FORUM REVIEW ARTICLE

GRK2 in the Heart: A GPCR Kinase and Beyond

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Abstract

Significance: Heart failure (HF) is a common end point for many underlying cardiovascular diseases. Down-regulation and desensitization of β -adrenergic receptors (β -AR) caused by G-protein-coupled receptor (GPCR) kinase 2 (GRK2) are prominent features of HF. **Recent Advances and Critical Issues:** Significant progress has been made to understand the pathological role of GRK2 in the heart both as a GPCR kinase and as a molecule that can exert GPCR-independent effects. Inhibition of cardiac GRK2 has proved to be therapeutic in the failing heart and may offer synergistic and additional benefits to β -blocker therapy. However, the mechanisms of how GRK2 directly contributes to the pathogenesis of HF need further investigation, and additional verification of the mechanistic details are needed before GRK2 inhibition can be used for the treatment of HF. **Future Directions:** The newly identified characteristics of GRK2, including the S-nitrosylation of GRK2 and the localization of GRK2 on mitochondria, merit further investigation. They may contribute to it being a pro-death kinase and result in HF under stressed conditions through regulation of intracellular signaling, including cardiac reduction-oxidation (redox) balance. A thorough understanding of the functions of GRK2 in the heart is necessary in order to finalize it as a candidate for drug development. *Antioxid. Redox Signal.* 21, 2032–2043.

Introduction

HEART FAILURE (HF) is the end stage of many underlying cardiovascular diseases such as congenital malformation, hypertension, and coronary artery disease. Despite recent improvements in therapy and clinical care, HF remains one of the leading causes of deaths in developed countries. In 2009, one in nine deaths in the United States was related to HF (31). The failing heart initially undergoes adaptive and compensatory changes to maintain cardiac output and eventually cardiac reserve decreases and decompensation develops, which is characterized by reduced left ventricular (LV) ejection fraction (EF%) and progressive myocardial remodeling, including fibrosis, enlarged LV dimension, and reduced wall thickness.

One of the most prominent changes in HF patients is sympathetic nervous system (SNS) activation and subsequent catecholamine (CA) overdrive, which is reflected by increased circulating levels of epinephrine (Epi, also named adrenaline) and norepinephrine (NEpi, also named noradrenaline). The elevated SNS activity increases heart rate and contractility in the short term through β -adrenergic receptors (β -AR) stimulation but has damaging effects in the

long run. As a matter of fact, clinical administration of β -AR agonists reduced the survival of patients with chronic HF despite producing temporary hemodynamic benefits (7). Alternatively, β -blocker treatment slows down HF progression and reduces morbidity and mortality, as these drugs protect the heart from the detrimental effects of exposure to elevated CAs (7). It is well accepted now that this continued CA stimulation ultimately contributes to the pathogenesis of HF.

In the current review, we will discuss the regulation of β -ARs by G-protein-coupled receptor kinase 2 (GRK2) and its role in the development of HF and the latest animal model studies exploring the potential of cardiac GRK2 inhibition as a viable target in cardiac disease. We will also include the newly identified functions of GRK2 in the heart beyond that of phosphorylating G-protein-coupled receptors (GPCRs), as well as the impact of β -ARs and GRK2 on the reactive oxygen species (ROS) and redox status which has been under active investigation in recent years.

β -ARs in the Heart

Three sub-types of β -ARs are identified in human cardiac myocytes: β 1, β 2, and β 3-ARs (8). Both β 1- and β 2-ARs are

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coupled to the downstream excitatory $G_{\alpha s}$ protein, which results in the activation of adenylyl cyclase (AC) and the generation of cyclic AMP (cAMP). cAMP plays an important role in positive chronotropic and inotropic responses. Studies in β_1 -AR overexpressing transgenic mice or using pharmacological manipulation in mice demonstrate that acute activation of β_1 -ARs results in positive inotropy and chronotropy (27). However, chronic continual activation plays a primary role in cell death. β_2 -ARs also couple to the inhibitory $G_{\alpha i}$ protein, which has been reported to exert a cardioprotective effect during cardiac injury because of the activation of the survival phosphatidylinositol 3-kinase (PI3K)-Akt pathway by the release of $G\beta\gamma$ subunits (15, 20). β_3 -ARs are also detected in the heart and have been reported to mediate cardioprotection induced by exercise (9). Unlike β_1 -ARs and β_2 -ARs, β_3 -ARs require a higher concentration of CAs for activation, and induce negative inotropic effects *via* activation of nitric oxide synthase (NOS) and nitric oxide (NO) release (29). In addition, the NO release has been reported to activate phosphodiesterase 2 (PDE2), which counteracted cAMP generation induced by β_1/β_2 -AR agonists and attenuated contraction (62). β_3 -AR selective agonists have been reported to preserve cardiac function after ischemia/reperfusion (I/R) injury and to prevent cardiac remodeling after transthoracic aorta constriction (TAC) (3, 63).

In nonfailing human left ventricles, about 80% of the β -ARs are β_1 -ARs. However, in HF samples, β_1 -ARs are selectively down-regulated by 50% and the remaining β_1 -ARs and β_2 -ARs are desensitized (7). This dampening process prevents uncontrolled stimulation from occurring. Desensitization is initiated by phosphorylation of the receptors by second-messenger kinases (*e.g.*, protein kinase A [PKA] and protein kinase C [PKC]). This process is known as heterologous desensitization, because it is not agonist specific, and PKA or PKC can be activated by other nearby receptors. In contrast, phosphorylation of β -ARs by GRKs is agonist specific and is referred to as homologous desensitization (39). Seven mammalian GRKs have been characterized so far, and they can be categorized into three subfamilies: (1) GRK1 and GRK7 (rhodopsin kinase sub-family); (2) GRK2 and GRK3, formerly β -adrenergic receptor kinase 1 and 2, respectively

(β ARK sub-family); and (3) GRKs 4, 5, and 6 (GRK4-like family). The subfamilies are different in terms of sequence similarities within the catalytic domain, expression, and receptor specificity (70). In the current review, we will focus on GRK2, which is the predominant GRK isoform in the heart, although GRK3, 5, and 6 are also expressed in the heart.

The pathway of homologous β -AR desensitization is best illustrated by the activity of the major GRK isoform in the heart, GRK2 (Fig. 1). GRK2 is distributed primarily in the cytosol under basal conditions and upon GPCR activation, GRK2 translocates to the location of the receptor (plasma membrane) *via* binding to the membrane-bound $G\beta\gamma$. After GRK phosphorylation, β -arrestins bind to phosphorylated receptors, sterically hinder the interactions between the receptors and the downstream G proteins, and result in uncoupling within milliseconds to minutes of GPCR activation (55). In addition, β -arrestin-bound receptors are internalized to intracellular lysosomes and degraded, resulting in receptor down-regulation at the plasma membrane (35). Importantly, the $G\beta\gamma$ -mediated translocation of GRK2 has been exploited to generate a potent inhibitor of GRK2 activation. A polypeptide comprising the last 194 amino acids of GRK2, known as the β ARKct, has been shown to compete with endogenous GRK2 for $G\beta\gamma$ binding and to prevent GPCR desensitization both *in vitro* and *in vivo* (48, 49). GRK5 is another major GRK isoform in the heart. Unlike GRK2, GRK5 does not need to undergo translocation before phosphorylating the receptor, as it is constitutively located at the plasma membrane (74).

The most likely mechanistic link between hyperactive SNS activity and β -AR dysfunction in HF is increased GRK expression, including GRK2 and possibly GRK5 (25). GRK2 was reported to be up-regulated and exhibited increased activity in the failing human myocardium (91). As a matter of fact, GRK2 expression increases almost immediately after cardiac injury, such as myocardial infarction (MI) induced by coronary artery ligation and hypertrophy induced by pressure overload, and causes β -AR desensitization (15, 16, 40, 58, 61, 92). As the heart begins to fail, it is unable to maintain sufficient cardiac output because of the desensitization of the β -ARs by GRKs, which results in activation of compensatory mechanisms, mainly increased CA secretion. This further up-

FIG. 1. GRK2 is primarily cytosolic under basal conditions and on GPCR activation, GRK2 translocates to membrane *via* binding to the membrane-bound $G\beta\gamma$. After GRK phosphorylation, β -arrestin binds to the receptor, sterically hindering interactions between the receptor and the downstream G proteins and resulting in desensitization within milliseconds to minutes of GPCR activation. In addition, β -arrestin-bound receptors are internalized to intracellular lysosomes and degraded, resulting in receptor down-regulation at the plasma membrane. GPCRs could also be recycled to the plasma membrane. GPCR, G protein-coupled receptor; GRK2, GPCR kinase 2. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

regulates GRK2 expression and facilitates β -AR desensitization. This vicious cycle eventually results in deterioration of heart function and HF progression. The success of β -blockade in treating chronic HF in clinical practice supports the maladaptive nature of β -AR desensitization (7). Indeed, chronic treatment of mice with β -blockers decreases GRK2 expression in the heart, and this could represent one of the possible mechanisms of β -blockers in treating HF (40). In another study, using a rabbit model of HF induced by coronary artery ligation, delivery of the GRK2 inhibitor β ARKct *via* adenovirus (discussed later in detail) at the time of infarction prevented increased GRK2 expression and activity, as well as β -AR down-regulation and decreased cAMP generation. The biochemical changes were accompanied by improved LV function and delayed development of HF (96). These results suggest that β -AR desensitization in HF is maladaptive, and suppression of GRK2 activity could revert the disease progression.

Structure and Function of GRKs

The GRKs share a tri-domain structure; the conserved central catalytic domain homologous to other serine/threonine kinases, and the amino terminus and carboxyl terminus that contain elements involved in regulation and membrane localization (70) (Fig. 2). Lys220 in the catalytic domain is universally conserved in serine/threonine kinases. When Lys220 is mutated to Arg, GRK2 loses its enzymatic activity and this mutant has been used as an effective dominant negative (50). The two flanking domains vary in structure in different GRK subfamilies. The amino-terminal domain has been proposed to be important for receptor recognition, as well as for regulation of GRK activity and subcellular localization (42, 70). GRK2's amino terminus contains a regulator of G-protein signaling (RGS)-like homology domain. RGS proteins have been shown to interact with G α subunits and stimulate GTPase activity, thus effectively turning off signaling (81, 83). *In vitro* studies showed that the GRK2-RGS domain can directly interact with G α_q and inhibit its signaling (11, 82, 83), although the mechanism does not involve inhibition of GTPase activity. The major function of the carboxyl-terminus is to determine the subcellular localization. Structural variations in this domain may account for differences in agonist-dependent translocation and receptor specificity. For example, the carboxyl-terminal domain of GRK2 contains a pleckstrin homology (PH) domain that binds the $\beta\gamma$ -subunit of G proteins (48, 72). Following GPCR activation, G α dissociates from G $\beta\gamma$, and the interaction with

G $\beta\gamma$ recruits GRK2 to the membrane from its basal location in the cytoplasm (6) (see Fig. 1). In contrast, GRK5 does not contain a PH domain, and it is constitutively bound to the plasma membrane *via* a carboxyl terminal amphipathic helix and phosphatidylinositol 4,5-bisphosphate (PIP2) binding domains (89). In addition, GRK5 contains a functional nuclear localization signal within its central catalytic domain (44). The structural differences between GRK2 and GRK5 determine the different substrates and localization in the heart and, hence, significance in diseases. There are breakthroughs in terms of understanding the roles of GRK5 in cardiac diseases in recent years (32, 53, 59), which is beyond the scope of the current review and is reviewed elsewhere (39).

GRK2 and Its Role in Cardiac Physiology and Pathophysiology

The critical role of GRK2 in the regulation of cardiac function has been documented primarily by studies in genetically engineered mice. Cardiac-specific GRK2 over-expressing mice (GRK2 Tg) were generated almost two decades ago (49). GRK2 Tg mice displayed a loss of β -AR-mediated inotropic reserve, as well as desensitized angiotensin II receptors, type I (AT1-Rs) in their hearts (49, 80). In contrast, cardiac-specific β ARKct Tg mice had increased function at baseline or in response to β -AR agonist isoproterenol (Iso) (49). The improved performance lasted throughout their lives without causing any myocardial damage. These mice also rescued several mouse models of HF (see Cardiac GRK2 as a Therapeutic Target). Over the years, these two lines of transgenic mice, exhibiting opposite levels of GRK2 activity, have been used as indispensable tools to investigate the role of GRK2 in the pathogenesis of multiple cardiac diseases. Mice with cardiac overexpression of an amino-terminal peptide of GRK2 (β ARKnt), which includes most of the RGS domain, exhibited cardiac hypertrophy after pressure overload induced by TAC, even though there was no dampening of Gq-mediated hypertrophic signaling, which is contrary to the theory that RGS turns off Gq signaling (47). Overall, these mouse models illustrate the importance of GRK2 in modulating myocardial contractile function and also growth.

The overall importance of GRK2 in cardiac regulation was further emphasized in mice in which GRK2 was disrupted globally (GRK2^{-/-}) by homologous recombination (43). The GRK2^{-/-} animals do not survive beyond gestational day 15.5, and GRK2^{-/-} embryos displayed pronounced ventricular myocardium hypoplasia. GRK2^{-/-} embryos exhibited a >70% decrease in cardiac ejection fraction by

FIG. 2. Linear diagram of GRK2 illustrating the typical tri-domain structure of GRK2. Amino-acid numbers are shown to specify defined interacting domains and regulatory sites. Key peptides used in the past to study the function of individual domains are displayed at the bottom. PH, pleckstrin homology; PKA, protein kinase A; PKC, protein kinase C; RGS, regulator of G-protein signaling.

in utero intravital microscopy, suggesting cardiac failure (43). Importantly, the heterozygous GRK2^{+/-} mice that have 50% of normal GRK2 expression were viable and had enhanced contractile function both *in vivo* and in isolated single myocytes (43). More recently, conditional cardiac specific GRK2 knockout (GRK2-KO) mice were generated by breeding GRK2-floxed mice and mice harboring Cre recombinase driven by the Nkx2.5 promoter (60). The cardiac-specific GRK2 KO mice developed normally, suggesting that the developmental defect in the global GRK2 KO mice may be attributed to extracardiac effects of GRK2. Chronic treatment with the β -AR agonist Iso increased cardiac damage in the cardiac-specific KO mice, demonstrating that intrinsic dampening of GPCR responses is essential for normal cardiac function, and that the loss of receptor desensitization can promote injury when myocytes are exposed to excess CA stimulation (60). In other studies with cardiac-specific GRK2^{-/-} mouse models, GRK2 has been deleted in myocytes using Cre recombinase driven by the α MHC promoter either constitutively or induced (MerCreMer) by tamoxifen. In these mice, loss of GRK2 before MI prevented HF development, while tamoxifen-induced loss of myocyte GRK2 after MI resulted in improved cardiac function and reversed remodeling (77). The contrasting results from the two studies are probably due to the different models used to induce HF, as with chronic Iso treatment there are no reflex mechanisms to shut off CA toxicity.

Another important cell type in the heart is the fibroblast, which constitute 60%–70% of total cell number in the heart (90). Fibroblasts are an important source of GRK2 in the heart and in the cardiac-specific GRK2 KO hearts, this residual GRK2 in the fibroblasts could be sufficient to account for the adverse remodeling after MI and HF progression (77). Interestingly, there was up-regulated GRK2 in fibroblasts isolated from failing human hearts, similar to the situation in myocytes (22). GRK2 knockdown by siRNA or inhibition by β ARKct in failing cultured fibroblasts decreased TGF β -induced collagen synthesis, and it restored the inhibition on collagen synthesis by β AR agonists. The study suggests that up-regulation of GRK2 in fibroblasts facilitates cardiac remodeling and HF development by increasing collagen synthesis (22).

Cardiac GRK2 as a Therapeutic Target

Since β ARKct mice and heterozygous mice with decreased GRK2 expression in the heart had enhanced cardiac contractile function, it was hypothesized that GRK2 may be a target for improving HF and β ARKct would be an appropriate candidate inhibitor. As a matter of fact, the β ARKct mice were protected from cardiac malfunction in surgically induced HF models. In a 12 week TAC model to induce LV pressure overload, high levels of myocardial β ARKct preserved β -AR density in cardiac membranes and Iso-stimulated AC activity, and these mice showed significantly less cardiac deterioration than control mice (84). In contrast, transgenic mice with a low level of β ARKct expression showed little protection, demonstrating that the extent of GRK2 inhibition determined cardiac functional improvements and outcomes (84). In a recent study, using an acute myocardial I/R injury model, GRK2 Tg mice had larger infarct sizes, accompanied by more cell death and less activa-

tion of the prosurvival kinase Akt and NO production than control nontransgenic mice (6). β ARKct mice, on the other hand, had significantly less infarct damage compared with control and GRK2 Tg mice, which was abrogated by the selective β 2-AR antagonist ICI 118551, suggesting that the pro-death effects of GRK2 in this model were *via* inhibition of the protective β 2-AR pathway (6) (Fig. 3). The data support the therapeutic potential of inhibiting GRK2 by β ARKct in cardiac diseases.

β ARKct has proved to be protective in chronic HF models induced by genetic manipulation as well. In the first rescue study, β ARKct mice were crossed with a mouse model of HF that was caused by knocking out the muscle-LIM protein (MLP^{-/-}) (4, 79). MLP promotes proper cardiomyocyte cytoarchitecture. The MLP^{-/-} mice had enlarged LV chambers and reduced fractional shortening. Those changes of HF were reversed in the hybrid mice bred with β ARKct mice (79). In another study, β ARKct mice were crossed with transgenic mice with cardiac overexpression of the sarcoplasmic reticulum Ca²⁺-binding protein calsequestrin (CSQ), which had a severe cardiomyopathy and a survival age of 9 $\pm$ 1 weeks. Overexpression of β ARKct in the CSQ mice improved the survival age to about 15 weeks, which was attributed to attenuated cardiac remodeling and improved LV fractional shortening. Even though there was no increase in membrane β -AR density, β ARKct induced an increase in the percentages of β -ARs in the high-affinity state in this model. More importantly, β ARKct and the β -blocker metoprolol had a synergistic effect to improve cardiac function and survival

FIG. 3. Proposed model of β ARKct cardioprotection. β 2-AR mediates cardioprotection through activation of Akt/eNOS and decreasing apoptosis when exposed to acute myocardial injury, which could be inhibited by GRK2. GRK2 inhibition by β ARKct blocks the negative effects, increases generation of NO, and activates other prosurvival signal, resulting in increased myocyte survival. eNOS, endothelial nitric oxide synthase; NO, nitric oxide. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(33). The synergistic effects appear to be counterintuitive, because β ARKct acts to inhibit GRK2 and re-sensitize β -ARs; while β -blockers block the newly re-sensitized β -ARs. However, the two approaches are acting to achieve the same end goal, which is to stop the up-regulation of GRK2 and down-regulation of β -ARs, thus stopping the damaging CA cycle and resulting in HF. Furthermore, β ARKct expression and GRK2 inhibition result in decreased CA levels, which halt the SNS activation cycle and enable β -ARs to normalize (76, 78). These data demonstrated that inhibition of GRK2 is beneficial in established HF models, and GRK2 could serve as a new drug target to bring additional benefits to β -blocker therapy. This carries prominent translational significance, as more patients now survive an acute cardiac attack and are likely to develop HF; so, it is important to find a new therapeutic strategy to improve cardiac performance or even to reverse the morphological changes.

Since β ARKct functions *via* $G\beta\gamma$ sequestration, the rescue effect of the β ARKct in HF could be due to mechanisms unrelated to GRK2 inhibition. Other cellular $G\beta\gamma$ targets include PI3K (63) and acetylcholine-activated K^+ channels ($I_{K,ACh}$) (38), and both could be protective when activated by $G\beta\gamma$ binding. Alternatively, β ARKct could inhibit the GRK2-mediated phosphorylation of GPCRs other than β -ARs, as GRK2 has many other GPCR targets in the heart. To further clarify that GRK2 is indeed the critical target of β ARKct, cardiac-specific GRK2 KO mice were subjected to permanent MI to induce HF. GRK2 KO mice with the loss of cardiomyocyte GRK2 at birth showed better survival and preserved β -AR signaling post-MI compared with control mice. GRK2 KO mice also had less remodeling and fetal gene activation. The protective phenotype was explained by the fact that GRK2 KO myocytes isolated from post-MI heart displayed improved contractility (77). In a different set of experiments, mice were first subjected to MI to induce LV dysfunction and remodeling, and then, GRK2 gene deletion was induced by tamoxifen injection. Loss of GRK2 in myocytes post-MI also significantly improved cardiac function and survival. More importantly, mice underwent an active reverse remodeling process (77). These data clearly show that in the setting of ischemia-induced HF, GRK2 is pathological and its loss actively reverses LV dysfunction. Moreover, the data indicate that the primary mechanism of β ARKct is through inhibition of GRK2, as GRK2 KO mice and β ARKct mice shared a similar beneficial phenotype in HF. Thus, β ARKct delivery and expression in the heart appears to represent a potential therapeutic strategy in HF.

In addition to β ARKct, other peptides targeting $G\beta\gamma$ -mediated signaling have also been proved to be effective in the treatment for HF. For example, the 197 aa PIK domain peptide of PI3K overexpression in the heart disrupted the GRK2/PI3K complex, prevented endogenous PI3K translocation to the membrane, and altered the intracellular trafficking of β ARs after prolonged agonist stimulation, eventually resulting in preserved β AR membrane levels and activity. More importantly, the PIK domain peptide overexpression in failing cardiomyocytes reversed already established β AR abnormalities (69). PIK overexpression differs from β ARKct overexpression in the fact that it does not prevent GRK2 membrane translocation and phosphorylation of β ARs, although it preserves levels and functions of agonist-accessible β ARs. In a recent study, two small mol-

ecules that selectively inhibit $G\beta\gamma$ interactions had been reported to block the progression of hypertrophy and cardiac dysfunction in murine models of HF. The major mechanism of the small molecules appears to be interfering with GRK2 membrane recruitment. Overexpression of small peptides or small molecules that are capable of disrupting the GRK2/ $G\beta\gamma$ /PI3K protein-protein interaction represents a novel approach to restore β AR function in HF (12).

Cardiac GRK2 Inhibition via Gene Delivery

At the moment, β ARKct gene delivery in the heart may represent a better option to inhibit GRK2 compared with pharmacological compounds. Although no specific GRK2 inhibitor has been identified, a recent paper demonstrated that the selective serotonin re-uptake inhibitor paroxetine is also a potent inhibitor of GRK2 (87), which could serve as a structural model for the design of new compounds to inhibit GRK2; however, that process may be rather time consuming. In addition, due to the ubiquitous expression of GRK2 throughout the body, for example in the brain, liver, and immune system, it would be difficult to avoid the unwanted effects due to the inhibition of GRK2 in other organs. In contrast, virus-mediated gene delivery of β ARKct could be more cardiac specific by using a cardiac myocyte-specific promoter or vectors that are more likely to accumulate in the heart.

Adenoviral-mediated β ARKct delivery has already been tested in larger animal models of HF to evaluate the ultimate clinical relevance of GRK2 inhibition. Adenoviral-mediated β ARKct overexpression in failing myocytes isolated from spontaneously hypertensive heart failure rats led to significant increases in basal and β -AR-stimulated cAMP production, which was even higher than in nonfailing myocytes (26). Single-cell contraction was also improved by β ARKct. Importantly, similar results were obtained in failing human ventricular myocytes (97). Adenoviral-mediated β ARKct overexpression has also improved cardiac function and β -AR signaling in *in vivo* studies using rat and rabbit HF models induced by MI, post cardioplegic arrest, or pacing (1, 86, 96), proving the efficacy of β ARKct overexpression even when the expression is only transient.

To improve on the limited chronic effects of adenoviral-mediated β ARKct levels, recent efforts have focused on the use of adeno-associated viral (AAV) vectors, which support long-term transgene expression (93). Another main advantage of using AAV vectors is they show limited immune responses, which makes them more amenable for human use especially in chronic diseases such as HF. Furthermore, AAV6 and AAV9 have been reported to have some cardiac tropism, which makes them an even more attractive carrier for treatment of cardiac diseases (100). Recently, a more clinically relevant study was undertaken in which AAV6- β ARKct was directly delivered to rat hearts at 12 weeks after cryo-infarction-induced HF, and the rats were followed chronically for 3 months. Echocardiography showed that β ARKct improved cardiac contractility and even reversed LV remodeling, while the hearts that received control virus continued to deteriorate. The circulating neurohormonal (CA and aldosterone) status of the chronic HF animals, as well as β AR signaling was normalized by β ARKct (78). Similar results were recently obtained in a porcine model of HF with AAV6-mediated expression of β ARKct using a percutaneous

catheter-mediated approach, which is clinically applicable (76). These results demonstrate the potential for β ARKct gene therapy in HF.

Regulation of GRK2 Activity

Since GRK2 appears to be crucial in cardiac physiology and pathophysiology, it is of great importance to elucidate the cellular mechanisms that regulate GRK2 activity. The far N terminus of GRK2 contains a Ca^{2+} /calmodulin binding site. Binding of Ca^{2+} /Calmodulin to this site inhibits GRK2 activity (18). Thus far, three major phosphorylation sites have been identified within GRK2; a PKC site at Ser29, an ERK site at Ser670, and a PKA site at Ser685. PKC phosphorylation activates GRK2 by relieving the tonic inhibition by Ca^{2+} /camodulin (17, 51) (see Fig. 2). It has recently been reported that mechanical stretch of cardiac myocytes activated G α_q and PKC, which resulted in the phosphorylation of GRK2 at Ser29 and was considered to account for impaired β -AR signaling afterward. The cross-talk between hypertrophic Gq and β -AR Gs pathways might be considered an important mechanism underlying the transition from hypertrophy to HF in that model (24, 56). PKA phosphorylation of GRK2 on Ser685 activates GRK2 by promoting G $\beta\gamma$ binding and, thus, enhances the ability of the kinase to translocate to the membrane (21). Although ERK phosphorylation of GRK2 at Ser670 was shown to decrease GRK2 kinase activity (73), it also enhanced GRK2 binding to heat shock protein 90 (Hsp90), which chaperoned GRK2 to mitochondria, where it was shown to play a role in cell death (13). Cyclin-dependent kinase 2 (CDK2) also phosphorylates GRK2 at Ser670, which impacts cell cycle arrest and possibly apoptosis (67).

Recent attention has been given to another form of post-translational modification of GRK2, S-nitrosylation. GRK2 was reported to be S-nitrosylated by NO with the primary site being Cys340, which resulted in the inhibition of GRK2 activity on β -AR signaling and other downstream targets. NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) treatment reduced *in vivo* NO production and accelerated Iso-mediated β -AR desensitization in the heart as determined by LV dP/dtmax decline within 30 min of Iso treatment (95). This suggests that NO bioavailability preserves β -AR signaling in the heart through GRK2 inhibition. Consistent with this, in s-nitrosoglutathione reductase knockout mice (GSNOR^{-/-}) where there was more endogenous NO due to the deficiency in the breakdown of active GSNO, there was increased membrane β -AR density (95). Mechanistic studies in cells demonstrated that the NO donor CysNO inhibited GRK2 mediated events, including β -AR phosphorylation and subsequent β -arrestin binding to the receptor as measured by FRET and receptor internalization (95). In addition to GRK2, other proteins involved in endocytosis such as β -arrestin (65) and dynamin (94) are also S-nitrosylated. This novel form of post-translational regulation has important therapeutic significance, as during HF there is both deficiency of NO and desensitized β -AR signaling. The significance of S-nitrosylation as an endogenous GRK2 inhibitor suggests new potential approaches to treat ischemic heart disease and HF.

GPCR Independent Functions of GRK2 in the Heart

In addition to its canonical GPCR kinase activity, emerging new data suggest that GRK2 may have other functions

which are independent from its actions on GPCRs. The recent new concept of an extensive “GRK2 interactome” refers to the possibility that GRK2 may interact with other intracellular proteins for its function. GRK2 has been reported to interact with tubulin (71), p38 (68), Akt (54), caveolin (10), ERK1/2 (73), and HDAC6 (66) in other tissues, which merits investigation in the heart, as those proteins are reported to be critical regulators of cardiac pathology.

As discussed earlier, during conditions of oxidative stress, ERK is activated and the phosphorylation of GRK2 by ERK facilitated the binding to Hsp90, which resulted in the translocation of GRK2 to the mitochondria. Increased mitochondrial GRK2 resulted in mitochondria with decreased Ca^{2+} uptake capacity, which was proposed to play a role in the pro-death effects of this kinase (13). Blocking the ERK-dependent phosphorylation of GRK2 by β ARKct resulted in cardioprotection post I/R (Fig. 4). The mechanistic details of how GRK2 exerts its negative effects on mitochondria need further investigation. In GRK2 Tg hearts, I/R resulted in less activation of Akt and endothelial NOS (eNOS); while β ARKct showed opposite effects and increased NO production (6) (Fig. 3). Interestingly, Akt, eNOS, and Hsp90 have been reported to interact with each other and converge on the production of NO (23, 28). It is possible that GRK2 is an important component of this complex and may be the eventual nodal point. GRK2 could also bind to and phosphorylate Smad2/3, which could have a significant impact on TGF β mediated fibrosis and remodeling post M/I (37). GRK2 interacts with and phosphorylates tubulin in bovine brain (71) and facilitates the polymerization of tubulin into microtubules (98). An increased ratio of microtubules to tubulin in the heart has been reported to be pro-hypertrophic (14). GRK2 has also been reported to directly associate with and phosphorylate HDAC6 to stimulate its α -tubulin deacetylase activity at the leading edge of epithelial cells, promoting local tubulin deacetylation and modulating motility and cellular spreading. Whether this GRK2-HDAC6 interaction has important impacts in the heart that are worth further studying needs to be investigated, as cardiac HDAC6 activity is also increased in cardiac fibroblasts under stressed conditions (52).

Another interesting and new finding of the GPCR independent functions of GRK2 is that GRK2 interacts with and phosphorylates insulin receptor substrate-1 (IRS1) at Ser307, which inhibits the binding of IRS1 to the insulin receptor and blocks the downstream signaling, including GLUT4 translocation to the cell membrane (19). Therefore, under stressed condition, increased expression of GRK2 would promote insulin resistance and impair the cardiac glucose metabolism, which would result in disease progression.

Role of β -AR and GRK2 in the Generation of Oxidative Stress

ROS, such as superoxide ($\text{O}_2^{\cdot-}$) and H_2O_2 , play an important role in the life and death of cardiac myocytes. Under pathological conditions, electron leakage from the electron transport chain in mitochondria is the major source of ROS, which is caused by malfunction of mitochondrial proteins (41). ROS are also generated by $\text{O}_2^{\cdot-}$ producing enzymes, such as NADPH oxidases (Noxs) (5), xanthine oxidase, cytochrome P450, and uncoupled NOS (30). NO contains an unpaired electron, and under certain conditions can react with

FIG. 4. Proposed model for the pro-death effects of mitochondrial GRK2 in the heart under stressed conditions. GRK2 interacts with Hsp90 under basal conditions (A). During periods of ischemic and oxidative stress (B), this interaction increases and facilitates the translocation of GRK2 to mitochondria. The translocation is dependent on the phosphorylation of GRK2 by ERK at Ser670. Increased GRK2 at the mitochondria results in decreased Ca^{2+} uptake capacity, resulting in opening of the MPTP at a lower Ca^{2+} threshold. Hsp90, heat shock protein 90; MPTP, mitochondria permeability transition pore; ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

O_2^- , which may result in adverse effects to the tissue because of the quenching of bioavailable NO. This reaction will form peroxynitrite (ONOO^-), an oxidant that also results in tissue damage. Under pathological conditions when the cellular antioxidant capacity is saturated, excessive ROS reacts with lipids in the cell membrane, DNA, and proteins and results in cell rupture and death (36). Finally, ROS results in the opening of the mitochondria permeability transition pore, which would trigger a series of events, including mitochondria matrix swelling, outer membrane rupture, release of cytochrome c, and apoptosis.

ROS overproduction plays significant roles in different cardiovascular disease models. During I/R, increased ROS during reperfusion results in tissue necrosis (99) and myocardial stunning (75). During the remodeling process that occurs after acute I/R, ROS facilitates development of HF by activating matrix metalloproteinases to restructure extracellular matrix (57), and participates in the development of compensatory hypertrophy by serving as signaling molecules downstream of α -AR activation (2).

β -ARs and GRK2 have been reported to regulate the generation of ROS in the heart, which may have important implications in the pathogenesis of cardiac diseases. ROS could be generated by either a β -AR-dependent mechanism or monoamine oxidases (MAOs), which have been reported to catabolize NEpi and generate hydrogen peroxide in a pressure-overload mouse model (45, 46). β -AR agonists stimulate apoptosis in cultured adult rat ventricular myocytes, which is inhibited by antioxidant enzyme overexpression, proving that ROS is necessary for the apoptosis observed in this model. β 3-AR activation in the heart is reported to be protective (64). As shown in a recent study, β 3-AR-specific agonist BRL 37344 treatment for 3 weeks post TAC protected the heart from developing hypertrophy and HF. The primary downstream mechanism is up-regulation of neuronal NOS (nNOS), increased generation of NO, and

decreased ROS production (64). β 3-AR and nNOS may, thus, function together to maintain NO and ROS balance in the failing heart. Recent investigation in the cardiac H9C2 cell line suggested that the ROS generated by β -AR stimulation was generated by NOX4, whose expression was up-regulated 2.5-fold by Iso. Surprisingly, the changes in ROS and Nox4 were blocked by β ARKct overexpression, suggesting that GRK2 might regulate apoptosis *via* Nox4 and oxidative stress in the heart (88). This is interesting and worthy of further exploration, as GRK2 was able to translocate to the mitochondria, which is also the location of Nox4. Lastly, as mentioned earlier, it has been reported that GRK2 regulates the generation of NO in the heart (6), which might affect the nitroso-redox balance. Regulating the nitroso-redox balance was proposed to be the mechanism for the beneficial outcome observed in a recent African American HF Trial (34, 85). In that trial, a combined therapy of isosorbide dinitrate and hydralazine, a vasodilator that also inhibits generation of O_2^- , decreased mortality by about 45% in African Americans with severe HF (85).

Innovation

This review is the first that discusses the mitochondrial localization and nitrosylation of GRK2, as well as the potential involvement of GRK2 in apoptosis *via* Nox4 and oxidative stress in the heart. A thorough understanding of the earlier hypothesis will further elucidate the contribution of GRK2 in apoptosis and HF, and potentially provide new avenues for therapeutic modulation of GRK2 in the heart.

Conclusion

As discussed in this review, β -ARs and GRK2 are intriguing targets for future HF therapy. Animal models with β ARKct overexpression prove the promising aspects of specifically targeting GRK2 in the heart to prevent and treat

HF. Small molecules that could specifically inhibit GRK2 activity or its interaction with downstream signaling complexes in a tissue-specific manner could obtain similar beneficial results. These strategies could offer synergistic beneficial effects to β -blocker, the current mainstay therapy for HF. Recent progress in investigating the noncanonical roles of GRK2, including an interaction with other signaling molecules in the heart, translocation to the mitochondria post injury, cross-talk with NOS and NO generation, and potential roles in ROS generation, opens up the possibility to develop new treatment options.

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Abbreviations Used

β -AR = β -adrenergic receptors
AAV = adeno-associated virus
AC = adenylyl cyclase
AT1-R = angiotensin II receptors, type1
CA = catecholamine
cAMP = cyclic AMP
CDK2 = cyclin-dependent kinase 2
CSQ = calsequestrin
EF% = ejection fraction
eNOS = endothelial nitric oxide synthase
Epi = epinephrine
GPCR = G-protein-coupled receptor
GRK2 = G-protein-coupled receptor kinase 2
HF = heart failure
Hsp90 = heat shock protein 90
I/R = ischemia/reperfusion
 $I_{K,Ach}$ = acetylcholine-activated K^+ channels
IRS1 = insulin receptor substrate-1
Iso = isoproterenol

L-NAME = N(G)-nitro-L-arginine methyl ester
LV = left ventricular
MAO = monoamine oxidase
MI = myocardial infarction
MPTP = mitochondria permeability transition pore
NEpi = norepinephrine
NO = nitric oxide
NOS = nitric oxide synthase
Nox = NADPH oxidase
ONOO⁻ = peroxynitrite
PDE = phosphodiesterase
PH = pleckstrin homology
PI3K = phosphatidylinositol 3-kinase
PIP2 = phosphatidylinositol 4,5-bisphosphate
PKA = protein kinase A
PKC = protein kinase C
RGS = regulator of G-protein signaling
ROS = reactive oxygen species
SNS = sympathetic nervous system
TAC = transthoracic aorta constriction