have now been evaluated for their effect on reducing cardiac fibrosis and improving overall cardiac function in certain small size clinical trials.

ACE inhibitors are the most commonly used drugs for treatment of hypertension and heart failure [35,36,58,59]. Enalapril was discovered as an ACE inhibitor that decreases levels of angiotensin II and leads to less vasoconstriction and lower blood pressure [35]. It was shown that enalapril can reduce the risk of death by 16% in patients with reduced ejection fraction [35,36]. ARBs have also been shown to reduce cardiac fibrosis in clinical trials [60]. In addition, animal models with pressure overload demonstrated that hypertension was able to induce cardiac fibrosis and lowering blood pressure attenuated cardiac fibrosis [61]. However, a clinical trial comparing different anti-hypertensive drugs showed that only the ACE inhibitor lisinopril reduced cardiac fibrosis measured by collagen volume fraction in tissue biopsies at 6 months after treatment, but hydrochlorothiazide failed to do so [3]. Spironolactone, an antagonist of aldosterone (another major component of RAAS), has also been shown to have potent effects in reducing cardiac fibrosis in clinical studies using serum levels of PICP and PIIINP as surrogate markers of fibrosis [62-64]. In addition to targeting RAAS, other categories of drugs including vasodilators, anti-inflammatory and anti-oxidative agents have been tested clinically for their effects on reducing cardiac fibrosis [65].

TGF-β and its downstream signaling components Smad proteins are main regulators of collagen synthesis [18]. TGF-β is synthesized in many cell types as a large latent complex, which can be activated by a variety of molecules. Thrombospondin-1 (TSP-1) can disrupt the interaction between the latency-associated peptide (LAP) and TGF-β resulting in activation of TGF-β. Active TGF-β binds to its receptor and induces expression of Smad 2/3 and phosphorylation of Smad 4, which are transcription factors [18,66]. Activation of the TGF-β signaling pathway enhances collagen types I and III synthesis, decrease collagenase expression, and induces integrin expression [67-69]. TGF-β was also a common pathway that regulates fibrosis-related microRNA expression [70,71]. Drugs targeting TGF-β and Smad proteins have been under active development. The Smad 3 inhibitor Halofuginone was shown to ameliorate radiation-induced fibrosis in mice [72]. A prespecified and pooled analysis of phase 3 clinical trials also indicated that the TGF-β inhibitor pirfenidone significantly reduced all-cause mortality and pulmonary fibrosis compared to the placebo group [73].

Using a combination of drugs from different categories has been shown to improve the treatment of heart failure. It was found that addition of spironolactone on top of the standard ACE inhibitor treatment decreased frequency of hospitalization due to worsened heart failure by 35% compared to ACE inhibitor treatment alone. The death rate was also decreased from 46% to 35% in spironolactone plus ACE inhibitor treatment [74]. Since aldosterone is a downstream component of RAAS, blocking aldosterone by spironolactone in addition to ACE inhibition was considered unnecessary and may predispose patients to side effects such as serious hyperkalemia [75]. However, studies found that ACE inhibition can only transiently decrease the production of aldosterone [76,77]. Spironolactone was also found to function as an antagonist of Na/K-ATPase signaling and attenuates cardiac fibrosis in experimental uremic cardiomyopathy [78]. Another important example of drug combination in the treatment of heart failure is the usage of LCZ696, which is composed of the neprilysin inhibitor LBQ657 and valsartan, an ARB drug [79]. A recent clinical study [80] found that LCZ696 reduced the hazard ratio of all-cause mortality and cardiovascular specific mortality compared to enalapril. Neprilysin

is a neutral endopeptidase, which degrades natriuretic peptides, bradykinin, and adrnomedullin [81-83]. Inhibition of neprilysin may increase the level of natriuretic peptides and thus counter act with the sodium retention and volume overload [83]. In a related study it was also found that the component of neprilysin inhibitor in LCZ696 had no effect on cardiac fibrosis, while the component of ARB in this drug reduces both cardiac fibrosis and hypertrophy [79].

Na/K-ATPase as a Novel Target for Cardiac Fibrosis

Despite the current advances in treatments, there remains a major gap in elucidating the mechanisms of cardiac fibrosis and its association with heart failure. Novel pathways and potential drug targets related with tissue fibrosis have been actively studied. A novel signaling pathway, Na/K-ATPase signaling, has been demonstrated in experimental animal models to contribute to the formation of cardiac fibrosis [84-90]. Reagents developed to target the components of Na/K-ATPase signaling have shown promising results in ameliorating cardiac fibrosis and improving cardiac functions [91-94].

Na/K-ATPase is a transmembrane protein that was discovered in 1957 by Dr. Skou [95]. It is a major ion transporter that helps maintain homeostasis of Na^+ and K^+ concentrations across the cell membrane by hydrolyzing ATP. The Na/K-ATPase α subunit has 10 transmembrane domain and 3 major cytosol domains namely actuator domain (A domain), nucleotide binding domain (N domain) and phosphate binding domain (P domain) [96,97]. Digitalis compounds, also called Cardiotonic Steroids (CTS), specifically bind to the extracellular portion of Na/K-ATPase α subunit and cause a conformational change which inhibits the ion transporting activity and ATP hydrolysis [98]. In addition to its canonical ion transporting function, the Na/K-ATPase was found to be able to associate with other signaling proteins and function as a signal transducer [5,99,100]. Treatment of cells with Na/K-ATPase ligands induces activation of Src, PI3K, NFκB, Erk, PLC and other signaling pathways as well as the generation of reactive oxygen species [101-104]. We and others have demonstrated that activation of Src, PI3K and NFκB signaling pathways may stimulate cell proliferation and protect cells from death [103,105,106], whereas prolonged activation of these signaling pathways in certain disease models was demonstrated to cause cardiac hypertrophy and fibrosis [84-90]. Digitalis drug such as digoxin have been used for treatment of congestive heart failure for centuries [107-109]. The endogenous digitalis-like compounds were only recognized a few decades ago and their physiological and pathological roles are just starting to be appreciated. It was found that ouabain and Marinobufagenin (MBG) exist in human blood with the same structure as plant-derived and toad gland-derived digitalis compounds, respectively [110-114]. The elevation of circulating levels of these endogenous compounds was reported in heart failure patients and was correlated with the severity of heart dysfunction [111,115-117]. Other diseases such as renal failure [118], preeclampsia [119], myocardial ischemia/infarction [120,121], and diabetes mellitus [122,123] were also observed in association with elevated levels of endogenous compounds in human plasma samples. However, the accurate measurement of endogenous digitalis compounds in human samples still faces big challenges [124].

Kennedy [125] showed that both 5/6th Partial Nephrectomy (PNx), which increase endogenous MBG levels, and MBG perfusion caused significant cardiac fibrosis, suggesting that digitalis compounds binding to Na/K-ATPase contributes to uremic cardiomyopathy. It has also been shown that immunization against MBG attenuated PNx-induced cardiac fibrosis [125]. Elkareh [90] later demonstrated that MBG stimulate procollagen-1 synthesis in rat cardiac fibroblasts and in MBG-perfused rat heart tissue. Interestingly, this study demonstrated that although it is required for MBG or PNx induced cardiac fibrosis, TGF-β and its downstream component Smad 2/3 or Smad 4 were not activated by MBG. The increased procollagen synthesis is rather associated with the activation of Src kinase and increased reactive oxygen species [90]. Further study [126] showed that MBG induces activation of PKCδ in a pathway involving phosphorylation of Friend leukemia integration-1 (Fli-1). Fli-1 is a transcription factor that negatively regulates collagen mRNA synthesis, while phosphorylation of Fli-1 can cause degradation of Fli-1 and subsequently induces collagen synthesis [127]. A recent study from our laboratory demonstrated that ouabain and MBG decrease miR-29b-3p, an anti-fibrotic microRNA, and led to an increase in collagen synthesis through a Src-related signaling pathway in cardiac fibroblasts [128]. These pathways may work in concert to stimulate collagen synthesis and fibrosis formation in response to digitalis compound treatment or in conditions of chronic renal dysfunction.

Since rodents express an ouabain-insensitive form of the Na/K-ATPase α1 subunit, Lingrel and his colleagues made a mutant mouse strain, in which its Na/K-ATPase α1 subunit was mutated to be sensitive to ouabain. These mutant mice and the wild type mice were then subjected to Transverse Aortic Coarctation (TAC). It was shown that TAC caused much more severe and earlier cardiac hypertrophy and fibrosis in mutant mice compared to that in wild type mice [85]. In the same study, they found that treatment with Digibind, a Fab fragment of an ovine anti-digoxin antibody, could prevent the development of cardiac fibrosis and hypertrophy in these animals. A more recent study demonstrated that a digitalis compound potentiates the myofibroblast differentiation through increased COX-2 expression and activation of PKA [129]. These studies clearly showed that Na/K-ATPase and its ligands are involved in the formation of cardiac fibrosis and the development of cardiomyopathy.

As the novel non-canonic role of Na/K-ATPase as a signaling transducer has been recognized, more evidences suggest that Na/K-ATPase signaling components can be important molecular targets for drug development [102]. Both passive immunization against MBG [90,91] and administration of anti-MBG or anti-digoxin antibodies [85,94,130] have been shown to attenuate tissue fibrosis in experimental models of renal and cardiac disease. A recent study by Haller [92] showed that rapamycin, an inhibitor of Akt/mTOR pathway, can reduce the synthesis of MBG in rats and block PNx-induced cardiac fibrosis. In addition, a novel patent product called NaKtide (US8981051 B2) was developed based on the discovery that Na/K-ATPase can directly interact with Src and keep Src in inactive states [131-133]. Na/K-ATPase binds with Src through two domain interactions, namely the A domain of Na/K-ATPase binds to the SH2 domain of Src, while the N domain of Na/K-ATPase interact with the kinase domain of Src. When digitalis compounds bind to Na/K-ATPase, the conformation changes cause the dissociation of Src kinase domain from the N domain of Na/K-ATPase and subsequently activates Src. The NaKtide is a peptide product that contains a 20-amino acid sequence derived from the N domain of Na/K-ATPase α1 subunit. By adding a leading sequence, it is then called pNaKtide. The pNaKtide can enter into cells and largely distribute on the plasma membrane [131]. Treatment of pNaKtide is hypothesized to disrupt the direct interaction between Na/K-ATPase and Src, and therefore Na/K-ATPase can no longer transduce the extracellular signal and activate downstream signaling pathways when digitalis compounds bind to it. *In vitro* study showed that pNaKtide can inhibit Src tyrosine 418 phosphorylation when directly incubated with purified Src [131]. Our recent studies found

that pNaKtide can block the ouabain-induced decrease in anti-fibrotic microRNA miR-29b-3p in cardiac fibroblasts [128] and significantly reduce cardiac fibrosis in mice subjected to PNx surgery by inhibiting the amplification of reactive oxygen species [93]. The latter study also showed that pNaKtide not only prevents PNx-induced cardiac fibrosis but also reduces cardiac fibrosis if administered after cardiac fibrosis already developed, suggesting a reversing effect in cardiac fibrosis [93]. However, the detailed molecular mechanism by which pNaKtide reduces cardiac fibrosis remains to be further explored. Even though Src activation has been indicated to be related with increased tissue fibrosis, blocking Src activation with different methods has yield inconsistent results in reducing tissue fibrosis [134-136]. It will be interesting to test if pNaKtide exerts its effect on cardiac fibrosis solely through inhibition of Src, or if additional mechanisms are involved. A direct comparison between pNaKtide and generic Src inhibitors will also be an interesting area of investigation.

Conclusion

In summary, our understanding of the pathology of cardiac fibrosis as well as other organ fibrosis has been greatly advanced. Some common mechanisms and major regulators were discovered and tested in experimental models and in clinical trials which showed promising results in reducing cardiac fibrosis and related cardiac disease. However, heart failure is still the leading cause of death worldwide. Novel drugs and novel strategies such as more specific targets, or a combination of different drugs should be explored to more effectively reduce the cardiac fibrosis and improve cardiac function.

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