PHLPP2 Negatively Regulates Phenylephrine (PE)-induced Cardiac Hypertrophy

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by

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DEDICATION

This is dedicated to my family:

To my father K.S, my mother, Lucy, and my brother, Wilson, for their endless support, encouragement, sacrifices, and love.
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ABSTRACT

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Crucial cellular events such as death, growth, metabolism, proliferation, and hypertrophy are regulated by phosphorylation and dephosphorylation of proteins. PH domain leucine-rich repeat protein phosphatase (PHLPP) is a serine/threonine phosphatase that has been shown to directly dephosphorylate several members of the AGC family of kinases. Removal of PHLPP2 in neonatal rat ventricular myocytes (NRVMs) induces hypertrophic growth and activates fetal gene expression at baseline and potentiates phenylephrine (PE)-induced gene expression 2 fold over
siControl. Since removal of PHLPP2 in NRVMs does not affect Akt phosphorylation PHLPP2 must have the ability to repress cellular hypertrophy through regulation of other unknown targets. G-protein coupled receptor kinase 5 (GRK5), an AGC kinase, has been shown to regulate cardiac hypertrophy through de-repression of gene transcription and by directly binding DNA. Upon down-regulation of PHLPP2, stimulation of NRVMs with the hypertrophic agonist PE induced GRK5 accumulation in the nucleus and export of HDAC5. Conversely, overexpression of PHLPP2 blocked PE-induced hypertrophic growth, re-expression of fetal genes and nuclear accumulation of GRK5 in cardiomyocytes. The translocation of GRK5 by PHLPP2 knockdown was dependent on calmodulin binding. Through siRNA studies it was found that GRK5 is necessary for PE-induced hypertrophy following PHLPP2 knockdown. Here we demonstrate for the first time in cardiomyocytes that knockdown of PHLPP2 regulates hypertrophic growth through GRK5. Understanding the signaling pathways affected by PHLPP2 has potential for new therapeutic targets in the treatment of cardiac hypertrophy and failure.
BACKGROUND

*Heart Failure and Significance*

Heart disease is the leading cause of death in the United States since 1935 [1]. Heart failure, which is a form of heart disease, is characterized by the heart’s inability to pump enough blood to the rest of the body, and can be caused by myocardial infarction, hypertension, atherosclerosis and valve disease [2]. In response to pressure or volume overload hearts adapt through a hypertrophic response, which allows the heart to increase cardiac output by contracting faster, develop more muscle mass, and enlarge the chambers [2, 3]. Hypertrophy is initially beneficial; however, prolonged hypertrophy can lead to decompensation and sudden death [2-4]. At the cellular level, cardiomyocyte hypertrophy is characterized by an increase in cell size, and enhanced protein synthesis [5]. At the molecular level, changes in growth are accompanied by reinduction of the so-called fetal gene program such as atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) because their pattern of gene expression mimic those seen during embryonic development [6, 7].

*Molecular pathways for pathological hypertrophy*

Considerable animal studies have shown that physiological and pathological cardiac hypertrophy is caused by an array of stimuli and various signaling pathways [3, 5, 8]. With the ongoing discovery of new proteins involved in these processes, the signaling cascades involved in cardiac hypertrophy are highly complex. Some of the known hypertrophic stimuli include angiotensin II (AngII), endothelin-1 (ET-1), phenylephrine (PE), and mechanical stress, which can signal through G-protein coupled receptors (GPCR) to trigger small G proteins to activate downstream signaling cascades [9-13]. Notable downstream targets of the activated GPCRs include; mitogen activated protein kinases (MAPKs), protein kinase C (PKC), protein kinase B (Akt), calmodulin activated kinase II (CamKII), and calcineurin [5, 9]. These signaling pathways
activate cellular responses that include transcriptional changes such as fetal gene re-expression, metabolism, cell death, and survival [2, 5, 14-16]. For instance, the signaling cascades can activate transcriptional regulators such as myocytes enhancer factor 2 (MEF2) and nuclear factor of activated T cells (NFAT), which in turn regulate fetal genes expression [3, 14, 17]. Reinduction of the fetal gene program regulates hypertrophic growth as well as cell survival [18].

**GRK5**

GRKs are a family of membrane bound serine/threonine kinases first found to desensitize GPCRs by decreasing the affinity of GPCR to their signaling molecules [19, 20]. Out of all seven GRKs, GRK2 and GRK5 have higher expression in cardiomyocytes [21]. All the GRK members contain a N-terminal domain that recognizes activated GPCRs while the rest of the domains vary across different isoforms [19, 22]. GRK5 contains two calmodulin binding sites that help translocate the protein, a kinase activity site at residue 215, a kinase domain that contains a nuclear export sequence (NES) and nuclear localization sequence (NLS) as well as two serine residues, and the C terminal that contains various phosphorylation sites (Scheme 1) [23]. In cardiomyocytes, stimulation of Gq dependent signaling leads to increased calcium/calmodulin and non-canonical signaling of GRK5, through translocation to the nucleus and phosphorylating histone deacetylase 5 (HDAC5), a repressor of MEF2 gene, or direct binding to the DNA to regulate hypertrophic growth (Scheme 2) [19].

**PHLPP family and Akt**

Akt, also known as Protein Kinase B (PKB), is a kinase that plays an important role in regulating protein synthesis, glucose metabolism, cell survival, angiogenesis, and cell growth [24, 25]. In the search for a phosphatase that directly dephosphorylates Akt on its hydrophobic motif, a serine/threonine phosphatase called PH (pleckstrin homology) domain Leucine rich repeat Protein Phosphatase (PHLPP) was discovered [26]. There are two isoforms in the PHLPP family: PHLPP1 and PHLPP2. PHLPP1 has two splice variants, PHLPP1α and PHLPP1β [27, 28]. All
PHLPP members contain a PH domain that allows it to bind to the membrane; a region of leucine-rich repeats (LRR) associated with protein-protein interaction; a PP2C phosphatase domain; and a C-terminal PDZ ligand domain that is important for its regulation of Akt. A putative Ras association (RA) domain is present on PHLPP1β and PHLPP2 (Scheme 3), which binds to Ras and results in decreased ERK signaling [27-29]. A homolog of PHLPP in yeast that retains PHLPP domain structures also contains an adenylate cyclase (AC) domain at the C-terminus [30].

PHLPP has been shown to dephosphorylate Akt and several other members of the AGC kinase family, which are a subgroup of ser/thr protein kinases that are closely related to cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinases (PKG), and PKC [26, 31]. In cancer cells, PHLPP regulates cell survival, suppressing tumor growth and cellular hypertrophy by dephosphorylating Akt on its hydrophobic motif and turning off Akt signaling [32, 33].

**PHLPP and cardiac hypertrophy**

Akt is also an important regulator of cardiac growth through the PI3K signaling pathway [19, 28, 29]. Growth factors and exercise activate Akt signaling in the heart to promote physiological hypertrophy [4, 30, 31]. However, prolonged activation of the Akt pathway leads to pathological hypertrophy [32, 33]. Although both PHLPP1 and PHLPP2 has been shown to dephosphorylate Akt in cancer cells, in cardiomyocytes, only removal of PHLPP1 alters Akt signaling [34]. Using PHLPP1 knockout mice, we found that removal of PHLPP1 increased Akt activity in the heart and promoted a more physiological type of hypertrophic response following pressure overload induced hypertrophy [34, 35]. While these studies suggest that removal of PHLPP1 would be beneficial in cardiomyocytes, the effect of PHLPP2 remains largely unknown. Our lab focuses on the PHLPP family in cardiomyocytes and their role in regulating physiological and pathophysiological stress leading to cardiac hypertrophy and heart failure.
This section, in part, is currently being prepared for submission for publication of the material. Yeh, Szu-Tsen; Zambrano, Cristina M.; Koch, Walter J.; Purcell, Nicole H. The thesis author was the primary investigator and author of this material.
INTRODUCTION

The balance between protein phosphorylation and dephosphorylation defines an important regulatory step in maintaining cellular homeostasis. The precise control between protein phosphatases and kinases is crucial for cellular decisions that lead to cell growth, metabolism, proliferation, and hypertrophy [2, 8, 32]. In cardiomyocytes, activation of G-protein coupled receptors (GPCRs) by hypertrophic agonists such as phenylephrine (PE) leads to activation of a number of intracellular signaling pathways that are important transducers of the hypertrophic response. These pathways include Calcineurin-nuclear factor of activated T cells (NFAT) [36], Ca++/calmodulin-dependent kinase II (CaMKII) [37, 38], mitogen activated protein kinases (MAPK) [39, 40], Protein Kinase C (PKC) [41], G-protein coupled receptor kinase 5 (GRK5) [42] and the Akt-mechanistic target of rapamycin (mTOR) pathway [25, 43] among many others.

Recently, the PHLPP family (PHLPP1 and PHLPP2) of serine/threonine phosphatases has been shown to directly target several members of the AGC family of kinases, including Akt, and PKC [14, 26, 30, 34, 44, 45]. Studies in cancer cells have revealed PHLPP’s ability to work as a tumor suppressor through targeting Akt and PKC ([28, 30, 46-48]. Genomic analysis of prostate cancer showed that both PHLPP1 and 2 genes are deleted at high frequency and increases the rate of prostate cancer [46], and is accompanied by high levels of Akt activity in cancer cells [28, 48]. Although in cancer cells, both isoforms of PHLPP are shown to dephosphorylate Ser473 on Akt [28, 45, 49], recent studies of the PHLPP isoforms in cardiomyocytes suggest they do not both target Akt [34].

In the heart, Akt is crucial for balancing cell survival and apoptosis in cardiomyocytes [50, 51]. Removal of PHLPP1 has been demonstrated to increase Akt activity and protect cardiomyocytes from oxidative damage [34]. Also using knockout mice, removal of PHLPP1 protects the heart from ischemic damage induced by ischemia/reperfusion injury ex vivo [34] and
pathological hypertrophy in vivo [35]. However, in contrast to cancer cell studies, down-regulation of PHLPP2 does not regulate Akt activity or other targets such as PKC [34, 52]. Although one in vitro study has shown in cardiac myocytes that dephosphorylation of Akt by PHLPP2 via beta-adrenergic stimulation occurs, the effect is within minutes and disappears rapidly [53]. Whether this occurs in vitro or in vivo to participate in pathological hypertrophy that is usually caused by prolonged exposure to beta-adrenergic stimulation is unknown. Since the function of PHLPP2 in cardiomyocytes is largely unknown, we wanted to determine the effect of PHLPP2 removal on cardiac hypertrophy in vitro. We found a novel interaction between PHLPP2 and the AGC kinase GRK5 in neonatal rat ventricular cardiomyocytes, which regulates hypertrophic growth.

GRK5 belongs to the AGC family of membrane bound kinases that classically phosphorylate GPCRs and lead to their desensitization [31, 42]. Unlike other GRKs, GRK5 translocates to the nucleus in cardiomyocytes by phenylephrine (PE) or angiotensin II (AngII) stimulation in a calmodulin-dependent manner to regulate cardiac hypertrophy [54, 55]. Accumulation of GRK5 interacts with several targets such as HDAC5, NFκB, or DNA directly, which in turn regulates transcription of genes involved in cardiomyocyte hypertrophy [54, 56-58].

In this study, we investigated GRK5 as a potential target of PHLPP2 and whether PHLPP2 modulates GRK5 induced cardiomyocyte hypertrophy. Removal of PHLPP2 showed accentuated cell growth, re-expression of fetal genes at baseline, and an elevation of PE-induced fetal gene expression. Besides physical evidence of the interaction between PHLPP2 and GRK5, removal of PHLPP2 also showed an increase in PE-induced nuclear translocation of GRK5 that could be blocked by overexpression of PHLPP2. An intact calmodulin-binding domain on the n-terminal of GRK5 was shown to be necessary for the ability of PHLPP2 knockdown to induce
translocation. Overall, our data revealed that PHLPP2 plays a role in regulating PE-induced cardiac hypertrophy via a GRK5 dependent pathway.

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MATERIALS AND METHODS

Reagents:

Phenylephrine ((R)-(−)-Phenylephrine hydrochloride) (PE) was purchased from Sigma-Aldrich (St.Louis, MO) and used at 50µM.

Animals

All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of University of California San Diego. Sprague-Dawley rats (1-2 days old) were used for cell isolation experiments.

Isolation of Neonatal Rat Ventricular Myocytes (NRVMs)

Neonatal rat ventricular myocytes were isolated from 1-2 day old Sprague-Dawley rat pups (Harlan, Indianapolis, IN). Myocytes were isolated using the Neonatal Cardiomyocyte Isolation System (Worthington, Lakewood, NJ) and plated at density of 3.5x10⁴/cm² in Dulbecco’s Modified Eagle’s Medium (DMEM) with 15% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100ug/ml streptomycin) at 37°C in 5% CO₂, as previously described [34]. After overnight culture, cell were placed in serum-free DMEM and transfected or infected as described below. DMEM, FBS, and antibiotics were purchased from Thermo Fisher Scientific (Waltham, MA).

Transfection and Adenoviral Infection

NRVMs were transfected with siRNA as previously described [59, 60]. Briefly, Pre-designed siRNA for PHLPP2 (ON-TARGET plus Rat PHLPP2, #J-104590-07-0050, Dharmacon, Lafayette, CO), siRNA for GRK5 (Qiagen rat Gqrk5, #SI01518650) and control siRNA (rat,
#1027281, Qiagen, Hilden, Germany) 2µM were transfected into NRVMs using DharmaFECT-1 (Dharmacon) transfection reagent in a 1:3 ratio respectively. Following 48 hours transfection, cells were treated with or without PE for various time points as described in figure legends.

For adenoviral infection, adenoviral vectors at 50 MOI we added to cells 24 hours after plating or after transfection as previously described [60]. Adenoviruses used expressed the following genes: full length GRK5, GFP, full length PHLPP2, and GRK5 (W30A/K31Q) which inhibits n-terminal CaM binding.

**Immunostaining**

Following 48 hours PE treatment, NRVMs were fixed in 4% paraformaldehyde. Cells were visualized using Alexa-488 Phalloidin antibody at 1:20 dilution (Cell Signaling Technology, Danvers, MA) and VECTASHIELD Antifade Mounting Medium with DAPI (Vector Lab, Burlingame, CA) for nuclear staining. Images were acquired at 20x magnification using a Leica DMI8 fluorescence microscope and DFC450C camera (Leica Microsystems, Wetzlar, Germany). Cell area was quantified using NIH Image J software (National Institutes of Health, Bethesda, Md) and area across cells with central nuclei was measured. For each condition at least 400 cells per experiment were measured for cell size analysis.

**Quantitative PCR (qPCR)**

For qPCR, following 48 hours PE treatment, total RNA was isolated using a micro-RNA isolation kit (Invitrogen) and cDNA synthesized using the Verso cDNA synthesis kit (Thermo Scientific) based on the manufacturer’s instructions as previously described [35]. Hypertrophic gene expression was analyzed using probe sets from Applied Biosystems for ANF (NPPA), BNP
(NPPB), and GAPDH as an internal control. Relative quantification was analyzed using the comparative threshold cycle (Ct) method normalized to GAPDH as previously described [35].

**Protein Isolation**

For protein analysis, whole cell extracts were isolated from NRVMs using RIPA buffer as previously described [34]. For fractionation experiments, cytosolic and nuclear fractions were isolated as previously described [34]. Protein concentration was measured using a Micro BCA Protein Assay Kit (Thermo Scientific) for further analysis. For immunoprecipitation (IP) of GRK5 or PHLPP2 for binding experiments, whole cell lysates (50-100µg) were incubated with 50µl of Protein A/G agarose-beads (Santa Cruz Biotechnology) in a 50% slurry along with specific antibodies for PHLPP2 (1µl/mg lysate, rabbit polyclonal, #A300-661A Bethyl Laboratories, Montgomery, TX,) or GRK5 (1µl/125µg, mouse, Millipore, Billerica, MA) at 4°C overnight. Immuno-complexes were washed and protein eluted in sodium dodecyl sulfate (SDS) followed by Western blot analysis.

**Western Blotting**

Electrophoresis and Western blotting were performed as previously described [34, 35]. Primary antibodies used are as follows: PHLPP2 from Bethyl Laboratories; LaminA/C (rabbit polyclonal, #2032S), RhoGDI (rabbit polyclonal, #2564S), and HDAC5 (rabbit polyclonal, #2082BC) antibodies were from Cell Signaling technology. The p-Ser/Thr (mouse monoclonal, #612548) was from BD Transduction laboratories. The GRK5 antibody (rabbit polyclonal, #sc-565) was from Santa Cruz Biotechnology (Santa Cruz, CA). PHLPP2 antibody was used at 1:2000 dilution and all other antibodies were used at 1:1000 dilution in 5% BSA/TBS-Tween-20. Secondary anti-rabbit antibody (Sigma, #A6154) was used at 1:8000 dilution and anti-mouse antibody (Sigma, #A5278) was at 1:2000 dilution in 5% milk/TBS-Tween-20.
**Statistical Analysis**

Researchers were blinded to the treatment group during analyses. Data are represented as mean ± SEM. Differences are considered statistically significant (p < 0.05) assessed using unpaired Student's t-test (for two groups) and ANOVA (for multiple comparisons involving two variables) with post hoc Tukey analysis using the GraphPad Prism software (GraphPad, La Jolla, CA, USA).

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RESULTS

PHLPP2 regulates hypertrophic growth and fetal gene expression in cardiomyocytes.

Since we have already demonstrated that removal of PHLPP1 protects myocytes from pathological hypertrophy [35], we determined the effect of PHLPP2 removal on cardiomyocyte hypertrophy. siRNA treated cells for siControl or siPHLPP2 (99% decrease in PHLPP2 mRNA versus control) were stimulated with the alpha-adrenergic agonist phenylephrine (50mM, PE) for 48 hours to induce myocyte hypertrophy. Removal of PHLPP2 significantly increased hypertrophic growth at baseline and following PE treatment compared to control (Figure 1A-B). Since fetal genes are re-induced during hypertrophic growth in response to adrenergic stimulation [6, 61], we assessed the mRNA levels of the fetal genes atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP). PHLPP2 removal significantly increased PE-induced ANF (Figure 2C) and BNP (Figure 2D) expression compared to siControl. This induction of the fetal genes by PE was blocked with overexpression of PHLPP2 (Figure 2C-D green bars). These findings suggest that PHLPP2 is a negative regulator of cardiomyocyte growth.

PHLPP2 binds GRK5 in cardiomyocytes.

Numerous studies have shown that activation of Akt leads to hypertrophic growth of cardiomyocytes in culture [4, 25, 62, 63]. Removal of PHLPP1 and PHLPP2 increases Akt activity in several cell types [26, 34, 35, 45, 52], however in cardiomyocytes, we have demonstrated that removal of PHLPP2 has no effect on Akt phosphorylation or activity [34]. Since PHLPPs mainly target AGC kinases, we determined whether PHLPP2 regulates myocyte hypertrophy through another kinase. G-protein couple receptor kinase 5 (GRK5) is an AGC kinase that regulates myocyte hypertrophy both in vitro and in vivo [54, 64, 65]. To investigate whether PHLPP2 and GRK5 interact in NRVMs, we performed a co-immunoprecipitation experiment. We found that immunoprecipitation of adenoviral overexpressed PHLPP2 bound to
overexpressed GRK5 (Figure 2A) in cardiomyocytes. To confirm that the interaction between PHLPP2 and GRK5 was present under normal conditions and not due to overexpression, we analyzed endogenous binding of PHLPP2 and GRK5 and confirmed the interaction (Figure 2B). This is the first evidence that PHLPP2 binds GRK5 in cardiomyocytes.

**PHLPP2 alters GRK5 nuclear localization**

It is well established that GRK5 through a non-canonical pathway downstream of Gq can regulate hypertrophic growth by localizing in the nucleus [42, 65]. This accumulation of nuclear GRK5 controls hypertrophic gene transcription via it acting as an HDAC kinase leading to MEF2 de-repression [23, 42, 65]. It is established that GRK5 translocates to the nucleus following PE stimulation in NRVMs. We examined whether removal of PHLPP2 altered nuclear accumulation of GRK5 following PE stimulation. Following knockdown with control or PHLPP2 siRNA, nuclear GRK5 accumulation in NRVMs treated with PE for 30 minutes was examined. Following stimulation, nuclear accumulation of GRK5 in the siRNA control samples was significantly increased (Figure 3A and B). Removal of PHLPP2 led to a greater accumulation of GRK5 in the nucleus basally and following PE stimulation compared to siControl (Figure 3B). Since translocation of GRK5 can reduce the amount of HDAC5 in the nucleus of cardiomyocytes [54], we examined HDAC5 levels as well. In accordance with the increased GRK5 in the nucleus, removal of PHLPP2 significantly decreased the amount of HDAC5 in the nucleus basally and following PE treatment (Figure 3A and C). Overexpression of PHLPP2 with GRK5 blocked the PE-induced nuclear accumulation of GRK5 (Figure 3D). Our data suggests that PHLPP2 is sufficient and necessary to block nuclear accumulation of GRK5 in cardiomyocytes.
PHLPP2 removal is unable to induce nuclear translocation of an n-terminal calmodulin GRK5 mutant.

GRK5 nuclear localization is a calmodulin-dependent event [65]. To investigate whether PHLPP2 removal affects GRK5 translocation through calmodulin binding, NRVMs were infected with a GRK5 mutant construct in which the n-terminal CaM domain was mutated (W30A) to inhibit translocation[65]. As shown in Figure 4, following PHLPP2 knockdown, the GRK5 W30A was unable to accumulate in the nucleus following PE stimulation. This finding indicates that PHLPP2 removal regulates PE-induced GRK5 translocation through calmodulin binding.

GRK5 mediates hypertrophy induced by PHLPP2 removal in cardiomyocytes.

We have demonstrated that PHLPP2 and GRK5 can interact in NRVMs and removal of PHLPP2 increases GRK5 nuclear translocation. To determine if GRK5 is necessary for the hypertrophic changes induced by PHLPP2 removal, siRNA for GRK5 was used. NRVMs were transfected with siRNA for control, PHLPP2, GRK5 or PHLPP2 and GRK5 and stimulated with or without PE for 48 hours. Hypertrophy was examined by measuring ANF mRNA expression. As expected, knockdown of PHLPP2 accentuated ANF gene expression induced by PE compared to control. Following GRK5 knockdown, PHLPP2 knockdown did not potentiate PE-induced ANF expression (Figure6, compare lane siCon+PE to lane siPH2+siGRK5+PE). Knockdown of GRK5 alone attenuated ANF gene expression induced by PE compared to control. Our data indicates that GRK5 is necessary for the hypertrophic response following PHLPP2 knockdown.

Knockdown of PHLPP2 increases Serine phosphorylation on GRK5.

The effect of phosphorylation or dephosphorylation of GRK5 in cardiomyocytes is unknown. Early studies on GRK5 demonstrate the ability of PKC to phosphorylate the c-
terminus and inhibit the ability of GRK5 to desensitize receptors [66]. Since PHLPP2 is a phosphatase known to dephosphorylate several different AGC kinases [32], the effect of PHLPP2 removal on GRK5 phosphorylation following PE stimulation was examined. Following knockdown with siControl or siPHLPP2, NRVMs were stimulated with PE for 30 minutes and phosphorylation of GRK5 analyzed. There are no commercially available phospho-specific antibodies for GRK5; alternatively endogenous GRK5 was immunoprecipitated and a phospho-Ser antibody used. As shown in Figure 6, PHLPP2 removal accentuated GRK5 phosphorylation at baseline and following PE stimulation compared to siControl. Our findings suggest that under hypertrophic stimulation GRK5 is phosphorylated and that removal of PHLPP2 increases this modification. Whether or not phosphorylation is needed for its translocation is unknown and further analysis is needed to determine the sites that are modified on GRK5.

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DISCUSSION

Hypertrophic agonists such as PE activate a myriad of intracellular signaling pathways within the cardiomyocyte to elicit growth [67, 68]. In a Gq-dependent process, increased calcium activates calmodulin (CaM) which in turn binds to the n-terminal region of GRK5 leading to nuclear translocation [54, 65, 69]. This non-canonical activation of GRK5 leads to hypertrophy both in vitro and in vivo [42, 56, 70]. Here we demonstrate for the first time that PHLPP2 acts as a negative regulator of PE-induced hypertrophy in NRVMs through its suppression of GRK5 nuclear translocation.

Both isoforms of PHLPP have been shown to target multiple AGC kinases including Akt and PKC in various cancer cells [26, 32, 49]. In primary astrocytes, removal of PHLPP1 increased Akt activity without altering PKC levels; however knockdown of PHLPP2 had no effect on the signaling pathway [52]. This is also the circumstance in neonatal cardiomyocytes where downregulation of PHLPP1 increased Akt activity while downregulation of PHLPP2 had no effect [34]. Here we demonstrate that removal of PHLPP2 in cardiomyocytes accentuates hypertrophic growth (Figure 1A-B) and activates re-expression of the fetal genes ANF and BNP (Figure 1C-D). Overexpression of PHLPP2 blocked the PE-induced re-expression of ANF and BNP (Figure 1C-D). We demonstrated that the removal of PHLPP1 in cardiomyocytes increases basal Akt phosphorylation both in vitro and in vivo [34, 35]. Under pathological stress, PHLPP1 knockout mice exhibited an attenuated pathologic hypertrophy and a more physiologic hypertrophic phenotype [35]. Based on the inability of PHLPP2 to regulate Akt in cardiomyocytes (Miyamoto) and the increased hypertrophic response with knockdown, we hypothesized that PHLPP2 may be targeting the pro-hypertrophic kinase GRK5.

We have established that not only adenoviral expressed but also endogenous GRK5 and PHLPP2 interact in cardiomyocytes (Figure 2). Upon PE stimulation, GRK5 translocates to the nucleus where it acts as an HDAC kinase to de-repress hypertrophic transcription [54]. By down-
regulating PHLPP2 in cardiomyocytes, we induced the translocation of GRK5 to the nucleus basally and accentuated PE-induced accumulation (Figure 3). Also conversely, the overexpression of PHLPP2 inhibited PE-induced translocation of GRK5 (Figure 3D). The only established mechanism for the non-canonical activation of GRK5 is the binding of CaM to the n-terminal region to induce translocation [65, 66]. By mutating the n-terminal CaM domain of GRK5 we revealed that downregulation of PHLPP2 was no longer able to induce nuclear accumulation of GRK5 (Figure 4). Taken together, these findings suggest that PHLPP2 removal may be increasing Ca\(^{++}\)/calmodulin signaling to elicit the increased nuclear translocation of GRK5.

Since the calmodulin-binding domain is important for the PHLPP2-GRK5 mediated cardiac hypertrophy, it is possible that the removal of PHLPP2 alters Ca\(^{++}\)/calmodulin flux, which is a major player in many signaling pathways such as Calcineurin/NFAT and CamKII that can also cause hypertrophy [36, 71]. NFAT proteins are a family of calcium level regulated transcriptional factors that activates a wide range of genes including fetal genes [71, 72]. Since GRK5 has been shown to enhance NFAT activity in cardiomyocytes [70] it is possible that NFAT may be regulating the induced fetal gene expression seen with the increased accumulation of GRK5 in nucleus. Even though we determined increased export of HDAC5 from the nucleus (Figure 3C), there may be multiple signaling pathways regulating the hypertrophic response following PHLPP2 knockdown.

Little is known about the effect of phosphorylation and dephosphorylation on the function of GRK5 let alone in cardiomyocyte hypertrophy. The Benovic lab has shown that PKC can phosphorylate GRK5 on its c-terminal domain and block canonical activity to desensitize receptors [73]. Activation of PKC isoforms downstream of phospholipase C (PLC) coupled to G, receptors stimulates cardiomyocyte hypertrophy \textit{in vitro} [41, 74, 75]. Whether in cardiomyocytes phosphorylation of GRK5 by PKC can attenuate its canonical and accentuate its
non-canonical activity is unknown. However, PHLPP 1 and 2 can target several PKC isoforms in cancer cells and cause its destabilization at the membrane [30, 33]. Whether removal of PHLPP2 will increase PKC levels and activity to alter cardiomyocyte growth is unknown. However, preliminary data from our lab suggests that knockdown of PHLPP2 does not alter the levels of PKCα and PKCβ in cardiomyocytes (data not shown), which rules out the possibility of elevated PKC as being the cause of the basal hypertrophic growth. Knockdown of PHLPP2 did increase the overall serine phosphorylation of GRK5 (Figure 6) but the exact cite or the relevance of this finding in promoting nuclear accumulation is under investigation.

Since PE can induce nuclear accumulation of GRK5 and hypertrophy independent of PHLPP2 removal in cardiomyocytes (Figure 1C-D; Figure 4), we used siRNA for GRK5 (Figure 5) to validate that GRK5 was necessary for the PE-induced hypertrophic response. Our data revealed GRK5 was necessary for the PE-induced hypertrophy and re-expression of the fetal gene program following PHLPP2 knockdown (Figure 5).

Our lab is the first to show that in vitro removal of PHLPP2 modulates PE-induced cardiomyocyte hypertrophy through GRK5. We demonstrated that removal of PHLPP2 accentuated PE-induced cardiomyocyte growth and re-expression of fetal genes associated with pathological hypertrophy by increasing GRK5 nuclear accumulation. Understanding the biological function and signaling pathways altered by PHLPP2 in cardiomyocytes may help delineate therapeutic targets for cardiac hypertrophy.

This section, in part, is currently being prepared for submission for publication of the material. Yeh, Szu-Tsen; Zambrano, Cristina M.; Koch, Walter J.; Purcell, Nicole H. The thesis author was the primary investigator and author of this material.
Scheme 1: Schematic of PHLPP2 and GRK5. (A) PHLPP is a serine/threonine phosphatase that directly dephosphorylates several AGC kinases including Akt and PKC. PHLPP2 contains a Ras association (RA) domain, pleckstrin homology (PH) domain, a leucine-rich repeats (LRR) region, a phosphatase domain (PP2c), and a PDZ binding domain. (B) GRK5 is an AGC kinase that contains two calmodulin binding site and a kinases domain that contains nuclear export sequence (NES) and nuclear localization sequence (NLS).
**Scheme 2: Regulation of HDAC5 nucleo-cytoplasmic shuttling.** GRK5 is an HDAC kinase in NRVMs. Upon stimulation with alpha-adrenergic agonists (PE), GRK5 translocates to the nucleus to phosphorylate HDAC5 on its N-terminal regulatory domain at two serine resides 259/498. Upon phosphorylation, 14-3-3 binds and removes HDAC from the nucleus and de-represses hypertrophic gene expression.
Scheme 3: PHLPP signaling pathway. PHLPP dephosphorylates several AGC kinases and regulates protein translation and cell survival. PHLPP levels are controlled by two negative feedback loops: high Akt activity blocks GSK-3-mediated PHLPP degradation; p70S6K activity increases PHLPP expression levels.
FIGURES

Figure 1: PHLPP2 regulates cell size and fetal gene expression in NRVMs. NRVMs were transfected with siControl (siCon) or siPHLPP2 (siPH2) in 2µM for 48 hours, or adenovirus PHLPP2 (adPH2, 50MOI) for 24 hours in the absence of serum. Cardiomyocytes were treated with the hypertrophic agonist phenylephrine (PE, 50µM) for 48 hours. (A) Immunohistochemistry was performed using Phalloidin (red) and Dapi (blue) to visualize cells. (B) Cell size was measured using NIH Image J Software. *p≤0.05 vs. siCon no treatment, n=4 independent experiments, 400 cells/experiment. (C) and (D) RNA was isolated and the level of ANF and BNP mRNA measured by RT-PCR. The graphs represent the fold change in ANF and BNP mRNA versus siControl (no PE). N=9 individual experiments for siControl and siPHLPP2. N=4 individual experiments for adPHLPP2. *p≤0.05 vs. siCon+PE, **p≤0.01 vs. siCon+PE.
Figure 2: PHLPP2 and GRK5 interact in NRVMs. (A) Whole cell extracts from NRVMs infected with PHLPP2 or with both PHLPP2 and GRK5 adenoviruses (50MOI) were immunoprecipitated (50µg) with anti-PHLPP2 antibody or IgG as control. Binding was visualized by immunoblotting for GRK5. PHLPP2 was blotted as control for immunoprecipitation. Levels of GRK5 and PHLPP2 from the whole cell extracts were blotted as input control. (B) Whole cell extracts from NRVMs were immunoprecipitated (75µg) with anti-GRK5 antibody and immunoblotted with anti-PHLPP2 antibody for endogenous binding. GRK5 levels from the immunoprecipitation were blotted as control. PHLPP2 from whole cell extract was immunoblotted for input control. IgG was used as a negative control. Data from one representative experiment out of 4 are presented.
Figure 3. PHLPP2 regulates GRK5 nuclear translocation. NRVMs were transfected with either siControl (siCon) or siPHLPP2 (siPH2) in the absence of serum. After 24 hours transfection, cells were infected with an adenovirus overexpressing GRK5 and cultured overnight. At 48-hour post-transfection, cells were treated with PE (50 µM) for 30 minutes and fractionated (A) Nuclear factions were immunoblotted for GRK5 and HDAC5. Lamin A/C and RhoGDI were blotted as purity controls. (B) The amount of GRK5 in the nucleus was normalized to lamin A/C and reported as fold change over control, *p ≤ 0.05 versus siCon, #p ≤ 0.05 versus siCon+PE. N=4 independent experiments. (C) The amount of nuclear HDAC5 was normalized to lamin A/C and cytosolic HDAC5 was normalized to RhoGDI. Nuclear to cytosol ratio of HDAC5 was normalized to baseline. N=4 independent experiments. (D) NRVMs were infected with GFP, PHLPP2 (adPH2), or GRK5 (adGRK5) (50MOI) adenoviruses over night. Following 30 minutes PE treatment (50µM), cells were fractionated and nuclear fractions were blotted for GRK5, which was normalized to lamin A/C and reported are fold change over control. *p ≤ 0.05 versus GFP, # p ≤ 0.05 versus GFP + PE.
Figure 4. PHLPP2 regulates PE induced GRK5 translocation through calmodulin binding.
NRVMs were transfected with either siControl (siCon) or siPHLPP2 (siPH2) (2µM) in the absence of serum. After 24 hours transfection, cells were infected with an adenovirus overexpressing a GRK5 calmodulin binding mutant (W30A, 50MOI) and cultured overnight. Cells were treated with 50 uM phenylephrine (PE) for 30 minutes and fractionated. (A) Nuclear fractions were immunoblotted for GRK5. Lamin A/C and Rho GDI were also blotted as purity controls. (B) The amount of W30A in the nucleus was normalized to lamin A/C and reported as fold change over baseline. N= 6 independent experiments.
Figure 5: GRK5 mediates hypertrophy induced by PHLPP2 knockdown in NRVMs. NRVMs were transfected with siControl (siCon), siPHLPP2 (siPH2), or siGRK5 (2µM) in the absence of serum. Cells were treated with PE (50µM) for 48 hours, and mRNA isolated. mRNA expression of ANF was represented as fold change over siControl (no PE). N=11 individual experiments for siControl and siPHLPP2; n=6 individual experiments for siGRK5. *p ≤ 0.05 vs siCon (no PE) or **p ≤ 0.05 versus siCon(+PE)
Figure 6. Knockdown of PHLPP2 increases GRK5 phosphorylation. NRVMs were transfected with either siControl (siCon) or siPHLPP2 (siPH2) (2µM). Following knockdown, cells were stimulated with PE (50µM) for 30 minutes and endogenous GRK5 immunoprecipitated (100µg). Representative Western blot for (A) phosphorylation of GRK5 and (B) graph of quantification. Total p-serine/threonine was blotted for GRK5 phosphorylation normalized to GRK5 IP control and reported as fold over control. n=4 independent experiments. *p≤0.05 versus siCon (no PE), #p≤0.05 versus siCon+PE.
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