UNIVERSITY OF CALIFORNIA SAN DIEGO

The Effect of PHLPP Isoform Removal on Cardiac Ischemia/Reperfusion Injury

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Kellie A. Lemoine is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

Chair

University of California San Diego

2020

DEDICATION

I dedicate this Thesis to my loving parents, whose unwavering support and encouragement helped me achieve this graduate degree.

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LIST OF ABBREVIATIONS

PHLPP	PH Domain Leucine-rich Repeat Protein Phosphatase
I/R	Ischemia/Reperfusion
WT	Wild-type
KO	Knockout
TTC	Triphenyltetrazolium chloride
IA	Infarct Area
AAR	Area at Risk
Akt	Protein Kinase B
PKC	Protein Kinase C
S6K	Ribosomal Protein S6 Kinase
Mst1	Macrophage Stimulating Protein
ERGF	Epidermal Growth Factor Receptor
ATP	Adenosine Triphosphate
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
GSK3	Glycogen Synthase Kinase 3
FoxO	Forkhead Box Proteins
TSC2	Tuberous Sclerosis Complex 2
MAPK	Mitogen-activated Protein Kinase
ERK	Extracellular Signal-Regulated Kinase
JNK	C-Jun N-Terminal Kinase
PGC1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 alpha
NRF1	Nuclear Respiratory Factor 1

NF-kB	Nuclear Factor-Kappa-Light-Chain-Enhance of Activated B Cells
IKKβ	Inhibitor of Nuclear Factor Kappa Beta Kinase Subunit Beta
ARE	Antioxidant Response Element
NRF2	Nuclear Factor Erythroid 2-Related Factor
HO-1	Heme Oxygenase 1
SOD	Superoxide Dismutase
NO	Nitric Oxide
iNOS/NOS2	Inducible Nitric Oxide Synthase
eNOS/NOS1	Endothelial Nitric Oxide Synthase
TNFα	Tumor Necrosis Factor Alpha
IL-6	Interleukin-6
IL-1β	Interleukin-1Beta
VEGF	Vascular Endothelial Growth Factor
HIF-1α	Hypoxia-Inducible Factor 1Alpha
TAC	Transverse Aortic Constriction
LAD	Left Anterior Descending Artery
PAGE	SDS-Polyacrylamide Gel
MOPS	N-morpholino propanesulfonic acid
PVDF	Polyvinylidene Fluoride
TBST	Tris-Buffered Saline-Tween-20
DPEC	Diethyl Pyrocarbonate

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I am tremendously grateful to Nicole Purcell, my PI and mentor, for all her advice and guidance during my graduate studies. Her knowledge and skills inspire me to be a better researcher in the biological sciences.

I am thankful for all the members of the Purcell Lab, as they were great peers to work with as well as wonderful friends.

I would also like to acknowledge my parents, family, and friends for supporting me on this academic journey. All of these individuals have played a valuable role in helping me pursue a graduate scientific degree and I appreciate their support.

ABSTRACT OF THE THESIS

The Effect of PHLPP Isoform Removal on Cardiac Ischemia/Reperfusion Injury

by

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Master of Science in Biology

University of California San Diego, 2020

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A myocardial infarction occurs when blood flow to the heart is restricted and is termed "ischemia." Reperfusion, the restoration of blood flow, is necessary for survival but can cause additional damage. PHLPP, a Ser/Thr protein phosphatase, includes two isoforms (PHLPP1 and

PHLPP2), and was originally identified as a tumor suppressor in cancer (Gao, 2005). In the heart, PHLPP1 removal was found to be cardio-protective through increased Akt phosphorylation/activation basally leading to cell survival following insult (Moc, 2015). PHLPP2 removal does not alter Akt activity basally and has been found to increase cardiomyocyte hypertrophy *in vitro* (Yeh, 2018). We hypothesize that PHLPP2 removal alters cardiomyocyte growth signaling and is detrimental to the heart following ischemic injury. Wild-type, PHLPP1 and PHLPP2 KO mice were subjected to 1hr ischemia via occlusion of the left descending artery followed by reperfusion for various times. Whole hearts were removed following reperfusion and the left ventricle sectioned into ischemic and non-ischemic regions and used for gene and protein expression analysis. The extent of I/R injury was determined using triphenyltetrazolium chloride and Evan's blue dye, followed by digitized imaging of the area of infarction (IA) versus the area at risk (AAR). Our initial studies demonstrate attenuation in infarct in PHLPP1 KO compared to WT with the same AAR; however PHLPP2 removal increased infarct injury. MRNA and protein expression analysis indicates PHLPP2 removal enhances injury through increased cell death, an imbalance in inflammation/antioxidant signaling, and dysregulated autophagy while PHLPP1 removal confers protection through apoptosis inhibition and increased mitochondrial biogenesis.

INTRODUCTION

Heart Disease

Heart disease is the leading cause of death in the United States. If heart disease goes unchecked it can cause heart failure, in which the heart's ability to pump blood is weakened. One form of cardiac disease is a myocardial infarction also known as a heart attack. According to the American Heart Association, an individual suffers from a heart attack every forty seconds in the US alone. This occurs when the blood supply is cut off to the heart and is termed "ischemia." Some common therapies used to treat ischemic damage include angioplasty, thrombolysis, and coronary bypass surgery (Hausenloy, 2004). Additionally, preventative measures including exercise and ischemic preconditioning have been shown to have cardio-protective effects on the heart in animal models (Brand, 2019). Many of these therapies involve reperfusion, the restoration of blood flow to the heart. Reperfusion is necessary to restore oxygen and nutrient flow to the heart, but it also has been shown to cause additional damage (Hausenloy, 2004). Given that reperfusion is necessary in order to ensure an individual's survival after a heart attack, it would be beneficial if measures to minimize the damage inflicted on the myocardium from reperfusion were taken.

Akt activity during ischemia/reperfusion

Following injury at the molecular level, protein kinase B (also known as Akt), plays a protective role in the heart. Akt is a serine/threonine kinase that promotes cell survival by regulating several key processes including cell growth, proliferation, glucose metabolism, apoptosis, cell motility and angiogenesis (Moc, 2015) (Gao, 2005) (Aviv, 2010). During simulated ischemia/reperfusion (I/R) in neonatal rat ventricular cardiomyocytes, Akt was

activated at reperfusion, but not during ischemia alone. (Mockridge, 2000). While moderate Akt activity is cardio-protective, as seen by its ability to promote cell survival and inhibit apoptosis during I/R, excessive Akt activity negatively affects the heart by accentuating injury following I/R (Brand, 2019). Therefore, it is important to regulate Akt activity in order to maintain proper heart function.

PHLPP: a negative regulator of Akt activity

A protein phosphatase termed PH domain leucine-rich repeat protein phosphatase (PHLPP) was originally identified as a tumor suppressor in cancer as it negatively regulates Akt activity by dephosphorylating its hydrophobic motif (Ser473). PHLPP has two isoforms, PHLPP1 and PHLPP2, with PHLPP1 including two splice variants, PHLPP1α and PHLPP1β. Its structure consists of a Ras association domain (only found in PHLPP1β and PHLPP2), PH domain, leucine-rich repeat domain, a PP2C phosphatase domain, and a PDZ ligand domain (see schematic 1) (Grzechnik, 2016) (Brognard, 2007) (Newton, 2014). In cancer cells, both PHLPP isoforms have been shown to selectively dephosphorylate Akt to regulate cell survival; PHLPP1 was found to act on the isoforms Akt2 and Akt3 whereas PHLPP2 acts on Akt1 and Akt3 (Miyamoto, 2010) (Grzechnik, 2016) (Brognard, 2007). In addition to Akt, PHLPP's other downstream substrates include protein kinase C (PKC), ribosomal protein S6 kinase (S6K), and macrophage stimulating protein (Mst1) (see schematic 2) (Moc, 2015) (Grzechnik, 2016) (Newton, 2014). Through dephosphorylation of these substrates, PHLPP inhibits cell growth and protein translation and promotes apoptosis and growth suppression. PHLPP1 removal in cancer cells has also been shown to increase signaling through the MEK/ERK cascade via increased transcription of the epidermal growth factor receptor (ERGF), thereby promoting cell survival (Grzechnik, 2016).

PHLPP in the heart

PHLPP has been extensively studied in the context of cancer, but very little is known regarding its role in the heart. While PHLPP isoforms are ubiquitously expressed, it is the highest in the brain with substantial levels in the heart (Gao, 2005). Importantly, recent studies suggest that PHLPP isoforms have different targets under physiological conditions compared to cancer cells. In the heart, PHLPP1 dephosphorylates the hydrophobic motif on Akt (Ser473), however PHLPP2 does not seem to act on Akt directly (Miyamoto, 2010) (Yeh, 2018). Additionally, PHLPP1 dephosphorylates all isoforms equally in contrast to just Akt2 and Akt3 in cancer cells (Miyamoto, 2010) (Aviv, 2010).

PHLPP1 in cardiac I/R

Given the evidence that activated Akt plays a significant role in protecting the heart against myocardial infarction, removal of PHLPP1 was examined in the context of I/R injury. It was found that removal of PHLPP1 in the heart accentuated Akt activation and reduced infarct size following *ex vivo* I/R (Miyamoto, 2010). Moreover, PHLPP1 knockdown in aged rats reduced infarct size following *in vivo* I/R (Xing, 2016). This data supports a protective role of PHLPP1 removal in the heart during I/R. However, the role of PHLPP2 during cardiac I/R is unknown.

The cellular effects of I/R

Ischemia and reperfusion injury have been shown to cause a wide range of cellular damage. Ischemia is characterized by low adenosine triphosphate (ATP) levels, reactive oxygen species (ROS) production, and cell death (Ma, 2014) (Hausenloy, 2004). ATP is a molecule that provides our cells with energy and is critical to maintain proper functioning. ROS are oxygen-

containing molecules that can react with other molecules in our cells and buildup of these species can cause molecular alterations or cell death. Reperfusion is necessary in order to restore blood flow to the ischemic tissue but it is unclear whether reperfusion injury exacerbates the damage caused by ischemia or if it induces additional damage irrespective of ischemic injury (Hausenloy, 2004). Previously published studies have shown that reperfusion is accompanied by increased ROS and reactive nitrogen species (RNS) production, inflammation, cell death, and mitochondrial dysfunction (Ma, 2014) (Bagheri, 2016). While I/R injury results in extensive cell dysfunction and is a major contributor to cardiac disease, the exact contribution of each of the above-mentioned factors is unclear and controversial. Many of these processes are necessary in order to maintain cellular homeostasis, but dysregulation can have detrimental effects on the myocardium.

I/R-induced cell death

Cardiac cell death can occur naturally or be induced by external factors such as disease or injury. Apoptosis is a type of programmed cell death in which the cell itself signals for old or damaged cells to die in order for the tissue or organ as a whole to survive. On the other hand, necrosis is caused by external factors like disease or injury and results in the premature death of some cells. Both apoptosis and necrosis have been observed during ischemia and reperfusion phases of myocardial infarction (Hausenloy, 2004). Akt works to promote cell survival and inhibit cell death processes through a multitude of downstream substrates including glycogen synthase kinase 3 (GSK3), forkhead box proteins (FoxO), and tuberous sclerosis complex 2 (TSC2) among others (Abeyrathna, 2015). PHLPP1 directly dephosphorylates and inactivates Akt in the heart thereby suppressing survival-signaling pathways. However, as mentioned earlier, PHLPP2 does not directly target Akt, suggesting an alternate role in the heart. Whether the two

PHLPP isoforms have redundant roles in the heart during I/R is unknown but of interest as it creates the potential for isoform-specific inhibitors.

The mitogen-activated protein kinase (MAPK) family, another regulator of cell death, consists of three members: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 kinases. Overexpression on ERK in the heart has been shown to cause cell death and ultimately heart failure (Nicol, 2011). Removal of PHLPP1 or PHLPP2 has been shown to increase agonist-induced ERK phosphorylation in cancer cells (Niederst, 2008). Whether the PHLPP isoforms regulate ERK phosphorylation in the heart during I/R is of interest as it could be a way to control cell death.

Meanwhile, previous studies report controversial roles of p38 and JNK signaling during I/R injury. Inhibition of p38 and JNK have been documented to both protect the heart and exacerbate I/R injury (Xu, 2015) (Shao, 2006) (Mackay, 1999). Therefore, more evidence is needed to understand how these factors are regulated during I/R. Interestingly, both PHLPP1 and PHLPP2 dephosphorylate macrophage stimulating 1 protein (Mst1), in order to activate p38 and JNK to induce apoptosis in cancer cells (Qiao, 2010). Whether both PHLPP isoforms also regulate these protein kinases during I/R and whether their functions are redundant could significantly affect I/R-induced injury to the myocardium.

Another form of cell death is autophagy. Autophagy is a natural process that is employed basally in the heart in order to maintain cellular homeostasis by removing and degrading damaged organelles or proteins from the cell. (Sai Ma, 2014) (Parzych, 2014). Whether autophagy plays a protective or detrimental role during I/R injury is highly debated. Autophagy can be protective during ischemia because this process allows for the recycling of amino acids and fatty acids, which are used in the TCA cycle to generate ATP. Additionally, autophagy could

be protective at reperfusion. Due to the influx of ROS, there is increased mitochondrial dysfunction. Mitophagy, a specialized form of autophagy, is increased at reperfusion and is beneficial in that it clears damaged mitochondria from the cell and prevents the release of cytochrome c which is toxic. However, excessive autophagy could also be detrimental and result in unnecessary cell death. In addition, it is also argued that autophagy is not increased in the reperfusion phase but instead there is a reduction in autophagosome clearance (Sai Ma, 2014). Beclin1 is both a downstream target of Akt and an inducer of autophagy. Beclin1 contains a BH3 domain, which interacts with BCl2, an anti-apoptotic factor, and thereby inhibits its activity (Kang, 2011). This suggests a mechanism in which the cell can switch between autophagy and apoptosis. When beclin1 is phosphorylated, this inhibition is removed and beclin1 can induce autophagy. If PHLPP1 could mediate Beclin1 phosphorylation through regulation of Akt activation, it could potentially negatively regulate autophagy (Mathur, 2017). This supports the cardio-protective phenotype of PHLPP1 removal. Whether this occurs in the heart during I/R is unknown. Moreover, whether PHLPP2 plays a redundant or opposing role is of interest.

I/R-induced mitochondrial dysfunction

In addition to cell death, I/R induces mitochondrial dysfunction. Mitochondria are the major source of energy production in our cells. It is responsible for producing ATP, is a source of ROS, and contains cytochrome c, a stimulus for cell death (Bagheri, 2016). When mitochondria are damaged during ischemia and reperfusion, multiple signaling pathways are activated including the antioxidant defense systems, cytoprotective pathways, biogenesis, autophagy, and apoptosis (Bartz, 2015). Activated Akt translocates to the mitochondria under stress conditions to prevent loss of function and cell death in the heart, suggesting PHLPP1 would promote mitochondrial dysfunction (Purcell, 2010). Furthermore, PHLPP1 knockdown in

colon cancer cells was found to promote the mitochondrial association of hexokinase 2, a regulator of glucose metabolism and an apoptosis inhibitor (Xiong, 2017). This supports a protective phenotype of PHLPP1 removal. Whether PHLPP2 plays a role in mitochondrial dynamics during I/R is unknown. Additionally, mitochondrial biogenesis is the process by which mitochondria increase ATP production, which is necessary for cell survival and is disrupted during I/R. A key player involved in regulating mitochondrial biogenesis is peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), which has shown to be reduced in response to ischemia and cause decreased biogenesis (Ahuja, 2010). Whether the PHLPP isoforms regulate mitochondrial biogenesis during cardiac I/R is unknown, but could be a potential mechanism to mediate cellular damage.

Antioxidant signaling during I/R

During I/R, there is increased oxidative stress in the form of ROS and RNS, which affects the electron transport chain as well as protein transcription and translation that can cause cellular damage (Bagheri, 2016), however mitochondria have endogenous antioxidant defense pathways in place in order to protect against oxidative stress. As long as this balance between oxidative stress and the antioxidant defense systems exist, the heart is protected. However, under conditions of excessive oxidative stress, for example during I/R injury, other redox pathways need to be upregulated in order to help combat the cellular damage inflicted from increased ROS/RNS production (Bartz, 2015).

One important pathway that is increased under excessive oxidative stress in the mitochondria is the antioxidant response element/nuclear factor erythroid 2-related factor 2 (ARE/NRF2) pathway. Increased oxidative stress stimulates Nrf2 to translocate to the nucleus where it binds to ARE and upregulates transcription of cytoprotective genes. A recent study

found that PHLPP2 removal reduced hypoxia-induced injury in cardiomyocytes by increasing the transcriptional activity of the NRF2/ARE pathway via GSK-3B phosphorylation and subsequent inactivation (Jin, 2019). Additionally, treatment with GYY4137, a H_2S donor thought to act as a cardiovascular autacoid, resulted in cardioprotection against myocardial ischemia/reperfusion by reducing PHLPP1 levels and increasing the nuclear localization of Nrf2 and subsequent expression of its downstream targets heme-oxygenase-1 (HO-1) and superoxide dismutase (SOD) (Qiu, 2017). This suggests removal of either PHLPP isoform is protective in cardiomyocytes. However, whether this effect is sustained in vivo is yet to be determined.

In addition to the production of ROS, RNS also play a large role in mitochondrial homeostasis. Myocardial ischemia causes the release of nitric oxide (NO), which can cause cellular damage. Inducible NO synthase (iNOS/NOS2) responds to this stress as well as inflammation. In previous studies, iNOS/NOS2 overexpression resulted in reduced infarct size following I/R as well as reduced ROS formation. Another key player, endothelial NO synthase (eNOS/NOS1) produces endogenous NO. A previous study has shown that Akt phosphorylates eNOS thereby increasing NO production and contributes to the insulin-induced anti-apoptotic effects seen in myocardial I/R (Gao, 2002). Given that Akt is a direct target of PHLPP, whether removal of either PHLPP isoform plays a role in mediating cardiac I/R injury via regulating NO production is of interest.

I/R-induced inflammatory response

Another hallmark of myocardial I/R injury is the inflammatory response. Inflammation is induced during I/R in order to signal the immune system to heal the damaged tissue. However, prolonged inflammation can cause cellular damage. Previous studies have shown that the inflammatory response is largely regulated by nuclear factor-kappa-light-chain-enhancer of

activated B cells (NF-kB), which is activated during I/R (Maimaitiaili, 2018). NF-kB is made up of two subunits, p65 and p50, and regulates the transcription of cytokines such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in the heart (Li, 1999) (Maimaitiaili, 2018). NF-kB activation is associated with ROS production and consequently has shown to be inhibited by the antioxidant response (Li, 1999). PHLPP2 was found to negatively regulate NF-kB transcriptional activity by inhibiting the phosphorylation/degradation of the NF-kB inhibitor, IKKβ, in cancer (Agarwal, 2014). Additionally, the NF-kB subunit, p65, and NRF1, another regulator of antioxidant signaling, have been shown to co-regulate each other, indicating a relationship between the inflammatory response and antioxidant defense signaling (Suliman, 2010). This suggests a potential role of PHLPP in regulating the inflammatory response after myocardial I/R-induced injury and implies a possible connection to a PHLPP-mediated antioxidant response.

Angiogenesis

Lastly, angiogenesis, a process that involves the development of new blood vessels, has been shown to be stimulated under ischemic conditions. When the heart is deprived of oxygen and nutrients, the creation of new blood vessels would allow necessary materials to reach the heart. One of the key players in promoting angiogenesis is vascular endothelial growth factor (VEGF). VEGF is activated by an upstream transcription factor, hypoxia-inducible factor 1-alpha (HIF-1α). HIF-1α is activated by ischemia where it translocates to the nucleus and binds to the hormone response element in order to upregulate the transcription of target genes including VEGF. Our lab previously found that PHLPP1 removal increases VEGF basally and following a transverse aortic constriction (TAC) (Moc, 2015). However, the role of PHLPP1 removal in

promoting angiogenesis during I/R is unknown as well as the role PHLPP2 plays in angiogenesis both basally and during I/R.

Aims of this study

In conclusion, I/R injury results in a serious health issue given the range of cellular damage it inflicts. PHLPP1 removal poses as a potential therapeutic target given its ability to promote cell survival. However, very little is known regarding the role of PHLPP1 in the heart, with even less known about PHLPP2. The studies to date on the role of PHLPP in I/R injury have only examined the effect of PHLPP1 removal on infarct and neglect to adequately address the other cellular effects such as inflammation and signaling. This study aims to further elucidate the distinct roles of the PHLPP isoforms in the heart, and contribute to the current knowledge of the effects that I/R injury has on the heart, and discover how PHLPP isoforms mediate cellular signaling in response to I/R injury.

MATERIALS AND METHODS

Animals

All animal procedures were performed in accordance with the National Institutes of Health 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. Age-matched wild-type (WT) and PHLPP1 and PHLPP2 global knockout (KO) mice were used. See previously described protocol for generation of PHLPP1 KO (Miyamoto, 2010) and PHLPP2 KO mice (Wen, 2015).

Ischemia/Reperfusion (I/R) surgery

According to a previously described protocol (Kaiser, 2004), mice were anesthetized with isoflurane and the heart was exposed via a left thoracotomy. A suture was tied around the left anterior descending artery (LAD) with a slipknot. The thoracotomy was closed and the mice were revived for 60mins of ischemia, after which the slipknot was released and the heart was reperfused for the appropriate time period (i.e. 1hr, 3hr, or 24hrs) (see schematic). After reperfusion, mice were sacrificed and the hearts were quickly removed and stored at -80˚C.

Heart homogenization

Following reperfusion, hearts were removed from WT, PHLPP1 and PHLPP2 KO mice. The left ventricle was divided into two regions: ischemic and non-ischemic (see schematic). The regions were snap frozen in liquid nitrogen. Tissue was homogenized in either 130 μL of Western buffer (in appendix) for protein analysis or 1 mL of TRIzol Reagent for RNA extraction. Protein and RNA concentration was determined as described below and samples were used for Western blot analysis or qPCR respectively.

Protein Isolation

Protein was isolated from the left ventricle using Western buffer (see appendix) according to the manufacturer's protocol. Briefly, tissue samples were homogenized in 130 µL of Western buffer and centrifuged at 14,000rpm for 20 minutes at -4˚C. The supernatant was removed and protein quantitation was determined by BCA assay. Briefly, 150µL of BCA reagent was combined with 1µL of sample in a 96-well plate and incubated for 1 hour at 37˚C. Following incubation, the protein concentration was determined by comparing the absorbance at 562λ using the Gen5 Data Analysis software (BioTek) of the protein samples to the standard curve generated from the absorbance of a known BSA standard.

Western Blotting

To measure changes in protein phosphorylation and expression following I/R in the left ventricle, Western blot was performed. From the protein assay, 15 µg of protein was prepared for analysis (see appendix). Samples were heated at 95˚C for 10min and loaded onto a SDS-polyacrylamide gel (PAGE) (Invitrogen NuPage) with a protein marker (BioRad). Depending on the molecular weights of the proteins being examined, either a 4-12%, 8%, or 12% Bolt-Bis-Tris gel was run in 1X-3-(N-morpholino)propanesulfonic acid (MOPS) buffer (see appendix). Gels were run at 150 volts for 1 hour. Following gel electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Sigma) that had been briefly soaked in methanol and run in 1X transfer buffer (see appendix) for 2 hours at 100 volts. Following transfer, membranes were cut according to the protein marker and blocked in 5% milk/0.1% Tris-buffered saline-Tween-20 (TBS-T) for 30 minutes on a rocker. Membranes were washed with 0.1 %TBS-T and primary antibodies in 5% BSA/0.1%TBS-Tapplied (see appendix for list of antibodies used and dilutions)at 4˚C overnight on a rocker. The following day, membranes

were washed three times in 0.1% TBS-T for 7 minutes each. Secondary antibodies diluted in 5% milk/0.1%TBS-T were added (see appendix) and membranes placed at room temperature for 1 hour on a rocker. Following the secondary antibody, membranes were washed three times in 0.1% TBS-T for 7 minutes each. In order to visualize the proteins, the membranes were briefly soaked in ProSignal West Femto Maximum Sensitivity Substrate, in a 1:1 dilution (Thermo Scientific), and exposed using a myECL imager (Thermo Scientific). The protein expression was quantified using AlphaView software and normalized to GAPDH as described previously (Moc, 2015).

RNA Isolation

To analyze changes in gene expression, RNA was isolated from left ventricle tissue using TRIzol Reagent (Thermo Scientific) according to the manufacturer's protocol. Briefly, tissue samples were homogenized in 1 mL of TRIzol. Samples were pipetted for 1minute per sample and then incubated at room temperature for 5 minutes. Following incubation, 200 µL of chloroform was added to each sample and samples were gently inverted for 5 minutes. Samples were centrifuged at 14,000rpm for 15 minutes at 4°C. Following centrifugation, the supernatant was removed and added to 500 µL of isopropyl alcohol. The samples were mixed and stored at -20˚C overnight in order to recover as much RNA as possible. Following RNA precipitation, the samples were centrifuged at 14,000rpm for 15 minutes at 4˚C. The supernatant was discarded and the RNA pellets were washed with 500 µL of 70% DPEC (diethyl pyrocarbonate)-treated ethanol. Following a centrifugation at 10,000rpm for 5 minutes at 4˚C, the supernatant was removed and the pelleted RNA was air-dried for 1 hour. Following drying, the pellets were dissolved in 25 µL of sterile water and heated at 55˚C for 10 minutes to completely dissolve the

pellet. The RNA concentration was determined from the 260/280 absorbance ratio using the Gen5 Data Analysis software (BioTek).

cDNA Synthesis

For cDNA synthesis, 0.1 µg of RNA was used. A cDNA mastermix solution was prepared from the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific) containing 10X RT buffer, 25X dNTP, 10X RT primers, reverse transcriptase, RNase inhibitor, and sterile water (see appendix). The 0.1 µg of RNA was prepared in a total volume of 10 µL and combined with 10 µL of the cDNA mastermix solution for a final 20 µL reaction. The samples were run on the Mastercycler Nexus PCR Machine (Eppendorf) following the manufacturers RT-PCR (reverse transcription polymerase chain reaction) protocol (see appendix). Following cDNA synthesis, each reaction was diluted with 20 µL of sterile water for a final volume of 40 µL.

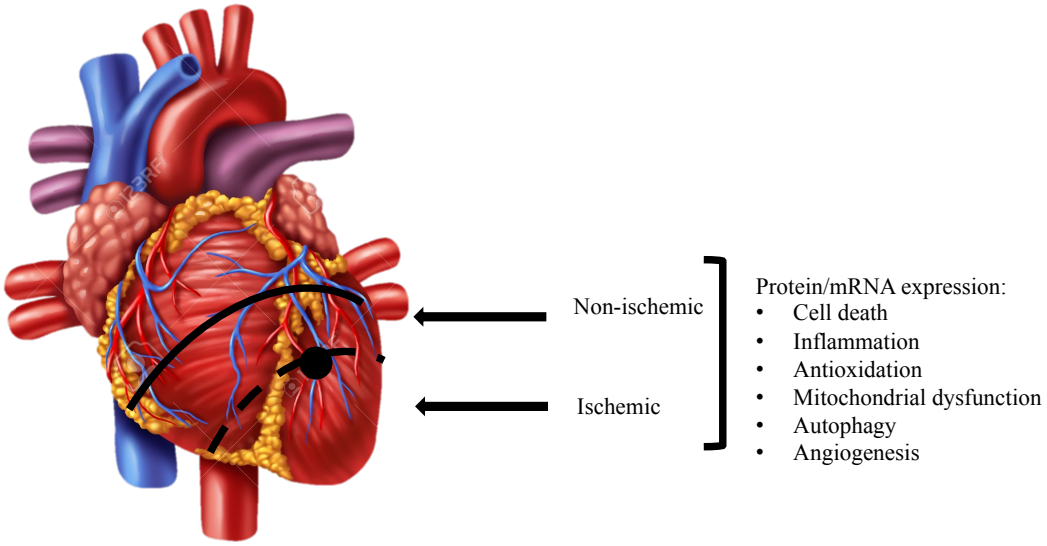
Quantitative Polymerase Chain Reaction (qPCR)

To determine the changes in gene expression following I/R, qPCR was performed. The cDNA samples were diluted 1:10 using sterile water. 9 µL of the diluted cDNA, 1 µL of primer (see appendix for complete list of primers used) and 10 µL of PCR Bio Probe Mix Lo-ROX (Genesee Scientific) were added to the wells of a 96-well plate. The plate was centrifuged at 700rpm for 15 seconds and run on a 7500 fast StepOne Real-Time PCR System (Thermo Scientific). Relative quantification was analyzed using the comparative threshold cycle (Ct) method normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described previously (Schmittgen, 2008).

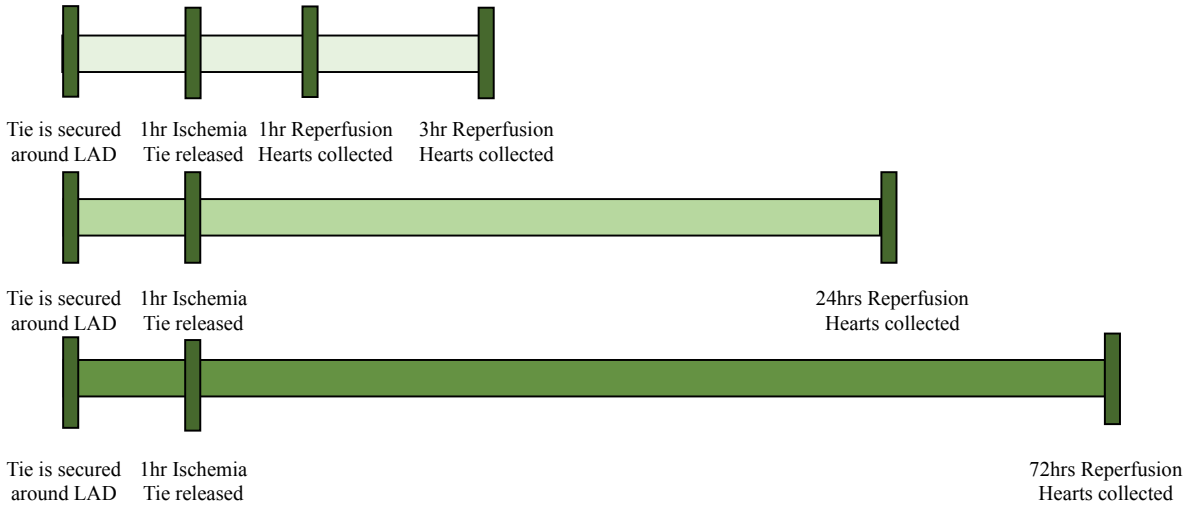
Statistical Analysis

All values are presented as mean ± standard error of the mean (SEM). The student's T-tests were performed with a probability<0.05 deemed significantly different.

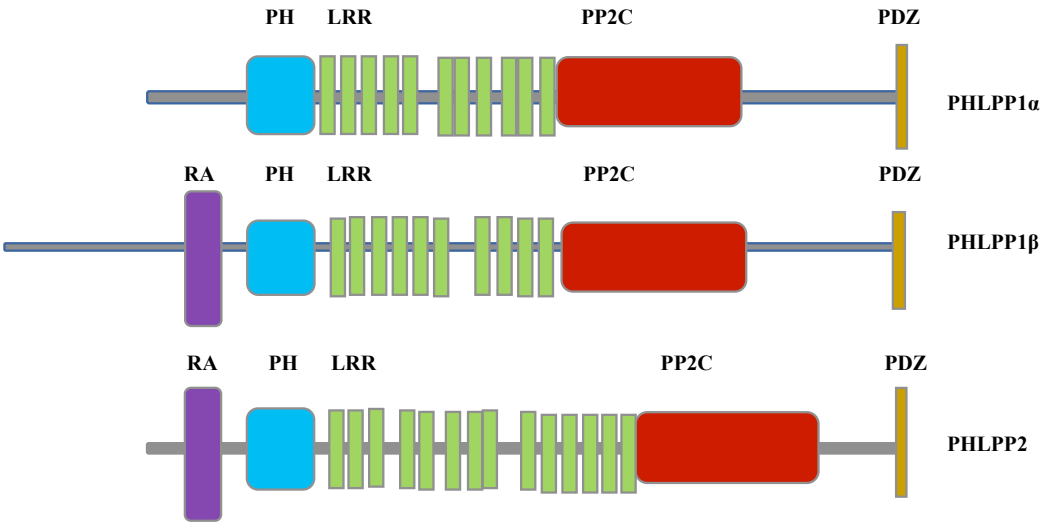
SCHEMES



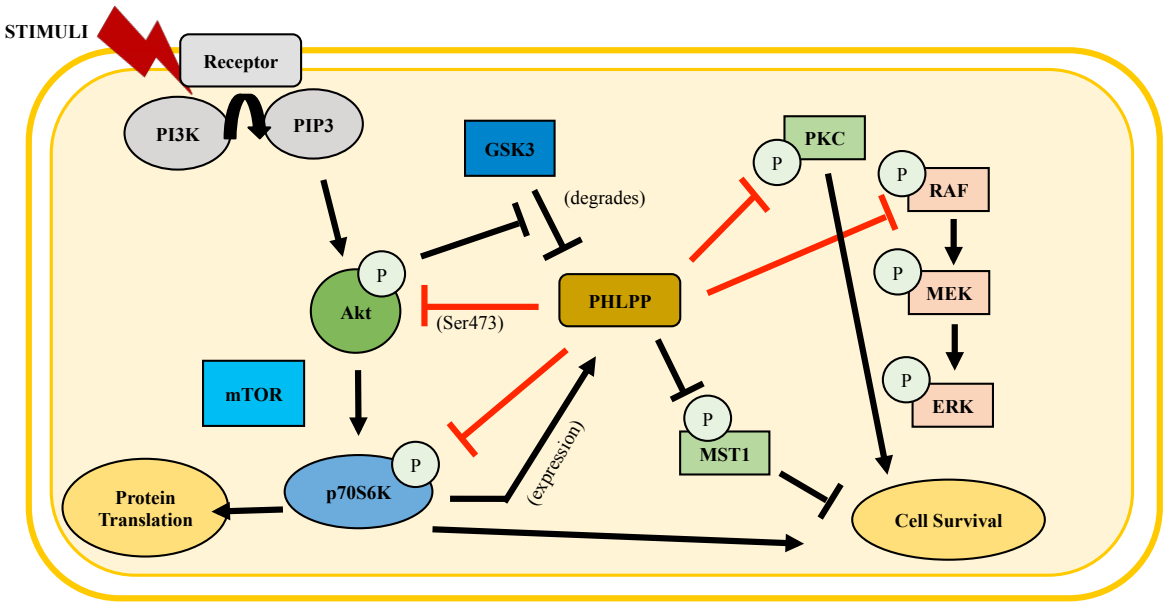
Scheme 1. Diagram of Experimental Aims. The tie securing the LAD is depicted with a solid black circle. The region below the tie is termed the "ischemic" region and the region above the tie is named the "non-ischemic region." The heart tissue was divided into the two regions and subsequently used for RNA and protein analysis to examine changes in inflammation, antioxidation, cell death, mitochondrial dysfunction, autophagy and angiogenesis.



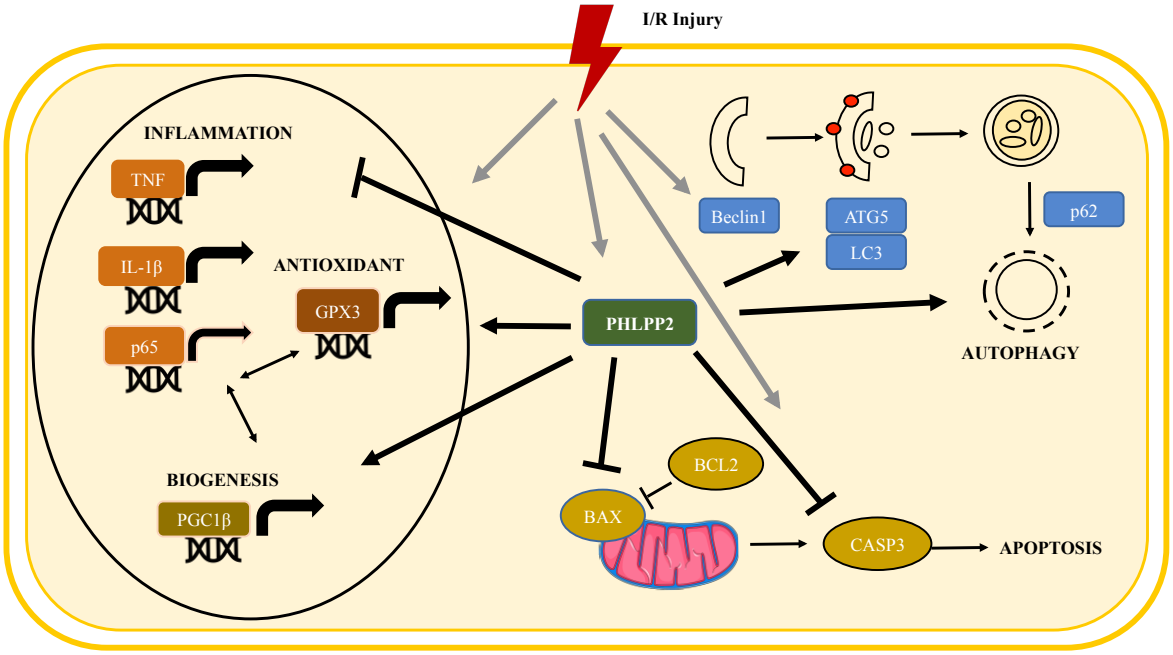
Scheme 2. Experimental Design of Ischemia/Reperfusion Time Course. A tie was secured around the left anterior descending artery (LAD) to initiate ischemia. Following one hour, the tie was released to initiate reperfusion and the duration lasted for several different time periods, which are depicted above. Hearts were collected following 1hr ischemia, 1hr reperfusion, 3hrs reperfusion, 24hrs reperfusion, and 72hrs reperfusion.



Scheme 3. Structures of PHLPP1 and PHLPP2. PHLPP contains two isoforms: PHLPP1 and PHLPP2, with PHLPP1 containing two splice variants: PHLPP1α and PHLPP1β. The structure consists of a PH domain, ras association (RA) domain, leucine-rich repeat (LRR) region, a protein phosphatase 2C (PP2C) domain and a PDZ ligand domain.



Scheme 4. PHLPP Signaling in Cancer. PHLPP selectively dephosphorylates Akt at the hydrophobic motif (Ser473) to inhibit cell survival. Other cellular targets of PHLPP include p70S6K, PKC, RAF, and MST1 and PHLPP regulation of these substrates works to reduce cell survival. Two feedback loops are depicted: 1) Increased PHLPP inhibits Akt phosphorylation and decreased Akt results in increased activation of GSK3 and subsequently increased PHLPP degradation. 2) PHLPP inhibits p70S6K which works to increase PHLPP protein translation.



Scheme 5. Experimental Findings. PHLPP2 removal was found to enhance I/R injury. Therefore, the endogenous role of PHLPP2 in the heart appears to be protective through its regulation of several interconnected cellular pathways including inflammation, antioxidant signaling, mitochondrial biogenesis, cell death, and autophagy.

RESULTS

Removal of PHLPP2 does not alter PHLPP1 or Akt activity in the heart.

We have previously demonstrated that removal of PHLPP1 increases basal Akt phosphorylation and activity in the heart without altering PHLPP2 levels (Moc, 2015). Here we demonstrate for the first time that in the global PHLPP2 KO mice, removal of PHLPP2 does not alter basal Akt phosphorylation in the heart or PHLPP1 levels (Figure 1A). However, removal of PHLPP2 significantly increased basal ERK phosphorylation (Figure 1B and 1C).

PHLPP1 removal attenuates while PHLPP2 removal enhances injury following I/R.

To determine the effect of PHLPP isoform removal on cardiac injury, WT and KO mice were subjected to 1-hour cardiac ischemia and 24 hours reperfusion. Following reperfusion the extent of injury was determined using triphenyltetrazolium chloride (TTC) and Evan's blue dye, followed by digitized imaging of the area of infarction (IA) versus the area at risk (AAR). Our initial studies for 1-hour ischemia and 24 hours reperfusion demonstrate a significant attenuation of infarct injury in PHLPP1 KO compared to WT with the same AAR; whereas PHLPP2 removal increased infarct injury (Figure 2A and 2B). Our data suggests that PHLPP isoforms do not have overlapping functions in the heart.

Reperfusion induces PHLPP2 gene expression and Akt protein expression

While PHLPP isoform removal may have unique functions in regards to I/R injury, how I/R injury affects PHLPP isoform expression in WT and KO mice is of interest. Analysis of the mRNA expression following 1-hour ischemia and 24 hours reperfusion indicates PHLPP2 gene expression is significantly increased and PHLPP1 mRNA is slightly decreased in the non-ischemic region from WT mice (Figure 3A). Removal of PHLPP1 reduces the gene expression

of PHLPP2 following reperfusion while loss of PHLPP2 does not alter PHLPP1 gene expression (Figure 3A). Upon examination of protein expression, PHLPP2 levels increase immediately following 1-hour reperfusion (data not shown) but are unchanged following 24 hours reperfusion in the non-ischemic region from WT mice (Figure 3C). PHLPP1 protein levels are not altered following 1-hour (data not shown) or 24 hours reperfusion in WT mice or PHLPP2 KO mice (Figure 3C

PHLPP2 removal increases cell death

To determine the mechanism of altered injury following I/R with PHLPP isoform removal, mRNA was isolated from the non-ischemic region following 1-hour ischemia and 24 hours reperfusion. Following reperfusion genes involved in cell death were significantly upregulated in the non-ischemic region however removal of PHLPP1 blocked the expression of the pro-apoptotic gene Bax and upregulated the anti-apoptotic gene Bcl-2 compared to WT (Figure 4A and 4B). Whereas removal of PHLPP2 significantly increased the gene expression of the pro-apoptotic factors Bax and Caspase3 (Figure 4D and 4E) and slightly reduced the anti-apoptotic Bcl-2 (data not shown) compared to WT following reperfusion.

PHLPP2 removal creates an imbalance between antioxidant and inflammatory signaling

In addition to increased cell death, mRNA analysis of the non-ischemic region following 1-hour ischemia and 24 hours reperfusion showed increased inflammatory gene expression in WT mice. Removal of PHLPP1 increased TGF-β gene expression compared to WT following reperfusion (Figure 4C) suggesting increased healing. In contrast, PHLPP2 removal significantly increased gene expression of NF-κB (p65) (Figure 4F), as well as gene expression of the inflammatory cytokines TNFα and IL-1β (data not shown).

Increases in inflammation have been accompanied by an increase in antioxidant signaling to prevent cellular injury. Analysis of mRNA expression in the non-ischemic region indicates that PHLPP2 removal significantly reduces the gene expression of the antioxidant enzyme Gpx3 both basally and in response to reperfusion, while PHLPP1 removal increases Gpx3 gene expression basally, which remains high following reperfusion (Figure 5A). Similarly, PHLPP1 removal significantly increases the basal gene expression of NRF1, a transcription factor that regulates antioxidant genes, compared to WT and this expression remains high following reperfusion while removal of PHLPP2 does not appear to alter NRF1 expression basally or following injury (Figure 5B). This data suggests the PHLPP isoforms have opposing roles in regulating antioxidant defense signaling.

Dysregulation of autophagy by PHLPP2 removal

Following I/R injury, autophagy is upregulated as a protective mechanism to clear damaged organelles from the cell (Sai Ma, 2014) (Parzych, 2014). Since removal of the PHLPP isoforms alter injury following reperfusion we examined whether changes in autophagy account for the cell damage that is occurring. We analyzed the protein expression of key autophagy regulators in the non-ischemic region of the heart following 1-hour ischemia and 1- or 3- hours reperfusion in WT and PHLPP2 KO mice. LC3II, an autophagosome structural protein, appeared to decrease at ischemia and then increase at reperfusion in WT mice (Figure 6A), indicating decreased autophagy at ischemia and increased autophagy at reperfusion. Phosphorylation of Beclin1, a promoter of autophagy, is unchanged at ischemia but increased at reperfusion in WT hearts (Figure 6A). Additionally, expression of the autophagy inhibitor, p62, is decreased at ischemia and stayed low at reperfusion in WT mice (Figure 6A), indicating autophagy is activated during I/R in the WT as previously reported. However, in the PHLPP2 KO, both p62

and phosphorylated Beclin1 increase at reperfusion (Figure 6B and 6C), while LC3II expression is not altered (data not shown), which indicates removal of PHLPP2 alters autophagic flux.

FIGURES

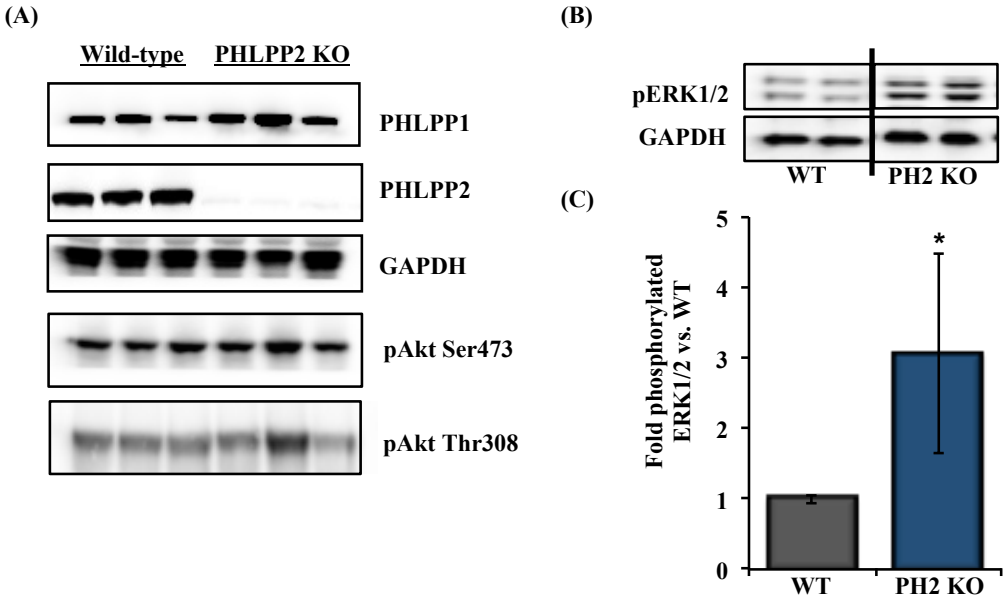


Figure 1. Removal of PHLPP2 in the heart does not effect PHLPP1 or Akt phosphorylation but increases phosphorylated ERK1/2 basally. (A) Heart extracts (15 μg) from wild-type (WT) and PHLPP2 knockout (PH2 KO) mice at 3 months of age were analyzed by Western blot for PHLPP1, PHLPP2, pAktSer473, and pAktThr308 levels (n=3/3 WT/PH2 KO). **(B)** Representative blot of basal phosphorylated ERK1/2 in WT and PH2 KO hearts and **(C)** quantitation of pERK1/2 from n=4 WT and PH2 KO heart extracts. GAPDH was probed as a loading control. *p<0.05 vs. WT

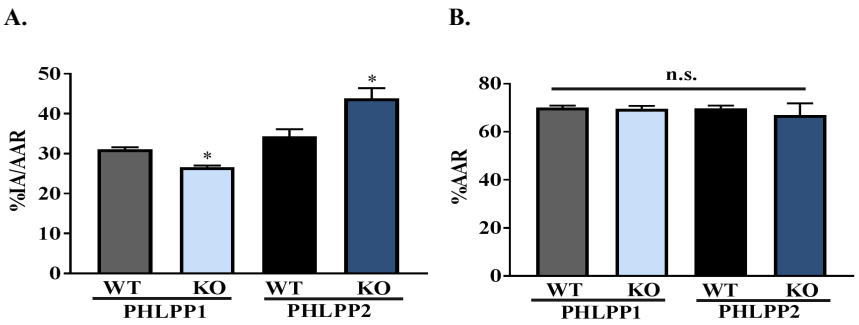


Figure 2. Removal of PHLPP1 attenuates while PHLPP2 removal potentiates injury following I/R. WT , PHLPP1 and PHLPP2 KO mice (3 months of age) were subjected to 1 hour ischemia and 24 hours reperfusion. **(A)** The percent infarct area (IA) versus area at risk (AAR) was determined by TTC staining. n=6/7 WT/PH1 KO and n=4/5 WT/ PH2 KO, *p ≤0.05 vs. WT **(B)** The percent area at risk (AAR) was not significantly different between the groups.

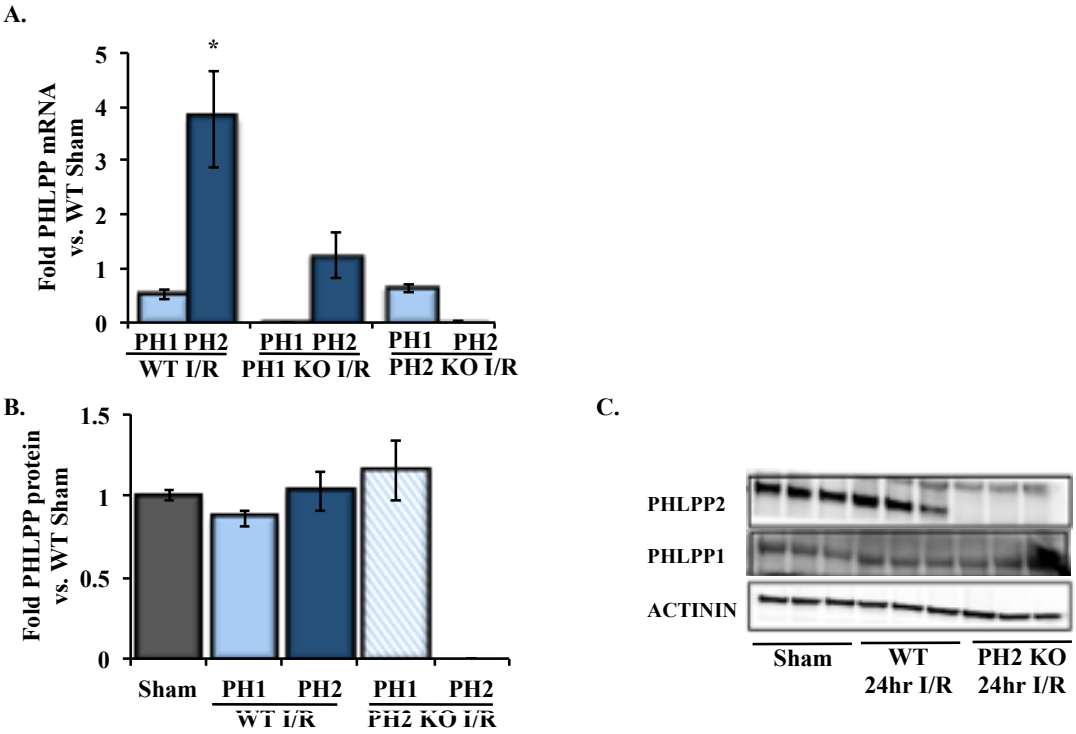


Figure 3. Examination of PHLPP expression following I/R injury. Following 24 hours reperfusion, WT and PH2 KO non-ischemic region extracts (50µg) were probed for PHLPP2 (n=3/3 WT/KO) and PHLPP1 protein expression (n=3/2 WT/KO). **(A)** Examination of PHLPP isoform gene expression following 24 hour I/R from non-ischemic RNA isolated from WT hearts. N=4/5 WT/KO **(B)** Quantitation and **(C)** representative Western blots. Actinin was probed as a loading control. *p<0.05 vs. WT Sham.

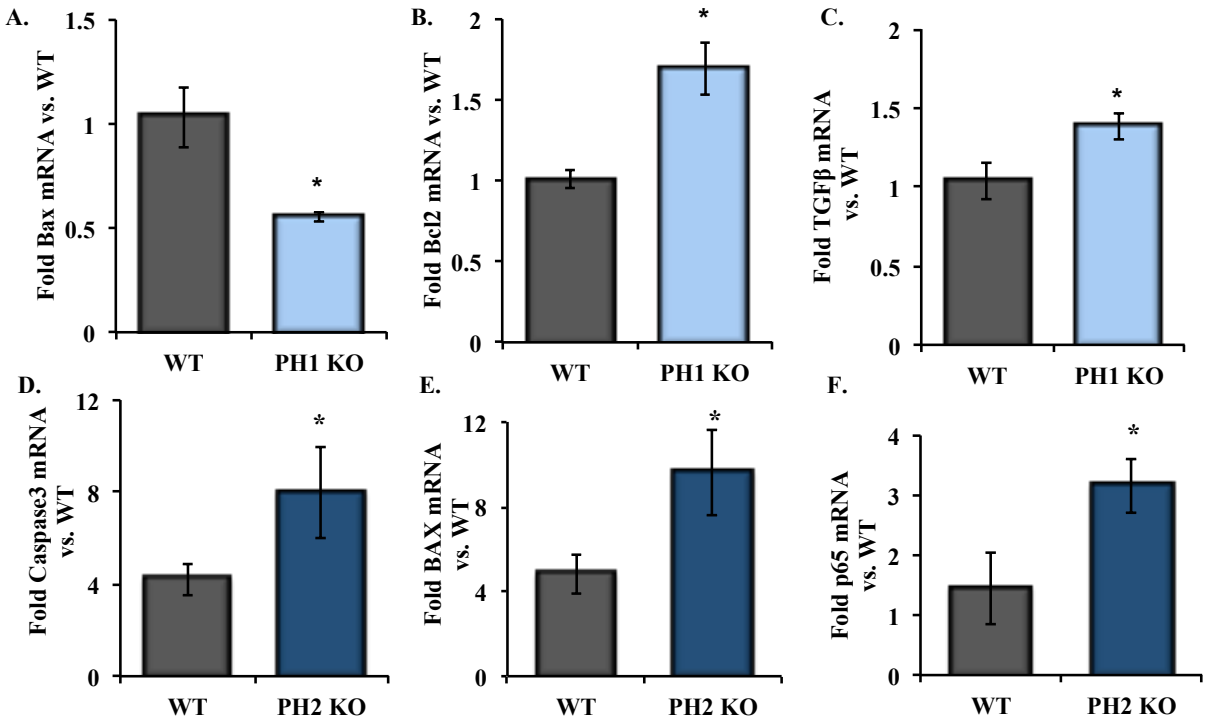


Figure 4: Removal of PHLPP isoforms alters apoptosis/inflammation pathways following I/R injury. WT, PH1 **(A-C)** and **(B-F)** PH2 KO (3 months) were subjected to 1 hour ischemia and 24 hrs reperfusion *in vivo*. The non-ischemic region was removed and RNA isolated. The level of the pro-apoptotic gene Bax (A&E) and Caspase 3 (D), anti-apoptotic Bcl2 (B), TGF-β (C) and NF-κB (p65) (F) were measured. Gene expression of WT, PHLPP1 and PHLPP2 KO mice were subjected to 1 hour ischemia and 1 day reperfusion *in vivo* (n=3/3 WT/KO). *p<0.05 vs. WT (I/R).

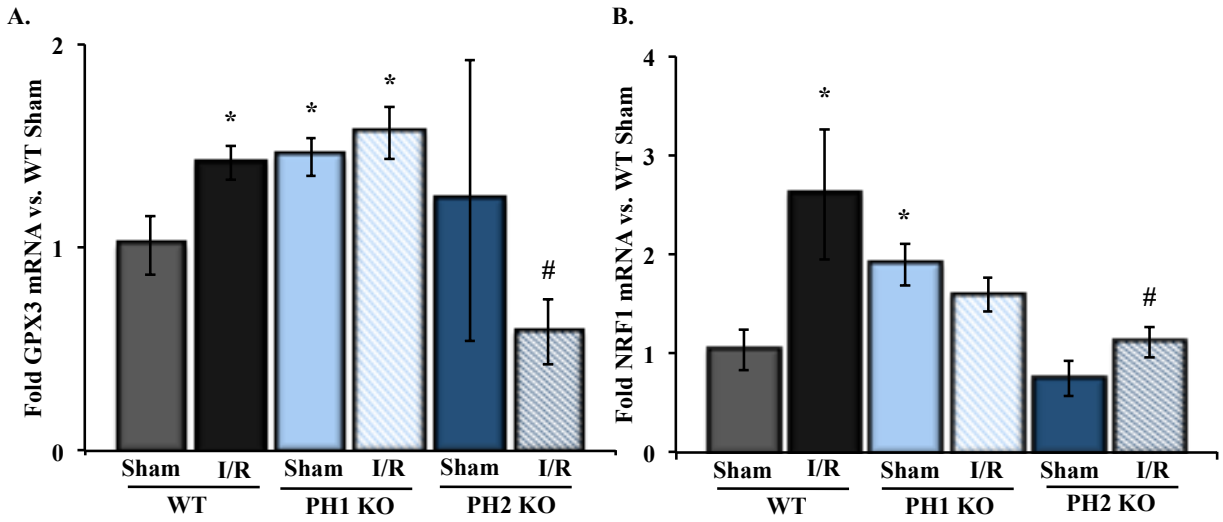


Figure 5. Removal of PHLPP isoforms alters antioxidant signaling. WT, PH1, and PH2 KO mice were subjected to 1hr ischemia and 24hrs reperfusion *in vivo*. The non-ischemic region was removed and RNA isolated. The gene expression of **(A)** GPX3 and **(B)** NRF1 were examined. *p<0.05 vs. WT Sham. #p<0.05 vs. WT I/R (n=3 WT and KO)

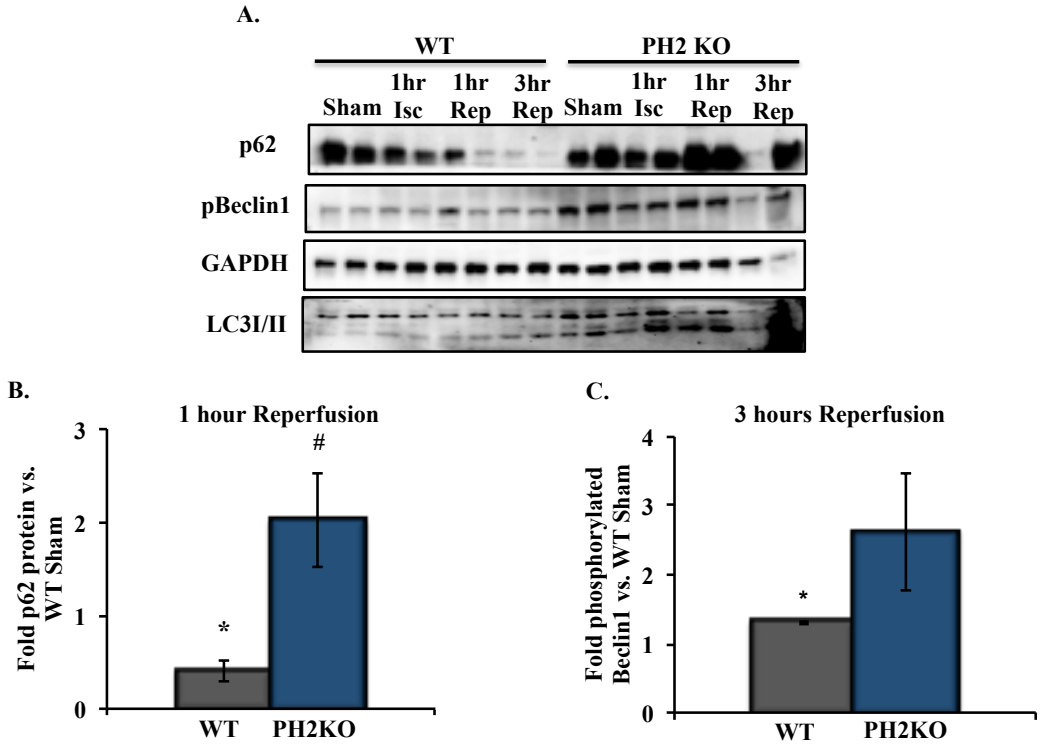


Figure 6. PHLPP2 removal dysregulates autophagy following reperfusion. WT and PH2 KO were exposed to 1hr ischemia and ischemia with 1hr and 3hrs reperfusion *in vivo.* The non-ischemic region (15μg) was analyzed for protein expression of markers of autophagy. **(A)** Representative Western blot of p62, phosphorylated Beclin1, and LC3I/II. GAPDH was probed as the loading control. Quantitation of **(B)** p62 at 1hr reperfusion and **(C)** phosphorylated Beclin1 at 3hrs reperfusion (n=3/3 WT/KO). *p<0.05 vs. WT Sham and #p<0.05 vs. WT I/R.

DISCUSSION

Heart disease is the leading cause of death in the United States. A myocardial infarction, or heart attack, occurs approximately every forty seconds and reperfusion is a necessary treatment but also causes additional injury to the myocardium (Hausenloy, 2004). PHLPP, a serine/threonine phosphatase, poses as a potential target in the treatment of myocardial infarction. PHLPP1 selectively dephosphorylates Akt at its hydrophobic motif (Ser473) and removal of PHLPP1 has been shown to be cardioprotective through increased Akt phosphorylation and activation leading to cell survival after insult (Moc, 2015) (Miyamoto, 2010). PHLPP2 removal does not alter Akt activity basally in the heart (unpublished data) and has been found to increase cardiomyocyte hypertrophy *in vitro* (Yeh, 2018). We hypothesize that removal of PHLPP2 alters cardiomyocyte growth signaling and is detrimental to the heart following ischemic injury. The data in this study supports our hypothesis and proposes that PHLPP1 removal confers protection against I/R injury through inhibition of cell death and increased mitochondrial biogenesis while PHLPP2 removal causes dysregulation of multiple signaling pathways following I/R that alter the balance of inflammation and antioxidant defense signaling leading to increased injury.

There is very little known regarding the role of PHLPP2 in the heart. Our lab has previously demonstrated that removal of PHLPP1 in the heart basally increases Akt phosphorylation and activity (Moc, 2015). Here we determined for the first time that removal of PHLPP2 in the heart does not affect the protein level of the PHLPP1 isoform or alters the phosphorylation of Akt at Ser473 or Thr308 basally (Figure 1A). Since PHLPP2 has a Ras association domain in its n-terminal domain, it has the ability to bind and inhibit the ERK signaling pathway. Upon examination we found that removal of PHLPP2 increased

phosphorylation of ERK1/2 basally (Figure 1B and 1C) which was not found with PHLPP1 removal. Given that overexpression of ERK in the heart can cause hypertrophy and lead to heart failure (Nicol, 2001), this data suggests that PHLPP2 removal may predispose the heart to increased injury by upregulation of ERK signaling. However, increased phosphorylation of ERK1/2 does not necessarily imply increased activity. Therefore, the kinase activity of ERK should be examined basally and following I/R injury.

We have previously shown that removal of PHLPP1 protects the heart from ischemic damage *ex vivo* (Miyamoto, 2010) however the effect of the PHLPP isoforms on ischemic damage *in vivo* has not been demonstrated. We determined for the first time that removal of PHLPP1 and PHLPP2 have different effects on the heart following I/R damage. TTC staining of hearts from 1hr ischemia and 24 hour reperfusion demonstrate a significant attenuation in infarct injury in PHLPP1 KO compared to WT with the same AAR; however PHLPP2 removal increased infarct injury (Figure 2A and 2B). This suggests that the two PHLPP isoforms have opposing roles in the heart but the pathways that are altered by these isoforms to elicit this response are unknown.

To determine the signaling pathways that are altered by PHLPP removal in the heart following I/R injury, it is important to understand how PHLPP isoforms are altered in WT mice following injury. Gene analysis of PHLPP1 and PHLPP2 indicates that loss of one isoform does not significantly affect the expression of the other basally, indicating the two PHLPP isoforms act independently in the heart and do not compensate for loss of the other. After 24 hours reperfusion, PHLPP2 mRNA was significantly increased while PHLPP1 mRNA slightly decreases in WT mice (Figure 3A). This could be a protective mechanism employed by the cell as reduced PHLPP1 levels and increased PHLPP2 levels may provide some protection against

I/R injury, given the observed effect that removal of PHLPP1 is protective on infarct injury discussed above. Analysis of the protein expression of the isoforms in the WT indicates that PHLPP2 expression is increased at 1-hour reperfusion and unchanged at 24 hours reperfusion, while PHLPP1 expression is not altered at either time point (Figure 3C). Therefore, upregulation of PHLPP2 may play a role immediately following reperfusion in trying to enhance protective mechanisms. Given that the mRNA is altered at 24 hours reperfusion but the protein level is not, suggests that the protein may be degraded or there is a problem with protein synthesis. It has been demonstrated that GSK3 can phosphorylate and degrade PHLPP1 (Li, 2009), we can postulate that while GSK3 degrades PHLPP1 to increase Akt activity which is needed for protection following I/R it may also degrade PHLPP2 that would be detrimental to the heart. Therefore overexpression of PHLPP2 in the presence of PHLPP1 removal may be protective and this could be tested both *in vitro* in cardiomyocytes *and in vivo* with the PHLPP1 KO. Furthermore, following 24 hours reperfusion, removal of PHLPP2 does not affect PHLPP1 mRNA expression, but removal of PHLPP1 blocks PHLPP2 mRNA expression (Figure 3A). This suggests that while the PHLPP isoforms may act independently under physiological conditions, following insult PHLPP1 removal negatively regulates PHLPP2, while PHLPP2 removal does not affect PHLPP1 expression. Examination of protein expression following 24 hours reperfusion indicates that removal of PHLPP2 does not affect PHLPP1 expression (Figure 3C) and removal of PHLPP1 does not alter PHLPP2 expression. Again, given the mRNA is altered at 24 hours reperfusion but the protein level is not further suggests that the protein may be degraded or there is a problem with protein synthesis. Overall, removal of one isoform does not appear to affect the other, which indicates that PHLPP1 and PHLPP2 act independently in the heart and mediate I/R injury through unique mechanisms.

Our studies presented here are from 24 hours reperfusion. Additionally, longer reperfusion time points (3-7 days) should be examined to determine the effect of PHLPP isoforms on cardiac function and remodeling and whether there are alterations in PHLPP2 expression over time as well as if PHLPP1 is altered at later times and if PHLPP1 removal blocks PHLPP2 expression. Also experiments in which PHLPP isoforms are individually overexpressed could be performed to determine if PHLPP2 overexpression protects against I/R injury and PHLPP1 expression is detrimental to the heart.

Given that I/R appears to increase PHLPP2 levels and slightly decrease PHLPP1 in WT mice following 24 hours reperfusion, changes in Akt phosphorylation were examined following reperfusion (1hr, 3hrs, 24hrs, and 72hrs). We found that in WT mice, Akt and GSK3β phosphorylation increased at 1-hour and 3 hours reperfusion and continued to increase at 24 hours and 72 hours reperfusion (data not shown), indicating increased Akt activity in response to I/R. Following PHLPP2 removal, phosphorylation of Akt at Ser473 was significantly increased during ischemia compared to WT but decreased at 1 hour and 3 hours reperfusion to similar levels as WT. A previous study found that myocardial ischemia increases activation of the Akt pathway to mediate survival in response to I/R injury (Wang, 2009), suggesting that the significant upregulation in phosphorylation of Akt may be due to the increased injury resulting from PHLPP2 removal. Additionally, removal of PHLPP2 appears to result in decreased Akt308 phosphorylation at both 1 hour and 3 hours reperfusion compared to WT; however, there is no change after 24 hours reperfusion compared to WT. Our data suggests that removal of PHLPP2 does not alter Akt activity basally, and following I/R Akt activity may be altered indirectly due to changes in cell survival. Whether removal of PHLPP2 alters Akt activity at later times following injury needs to be examined since Akt phosphorylation appears to increase over

reperfusion time in WT mice and changes in Akt phosphorylation does not necessarily indicate changes in overall activity.

We found that one of the mechanisms through which PHLPP isoform removal mediates I/R injury is through alterations in cell death signaling. Cell death, specifically apoptosis, is a large component of infarct injury (Hausenloy, 2004). This is evident by increased BAD and BAX mRNA expression seen in WT mice following 1-hour ischemia and 24 hours reperfusion. PHLPP1 removal significantly decreased pro-apoptotic BAX mRNA levels (Figure 4A) while upregulating anti-apoptotic Bcl-2 (Figure 4B) compared to WT, indicating PHLPP1 removal may protect against I/R injury by inhibiting apoptosis. This could be through alterations in Akt signaling since we have previously demonstrated in the brain that PHLPP1 removal decreased infarct following I/R injury through increased Akt activity (Chen, 2014). Meanwhile, PHLPP2 removal increased Caspase3 and BAX mRNA expression (Figure 4D and 4E) and slightly decreased Bcl-2 following I/R compared to WT, demonstrating that removal of PHLPP2 acts in opposition to PHLPP1 removal and may increase I/R injury through increased cell death. Initial studies of apoptotic protein expression show PHLPP2 removal increases BAX expression (data not shown) following 1-hour reperfusion; further supporting PHLPP2 removal increases apoptosis. Interestingly, the anti-apoptotic Bcl-2 protein was increased at ischemia in the PHLPP2 KO in both the ischemic and non-ischemic regions (data not shown). One possible explanation is that Bcl-2 is initially upregulated as a secondary response to PHLPP2 removal in an effort to combat the I/R-induced injury and is not directly regulated by PHLPP2. Given that there is no significant difference in Bcl-2 protein expression between the WT and PHLPP2 KO at 3 hours reperfusion suggests that the cells attempt to protect against the increased injury is

reduced over time. This is also supported by the unchanged Bcl-2 mRNA expression in the PHLPP2 KO at 24hrs reperfusion.

In addition to apoptotic markers, the MAPK pathways, another regulator of cell death, were examined as a potential mechanism through which PHLPP2 removal enhances I/R injury. As previously mentioned, PHLPP2 removal basally increased ERK1/2 phosphorylation (Figure 1B and 1C); however, initial studies indicate PHLPP2 removal does not basally alter p38 phosphorylation, another member of the MAPK family known to regulate cell death. Analysis of ERK1/2 and p38 phosphorylation following 1hr ischemia and 1 and 3-hour reperfusion demonstrates that I/R increases phosphorylation and activation of both of these MAPK kinases in the WT (data not shown) and phosphorylation remained high in the PHLPP2 KO without differing significantly from the WT. At 24 hours reperfusion, phosphorylated ERK1/2 is still increased in the WT but not to the same degree as seen in the shorter reperfusion time points, while p38 phosphorylation is not altered. Following PHLPP2 removal and 24 hours reperfusion, ERK1/2 phosphorylation remains remain high compared to WT while p38 is unchanged. This suggests that PHLPP2 may act selectively on ERK through its RAS association domain and not on the other MAPK family members to alter survival. Since ERK activity may be heightened following I/R in the KO, the ERK inhibitor UO126 can be given prior to I/R or at reperfusion to determine if it alters infarct injury in the PHLPP2 KO. In addition to these two kinases, JNK is another member of the MAPK family that has been shown to regulate cell death. Myocardial I/R increases mitochondrial JNK activation, which results in increased cell death (Xu, 2015). JNK has also been shown to increase ROS production leading to cardiomyocyte cell death (Chambers, 2013). In cancer cells, PHLPP dephosphorylates and activates Mst1, which in turn activates its downstream effector JNK to inhibit cell survival (Qiao, 2010). Therefore, JNK phosphorylation

following PHLPP2 removal and I/R should be examined to determine if PHLPP2 acts selectively on this kinase to enhance I/R injury through increased cell death.

Our laboratory and others has shown that PHLPP1 removal does not alter ERK1/2 phosphorylation basally or following stimulation in both the heart and brain (Huang, 2009) (Chen, 2013). Our initial studies indicate PHLPP1 removal does not alter JNK phosphorylation following 24 hours reperfusion as well (data not shown). However, PHLPP1 knockdown increased LPS-induced p38 phosphorylation in macrophages leading to increased inflammation (Alamuru, 2014), which suggests PHLPP1 may affect the inflammatory cells involved in the immune response. Whether PHLPP1 removal affects p38 activity during cardiac I/R injury to inhibit cell death should be investigated.

In addition to cell death, PHLPP2 removal appears to increase I/R injury through an imbalance in inflammation/antioxidant signaling. In response to I/R, inflammation is induced and can be both protective and detrimental (Ong, 2018). While the influx of inflammatory cells is beneficial because these cells work to heal the I/R-induced damage to the myocardium, they are also a source of ROS, which can damage the tissue (Hu, 2016). Our data shows that I/R increases inflammatory gene expression in WT mice following 24hrs reperfusion. In the non-ischemic region, PHLPP2 removal enhances inflammatory gene expression following I/R compared to WT, as seen by the increased expression of the transcription factor NF-κB (p65) (Figure 4F) as well as its downstream cytokines TNFα and IL-1β. In contrast, PHLPP1 removal blocks inflammatory gene expression following injury (data not shown) in the non-ischemic region yet increases TGF-β mRNA (Figure 4C) following I/R, suggesting increased healing.

It has been previously reported that inflammatory and antioxidant signaling co-regulate each other (Li, 2008). Inflammation, as previously mentioned, is also a large source of ROS, in

addition to the influx of ROS from reperfusion. Therefore, during I/R the antioxidant defense signaling pathways also increase as a way to protect against the increased oxidative stress from both inflammation and reperfusion injury (Li, 2008). Analysis of redox gene expression following 1-hour ischemia and 24 hours reperfusion shows that PHLPP2 removal decreases GPx3 expression, which is a detoxifier of oxidative stress, while PHLPP1 removal increases GPx3 expression basally and following reperfusion remains high and not significantly different from WT (Figure 5A). Similarly, PHLPP1 increases NRF1 gene expression basally which is downstream of GPx3 and its expression remains high as induced by reperfusion in the WT while PHLPP2 removal does not appear to alter NRF1 mRNA at reperfusion (Figure 5B). Taken together, I/R increases inflammatory gene expression in the hearts of WT and PHLPP2 KO that may be reduced with PHLPP1 removal; however, the antioxidant defense system, which is needed to counteract the inflammatory response, is inhibited following reperfusion with PHLPP2 removal and basally increased with PHLPP1 removal. An increase in inflammation has been shown to alter binding of the transcription factor NF-κB to the NRF1 promoter to increase transcription and increase redox signaling (Suliman, 2010). Following 24 hours reperfusion, PHLPP2 removal significantly increases inflammation without the associated increase in NRF1, indicating an imbalance in inflammatory regulation of redox signaling. These alterations in the balance could explain why PHLPP1 removal provides protection following I/R and PHLPP2 removal potentiates injury. Interestingly, a previous study examining the role of PHLPP2 in hypoxia-induced cardiomyocyte injury *in vitro* found that PHLPP2 inhibition resulted in increased GSK3β phosphorylation/inactivation, which allowed for NRF2 to translocate to the nucleus, bind its transcriptional response element, and regulate gene expression of antioxidant enzymes to alleviate injury (Jin, 2019). We show that *in vivo* PHLPP2 removal exacerbates

injury by reducing the gene expression of antioxidant enzymes. Our initial studies show that PHLPP2 removal decreases GSK3β phosphorylation following 24 hours reperfusion. Under *in vivo* conditions, global PHLPP2 removal does not increase GSK3β phosphorylation/inactivation in the heart resulting in reduced NRF2 translocation to the nucleus and subsequent regulation of antioxidant gene expression. The effect of PHLPP2 removal in cardiomyocytes may be different than in the whole heart and using the cardiac specific PHLPP2 KO mice for the I/R studies would be necessary to determine the cell specific changes.

Furthermore, I/R has been shown to alter mitochondrial ROS and mitochondria are a major source of both energy production and ROS formation and the balance is important for maintaining cell health. The lack of regenerative capabilities of cardiac cells along with the high amount of mitochondria present poses a serious problem in the face of mitochondrial damage following ischemic injury. As demonstrated earlier, PHLPP2 removal significantly increased BAX and Caspase3 gene expression (figure 4D and 4E), suggesting PHLPP2 removal may cause increased Bax at the mitochondria which alters mitochondrial permeability transition pore (mPTP) and the release of ROS to increase injury and cell death. Future experiments to look at the amount of ROS formation in the tissues from WT and PHLPP2 KO mice following I/R injury need to be examined. Also, mitochondria from WT and KO hearts exposed to ischemia and various times of reperfusion can be examined for changes in mitochondrial function and pore opening. Following reperfusion, PHLPP1 removal reduces BAX expression which suggests that PHLPP1 removal protects the mitochondria by preventing excessive mPTP opening and the release of ROS. Akt, which is regulated by PHLPP1, also plays a role in protecting the mitochondria against hypoxic injury. Miyamoto et. al found that activated Akt translocates to the mitochondria and binds hexokinase II to prevent opening of the mPTP and release of

cytochrome-c. Given that PHLPP1 removal results in increased Akt activity, loss of PHLPP1 could confer protection against I/R injury by protecting the mitochondria from oxidative damage and reducing the release of cytochrome c, a stimulus for cell death.

Another key regulator of mitochondrial ROS is the PGC-1 family of transcriptional co-activators, including PGC-1α and PGC-1β, are known to be master regulators of mitochondrial biogenesis as well as play a role in antioxidant defense signaling (Schilling, 2011). Specifically, PGC-1α appears to uniquely regulate fatty acid oxidation while both PGC-1α and PGC-1β regulate ROS suppression (St-Pierre, 2003) (St-Pierre, 2007) (Riehle, 2012). We found that 24 hours reperfusion upregulates PGC-1α mRNA expression without altering PGC-1β mRNA levels in the non-ischemic region of WT mice (data not shown). PHLPP1 removal increases PGC-1α basally and does not affect PGC-1β, while PHLPP2 removal decreases PGC-1β basally and does not alter PGC-1α (data not shown), suggesting the PHLPP isoforms uniquely target the gene expression of PGC-1 isoforms. Moreover, PHLPP1 removal may confer protection by basally increasing mitochondrial biogenesis and antioxidant defense signaling as seen by the increase in PGC-1α expression. On the other hand, PHLPP2 removal basally reduces antioxidant defense signaling as seen by the decrease in PGC-1β levels along with the reduced GPX3 expression discussed above. Following 24 hours reperfusion, PHLPP1 removal blocks PGC-1α expression and PHLPP2 removal sustains low expression of PGC-1β (data not shown). It is unexpected that PHLPP1 removal would block PGC-1α, as this would indicate reduced mitochondrial biogenesis and antioxidant defense signaling and does not support the cardio-protective phenotype seen in PHLPP1 removal. However, a previous study found that PGC-1α upregulation in cardiac-derived H9c2 cells increased cell death following simulated I/R (Lynn, 2010). This study, coupled with the data we obtained, suggests that PHLPP1 removal may confer protection against I/R injury by

reducing PGC-1α and thereby inhibiting I/R-induced cell death. Whereas PHLPP2 removal maintained low levels of PGC-1β following injury, supporting the finding that PHLPP2 removal reduces antioxidant defense signaling.

Future studies should examine mitochondrial function in more depth as this organelle plays a large role in I/R injury. Other markers specific to mitochondrial damage, such as cytochrome c release or BAD protein expression should be examined following I/R injury in the WT and KO mice. Also, electron microscopy should be performed to analyze the mitochondrial structure in the hearts basally and following injury in the mice. This would allow us to determine if there are any alterations in the mitochondria that would predispose the PHLPP2 KO mice to injury.

Following reperfusion, autophagy, a pro-survival mechanism employed in order to remove damaged organelles from the cell, is activated. However, dysregulation of autophagy can be detrimental to the cell and cause further damage. In the non-ischemic region, autophagy was increased at 1 hour and 3 hours reperfusion in WT mice, as seen by accumulation of LC3II, increased phosphorylation of Beclin1, and decreased p62 protein levels (Figure 6A), due to the influx of ROS and ensuing cell damage following reperfusion. PHLPP2 removal increased both p62 and phosphorylated Beclin1 protein levels in response to 1-hour and 3 hours reperfusion respectively (Figure 6B and 6C), while there was no significant accumulation of LC3II. If autophagy is activated there should be a decrease in p62 with an accumulation of LC3II. Therefore, PHLPP2 removal most likely affects autophagic flux. Autophagic flux refers to the entire autophagy process starting with induction and leading to maturation, lysosomal fusion, and degradation (Zhang, 2013), suggesting that there may be an impairment in autophagy following I/R with PHLPP2 removal. Additionally, autophagy is induced by increased oxidative stress. An

increase in p62 levels leads to KEAP1 sequestration and subsequent activation of NRF2 and its target genes, which promote antioxidant signaling (Ichimura, 2013). This data is evidence that PHLPP2 removal reduces antioxidant signaling and increases oxidative stress, which should result in increased autophagy. Therefore, the reduction in antioxidant signaling and resulting increase in oxidative stress coupled with dysregulation of autophagy could explain why PHLPP2 removal potentiates I/R injury.

In conclusion, a myocardial infarction is a huge health burden that currently does not have an ideal therapeutic approach. Ischemia causes extensive damage to the myocardium and reperfusion, while necessary for survival, can cause additional tissue damage. Therefore, the need for novel therapeutic approaches in the treatment of myocardial infarction, as well as other forms of heart disease, is pertinent. PHLPP has been suggested as a therapeutic target in the treatment of cancer given its role as a tumor suppressor. In the heart, PHLPP may be a therapeutic target given the established cardio-protective role of PHLPP1 removal. This study shows that following I/R, PHLPP1 removal reduces infarct area by reducing apoptotic signaling whereas PHLPP2 removal enhances infarct through disruption of multiple interconnected cellular pathways. PHLPP2 removal inhibits antioxidant signaling by reducing GPX3 expression while increasing oxidative stress via upregulation of inflammatory cytokines, coupled with dysregulation of autophagy and lack of upregulation of mitochondrial biogenesis. These signaling pathways are connected and require a delicate balance in order to protect the cell. While PHLPP1 removal maintains this balance, PHLPP2 removal disrupts it by acting on multiple signaling pathways. Currently, the need for specific PHLPP isoform inhibitors is required to understand the therapeutic potential of either isoform in disease. The opposing roles

of these two isoforms found in this study support the potential development of isoform-specific inhibitors in an effort to reduce I/R-induced injury to the myocardium following a heart attack.

This study lays the groundwork for future experiments concerning the role of the PHLPP isoforms in mediating cardiac injury. Future studies should focus on a cardiomyocyte-specific PHLPP2 knockout to rule out the possibility of the global PHLPP2 knockout affecting inflammatory cells themselves rather than interfering with the signaling pathways. Additionally, given that previous studies show a relationship between PHLPP removal and cardiac hypertrophy, experiments should be conducted to determine whether the hypertrophic response is altered in the remote region following injury with PHLPP removal. Lastly, cardiac remodeling and the progression to heart failure following I/R and PHLPP removal is a potential avenue of interest and would require examination of long-term effects. In all, PHLPP1 may pose as a potential therapeutic target in the treatment of myocardial infarction as a way to mitigate the damage to the myocardium.

APPENDIX

A. Western Blot Solutions

Western Buffer

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
Sodium phosphate (ph 7)	Sigma Aldrich	0.5 M	20 mL	20 mM
Sodium chloride	Fischer Scientific	5 M	15 mL	150 mM
Magnesium chloride	Acros Organics	1 M	1 mL	2 mM
Nonidet P40	Sigma Aldrich	100%	0.5 mL	0.1%
Glycerol	Fischer Scientific	100%	50 mL	10%
Okadaic acid	Sigma Aldrich	10 μM	0.5 mL	10 nM
Sodium fluoride	Sigma Aldrich	1 M	5 mL	10 mM
Sodium pyrophosphate	Sigma Aldrich	0.5 M	10 mL	10 mM
Dithiothreitol	Sigma Aldrich	1 M	0.5 mL	1 mM
Sodium orthovanadate	Sigma Aldrich	200 mM	0.0092g	0.1 mM
Pepstatin	Sigma Aldrich	1 mg/mL	5 mL	10 μg/mL
Leupeptin	Sigma Aldrich	1 mg/mL	5 mL	10 μg/mL

Aprotinin	Sigma Aldrich	25 mg/mL	200 µL	10 µg/mL
N α-Tosyl-L-lysine chloromethyl ketone hydrochloride	Sigma Aldrich	5 mg/mL	1 mL	10 µg/mL
L-1-Toslamide-2-phenylethyl chloromethyl ketone	Sigma Aldrich	5 mg/mL	1 mL	10 µg/mL

Combine reagents in 250 mL of water and then fill with water to a total volume of 500 mL.

5X SDS Loading Dye

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
Tris (pH 7)	Fischer Scientific	1 M	12 mL	0.25 M
Glycerol	Fischer Scientific	92.09	19.2 mL	40%
SDS	Hoefer	288.38	3.84 g	277 mM
2-Mercaptoethanol	Sigma-Aldrich	78.13	9.6 mL	20%
Bromophenol blue	Fischer Scientific	669.96	0.048 g	0.0015 mM

Combine above reagents and fill with water to a total volume of 48 mL.

MOPS Buffer

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
MOPS	Spectrum Chemical	209.26	836.8 g	1 M
Tris-base	Gentrox	121.14	484.8 g	1 M
SDS	Hoefer	288.38	80 g	69.4 mM
EDTA	Aldrich Chemical Company	372.24	24 g	16.1 mM

Dissolve above reagents in 2 L of water then fill with additional water to a total volume of 4 L. Dilute with water in a 1:20 ration to prepare 1X MOPS Buffer.

TA Buffer

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
TA Buffer	Novex Life Technologies	20X	50 mL	1X

Dissolve above reagents in 950 mL of water.

20X Transfer Buffer

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
Tris-base	Gentrox	121.14	145.6 g	0.24 M
Glycine	Gentrox	75.07	720 g	1.92 M

Combine above reagents in 2 L of water and fill with water to a total volume of 5L.

1X Transfer Buffer

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
20X Transfer Buffer	See above table	20X	50 mL	1X
Methanol	Fischer Scientific	32.04 g/mol	200 mL	20%

Combine above reagents and fill with water to a total volume of 1 L.

10X TBS

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
Tris-hydrochloride	Fischer Scientific	157.6	63g	0.1 M
Sodium Chloride	Fischer Scientific	58.44 g/mol	10.2 g	0.3 M

Combine above reagents in 2L of water. Bring the pH to 7.5. Fill with water to a total volume of 4 L.

0.1% TBS/Tween-20

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
10X TBS	See above table	10X	2 L	10%
Tween-20	Chem-Impex International	1227.54	20 mL	0.1%

Combine above reagents and fill with water to a total volume of 20L.

Western Blot Primary Antibody List

Antigen	*Product Source/Catalog No.*	*Dilution*
PHLPP1	Bethyl/A300-660A	1:1000
PHLPP2	Bethyl/A300-661A	1:2000
pAkt(Ser473)	Cell Signaling Technology/9271L	1:1000
pAkt(Thr308)	Cell Signaling Technology/13038S	1:1000
pERK1/2	Cell Signaling Technology/9101S	1:1000
pp38	Cell Signaling Technology/4511S	1:1000
BCL2	Cell Signaling Technology/3498S	1:1000
BAX	Cell Signaling Technology/14796S	1:1000
p62	Cell Signaling Technology/5114S	1:1000
LC3β	Cell Signaling Technology/3868S	1:1000
pBeclin1(S93)	Cell Signaling Technology/14717S	1:1000
PULK1(S757)	Cell Signaling Technology/14202	1:1000
PMTOR(S2448)	Cell Signaling Technology/5536T	1:1000
pGSK3α/β(S21/S9)	Cell Signaling Technology/9331S	1:1000
p70S6K	Cell Signaling Technology/9202S	1:1000

ATG5	Cell Signaling Technology/12994S	1:1000
NRF1	Cell Signaling Technology/46743S	1:1000
Gapdh	Cell Signaling Technology/2118S	1:1000
Actinin	Cell Signaling Technology/6487S	1:1000

Primary antibodies diluted in 5% Bovine Serum Albumin/0.1%TBST with NaN3

Western Blot Secondary Antibody List

Animal	*Product Source/Lot No.*	*Dilution*
Rabbit	Sigma Life Sciences/SLBV9141	1:8000
Mouse	Sigma Life Sciences/SLBV2305	1:2000

Secondary antibodies diluted in 5% milk

B. qPCR Solutions

cDNA Synthesis Mix

Reagent	*Source*	*FW or Stock Concentration*	*Quantity*	*Final Concentration*
10X RT random primers	Fischer Scientific	10X	2 µL	20%
dNTP Mix	Fischer Scientific	100 mM	0.8 µL	8%
RNAse Inhibitor	Fischer Scientific	20X	1 µL	10%
MultiScribe Reverse Transcriptase	Fischer Scientific	20X	1 µL	10%
10X RT Buffer	Fischer Scientific	10X	2 µL	20%

Combine above reagents with 3.2 µL of sterile water. Add to 0.1 µg RNA (10 µL volume) for a final volume of 20 µL.

RT-PCR Protocol

Time	*Temperature*
10:00	25˚C
2:00:00	37˚C
5:00	85˚C
Hold	4˚C

qPCR Mouse Primer List: PrimeTime Std. qPCR assay IDT

Gene Symbol	*Primer ID*
GAPDH	Mm.PT.39a.1
PHLPP1	Mm.PT.58.9003883
PHLPP2	Mm.PT.58.13356316
Nrf1	Mm.PT.58.13216611
PGC1α	Mm.PT.58.16192665
PGC1β	Mm.PT.58.29600686
VEGFα	Mm.PT.58.14200306
BAX	Mm.PT.58.4012210
BCL2	Mm.PT.58.7362966
Caspase3	Mm.PT.58.13460531
GPX3	Mm.PT.58.29885432
IL-1β	Mm.PT.58.41616450
TNFα	Mm.PT.58.12575861
NF-kB (p65)	Mm.PT.58.29633634
TGFβ	Mm.PT.58.11254750

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