GRK5-Mediated Exacerbation of Pathological Cardiac Hypertrophy Involves Facilitation of Nuclear NFAT Activity


Rationale: G protein–coupled receptor kinases (GRKs) acting in the cardiomyocyte regulate important signaling events that control cardiac function. Both GRK2 and GRK5, the predominant GRKs expressed in the heart, have been shown to be upregulated in failing human myocardium. Although the canonical role of GRKs is to desensitize G protein–coupled receptors via phosphorylation, it has been demonstrated that GRK5, unlike GRK2, can reside in the nucleus of myocytes and exert G protein–coupled receptor–independent effects that promote maladaptive cardiac hypertrophy and heart failure.

Objective: To explore novel mechanisms by which GRK5 acting in the nucleus of cardiomyocytes participates in pathological cardiac hypertropy.

Methods and Results: In this study, we have found that GRK5-mediated pathological cardiac hypertrophy involves the activation of the nuclear factor of activated T cells (NFAT) because GRK5 causes enhancement of NFAT-mediated hypertrophic gene transcription. Transgenic mice with cardiomyocyte-specific GRK5 overexpression activate an NFAT-reporter in mice basally and after hypertrophic stimulation, including transverse aortic constriction and phenylephrine treatment. Complimentary to this, GRK5 null mice exhibit less NFAT transcriptional activity after transverse aortic constriction. Furthermore, the loss of NFATc3 expression in the heart protected GRK5 overexpressing transgenic mice from the exaggerated hypertrophy and early progression to heart failure seen after transverse aortic constriction. Molecular studies suggest that GRK5 acts in concert with NFAT to increase hypertrophic gene transcription in the nucleus via GRK5’s ability to bind DNA directly without a phosphorylation event.

Conclusions: GRK5, acting in a kinase independent manner, is a facilitator of NFAT activity and part of a DNA-binding complex responsible for pathological hypertrophic gene transcription. (Circ Res. 2014;115:976-985.)

Key Words: G protein–coupled receptor kinase ■ heart failure ■ nuclear factor of activated T cells

Heart failure (HF) is a clinical end point defined by the heart’s inability to perfuse the body with blood adequately. This condition affects >5 million Americans with 82,500 new cases annually.1 Although HF can be the result of many diverse etiologies, there seems to be common underlying molecular mechanisms, including the dysfunction of the β-adrenergic receptor (β-AR) system, dysregulation of myocyte calcium handling and activation of the fetal gene program among which are genes that can lead to pathological cardiac hypertrophy.2 β-ARs act to drive the contractile function but become dysfunctional after chronic catecholamine stimulation, which occurs in HF. G protein–coupled receptor (GPCR) kinases (GRKs) phosphorylate these receptors leading to their desensitization and down-regulation.3 GRK2 and GRK5, the 2 major GRKs in the heart, are in fact upregulated in HF leading to a loss of the heart’s inotropic reserve.4,5 In fact, GRK2 inhibition and the improved resensitization of β-AR signaling in the failing heart have led to HF reversal and a potential therapeutic strategy.6 The role of GRK5 on cardiac β-AR signaling is less understood although GRK5 can also desensitize these GPCRs.6

Recently, many non-GPCR functions of GRKs have been discovered and some of these seem to be physiologically important. For example, GRK2 is a prodeath kinase in myocytes acting at the level of mitochondria in a non-GPCR fashion mediated by oxidative stress.8 Although GRK5 is not found in the mitochondria, it does contain a nuclear localization sequence homologous to homeobox-containing transcription factors.

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which allows it to translocate to the nucleus where it has been shown to have DNA-binding properties in some cells.\textsuperscript{10,11} In cardiomyocytes, it has been demonstrated that GRK5 can accumulate in the nucleus in a Gq-dependent manner either through pharmacological hypertrophic stimulation or left ventricular (LV) pressure-overload.\textsuperscript{12–14} Once in the nucleus, GRK5 has been shown to phosphorylate histone deacetylase-5 (HDAC5) leading to derepression of myocyte enhancer factor 2 (MEF2)–mediated hypertrophic gene transcription.\textsuperscript{14} This was best shown in transgenic mice with cardiac GRK5 overexpression because these mice, but not mice overexpressing nuclear localization sequence-mutant GRK5 that cannot accumulate in the nucleus, displayed exaggerated cardiac hypertrophy and early onset HF after pressure-overload.\textsuperscript{14} Not only did the nuclear localization sequence–GRK5 mutant mice not have maladaptive cardiac hypertrophy but also GRK5 knockout mice are protected against pressure-overload stress.\textsuperscript{12}

In addition to the phosphorylation of HDAC5, nuclear GRK5 has been shown to activate the nuclear factor-xB (NF-xB) pathway through phosphorylation of IkB-Bcl and regulate cell cycle progression through direct phosphorylation of nucleophosmin.\textsuperscript{15–17} Thus, there could be additional targets in the nucleus of hypertrophic cardiomyocytes. One of the key transcription factors involved with myocardial hypertrophy and subsequent HF is the nuclear factor of activated T-cells (NFAT). NFAT resides in the cytoplasm in a hyperphosphorylated state and then on increased calcium entry, NFAT is dephosphorylated by the calcium-sensitive phosphatase calcineurin and then it can translocate to the nucleus where it controls transcription of several hypertrophic and maladaptive genes.\textsuperscript{18} The NFAT pathway is an interesting therapeutic target because it is involved in pathological, but not in physiological hypertrophy and several drugs exist that can regulate this pathway including cyclosporine.\textsuperscript{19,20}

Herein, we provide evidence that GRK5 is able to activate the NFAT transcriptional pathway after hypertrophic stress. Our data reveal that the GRK5 localization in the nucleus and not in the cytosol facilitates the transcriptional activity of NFAT in vitro and in vivo. Furthermore, NFATc3 expression is needed for the induction of early pathology after hypertrophic stress with enhanced GRK5 expression. Molecular studies suggest that GRK5 acts in concert with NFAT at the level of chromatin to facilitate hypertrophic gene transcription in a kinase independent manner. Thus, nuclear GRK5 seems to be a key player and the determinant of pathological cardiac hypertrophy, and limiting its accumulation in the nucleus may allow for the development of novel therapeutics and strategies to prevent and reverse the progression of maladaptive cardiac hypertrophy and HF.

### Methods

#### Cell Culture

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day-old rats as previously described.\textsuperscript{21} H9c2 cells, a rat myoblast cell line, were cultured in Dulbecco’s modified Eagle’s medium from ATCC supplemented with 10% bovine calf serum and penicillin-streptomycin in a humidified chamber with 5% CO\textsubscript{2} at 37 °C.

#### Adenovirus, Plasmids, and Transfection

NRVMs were infected with recombinant, replication-deficient adenoviruses at a multiplicity of infection from 1 to 10 viral particles per cell. Plasmids encoding wild-type full-length bovine GRK5, GRK5 K215R kinase dead and the GRK5 NES nuclear exclusion sequence mutant (a kind gift of Dr Julie Pitcher at University College London) in the pRK5 vector were transiently transfected into H9c2 cells using Lipofectamine 2000 as described.\textsuperscript{11,22–24}

#### Luciferase Assay

Cells were harvested 48 hours after infection in lysis buffer according to the manufacturer’s protocol (Promega).\textsuperscript{14}

#### Real-Time Polymerase Chain Reaction

Total RNA was extracted by the TRIzol method and reverse transcription polymerase chain reaction (RT-PCR) was performed as described.\textsuperscript{12}

#### Experimental Animals

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University.

#### Echocardiography

To measure the global cardiac function, echocardiography was performed with the VisualSonics VeVo 2100 imaging system in anesthetized animals as described.\textsuperscript{12}

#### Transverse Aortic Constriction

Transverse aortic constriction (TAC) was performed as described previously.\textsuperscript{14}

#### Miniosmotic Pumps

Chronic infusion of phenylephrine (phenylephrine, purchased from Sigma) was done using Alzet 3-day miniosmotic pumps (model 1003D; DURECT Corporation) following manufacturer’s specifications.

#### Cardiomyocyte Cross-Sectional Area

Axial cut tissue sections were stained with Alexa Fluor 594 conjugated of wheat germ agglutinin. Cell borders were planimetered manually by an operator who was blinded to the treatment group.

#### Electrophoretic Mobility Shift Assay

IRDye 700 end labeled NFAT consensus oligonucleotides were incubated with 2.5 μg NRVM nuclear extracts. For antibody-mediated super shift assay, 1 μg antibody was incubated with 5 μg nuclear extract in a reaction mixture. Protein–DNA complexes were separated on 4%
nondenaturing polyacrylamide gel and visualized using an Odyssey infrared imaging system.17

Statistics
All the values in the text and figures are presented as mean±SEM. Statistical significance was determined by Student t test or ANOVA. P values of <0.05 were considered significant.

Results
GRK5 Enhances Cardiac NFAT Transcriptional Activity In Vitro
Recent data from our laboratory have shown that GRK5 acting in the nucleus is a key mediator of pathological cardiac hypertrophy and this is because, in part, of its actions as a HDAC kinase.12–14 In this study, we sought other actions of GRK5 in the nucleus, because in addition to its kinase activity in the nucleus, GRK5 has been shown to have potential DNA-binding capabilities10,11 and to interact with other nuclear proteins.25 It is well known that in addition to hypertrophic gene regulation.26 To investigate whether GRK5 may influence NFAT activity, we first used an NFAT reporter assay in myocytes. In NRVMs expressing an NFAT-luciferase reporter adenovirus, we overexpressed GRK5 and found significantly increased luciferase levels after stimulating the cells with the hypertrophic α1-adrenergic agonist, phenylephrine (Figure 1A).

Figure 1. G protein–coupled receptor kinase 5 (GRK5) enhances cardiac nuclear factor of activated T cell (NFAT) transcriptional activity in vitro. A, NFAT luciferase activity in neonatal rat ventricular myocytes (NRVMs) infected with an NFAT luciferase reporter virus and a GRK5 or LacZ control virus. Cells were stimulated for 24 hours with 50 μmol/L phenylephrine (PE; n=6; ***P<0.01; ***,P<0.001 by ANOVA). B, Quantitative reverse transcription polymerase chain reaction (RT-PCR) for NFAT target gene RCAN1.4 (regulator of calcineurin [RCAN]) in NRVMs overexpressing GRK5 or LacZ control for 48 hours and stimulated with 50 μmol/L PE for 16 hours (n=3; *P<0.05; **P<0.01 by ANOVA). C, Quantitative RT-PCR for NFAT target gene RCAN in NRVMs overexpressing GRK5 or LacZ control adenovirus with or without constitutively active Grq (CAM-Grq) adenovirus 48 hours after adenoviral infection (n=3; **P<0.01; ***P<0.001 by ANOVA).

To confirm that GRK5-mediated increases in NFAT luciferase activity postphenylephrine was because of a true induction in NFAT transcriptional activity, we performed an additional and complementary experiment where we measured expression levels of the NFAT target gene regulator of calcineurin (RCAN1.4; henceforth referred to only as RCAN) in myocytes after phenylephrine stimulation with varying levels of GRK5. NRVMs were subjected to viral overexpression of GRK5 or LacZ as a control followed by stimulation with 50 μmol/L phenylephrine for 16 hours, and RCAN levels were assessed by quantitative RT-PCR. Although phenylephrine was able to increase RCAN expression in myocytes, we found a significant increase in RCAN mRNA levels when GRK5 levels were enhanced (Figure 1B). This confirmed that GRK5 overexpression is able to induce increased NFAT transcriptional activity with RCAN expression serving as an endogenous reporter of NFAT activity.

Finally, phenylephrine is known to activate the α1-AR and hypertrophic signaling occurring downstream of Gαq activation. To confirm that GRK5’s actions on NFAT after phenylephrine were because of Gαq activation, we performed RCAN expression assays in NRVMs expressing a constitutively active mutant of Gαq and found that when GRK5 is elevated there is significantly more RCAN expression induced with constitutively active Gαq (Figure 1C).

In separate experiments using immunofluorescence and confocal microscopy, we found that GRK5 overexpression in myocytes does not cause more NFAT to accumulate in the nucleus after phenylephrine stimulation. This indicates that the induction of NFAT activity by GRK5 is not because of more NFAT translocating to the nucleus, but rather somehow enhancing activity within the nucleus (Online Figure I). Supporting this are studies where overexpressing GRK5 had no effect on cytoplasmic calcineurin activity (Online Figure IC and ID).

GRK5 Enhances NFAT Transcriptional Activity In Vivo in Models of Cardiac Pathology
After demonstrating in vitro that increased GRK5 expression in myocytes can enhance NFAT activity and hypertrophic gene transcription, we sought to confirm these findings in vivo. To do so, we crossed cardiac-specific NFAT luciferase reporter mice previously characterized20 with mice that our laboratory previously created with cardiac-specific transgenic overexpression...
of GRK5 (TgGRK5). Using in vivo bioluminescence imaging techniques, we found that cardiac GRK5 overexpression causes increased NFAT activity in the upper thoracic cavity consistent with the heart (Online Figure II). Taking hearts from TgGRK5 mice, luciferase reporter mice and luciferase reporter/TgGRK5 hybrid mice, we assessed the ex vivo luciferase activity and found approximately twice as much NFAT activity in the TgGRK5 mice compared with those with endogenous levels of GRK5 (Figure 2A). Somewhat surprisingly, these results indicate that only by overexpressing GRK5 in the myocytes, NFAT activity is enhanced. Overall, this activity, although significant, must not be robust enough to drive hypertrophy because the luciferase reporter/TgGRK5 mice do not exhibit altered cardiac mass at this age in the absence of hypertrophic stress.

To determine whether the increase in NFAT luciferase activity seen in TgGRK5 mice was relevant in the context of pathology, these mice were subjected to left ventricular pressure-overload through surgical TAC. Cardiac function in these animals was assessed 14 days after TAC by echocardiography. The ejection fraction in the TgGRK5/luciferase reporter mice that were subjected to TAC was significantly decreased (Figure 2B), consistent with early post-TAC HF seen with TgGRK5 mice previously. Importantly, after 14 days of pressure-overload, an ex vivo NFAT luciferase assay was performed on heart tissue and NFAT activity via luminescence readings was significantly higher in luciferase reporter mice as expected; however when GRK5 was elevated in these mice (luciferase reporter/TgGRK5), there was significantly more NFAT activity (Figure 2C).

To confirm that this increase in luciferase activity was indeed a result of increased NFAT transcriptional activity, we performed RT-PCR from the hearts of these mice for the NFAT target gene RCAN. After TAC, animals with endogenous levels of GRK5 (luciferase reporter mice) showed an increase in RCAN expression, whereas luciferase reporter/TgGRK5 hybrid animals demonstrated a significant increase in RCAN expression compared with all other groups (Figure 2D). Therefore, using an endogenous gene targeted readout, we were able to confirm that NFAT transcriptional activity is increased in TgGRK5 mice in a model of pathology.

It is known that TAC causes hypertrophy through the Gαq pathway and has been shown to drive GRK5 into the nucleus; therefore, we wanted to test whether GRK5 overexpression would enhance NFAT activity after stimulation of the Gαq pathway via the α1-adrenergic hypertrophic agonist phenylephrine in these animals. Miniosmotic pumps were used to deliver a subpressor dose of phenylephrine subcutaneously for 24 hours in luciferase reporter mice and luciferase...
reporter/TgGRK5 mice. As seen previously, TgGRK5 mice alone pumped with vehicle control have an increase in NFAT luciferase activity as compared with luciferase reporter-alone mice with endogenous levels of GRK5 (Figure 2E). After phenylephrine treatment, luciferase reporter/TgGRK5 mice demonstrate a significant increase in NFAT activity over all other groups (Figure 2E). Thus, in this in vivo mouse model, we demonstrate that GRK5 is able to increase NFAT transcriptional activity basal and down-stream of the Gαq hypertrophic signaling pathway.

**GRK5 Knockout Mice Demonstrate Attenuated NFAT Transcriptional Activity After Hypertrophic Stress**

To determine that GRK5-mediated activation of the NFAT pathway is physiologically relevant, we subjected GRK5 null (GRK5 knockout) mice to LV pressure-overload via TAC. At baseline, 2 and 4 weeks, post-TAC echocardiography was performed to assess LV posterior wall thickness of these animals as a marker of hypertrophy. At baseline, we observed no difference in wall thickness; however, at 4 weeks, post-TAC wild-type mice showed a significant increase in wall thickness compared with all other groups (Figure 3A). As expected from previous work in our laboratory, the increase in wall thickness seen after TAC was significantly attenuated in GRK5 knockout mice (Figure 3A). After 4 weeks, hearts were harvested and RT-PCR was performed on heart tissue to determine the level of transcription of the NFAT target gene RCAN and we found a significant increase in RCAN transcription in wild-type mice subjected to TAC as compared with all other groups (Figure 3B). Interestingly, we found less RCAN transcription in GRK5 knockout mice subjected to TAC when compared with wild-type littermate mice that also underwent TAC surgery (Figure 3B), indicating that endogenous levels of GRK5 are required to get a normal hypertrophic response that includes NFAT-mediated RCAN expression. These results are consistent with GRK5 being relevant for normal NFAT activation after pressure-overload.

**NFATc3 Knockout Attenuates GRK5-Mediated Cardiac Pathology After Pressure-Overload Stress**

To determine whether NFAT activity is required for GRK5-mediated cardiac pathology after TAC, we bred TgGRK5 mice with mice null for NFATc3 (NFATc3 knockout). We found significant differences in mice overexpressing GRK5 in the hearts with and without NFATc3 as early as 1 week after TAC as determined initially by a simple heart weight to body weight ratio (Figure 4A; Online Figure III). As expected, we observed a significant increase in the heart weight to body weight ratio in TgGRK5 mice subjected to TAC compared with control nontransgenic, wild-type animals that have endogenous levels of GRK5 (Figure 4A). Interestingly, NFATc3 deletion significantly attenuated the observed increase in heart weight seen in the TgGRK5 mice after TAC (Figure 4A). We also assessed cardiac function in these animals by echocardiography at 1 week post-TAC and found EF% significantly decreased in the TgGRK5 mice compared with control mice supporting early onset HF (Figure 4B; Online Figure IV). However, importantly, this LV dysfunction that was present in TgGRK5 mice at 1 week was not present in TgGRK5 mice where NFATc3 is deleted, including LV dilatation (Figure 4B and 4C). Thus, the loss of NFATc3 can prevent early progression to HF after TAC when GRK5 is overexpressed in myocytes. Chronically, at 4 weeks post-TAC, these TgGRK5/NFATc3 knockout mice were dysfunctional consistent with GRK5 overexpression exerting HDAC kinase effects (data not shown). We also assessed the cardiomyocyte cross-sectional area in these animals to confirm that hypertrophy occurs at the cellular level using wheat germ agglutinin staining and indeed TgGRK5 mice display a significantly increased cell size after pressure-overload compared with wild-type littermate mice although NFATc3 deletion leads to an attenuation of cardiomyocyte hypertrophy as shown by a significant decrease in the cross-sectional area after TAC in TgGRK5 mice (Figure 4D).

Finally, we assessed the transcription of selected genes by quantitative RT-PCR to determine on a molecular level whether NFATc3 deletion protects the TgGRK5 mice from transcriptional changes associated with the post-TAC GRK5-mediated HF phenotype (Figure 4E). Induction of the fetal gene program was assessed by transcription of genes for the contractile proteins skeletal muscle actin-1 and β-myosin heavy chain. To assess remodeling and fibrosis of the heart, transcription of the connective tissue growth factor gene was also determined. Finally, the transcription of the NFAT target gene RCAN was analyzed to show specificity for NFAT. All of...
these transcripts were similarly upregulated in TgGRK5 mice compared with wild-type mice after TAC; however, expression of all these genes is attenuated when NFATc3 is deleted (Figure 4E). Therefore, NFATc3 deletion in cardiac GRK5 overexpressing mice leads to a more favorable genetic profile after LV pressure-overload.

GRK5 Interacts With NFAT in a Kinase Independent Manner Through DNA-Binding

To assess the mechanism by which GRK5 activates the NFAT pathway, we used H9c2 myoblasts to introduce plasmids carrying wild-type GRK5 and mutants that render GRK5 kinase-dead (K215R) or incapable of being exported from the nucleus (nuclear export signal); (Online Figure VII). All of these GRK5 proteins can accumulate in the nucleus after hypertrophic stimulation, and we tested whether kinase activity and nuclear localization are required for NFAT activation via GRK5. These cells were also infected with the constitutively active Gαq mutant adenovirus to activate hypertrophic signaling, and RT-PCR was performed for the NFAT target gene RCAN. We found that the nuclear export signal mutant that becomes trapped in the nucleus caused the greatest activation of NFAT (Figure 5A). This is not surprising because nuclear GRK5 is responsible for the exaggerated cardiac pathology seen in TgGRK5 mice after pressure-overload.6 Surprisingly, this experiment also demonstrates that the kinase dead
K215R GRK5 was able to activate NFAT to a similar degree as wild-type GRK5 (Figure 5A). This proves that the activation of NFAT by GRK5 does not involve a phosphorylation event and, therefore, segregates the activation of MEF2 by GRK5 via HDAC5 phosphorylation from the activation of the NFAT pathway.

Because the activation of NFAT by GRK5 seems to occur within the nucleus (Figure 5A), we decided to perform an electrophoretic mobility shift assay to determine whether GRK5 was able to alter DNA-binding by NFAT. The assay was performed by incubating NFAT-specific DNA probes with nuclear lysates from NRVMs overexpressing GRK5 or a LacZ control and stimulated with phenylephrine for 5, 15, or 30 minutes (Figure 5B). We found that GRK5 was able to potentiate NFAT:DNA binding because GRK5 overexpression led to an increase in the amount of NFAT-specific DNA probe that was bound after phenylephrine stimulation (Figure 5B).

Next, we hypothesized that GRK5 may be interacting with NFAT at the level of DNA as GRK5 has previously been shown to bind DNA in a kinase independent manner.10,11 Accordingly, we performed an electrophoretic mobility shift assay with an antibody-mediated supershift to determine whether GRK5 was present in a complex with NFAT at the level of DNA (Figure 5C). Nuclear lysates from NRVMs overexpressing GRK5 and stimulated with phenylephrine for 30 minutes were incubated with IgG, GRK2, or GRK5 antibodies. Importantly, a shift band was observed in the lysate incubated with the GRK5 antibody, but not with IgG or GRK2 negative controls (Figure 5C). This suggests that GRK5 interacts with NFAT at the level of DNA to potentiate the binding of the NFAT:DNA complex and adds a new mechanism for GRK5 in the facilitation of hypertrophic gene transcription (Figure 6).

Discussion

Although the canonical role of GRKs is to phosphorylate activated seven transmembrane receptors leading to their desensitization and down-regulation, a growing non-GPCR interactome is emerging.25 GRK5 and GRK6 have the unique properties among GRK family members to translocate and localize to the nucleus, whereas in myocytes it has been shown that GRK5 has the non-GPCR activity of acting as a class II HDAC kinase facilitating maladaptive cardiac

Figure 5. G protein–coupled receptor kinase 5 (GRK5) interacts with the nuclear factor of activated T cell (NFAT) in a kinase independent manner through DNA binding. A, Quantitative reverse transcription polymerase chain reaction for NFAT target gene regulator of calcineurin (RCAN) in H9c2 cells transfected with wild-type GRK5, kinase dead (K215R) GRK5 mutant, nuclear export sequence (NES) GRK5 mutant or vector (pRK5) control for 72 hours and infected with the constitutively active Gαq (CAM-Gq) virus for 24 hours (n=3; *P<0.05; **P<0.01; ***P<0.01 by ANOVA). B, Electrophoretic mobility shift assay was performed with 2 μg of nuclear extract (NE) from cultured neonatal rat ventricular myocytes (NRVMs) infected with GRK5 or LacZ control and stimulated with hypertrophic agonist phenylephrine (PE) for 0, 5, 15 or 30 minutes to analyze the DNA binding activity of NFAT using IR dye-labeled oligonucleotides containing the consensus NFAT binding sequence. Lane 1 is free probe no NE; lanes 2 to 9, GRK5 overexpression and 50 μmol/L PE treatment as indicated; lane 10, NE from GRK5 overexpressing myocytes stimulated with 50 μmol/L PE for 30 minutes and 200-fold excess nonlabeled consensus oligonucleotides. Shown is a representative image from 3 independent experiments. C, Antibody-mediated supershift EMSA using 5 μg NE from AdGRK5 infected (NRVMs) stimulated with PE for 30 minutes. Samples were incubated with 1 μg specific antibodies to GRK2, GRK5, or rabbit IgG. Shown is a representative image from 3 independent experiments.
GRK5 Enhances NFAT Activity in Cardiac Hypertrophy

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Nuclear translocation of GRK5 occurs via stimulation of the Gq pathway after transaortic constriction (TAC) or phenylephrine stimulation via activated calmodulin (CaM) binding the N-terminus of GRK5. CaM binding causes GRK5 to dissociate from the plasma membrane and translocate to the nucleus.13 Once in the nucleus, GRK5 phosphorylates histone deacetylase 5 (HDAC5) leading to its nuclear export and derepression of the transcription factor myocyte enhancer factor 2. In parallel, CaM also binds to and activates the phosphatase calcineurin, which dephosphorylates the nuclear factor of activated T cells (NFAT) leading to its nuclear translocation. At the level of the DNA, GRK5 potentiates NFAT:DNA binding and enhances the transcription of hypertrophic genes and subsequent maladaptive cardiac hypertrophy.12–14 This novel nuclear activity of GRK5 was confirmed as playing a role in the normal hypertrophic response of the heart since GRK5 knockout mice (global and cardiac) are protected from pathological hypertrophy.12–14 This novel nuclear activity of GRK5 was considered in Figure 6.

Figure 6. Schematic depicting the facilitation of hypertrophic transcription by G protein–coupled receptor kinase 5 (GRK5). Nuclear translocation of GRK5 occurs via stimulation of the Gq pathway after transaortic constriction (TAC) or phenylephrine stimulation via activated calmodulin (CaM) binding the N-terminus of GRK5. CaM binding causes GRK5 to dissociate from the plasma membrane and translocate to the nucleus.13 Once in the nucleus, GRK5 phosphorylates histone deacetylase 5 (HDAC5) leading to its nuclear export and derepression of the transcription factor myocyte enhancer factor 2. In parallel, CaM also binds to and activates the phosphatase calcineurin, which dephosphorylates the nuclear factor of activated T cells (NFAT) leading to its nuclear translocation. At the level of the DNA, GRK5 potentiates NFAT:DNA binding and enhances the transcription of hypertrophic genes and subsequent maladaptive cardiac hypertrophy.

In this study, we used mutant constructs of GRK5 to determine a possible mechanism for the regulation of the NFAT pathway by GRK5 and indeed confirmed a nuclear-dependent effect that happens to be kinase independent. We found that the nuclear export signal mutant of GRK5, which lacks a nuclear export sequence and is therefore trapped in the nucleus, leads to the greatest activation of NFAT activity. This is logical because in vivo experiments performed previously in our laboratory found nuclear GRK5 to be the cause of cardiac pathology after TAC.14 Surprisingly, the kinase-dead GRK5 K215R mutant was able to activate NFAT similar to wild-type GRK5 providing the first hint of kinase independent regulation of this transcription factor by GRK5. Previously, GRKs have been shown to exert kinase independent effects, including GRK5, which was shown recently to promote filamentous actin bundling through a kinase independent manner by interacting with F-actin and phosphatidylinositol 4,5-bisphosphate.25,29 This finding with GRK5-K215R limits the possibility of NFAT regulation through HDAC kinase activity or other phosphorylation events, which is different from how GRK5 can regulate MEF2 hypertrophic gene transcription. The kinase-independent activation of NFAT by GRK5 seems logical when one considers that NFAT is negatively regulated by phosphorylation yet GRK5, a kinase, is able to increase its activity. Other kinases, such as extracellular signal-regulated kinase, casein kinase II, c-Jun N-terminal kinase, p38, protein kinase A, and glycogen synthase kinase-3β, negatively regulate the NFAT pathway through phosphorylation of NFAT.26–30,34 In addition, CAMKII is able to oppose the NFAT pathway by direct phosphorylation of calcineurin.15

Contrary to the above kinases, which oppose NFAT activity, p90 ribosomal S6 kinase has been shown to potentiate NFAT:DNA binding through interaction with the NFAT:DNA complex.36 This is of particular interest to this study because both p90 ribosomal S6 kinase and GRK5 belong to the AGC protein kinase subfamily.37 Overall, our findings add significantly to the understanding of GRK5 in cardiac hypertrophic gene transcription through the 2 most important pathways (NFAT and MEF2). Importantly, these 2 transcriptional regulation pathways influenced by nuclear GRK5 occur because of either kinase-dependent effects (HDAC5 kinase) or kinase independent actions (via NFAT:DNA binding and induction of NFAT transcriptional activity). These mechanisms are illustrated in Figure 6.

Although we are not the first to suggest that GRK5 can exert effects through its DNA binding properties, we are the first to show that this can lead to positive regulation of transcription.30–38 It has been demonstrated previously that GRK5 and GRK4 subfamily member GRK6 can bind directly to DNA in vitro.30,31 Liu et al38 found that GRK5 binds directly to the Bcl-2 promoter and inhibits the transcription of Bcl-2 in vivo. Because NFAT is known to have weak DNA-binding ability and cooperates with other transcription factors in a complex,39 we believe our data are consistent with GRK5 being present in this transcriptional complex and is, therefore, able to enhance the transcription of NFAT target genes (Figure 6). This is interesting because NF-κB, which shares the DNA-binding Rel homology domain with NFAT, is also regulated by GRK5 although controversy surrounds the mechanism by which this occurs.40,41 Sorrentino et al42,43 believe that GRK5 is a negative regulator of NF-κB through forced nuclear accumulation of NF-κB inhibitor IκB-α. Islam et al43 and Patial et al44 found that GRK5 is a positive regulator of NF-κB signaling through
either upregulation of protein levels of NF-xB subunits p50 and p65 or phosphorylation of IkxB. It is possible that GRK5 is able to regulate the NF-xB pathway through interactions with p50 and p65 at the level of chromatin further.

It remains to be seen whether GRK5 binds directly to DNA to potentiate NFAT-DNA binding and transcription or whether GRK5 is simply present in a transcriptional complex with NFAT. Although we cannot rule out a role for HDACs in the regulation of NFAT activity by GRK5, we found that GRK5 acts in a kinase independent fashion and therefore the phosphorylation of HDAC5 by GRK5 is not the mechanism at work (Figure 6). Future experiments involving chromatin immunoprecipitation (ChIP) for GRK5 would allow for the identification of novel GRK5 targets and interactions. These data could then be used along with gene expression to determine which promoters are positively or negatively regulated by GRK5.

In summary, we provide evidence that GRK5 is able to activate the NFAT transcriptional pathway leading to the upregulation of hypertrophic genes. We found that the cardiac-specific NFAT luciferase reporter mice crossed with mice that overexpress wild-type GRK5 in a cardiomyocyte-specific manner exhibit an increase in NFAT activity both in the basal state and after the hypertrophic stress TAC and phenylephrine administration. Complimentary to this, GRK5 null mice exhibit less NFAT transcriptional activity after left ventricular pressure-overload as shown by the expression of the NFAT target gene RACN. Importantly, the loss of NFATc3 expression protected GRK5 overexpressing mice from the exaggerated hypertrophy and early progression to HF seen acutely after TAC. Molecular studies suggest that GRK5 acts in concert with NFAT to increase hypertrophic gene transcription in the nucleus and this is a kinase independent action of GRK5 that involves its known properties of DNA binding. The overall translatational significance of these findings is substantial as simply finding a kinase inhibitor of GRK5 as a potential therapeutic for maladaptive cardiac hypertrophy would block its HDAC kinase activity in the nucleus, but it would not prevent the activation of the NFAT pathway as our data show that GRK5 acts in a kinase independent manner. We suggest that a more effective strategy would be to develop a therapy that is capable of inhibiting the nuclear accumulation of GRK5 allowing this enzyme to exert protective effects at the sarcolemma through transactivation of the β-AR and EGF receptors although preventing its activation of MEF2 and NFAT in the nucleus.45 By developing novel therapeutics to inhibit GRK5 nuclear accumulation, it may be possible to prevent and reverse the progression of HF.

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Disclosures
None.

References
GRK5 enhances NFAT activity in cardiac hypertrophy

Novelty and Significance

What Is Known?

- GRK5 can localize to the nucleus of cardiomyocytes and this localization increases under conditions of hypertrophic stress.
- Nuclear accumulation of GRK5 promotes maladaptive cardiac hypertrophy and HF at least in part because of novel histone deacetylase kinase activity. However, in this study, we sought additional mechanisms involved in GRK5-mediated cardiac pathology. We found that GRK5 can also facilitate the activation of the hypertrophic transcription factor, NFAT.
- Studies in NFATc3 knockout mice with cardiac GRK5 overexpression demonstrate that GRK5-mediated cardiac pathology after pressure-overload requires NFAT expression.
- Molecular studies demonstrate that GRK5 acts in concert with NFAT to increase the hypertrophic gene transcription in the nucleus in a noncanonical, kinase independent manner via GRK5’s ability to directly bind DNA.

What New Information Does This Article Contribute?

- GRK5-mediated pathological cardiac hypertrophy involves the activation of the nuclear factor of activated T cells (NFAT) within the nucleus in a kinase independent manner.
- Molecular studies suggest that GRK5 acts in concert with NFAT to facilitate and increase hypertrophic gene transcription in the nucleus via GRK5’s ability to bind DNA directly.
- GRK5-dependent cardiac maladaptive hypertrophy and early onset HF after pressure overload are dependent on NFAT activity as the loss of NFATc3 expression in the heart protected GRK5 overexpressing transgenic mice from early progression to HF.

- GRK5 enters the nucleus of cardiomyocytes via a functional nuclear localization sequence in response to hypertrophic stimuli where it can act in a non-GPCR manner as a histone deacetylase 5 kinase enhancing myocyte enhancer factor 2-dependent hypertrophic gene transcription.
- Increased nuclear GRK5 is pathological in the setting of chronic pressure-overload, although GRK5 ablation significantly delays the onset of HF after pressure-overload.


GRK5-Mediated Exacerbation of Pathological Cardiac Hypertrophy Involves Facilitation of Nuclear NFAT Activity
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SUPPLEMENTAL MATERIAL

Detailed Methods:

Cell Culture:
Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- to 2-day old rats as previously described.
NRVMs were cultured in DMEM supplemented with penicillin/streptomycin (100 units/ml) and 5% bovine calf serum at 37°C in a 5% humidified atmosphere for 2–3 days. H9c2 cells, a rat myoblast cell line, were cultured in DMEM from ATCC supplemented with 10% bovine calf serum and penicillin-streptomycin in a humidified chamber with 5% CO2 at 37°C.

Adenovirus, Plasmids and Transfection:
At 24 hrs post-isolation, NRVMs were infected with recombinant, replication-deficient adenoviruses at an MOI from 1-10 viral particles per cell. Equal particles of an adenovirus expressing LacZ were used to control for non-specific adenoviral effects when GRK5 viruses were used. Plasmids encoding wild-type full length bovine GRK5, GRK5 K215R kinase dead and the GRK5 NES nuclear exclusion sequence mutant (a kind gift of Dr. Julie Pitcher at University College London) in the pRK5 vector were transiently transfected into H9c2 cells using Lipofectamine 2000 as described. Cells were harvested 72 hrs after transfection.

Luciferase Assay:
Cells were harvested 48 hrs after infection in passive lysis buffer (Promega). Luciferase activity corresponding to NFAT activity was measured according to manufacturer’s protocol (Promega) using a Victor plate reader. Lysates were loaded as equal concentrations of protein in equal volumes of buffer.

Real-time PCR:
Total RNA from mouse hearts or NRVMs was extracted with TRIzol (Life Technologies). After RNA isolation, cDNA was synthesized from RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Semiquantitative PCR was carried out on cDNA using 100 nM of the following gene-specific oligonucleotides—18s, RCAN1.4, βMHC, Acta-1. Real-time quantification was performed using Sybr Select Master Mix (Applied Biosystems) with the Applied Biosystems StepOnePlus Real-Time PCR system. Relative gene expression was normalized to that of 18s and compared using the ddCt method as described.

Experimental Animals:
Transgenic mice with cardiac-specific GRK5 overexpression (TgGRK5 mice), cardiac-specific NFAT luciferase reporter mice (a kind gift of Dr. Molkentin at the Cincinnati Children’s Hospital), global GRK5 gene ablation (GRK5 KO mice), and NFATc3 global gene ablation (NFATc3 KO mice, purchased from The Jackson Laboratory) have all been described previously. TgGRK5 mice in a C57BL/6 background exhibit ~30 fold cardiac overexpression of GRK5. These animals were backcrossed for 9 generations with NFAT luciferase reporter mice which are also in a C57BL/6 background. Male mice between 8-12 weeks of age were utilized. GRK5 KO mice in a C57BL/6 background were backcrossed for >10 generations. Male mice between 8-12 weeks of age were utilized. NFATc3 KO mice in a C57BL/6 background were backcrossed with TgGRK5 mice for 5 generations. Both male and female mice between 8-12 weeks of age were utilized.

For all in vivo experiments non-transgenic littermate control (NLC) mice or corresponding wild-type (WT) mice were used as controls. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University School of Medicine.
**Echocardiography:**

To measure global cardiac function, echocardiography was performed with the VisualSonics VeVo 2100 imaging system in anesthetized animals (1.5% isoflurane, vol/vol). The internal diameter of the left ventricle was measured in the short-axis view from M-mode recordings in end diastole and end systole as described 6.

**Transverse aortic constriction:**

Transverse aortic constriction (TAC) was performed as described previously 10. Briefly, 8 week-old mice were 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 needle 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 mm Hg were used.

**Mini-osmotic Pumps:**

Chronic infusion of phenylephrine (PE, purchased from Sigma) was done using Alzet 3-day mini-osmotic pumps (model 1003D, DURECT Corporation). Pumps were filled following the manufacturer’s specifications with sterile PBS or PE (35 mg/kg/day). Briefly, Mice were anesthetized with isoflurane (2.5% vol/vol) and pumps were implanted subcutaneously through a subscapular incision, which was then closed using 4.0 silk suture (Ethicon). The contents of the pumps were delivered at a rate of 1.0 μl/hour for 24 hrs.

**Cardiomyocyte Cross-Sectional Area:**

Axial cut tissue sections were stained with Alexa Fluor 594 conjugated of wheat germ agglutinin. For each treatment group 3 hearts were imaged using a Nikon Eclipse Ti microscope with the 20X objective. Only myocytes cut in short axis with visible nucleus were measured, minimum 50 cells per heart. Cell borders were planimetered manually by an operator who was blinded to treatment group. Nikon NIS Elements software was used to calculate 2-dimensional cross-sectional areas.

**Electrophoretic Mobility Shift Assay (EMSA):**

Nuclear extracts were prepared from NRVMs using a nuclear/cytosol fractionation kit (BioVision). Protein concentrations were determined by a Pierce BCA protein assay kit (Thermo Scientific). IRDye 700 end labeled NFAT consensus oligonucleotides (5’-ACG CCC AAA GAG GAA AAT TTG TTT CAT-3’ and 5’-TGT ATG AAA CAA ATT TTC CTC TTT GGG -3’) were used. 2.5 μg nuclear extracts were incubated with IRDye-labeled NFAT oligonucleotide in the dark for 30 min at room temperature in reaction buffer (10mM Tris, 50mM KCl, 1mM DTT; pH 7.5) and 1 μg of poly(dI-dC)-poly(dI-dC) (Li-Cor) as nonspecific competitor. For antibody mediated super shift assay, 1 μg of GRK2 (Santa Cruz sc-562), GRK5 (Santa Cruz sc-565) or rabbit IgG (Santa Cruz sc-2027) antibody was incubated with 5 μg NE in reaction mixture. Protein-DNA complexes were separated from free probe on 4% non-denaturing polyacrylamide gel. Gel was visualized using an Odyssey infrared imaging system.

**Calcineurin Assay:**

Whole heart from TK45, GRK5 KO or NLC animals was homogenized in lysis buffer (Enzo Life Sciences). Calcineurin phosphatase activity was measured according to manufacturer’s protocol (Enzo Life Sciences) using a Tecan plate reader. Lysates were loaded as equal concentrations of protein in equal volumes of buffer.
Statistics:

All the values in the text and figures are presented as mean +/- SEM. Statistical significance of multiple treatments was determined using GraphPad Prism Software Version 5 (San Diego, Calif) by Student's t-test or ANOVA followed by the Tukey’s multiple comparison test when appropriate. P values of <0.05 were considered significant.
Supplemental Figures:

**Supplemental Figure I:** *GRK5 does not alter NFAT translocation of calcineurin activity.* (A) Representative confocal images of feline myocytes infected with a NFATc3-GFP adenovirus and either GRK5 or LacZ control adenovirus for 48 hours. Cells were stimulated with 50 μmol/L phenylephrine (PE) for 1 hr before fixation and immunostaining with anti-GRK5 antibody (sc-565). Dapi is used as a nuclear marker. (B) Quantification of NFATc3 nuclear to cytoplasmic localization following 50 μmol/L PE stimulation for 0, 1 or 2 hours in feline myocytes infected with NFATc3-GFP and either GRK5 or LacZ control adenovirus. (n>180 cells per condition. ***, p<0.001 by t-test). (C) Calcineurin activity of whole heart lysates from TgGRK5 mice 1 week post-TAC and (D) GRK5 KO mice after 24 hr administration of PE as measured by calcineurin phosphatase activity assay kit. (n=3. Not significant by t-test).

A.
Supplemental Figure II: *In vivo* bioluminescence of transgenic NFAT-luciferase reporter mice. In vivo bioluminescence of transgenic NFAT-luciferase reporter mice (LUC) with endogenous levels of GRK5 or crossed with transgenic mice which overexpress GRK5 in a cardiac-specific manner (TgGRK5/LUC). Non-transgenic littermate control (NLC) does not contain the NFAT-luciferase transgene. Mice were anesthetized (1.5% isoflurane, vol/vol), injected with luciferin substrate and luciferase activity was measured using a Xenogen IVIS system.
Supplemental Figure III: Gross morphology of TgGRK5 x NFATc3 KO mice 7 days post-TAC. Hematoxylin and eosin stained axial sections of hearts from wild type, TgGRK5, NFATc3 KO or TgGRK5/NFATc3 KO mice 7 days post-TAC or post-sham to demonstrate heart size and morphology.
**Supplemental Figure IV:** *Echocardiography from TgGRK5 x NFATc3 KO mice 7 days post-TAC.*
Representative short axis M-mode images of echocardiography performed 7 days post-TAC on wild type, TgGRK5, NFATc3 KO or TgGRK5/NFATc3 KO mice.
**Supplemental Figure V:** RT-PCR of NFAT isoforms in NFATc3 KO x TgGRK5 mice 1 week post-TAC. RT-PCR from whole heart of NFATc3 KO x TgGRK5 mice 1 week post-TAC or Sham for expression of NFAT isoforms: (A) NFATc1, (B) NFATc2, (C) NFATc3 and (D) NFATc4. (n=3 per group).
**Supplemental Figure VI**: Quantification of bound NFAT specific DNA probe (Figure 5A) from nuclear fractions of NRVMs overexpressing GRK5 or LacZ control and stimulated with PE for 30 min. (n=3. **, p<0.01 by ANOVA). (B) A representative EMSA from nuclear extracts of H9c2 cells transfected with GRK2, GRK5, GRK6 or control plasmid pRK5 and infected with CAM-Gq adenovirus and incubated with IRdye700 labeled NFAT specific DNA probe. (C) EMSA was performed using an IRdye700 labeled NFAT specific DNA probe from heart nuclear lysates of WT or GRK5 knockout (KO) mice 4 weeks post-TAC or sham.
Supplemental Figure VII: Cellular localization of GRK5 mutants. Confocal images of H9c2 cells transfected with wild type, kinase dead (K215R) or nuclear exclusion mutant (NES) GRK5 for 72 hrs. Cells were fixed and immunostained with anti-GRK5 antibody (Millipore) to demonstrate GRK5 cellular localization. Dapi was used as a nuclear marker.
Supplemental References:


