G\textsubscript{i}-Biased \(\beta_2\text{-AR} \) Signaling Links GRK2 Upregulation to Heart Failure


Rationale: Phosphorylation of \(\beta_2\)-adrenergic receptor (\(\beta_2\text{-AR} \)) by a family of serine/threonine kinases known as G protein–coupled receptor kinase (GRK) and protein kinase A (PKA) is a critical determinant of cardiac function. Upregulation of G protein–coupled receptor kinase 2 (GRK2) is a well-established causal factor of heart failure, but the underlying mechanism is poorly understood.

Objective: We sought to determine the relative contribution of PKA- and GRK-mediated phosphorylation of \(\beta_2\text{-AR} \) to the receptor coupling to G\textsubscript{i} signaling that attenuates cardiac reserve and contributes to the pathogenesis of heart failure in response to pressure overload.

Methods and Results: Overexpression of GRK2 led to a G\textsubscript{i}-dependent decrease of contractile response to \(\beta\text{-AR} \) stimulation in cultured mouse cardiomyocytes and in vivo. Importantly, cardiac-specific transgenic overexpression of a mutant \(\beta_2\text{-AR} \) lacking PKA phosphorylation sites (PKA-TG) but not the wild-type \(\beta_2\text{-AR} \) (WT-TG) or a mutant \(\beta_2\text{-AR} \) lacking GRK sites (GRK-TG) led to exaggerated cardiac response to pressure overload, as manifested by markedly exacerbated cardiac maladaptive remodeling and failure and early mortality. Furthermore, inhibition of G\textsubscript{i} signaling with pertussis toxin restores cardiac function in heart failure associated with increased \(\beta_2\text{-AR} \) to G\textsubscript{i} coupling induced by removing PKA phosphorylation of the receptor and in GRK2 transgenic mice, indicating that enhanced phosphorylation of \(\beta_2\text{-AR} \) by GRK and resultant increase in G\textsubscript{i}-biased \(\beta_2\text{-AR} \) signaling play an important role in the development of heart failure.

Conclusions: Our data show that enhanced \(\beta_2\text{-AR} \) phosphorylation by GRK, in addition to PKA, leads the receptor to G\textsubscript{i}-biased signaling, which, in turn, contributes to the pathogenesis of heart failure, marking G\textsubscript{i}-biased \(\beta_2\text{-AR} \) signaling as a primary event linking upregulation of GRK to cardiac maladaptive remodeling, failure and cardiodepression. (Circ Res. 2012;110:265-274.)

Key Words: \(\beta_2\)-adrenergic receptor • G protein–coupled receptor kinase • heart failure • hyertrophy

Despite major developments in both diagnosis and treatment, heart failure (HF) continues to be a leading cause of death and disability in Western countries and will become the number-1 killer worldwide in 2020.\(^1\) It has been controversial as to whether increased cAMP–protein kinase A (PKA) signaling is beneficial or detrimental in the context of HF. Patients with HF exhibit chronically enhanced PKA signaling,\(^2,3\) and transgenic mouse models with cardiac-specific overexpression of \(\beta_1\) adrenergic receptor (AR),\(^3\) the \(\alpha\)-subunit of G\textsubscript{i},\(^4\) and the catalytic subunit of PKA\(^5\) display HF phenotypes, suggesting that overtly enhanced PKA signaling is cardiac detrimental. Over the past 2 decades, compelling evidence indicates that phosphorylation of \(\beta\text{ARs} \) by another family of serine/threonine kinases known as GPCR kinases (GRKs) in the heart is a critical determinant of cardiac function and has been implicated in many pathological conditions including HF.\(^6\) In humans or animal models with HF, chronic catecholamine elevation causes marked dysregulation of \(\beta\text{ARs} \), resulting in various molecular abnormalities, including upregulation of GRK2 and pertussis toxin (PTX)-sensitive G\textsubscript{i} proteins. Upregulation of both of these proteins have been implicated as causal factors in the deven-
opment of HF. In particular, GRK2 is the most abundant and best-characterized GRK in the heart.7 GRK2 expression and activity are markedly elevated and play a central role in the HF-associated defect in βAR signaling and cardiac dysfunction. Myocardial ischemia and hypertension in humans and animal models have also been associated with elevated GRK2 expression and activity.6 These previous studies have defined GRK2 upregulation as an early common event in cardiac maladaptive remodeling and HF.

It has been shown that phosphorylation of β2AR plays a crucial role in regulating differential G protein coupling of the receptor. Specifically, β2AR phosphorylation by PKA mediates the switch of coupling from Gi to Gs.8,9 Targeted transgenesis reveals discrete attenuator functions of GRK and PKA in airway β2AR physiological signaling.10 Further studies have demonstrated that β2AR coupling to Gi may be also dependent on the receptor internalization and recycling.11–13 However, it is unclear whether GRK-mediated phosphorylation of β2AR is involved in the regulation of β2AR-coupled Gi, signaling in heart. Because both GRK2 and Gi proteins are significantly elevated in HF caused by a multitude of etiologies,14–19 we hypothesize that the well-documented detrimental effects of GRK2 in the failing heart may causatively link to enhanced β2AR-coupled Gi signaling.

In the present study, we explored the mechanism that links pathological upregulation of GRK2 to the development of HF. We found both in vivo and in vitro that enhanced β2AR phosphorylation by GRK2 leads the receptor to Gi-biased signaling and that inhibition of the Gi signaling blocks HF in transgenic (TG) mice with cardiac-specific overexpression of GRK2 (GRK2-TG) or of a β2AR mutant lacking all of the PKA phosphorylation sites (PKA-TG) subjected to pressure overload, marking Gi-biased β2AR signaling as a primary event linking upregulation of GRK2 to cardiac maladaptive remodeling and failure.

Methods

An expanded Methods section is provided in the Online Supplement at http://circres.ahajournals.org.

Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>β-AR</td>
<td>β-adrenergic receptor</td>
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<tr>
<td>CGP</td>
<td>CGP20712A</td>
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<td>GRK-TG</td>
<td>transgenic mice expressing a human β2AR mutant lacking GRK phosphorylation sites</td>
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<td>GPCR</td>
<td>G protein–coupled receptor</td>
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<td>ICI</td>
<td>ICI 118,551</td>
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<tr>
<td>[125I]-ICYP</td>
<td>[125I]-iodocyanopindolol</td>
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<tr>
<td>ISO</td>
<td>isoproterenol</td>
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<tr>
<td>LV</td>
<td>left ventricular</td>
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<td>NTG</td>
<td>nontransgenic mice</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
<td>PKA-TG</td>
<td>transgenic mice expressing a human β2AR mutant lacking PKA phosphorylation sites</td>
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<tr>
<td>PKI</td>
<td>protein kinase inhibitor</td>
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<tr>
<td>PTX</td>
<td>pertussis toxin</td>
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<tr>
<td>WT-TG</td>
<td>transgenic mice overexpressing wild-type human β2-AR</td>
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Generation of TG Mice

Flag-tagged human β2-AR mutants lacking either the putative GRK phosphorylation sites (GRK−) or the putative PKA phosphorylation sites (PKA−) (Figure 2A and 2B) were subcloned into a pBuescript-based transgenic vector downstream of α-myosin heavy chain gene promoter and upstream of the SV40 polyadenylation site. The detail sequences of the β2AR PKA− and GRK− mutants were presented in Figure 2B. Transgenic mice with cardiac-specific overexpression of wild-type human β2-AR (WT-TG) were imported from Dr Gerald Dorn’s Laboratory.

Animal Models

We used male nontransgenic mice (NTG), transgenic mice with cardiac-specific overexpression of wild-type human β2-AR (WT-TG) or PKA-phosphodeficient β2-AR (PKA-TG), or GRK-phosphodeficient β2-AR (GRK-TG) and their littermate controls at 12–16 weeks of age. In addition, male transgenic mice with cardiac-specific overexpression of GRK2 (GRK2 TG) and their littermate control mice (LC) were used in a subset of experiments. Pressure overload was produced by transverse aortic constriction (TAC) as previously described.20

Supplemental Materials on Detailed Methods

See the Online Supplement Materials for detailed methods regarding in vivo assessment of mouse cardiac contractility by echocardiography and Millar system, radioligand binding assay, Western blot analysis, adult mouse cardiomyocyte culture and adenosine gene transfer, cardiomyocyte contraction measurements, histological analysis, cAMP assay, terminal deoxynucleotidyl transfer, cardiomyocyte contraction measurements, histological analysis, CAMP assay, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and statistical analysis.

Results

Overexpression of GRK2 Causes βAR Dysfunction by Enhancing Gi Signaling in Cultured Cardiomyocytes and In Vivo

We have recently demonstrated that Gi-biased β2AR signaling is dependent on agonist stimulation and that prolonged absence of agonist stimulation leads to uncoupling of β2AR from the Gi signaling, as is the case in adult mouse cardiomyocytes cultured for 24 hours.21 Consistent with the previous notion, in adult mouse cardiomyocytes cultured and infected by adenosine-β-Gal for 24 hours, β2AR stimulation with zinterol led to a full contractile response which was insensitive to PTX treatment (Figure 1A and 1B). Importantly, overexpression of GRK2 with adenosine gene transfer suppressed β2AR-mediated contractile response and the inhibitory effect of GRK2 was fully abolished by disrupting Gi signaling with PTX (Figure 1A and 1B). Prolonged stimulation of cardiomyocytes with isoproterenol (ISO, 1 nmol/L) in the presence or absence of PTX did not change the expression of GRK2 (Online Figure I). These results demonstrate, for the first time, that overexpression of GRK2 enhances Gi-biased β2AR signaling. To further determine whether in vivo overexpression of GRK2 in the heart can facilitate β2AR-coupled Gi signaling and, if so, whether the enhanced Gi signaling is involved in GRK2-mediated βAR dysfunction, we took advantage of transgenic mice with cardiac-specific overexpression of GRK2.22 Consistent with our previous studies on these mice,22 in vivo experiments revealed that βAR-induced increases in cardiac contractility and relaxation, as measured by left ventricular (LV) +dP/dtmax and −dP/dtmin, respectively, were markedly suppressed in mice overexpressing GRK2 (GRK2 TG mice) compared with wild-type littermate controls (Figure 1C and 1D). Remarkably, disruption of Gi signaling with
PTX fully restored cardiac contractile response to βAR stimulation with ISO in GRK2 TG mice without altering the effect of ISO in littermate control mice (Figure 1C and 1D), indicating that overexpression of GRK2-induced cardiac βAR dysfunction is mediated by enhanced G_{i} signaling.

**Augmentation of GRK-Mediated βAR Phosphorylation Leads to Exacerbated Cardiac Hypertrophy, HF, and Early Mortality in Response to Pressure Overload**

Because β2AR can be phosphorylated by at least 2 types of protein kinases, PKA and GRK (Figure 2A), we sought to distinguish the potential role of GRK- versus PKA-mediated βAR phosphorylation in the pathogenesis of HF in vivo. The experiments took advantage of 3 different transgenic mouse models that we developed: mice cardiac-specifically overexpressing WT human β2AR (WT-TG) or β2AR mutants in which all of the PKA phosphorylation sites or all of the GRK phosphorylation sites were substituted by alanine (A) or glycine (G) (namely PKA-TG or GRK2-TG, respectively) (Figure 2A and 2B) at a matched receptor density (Figure 2C). The receptor densities in hearts from WT-TG, PKA-TG (Figure 2 A and 2B) at a matched receptor density (Figure 2C). As compared with NTG mice, overexpression of the WT β2AR led to profoundly increased phosphorylation of β2AR at both GRK and PKA sites assayed by site-specific antibodies (Figure 2D). As expected, PKA-TG mice showed a clear increase in phosphorylation of β2AR by GRK but not PKA, whereas GRK-TG mice exhibited augmented phosphorylation of β2AR by PKA but not GRK (Figure 2D).

Thus, GRK- and PKA-mediated phosphorylation of β2AR was selectively enhanced in PKA-TG and GRK-TG mice, respectively.

To investigate whether β2AR phosphorylation by GRK and PKA is differentially involved in the regulation of cardiac structure and function, NTG or PKA-TG, GRK-TG, or WT-TG mice were subjected to pressure overload by TAC. There was no obvious phenotypic difference in cardiac anatomy and function among the genotypes under resting conditions at 4–5 months old of age, as assessed by echocardiography (Figure 3A and 3B). However, after TAC for 5 weeks, PKA-TG mice developed severe LV dilation and contractile dysfunction (Figure 3A and 3B) and increased mortality rate (Figure 3C). Cardiac dysfunction occurred as early as 1 week after TAC and was progressively worse as TAC continued only in PKA-TG mice (Figure 3B). In contrast, GRK-TG mice were not different from NTG or WT-TG mice in terms of cardiac anatomy and function in response to TAC. Furthermore, PKA-TG mice displayed markedly exaggerated cardiac hypertrophic response to TAC, as manifested by significantly increased heart size (Figure 4A), heart/body ratio (Figure 4B), and cardiomyocyte size (Online Figure II). It is also important to note that PKA-TG mice but not NTG or WT-TG or GRK-TG showed increased fibrosis after TAC for 5 weeks (Figure 4C and 4D). Thus, pressure overload led to more severe ventricular dilation with increased end-diastolic and end-systolic dimensions, reduced fractional shortening (Figure 3) and exacerbated cardiac maladaptive remodeling (Figure 4) in PKA-TG mice but not in other groups including NTG, WT-TG, and GRK-TG mice.
Accelerated HF of PKA-TG Mice Is Associated With Enhanced βAR-Coupled Gi Signaling

More detailed examination of cardiac function was carried out by invasive pressure-volume analysis. Pressure-volume loops were measured before and during transient reduction of chamber preload to generate specific end-systolic (ESPVR) and end-diastolic (EDPVR) pressure-volume relations (Figure 5). After TAC for 5 weeks, NTG hearts had enhanced effective arterial elastance (Ea; an index of total ventricular afterload) and slope of end-systolic pressure-volume relation with reduced ejection fraction, characteristics of hypertrophy induced by sustained pressure overload. Under resting conditions, PKA-TG, GRK-TG, and WT-TG mice had similar increases in LV peak systolic pressure and basal systolic function with a slight upward and leftward shift of their ESPV compared with NTG mice. TAC for 5 weeks triggered distinct phenotypes between PKA-TG mice and GRK-TG or WT-TG mice in terms of the progression of cardiac dysfunction. As shown by the representative examples, in the GRK-TG and WT-TG mice, enhancements in resting Ea and slopes of ESPVR before TAC were slightly increased after 5 weeks of TAC, consistent with a functional response with feature of cardiac hypertrophy (Figure 5A). This did not occur in PKA-TG mice. Notably, PKA-TG mice displayed ventricular dilation, suppressed +dP/dt, reduced Ea, decreased ejection fraction associated with a rightward shift of pressure-volume relation, and a decrease in the slope of end-systolic pressure-volume relation (Figure 5A and 5B). After 5 weeks of TAC, PKA− mice demonstrated a more significant decrease in maximal rate of pressure decline (−dP/dt), although a relaxation index tau was comparable in GRK-TG and PKA-TG mice (Figure 5C).

The aforementioned data clearly indicate that PKA-TG mice are more vulnerable to pressure overload, suggesting that selectively enhancing βAR phosphorylation by GRK but not PKA profoundly exacerbates pressure overload-induced cardiac maladaptive remodeling and dysfunction. Next, we sought to decipher the mechanism underlying the distinct phenotypes of PKA-TG mice versus those of GRK-TG mice. Western blotting revealed that the increases in GRK2 and Gi protein abundance were significantly greater in hearts from PKA-TG mice relative to hearts from NTG or WT-TG or GRK2-TG mice, although both GRK2 and Gi proteins were markedly elevated in all genotypes after 5 weeks TAC (Figure 6A and 6B). Concomitantly, hearts from PKA-TG mice failed to compensate pressure overload even 1 week after TAC, resulting in significantly diminished cardiac contractility in PKA-TG mice compared with that in other genotypes (NTG or WT-TG or GRK2-TG) (Figure 6C). Importantly, disruption of the Gi signaling with PTX treatment minimized the difference between PKA-TG mice and other groups (Figure 6C). In fact, in an early stage of HF (1 week after TAC), PTX treatment fully restored cardiac function in PKA-TG mice, whereas in the later stage of HF (5
weeks after TAC), PTX substantially improved cardiac function in PKA-TG mice (Figure 6C), highlighting that enhanced GRK2 and subsequent enhancement of Gi-biased β2AR signaling play a crucial role in the triggering and worsening TAC-induced cardiac maladaptive remodeling and failure.

In principle, the cardiac dysfunction in PKA-TG mice could be due to an enhancement in the receptor to Gi signaling for attenuation of tonic cAMP signaling or due to cardiac adaptive remodeling for structure changes in myocardium or both alterations. To further investigate this issue, we measured basal and ISO-induced cAMP formation in cardiomyocytes from NTG or TG mice expressing WT or mutant β2AR. The present data demonstrated that spontaneous β2AR activity is increased in all of the transgenic mice expressing WT or mutant β2AR, as evidenced by their increased cAMP baselines (Figure 6D and Online Figure III), consistent with the hemodynamic data (Figure 5). It is noteworthy that the decay of ISO-induced cAMP accumulation declines faster in cells from PKA-TG mice than that in cells from WT-TG or GRK-TG mice. Because the decay of ISO-induced cAMP accumulation is sensitive to PTX, our data indicates that β2AR/Gi coupling is enhanced in PKA-TG mice. Thus, the cardiac dysfunction of PKA-TG mice probably is due to enhanced Gi signaling, which, in turn, contributes to cardiac adaptive remodeling and the progression of HF.

Discussion
The present study has revealed 3 major findings. First, phosphorylation of β2AR by GRK as well as PKA is a primary determinant of the receptor-coupled Gi signaling. Second, overexpression of GRK2 enhances Gi-biased β2AR signaling, which subsequently negates cardiac contractile response to βAR stimulation in a PTX-sensitive manner in GRK2 transgenic mice and cultured mouse cardiomyocytes. Third, selective augmentation of GRK-mediated β2AR phosphorylation in PKA-TG mice renders the heart more vulnerable to pressure overload and inhibition of Gi signaling can restore cardiac function of PKA-TG mice. In contrast, a selective increase in PKA-mediated phosphorylation of β2AR does not affect cardiac response to pressure overload in GRK-TG mice compared with NTG or WT-TG mice. Therefore, we conclude that cardiac detrimental effects of GRK2 upregulation are mediated, at least in part, by enhanced β2AR-coupled Gi signaling, which, in turn, contributes to the pathogenesis and progression of HF. Thus, both GRK2 and β2AR-coupled Gi signaling may offer novel therapeutic opportunities for the treatment of HF.

Role of GRK2 in Normal and Failing Hearts
In our previous studies, we have shown that cardiac-specific overexpression of GRK2 to the levels seen in human HF
Figure 4. Overexpression of βAR mutant lacking PKA phosphorylation sites exaggerated cardiac maladaptive remodeling after pressure overload. A, Cross sections of hearts subjected to sham operation (Sham) or TAC for 5 weeks (5w TAC); B, ratio of heart/body weight (n=8-12, *P<0.01 versus sham; †P<0.01 versus the other 3 groups with TAC). C and D, Representative examples of myocardial connective tissue staining (E.P.S. R) to show cardiac fibrosis (top) and average data of fibrosis area (bottom) (n=5 for each group, †P<0.01 versus sham; †P<0.01 versus the other 3 groups with TAC).

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The beneficial effect of restoration of βAR signaling through inhibiting GRK2 activity appears to contradict clinical convention that β-blockade is widely used to treat patients with HF and that chronic β-agonist (ie, catecholamine) stimulation leads to deleterious effects. However, a close inspection reveals that the detrimental effects of catecholamines are mainly mediated by stimulation of β1-ARs, which triggers myocyte apoptosis and arrhythmogenic events. In contrast, the present study has shown that inhibition of GRK2-mediated β2-AR-coupled Gi signaling can normalize cardiac contractile response and ameliorate maladaptive remodeling. In this regard, our recent in vivo studies on a rat ischemic HF model have demonstrated that selective activation of β2-AR can, indeed, improve cardiac function and reduce maladaptive remodeling as well as arrhythmogenic events. Taken together, we propose that a combination of GRK2 inhibition and β2-AR activation with β1-blocker therapy may provide a more effective therapy for the treatment of HF.

Role of GRK-Mediated βAR Phosphorylation in Promoting Gi-Biased Signaling and Its Implications in HF

Although βAR is classically viewed as a prototypical Gi-coupled receptor, compelling evidence indicates that β2-AR couples dually to Gi and Gs proteins. We and others have previously shown that Gi-biased β2AR signaling may protect cardiomyocytes against various insulting stimuli induced apoptosis. In addition, it has been shown that inhibition of Gi signaling worsens cardiac outcomes in response to ischemia/reperfusion injury and myocardial infarction and that GRK inhibition with the peptide inhibitor GRK2-ct is cardioprotective, at least in part, due to increasing Gi-biased β2AR signaling. Furthermore, recent studies have shown that ablation of β2AR, indeed, worsens pressure overload–induced cardiac hypertrophy and remodeling in mice. These previous studies seem to contradict the present conclusion that an augmentation in GRK-mediated β2AR-Gi signaling contributes to cardiac maladaptive remodeling and failure under TAC in PKA-TG mice. To address this question, we have examined the potential effect of TAC on myocardial cell apoptosis in all 4 groups of mice (NTG, WT-TG, GRK2-TG, and PKA-TG) and found no genotype-related difference in TAC-triggered myocardial apoptosis (Online Figure IV). In addition, we have shown that PTX enhances ISO-induced cardiomyocyte death, whereas β2AR stimulation with zin- terol protects cardiomyocytes in PTX-sensitive manner (Online Figure V), consistent with the previous notion that β2AR-Gi signaling is cardiac protective. However, cardiomyocytes from PKA-TG mice are more vulnerable than those from other groups (Online Figure V), although β2AR-Gi signaling is markedly enhanced in PKA-TG mice. Thus, it merits future investigation to elucidate the exact mechanism underlying cardiac maladaptive remodeling and failure associated with enhanced GRK-mediated β2AR/Gi signaling in PKA-TG mice under TAC.

During prolonged agonist stimulation, GRK2 plays a predominant role in desensitizing βARs and has been implicated as a causal factor of HF, but the underlying etiologic...
mechanism is unknown until now. Traditionally, it has been shown that GRK-mediated phosphorylation of βAR inhibits the interaction between activated receptor and G proteins through recruiting β-arrestins, which bind to phosphorylated βAR and sterically block the receptor coupling to the G subunit of G protein.43 In this study, however, we have provided multiple lines of evidence to show that increased phosphorylation of βAR by GRK facilitates the receptor to G_i signaling, which, in turn, results in cardiac contractile dysfunction and maladaptive remodeling. The present findings have also unraveled a novel function of the prototypical GRK, GRK2, in switching G_s to G_i-biased βAR signaling. Thus, in addition to PKA, GRK plays an important role in sorting βAR to G_i-biased signaling pathway in response to

**Figure 5. In vivo cardiac pressure-volume relations in mice with sham operation or 5 weeks after TAC.** A, Left ventricular pressure-volume loops show TAC-induced increase in systolic load. In NTG-, WT-TG, and GRK-TG mice, TAC leads to rightward shift of the loops and end-systolic pressure-volume relation, marking hypertrophy remodeling. However, in PKA-TG mice, TAC induces heart failure (HF). B and C, Summary data on systolic function (B) and diastolic function (C). LVSP indicates left ventricular systolic pressure; SV, stroke volume; Ea, arterial elastance (measure of ventricular afterload); EF, ejection fraction; +dP/dt, maximum dP/dt; Ees, end-systolic pressure-volume relationship (ESPVR); Eed, end-diastolic pressure-volume relationship (ESDPR); EDP, end-diastolic pressure; Tau, regression of log (pressure) versus time (Weiss method). All values present mean±SEM (n=8–13 for each group; *P<0.05, **P<0.01 versus respective sham; †P<0.05, ‡P<0.01 versus respective TAC).
enhanced catecholamine stimulation, as is the case in the failing heart.

It is noteworthy that whereas the β1AR subtype does not couple to Gι under normal conditions, β2AR-mediated contractile response is cross-inhibited by enhanced Gι-biased β2AR signaling in the failing heart.29,44 The reinforced GRK-dependent Gι-biased β2AR signaling probably is also responsible for GRK2 overexpression-induced dysfunction of β1AR in addition to the defect of β2AR contractile response in these transgenic mice. This idea is, indeed, corroborated by the fact that disruption of Gι signaling fully rather than partially restores the nonselective βAR agonist, ISO, induced positive inotropic effect in transgenic mice overexpressing cardiac GRK2 (Figure 1C and 1D). Thus, similar to the positive inotropic effect in transgenic mice overexpressing β2AR in facilitating Gi-biased cardiac reserve function,13 although the present study has used WT-TG and GRK2-TG mice for comparison. This technical limitation should be taken into consideration, when the present data are interpreted.

In summary, we have revealed a novel function of GRK in promoting Gι-biased β2AR signaling that compromises cardiac reserve and contributes to the pathogenesis of HF. In the failing heart, enhanced expression and activity of GRK2 and Gι proteins further promote Gι-biased β2AR signaling, thus negating β1AR- and β2AR-mediated cardiac reserve function, resulting in cardiac maladaptive remodeling and failure. These in vitro and in vivo results have not only revealed a fundamental function and new mechanism of action of GRK2, the best characterized GRKs, in facilitating Gι-biased β2AR signaling but also defined the β2AR-Gι signaling as an essential link between pathological upregulation of GRK and the development of HF. As a well-established pathogenic factor of HF, GRK2 and resultant Gι-biased β2AR signaling
may present important therapeutic targets for the treatment of HF caused by various etiologies.

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We thank Dr Robert J. Lefkowitz, Department of Medicine and Howard Hughes Medical Institute, Duke University Medical Center, for kindly providing cDNAs encoding wild-type human β2-AR, β2AR mutant lacking putative phosphorylation sites for PKA (PKA−, point mutations of serine residues at 261, 262, 345, and 346), and for GRK (GRK−, 11 point mutations of serine or threonine residues in the C-terminus substituted with alanines or glycine), and Dr Richard B. Clark, The University of Texas Health Science Center, for antipSer262 PKA site and the anti-pS (Ser355, 356) GRK sites. We are also grateful to Dr Harold Spurgeon and Bruce Ziman for excellent technical support.

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Disclosures
None.

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### Novelty and Significance

**What Is Known?**

- Phosphorylation of G protein–coupled receptor alters the selectivity of the receptor for G protein coupling.
- Beta2-adrenergic receptor (beta2-AR) phosphorylation by protein kinase A (PKA) is a critical determinant in its signaling to distinct G proteins such as Gs versus Gi.
- Beta2-AR–mediated Gi signaling elicits cardiac protective effects.
- Upregulation of G protein–coupled receptor kinase (GRK) is a well-established causal factor of heart failure.

**What New Information Does This Article Contribute?**

- Overexpression of GRK2 profoundly enhances Gi-biased beta2-AR Gi signaling in vivo and in vitro, whereas inhibition of GRK2 markedly suppresses the beta2-AR-Gi signaling.
- Phosphorylation of beta2-AR by GRK as well as PKA is an important determinant for the Gi-biased beta2-AR signaling.
- In transgenic mice with cardi-specific overexpression of a beta2-AR mutant lacking PKA phosphorylation sites (PKA-TG), augmentation of beta2-AR phosphorylation by GRK leads to exaggerated cardiac response to pressure overload, resulting in increased Gi-biased beta2-AR, markedly exacerbated cardiac maladaptive remodeling and failure, leading to early mortality.

- In contrast, enhancement of PKA-mediated phosphorylation of beta2-AR in mice overexpressing a beta2-AR mutant lacking GRK phosphorylation sites (GRK-TG) does not alter cardiac anatomy or function when compared with nontransgenic mice or transgenic mice overexpressing the wild-type beta2-AR (WT-TG) at a matched receptor density, indicating that phosphorylation of beta2-AR by GRK but not by PKA contributes to the pathogenesis of heart failure.

- Inhibition of Gi signaling with pertussis toxin restores cardiac function in PKA-TG mice with pressure overload-induced heart failure and in GRK transgenic mice.

- Enhanced beta2-AR coupling to Gi signaling may induce cardiac protective or detrimental effects.

Our in vitro and in vivo results reveal a fundamental function and new mechanism of action of GRK in facilitating Gi-biased beta2-AR signaling by phosphorylation of beta2-AR. We found that beta2-AR-Gi signaling is an essential linker between pathological upregulation of GRK and the development of heart failure. As a well-established pathogenic factor of heart failure, GRK and resultant Gi-biased beta2-AR signaling may be important therapeutic targets for the treatment of heart failure.
Gβ1-Biased β2AR Signaling Links GRK2 Upregulation to Heart Failure

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**Generation of Transgenic Mice**  HA-Flag-tagged human β2AR mutants lacking either the putative GRK phosphorylation sites (GRK-) or the putative PKA phosphorylation sites (PKA-) were subcloned into a vector downstream of myosin heavy Chain (MHC) gene promoter and upstream of the SV40 polyadenylation site (Fig. 2A&B). The GRK-β2AR mutant was generated by mutating the serine/threonine amino acids residues in the carboxyl tail to alanine or glycine at positions 345, 346, 355, 360, 364, 384, 393, 396, 401, 407, 408, 411 and the PKA-β2AR mutant was generated by mutating the serine residues in the third intracellular loop to alanine at positions 261, 262, 345, and 346. The pBluescript II vector harbors a 5.5-kb murine α-MHC promoter (pMHC) and a SV-40 polyadenylation sequence (a gift from Yibin Wang, University of California Los Angeles). For convenient cloning of GRK- and PKA- mutant β2AR, a polylinker containing more sites of restriction endonucleases were inserted between Hind III and Eco RV. As a result, Mlu II, Afl II and Nhe I were introduced in the multiple clone sites by the polylinker sequence of AAG CTT ACG GCT TTT AAG ATC ATC GCC CTG; GRK- and PKA- mutated β2AR cDNA sequences with N-terminal HA-Flag tags were amplified from pCDNA3-GRK and PBK-CMV-PKA using the primers of HA forward/GRK reverse and HA forward/PKA reverse respectively, and cloned into pBluescript II-MHC by Hind III/Nhe I. The primer sequences are: HA forward: atg ctc AAG CTT atg aag acc atc atc gcc ctg; PKA reverse: agt gca GCT AGC CAG CAG TGC GTC ATT TGT ACT AC; and GRK2 reverse: agt gca GCT AGC CAG CAG TGC GTC ATT TGC ACC AC. The expression cassettes (pMHC-β2AR-PolyA) were released by Xho I/Not I from endotoxin-free prepared plasmid, Purified by agarose gel and injected into the fertilized mouse oocytes. Transgenic founders were identified by Southern blot analysis of tail DNA using the SV40 poly(A) as a probe. Transgenic founder mice were backcrossed to C57BL/6 mice for at least 7 generations before being used in experiments. Mice were screened by PCR with sense primer 5'-gac ctc tga cag aga agc agg c-3', located in the MHC (5264-5285), and an antisense primer, 5'-ggt acc agt gca tet gaa tgg g-3', located in β2AR coding sequence (502-530). The product size is 810 bp. Transgenic mice with cardiac-specific overexpression of WT human β2AR or its PKA- or GRK2- mutant were named WT TG, PKA- TG or GRK- TG mice, respectively. WT TG mice were imported from Dr. Gerald Dorn’s lab. The founder of WT-β2-AR TG is FVB background. The founder and its offspring bred with C57 background mice more than 7 generations. The offspring of 7th generation were used for experiments. Other mice strains are C57 background.

**Animal models** We used NTG, WT TG, PKA- TG, GRK- TG mice and GRK2 TG mice or their littermate controls (negative genotyping) at 12-16 weeks of age. Pressure overload was produced by transverse aortic constriction (TAC). Mice were anesthetized with isoflurane (2.5%) and pressure overload was produced by TAC as previously described. Mice were placed supine on a heating pad (37%). A horizontal skin incision 0.5-1.0 cm was made at the level of suprasternal notch. To allow visualization of the aortic arch, a 2-3 mm longitudinal cut was made in the proximal portion of the sternum. A 6.00 silk suture was passed under the aorta. A 26-gauge needle was placed next to the aortic arch, and suture was tied around the needle and the aorta. After ligation, the needle was quickly removed. The chest was closed and the animal was allowed to recover after anesthesia. Doppler velocity was measured in the right and the left carotid arteries (RCA...
and LCA) and RCA/LCA velocity ratio was calculated to ensure that TAC produced equal aortic pressure gradient in all experimental groups. Acute mortality within 24 hour after operation is not included in survival curves. National Institute of Aging Animal Care and Use Committee approved all animal experimental protocols.

Echocardiography In vivo cardiac morphology and function were assessed on anesthetized (2.0 isoflurane) mice by transthoracic echocardiography (Vevo770 echocardiograph with 704B probe, Visualsonics, Toronto, CA. USA). The heart was imaged in the two-dimensional mode (M-mode) in the parasternal long axis views. The measurements of intra-ventricular septal (IVS) thickness, left ventricular posterior wall thickness (LWPW), and left ventricular internal diameters were made in systole and diastole. Left ventricular percent fractional shortening, ejection fraction, chamber volume and mass were calculated using methods as previously described. 4

In Vivo Assessment of Mouse Left Ventricular Contractile Function In vivo LV function was assessed by PV catheter. Briefly, mice were anesthetized with pentobarbital (50 mg/kg), incubated and ventilated with a custom-designed constant-pressure ventilator with 100% oxygen at 120 breaths/min and a tidal volume of 200 µl. The LV apex was exposed and 1.4 F PV catheter (SPR 839; Millar Instruments, Houston,TX) was advanced through apex along the longitudinal axis. PV data were measured at steady state and during transient reduction in venous return by occluding the inferior vena cava 5. For carotid approach, GRK2 transgenic mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) as previously described6,7. A cervical middle line skin incision was made; right common carotid artery was isolated or chest opened and cannulated with 1.4 F PV catheter through right carotid artery. LV pressures, LV end-diastolic pressure (LVEDP) and heart rate (HR) were measured by this catheter advanced into the LV cavity, and data was recorded and analyzed on a PowerLab System (AD Instruments Pty Ltd., Mountain View, CA) to obtain dP/dt max as a measure of global systolic function and dP/dt min as a measure of global diastolic function. The right jugular vein was canulated with PE10 tubing and after recording the baseline hemodynamic data, the βAR agonist, isoproterenol (ISO, 0.5 ~ 7.5 ng) was administered through i.v. delivery line.

Radioligand Binding Heart tissues were harvested in lysis buffer (5 mM Tris-HCl, pH 7.4, with 5 mM EGTA) and homogenized with 15 strokes on ice. Samples were centrifuged at 30,000 g for 15 min to pellet membranes. The membrane proteins were then resuspended in binding buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM MgCl2, 2 mM EDTA) and stored in aliquots at -80°C. Binding assays were performed on 5-25 µg of membrane proteins using saturating amounts of the βAR-specific ligand [125I]-iodocyanopindolol ([125I]-CYP), as described previously 8. Saturation experiments were performed with [125I]-CYP concentrations ranging from 1 to 300 pM in the absence or presence of 1 µM propranolol to determine total and non-specific binding, respectively. The competition binding was performed with ICI118551 in the presence of 50 pM of 125I-CYP. Radioligand binding reactions were terminated by dilution and rapid filtration over glass fiber filters. All assays were performed in duplicate, and receptor density was normalized to milligrams of membrane proteins.
Western Blot Analysis  For western blot analysis, cell extracts were electrophoresed on a 4-12% NuPAGE gel (Invitrogen) and transferred to the polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline-0.1% Tween-20 (TBS-T) and incubated overnight with an anti-GRK2 (Santa Cruz, SC-562), or anti-phospho-ERK1/2 (New England Biolabs), or anti-actin (Santa Cruz, SC-1616) Anti-Giα3 (Santa Cruz, SC-262), Anti-β2-AR (Santa Cruz, SC-569). The antibodies for phosphorylated β2AR were raised against the peptides CDRTGHLRRSpSKF-NH2 for the anti-pSer262 PKA site (clone 2G3) and CKAYGNGYpSpSNGN-NH2 for the anti-pS (Ser355, 356) (clone 5C3). They were a gift from Dr. Richard B. Clark. Membranes were washed and then incubated with secondary antibody conjugated with horseradish peroxidase in 5% non-fat milk in TBS-T buffer for 1-2 h. Detection was carried out using a chemiluminescence detection kit (Cell Signaling Technology) and quantified by scanning laser densitometry.

Adult Mouse Cardiac Myocytes Culture, Adenoviral Gene Transfer, and Survival Assay  Single mouse cardiac myocytes were isolated from 2~3-month-old C57/B6 mice with an enzymatic technique. Cells were then cultured and infected with adenoviral vectors, as described previously. Before culture, myocytes were washed three times with minimal essential medium (MEM) containing 1.2 mM Ca2+, 2.5% fetal bovine serum (FBS), and 1% penicillin-streptomycin and then plated with the same medium in the culture dishes pre-coated with 10 µg/ml mouse laminin. Adenovirus-mediated gene transfer was implemented by adding a minimal volume of the FBS-free MEM containing an appropriate titer of gene-carrying adenovirus. The full volume of FBS-free MEM was supplied after culture for another 1 to 2 h. All experiments were performed after 24 h of adenoviral infection. The cultured myocytes was stained with 1 µg/ml of Propidium iodide (Calbiochem, San Diego, CA).

Cardiac Myocytes Contraction Measurements Cells were placed on the stage of an inverted microscope (Zeiss, model IM-35), perfused with a Heps-buffered solution containing (in mM) 137 NaCl; 5.4 KCl; 1.2 MgCl2; 1 NaH2PO4; 1 CaCl2; 20 Glucose and 20 Heps (pH 7.4), and electrically stimulated at 0.5 Hz at 23°C. Cell length was monitored by an optical edge-tracking method using a photodiode array (Model 1024 SAQ, Reticon) with a 3 ms time resolution. Cell contraction was measured by the percent shortening of cell length following electrical stimulation. To inhibit G1 signaling, subset of freshly isolated or cultured myocytes were incubated with PTX (1.5 µg/ml, at 37°C for at least 3 hr), prior to contraction measurements. PTX-treated cells were compared with myocytes which had been kept at 37°C in the absence of PTX for an equal period of time.

Histological analysis Heart specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5-µm thickness. Sections were stained with H&E and Elastic Picro Sirius Red (E.P.S.R). We determined cardiomyocyte diameter and interstitial collagen fraction using computer-assisted image analysis (Image J).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Staining
CardioTACS in Situ apoptosis detection kit (R and D Systems Inc. no. TA5353; Minneapolis, MN) was used to detect DNA fragmentation in tissue sections. For each sample, two slides were stained. From each slide, $16 \times 10$ fields of view were digitized for analysis. TUNEL positive nuclei and total nuclei were then counted for each image, tallied for each slide, and averaged for each sample.

**cAMP Measurement** Intracellular cAMP levels were assayed by radioimmunoassay. The cardiomyocytes from NTG, WT-, PKA-, GRK- TG $\beta_2$AR mice were isolated and plated in laminin-coated 6-well plates. Four hours after cell were seeded, cells were stimulated with 10 mM of isoproterenol at designed time point. Five minutes before stopping reaction at designed time points, cells were treated with 200 mM of IBAX (3-isobutyl-1-methylxanthine) to accumulate cAMP. The cell lysates were committed to cAMP assay according to the Assay kit (Parameter TM, R&D Systems, KGE002B) with a duplicate in each experiment. Protein content was measured using the Piece BCA (Thermo Scientific 23228).

**Statistical Analysis** Data were expressed as mean ± s.e.m. Differences between multiple groups were compared by analysis of variance (ANOVA) followed by a Bonferroni’s multiple comparisons test. Two-group analysis was performed by t-test. Serial studies were tested by repeated measures ANOVA. Survival curves were analyzed by Kaplan-Meier Survival Analysis.

References


Online Figure I. ISO or PTX incubation did not change the GRK2 expression in NTG mice cardiomyocytes cultured for 24 hours. After cells were plated, cells were further cultured in the presence of ISO (1 nM) with or without PTX (0.5 µg/ml) for 24 hours. At this concentration of ISO or PTX, no cardiomyocytes was died compared to no any treatment cells, as evidenced by PI staining. n=3, *p<0.01 v.s. β-gal group.

Online Figures II: Overexpression of β2AR mutant lacking PKA phosphorylation sites exaggerated cardiomyocyte hypertrophy in response to pressure overload. (A) Representative photomicrographs illustrate ventricular myocyte cross sections in NTG, WT TG, PKA- TG and GRK2-TG mice. (B) Average data on ventricular myocytes cross sections (369 and 412 cells from 4 hearts for NTG Sham and TAC, respectively; 425 and 434 cells from 4 hearts for WT TG Sham and TAC, respectively; 484 and 450 cells from 4 hearts for GRK2- TG Sham and TAC, respectively; 351 and 451 cells from 5 hearts for PKA- TG Sham and TAC, respectively; *p<0.001 v.s. respective control; †p<0.001 v.s. other three groups with TAC).
Online Figure III. The cardiac myocytes from PKA-TG mice were facilitated to decay of cAMP accumulation through PTX sensitive pathway. The basal cAMP (A), maximum response (initial 5 min) (B), and decay of cAMP accumulation (C) in the cardiac myocytes from NTG, WT-, PKA-, GRK-TG mice. The cardiomyocytes from NTG, WT-, PKA-, GRK-TG mice were isolated and plated in laminin-coated 6-well plates. Four hours after cell were plated, cells were stimulated with 10 µM of isoproterenol (ISO) at designed time. 5 minutes before stopping reaction at designed time points, cells were treated with 200 µM of IBMX (3-isobutyl-1-methylxanthine) to accumulate cAMP. The cell lysates were committed to cAMP assay according to the assay kit. *P<0.05 v.s. NTG , #P<0.05 v.s. NTG, GRK-, WT-TG, n=3-4.
Online Figure IV. Pressure over-load induced the myocardium apoptosis in NTG, WT-, PKA-, GRK-β2AR TG mice, but no difference between different transgenic β2AR mice. Heart specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5-μm thickness. For each sample, two slides were stained. From each slide, 10 fields of view (200X) were digitized for analysis. TUNEL positive nuclei and total nuclei were then counted for each image, tallied for each slide, and averaged for each sample. *P<0.01 vs sell sham group, n=3 hearts.
Online Figure V. Cardiomyoctes with high expression of phosphorylation of β2AR in GRK sites (PKA-β2AR) were vulnerable to βAR agonist stimulation when inhibition of G_i-signaling with PTX. The cardiomyocytes from NTG, WT-, PKA-, GRK-TG β2AR mice were cultured for 24 h with or without isoproterenol (1 μM) or zinterol (10 μM) in the absence or presence PTX (0.5 μg/ml). The cells were stained with Propidium iodide (1 μg/ml). The death cell was seen as nuclear positive staining. N=3-4, * P<0.01 vs NTG, WT, GRK-TG; # P<0.05 v.s. ISO PKA-TG and control PKA-TG group; $ P<0.05 v.s. ISO alone self-strain; & P<0.05 v.s. Zinterol alone.
### Table I. Characteristics of β₂AR in hearts from NTG, WT-TG, PKA-TG, and GRK-TG mice

<table>
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<tr>
<th>Groups</th>
<th>β-AR (fmol/mg protein)</th>
<th>K_d (pM)</th>
<th>β₂AR %</th>
<th>β₂-AR (fmol/mg protein)</th>
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<tr>
<td>NTG Sham (4)</td>
<td>28.6±3.4</td>
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N=3-4, mean ± SE, *P<0.05 vs NTG sham

### Table II. Affinity of β₂AR for ICI 118551

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<th>Groups</th>
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<th>kL (nM)</th>
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<td>NTG TAC (3)</td>
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N=3-4, mean ± SE, *P<0.05 vs unity