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Determining the absolute requirement of G protein-coupled receptor kinase 5 for pathological cardiac hypertrophy: short communication.

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Determining the Absolute Requirement of G Protein–Coupled Receptor Kinase 5 for Pathological Cardiac Hypertrophy

Short Communication

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Rationale: **Heart failure (HF) is often the end phase of maladaptive cardiac hypertrophy. A contributing factor is activation of a hypertrophic gene expression program controlled by decreased class II histone deacetylase (HDAC) transcriptional repression via HDAC phosphorylation. Cardiac-specific overexpression of G proteinen–coupled receptor kinase-5 (GRK5) has previously been shown to possess nuclear activity as a HDAC5 kinase, promoting an intolerance to in vivo ventricular pressure overload; however, its endogenous requirement in adaptive and maladaptive hypertrophy remains unknown.**

Objective: **We used mouse models with global or cardiomyocyte-specific *GRK5* gene deletion to determine the absolute requirement of endogenous GRK5 for cardiac hypertrophy and HF development after chronic hypertrophic stimuli.**

Methods and Results: **Mice with global deletion of *GRK5* were subjected to transverse aortic constriction. At 12 weeks, these mice showed attenuated hypertrophy, remodeling, and hypertrophic gene transcription along with preserved cardiac function. Global *GRK5* deletion also diminished hypertrophy and related gene expression due to chronic phenylephrine infusion. We then generated mice with conditional, cardiac-specific deletion of *GRK5* that also demonstrated similar protection from pathological cardiac hypertrophy and HF after transverse aortic constriction.**

Conclusions: **These results define myocyte GRK5 as a critical regulator of pathological cardiac growth after ventricular pressure overload, supporting its role as an endogenous (patho)-physiological HDAC kinase. Further, these results define GRK5 as a potential therapeutic target to limit HF development after hypertrophic stress. (*Circ Res*. 2012;111:1048–1053.)**

Key Words: G protein–coupled receptor kinases ■ cardiac hypertrophy ■ conditional transgenic mouse

Heart failure (HF), a leading cause of death in the Western world, often occurs as an end phase of pathological myocardial hypertrophy.[1] Hypertrophy is initially an adaptive response to stresses ranging from hypertension, valve disease, or cardiac injury.[2,3] In an attempt to normalize wall stress, cardiomyocytes enlarge, sarcomeres reorganize, fibroblasts proliferate, and hypertrophic genes, including the so-called fetal gene program, are upregulated. If prolonged, these responses lead to chamber dilation, myocardial apoptosis, and HF.[2,3]

In pathological cardiac growth, the molecular pathways affecting transcription lie downstream of the nodal hypertrophic signal transducer, Gq.[1] Among these complex pathways are the class II histone deacetylases (HDACs). Physiologically opposed to cardiac growth, the HDACs repress expression of key hypertrophic genes, primarily through inhibition of myocyte enhancer factor 2 (MEF2).[3] Genetic deletion of the class II HDAC, HDAC5, sensitizes mice to cardiac stress, whereas murine deletion of MEF2 confers cardioprotection, decreasing pathological hypertrophy and attenuating upregulation of the fetal gene program.[4] HDAC kinases control nuclear HDAC activity as phosphorylation of HDAC5 induces its nuclear export and MEF2 derepression.[3–5]

In This Issue, see p 947
Editorial, see p 957

We recently identified G protein-coupled receptor (GPCR) kinase-5 (GRK5) as a nuclear HDAC kinase, joining protein kinase D (PKD) and calmodulin-dependent kinase II (CaMKII) as HDAC-mediated facilitators of cardiac growth after hypertrophic stimuli.[5–7] This represents a novel, non-GPCR cardiac role for this GRK. We showed that mice with cardiac overexpression of GRK5 demonstrate Gq-dependent nuclear translocation of GRK5, where it can phosphorylate HDAC5 and induce

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Non-standard Abbreviations and Acronyms

AR	adrenergic receptor
CaMKII	calmodulin-dependent kinase II
%EF	ejection fraction percent
GPCR	G protein–coupled receptor
GRK	G protein–coupled receptor kinase
HDAC	histone deacetylase
HF	heart failure
HW/BW	heart weight–to–body weight ratio
KO	knockout
LV	left ventricular
LVIDs	systolic left ventricular internal diameter
LVPWT	left ventricular posterior wall thickness
MEF2	myocyte enhancer factor 2
PBS	phospho-buffered saline
PE	phenylephrine
PKD	protein kinase D
TAC	transverse aortic constriction
WT	wild-type

MEF2 activity.[6] Moreover, cardiac GRK5-overexpressing mice displayed an intolerance to ventricular pressure overload with potentiated maladaptive hypertrophy and accelerated HF after transverse aortic constriction (TAC).[6] Importantly, these transgenic studies did not address whether endogenous GRK5 plays a role in the hypertrophic response.

In the present study, we used global and cardiomyocyte-specific GRK5 knockout (KO) mice to directly address the importance of endogenous GRK5 in cardiac hypertrophy. We found that GRK5 in cardiomyocytes is absolutely required for hypertrophic responses after stress. Further, our data indicate that limiting GRK5 expression in the heart can protect against maladaptive cardiac growth and HF development.

Methods

Generation of GRK5cKO Mice

Detailed mouse protocols are described in the Online Data Supplement Methods.

Surgical Procedures and Echocardiography

TAC methodology, minipump implantation, and determination of in vivo cardiac function and morphology are described in the Online Data Supplement and Methods.

RNA Analysis

Methods for RNA analysis are described in the Online Data Supplement.

Immunoblot Analysis

Detailed methods for heart subfractionation and immunoblotting are described in the Online Data Supplement.

Results

Global GRK5 Deletion Diminishes In Vivo Cardiac Hypertrophy

We subjected male global *GRK5* gene knockout mice (GRK5gKO)[8] and littermate wild-type (WT) control mice to TAC. Constitutive *GRK5* deletion attenuated pressure-overload-induced cardiac growth seen in WT mice at 12 weeks after TAC (Figure 1A). Cardiac dimensions were measured serially by echocardiography to track development of hypertrophy and left ventricular (LV) dilatation over 12 weeks. WT mice showed a quick rise in LV posterior wall thickness (LVPWT), with a peak thickness of 1.99±0.05 mm at 4 weeks after TAC, which then decreased rapidly, indicative of adverse remodeling (Figure 1B). Interestingly, global *GRK5* deletion significantly delays the initiation of cardiac hypertrophy after TAC, as GRK5gKO mice do reach a similar LVPWT (1.89±0.09 mm) as WT mice but not until 12 weeks after TAC (Figure 1B). At the end of 12 weeks, GRK5gKO mice had significantly less cardiac hypertrophy as determined by smaller heart weighten-to-enbody weight (HW/BW) ratios (Figure 1C). Importantly, GRK5gKO mice show no signs of LV dilatation via echocardiographic measurements of systolic LV interior diameter (LVIDs), which were significantly increased in post-TAC WT mice (Figure 1D and Online Figure I). Further, post-TAC GRK5gKO mice showed preserved cardiac function as determined by ejection fraction percent (%EF) compared with post-TAC WT mice that had significant LV dysfunction (Figure 1E and Online Figure I).

We have previously shown that elevated GRK5 plays a critical role in myocytes as an HDAC5 kinase, derepressing hypertrophy-related transcription.[6] Upregulation of generalized hypertrophy markers, including those of the fetal gene program—atrial natriuretic factor (ANF), β-myosin heavy chain (βMHC), and procollagen, type Iα2 (Col1a2)—were significantly attenuated in the GRK5gKO mice compared with WT mice 12 weeks after TAC (Figure 1F). Loss of GRK5 expression globally also prevented the post-TAC upregulation of hypertrophic genes directly regulated by MEF2[9]: brain natriuretic peptide (BNP), actin-α1 (Acta-1), and connective tissue growth factor (CTGF) (Figure 1G). Thus, deletion of GRK5 is protective against hypertrophy at the molecular level, consistent with the in vivo phenotype.

Grk5gKO Mice Are Resistant to Phenylephrine-Dependent Hypertrophy

Phenylephrine (PE), acting through α-adrenergic receptors (αARs), induces cardiomyocyte hypertrophy in vitro and in vivo. Previously, we have found that PE causes GRK5 nuclear translocation and increased MEF2 activity in myocytes.[6] Therefore, we tested whether endogenous GRK5 was necessary for development of PE-induced cardiac hypertrophy. Male mice (WT and GRK5gKO) were treated with a subpressor dose of PE (35 mg/kg per day) or phospho-buffered saline (PBS) for 14 days via osmotic minipumps (Figure 2A). This period covers only an initial hypertrophy stage without decompensation, thereby testing the requirement of endogenous GRK5 in myocardial αAR-mediated hypertrophy. PE treatment caused cardiac growth in WT mice, with a significant 26.9±8% increase in HW/BW ratio at 2 weeks, whereas GRK5gKO mice subjected to PE had only an 11.2±7% increase in HW/BW, insignificant compared with PBS-treated GRK5gKO mice (Figure 2B). Two weeks of PE treatment does not change cardiac function, which is what we found for %EF (Figure 2C), although PE significantly altered morphology in WT mice (Figure 2D and 2E). Importantly, PE-treated GRK5gKO mice showed no changes in cardiac dimensions (Figure 2D, 2E).

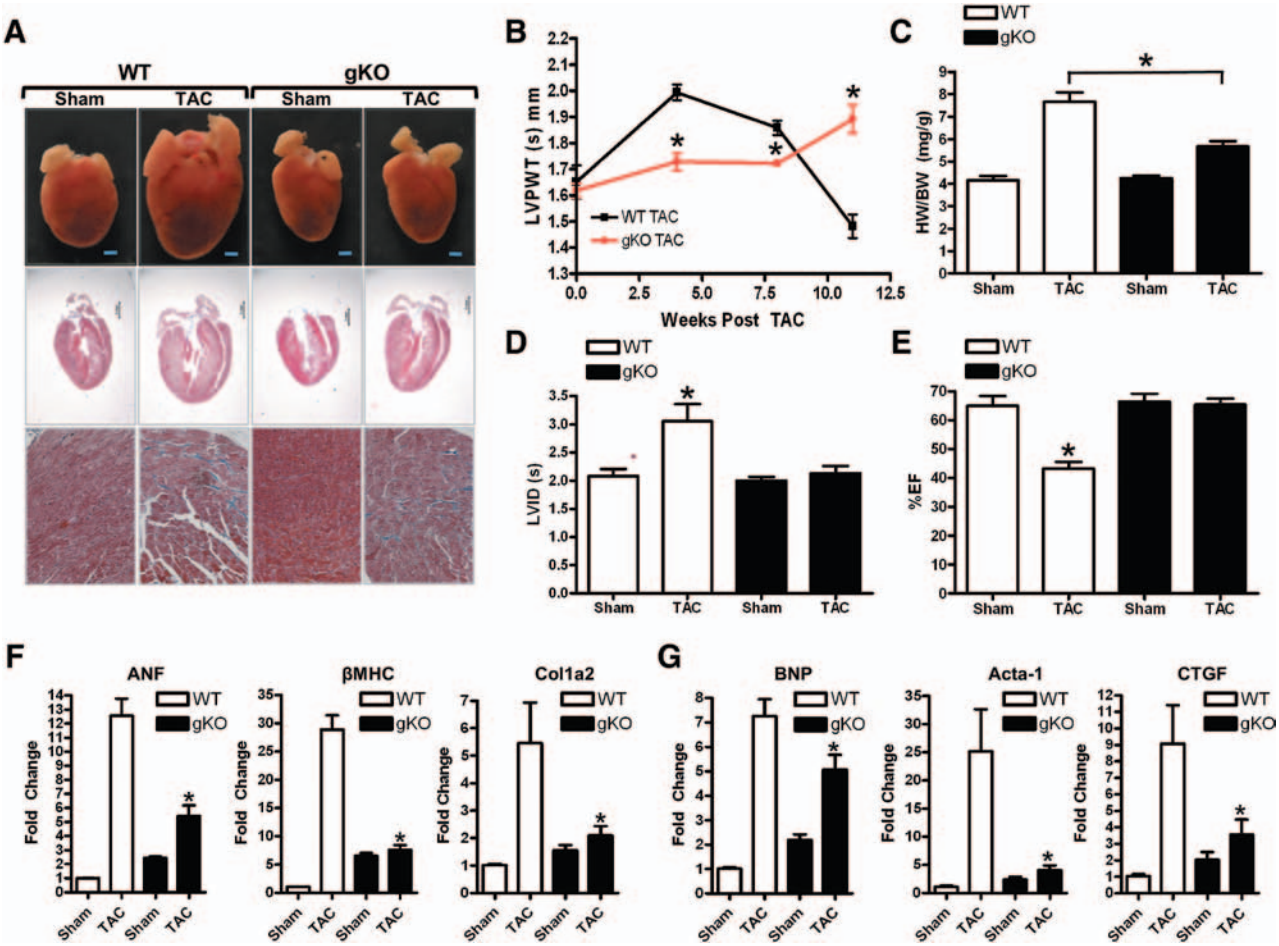

Figure 1. Attenuated hypertrophy seen in GRK5gKO mice after TAC. **A**, Hearts from WT and GRKgKO mice subjected to a sham operation or TAC **(top)**. Histological sections stained are with Masson trichrome for fibrosis **(middle; bottom)**. WT sham, n=6; WT TAC, n=9; gKO sham, n=6; gKO TAC, n=11. **B**, Systolic LVPWT measured serially by echocardiogram after sham or TAC operations. *P<0.05. **C**, HW/BW ratios were measured 12 weeks after TAC. *P<0.01. LVIDs **(D)** or %EF **(E)** as measured by echocardiogram at 12 weeks after TAC. *P<0.01. RT-PCR was used to measure mRNA expression of known markers of cardiac hypertrophy **(F)** and genes directly regulated by MEF2 **(G)**; n=8, *P<0.05.

As with post-TAC, *GRK5* deletion significantly decreased PE-mediated upregulation of hypertrophy markers. Indeed, whereas PE led to robustly increased expression of our panel of hypertrophic genes in WT mice, no such upregulation was seen in GRK5gKO mice (Figure 2F and 2G). Overall, these data demonstrate that GRK5 plays a key role in cardiac hypertrophy downstream of PE, identical to post-TAC phenotype.

Global GRK5 Ablation Decreases Nuclear HDAC5 Export After Hypertrophic Stimulus

The diminished upregulation of genetic hypertrophy markers seen in GRK5gKO mice after TAC suggests that GRK5 can regulate cardiac gene transcription. This is probably due to the ability of GRK5 to phosphorylate HDAC5, inducing its export from the nucleus.[6] Hence, we examined location and phosphorylation of HDAC5 after hypertrophic stress in GRK5gKO and WT mice. Hearts from the above experiments were subjected to subcellular fractionation. After TAC, WT mice showed a significantly increased amount of phosphorylated HDAC5 in the non-nuclear subcellular fraction compared with GRK5gKO TAC mice and WT sham mice (WT TAC: 1.94±0.13;GRK5gKO TAC: 0.91±0.14; WT sham: 1.02±0.17) (Figure 3A and 3B). Similar results were seen in the non-nuclear fraction from WT mice that had received chronic infusion of PE. These mice showed significantly greater non-nuclear phosphorylated HDAC5 than PE-infused GRK5gKO mice and PBS-infused WT mice (Figure 3C), further reinforcing the role of endogenous GRK5 as an HDAC5 kinase.

Cardiac-Specific Deletion of GRK5 Attenuates Hypertrophy After TAC

The above results show that complete GRK5 ablation attenuates the cardiac hypertrophic response but does not address the specific role of myocyte GRK5 in maladaptation and post-TAC HF. Therefore, we developed conditional GRK5KO mice in which *GRK5* deletion was cardiac-specific. We bred *floxed*GRK5 mice with transgenic mice expressing Cre-recombinase under control of the αMHC promoter.[10] These conditional GRK5 KO (GRK5cKO) mice had >50% loss of cardiac GRK5 determined by either protein immunoblotting or RT-PCR (Online Figure II). As above, we stressed GRK5cKO mice and WT control mice (GRK5floxed) via TAC and studied these groups alongside sham-operated mice for 12 weeks (Figure 4A). Serial echocardiography showed significantly attenuated hypertrophy

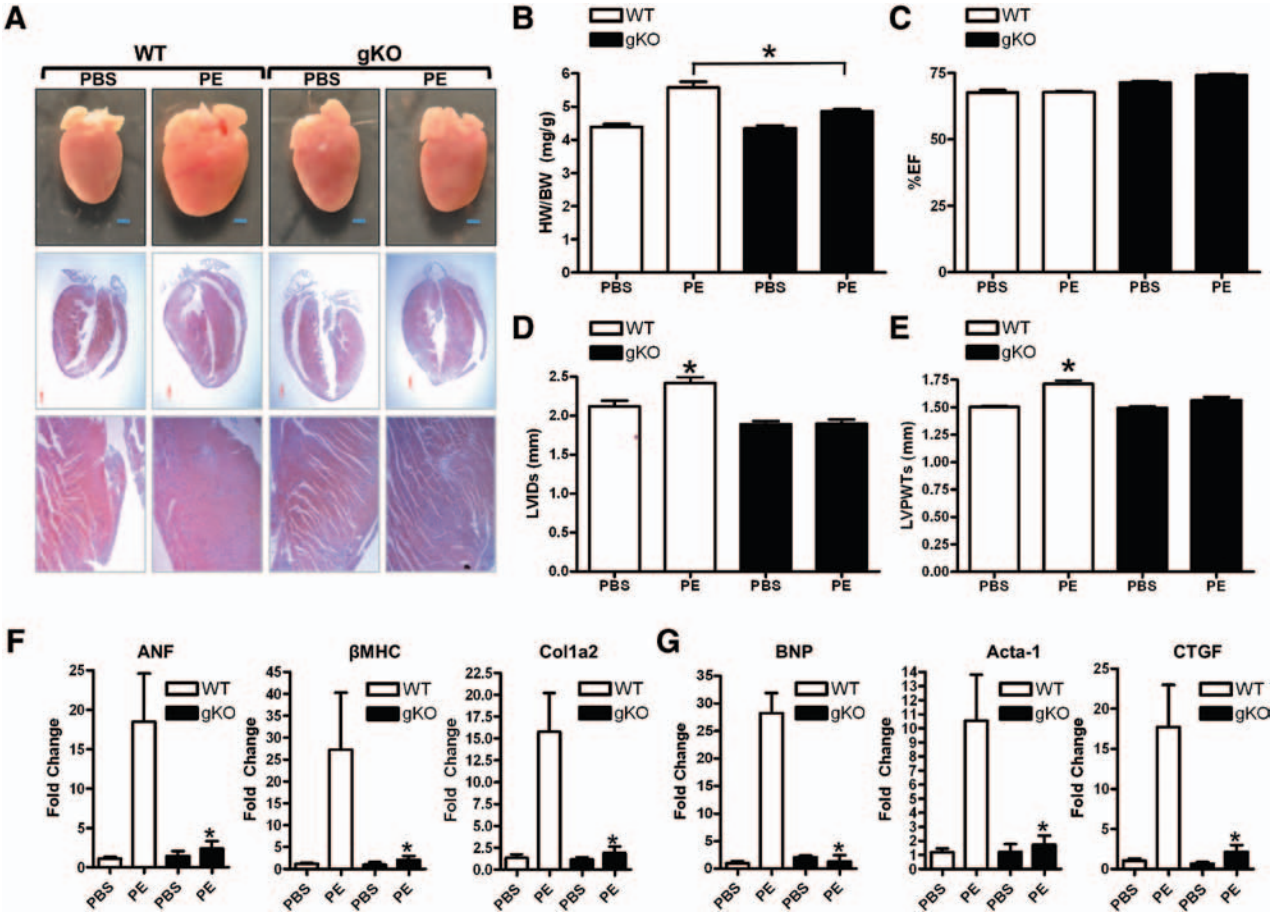

Figure 2. PE induces less hypertrophy in GRK5gKO mice. A, Hearts from WT and GRK5 gKO mice subjected to 2 weeks of chronic infusion of PBS or subpressor PE (35 mg/kg per day) **(top)**. Histological sections are stained with Masson trichrome for fibrosis **(middle; bottom)**. WT PBS, n=8; WT PE, n=10; gKO PBS, n=9; gKO PE, n=11. **B**, HW/BW ratios for WT and GRK5gKO mice were measured after 2 weeks of chronic PBS or PE infusion. **P*<0.05. %EF **(C)**, LVIDs **(D)**, and LVPWT **(E)** as measured by echocardiogram at 2 weeks. **P*<0.05. RT-PCR was used to measure mRNA expression of known markers of cardiac hypertrophy **(F)** or MEF2-regulated genes **(G)**; n=8, **P*<0.05.

in GRK5cKO mice with maximally increased cardiac mass at 12-weeks when WT mice are clearly decompensated after their peak hypertrophy 4 weeks after TAC (Figure 4B). This delayed compensatory hypertrophy led to a trend toward lower HW/BW ratio in GRK5cKO mice at 12 weeks after TAC compared with WT mice (Figure 4C). This effect is less robust than the GRK5gKO mice but no doubt due to incomplete GRK5 ablation. However, from in vivo functional studies, it is clear that the myocyte GRK5 loss protects these hearts from adverse LV remodeling and HF, as GRK5cKO mice displayed no increased LV dilatation at the study's end, compared with significantly increased LVIDs in WT mice (Figure 4D and Online Figure III). This protection against HF was also evident in global in vivo cardiac function as 12-week post-TAC WT mice had a significant loss of LV %EF compared with sham WT mice, while there was absolutely no drop in %EF in 12-week post-TAC GRK5cKO mice (Figure 4E).

Again, we examined our panel of hypertrophy-related genes. At 12 weeks after TAC, hearts from GRK5cKO mice showed significantly diminished upregulation of these common cardiac hypertrophy markers, including those of the fetal gene program (Figure 4F), and specific MEF2-regulated genes (Figure 4G). Overall, these data demonstrate that GRK5 expression in cardiomyocytes alone is required for WT molecular, functional, and morphological responses after TAC.

Discussion

Our results indicate an absolute requirement of cardiomyocyte GRK5 for normal hypertrophic responses and that this kinase plays a critical pathological role in ventricular decompensation and transition to HF after ventricular pressure overload. Importantly, merely decreasing cardiomyocyte GRK5 in mice blunts hypertrophic myocardial growth and prevents HF after TAC. Importantly, these data demonstrate that endogenous myocyte GRK5 plays a crucial role in adaptive and maladaptive hypertrophy and is required for WT response to stress. Thus, increased GRK5 in the failing human heart[11] has pathological significance, since lowering GRK5 or inhibiting its activity appears to offer novel beneficial effects against maladaptive cardiac growth.

These results in global and cardiac-specific GRK5KO mice, coupled with our previous results showing that GPCR-independent nuclear activity of GRK5 can facilitate hypertrophy,[6] indicate that nuclear targeting of endogenous GRK5 as a class II HDAC kinase is physiologically significant in normal and abnormal cardiac growth after stress. Interestingly, loss of nuclear GRK5 activity can delay HF onset through slower cardiac growth, although its deletion does not completely ameliorate hypertrophy. This may be due to compensatory and discrete roles of the other known HDAC5 kinases, CAMKII, and PKD,

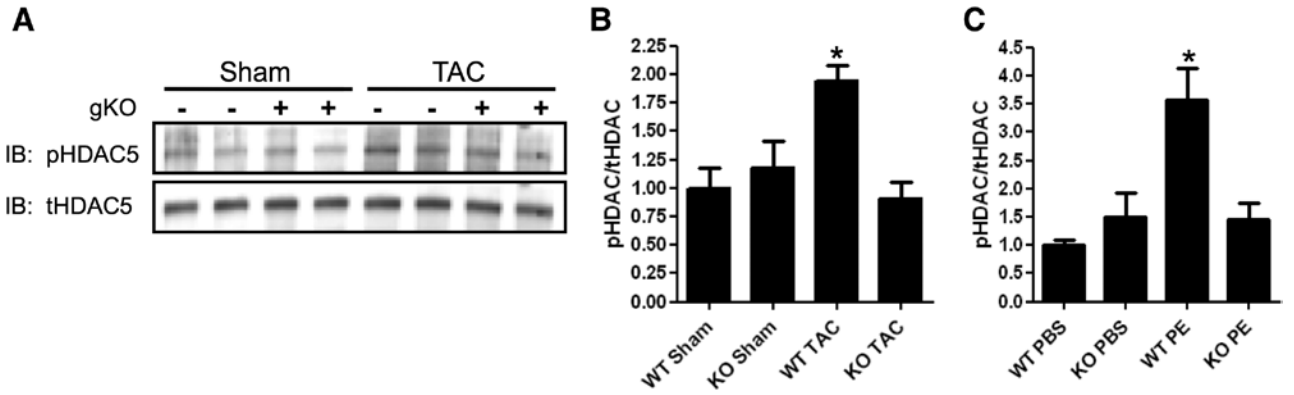

Figure 3. Global GRK5 ablation decreases phosphorylated HDAC5 in the cytoplasm. **A**, Twelve weeks after TAC or a sham operation, hearts from WT and GRK5gKO mice were fractionated into non-nuclear and nuclear fractions. HDAC5 was immunoprecipitated from the non-nuclear fraction and immunoblotted for phosphorylated and total amounts (pHDAC5 and tHDAC5, respectively). **B**, Denistometric quantification of pHDAC5 normalized to tHDAC. **P*<0.01 versus all groups, n=6. **C**, Denistometric quantification of pHDAC5 normalized to tHDAC5 after immunoprecipitation and immunoblotting for pHDAC5 in WT and GRK5gKO mouse hearts after 2 weeks of chronic PBS or PE infusion. **P*<0.01 versus all groups, n=6.

acting downstream of hypertrophic signaling.[5,7] Each kinase has been shown to cause HDAC5 nuclear export after select receptor activation such as endothelin-1 receptor for CAMKII and αAR for PKD.[5,12] We now can add GRK5 to the list of physiological HDAC kinases downstream of TAC and αAR stimulation. These distinct activators of HDAC kinases appear to underlie a complex network of parallel signaling converging on the same target, HDAC5. Overall, our data demonstrate that targeting only 1

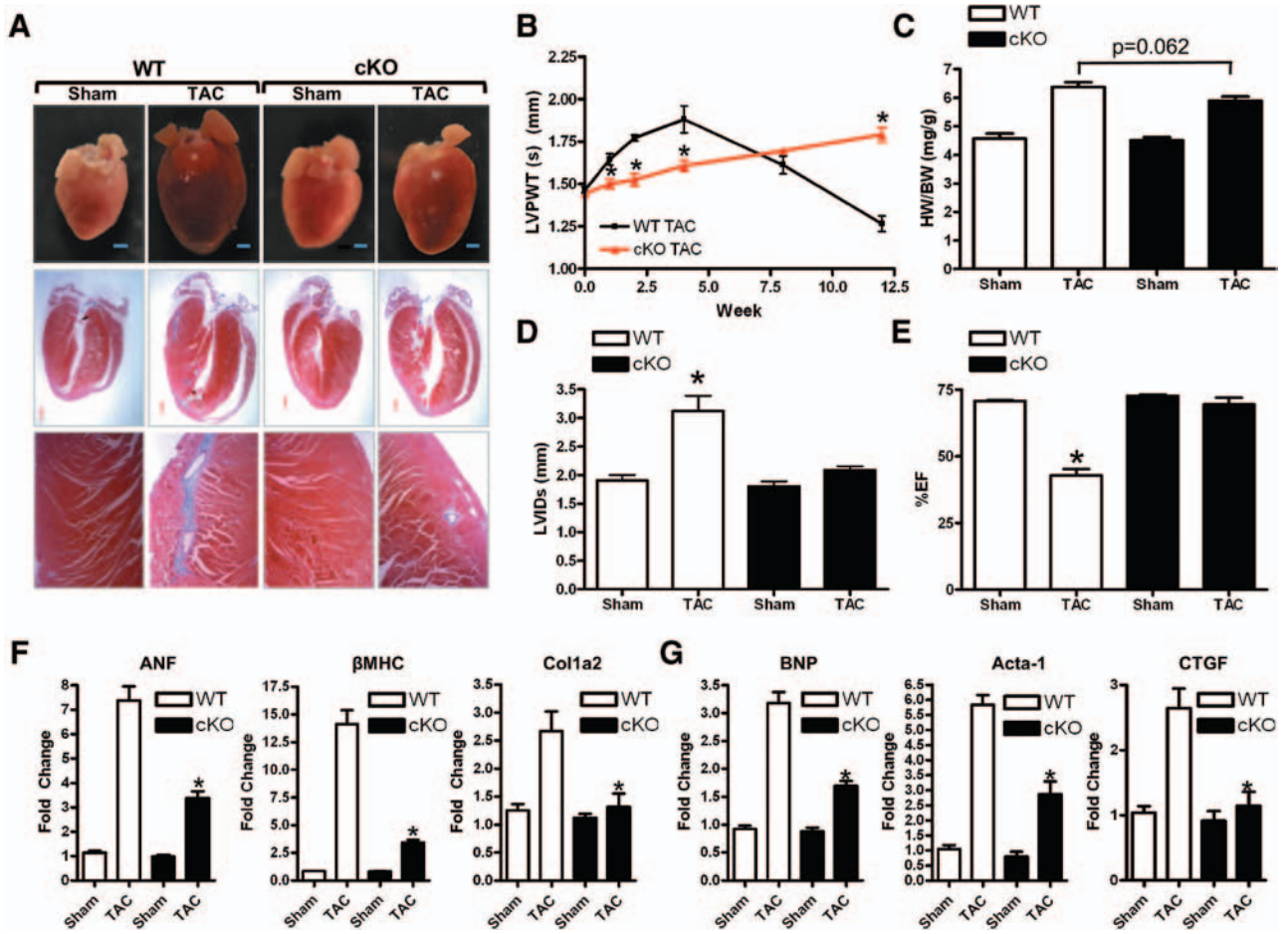

Figure 4. Attenuated hypertrophy seen in GRK5cKO mice after TAC. **A**, Hearts from WT and GRK5cKO mice subjected to a sham operation or TAC **(top)**. Histological sections are stained with Masson trichrome for fibrosis **(middle; bottom)**. WT sham, n=9; WT TAC, n=14; cKO sham, n=8; cKO TAC, n=9. **B**, Systolic LVPWT measured serially by echocardiogram after sham or TAC operations. **P*<0.05. **C**, HW/BW ratios for WT and GRK5cKO mice were measured 12 weeks after TAC. LVIDs **(D)** or %EF **(E)** as measured by echocardiogram at 12 weeks after TAC. **P*<0.01. RT-PCR was used to measure mRNA expression of known markers of cardiac hypertrophy **(F)** and MEF2-regulated genes **(G);** n=8, **P*<0.05.

HDAC kinase can delay HF and may be advantageous in therapeutic intervention by allowing some signaling.

Because GRK5 plays a dual role in the cardiomyocyte—membrane-associated GPCR desensitizing kinase and nuclear kinase facilitating transcription—the most critical activities for cardiac signaling and function are uncertain. Classic GRK5 activity toward β-adrenergic receptors (βARs) has been shown to induce transactivation of the cardioprotective epidermal growth factor receptor.[13] A human polymorphism of GRK5 (Q41L) appears to increase βAR desensitization, protecting some HF patients chronically.[14] Additionally, transgenic overexpression of Gαq caused a slight cardiac dilatation in GRK5gKO mice compared with WT controls.[15] Therefore, there is some question as to whether the increased GRK5 is protective or injurious.

In the present study, simply decreasing myocyte GRK5, either completely in GRK5gKO mice or significantly in GRK5cKO mice, attenuated cardiac hypertrophy and prevented pathogenesis of HF. We show that GRK5 ablation does not completely prevent hypertrophy but significantly delays it. Therefore, despite potential beneficial activities at the plasma membrane, removing cardiomyocyte GRK5 has a profound positive effect on outcomes after pressure overload and chronic α-adrenergic stress. Clearly, our data now indicate that GRK5, and, most likely, its nuclear HDAC kinase activity, represent a novel target to prevent maladaptive cardiac hypertrophy and protect against ventricular decompensation and HF.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- G protein-ncoupled receptor kinase 5 (GRK5) is upregulated in numerous models of heart failure (HF), as well as in the failing human heart.
- GRK5 enters the nucleus and acts as a histone deacetylase 5 (HDAC5) kinase, increasing transcription of cardiac hypertrophy genes.
- Increased nuclear GRK5 is pathological in the setting of chronic pressure overload.

What New Information Does This Article Contribute?

- Ablation of GRK5 significantly delays maladaptive cardiac remodeling and HF after chronic pressure overload or α-adrenergic receptor (αAR) stimulation.
- Removing nuclear GRK5 by global ablation decreases HDAC5 export.
- Deletion of GRK5 in cardiomyocytes alone significantly delays the onset of HF.

Pathological cardiac hypertrophy, a process commonly ending in HF, occurs through activation of nodal signal transducer, Gq. Downstream of Gq, class II HDACs represses hypertrophic gene transcription. GRK5, a recently identified HDAC kinase, has been shown to be upregulated in human HF, although the is unknown. Here, we investigated the role of endogenous GRK5 in maladaptive cardiac remodeling. Our results show that global and cardiomyocyte-specific ablation of GRK5 significantly attenuates pathological cardiac hypertrophy, delaying HF onset. This cardioprotection may be attributed to decreased nuclear HDAC5 export after GRK5 deletion. Overall, this study suggests that endogenous nuclear GRK5 plays an injurious role in maladaptive hypertrophy and may represent a novel therapeutic target.

Supporting Information Materials and Methods

Generation of Conditional GRK5 knockout mice. . All animal procedures and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Thomas Jefferson University. The GRK5/flox line was created as described in Gainetdinov et al, 1999[1], except that a Cre transfected, GRK5-targeted ES cell line was selected for deletion of the TK-NEO marker cassette but maintenance of the exon 7/8 region of the GRK5 gene flanked by loxP sites. These GRK5/flox cells were microinjected into day 3.5 blastocysts (c57BL/6J) and implanted into pseudopregnant mice to generate founder chimeric pups. After germline transmission was verified, GRK5/flox mice were backcrossed with c57BL/6J mice for >12 generations. An initial cross between $GRK5^{flox/flox}$ and αMHC-Cre mice led to a cardiac-specific GRK5 deletion. This conditional deletion was maintained by continued breeding of the $GRK5^{flox/flox}$ with $GRK5^{flox/flox}$/αMHC-$Cre^{+/-}$. Only 8 week-old males from the above cross were utilized in our experiments. Genotyping was performed using specific PCR primers designed for the loxP site.

TAC. Transverse aortic constriction was performed as described previously[2]. Briefly, 8 week-old mice were sedated in an isoflurane sedation box (induction 3% and maintenance 5%) and anesthetized to a surgical plane with an i.p. dose of ketamine (50mg/kg) and xylazine (2.5mg/kg). Anesthetized mice were intubated using a blunt 20-gauge needed that was connected to a volume-cycle rodent ventilator on supplemental oxygen at a rate of 1 L/min and respiratory rate of 140 bpm/min. A midline cervical incision was made to expose the trachea, carotid arteries and rib cage. Aortic constriction was performed by tying a 7.0 nylon suture ligature against a 27-gauge needle that was promptly removed to yield a 0.4 mm constriction. Pressure gradients were determined by *in vivo* echocardiography of the transverse aorta and mice with gradients greater than 30 mmHg were used.

Echocardiography. Echocardiography was performed as previously described[3]. To measure global cardiac function, echocardiography was performed at 8 weeks of age prior to TAC and at 1, 2, 4, 8, and 12 weeks post-TAC by use of the VisualSonics VeVo 770 imagingsystem with a 707 scan head in anesthetized animals (1.5% isoflurane, vol/vol). The internal diameter of the left ventricle was measure in the short-axis view from M-mode recordings in end diastole and end systole. At 1-week post-TAC, pressure gradients were determined by PW Doppler mode.

Mini-osmotic pumps. Chronic infusion of phenylephrine (Sigma) was achieved using Alzet 2 week osmotic minipumps (model 2002, DURECT Corporation). Pumps were filled following the manufacturer's specifications with sterile PBS, or PE (35μM/kg/day). Briefly, Mice were anesthetized with isofluorane (2.5% vol/vol) and pumps were implanted subcutaneously through a sub-scapular incision, which was then closed using 4.0 silk suture (Ethicon). The contents of the pumps were delivered at a rate of 0.5μl/hour for 2 weeks. Mice were followed by echocardiogram prior to pump implantation and then weekly until the end of the 2 week period when mice were euthanized.

RNA Isolation and Semiquantitative PCR. RNA isolation and analysis was performed as previously described[2]. Total RNA isolation was performed using the Ultraspec RNA Isolation System (Biotecx), according to the manufacturer's protocol and as previously

described[2]. After RNA isolation, cDNA was synthesized from 200ng of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Semiquantitative PCR was carried out on cDNA using iQ SYBR Green Supermix (Bio-Rad Laboratories) and 100 nM of the following gene-specific oligonucleotides—*18S*, *ANF*, *βMHC*, *BNP*, *Col1a2*, *CTGF*, and *Acta-1*. Quantitation was established by comparing 18s rRNA, which was similar between groups, for normalization. For each run, saturation of amplification cycles was controlled by the use of MyiQ software (version 1.0); subsequently, a melting curve was generated by heating the product to 95°C, cooling to and maintaining at 55°C for 20 seconds, and then slowly (0.5°C/s) heating to 95°C to determine the specificity of PCR products, which was then confirmed by gel electrophoresis.

Immunoblot Analysis. Isolated cardiac tissue was first homogenized using a Dounce homogenizer in a buffer containing: 4mM Hepes, 320mM sucrose, 10mM KCL, 5mM EDTA, 2mg NaF, 8mg $MgCl_2$, .1% Triton x-10, 1.094g DTT, and protease inhibitors. The homogenate was filtered through a 70μm cell filter. Total cell lysate was taken at this point. Then the lysate was subjected the same protocol as previously described for NRVM[2]. Following subcellular fractionation, 500 μg of non-nuclear protein was immunoprecipitated with protein A/G and anti-HDAC5 (sc-133106, Santa Cruz). Proteins were subjected to SDS-PAGE, and immunoblotted with antiphospho-HDAC5 (PA1-14187, Thermo Scientific), and anti-HDAC5 (sc-133106, Santa Cruz). Visualization of Western blot signals was performed using secondary antibodies coupled to Alexa Fluor 680 or 800 (Molecular Probes) on a LI-COR infrared imager (Odyssey). Pictures were processed by Odyssey version 1.2 infrared imaging software. All densitometry scans were carried out in the linear range of detection.

Statistics. All the values in the text and figures are presented as mean ± SEM from given *n* sizes. Statistical significance of multiple treatments was determined by one-way ANOVA followed by the Bonferroni's post hoc test when appropriate. Statistical significance between two groups was determined using the two-tailed Student's *t* test. *P* values of <0.05 were considered significant.

Supplemental References

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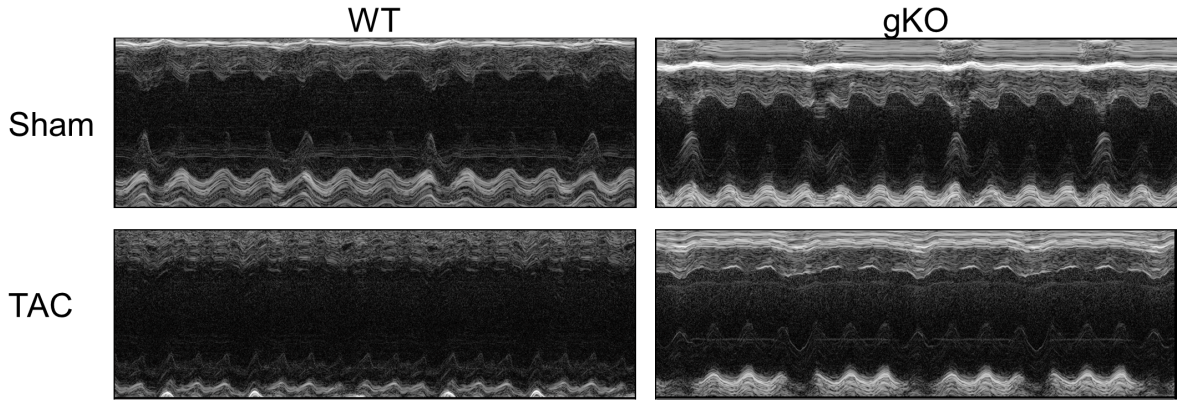
Online Supplemental Figure Legends

Online Fig. I Representative Echocardiogram tracings from WT and GRK5gKO mice 12 weeks following sham or TAC operations

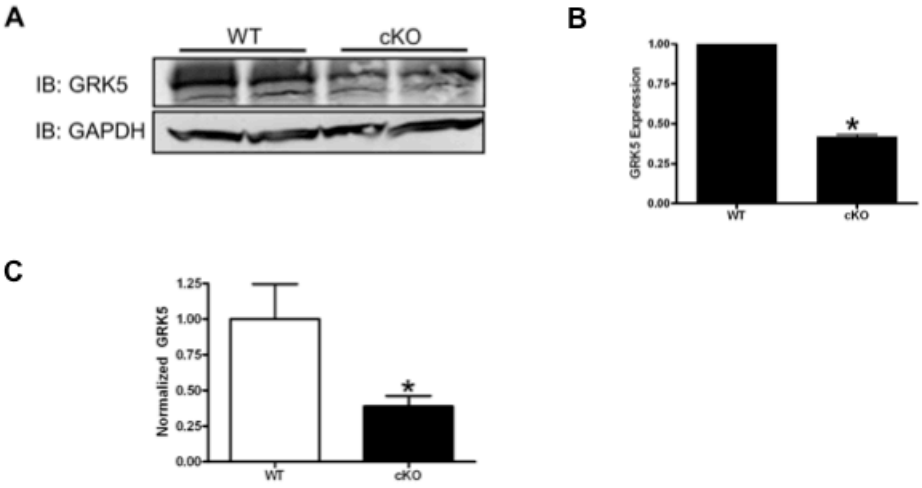
Online Fig. II GRK5Flox/αMHC-Cre^{+} mice have a greater than 50% knockdown of GRK5. (A) To determine GRK5 expression in our αMHC-Cre system, hearts from GRK5Flox/αMHC-Cre^{-} (WT) and GRK5Flox/αMHC-Cre^{+} (cKO) were homogenized and immunoblotted for GRK5 and GAPDH. (B) Quantitative analysis of immunoblots for GRK5 expression in the hearts of (A). n=4, *p<0.01. (C) Semi-quantitative PCR was used to assess GRK5 mRNA levels in the hearts of WT or cKO mice. n=6, *p<0.05

Online Fig. III Representative Echocardiogram tracings from WT and GRK5cKO mice 12 weeks following sham or TAC operations.

Online Supplemental Figure I



Online Supplemental Figure II



Online Supplemental Figure III