Low molecular weight fibroblast growth factor 2 signals via protein kinase C and myofibrillar proteins to protect against post-ischemic cardiac dysfunction

Janet R. Manning1, Sarah O. Perkins2, Elizabeth A. Sinclair2, Xiaoqian Gao1, Yu Zhang1, Gilbert Newman1, W. Glen Pyle2, Jo El J. Schultz1

1Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267, 2Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario Canada N1G 2W1

Running Title: Myofibrillar function, PKCa/ε in LMW FGF2 cardioprotection

Corresponding Author: Jo El J. Schultz, Ph.D.

Department of Pharmacology and Cell Biophysics
University of Cincinnati, College of Medicine
231 Albert Sabin Way, ML 0575
Cincinnati, OH 45267
Phone: (513) 558-9754
Fax: (513) 558-9969
Email: schuljo@ucmail.uc.edu

Copyright © 2013 by the American Physiological Society.
Abstract

Aims: Among its many biological roles, fibroblast growth factor 2 (FGF2) acutely protects the heart from dysfunction associated with ischemia/reperfusion (I/R) injury. Our laboratory has demonstrated that this is due to the activity of the low molecular weight (LMW) isoform of FGF2, and that FGF2-mediated cardioprotection relies on the activity of protein kinase C (PKC), which PKC isoforms are responsible for LMW FGF2-mediated cardioprotection, and their downstream targets, remain to be elucidated. Methods and Results: To identify the PKC pathway(s) that contributes to post-ischemic cardiac recovery by LMW FGF2, mouse hearts expressing only LMW FGF2 (HMWKO), were bred to mouse hearts not expressing PKC alpha (PKCαKO), or subjected to a selective PKC epsilon inhibitor (εV1-2), prior to and during I/R. Hearts only expressing LMW FGF2 showed significantly improved post-ischemic recovery of cardiac function following I/R (p<0.05), which was significantly abrogated in the absence of PKCα (p<0.05) or presence of PKCε inhibition (p<0.05). Hearts only expressing LMW FGF2 demonstrated differences in actomyosin ATPase activity as well as increases in the phosphorylation of troponin I and T during I/R compared to wildtype hearts; several of these effects were dependent upon PKCα activity. Conclusions: This evidence indicates that both PKC alpha and epsilon play a role in LMW FGF2-mediated protection from cardiac dysfunction, and that PKCα signaling to the contractile apparatus is a key step in the mechanism of LMW FGF2-mediated protection against myocardial dysfunction.

Keywords: low molecular weight FGF2; PKC alpha and PKC epsilon; troponin; actomyosin ATPase; post-ischemic cardiac function
Introduction

Cardiovascular disease is the number one killer in the U.S., and every year, over eight million people in the U.S. suffer from myocardial infarction. Often, myocardial ischemia is characterized by lowered post-ischemic left ventricular function, which is associated with increased morbidity and mortality. There is a profound and immediate need to address the problem of myocardial dysfunction and infarction associated with cardiac ischemia and reperfusion (I/R). One promising therapeutic strategy to alleviate I/R-induced cardiac injury is fibroblast growth factor 2 (FGF2).

FGF2, found at elevated levels in the serum of I/R patients, has both angiogenic and acute cardioprotective effects. This makes FGF2 an outstanding candidate for the treatment of cardiac patients; however, while in recent years, there have been a number of noteworthy developments characterizing the mechanism of action of FGF2 in the ischemic heart, there are several unanswered questions that must be addressed prior to FGF2 becoming a therapeutic molecule. Yet to be determined is the distinct role each FGF2 isoform plays in the ischemic heart.

FGF2 includes two classes of protein isoforms, the low molecular weight (LMW) and the high molecular weight (HMW). LMW FGF2 is approximately 18 kDa in both mouse and human, and is found primarily in the cytosol of the cell and extracellular fluid as well as nucleus. HMW FGF2 differs from LMW in that it includes a nuclear localization signal, and encompasses two isoforms in mouse (21-22 kDa) and four in humans (21-34 kDa). Our laboratory, using gain-of-function and loss-of-function genetic mouse models, has demonstrated that all endogenous FGF2 isoforms are necessary for protection against myocardial infarction; whereas, HMW and LMW FGF2 isoforms play opposing roles in
the recovery of the heart from I/R injury(40, 41). Studies using genetically-modified mice with only LMW FGF2 expression (HMWKO), or wildtype mouse hearts with LMW FGF2 exogenously added, have shown that this isoform is protective against post-ischemic dysfunction(30, 40, 41), while hearts with only the HMW isoforms (LMWKO), or with HMW FGF2 overexpressed, have significantly lower post-ischemic cardiac function(40). However, the pathways involved in signal transduction for each isoform subtype during I/R–induced cardiac dysfunction have not yet been elucidated; this information is critical for understanding the actions of FGF2 in the post-ischemic heart.

Our laboratory has previously identified protein kinase C (PKC) as necessary for mediating the protection against cardiac dysfunction seen in mouse hearts overexpressing all LMW and HMW isoforms of FGF2(27), which suggests that PKC is critical to the signaling mechanisms of LMW and HMW FGF2’s effects on the heart during I/R. Among the isoforms that are activated by overexpression of FGF2 in the mouse heart that may also phosphorylate targets modulating cardiac function are PKCε and α(1, 5, 9, 17, 22, 27, 33, 34, 46, 53). PKCε has been shown to localize to the cross-striated structures when activated(17, 27, 52) and is known to phosphorylate troponin I (TnI) and troponin T (TnT), as well as myosin light chain and myosin binding protein C (MyBPC), which modulates calcium sensitivity of the myofilaments in the heart(34). Similarly, PKCα is known to translocate to myofibrillar structures(67) and can modulate the function of TnT, TnI, and MyBPC(34). Finally, PKCε and PKCα have been shown to be necessary for ischemic preconditioning (IPC)(23, 32), and PKCα has been shown to be activated in the heart associated with sevofluorane- and sildenafil-induced cardioprotection(16, 32). However, these kinase cascades are complex, and stimulus-dependent(21, 29), which is
why it is essential to delineate the isoforms of PKC, as well as their downstream targets, that
mediate LMW FGF2’s protective actions.

Based on our previous findings which demonstrated that the individual FGF2 isoforms
have opposing action on post-ischemic cardiac function, but not on infarct size(40, 41), the
current study focuses on the cardiac function effects modulated by LMW FGF2 during acute
ischemia-reperfusion injury. Here, our laboratory seeks to elucidate, for the first time, a
mechanism by which endogenously expressed LMW FGF2 protects the heart, identifying the
isoforms of PKC that are directly responsible and determining their role in modulating
myofibrillar protein function. Our study reveals that PKCα and ε are selectively activated and
translocate to the myofibril at early ischemia or reperfusion, respectively, and that ablation of
PKCα, and inhibition of PKCε abrogates the protective effects of LMW FGF2 against post-
ischemic cardiac dysfunction. These data also demonstrate that expression of only LMW FGF2
results in differential phosphorylation of troponin I and T and alteration of actomyosin
MgATPase activity during I/R, which is dependent on PKC and suggests that the improvement in
post-ischemic contractile function mediated by LMW FGF2 is due to direct targeting of the
PKCα and ε to the myofibril.
Methods

Mouse models: Generation and characterization of HMW FGF2 knockout (HMWKO) mice, which only express LMW FGF2, on a SV 129/Black Swiss mixed background, have been previously described(3, 40). To test the role of PKCα, HMWKO mice were bred to mice lacking expression of PKCα (PKCαKO) on a mixed background (SV 129/Black Swiss/FVBN), which have been previously described(9). Mice that did not express either the HMW isoform of FGF2 or PKCα (HMWKOxPKCaKO) were found to have no changes in the expression of other PKC isoforms or LMW FGF2 compared to HMWKO hearts (Figure 1). Similarly, there were no differences in fibroblast growth factor receptor (FGFR1) expression, the predominant FGFR in mouse heart, in these genotypes (Figure 1) and as previously demonstrated by our laboratory(40). All mice were randomly assigned to studies and compared with their wildtype littermates (n= 3-16 per study/genotype). All animal protocols were submitted to and approved by the University of Cincinnati Institutional Animal Care and Use Committee. Mice were housed in pathogen-free housing according to standard use protocols, animal welfare regulations, and the NIH Guide for the Care and Use of Laboratory Animals.

Exclusion criteria: In the I/R studies, aortic leak (pressure <60 mmHg on retrograde perfusion), pulmonary vein leak (aortic flow of <2.0 mL/min and a perfusate gas pO₂ >380 mmHg, with low atrial pressure), or a visible leak in the heart itself were the basis for exclusion, and a total of 17 mice were excluded. Aortic leak was represented as an aortic pressure <60 mmHg on Langendorff, retrograde perfusion mode. Pulmonary vein leak was demonstrated as an aortic flow <2.0 ml/min, low (<4 mmHg) atrial pressure, and a perfusate gas pO₂ >380 mmHg or a visible leak (i.e., hole in ventricle or atrium) in the heart.
Ischemia/Reperfusion injury: 10-12 week old sex-matched mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.), and anesthesia was confirmed by a lack of response to painful stimulus (toe pinch). Hearts were rapidly excised, the aorta and pulmonary vein were rapidly cannulated to induce a working-heart system with a cardiac output of 5 mL/min and a constant afterload of 50 mmHg. and establishment of baseline cardiac and hemodynamic parameters of cardiac output, aortic pressure, aortic and coronary flow, left ventricular systolic and diastolic pressure, and contractility (+dP/dt, positive derivative of the left ventricular pressure with respect to time), and relaxation (-dP/dt, negative derivative of the left ventricular pressure with respect to time) occurred. Mouse hearts were subjected to global low-flow I/R injury as previously described(26). Ischemia was induced by reducing total cardiac output from 5 mL/min to 1 mL/min, for 1 hour resulting in a >90% reduction in coronary flow, after which the heart was reperfused at 5 mL/min for 2 hours. Post-ischemic recovery of contractility and relaxation was depicted as a percentage of ±dP/dt at 2 hours reperfusion normalized to baseline ±dP/dt. An ex vivo model has been chosen for these experiments to evaluate cardiac muscle function in the absence of neural or humoral influences. Following I/R, hearts were subsequently frozen and analyzed for phosphorylation of downstream targets in LMW FGF2-mediated cardioprotection. Lactate dehydrogenase (LDH) levels released into coronary effluent were determined at designated time points of baseline, ischemia and reperfusion (Figure 2). Protease inhibitor tablets (Roche) were added to the collected coronary effluent. The amount of LDH release was determined using the Promega CytoTox 96 Cytotoxicity colorimetric assay per the manufacturers’ instructions.
Pharmacological agents: To examine the role of PKCε, TAT-conjugated translocation inhibitor εV_{1,2} and the TAT peptide (vehicle) were dissolved in water, and administered in Kreb’s perfusate buffer 15 minutes prior to ischemia to 15 minutes after the onset of ischemia, as well as 15 minutes prior to reperfusion to 15 minutes after the onset of reperfusion (Figure 2). A concentration response curve (10 nM-1 μM) was performed to identify the maximal efficacy of inhibitor without adverse effects. A concentration of 100 nM was found to effectively block the translocation of PKCε, while not affecting the activation of PKC isoforms α, δ, and ζ (data not shown). Cardiac preparation and immunoblotting for detection of FGF2: Snap-frozen non-ischemic hearts were powdered and homogenized in homogenization buffer (20mM Tris, 2mM EDTA, 2M NaCl, 1% NP40, PMSF), and FGF2 was extracted as previously described(26). Western blot analysis of the extracted FGF2 was performed with a rabbit polyclonal antibody to FGF2 (1:1000, Santa Cruz Biotechnology). Levels of FGF2 protein isoforms were visualized by chemiluminescence (Amersham Bioscience). Densitometry of protein bands was performed using a Fluorchem 8800 gel imager (Alpha Innotech).

Whole heart preparation to detect FGFR activation: Snap-frozen non-ischemic hearts (Wt, were powdered and homogenized in homogenization buffer (25 mM HEPES, pH 7.5, 1% glycerol, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM sodium orthovandate, 25 mM β-glycerolphosphate, 50 mM sodium fluoride solid, 0.5 μM okadaic acid, diisopropylfluorophosphate, 100 μM calpain inhibitor, Pefabloc stock 1 and 2, SIGMA phosphatase inhibitor) as previously described (30, 40, 41). The homogenate was centrifuged at 13,000g for 15 minutes and the supernatant collected. Protein concentration was determined via Bio-Rad Lowry protein assay (Bio-Rad, Hercules, CA).
**Western immunoblotting for FGFR1 expression:** One hundred micrograms of whole cell homogenate were loaded onto an 8% SDS-PAGE gel then transferred onto 0.2 μm nitrocellulose membrane. Transfer efficiency and loading equality were examined by staining the membrane with 0.1% Ponceau S in 5% acetic acid. The membrane was blocked in 5% dry milk and then incubated with primary antibody against total FGFR1 (1:500, Cell Signaling) followed by incubation with HRP-conjugated secondary mouse (1:10000, Bio-Rad) antibody. FGFR1 expression was visualized by ECL and densitometry of protein bands were quantitated using a Fluorchem 8800 gel imager, and normalized to calsequestrin (primary antibody dilution 1:2500, Thermo Scientific; HRP-conjugated secondary goat anti-rabbit antibody dilution 1:5000, Santa Cruz Biotechnology).

**PKC phosphorylation and translocation to the myofibril:** Activation of PKC isoforms by phosphorylation or translocation to the myofibril was measured as previously described(68). Hearts were snap-frozen at key time points during I/R (see Figure 2). PKC activation in the whole heart samples or myofilaments was analyzed with immunoblotting, as described below. The time points were chosen to reflect times when PKCα, δ, and ε have been shown by our laboratory to be activated during I/R(26).

**PKC phosphorylation via immunoblotting (whole heart):** Hearts were homogenized in a Tris buffer containing a nonionic detergent (Tween-20) with protease and phosphatase inhibitors, and analyzed with SDS-PAGE electrophoresis using 12% separating gels. Nitrocellulose membranes were probed for antibodies for phosphorylated PKCε, PKCα and PKCδ (1:500,
Santa Cruz) then stripped and reprobed with antibodies against the total form of the kinase (1:500, Santa Cruz). Activation is indicated as a ratio of phosphorylated kinase/total kinase.

PKC translocation via immunoblotting (myofilament): Myofilament proteins (75 μg) were resolved by SDS-PAGE electrophoresis using 10% separating gels. Proteins were transferred to nitrocellulose membranes and probed with antibodies for PKCα, PKCδ, and PKCe (BD Biosciences, Mississauga, Ontario, Canada, and Upstate, Mississauga, Ontario, Canada). Protein loading was assessed by probing with an anti-actin antibody (Millipore, Etobicoke, Ontario, Canada). Secondary antibodies were conjugated to horseradish peroxidase. Primary antibodies were used at 1:1,000 dilution, except for anti-actin, which was 1:25,000. Secondary antibodies were used at 1:5,000 dilution. PKC bands were detected using Western Lightning (PerkinElmer Life and Analytical Sciences, Woodbridge, ON), and analysis of band density was done using Image J software. All bands were normalized to their respective controls and are expressed as a percent change in band density. As the PKC immunoblots were conducted using myofilament isolations, sarcomeric β-actin was used as a loading control.

Myofilament isolation: Cardiac myofilaments were prepared as previously described.33 Hearts collected at specific time points of I/R (see Figure 2) were homogenized in ice cold Standard Buffer (60 mM KCl, 2 mM MgCl₂, 30 mM imidazole (pH 7.0), 0.2 mM PMSF, 0.1 mM Leupeptin, 0.01 mM Pepstatin A). After centrifugation (12,000g for 15 min at 4 °C) the pellets were resuspended in ice-cold skinning buffer (1% Triton X-100, 10 mmol/L EGTA, 8.2 mmol/L MgCl₂, 14.4 mmol/L KCl, 60 mmol/L imidazole (pH 7.0), 5.5 mmol/L ATP, 12 mmol/L creatine phosphate, 10 U/mL creatine phosphokinase, 0.2 mmol/L PMSF, 0.1 mmol/L Leupeptin, 0.01 mmol/L Pepstatin A) and gently mixed for 45 minutes at 4°C. The suspension was centrifuged
(1100g for 15 minutes at 4°C) and pellets washed 3 times in cold Standard Buffer. Protein concentration was measured using a Bradford assay.

**Actomyosin MgATPase analysis:** Myofilaments isolated from snap-frozen hearts (see Figure 2) were subjected to an actomyosin MgATPase activity as previously described(68). Purified myofilaments (50 μg) were incubated in activating buffers containing various amounts of free Ca$^{2+}$ at 32°C for 5 minutes. Activating buffers were made by mixing maximally activating and relaxing ATPase buffers. Maximally activating ATPase buffer contained 23.5 mmol/L KCl, 5 mmol/L MgCl$_2$, 3.2 mmol/L ATP, 2 mmol/L EGTA, 20 mmol/L imidazole, 2.2 mmol/L CaCl$_2$, 0.2 mmol/L PMSF, 0.1 mmol/L Leupeptin, and 0.01 mmol/L Pepstatin A (pH 7.0). The relaxing ATPase buffer contained 26 mmol/L KCl, 5.1 mmol/L MgCl$_2$, 3.2 mmol/L ATP, 2 mmol/L EGTA, 20 mmol/L imidazole, 4.9 μmol/L CaCl$_2$, 0.2 mmol/L PMSF, 0.1 mmol/L Leupeptin, and 0.01 mmol/L Pepstatin A (pH 7.0). Free Ca$^{2+}$ was calculated using the program of Patton and colleagues(48). Reactions were quenched with equal volumes of 10% trichloroacetic acid. The samples were centrifuged (14,100g for 3 minutes) and the supernatant removed for phosphate analysis. The amount of inorganic phosphate released was determined colorimetrically by adding an equal amount of developing solution (0.5% FeSO$_4$ – 0.5% ammonium molybdate in 0.5 mol/L H$_2$SO$_4$) to the supernatant and measuring absorbance at 630 nm.

**Myofibrillar protein phosphorylation analysis:** Myofilament proteins (40 μg) were separated on 12.5% SDS-PAGE gels. Gels were fixed in 50% methanol-10% acetic acid at room temperature overnight, and phosphorylated proteins were stained with Pro-Q Diamond (Molecular Probes, Eugene, OR), according to the manufacturer's instructions. Imaging was done
using a Typhoon gel scanner (GE Healthcare, Baie d’Urfé, PQ), and analysis was done using Image J software (NIH, Bethesda, MD). Protein loading was assessed by Coomassie staining of gels after imaging.

Statistical analysis: Values are presented as mean ± SEM of n independent experiments. The number of individuals for each study was determined by power analysis referencing previous studies that made use of each method. One-way analysis of variance (ANOVA) was performed for the post-ischemic functional recovery of hearts before and after I/R injury, followed by a post-hoc Students' t-test. One- or two-way ANOVA was used to analyze the significance of the results among three or more independent groups within groups of untreated samples (one-way) or the presence or absence of treatments for evaluation of time course data (two-way). Probabilities of 0.05 or less are considered statistically significant.
**Results**

Expression of only LMW FGF2 results in differential activation of PKC isoforms during I/R injury. Previous studies from our laboratory have established that PKC is required for cardioprotection induced by overexpression of all isoforms of FGF2(26), but it is unclear if this is also the case for the improvement in post-ischemic function seen when only LMW FGF2 is expressed. To evaluate changes in PKC activation at key time points during ischemia/reperfusion, the phosphorylation of PKC isoforms was examined in HMWKO and wildtype hearts (Figure 2). Hearts expressing only the LMW isoform of FGF2 and wildtype hearts showed similar activation of PKC\(\alpha\), \(\delta\), and \(\epsilon\) at baseline (Figure 3). At the onset of ischemia, PKC\(\alpha\) showed higher activation (approximately 20%) in hearts only expressing LMW FGF2 (HMWKO) compared to wildtype; this degree of PKC activation correlates to that seen in other forms of cardioprotection sufficient to significantly reduce I/R injury, both in terms of activation by phosphorylation(62) and translocation(49). Similarly, HMWKO hearts showed higher activation of PKC\(\epsilon\) compared to wildtype at early reperfusion (p<0.05).

Improved post-ischemic functional recovery of LMW expressing hearts is ablated in the absence of PKC alpha or presence of PKC epsilon inhibition. It was next determined whether these changes in the phosphorylation state of PKC\(\alpha\) at early ischemia, and PKC\(\epsilon\) at early reperfusion (Figure 3), play a role in LMW FGF2-mediated protection from cardiac dysfunction. As previously seen(40), HMWKO hearts showed a significantly higher post-ischemic recovery of cardiac function than wildtype (Figure 4A and B). Breeding these mice with genetically-modified mice that lack PKC\(\alpha\) expression (PKC\(\alpha\)KO) resulted in a complete abrogation of this protection (p<0.05, Figure 4A). No difference in post-ischemic functional recovery was seen in
hearts with PKCα ablation, but with HMW FGF2 expression intact (PKCαKO) compared to wildtype cohorts, suggesting that PKCα has little influence on function following I/R injury in our models, without activation by LMW FGF2. Similarly, while a TAT peptide alone had no effect on either wildtype or HMWKO hearts, a TAT-conjugated PKCe inhibitor administered during I/R attenuated the recovery of +dP/dt, but not -dP/dt, of HMWKO heart (p<0.05; Figure 4B). Other preischemic and post-ischemic functional parameters for normal PKCα expression and PKCα ablated or untreated and PKCe inhibitor-treated Wt and HMWKO hearts are shown in Table 1 and Table 2, respectively. There were no differences in pre-ischemic functional parameters among all genotypes or treatment groups. Following ischemia-reperfusion injury, there was significant systolic and diastolic dysfunction as measured by left ventricular pressure, +dP/dt and -dP/dt in mice expressing LMW FGF2 but not PKCα (HMWKOxPKCa, p<0.05, Table 1A). Similarly, inhibition of PKCe in mice expressing only LMW FGF2 resulted in post-ischemic systolic and diastolic dysfunction as represented by +dP/dt and -dP/dt (p<0.05, Table 2A).

Myocardial cell death was represented as lactate dehydrogenase (LDH) release measured from coronary effluent at designated time points of baseline, ischemia, and reperfusion (Figure 2). There was a significant increase in LDH release at early reperfusion compared to baseline values in all groups (p<0.05, Tables 1B and 2B), suggesting this is an irreversible I/R model as previously described by our lab(26). Yet, there was no difference in LDH release at early reperfusion between the groups. These findings confirm our previously published studies, suggesting that the primary protective effects of this FGF2 isoform lies in the prevention of contractile dysfunction; whereas, both the LMW and HMW are involved in infarct reduction(40, 41).
Crosstalk between PKCα and ε in HMWKO hearts does not occur during I/R injury. As both PKCε and PKCα are necessary for the protection of the heart from I/R-mediated functional injury in HMWKO mice, crosstalk between the two was evaluated by measuring the activation of PKCε in the absence of PKCα, and the activation of PKCα in the presence of PKCε inhibition. There was no decrease in PKCε activation in the absence of PKCα, and in fact was a slight increase, indicating that PKCα does not activate PKCε further downstream (Figure 5). Similarly, there was no change in the phosphorylation of PKCα in the presence of PKCε inhibition (Figure 5), suggesting that the activation of PKCα is not dependent on the epsilon isoform.

Translocation of PKC isoforms to the myofibril is altered in LMW expressing hearts during ischemia and reperfusion. As LMW FGF2 expression increases the function of the heart after I/R injury, the relevance of myofibrillar proteins in LMW FGF2-mediated PKC signaling during cardiac ischemia was investigated. Translocation of PKC isoforms to the myofibrils was measured at key time points during I/R. In sham hearts, significant differences in the levels of PKC δ, ε, and α at the myofibrils were seen in the HMWKO compared to wildtype (Figure 6), although no difference in the phosphorylation of these isoforms was seen at this time point (Figure 3), suggesting that the localization to the myofibril, but not phosphorylation of these PKCs is altered. After the onset of ischemia, myofilament PKCα levels increased, and levels of PKCδ decreased in HMWKO hearts compared to wildtype cohorts. After reperfusion, levels of PKCε at the myofibril increased. The timing of the translocation of PKCα and ε mirrors the phosphorylation of these isoforms (Figure 3), suggesting that upon phosphorylation, PKCα...
and ε translocate to the myofilaments during ischemia/reperfusion and may target proteins that
are relevant to cardiac function.

**Phosphorylation of myofilament proteins is increased during I/R in LMW expressing**
hearts. Given that both PKCα and ε translocate to the myofibrils during I/R injury (Figure 6),
the phosphorylation state of the myofibrillar proteins troponin I and T were analyzed (Figure 7),
which can affect calcium binding and crossbridge cycling of the myofibrils(59). This is total
protein phosphorylation, which may be the result of changes in the activity of several
kinases/phosphatases. Interestingly, the phosphorylation of both troponins increased significantly
in hearts expressing LMW FGF2 after the onset of ischemia through early reperfusion (Figure
7A and C). The initial increase in phosphorylation was seen after five minutes of ischemia, which
corresponds to the same time of activation and translocation of PKCα, suggesting that this kinase
may be responsible for these changes. To test this, the phosphorylation of troponin I and T at
early ischemia were measured in the absence of PKCα, and found to be significantly reduced in
the HMWKO hearts at the onset of ischemia, back to wildtype levels (Figure 7B and D).
Phosphorylation of the troponins was also increased at early reperfusion, the point that
corresponds to the time of PKCε activation (Figure 3). Interestingly, PKCε inhibition with εV1-2
did not lower the phosphorylation state of the troponins (data not shown). Therefore, this data
indicates that this PKC isoform was not involved in the post-translational modifications of
troponin I or T at early reperfusion, when PKCε is activated and translocated to the myofilament.
This does not rule out a role, however, for PKCε in post-translational modification of other
myofibrillar proteins.
Actomyosin MgATPase activity is altered in HMWKO hearts during I/R. To determine if LMW FGF2-mediated activation of PKC isoforms during I/R injury affects the crossbridge cycling activity of the myofibrils, actomyosin MgATPase activity was evaluated (Figure 8). The maximum activity of actomyosin MgATPase was elevated in HMWKO hearts during reperfusion (Figure 8), which was not abrogated by the absence of PKCα or inhibition of PKCε (Figure 8 and Figure 9, respectively). Alternately, the EC50 was raised in HMWKO hearts compared to wildtype at baseline, early ischemia, and early reperfusion, suggesting that calcium activates the myofibril with lowered potency; these changes in myofilament calcium sensitivity in HMWKO hearts were ablated in the absence of PKCα at baseline and early ischemia, indicating that this decreased potency is dependent on PKCα (Figure 8). However, inhibition of PKCε did not have an effect (Figure 9). Overall, this suggests that in hearts only expressing LMW FGF2, the presence of PKCα results in a decrease in myofilament calcium sensitivity.
Our laboratory has previously demonstrated opposing roles for HMW and LMW FGF2 in the functional recovery of the heart after I/R(40, 41). This study is the first to show that LMW FGF2 requires the activity of two isoforms of PKC, PKCε and α, which are differentially activated during I/R through distinct parallel pathways. Additionally, the expression of only LMW FGF2 alters the activity of the actomyosin MgATPase in ischemic hearts, corresponding to differences in the phosphorylation of troponin I and T. These results describe a novel mechanism of FGF2-mediated protection from I/R injury through activation of protective PKC isoforms, which then phosphorylate myofibril proteins to modulate post-ischemic cardiac function (Figure 10).

As we have previously described using gain-of-function and loss-of-function models, expression of only LMW FGF2 improves post-ischemic cardiac function compared to wildtype and Fgf2 KO hearts. These protective effects are dependent on the activity of the FGF receptor 1 (FGFR1)(40), which can activate PKCs via phospholipase C (PLC)(45, 47) or other scaffolding proteins such as FRS2/Grb(35) which also have the potential to interact with PKCs(42). Here, evidence suggests that expression of only LMW FGF2 results in the differential activation of PKCα and ε to protect the heart from post-ischemic dysfunction.

While our model identified PKC as necessary for FGF2-induced cardioprotection, early studies examining the role of PKC in protection against I/R injury, particularly ischemic preconditioning, produced conflicting findings (see review(56)). This phenomenon has, in part, been attributed to the different and sometimes opposing roles of multiple isoforms of PKC that have been uncovered in recent years (see reviews(10, 57)). For example, while inhibition of all isoforms of PKC with staurosporine fails to prevent ischemic preconditioning in porcine(65),
activation of simply the epsilon isoform of PKC protects the porcine heart from arrhythmia and infarct development (28). It was later determined that the activation of various isoforms of PKC may result in diverse and opposing effects during I/R, with PKC delta acting as an injurious element and PKC epsilon behaving as a protective molecule in infarct development during IPC (29). For this reason, a thorough and complete characterization of the signaling mechanisms of FGF2 must include a description of the specific PKC isoforms that are activated. Therefore, investigation of the targets of PKC\(\alpha\) and \(\varepsilon\) was driven by the observation that, while hearts that only express LMW FGF2 are protected from I/R-induced dysfunction, they are not similarly protected from infarct development and tissue death (40, 41) (and see Tables 1b and 2b).

The data presented indicate that PKC\(\alpha\) is activated to a higher degree during ischemia, while PKC\(\varepsilon\) is activated after reperfusion in hearts only expressing LMW FGF2 compared to wildtype and \(Fgf2\) KO (data not shown and previously published (27)) hearts. It should be noted that this differs from a mouse model overexpressing all isoforms of FGF2, where the activity of PKC\(\alpha\) is reduced during I/R (26), suggesting that this increase in PKC\(\alpha\) activity is a unique result of only LMW FGF2. Conversely, in both models, PKC\(\varepsilon\) activity is increased at early reperfusion, suggesting that this isozyme may contribute to protection from both post-ischemic cardiac dysfunction and infarct development (seen when all isoforms of FGF2 are overexpressed). While some PKC isoforms can activate one another (58) and crosstalk between PKC\(\alpha\) and \(\varepsilon\) has been shown to occur in ischemic preconditioning models of cardioprotection (23), this does not appear to be the case here as PKC\(\alpha\) and \(\varepsilon\) are activated independently of one another (Figure 5).

Paradoxically, while it is demonstrated in this study that expression of PKC\(\alpha\) results in higher contractility after I/R injury in HMWKO hearts, it has been established that PKC\(\alpha\) expression is associated with lowered contractility in non-ischemic hearts (9). Furthermore,
phosphorylation of myofibril targets by PKCα is maladaptive for heart failure and remodeling(5). This phenomenon illustrates the importance of the conditions under which PKC isoforms are activated. PKCα was activated differentially by LMW FGF2 only after the onset of ischemia, an event that precipitates a unique set of intracellular conditions, characterized by acute loss of ATP, and transient but substantial increases in intracellular acidity, and sodium and calcium concentrations, which leads to a distinct manner of injury associated with calcium overload(25). It is well-characterized that PKC isoforms can play varying roles under different circumstances in the heart; for example, PKCδ is necessary for cardioprotection from I/R triggered by opioid agonists(21), and adenosine-mediated late preconditioning(36), while this same isoform is detrimental in conditions of ischemic preconditioning(29) and ethanol-induced ischemic protection(13). Similarly, it is during the unique events of I/R injury in the presence of LMW FGF2 that the activation of PKCα protects the post-ischemic heart and results in improved cardiac function. We speculate that the reduced levels of myofilament PKCα seen in the HMWKO sham mice represent a compensatory change in which myofilament inhibition by PKCα is reduced, allowing for a more efficient contractile state. Paradoxically, the increase in myofilament-associated PKCα following an acute ischemic challenge may also represent a compensatory change. Molnar and colleagues(46) found that PKCα maintains cardiac myofilament force development under conditions that occur during I/R. By contrast, Shintani-Ishida and Yoshida(55) report that post-I/R activation of PKCα at the sarcoplasmic reticulum exacerbates I/R damage, arguing for subcellular-specific effects of PKCα. We propose that the reduction in myofilament-associated PKCα allows the myofilament complex to function with less hindrance by PKCα, but that transient activation of myofilament-associated PKCα during I/R provides a cardioprotective advantage, possibly through an energy sparing mechanism(51).
This hypothetical model incorporates the importance of both timing and subcellular localization of PKC activation, as has been advanced elsewhere (14, 69). The time-dependent changes in PKC isoform translocation to the myofilaments are interesting and suggestive of a dynamic system during I/R. Although this represents the first report showing time-dependent changes in myofilament-associated PKC isoforms, earlier work by Yoshida and investigators (70) have reported similarly dynamic alterations in membrane-associated PKC isoforms. The significance of these changes is difficult to assess, as it would require inhibiting not only the specific isoforms, but at certain times and within the myofilament compartment, in order to determine the significance of the specific changes. At the moment, this represents an untenable technical challenge, and is beyond the scope of the current paper.

The impact of PKCs on cardiac functional recovery after ischemia/reperfusion is manifested by changes in myofibrillar proteins. As shown here for the first time, alterations in both actomyosin MgATPase activity, and troponin I and T phosphorylation, in hearts only expressing LMW FGF2 are dependent on the presence of PKCα. Troponin I and T have several PKC phosphorylation sites that regulate the calcium affinity of the thin filaments and contractile force (59). Phosphorylation of the known PKC sites on troponin T produce a reduction in contractile force and calcium sensitivity (60, 61), while the PKC-targeted residues in troponin I result in a more complex response. Phosphorylation of S23/24, a PKA and PKCδ target, increases lusitropy and inotropy, while the PKC targets T144 and S43/45 reduce calcium binding cooperativity and contractile force, respectively (34, 38, 46, 60). Unfortunately, the specific residues that contribute to increased phosphorylation of TnI remain unknown until the availability of reliable and specific phospho-antibodies.
The molecular basis of myocardial dysfunction has been under investigation for a significant period of time, but a singular, definitive mechanism has yet to be identified. Amongst the most well-supported theories are the “oxyradical” and “calcium hypotheses”(8, 24). Although each model has component elements that are unique, both hold cardiac myofilaments as central players in the development of the contractile dysfunction that underlies stunning(24). MacGowan and colleagues(44) support a key role for myofilament protein phosphorylation in the response to acute myocardial ischemia by demonstrating that alterations in troponin I phosphorylation alter the susceptibility of hearts to ischemic injury. However, a recent study by Avner and colleagues(2) shows that myofilament protein oxidation with ischemia mediates a similar reduction in myofilament calcium sensitivity. In chronic models of heart failure, protein oxidation has been linked to functional impairment(12), suggesting that a prolonged reduction in myofilament calcium sensitivity is not beneficial. Together, these studies suggest that acute changes in the phosphorylation of myofilaments to temporarily decrease myofilament calcium sensitivity confers a protective effect, but that long-term damage to cardiac myofilaments by oxidation is associated with impaired function.

Phosphorylation of regulatory proteins, including troponin I and T, myosin light chain, and myosin-binding protein C, have been implicated in myofilament calcium sensitivity, which subsequently affects force development(15, 34, 44, 60, 61, 66). In the studies presented here, increased troponin phosphorylation during I/R mirrors an increase in the EC_{50} of actomyosin MgATPase, suggesting that LMW FGF2 activation of PKC\(\alpha\) results in a lowered potency of calcium-mediated activation of the ATPase. This is particularly important during ischemia, where calcium overload is a primary effector of the cardiac dysfunction seen during late reperfusion; by requiring a higher calcium concentration to achieve the same level of ATPase activity,
myofilament activity and ATP consumption is reduced, and the damage caused by calcium
overload is slowed. Thus, a temporary increase in EC$_{50}$ during ischemia results in a mitigation of
calcium-induced injury and stunning. This PKC-dependent transient depression of actomyosin
MgATPase calcium sensitivity has previously been demonstrated to result in
cardioprotection(51).

The effects of PKC on actomyosin MgATPase activity are complex, dependent on the site
of phosphorylation and the conditions of activation. Burkart and colleagues(11) showed that
mutating S43 and S45 in cardiac troponin I to E43 and E45 to mimic PKC phosphorylation
decreased maximum actomyosin MgATPase activity. By contrast, a similar mutation at T144 had
no effect on maximum myofilament ATP consumption, demonstrating that the PKC-dependent
depression of maximum actomyosin MgATPase activity is specific to T144 phosphorylation.
More recently, Engel and group(19) found that myofilament regulation via PKC preferred sites is
altered under conditions that occur during prolonged ischemia. Thus, while physiological and
biochemical investigations of PKC-dependent regulation of myofilament function present a
sound basis from which to build an understanding of myocardial PKC signaling, understanding
the role of this molecular messenger in disease requires that its regulation and impact be
investigated in pathological models similar to that used here.

In addition, increased levels of intracellular ATP during ischemia have been seen when
recombinant FGF2 is administered to ischemic hearts(30). Further studies are currently
determining whether depressed activity of contractile proteins mediated by PKC$_{\alpha}$ during I/R
may slow ATP depletion and intracellular calcium overload.

The model used here studied the effects of the LMW isoform of FGF2 by ablating HMW
FGF2 expression. It should be noted that studies by Jiang and colleagues(31), examining the
response of the ischemic heart to exogenously-added recombinant rat HMW FGF2 protected the heart from ischemic injury and activated PKCα. This result, while contradictory on the surface, is not surprising in view of the fact that exogenous HMW FGF2 shares LMW FGF2’s FGFR1 binding domain, and is expected to act similarly on the extracellular domain of the FGFR1 receptor, when introduced as an external source(50). We have determined previously that LMW FGF2, and not HMW FGF2, is released outside of the cell, and this is the case both under normal circumstances and during I/R injury(26). Our investigations seek to determine the activities of endogenous HMW and LMW FGF2 on I/R in a physiological setting, and it is clear that HMW FGF2 expressed by the cardiomyocyte, as demonstrated here and previously by our laboratory(39), plays a different role than HMW FGF2 applied exogenously, specifically, producing a decrease in cardiac function.

An important caveat for the interpretation of these studies is that the use of global knockouts does not preclude the possibility for non-cardiomyocyte effects on post-ischemic function. We have minimized potential systemic influence by conducting the entire ischemia and reperfusion event in an ex vivo working heart system. However, the use of the intact heart indicates that there may be potential roles for fibroblasts, vascular tissue and nerve tissue in influencing the outcome of the I/R injury via a paracrine effect. These other cell types, while making up the minority of tissue present in the heart, are still present(4) and cannot be ruled out as possible sources contributing to the effects described in this manuscript.

While these studies elucidate a key component of the LMW FGF2-induced cardioprotection signal transduction pathway, ongoing work is addressing some remaining questions, in particular the effect of calcium handling proteins in LMW FGF2-mediated protection. As Braz and colleagues(9) have elegantly shown, PKCα can modulate cardiac
contractility by altering the affinity of phospholamban for the sarco-endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA), through phosphorylation of protein phosphatase 1’s inhibitor-1. Examination of the LMW FGF2-induced effects of PKC$\alpha$ during I/R on SR protein activity may shed further light into the differences in post-ischemic function observed between the wildtype and HMWKO groups.

The findings presented here are the first to demonstrate that LMW FGF2-induced cardioprotection against post-ischemic cardiac dysfunction depends on the activation of both PKC$\alpha$ and PKC$\epsilon$. Moreover, the activation of PKC$\alpha$ is associated with the phosphorylation and regulation of proteins at the myofibril, leading to the modulation of the activity of actomyosin ATPase during ischemia. This understanding will better enable the development of LMW FGF2 as a therapeutic target for ischemic heart disease.
Acknowledgments

The authors would like to acknowledge J. Molkentin for the gift of the PKCα knockout mouse and C. York for excellent animal husbandry.
This work was supported by the National Institutes of Health/National Heart, Lung, and Blood Institute [grant number NIH/NHLBI R01 (HL075633) to JJS]; the Canadian Institute of Health Research [CIHR Operating Grant and Heart and Stroke Foundation of Canada New Investigator Award to WGP]; the Pharmaceutical Research and Manufacturers of America (PhRMA) Foundation [predoctoral fellowship to JRM].
Disclosures

None declared.
Author Contributions

J.R.M. W.G.P., and J.J.S. involved with conception and experimental design; J.R.M., G.N. and J.J.S. performed the ischemia-reperfusion studies with pharmacological or genetic evaluation of protein kinase C; J.R.M. and X.G. performed the protein kinase C activation studies; J.R.M. and Y.Z. performed the FGFR1 immunoblots; S.O.P, E.A.S. and W.G.P. performed the troponin phosphorylation, protein kinase C translocation to myofibrils and actomyosin ATPase experiments; J.R.M., W.G.P., and J.J.S. performed data analysis and interpretation; J.R.M. and W.G.P. drafted manuscript; J.R.M., W.G.P., and J.J.S. revised manuscript; J.J.S. edited and organized final manuscript. All authors have reviewed and approved the final manuscript.
References


31. **Jiang ZS, Wen GB, Tang ZH, Srisakuldee W, Fandrich RR, and Kardami E.** High molecular weight FGF-2 promotes postconditioning-like cardioprotection linked to activation of


38. Li MX, Wang X, Lindhout DA, Buscemi N, Van Eyk JE, and Sykes BD. Phosphorylation and mutation of human cardiac troponin I deferentially destabilize the


44. **MacGowan GA, Du C, Cowan DB, Stamm C, McGowan FX, Solaro RJ, Koretsky AP, and Del Nido PJ.** Ischemic dysfunction in transgenic mice expressing troponin I lacking


47. Murphy S and Frishman WH. Protein kinase C in cardiac disease and as a potential therapeutic target. *Cardiol Rev* 13: 3-12, 2005.


Figure Legends

Figure 1. Protein expression levels of FGF2 isoforms, PKC isoforms and FGFR1 in wildtype (WT), HMWKO, PKCαKO, and HMWKOxPKCαKO hearts. (A) Representative immunoblot of FGF2 and PKC isoform protein expression. Both PKCα and HMW FGF2 expression is ablated in HMWKOxPKCαKO hearts, with no alteration in the expression of PKCε or δ, or LMW FGF2. (B) Representative immunoblot of FGF receptor 1 (FGFR1) protein expression normalized to calsequestrin (CSQ). There is no significant difference in FGFR1 expression in all groups similar to that previously published by our lab(40). n= 6-11 hearts per genotype.

Figure 2. Isolated working heart protocols for analysis of cardiac function (arrowhead) and LDH release (line) (A) and for examination of the activity and phosphorylation of myofibrillar proteins (B) at baseline and during the course of I/R injury. CE, coronary effluent collection for LDH levels.

Figure 3. The phosphorylation of PKC isoforms during ischemia and reperfusion as a measure of kinase activation for PKCα (A), ε (B), and δ (C) in wildtype hearts (diamond, solid line) and HMWKO (square, solid line). Phosphorylation is normalized to total amounts of each kinase and given as arbitrary values (n=5-8 per group). All samples were derived at the same time and processed in parallel.*p<0.05 vs. wildtype cohorts.

Figure 4. Recovery of contractile function as measured by +dP/dt in wildtype hearts (open circle and for inset: black bar) and hearts expressing only LMW FGF2 (HMWKO, open triangle...
and for inset: striped bar) in the absence (square) and presence of PKCα expression (A), and in
the absence and presence (solid circle and triangle for WT and HMWKO, respectively) of a
selective inhibitor of PKCε (B). Insets: Percent (%) recovery of contractility (+dP/dt) is
normalized to baseline +dP/dt (n=5-10 per group). *p<0.05 vs. wildtype cohorts, †p<0.05 vs.
HMWKO hearts with normal expression of PKCα or treated with TAT-vehicle.

Figure 5. Crosstalk between PKCα and ε. Phosphorylation of PKCε in the absence of PKCα at
early reperfusion (A) or PKCα in the presence of PKCε inhibition (B). Phosphorylated PKC is
normalized to total amounts of each kinase and given as arbitrary values (n=6-8 per group). All
samples were derived at the same time and processed in parallel.*p<0.05 vs. HMWKO hearts
with PKCα present.

Figure 6. Myofilament-associated PKC isoforms during I/R. PKCα (A), ε (B), and δ (C) in
wildtype (black bar) and HMWKO (striped bar) hearts (n=3-16 per group). All samples were
derived at the same time and processed in parallel. *p<0.05 vs. wildtype cohorts. †p<0.05 vs.
sham.

Figure 7. Phosphorylation of troponin I (A and B) and T (C and D) during ischemia and
reperfusion in wildtype (black bar) and HMWKO (striped bar) hearts expressing or not
expressing PKCα (DKO, white bar) (n=3-16 per group). All samples were derived at the same
time and processed in parallel.*p<0.05 vs. wildtype cohorts. †p<0.05 vs. sham.
Figure 8. Actomyosin MgATPase activity in wildtype (black bar) and HMWKO (striped bar) hearts (A, B and C). Activity at maximum calcium levels (D), Hill coefficient (E), and EC$_{50}$ (F) were measured in hearts expressing or not expressing PKC$_{\alpha}$ (DKO, white bar) (n=3-16 per group). All samples were derived at the same time and processed in parallel.*p<0.05 vs. wildtype cohorts. #p<0.05 vs. HMWKO hearts with PKC$_{\alpha}$ present.

Figure 9. Actomyosin MgATPase activity in wildtype (black bar) and HMWKO (striped bar) hearts (A and B). Activity at maximum calcium levels (C), Hill coefficient (D), and EC$_{50}$ (E) were measured in hearts in the presence of vehicle treatment or a PKC$_{\varepsilon}$ inhibitor (wildtype, dotted bar; HMWKO, white bar) (n=3-16 per group). All samples were derived at the same time and processed in parallel. *p<0.05 vs. wildtype cohorts.

Figure 10. Schematic of the hypothesized role of PKC$_{\alpha}$ and $\varepsilon$ at the myofibril in LMW FGF2-mediated protection from post-ischemic dysfunction. LMW FGF2 activates PKC$_{\alpha}$, which phosphorylates troponin I and T at the myofibril and transiently reduces calcium sensitivity (EC$_{50}$), to protect the heart during ischemia.
A.

<table>
<thead>
<tr>
<th></th>
<th>Fg2 KO</th>
<th>WT</th>
<th>HMWKO</th>
<th>PKCαKO</th>
<th>HMWKOPKCaKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW FGF2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LMW FGF2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PKCα</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B.

**FGFR1**

**CSQ**

**FGFR1 protein expression normalized to calcequestrin**

- **WT:**
- **HMWK0:**
- **PKCαKO:**
- **HMWK0PKCαKO:**
Heart snap frozen for PKC activation and myofibril protein level and activity
### A.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>5'I</th>
<th>60'I + 5'R</th>
<th>60'I + 120'R</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>HMW</td>
<td>HMW</td>
<td>KO</td>
</tr>
<tr>
<td>pPKCα</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PKC α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPKCε</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PKC ε</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPKCδ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total PKC δ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B.

![Graph B]

* p<0.05 vs. WT

### C.

![Graph C]

* p<0.05 vs. WT

### D.

![Graph D]

* p<0.05 vs. WT
A.

- Wildtype
- HMWKO
- PKCαKO
- HMWKOxPKCαKO

% Contractile Recovery (+dP/dt)

* p<0.05 vs. WT
† p<0.05 vs. HMWKO

PKCα present
PKCα absent

Baseline

Ischemia (minutes) Reperfusion (minutes)
B. *p<0.05 vs. WT
†p<0.05 vs. HMWKO
A. *p<0.05 vs. HMWKO

Phospho/total PKCε (arbitrary units)

- HMWKO
- HMWKO x PKCαKO

B. Phospho/total PKCα (arbitrary units)

- HMWKO + TAT vehicle
- HMWKO + PKCε inhibitor

PKCα present
PKCα KO

pPKCε
PKCε

TAT vehicle
PKCε inhibited

pPKCα
PKCα
A. PKCα

*\( p<0.05 \) vs. WT
†*\( p<0.05 \) vs. Sham

![Bar graph showing myofilaent associated PKC (arbitrary units) for WT and HMWKO groups under different conditions.](image)

B. PKCε

*\( p<0.05 \) vs. WT
†*\( p<0.05 \) vs. Sham

![Bar graph showing myofilaent associated PKC (arbitrary units) for WT and HMWKO groups under different conditions.](image)

C. PKCδ

*\( p<0.05 \) vs. WT
†*\( p<0.05 \) vs. Sham

![Bar graph showing myofilaent associated PKC (arbitrary units) for WT and HMWKO groups under different conditions.](image)
**A.** Troponin I

- **Sham**
- 5'I
- 60'I + 5'R
- 60'I + 120'R

**B.** Troponin I

- **Sham**
- 5'I

**C.** Troponin T

- **Sham**
- 5'I
- 60'I + 5'R
- 60'I + 120'R

**D.** Troponin T

- **Sham**
- 5'I

* *p<0.05 vs. WT*
A. Sham

B. 5' I

C. 60' I + 5' R

D. Maximum Efficacy

E. Hill Coefficient

F. EC50

* p<0.05 vs. WT
† p<0.05 vs. HMWKO
A. Sham

B. 60' I + 5' R

C. Maximum Efficacy

D. Hill Coefficient

E. EC₅₀

* p<0.05 vs. WT
LMW FGF2 → FGFR1 → PKCε → PKCα → Myofibril → Transient decrease in calcium sensitivity during ischemia → Improved post-ischemic function
Table 1A. Pre-ischemic and Post-ischemic Functional Parameters for WT and HMWKO Hearts in the presence or absence of PKCalpha expression

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>MAP</th>
<th>LVP</th>
<th>LVEDP</th>
<th>+dP/dt</th>
<th>-dP/dt</th>
<th>TPP</th>
<th>RT 1/2</th>
<th>LAP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-Ischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>367±4</td>
<td>50.3±0.3</td>
<td>97±4</td>
<td>8±2</td>
<td>3847±249</td>
<td>-3023±200</td>
<td>51±9</td>
<td>54±15</td>
<td>11±3</td>
</tr>
<tr>
<td>HMWKO</td>
<td>381±8</td>
<td>50.9±0.2</td>
<td>98±2</td>
<td>10±2</td>
<td>3998±90</td>
<td>-3425±220</td>
<td>35±2</td>
<td>29±1</td>
<td>7±1</td>
</tr>
<tr>
<td>PKCaKO</td>
<td>376±9</td>
<td>50.5±0.1</td>
<td>97±4</td>
<td>8±2</td>
<td>3892±192</td>
<td>-3285±249</td>
<td>37±1</td>
<td>30±2</td>
<td>10±2</td>
</tr>
<tr>
<td>HMWKOxPKCaKO</td>
<td>363±5</td>
<td>50.5±0.1</td>
<td>104±3</td>
<td>10±1</td>
<td>4401±154</td>
<td>-3385±136</td>
<td>36±1</td>
<td>27±1</td>
<td>8±1</td>
</tr>
<tr>
<td><strong>60 minutes Ischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>359±1</td>
<td>10.9±2.1*</td>
<td>18±3*</td>
<td>11±2</td>
<td>536±60*</td>
<td>-369±58*</td>
<td>27±1*</td>
<td>35±1*</td>
<td>9±3</td>
</tr>
<tr>
<td>HMWKO</td>
<td>381±5</td>
<td>12.3±0.9*</td>
<td>22±2*</td>
<td>13±1</td>
<td>943±230*</td>
<td>-749±147*</td>
<td>21±2*</td>
<td>23±8#</td>
<td>7±1</td>
</tr>
<tr>
<td>PKCaKO</td>
<td>377±9</td>
<td>11.8±0.8*</td>
<td>17±2*</td>
<td>10±1</td>
<td>709±104*</td>
<td>-510±45*</td>
<td>15±3*</td>
<td>34±5</td>
<td>8±1</td>
</tr>
<tr>
<td>HMWKOxPKCaKO</td>
<td>365±6</td>
<td>13.5±0.8*</td>
<td>22±1*</td>
<td>13±1</td>
<td>775±62*</td>
<td>-597±52*</td>
<td>21±3*</td>
<td>29±2</td>
<td>9±1</td>
</tr>
<tr>
<td><strong>60 minutes Ischemia + 120 minutes Reperfusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>377±4</td>
<td>35.4±6.5*</td>
<td>59±9*</td>
<td>27±7*</td>
<td>1529±678*</td>
<td>-1310±326*</td>
<td>56±16</td>
<td>15±8*</td>
<td>30±9*</td>
</tr>
<tr>
<td>HMWKO</td>
<td>388±8</td>
<td>45.7±1.2*#</td>
<td>76±3*#</td>
<td>27±3*</td>
<td>3008±140*</td>
<td>-2128±140*</td>
<td>26±3*</td>
<td>26±1*#</td>
<td>20±2*</td>
</tr>
<tr>
<td>PKCaKO</td>
<td>379±13</td>
<td>34.9±2.9*</td>
<td>56±8*</td>
<td>28±3*</td>
<td>1523±485*</td>
<td>-1284±335*</td>
<td>27±3*</td>
<td>25±3*</td>
<td>29±4*</td>
</tr>
<tr>
<td>HMWKOxPKCaKO</td>
<td>366±7</td>
<td>35.6±3.2‡ †</td>
<td>55±5‡ †</td>
<td>32±3‡</td>
<td>1437±311‡ †</td>
<td>-1134±155‡ †</td>
<td>33±4*</td>
<td>24±2*</td>
<td>32±3†</td>
</tr>
</tbody>
</table>

HR indicates heart rate (bpm); MAP, mean aortic pressure (mm Hg); LVP, peak systolic left ventricular pressure (mm Hg); LVEDP, left ventricular end-diastolic pressure (mm Hg); +dP/dt, rate of contractility (mm Hg per second); -dP/dt, rate of relaxation (mm Hg per second); TPP, time-to-peak left ventricular pressure normalized to contractility (ms/mm Hg); RT 1/2, half relaxation time normalized to relaxation (ms/mm Hg); and LAP, left atrial pressure (mm Hg). All values are mean±SEM. n=10 per genotype and treatment. *p<0.05 vs. pre-ischemic cohort. #p<0.05 vs. WT cohort. †p<0.05 vs. HMWKO cohort.
Table 1B. Lactate dehydrogenase (LDH) release for WT and HMWKO hearts in the presence or absence of PKCalpha expression

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ischemia</th>
<th>Early Reperfusion</th>
<th>Late Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.039±0.006</td>
<td>0.019±0.010</td>
<td>0.094±0.010*</td>
<td>0.023±0.011</td>
</tr>
<tr>
<td>HMWKO</td>
<td>0.028±0.004</td>
<td>0.037±0.016</td>
<td>0.080±0.029*</td>
<td>0.022±0.007</td>
</tr>
<tr>
<td>PKCaKO</td>
<td>0.019±0.004</td>
<td>0.029±0.015</td>
<td>0.068±0.011*</td>
<td>0.025±0.007</td>
</tr>
<tr>
<td>HMWKOxPKCaKO</td>
<td>0.042±0.016</td>
<td>0.034±0.010</td>
<td>0.082±0.008*</td>
<td>0.022±0.004</td>
</tr>
</tbody>
</table>

All values depicted as mean±SEM of n independent experiments. Early reperfusion: 0-14 minutes of reperfusion. Late reperfusion: 114-120 minutes of reperfusion. n=10-16 per genotype or treatment. *p<0.05 vs. baseline.
Table 2A. Pre-ischemic and Post-ischemic Functional Parameters for vehicle-treated and PKCepsilon inhibitor-treated WT and HMWKO Hearts

<table>
<thead>
<tr>
<th></th>
<th>HR</th>
<th>MAP</th>
<th>LVP</th>
<th>LVEDP</th>
<th>+dP/dt</th>
<th>-dP/dt</th>
<th>TPP</th>
<th>RT 1/2</th>
<th>LAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vehicle (TAT)-treated WT</td>
<td>386±6</td>
<td>50.4±0.1</td>
<td>100±4</td>
<td>5±2</td>
<td>4174±179</td>
<td>-3433±165</td>
<td>37±1</td>
<td>27±1</td>
<td>10±2</td>
</tr>
<tr>
<td>vehicle (TAT)-treated HMWKO</td>
<td>382±13</td>
<td>50.2±0.1</td>
<td>103±2</td>
<td>5±1</td>
<td>4358±111</td>
<td>-3594±128</td>
<td>40±4</td>
<td>34±8</td>
<td>8±1</td>
</tr>
<tr>
<td>PKCe inhibitor-treated WT</td>
<td>360±14</td>
<td>50.4±0.1</td>
<td>102±2</td>
<td>5±1</td>
<td>4193±89</td>
<td>-3468±117</td>
<td>38±1</td>
<td>28±1</td>
<td>7±2</td>
</tr>
<tr>
<td>PKCe inhibitor-treated HMWKO</td>
<td>410±1</td>
<td>50.4±0.1</td>
<td>102±3</td>
<td>4±1</td>
<td>4316±100</td>
<td>-3424±240</td>
<td>38±1</td>
<td>27±2</td>
<td>8±1</td>
</tr>
<tr>
<td>60 minutes Ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vehicle (TAT)-treated WT</td>
<td>387±5</td>
<td>10.2±0.7*</td>
<td>15±2*</td>
<td>10±3</td>
<td>473±46*</td>
<td>-337±4*</td>
<td>17±3*</td>
<td>27±4</td>
<td>10±2</td>
</tr>
<tr>
<td>vehicle (TAT)-treated HMWKO</td>
<td>367±3</td>
<td>10.9±1.2*</td>
<td>16±1*</td>
<td>11±1*</td>
<td>581±99*</td>
<td>-411±88*</td>
<td>19±5*</td>
<td>22±8</td>
<td>10±1</td>
</tr>
<tr>
<td>PKCe inhibitor-treated WT</td>
<td>369±7</td>
<td>10.1±0.8*</td>
<td>15±1*</td>
<td>9±1</td>
<td>452±28*</td>
<td>-357±52*</td>
<td>17±3*</td>
<td>22±2</td>
<td>9±1</td>
</tr>
<tr>
<td>PKCe inhibitor-treated HMWKO</td>
<td>412±9</td>
<td>14.6±0.8*#</td>
<td>22±1*#</td>
<td>16±1*</td>
<td>532±65*</td>
<td>-360±53*</td>
<td>20±6*</td>
<td>30±4</td>
<td>11±2</td>
</tr>
<tr>
<td>60 minutes Ischemia + 120 minutes Reperfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vehicle (TAT)-treated WT</td>
<td>390±5</td>
<td>29.6±3.0*</td>
<td>48±6*</td>
<td>23±7*</td>
<td>1246±198*</td>
<td>-1128±116*</td>
<td>31±7</td>
<td>22±2*</td>
<td>31±5*</td>
</tr>
<tr>
<td>vehicle (TAT)-treated HMWKO</td>
<td>367±5</td>
<td>43.3±2.2*#</td>
<td>77±7*#</td>
<td>18±3*</td>
<td>3088±283*#</td>
<td>-2007±159*#</td>
<td>39±8</td>
<td>36±10</td>
<td>20±3*#</td>
</tr>
<tr>
<td>PKCe inhibitor-treated WT</td>
<td>382±10</td>
<td>29.1±4.4*</td>
<td>51±8*</td>
<td>16±2*</td>
<td>1426±306*</td>
<td>-1316±196*</td>
<td>28±1*</td>
<td>24±1*</td>
<td>17±2†</td>
</tr>
<tr>
<td>PKCe inhibitor-treated HMWKO</td>
<td>410±1</td>
<td>35.1±11.0*</td>
<td>62±9*</td>
<td>28±6*</td>
<td>2170±333*#†</td>
<td>-1666±165†</td>
<td>27±4*</td>
<td>25±5</td>
<td>39±11*#†</td>
</tr>
</tbody>
</table>

HR indicates heart rate (bpm); MAP, mean aortic pressure (mm Hg); LVP, peak systolic left ventricular pressure (mm Hg); LVEDP, left ventricular end-diastolic pressure (mm Hg); +dP/dt, rate of contractility (mm Hg per second); -dP/dt, rate of relaxation (mm Hg per second); TPP, time-to-peak left ventricular pressure normalized to contractility (ms/mm Hg); RT 1/2, half relaxation time normalized to relaxation (ms/mm Hg); and LAP, left atrial pressure (mm Hg). All values are mean±SEM. n=10 per genotype and treatment. *p<0.05 vs. pre-ischemic cohort. #p<0.05 vs. WT cohort. †p<0.05 vs. HMWKO cohort.
Table 2B. Lactate dehydrogenase (LDH) release for vehicle-treated and PKCepsilon inhibitor-treated WT and HMWKO hearts

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Ischemia</th>
<th>Early Reperfusion</th>
<th>Late Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle (TAT)-treated WT</td>
<td>0.030±0.005</td>
<td>0.018±0.010</td>
<td>0.095±0.018*</td>
<td>0.018±0.007*</td>
</tr>
<tr>
<td>vehicle (TAT)-treated HMWKO</td>
<td>0.016±0.005</td>
<td>0.020±0.003</td>
<td>0.088±0.009*</td>
<td>0.011±0.003</td>
</tr>
<tr>
<td>PKCe inhibitor-treated WT</td>
<td>0.047±0.016</td>
<td>0.054±0.017</td>
<td>0.112±0.026*</td>
<td>0.019±0.007*</td>
</tr>
<tr>
<td>PKCe inhibitor-treated HMWKO</td>
<td>0.030±0.008</td>
<td>0.047±0.011</td>
<td>0.094±0.010*</td>
<td>0.016±0.004</td>
</tr>
</tbody>
</table>

All values depicted as mean±SEM of \( n \) independent experiments. Early reperfusion: 0-14 minutes of reperfusion. Late reperfusion: 114-120 minutes of reperfusion. \( n=8-10 \) per genotype or treatment. *\( p<0.05 \) vs. baseline.