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A peptide of the RGS domain of GRK2 binds and inhibits G_{α_q} to suppress pathological cardiac hypertrophy and dysfunction

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Abstract

G protein-coupled receptor (GPCR) kinases (GRKs) play a critical role in cardiac function by regulating GPCR activity. GRK2 suppresses GPCR signaling by phosphorylating and desensitizing active GPCRs, and through protein-protein interactions that uncouple GPCRs from their downstream effectors. Several GRK2 interacting partners, including G_{α_q} , promote maladaptive cardiac hypertrophy, which leads to heart failure, a leading cause of mortality worldwide. The regulator of G protein signaling (RGS) domain of GRK2 interacts with and inhibits G_{α_q} in vitro. We generated Tg β ARKrgs mice with cardiac-specific expression of the RGS domain of GRK2 and subjected these mice to pressure overload to trigger adaptive changes that lead to heart failure. Unlike their nontransgenic littermate controls, the Tg β ARKrgs mice exhibited less hypertrophy as indicated by reduced left ventricular wall thickness, decreased expression of genes linked to cardiac hypertrophy, and less adverse structural remodeling. The β ARKrgs peptide, but not endogenous GRK2, interacted with G_{α_q} and interfered with signaling through this G protein. These data support the development of GRK2-based therapeutic approaches to prevent hypertrophy and heart failure.

INTRODUCTION

GRK2 [G protein-coupled receptor (GPCR) kinase 2, also known as β -adrenergic receptor kinase 1 (β ARK1)] regulates GPCR-mediated intracellular signaling by phosphorylating agonist-occupied receptors to promote the binding of β -arrestin, thereby sterically blocking further G protein activation and attenuating downstream effector signaling (1–3). Ongoing

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research has demonstrated great diversity in the functional roles of GRK2, including phosphorylation of non-GPCR substrates and numerous phosphorylation-independent regulatory protein-protein interactions (3–5). GRKs have a tridomain structure with a central catalytic domain, flanked by N- and C-terminal domains containing elements involved in GRK regulation as well as key binding elements for effectors and protein-protein interactions. One such binding element is the regulator of G protein signaling (RGS)–like domain that has been identified within the N terminus (residues 51 to 173) of GRK2, which binds to and regulates G_{α_q} activity in vitro (6–9). Classically, RGS proteins bind to G_{α} proteins, allosterically increasing their rate of guanosine 5'-triphosphate (GTP) hydrolysis and thereby inhibiting their activity (8, 10). Unlike canonical RGS proteins, the RGS domain of GRK2 appears to directly interact with G_{α_q} and inhibit signaling without altering its GTPase activity (9, 11, 12). The domain of GRK2 responsible for G_{α_q} binding appears to be distinct from the binding sites for RGS4 and RGS9, suggesting potential distinct functions of the GRK2 RGS peptide within its N terminus (8, 13).

G_{α_q} is the final common trigger of maladaptive cardiac hypertrophy after pressure overload (14). Left ventricular hypertrophy and remodeling are often seen in hypertensive patients as an adaptive response to normalize cardiac wall stress in response to the increased pressure required to overcome systemic hypertension and achieve appropriate delivery of blood to the tissues (14–17). Left ventricular hypertrophy alters cellular signaling within cardiomyocytes and cardiac fibroblasts, increasing gene transcription, apoptosis, and fibrosis as well as altering coronary circulation (14–17). This cardiac remodeling, particularly in the presence of diabetes, obesity, high salt intake, or genetic factors, may become maladaptive and contribute to cardiac decompensation with thinning of the left ventricular wall, chamber dilation, and contractile dysfunction, resulting in heart failure (14–17). Transgenic mice with cardiac-specific overexpression of G_{α_q} have a phenotype of decompensated cardiac hypertrophy (18–20), whereas cardiac overexpression of RGS4 blocks G_{α_q} -mediated hypertrophy and heart failure (10). Furthermore, transgenic mice with cardiac expression of a G_{α_q} inhibitor ($G_{\alpha_q}I$), which is the last 50 amino acids of murine G_{α_q} that targets the G_{α_q} -activated $G_{\alpha_q}PCR$ interface, have significantly lessened ventricular hypertrophy and ventricular dysfunction after pressure overload (21–23). Therefore, a selective interaction of the GRK2 RGS domain with G_{α_q} may alter hypertrophic responses, revealing a role for GRK2 and a potential therapeutic target to limit maladaptive cardiac hypertrophy. We have begun to address this by generating transgenic mice with cardiac-specific expression of the RGS domain of GRK2 (we have termed this peptide $\beta ARKrgs$). Further studies use transgenic lines representing a G_{α_q} -mediated antihypertrophic phenotype as well as lines that are susceptible to or protected from heart failure to further elucidate the mechanism of action of the $\beta ARKrgs$ peptide. Using a transaortic constriction (TAC) model of pressure overload to trigger cardiac hypertrophy followed by chronic de-compensation and heart failure in these mouse lines, we investigated whether the $\beta ARKrgs$ could serve as a noncanonical inhibitor of G_{α_q} -mediated hypertrophic signaling in the heart and pave the way for novel GRK2-based therapeutic approaches to prevent maladaptive hypertrophy.

RESULTS

β ARKrgs transgenic mice exhibit an antihypertrophic phenotype after pressure overload

To generate the Tg β ARKrgs line, the cDNA that encodes bovine GRK2 residues 50 to 185 was cloned into a vector driven by the α -myosin heavy chain (*MYH6*) promoter with a C-terminal Flag tag. Transgenic mice did not show gross phenotypic changes compared to nontransgenic littermate control mice. Transgenic expression was confirmed by Western blotting of cardiac lysates using a mouse monoclonal Flag antibody to detect the ~17-kD band (Fig. 1A). We analyzed the cardiovascular function and adrenergic responsiveness of the Tg β ARKrgs mice using terminal hemodynamics at baseline and upon challenge with increasing doses of the β AR agonist isoproterenol in transgenic and nontransgenic mice. No difference was observed in mean systemic pressure (Fig. 1B), heart rate responses to isoproterenol (Fig. 1C), or dP/dt maximum and minimum, measures of the rate of increase in left ventricular pressure during systole and decrease in pressure during diastole that indicate cardiac contractility and relaxation, respectively, at baseline and in response to isoproterenol (Fig. 1, D and E). These data demonstrate that β AR responsiveness was not altered in the Tg β ARKrgs animals.

To determine whether the expression of the RGS peptide of GRK2 could alter $G\alpha_q$ -mediated signaling and hypertrophy in an animal model of pressure overload, Tg β ARKrgs and nontransgenic mice underwent TAC or sham surgery (24), and echocardiography was performed at baseline and at 2 and 4 weeks to monitor cardiac function and dimensions. The systolic pressure gradient as measured by pulsed wave Doppler of the aortic arch 24 hours after surgery confirmed TAC and showed no differences between nontransgenic and transgenic groups (fig. S1A). Over this time course, we typically found no effect of TAC on left ventricular ejection fraction (Fig. 2A) or fractional shortening (fig. S1B), two parameters that measure the efficiency of the heart to eject blood with each contraction. However, there was hypertrophic growth of the heart in this time period as determined by several parameters. Left ventricular posterior wall thickness during systole increased soon after TAC by about 47% in non-transgenic mice compared to an about 19% increase in the Tg β ARKrgs animals (Fig. 2B). Further, the left ventricular posterior wall thickness during diastole significantly increased in nontransgenic mice but not in transgenic mice, despite similar systolic pressure gradients (Fig. 2C). The pronounced post-TAC growth in the left ventricular mass in the nontransgenic mice (a 109% increase) was also significantly blunted in the β ARKrgs mice (a 47% increase) (Fig. 2D). Tg β ARKrgs mice also had reduced heart weight normalized to tibia length after TAC, although there was no difference between transgenic and nontransgenic sham controls (Fig. 2E) or in tibia length (fig. S1C). Furthermore, left atrial weight was preserved in Tg β ARKrgs animals (Fig. 2F). These data support a notable antihypertrophic effect of β ARKrgs peptide expression after TAC.

We next investigated whether the antihypertrophic phenotype of the Tg β ARKrgs mice was consistent with inhibition of $G\alpha_q$ -mediated signaling. We used mice with *MYH6*-driven expression of the C-terminal 50 amino acids of murine $G\alpha_q$ (G_qI), a peptide that competes with $G\alpha_q$ for binding to GPCRs (21). This genetic manipulation inhibits $G\alpha_q$ -mediated signaling, particularly 7 days after TAC, and the mice show blunted responses to $G\alpha_q$ -

coupled GPCR agonists, as measured by diminished G_{α_q} -mediated activation of phospholipase C (PLC) and production of the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) and downstream activation of mitogen-activated protein kinase (MAPK), and reduced left ventricular weight (21–23). Similar to the Tg β ARKrgs mice, the Tg G_{α_q} I animals showed decreased hypertrophy 4 weeks after TAC because the significant increases in left ventricular posterior wall thickness during systole (Fig. 2G) and diastole (fig. S1D) and left ventricular mass (Fig. 2H) in nontransgenic mice were decreased in G_{α_q} I mice. Furthermore, heart weight normalized to tibia length was also reduced to a similar extent in Tg G_{α_q} I mice after TAC as in β ARKrgs mice (Fig. 2I). These data are consistent with the β ARKrgs peptide inhibiting pressure overload–induced hypertrophy through altered G_{α_q} -mediated signaling, thereby imparting a similar degree of protection as that seen with G_{α_q} I.

To further elucidate whether the phenotype of the mice resulted from a selective interaction of β ARKrgs with G_{α_q} rather than a GRK2-mediated effect, we used two different mouse lines. The first line was cardiac-targeted β ARKct transgenic mice (Tg β ARKct), which express a peptide inhibitor of GRK2 that elicits cardioprotection by binding to $G\beta\gamma$ in place of GRK2. Thus, $G\beta\gamma$ facilitates the membrane translocation of β ARKct, which does not contain the catalytic domain, rather than the full-length GRK2 (25). The second line was cardiac GRK2 overexpressing mice (TgGRK2) in which GRK2 abundance is similar to that observed in failing human hearts (26). Unlike β ARKrgs and G_{α_q} I transgenic mice, cardiac hypertrophy was unaltered in Tg β ARKct or TgGRK2 mice after TAC relative to their nontransgenic controls (Fig. 3). Left ventricular posterior wall thickness during systole and left ventricular mass significantly increased to a similar extent in Tg β ARKct or TgGRK2 mice and their respective nontransgenic controls after TAC (Fig. 3, A to D). Heart weight normalized to tibia length and left atrial weight increased to a similar extent in Tg β ARKct and TgGRK2 mice as their nontransgenic controls (Fig. 3, C to H). Together, these data demonstrate that peptide inhibition or, conversely, overexpression of full-length GRK2 does not alter cardiac hypertrophy, further supporting our hypothesis that the RGS domain of GRK2 is a distinct interaction that prevents hypertrophic signaling in the heart. These data also suggest that the RGS domain within full-length GRK2 does not appear to substantially interact with G_{α_q} in cardiomyocytes even when overexpressed.

β ARKrgs expression protects against decompensated hypertrophy and heart failure after pressure overload

To investigate whether the antihypertrophic effect of β ARKrgs expression could impart cardioprotection, we monitored cardiac function in a cohort of mice up to 14 weeks after TAC with serial echocardiography. Nontransgenic mice exhibited pronounced left ventricular decompensation from weeks 10 to 14, exhibiting significant chamber dilation, thinning of the ventricular wall, and contractile dysfunction with a reduction in ejection fraction from ~60% at baseline to 31% at 14 weeks after TAC (Fig. 4A) and a corresponding reduction in fractional shortening (fig. S2A). In contrast, ejection fraction was stable in Tg β ARKrgs mice throughout the chronic time course and similar to the sham controls, despite similar systolic pressure gradients (fig. S2B). Although left ventricular mass continually expanded in nontransgenic mice, this measure increased much more slowly in

Tg β ARKrgs mice from 4 to 14 weeks after TAC (Fig. 4B). Similar trends were observed for left ventricular internal diameter during systole and left ventricular posterior wall thickness during diastole (fig. S2, C and D). The progressive reduction in left ventricular posterior wall thickness during systole that began in nontransgenic mice at 8 weeks after TAC, an index of decompensation, was absent in the Tg β ARKrgs mice (Fig. 4C). Furthermore, the increase in lung weight due to pulmonary congestion, which is characteristic of heart failure, was prevented in Tg β ARKrgs mice, and the left atrial weight was preserved (Fig. 4, D and E). Also, heart weight normalized to tibia length did not increase to the same extent in Tg β ARKrgs mice as in nontransgenic mice, and the tibia length did not differ between these two groups (fig. S2, E and F). These data confirm that the reduction in hypertrophy triggered by β ARKrgs expression was sufficient to prevent decompensation and heart failure.

The β ARKrgs peptide limits adverse ventricular remodeling after pressure overload

Reverse transcription polymerase chain reaction (RT-PCR) revealed that hypertrophic gene expression, including members of the fetal gene program such as *ANF* (which encodes atrial natriuretic factor), *BNP* (which encodes brain natriuretic peptide), and β *MHC* (which encodes β -myosin heavy chain), was increased after TAC to a lesser extent in both Tg β ARKrgs and TgG_qI mice than in their nontransgenic controls (Fig. 5, A to F, and fig. S3, A to C). These data indicate that the β ARKrgs peptide prevented the molecular alterations that occur during hypertrophy and are consistent with our echocardiography results. At the protein level, the abundance and activity of GRK2 are increased after cardiac injury, when it serves as a prodeath kinase in the heart (27–29). We therefore measured GRK2 abundance by Western blot and found that the increase in GRK2 in nontransgenic mice was attenuated in Tg β ARKrgs mice (Fig. 5G and fig. S3D). GRK2 abundance was also lower after TAC in Tg β ARKct mice (Fig. 5H and fig. S3E), although hypertrophic gene induction was not altered in these mice after TAC (fig. S3, A to C). Thus, the β ARKct-mediated decrease in GRK2 abundance and activity after TAC was not sufficient to prevent maladaptive hypertrophy, further suggesting that the β ARKrgs peptide prevents hypertrophic signaling through a distinct mechanism.

To visualize the effect of hindering molecular responses to TAC on myocardial structure, we performed wheat germ agglutinin (WGA) staining to measure cardiomyocyte cross-sectional area (CSA) in these Tg β ARKrgs and nontransgenic mice 4 weeks after TAC. Although we found no difference between the sham groups, the increase greater than twofold in CSA in the nontransgenic mice was significantly blunted with β ARKrgs peptide expression (Fig. 6, A and B), confirming reduced cardiomyocyte hypertrophy. Furthermore, we performed Masson trichrome staining on paraffin sections taken at the level of the aortic outflow tract where left ventricular dimensions are at their maximum. Four chamber images of the myocardium revealed no difference in sham hearts but a partial preservation of left ventricular architecture in the Tg β ARKrgs hearts 4 weeks after TAC compared to nontransgenic mice (Fig. 6C). To further investigate the integrity of the myocardium, we analyzed higher-magnification images of the left ventricle (Fig. 6D) and found that interstitial fibrosis was significantly reduced in Tg β ARKrgs mice compared to nontransgenic mice 4 weeks after TAC, with no difference in the total tissue area (Fig. 6E

and fig. S4). Together, these data demonstrate that β ARKrgs peptide expression reduces both adaptive and maladaptive left ventricular remodeling after TAC.

β ARKrgs sequesters $G\alpha_q$ from effector binding to prevent downstream signaling

To confirm our hypothesis that the β ARKrgs peptide inhibits $G\alpha_q$ -mediated hypertrophic signaling by directly interacting with $G\alpha_q$, we performed immunoprecipitation assays on nontransgenic and Tg β ARKrgs mice at baseline and 4 weeks after sham or TAC surgery. Flag-tagged β ARKrgs coimmunoprecipitated with $G\alpha_q$ (Fig. 7A), and this coimmunoprecipitation was significantly increased after pressure overload with no difference in total immunoprecipitated β ARKrgs-Flag (Fig. 7, B and C, and fig. S5, A to C), indicating that $G\alpha_q$ -coupled GPCR activation and dissociation of $G\alpha_q$ facilitate binding of the β ARKrgs peptide to $G\alpha_q$. Further, Flag-tagged β ARKrgs did not coimmunoprecipitate with $G\alpha_s$ or $G\alpha_i$ at baseline or 4 weeks after TAC (fig. S5, D to G), demonstrating the specificity of the binding of β ARKrgs to $G\alpha_q$. That GRK2 overexpression did not alter the cardiac hypertrophic response suggests that the RGS domain within full-length GRK2 does not appear to interact with $G\alpha_q$ in cardiomyocytes even when overexpressed. Therefore, it was important to determine the extent of the interaction between full-length GRK2 and $G\alpha_q$ and how this differed from that of β ARKrgs. We performed immunoprecipitations on cardiac tissue from nontransgenic and Tg β ARKrgs mice at baseline and 4 weeks after TAC and found that despite efficient immunoprecipitation, $G\alpha_q$ did not associate with full-length GRK2 in sham or post-TAC animals (Fig. 7D and fig. S5, A and B). We next investigated whether this interaction translated to a functional effect by measuring $G\alpha_q$ -mediated activation of PLC by quantifying IP_3 . As expected, IP_3 concentrations were increased in nontransgenic mice after TAC, presumably as a result of enhanced $G\alpha_q$ -coupled GPCR activation; however, this increase was partially attenuated by β ARKrgs peptide expression (Fig. 7E). Together, these data support our hypothesis that the β ARKrgs peptide inhibits $G\alpha_q$ -mediated hypertrophic signaling by directly interacting with $G\alpha_q$.

DISCUSSION

Here, we investigated whether the RGS domain within the N terminus of GRK2 inhibited $G\alpha_q$ -mediated hypertrophic signaling in the heart. Mice with cardiac-specific Tg β ARKrgs expression were not only resistant to hypertrophic growth after TAC, but also protected from heart failure during chronic pressure overload. Tg β ARKrgs expression provided the same antihypertrophic effect as inhibition of $G\alpha_q$ receptor association (Tg G_qI) and reduced several markers of maladaptive remodeling after TAC. The ability to selectively inhibit $G\alpha_q$ -mediated signaling may offer an advancement in the treatment of human hypertrophy and heart failure because $G\alpha_q$ represents the common driving force for maladaptive cardiac hypertrophy (Fig. 8) (14, 21).

Activated $G\alpha_q$ interacts with the N terminus of GRK2, which in vitro inhibits $G\alpha_q$ -mediated activation of PLC β (6). This inhibition is independent of the kinase activity of GRK2, is enhanced in the presence of agonist, and occurs not only upon GPCR stimulation but also in the presence of constitutively active $G\alpha_q$, supporting the concept that it is due to a direct interaction with $G\alpha_q$ (6). Biochemical and structural studies have mapped the residues

important for this physical interaction, specifically identifying a critical proline residue unique to $G\alpha_q$ that is absent in $G\alpha_i$ or $G\alpha_s$ (13, 30). Mutating the residues important for association with GRK2 does not alter the binding of RGS4 or RGS2 to $G\alpha_q$ because GRK2 does not use the RGS binding domain on $G\alpha_q$ (30). Rather, GRK2 binds within the effector binding domain of $G\alpha_q$ that facilitates the association of $G\alpha_q$ with effector proteins such as PLC for downstream signaling (30, 31). Further, the RGS domain of GRK2 binds $G\alpha_q$ through an α -helix domain similar to that in both PLC β and Rho-GEF, using a conserved leucine structurally similar to their ALXXPI binding motif to associate with the effector binding domain of $G\alpha_q$ (31). These studies suggest that the direct interaction of the RGS domain of GRK2 with $G\alpha_q$ acts in competition with binding to other $G\alpha_q$ effector proteins, thereby inhibiting downstream signaling. This is consistent with our data demonstrating reduced IP₃ production 4 weeks after TAC in the Tg β ARKrgs compared to nontransgenic mice, suggesting an inhibition of $G\alpha_q$ -mediated signaling through PLC. In particular, this $G\alpha_q$ -centric mechanism of inhibition could apply to any GPCR coupled to $G\alpha_q$ and various effector proteins within a given cell type. Although we did not see adverse phenotypes in our cardiac-specific Tg β ARKrgs animals, it will be important to determine the potential effects of such an inhibitor of $G\alpha_q$ signaling within other cell types to determine the optimal path for therapeutic development.

Notably, we observed no antihypertrophic effect with cardiac GRK2 over-expression up to 4 weeks after TAC. These data are in contrast to numerous in vitro studies showing that binding of GRK2 as well as the N-terminal RGS domain inhibit effector signaling to an equal extent and that increased $G\alpha_q$ -coupled GPCR activation acts as a driving force to enhance GRK2 association with $G\alpha_q$ (6). It is difficult to extrapolate whether such interactions will occur in the complex milieu in vivo, as opposed to the overexpression of a limited number of interacting partners in an in vitro system. The lack of interaction between full-length GRK2 and $G\alpha_q$ at baseline or during pressure overload was consistent with our data demonstrating the lack of anti-hypertrophic effect of GRK2 overexpression. It is possible that whereas GRK2 can bind to $G\alpha_q$, as demonstrated in vitro, other protein-protein interactions that occur within the holoenzyme sterically or allosterically block this interaction in vivo. Two such presumed N-terminal GRK2 binding partners are caveolin (residues 63 to 71) (32) and calmodulin (residues 18 to 37) (33), but these interactions and their functional consequences have not been confirmed in vivo or characterized. Notably, the in vitro interaction between GRK2 and $G\alpha_q$ is enhanced by c-Src kinase-mediated phosphorylation of GRK2 that can be regulated by $G\alpha_q$ -coupled GPCR activation, such as the M1-muscarinic receptor (34). Of the tyrosine residues targeted by c-Src (Tyr¹³, Tyr⁸⁶, and Tyr⁹²), two are within the β ARKrgs. Therefore, it may be interesting to elucidate any differences in the tyrosine phosphorylation of full-length GRK2 compared to the β ARKrgs peptide after TAC. Investigation of the endogenous protein binding partners for β ARKrgs, compared to full-length GRK2, and the regulation of these interactions during cardiovascular disease will be a major focus of future studies.

Given the mild hypertrophy observed in the Tg β ARKrgs mice that is preserved 14 weeks after TAC and does not decompensate to heart failure, one consideration is whether the stress response in these animals represents a “physiological hypertrophy.” Pathological hypertrophy occurs in response to cardiac stress or disease and is irreversible, whereas

physiological hypertrophy occurs with chronic exercise or during pregnancy and is reversible. Numerous functional and biochemical differences have been well characterized, with pathological hypertrophy often identified by interstitial fibrosis, cell death, and dysfunction, whereas physiological hypertrophy presents with a proportional increase in wall thickness and chamber dimension (35, 36). Consistent with this idea is the fact that we observed significantly reduced interstitial fibrosis in our Tg β ARKrgs compared to nontransgenic mice after TAC. Although reduced fibrosis in the Tg β ARKrgs mice after TAC supports the notion of beneficial cardiac growth, this may simply be due to enhanced cardiomyocyte survival in the Tg β ARKrgs heart during pressure overload. Gene profiling may be necessary to assess the type of hypertrophy that occurs when this RGS domain of GRK2 is expressed in the heart.

Moving forward, a main focus of this research will be to determine the therapeutic potential of β ARKrgs to reverse established hypertrophy or heart failure. This work will follow two lines of investigation: one into the gene therapy potential of this peptide delivered exogenously to cardiac injury models, and the other elucidating whether a small-molecule inhibitor could be developed to mimic β ARKrgs activity. Our laboratory has successfully translated the study of the β ARKct peptide of GRK2 from a transgenic murine model to a successful gene therapy in a porcine model of heart failure (37, 38). Furthermore, the use of gene therapy is expanding and is the subject of numerous clinical trials worldwide (39). Alternatively, the concept of a small-molecule mimic of a peptide inhibitor has been supported by work with gallein, a drug that mimics the effect of β ARKct by inhibiting GRK2 coupling to, and translocation by, G $\beta\gamma$ (40, 41). This concept of peptidomimetics is gaining ground as a viable method for structure-based drug design in numerous fields, particularly as an approach to inhibiting highly conserved and yet selective effector sites, such as within large families of related kinases (42).

In summary, we found that transgenic expression of the RGS domain of GRK2 (β ARKrgs) demonstrates a marked antihypertrophic effect in a murine model of pressure overload. The reduction in hypertrophy was similar to that induced by inhibition of G α_q activation and was not replicated by β ARKct or GRK2 overexpression. Further, this inhibition of G α_q signaling was cardioprotective, preventing decompensation to heart failure and supporting a therapeutic role for noncanonical inhibition of G α_q signaling by β ARKrgs. Moreover, β ARKrgs inhibited induction of the fetal gene program, preserved left ventricular structure, and reduced G α_q -mediated downstream signaling through a selective functional interaction. This study emphasizes that investigation of distinct protein-interacting domains of GRK2 may enable the development of GRK2-based therapeutic approaches to prevent hypertrophy and heart failure, and highlights the importance of understanding the functional relevance of the expanding GRK2 interactome.

MATERIALS AND METHODS

Study design

Our central hypothesis was that the RGS domain of GRK2 would act as a noncanonical inhibitor of G α_q -mediated hypertrophic signaling in the heart after TAC. Statistical powering was initially performed using the nQuery Advisor 3.0 software (Statistical

Solutions) for estimation of sample size. For all experiments, the calculations use $\alpha = 0.05$ and $\beta = 0.2$ (power, 0.80). On the basis of these calculations, our target was a minimum of 5 animals (powered for indices of TAC) per group to attain statistical significance, with a preference for 10 or more. Each experiment was performed a minimum of three times. The number and composition of the replicates were determined on the basis of the availability of transgenic and non-transgenic animals, the surgeon, and echocardiography and hemodynamic machinery. All animals and resulting samples were monitored by mouse number only until data quantification was complete, meaning that all data analyses were blinded. Data were then decoded by gene expression and surgical group for statistical analysis. All results were substantiated by repetition. Data were only excluded if their validity was undermined by the condition of the animal or cells before or during the experiment, such as loss of the specimen.

Experimental animals

All animal procedures were carried out according to National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Animal Care and Use Committee of Temple University. To generate the Tg β ARKrgs line, the cDNA that encodes bovine GRK2 residues 50 to 185 was cloned into a vector driven by the *MYH6* promoter with a C-terminal Flag tag, as previously described for β ARKnt (43). For all in vivo experiments, nontransgenic littermate control mice were used. Mice with α MHC-targeted expression of the β ARKct peptide (C-terminal 194 residues) containing the G $\beta\gamma$ binding domain were used as a control for GRK2 inhibition, whereas mice with *MYH6*-targeted expression of GRK2, causing a two- to threefold increase in protein abundance similar to the increase observed during human heart failure, were used as a control for enhanced GRK2 activity. Mice with *MYH6*-targeted expression of the G α I peptide, the last 50 amino acids of murine G α_q , which targets the G α_q -activated GPCR interface, were used as a control for inhibition of G α_q activation.

TAC was performed as described previously (24). To measure global cardiac function, echocardiography was performed at 8 to 10 weeks of age before TAC and at 2 and 4, or 2, 4, 8, 10, 12, and 14 weeks after TAC. In vivo cardiac hemodynamic function was measured at baseline and after administration of the β AR agonist isoproterenol (0.1, 0.5, 1, 5, and 10 ng) as described (44). Echocardiography was performed using the Vevo 2100 imaging system from VisualSonics as described (27). Briefly, two-dimensional echocardiographic views of the midventricular short axis were obtained at the level of the papillary muscle tips below the mitral valve. M-mode measurements were determined at the plane bisecting the papillary muscles according to the American Society of Echocardiography leading-edge method.

RNA isolation and semiquantitative PCR

RNA isolation and analysis was performed as previously described (45). Total RNA isolation was performed using TRIzol reagent (Life Technologies) and a VCX 130PB ultrasonic processor from Sonics and Materials Inc. for homogenization. After RNA isolation, cDNA was synthesized from 1 mg of total RNA using the iScript cDNA Synthesis Kit from Bio-Rad Laboratories. Semiquantitative PCR was carried out on cDNA using SYBR Green (Bio-Rad) and 150 nM gene-specific oligonucleotides for *18S*, *ANF*, *BNP*,

and β MHC on CFX96 Real-Time System with Bio-Rad CFX Manager 2.1 software. Quantitation was established by comparing 18S mRNA, which was similar between groups, for normalization and was compared using the C_t method.

Histological sectioning and staining

Subgroups of hearts were harvested at 4 and 14 weeks after TAC for analyses of structure, fibrosis, and biochemistry. Trichrome staining was performed as previously described (44). Briefly, mice were euthanized, and after cardioperfusion, hearts were fixed for 1 to 3 days in 4% paraformaldehyde at 4°C. Hearts were dehydrated and paraffinized using the Microm STP 120 from Thermo Fisher Scientific, embedded in paraffin using a HistoStar apparatus (Thermo Fisher), and sectioned (4 to 6 μ m) using the Microm HM 325 (Thermo Fisher). Tissue sections were then stained with Weigert's iron hematoxylin and Masson trichrome (Sigma-Aldrich) according to the manufacturer's instructions. Interstitial fibrosis was quantified by color threshold measures using ImageJ. Alternatively, tissue sections were deparaffinized and rehydrated according to the trichrome staining protocol, but after the wash with deionized water, heart sections were washed three times for 5 min with 1 $\times$ phosphate-buffered saline (PBS) followed by staining with Alexa Fluor 488-conjugated WGA (1:10 in 1 $\times$ PBS) (Invitrogen) for 1 hour at room temperature in a humidified chamber in the dark. Sections were again washed three times for 5 min with 1 $\times$ PBS followed by mounting with coverslips using Fluoromount-G mounting media containing DAPI nuclear stain (Southern Biotech).

Immunoblotting and immunoprecipitation

Immunoprecipitation was performed as described previously (6). Briefly, cardiac lysates were centrifuged at 13,000g at 4°C for 30 min, protein concentration was measured using a bicinchoninic acid assay (Pierce), and 1 mg of protein was used for each immunoprecipitation. GRK2 and β ARKrgs-Flag immunoprecipitations were conducted using anti-GRK2 agarose conjugate (Santa Cruz Biotechnology) and anti-Flag M2 affinity gel (Sigma-Aldrich), respectively. Control immunoprecipitations were conducted using normal rabbit or mouse immunoglobulin G-agarose conjugates (Santa Cruz Biotechnology). Western blotting was performed as described previously (45). After SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes, primary antibody incubations were performed overnight at 4°C. Primary antibodies used were as follows: rabbit polyclonal GRK2 (Santa Cruz Biotechnology), monoclonal GRK2 (Millipore), monoclonal GAPDH (Santa Cruz), monoclonal and polyclonal Flag (Sigma-Aldrich), goat polyclonal $G\alpha_{q/11}$ (Santa Cruz Biotechnology), rabbit polyclonal $G\alpha_q$ (Santa Cruz Biotechnology), mouse monoclonal $G\alpha_s$ (Santa Cruz Biotechnology), and rabbit polyclonal $G\alpha_i$ (Santa Cruz Biotechnology). Visualization of Western blot signals was performed using secondary antibodies coupled to Alexa Fluor 680 (Invitrogen Molecular Probes) or IRDye 800 (LI-COR Biosciences) and imaged using the Odyssey CLx infrared imager (LI-COR Biosciences). Odyssey version 1.2 imaging software was used to process all images.

IP₃ quantification

IP₃ concentration was quantified using a mouse IP₃ enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (MyBioSource).

Statistical analysis

All values in the text and figures are presented as means $\pm$ SEM of independent experiments for given n sizes. Statistical significance was determined by two-way ANOVA with Bonferroni post hoc, one-way ANOVA with Tukey post hoc, or Student's t test, as appropriate. Probabilities of 0.05 or less were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Cardiac-specific βARKrgs expression does not alter hemodynamic function

(A) Representative Western blot of TgβARKrgs (Tg) and nontransgenic littermate control (NLC or N) ventricular lysates probed for Flag-tagged βARKrgs and the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (B to E) Hemodynamics were recorded from 10- to 12-week-old TgβARKrgs and nontransgenic mice. Quantification of mean systemic pressure (B), heart rate (C), left ventricular +dP/dt average maximum (D), and left ventricular -dP/dt average minimum (E) at baseline and with increasing doses of isoproterenol. Statistics are relative to non-transgenic littermate control mice by nonparametric Student's *t* test for (B) and two-way analysis of variance (ANOVA) with repeated measures and Bonferroni post hoc test for (C) to (E) as appropriate. The genotype of all animals was confirmed by Western blot before unblinding the analysis and results, meaning *n* > 10 independent experiments for (A); *n* = 12 mice per group for (B) to (E).

Fig. 2. TgβARKrgs mice exhibit a similar decrease in cardiac hypertrophy as TgGqI mice after TAC

(A to F) TgβARKrgs and nontransgenic littermate control sham (Sh) and post-TAC animals were analyzed at baseline and at 2 and/or 4 weeks after surgery. Percent left ventricular ejection fraction (A). Serial measures for left ventricular (LV) posterior wall thickness during systole (LVPWs) (B), diastole (LVPWd) (C), and left ventricular mass (D). Measures of heart weight (HW) normalized to tibia length (TL) (HW/TL) (E) and left atrial weight (F) 4 weeks after surgery. (G and H) TgGqI and nontransgenic sham and post-TAC animals were analyzed at baseline and at 2 and/or 4 weeks after surgery. Serial measures for LVPWs (G) and LV mass (H). (I) Measures of HW/TL. * $P=0.01$; ** $P=0.001$; *** $P<0.0001$ as determined by two-way ANOVA with repeated measures and Bonferroni post hoc test for (A) to (D) and (G) to (H) and by one-way ANOVA with Tukey post hoc test for (E), (F), and (I) relative to nontransgenic sham mice. $^{\text{†}}P=0.01$; $^{\text{†††}}P<0.0001$ by two-way ANOVA with repeated measures and Bonferroni post hoc test relative to nontransgenic TAC mice. $^{\text{†}}P=0.01$ by one-way ANOVA with Tukey post hoc test relative to nontransgenic TAC mice. $n=10$ to 16 mice per group for (A) to (I).

Fig. 3. Hypertrophy is unaltered in Tg β ARKct and TgGRK2 mice after TAC

(A and B) Serial measures of Tg β ARKct and nontransgenic littermate control sham and post-TAC animals for LVPWs (A) and LV mass (B). (C and D) Serial measures of TgGRK2 and nontransgenic sham and post-TAC animals for LVPWs (C) and LV mass (D). (E and F) Measures of HW/TL (E) and left atrial weight (F) in Tg β ARKct and nontransgenic sham and post-TAC animals 4 weeks after surgery. (G and H) Measures of HW/TL (G) and left atrial weight (H) in TgGRK2 and nontransgenic sham and post-TAC animals 4 weeks after surgery. *** $P < 0.0001$ by two-way ANOVA with repeated measures and Bonferroni post hoc test for (A) to (D). * $P = 0.01$; ** $P = 0.001$; *** $P < 0.0001$ by one-way ANOVA with Tukey post hoc test (E to H) relative to corresponding nontransgenic sham mice. $n = 8$ to 14 mice per group for (A) to (H).

Fig. 4. Chronic pressure overload-induced heart failure is inhibited by cardiac β ARKrgs expression

(A to E) Serial measures of nontransgenic littermate control and Tg β ARKrgs sham and post-TAC animals for percent LV ejection fraction (A), LV mass (B), and LVPWs (C). Lung weight normalized to TL (LW/TL) (D) and left atrial weight (E) 14 weeks after surgery. * $P = 0.01$; ** $P = 0.001$; *** $P < 0.0001$ by two-way ANOVA with repeated measures and Bonferroni post hoc test for (A) to (C). *** $P < 0.0001$ by one-way ANOVA with Tukey post hoc test for (D) to (E) relative to nontransgenic sham mice. ††† $P < 0.0001$ by two-way ANOVA with repeated measures and Bonferroni post hoc test relative to nontransgenic TAC mice. $n = 8$ to 15 mice per group for (A) to (E).

Fig. 5. Molecular markers of left ventricular dysfunction are reduced in TgβARKrgs and TgG_qI mice but not in TgβARKct mice

(A to F) Quantification of RT-PCR data in nontransgenic littermate control and transgenic sham and post-TAC mice 4 weeks after surgery showing fold change in mRNA expression of atrial natriuretic factor (*ANF*) in TgβARKrgs (A) and TgG_qI (B) mice, brain natriuretic peptide (*BNP*) in TgβARKrgs (C) and TgG_qI (D) mice, and β-myosin heavy chain (β*MHC*) in TgβARKrgs (E) and TgG_qI (F) mice. ***P* = 0.001; ****P* < 0.0001 by one-way ANOVA with Tukey post hoc test relative to nontransgenic sham mice. ^t*P* = 0.001; ^{tt}*P* < 0.0001 by one-way ANOVA with Tukey post hoc test relative to nontransgenic TAC mice. *n* = 8 to 10 mice per group for (A), (C), and (E), and *n* = 12 to 13 mice per group for (B), (D), and (F). (G and H) Quantification of GRK2 protein abundance normalized to GAPDH from Western blots in TgβARKrgs (G) and TgβARKct (H) sham and post-TAC animals 4 weeks after surgery. See fig. S3, D and E, for representative Western blots. **P* = 0.01; ***P* = 0.001 by nonparametric one-way ANOVA with Dunn post hoc test relative to nontransgenic sham mice. *n* = 5 to 10 mice per group and 4 blots for (G) and 3 blots for (H).

Fig. 6. Left ventricular structural remodeling is inhibited in TgβARKrgs mice

(A) Representative images of WGA and 4',6-diamidino-2-phenylindole (DAPI)-stained murine heart sections from nontransgenic littermate control and TgβARKrgs hearts 4 weeks after sham or TAC surgery. Scale bar, 50 μm. (B) Quantification of cardiomyocyte CSA in these animals. $^{*t}P = 0.0001$ by one-way ANOVA with repeated measures and Tukey post hoc test relative to nontransgenic sham and TAC animals, respectively. $n = 5$ to 9 hearts per group, 40 images per heart. (C) Representative images of Masson trichrome-stained murine heart sections from nontransgenic and TgβARKrgs hearts 4 weeks after sham or TAC surgery. Scale bar, 2000 μm. (D) Representative higher magnification (×40) images of Masson trichrome-stained post-TAC murine heart sections from nontransgenic and TgβARKrgs mice demonstrating interstitial fibrosis. Scale bar, 200 μm. (E) Quantification of the percent of fibrotic tissue in the left ventricular area in nontransgenic and TgβARKrgs hearts 4 weeks after TAC. $***P < 0.0001$ by Student's t test relative to non-transgenic TAC mice. $n = 108$ images from nine hearts for nontransgenic mice (~11 images per heart) and 78 images from seven hearts for TgβARKrgs mice.

Fig. 7. The βARKrgs peptide interacts with endogenous Gα_q in the heart

(A) Representative image of a monoclonal Flag immunoprecipitation (IP) followed by Western blotting probing for rabbit polyclonal Gα_q and rabbit polyclonal Flag-tagged βARKrgs in cardiac lysates from nontransgenic littermate control and TgβARKrgs mice. (B and C) Quantification of (B) Gα_q normalized to βARKrgs-Flag and (C) total βARKrgs in TgβARKrgs lysates after monoclonal Flag IP 4 weeks after sham or TAC (T) surgery. (D) Representative image of a polyclonal GRK2 IP followed by Western blotting probing with goat polyclonal Gα_{q/11} and mouse monoclonal GRK2 in cardiac lysates from nontransgenic and TgβARKrgs mice. ***P* < 0.001 by non-parametric Student's *t* test relative to TgβARKrgs TAC mice. *n* = 6 sham hearts and 8 TAC hearts and 4 Western blots each with sham and TAC hearts run side by side. (E) Quantification of IP₃ concentrations from non-transgenic and TgβARKrgs lysates 4 weeks after sham or TAC surgery. **P* < 0.01 by nonparametric one-way ANOVA with Dunn post hoc test relative to nontransgenic sham mice. *n* = 6 to 8 mice per group.

Fig. 8. Model for GPCR signaling in the heart

Agonist binding to GPCRs in the heart leads to activation and dissociation of the heterotrimeric G protein complex. $G\alpha$ and $G\beta\gamma$ subunits then bind to downstream effector proteins triggering signaling cascades that control cardiac function. Catecholamine binding to β_1 -adrenergic receptors (β_1 ARs) leads to activation of $G\alpha_s$, which directly stimulates adenylyl cyclase at the membrane, altering heart rate and contractility. Concomitantly, GRK2 associates with dissociated $G\beta\gamma$ subunits and translocates to the membrane where it phosphorylates the agonist-bound receptor, leading to receptor desensitization and internalization. Agonist binding to $G\alpha_q$ -coupled receptors leads to activation of multiple $G\alpha_q$ effector proteins involved in cardiomyocyte hypertrophy and vascular function. This study suggests that a direct interaction of the β ARKrgs peptide, but not the RGS domain within endogenous GRK2, with $G\alpha_q$ competes for binding to other $G\alpha_q$ effector proteins, inhibiting downstream signaling and cardiac hypertrophy.