**Integrative Physiology**

**Cardiac Fibroblast GRK2 Deletion Enhances Contractility and Remodeling Following Ischemia/Reperfusion Injury**

Meryl C. Woodall, Benjamin P. Woodall, Erhe Gao, Ancai Yuan, Walter J. Koch

**Rationale:** G protein–coupled receptor kinase 2 (GRK2) is an important molecule upregulated after myocardial injury and during heart failure. Myocyte-specific GRK2 loss before and after myocardial ischemic injury improves cardiac function and remodeling. The cardiac fibroblast plays an important role in the repair and remodeling events after cardiac ischemia; the importance of GRK2 in these events has not been investigated.

**Objective:** The aim of this study is to elucidate the in vivo implications of deleting GRK2 in the cardiac fibroblast after ischemia/reperfusion injury.

**Methods and Results:** We demonstrate, using Tamoxifen inducible, fibroblast-specific GRK2 knockout mice, that GRK2 loss confers a protective advantage over control mice after myocardial ischemia/reperfusion injury. Fibroblast GRK2 knockout mice presented with decreased infarct size and preserved cardiac function 24 hours post ischemia/reperfusion as demonstrated by increased ejection fraction (59.1±1.8% versus 48.7±1.2% in controls; P<0.01). GRK2 fibroblast knockout mice also had decreased fibrosis and fibrotic gene expression. Importantly, these protective effects correlated with decreased infiltration of neutrophils to the ischemia site and decreased levels of tumor necrosis factor-α expression and secretion in GRK2 fibroblast knockout mice.

**Conclusions:** These novel data showing the benefits of inhibiting GRK2 in the cardiac fibroblast adds to previously published data showing the advantage of GRK2 ablation and reinforces the therapeutic potential of GRK2 inhibition in the heart after myocardial ischemia. (Circ Res. 2016;119:1116-1127. DOI: 10.1161/CIRCRESAHA.116.309538.)

**Key Words:** cardiac fibroblast ■ fibrosis ■ G protein–coupled receptor kinase 2 ■ inflammation ■ myocardial ischemia/reperfusion injury

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Cardiac fibroblasts represent the largest population of interstitial cells in myocardium. They are responsible for secreting and maintaining the extracellular matrix forming a 3-dimensional scaffold supporting the surrounding myocytes. Although not contractile, fibroblasts are directly connected to myocytes through cell junctions and may act as bridges to connect myocytes electrically isolated by connective tissue. Unlike myocytes, cardiac fibroblasts are particularly resistant to hypoxia and so play multiple roles in the cascade of events after ischemic injury. After ischemic injury, cardiac fibroblasts transform into a specialized cell type called a myofibroblast marked by increased amounts of α-smooth muscle actin (α-SMA) and characterized as being hyper-secretory. Myofibroblasts have a critical role in cardiac remodeling by forming a collagen-rich scar that allows the infarcted area to maintain structural integrity after cardiomyocyte death. Additionally, in response to injury, fibroblasts secrete cytokines and chemokines that have important roles in the immediate inflammatory stage. Fibroblasts are responsive to a variety of stimuli for these above functions, including agents that activate membrane-bound G protein–coupled receptors (GPCRs). Thus, regulation of these receptors is important in the overall process of myofibroblast activation after injury.

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**Editorial, see p 1049**
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Ischemia/reperfusion (I/R) injury activates a sterile inflammatory response where neutrophils are recruited to the ischemic area by necrotic myocytes and reactive oxygen species. Excessive neutrophil infiltration in the infarcted site is thought to be detrimental to myocyte survival because neutrophils also secrete reactive oxygen species, further exacerbating cell and tissue destruction. Understanding inflammation and remodeling after I/R injury is important for developing novel therapies; multiple studies have demonstrated that inhibiting neutrophil extravasation reduces tissue damage and decreases infarct size. We are interested in the cardiac signals and...
cellular communication between myocytes and fibroblasts that may regulate or alter the tissue damage after I/R injury.

Although the regulation of GPCRs in myocytes during ischemic injury has been extensively studied, fibroblast GPCR signaling during I/R injury and the focus of this study, GPCR kinase 2 (GRK2), has not been well characterized. The primary role of GRK2 is to phosphorylate agonist-activated GPCRs and target them for β-arrestin-mediated internalization; in the heart, GRK2 is upregulated in the myocyte after injury, and its enhanced activity targets the β-adrenergic receptor (βAR) to modulate contractility. Decades of research have uncovered the detrimental effects of increased GRK2 activity in heart disease and provided sweeping evidence that loss of myocyte GRK2 expression or its inhibition either before or after cardiac insult is beneficial for myocardial contractility and cardiac remodeling. More recent studies have also expanded on the versatility of GRK2 in freshly isolated neonatal rat and adult mouse cardiac fibroblasts to gain insight into how the fibroblast may be influencing myocyte survival and cardiac inflammation in vivo.

**Methods**

**Experimental Animals**

To obtain inducible, fibroblast-specific GRK2 KO mice, collagen1α2-CreER(T)/GRK2fl/fl mice were crossed with GRK2fl/fl mice (referred to as GRK2 fKO). GRK2fl/fl mice were used as wild-type (WT) littermate controls. Tamoxifen (Sigma T5648) was injected intraperitoneally daily for 10 days at 40 mg/kg per day. All animal studies were conducted with the approval of the Animal Care and Use Committee at Temple University.

**Isolation of Adult Mouse Cardiac Fibroblasts and Myocytes**

Hearts were removed from >2- to 3-month-old mice and processed as previously described.

**Isolation of Bone Marrow Cells and Lysate Preparation**

Thoracic aortas were isolated from >2- to 3-month-old mice. After washing in 1x PBS, aortas were responded in RIPA buffer supplemented with protease inhibitors, sonicated, and centrifuged to remove cell debris.

**Isolation of Neonatal Rat Cardiac Fibroblasts**

Neonatal rat cardiac fibroblasts are isolated as a byproduct of neonatal rat cardiac myocyte isolation, performed as previously described.

**Immunoblotting**

GRK2 and GAPDH antibodies were from Santa Cruz. Phospho-protein kinase B (AKT) and total AKT antibodies were from Cell Signaling.

**Measurement of cAMP Levels**

Neonatal rat cardiac fibroblasts were treated with 10 μmol/L isoproterenol for 15 minutes. cAMP levels were measured using the cyclic AMP XP Assay Kit (Cell Signaling 4339) without modifications.

**Immunofluorescence**

Hearts were harvested 72 hours post reperfusion, fixed overnight in 4% paraformaldehyde, embedded in paraffin, and cut on the long axis into 6 μm sections. After deparaffinization, rehydration, and antigen retrieval using Vector Antigen Unmasking Solution (Vector Laboratories H3300), sections were blocked in 10% FBS/1× PBS. Immunofluorescence was performed as previously described.

**Assessment of Myocardial Fibrosis**

Collagen levels were measured using the Masson’s Trichrome staining kit (Sigma HT15) and the Hydroxyproline assay kit (Sigma MAK008) without modifications.

**Terminal Hemodynamic Analysis of Cardiac Function**

Hemodynamic analysis was conducted blinded as described previously.

**In Vivo Model of I/R Injury**

I/R injury was performed as described previously. Infarct size was measured as previously described.

**Transthoracic Echocardiographic Analysis**

Transthoracic 2-dimensional echocardiography was performed blinded as described.

**Measurement of Myocardial Apoptosis**

Myocardial apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining.

**Hemodynamic Analysis**

Hemodynamic analysis was conducted blinded as described.

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intensity was measured using ImageJ. For staining of neonatal rat cardiac fibroblasts, cells were treated with methanol for 15 minutes at −20°C, washed, and blocked for 1 hour at room temperature in 5% BSA/PBS. P65 antibody (Cell Signaling 6956) was incubated overnight at 4°C, and secondary antibody (Life Technologies) was applied for 1 hour at room temperature. Sections were treated with DAPI mounting media and coverslipped. Cells were counted using Image J over at least 5 fields per group. All immunofluorescence images were taken with a Nikon Ti microscope.

**Myeloperoxidase Staining**
Mice were euthanized after 6 and 24 hours of reperfusion, and hearts were fixed in 4% paraformaldehyde. The hearts were embedded in paraffin and cut into 5-μm-thick sections. Immunohistochemistry was performed on sections, including antigen retrieval with Vector Antigen Unmasking Solution. Myeloperoxidase (MPO) primary antibody and secondary antibody were sourced from Santa Cruz (sc16129, sc2354, respectively). Images of the infarct area of the left ventricular (LV) wall were taken from at least 2 randomly chosen fields on a Nikon DS-Ri1 and quantified in a blind manner using ImageJ.

**Cytokine Microarray**
Adult mouse cardiac fibroblasts were isolated as described earlier. Cells were subjected to in vitro ischemia buffer,27 and supernatant was harvested for use with the Proteome Profiler Mouse Cytokine Array Panel A (R&D ARY006) with one modification: IRDye 800CW Streptavidin (Rockland S000-31) was used.

**Conditioned Media Experiment**
Neonatal cardiac fibroblasts were treated with β-galactosidase (AdLacZ) or short hairpin GRK2 (AdshGRK2) containing adenovirus. After 24 hours, fibroblasts were serum-starved overnight, treated for 30 minutes with in vitro ischemia buffer, and then reperfused in 0.1% DMEM for 3 hours. Supernatant was collected and placed on neonatal cardiac myocytes for 10 minutes. Fibroblasts were collected to ensure GRK2 loss, and myocytes were collected for whole cell lysate.

**In Vitro I/R Buffer**
This buffer simulates the extracellular milieu of an ischemic environment because it is glucose-deficient, hyperkalemic, and acidic/lactate rich. It is composed of 137 mmol/L NaCl, 3.8 mmol/L KCl, 0.49 mmol/L MgCl₂, 0.9 mmol/L CaCl₂, 4.0 mmol/L Hepes supplemented with 10 mmol/L 2-deoxyglucose, 20 mmol/L sodium lactate, 1 mmol/L sodium dithionite, and 12 mmol/L KCL, pH 6.5.

**Statistical Analysis**
Data are expressed as means±SE. Statistical significance was determined by unpaired t test on experiments comparing 2 groups or analysis of variance and Tukey test for experiments involving 4 comparisons using vassarstats.net. P values <0.05 were considered significant.

**Results**
**Decreased Levels and Activity of GRK2 in Fibroblasts Are Beneficial to Post-I/R Myocardium**
Eight-week-old adult male GRK2 fKO mice and their control littermates (WT) were injected intraperitoneally with tamoxifen (40 mg/kg per day) for 10 days (Online Figure 1). After a 2-week washout period, freshly isolated fibroblasts from GRK2 fKO mice demonstrated 60% protein loss compared with fibroblasts from WT mice (Figure 1A). GRK2 knockdown was specific for fibroblasts because protein levels were maintained in myocytes, bone marrow cells, and vascular smooth muscle cells after tamoxifen treatment in both groups (Figure 1A). This finding correlates with previously published reports using this model for other gene products.24,28 We can infer negligible interference from knockdown in circulating fibrocytes. Although this cell type has fibroblast qualities, fibrocytes are monocyte-derived, and GRK2 levels are maintained in the bone marrow cells. LV infarct size subsequent to 30 minutes of left anterior descending coronary artery occlusion and 24 hours of reperfusion was measured in GRK2 fKO mice, and significant differences were found compared with control mice (Figure 1). In WT littermates, LV infarct size was 34.17±3.98% of the area at risk (Figure 1B and 1C). However, fibroblast-specific ablation of GRK2 caused a decreased infarct size of 20.67±0.35%. The LV area at risk was similar in both groups, indicating the same amount of ischemic trauma (Figure 1D). These results establish that loss of fibroblast GRK2 during acute ischemic injury confers a protective advantage to reperfused myocardial tissue.

Echocardiography was performed on WT and GRK2 fKO mice after 30 minutes of ischemia and 24 hours of reperfusion (Figure 2A and 2B). Control mice presented with decreased LV ejection fraction of 48±2.1% compared with 62±2.5% at baseline (Figure 2A). WT mice also exhibited loss of fractional shortening (24±1.2% versus 33±1.7% in WT baseline group; Figure 2B). However, GRK2 fKO mice displayed partial but significantly restored cardiac function with an ejection fraction of 59±1.7% and fractional shortening of 31±1.1%. Both groups displayed similar baseline ejection fractions, demonstrating equal cardiac function before surgery. To correlate echocardiography data and to measure intact cardiac pressures, terminal hemodynamics was done 24 hours post I/R injury, and mice were infused with increasing doses of isoproterenol to assess their inotropic reserve (Figure 2C). WT mice presented with compromised contractility demonstrated by decreased dP/dTmax and −dP/dTmin compared with GRK2 fKO mice, which maintained contractility levels close to baseline. In agreement with the systolic function data, we also noted decreased cardiac output and stroke volume in the WT mice compared with GRK2 fKO mice after 24 hours (Table). We also assessed cardiac function at 1 and 4 weeks post I/R to determine the chronic effects of fibroblast GRK2 deletion. The WT I/R group maintained a significantly reduced ejection fraction at 1 week and 4 weeks post I/R (51.9±2.3 and 53.3±0.7, respectively), whereas the GRK2 fKO mice presented with rescued cardiac function at both time points (62.1±1.2 and 60.3±1.7; Table). There were no survival differences between the I/R groups after 4 weeks of reperfusion (Table).

Because myocardial apoptosis is a contributing factor to I/R injury and the loss of viable myocardium, we evaluated TUNEL positivity in myocardial sections of post-I/R mice (Figure 3). WT and GRK2 fKO mice were subjected to 30 minutes of myocardial ischemia and 6 hours of reperfusion. TUNEL staining, along with α-sarcomeric actin staining for cardiomyocytes, was performed on heart sections, and apoptosis levels were measured in the ischemic border zone of the infarcted area (Figure 3A). Quantification of these findings revealed that WT mice exhibited significantly more apoptotic cardiomyocytes, with nearly 3× the TUNEL-positive myocytes of GRK2 fKO littermates (Figure 3B).

We also performed conditioned media experiments using both isolated myocytes and fibroblasts from neonatal rats (Figure 3C and 3D). Fibroblasts were treated with adenovirus...
containing an shRNA for GRK2 or a LacZ control adenovirus (Figure 3C, top panel) exposed to an in vitro Kreb’s buffer and reperfused with DMEM for 3 hours. The supernatant was collected, placed on myocytes, and AKT signaling was measured (Figure 3C, bottom panel). Conditioned media from LacZ adenovirus and in vitro I/R-treated fibroblasts increased phosphorylated AKT in myocytes compared with media from fibroblasts treated only with LacZ adenovirus. Knockdown of GRK2 in cardiac fibroblasts potentiated this increase in phosphorylated AKT in myocytes after treatment with in vitro I/R fibroblast–conditioned media, indicating that in vitro loss of GRK2 in fibroblasts is able to modify the fibroblast secretome to enhance survival signaling in myocytes (Figure 3D).

**GRK2 Ablation in Fibroblasts Inhibits I/R-Induced Myocardial Fibrosis and Fibrotic Gene Upregulation**

Because collagen deposition by cardiac fibroblasts is linked to myocardial apoptosis, we wanted to investigate whether

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**Figure 1.** G protein–coupled receptor kinase 2 (GRK2) levels and post-ischemia/reperfusion (I/R) left ventricular (LV) infarct size in collagen1α2-CreER(T)/GRK2fl/fl mice (GRK2 fKO) mice. A, Western blots and GRK2 protein quantification of freshly isolated cardiac fibroblast (F), myocyte (M), bone marrow (BM), and vascular smooth muscle cell (VSMC) lysate in Tamoxifen-treated wild-type (WT) and GRK2 fKO hearts. *P<0.05 vs WT. N=3 to 4. B, Representative photographs of post-I/R Evan’s Blue/triphenyl tetrazolium chloride–stained sections from WT and GRK2 fKO hearts. C, LV infarct size expressed as a percentage of the area at risk. D, LV area at risk expressed as a percentage of the total LV. *P<0.05 vs WT. N=6.

**Figure 2.** Cardiac function post ischemia/reperfusion (I/R) in wild-type (WT) and collagen1α2-CreER(T)/GRK2fl/fl mice (GRK2 fKO) mice. Cardiac function shown by ejection fraction (%) in A and fractional shortening (%) in B from echocardiography on WT and GRK2 fKO mice at baseline and 24 hours after I/R. *P<0.05 and #P<0.01 vs WT post I/R, **P<0.01 vs WT baseline. N=8. C, In vivo contractility demonstrated by +dP/dT max and −dP/dT min measured with terminal hemodynamics. *P<0.05 and **P<0.01 vs WT Sham. N=8. GRK2 indicates G protein–coupled receptor kinase 2.
the decrease in apoptotic myocytes led to diminished collagen deposition. Masson’s Trichrome staining was performed on heart sections after 30 minutes of ischemia and 72 hours of reperfusion. Fibrosis was measured in the infarcted area of the LV. As shown in Figure 4A, WT mice had a robust amount of reparative fibrosis (16.1±1.4% of the total area quantified) after I/R as compared with Sham-treated animals. Correlating with decreased myocardial cell death, the GRK2 fKO group also presented with diminished collagen deposition (4.9±1.8% of the total area quantified). Independently, we also used the hydroxyproline assay to measure collagen levels in equal weights of heart tissue taken from the LV 72 hours after reperfusion (Figure 4B). WT hearts exhibited an increase in hydroxyproline levels (0.2049±0.0148 μg/mg tissue) compared with sham animals (0.1347±0.0013 and 0.1285±0.0073 μg/mg tissue). Like the Masson’s Trichrome results, myocardial tissue from the GRK2 fKO group had decreased hydroxyproline levels (0.1407±0.0037 μg/mL tissue) compared with the WT post I/R. We also measured mRNA levels of collagen I and collagen III in myocardium taken from the LV and found that although both collagen types were significantly upregulated (3-fold and 27-fold increase, respectively) in the WT mice, the GRK2 fKO mouse heart tissue contained significantly decreased levels of both collagen I and collagen III (1.25-fold and 11-fold increase, respectively; Figure 4C). Attenuated levels of fibrosis were maintained in the GRK2 fibroblast KO mouse (10.02±0.79%) after 4 weeks of reperfusion compared with wild type littermates (17.2±1.5%; Figure 4D). Importantly, decreased fibrosis levels did not cause cardiac rupture, and benefits to systolic function were still detectable in the GRK2 fKO mouse 1 week and 4 weeks after I/R injury (Table).

After myocardial injury, fibroblasts undergo phenotypic changes that result in the transformation to the α-SMA expressing myofibroblast; this change is also important for the increase in collagen production necessary for wound healing. To investigate whether the decrease in collagen production seen in the GRK2 fKO mice is caused by the inability of the fibroblasts to transform into myofibroblasts, we performed immunohistochemistry and reverse transcriptase polymerase chain reaction. After 72-hour reperfusion, mouse hearts were sectioned and probed for α-SMA, and the level of fluorescence intensity was measured using ImageJ software (Figure 5A). Interestingly, we found that both WT and GRK2 fKO mice expressed equal amounts of α-SMA protein, with both groups demonstrating a 2.64-fold increase (Figure 5B). We also found similar increases in α-SMA mRNA expression in both the WT and GRK2 fKO mice (Figure 5C).

The first 48 hours after myocardial injury are marked by inflammation and diapedesis of immune cells; of these cells, neutrophils are the first to migrate to the infarcted area and have been implicated in negative repercussions for cardiomyocyte survival. Therefore, we assessed whether the decreases in infarct size in the GRK2 fKO mouse could be correlated to a reduction in neutrophil extravasation. To answer this question, we performed immunohistochemistry for MPO, an enzyme abundantly expressed by neutrophils. After 6 and 24 hours of reperfusion, hearts were stained for MPO; MPO-positive cells were quantified (Figure 6; Online Figure II). After 6 hours, WT mice exhibited a robust accumulation of MPO-positive neutrophils in both the border zone and the infarct area (557±59 and 176±17, respectively, compared with sham groups; Figure 6B and 6C). Similar results were seen at 24 hours post reperfusion for the border zone and infarct area (183±22 and 169±42, respectively, compared with sham groups). Conversely, the GRK2 fKO hearts displayed attenuated neutrophil accumulation at 6 hours post I/R (329±56 in the border zone and 80±16 in the infarct area). The GRK2 fKO group also had decreased neutrophil counts at 24 hours post I/R in the border and infarct zones (132±18 and 61±20, respectively). The same experiment performed at 6 hours in the myocyte-specific KO αMHC-Cre/GRK2flox mouse line showed no differences between groups, indicating that this is a fibroblast-specific event (Online Figure IIIA and IIIB).

We first explored the possibility that expression levels of the neutrophil-recruiting CXCL chemokines were affected by GRK2 ablation. Surprisingly, we found no changes in mRNA levels of CXCL1, CXCL2, or CXCL5 (Online Figure IV). Because inflammation is a complex process characterized by the activation of multiple cytokines, we turned to the Mouse Cytokine Antibody Array to glean further insight into what cytokines may be altered in the GRK2 fKO mouse after I/R injury. Adult cardiac fibroblasts were isolated from WT and GRK2 fKO mice, treated with in vitro Kreb’s buffer, and reperfused with DMEM. After 6 hours, the DMEM was collected

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**Table. Functional Measurements Obtained From Echocardiography and Survival Rate on WT and GRK2 fKO Mice Subjected to Sham and I/R Surgery**

<table>
<thead>
<tr>
<th>Animal, Time Post I/R</th>
<th>Heart Rate, bpm, 24 h</th>
<th>LVEDD, mm, 24 h</th>
<th>Stroke Volume, μL, 24 h</th>
<th>Cardiac Output, mL/min, 24 h</th>
<th>Ejection Fraction, %</th>
<th>Survival Rate, %</th>
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<td></td>
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<tr>
<td>WT Sham</td>
<td>504±14.1</td>
<td>4.0±0.18</td>
<td>47.5±2.6</td>
<td>23.2±1.5</td>
<td>63.8±2.2</td>
<td>60.8±1.7</td>
</tr>
<tr>
<td>GRK2 fKO Sham</td>
<td>535±22.8</td>
<td>3.97±0.14</td>
<td>46.0±3.3</td>
<td>25.1±2.1</td>
<td>60.4±0.9</td>
<td>59.2±2.0</td>
</tr>
<tr>
<td>WT I/R</td>
<td>470±12.3</td>
<td>4.07±0.14</td>
<td>35.3±1.7*</td>
<td>16.6±0.7*</td>
<td>51.9±2.3*</td>
<td>53.3±0.7</td>
</tr>
<tr>
<td>GRK2 fKO I/R</td>
<td>489±9.9</td>
<td>4.01±0.12</td>
<td>42.2±1.6†</td>
<td>20.7±1.1†</td>
<td>62.1±1.2§</td>
<td>60.3±1.7§</td>
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GRK2 indicates G protein–coupled receptor kinase 2; GRK2 fKO, collagen1α2-CreER(T)/GRK2flox mice; I/R, ischemia/reperfusion; LVEDD, left ventricular end diastolic diameter; and WT, wild-type.

*P<0.01 vs WT Sham.
†P<0.05 vs WT Sham.
‡P<0.05 vs WT I/R.
§P<0.01 vs WT I/R. N=4–7 for Sham groups, N=6–9 for I/R groups.
and used in the Cytokine Antibody Array kit (Figure 7; Online Figure V). We noted that tumor necrosis factor-α (TNFα), a potent activator of inflammation, was downregulated by 30% in the media obtained from postischemic GRK2 fKO purified adult mouse fibroblasts (Figure 7A). Using reverse transcriptase polymerase chain reaction, we investigated mRNA levels of TNFα in myocardial tissue taken from 6-hour post-ischemic hearts and found that although the WT hearts had a 3.7-fold increase in TNFα mRNA, the GRK2 fKO hearts presented with reduced TNFα, with only a 1.3-fold increase (Figure 7B). There were other changes in the microarray, including decreases in interleukin (IL)6 and IL1β, both important for inflammation after myocardial I/R injury. However, we were not able to resolve differences in mRNA expression levels as with TNFα (data not shown). There were also decreases in IL4 and IL5; however, the role of these cytokines in I/R injury is less defined, and so we focused on TNFα. Next, we purified GRK2 flox+− adult mouse cardiac fibroblasts treated with Cre adenovirus to knockdown GRK2 levels. In agreement with our in vivo reverse transcriptase polymerase chain reaction data, we found that levels of secreted TNFα were also decreased after in vitro I/R, compared with control fibroblasts treated with LacZ adenovirus (Figure 7C and 7D). Nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) is known to regulate TNFα transcription; therefore, we were curious to see if GRK2 was affecting the ability of NFκB to enter the nucleus.

We treated neonatal rat cardiac fibroblasts with shGRK2 adenovirus to knockdown GRK2 expression, subjected the cells to in vitro I/R, and used immunofluorescence to track the movement of the p65 subunit of NFκB. Only in postischemic cells treated with control LacZ adenovirus was NFκB able to move into the nucleus. Nuclear translocation was prevented in

Figure 3. Apoptosis and survival signaling after fibroblast G protein–coupled receptor kinase 2 (GRK2) ablation. A, Representative photographs of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and α-sarcomeric actin (α-SA)–stained sections from the border zone of 6-hour post-ischemia/reperfusion (I/R) wild-type (WT) and collagen1α2-CreER(T)/GRK2fl/fl mice (GRK2 fKO) hearts, including 40× image showing TUNEL and α-SA+ cardiomyocytes (white arrows). Scale bar, 100 μm. α-SA (green), cardiomyocytes; TUNEL (red), apoptotic nuclei; DAPI (blue), total nuclei. B, TUNEL+ cardiomyocyte quantification represented as the percentage of TUNEL and αSA+ cells in WT and GRK2 fKO. *P<0.05 vs WT, N=4. C, Western blot demonstrating GRK2 knockdown in neonatal rat cardiac fibroblasts using an shRNA-containing adenovirus for GRK2 (top blot) and protein kinase B (AKT) levels in neonatal rat cardiac myocytes after conditioned media treatment (CM) from LacZ and shGRK2 adenovirus-treated fibroblasts at basal and after in vitro I/R (bottom blot). D, Quantified phosphorylated AKT normalized to total AKT in myocytes. "P<0.01 vs basal CM, #P<0.05 vs AdLacZ in vitro I/R CM. N=3 separate experiments.
postischemic cells lacking GRK2 (Figure 7E). Importantly, increased levels of cAMP, a second messenger that is indirectly affected by GRK2 activity, have been shown to prevent TNFα expression. To ascertain if this is a potential mechanism, we infected neonatal rat cardiac fibroblasts with shGRK2 adenovirus, treated with isoproterenol, and measured fold changes in cAMP versus the basal control. Fibroblasts lacking GRK2 had an enhanced fold change compared with the isoproterenol-treated fibroblasts infected with LacZ adenovirus (Figure 7F).

**Discussion**

Fibroblasts comprise a large proportion of the nonmyocyte cells in the heart, which varies from species to species but constitutes ≈26% of the total cells in the murine heart. Initially thought to be a relatively inert stromal cell, recent publications have expanded on favorable and adverse fibroblast contributions after myocardial I/R injury. For example, after ischemic insult, cardiac fibroblasts undergo mesenchymal to endothelial transition, contributing to beneficial neovascularization of the injured myocardium. Additionally, inflammasome activation in the cardiac fibroblast has been shown to be critical for the detrimental inflammatory response seen after I/R injury. To our knowledge, this is the first study to investigate the in vivo effects of GRK2 ablation in the fibroblast after myocardial ischemic injury. This mouse model is beneficial because the Cre is driven by a fibroblast-specific regulatory sequence, which prevents off-target effects to other collagen-expressing cell types, like vascular smooth muscle cells. Our mouse model is not cardiac specific and will incur the loss of GRK2 in all fibroblasts. However, this study will focus on myocardial-specific effects of GRK2 ablation in the fibroblast.
effects of GRK2 ablation in cardiac fibroblasts after ischemic injury. We have shown here, using an inducible and cell-selective transgenic mouse model, that the absence of fibroblast GRK2 in the postischemic heart confers a protective advantage for cardiomyocyte survival and cardiac function. In the setting of I/R, these cardioprotective effects can be attributed to decreased neutrophil infiltration and reduced TNFα production in the infarcted tissue, resulting in smaller infarct size and enhanced contractility because of more viable myocardium.

Neutrophils are the most abundant type of white blood cell and are a part of the innate immune system; they are the first immune cells to respond to injury or infection. Multiple
studies have demonstrated that neutrophils are the main source of reactive oxygen species in the reperfused heart, lending a negative connotation to the presence of this cell type that is typically thought of as a beneficial member of the inflammatory environment.2,3 TNFα is a pleiotropic and potent activator of inflammation and is involved in neutrophil chemotaxis to the injury area.3 Cardiac fibroblasts secrete TNFα in response to different types of injury, like mechanical strain and hypoxia.2 Although latent TNFα is tethered to the external plasma membrane for immediate availability via proteolytic cleavage, nascent production of TNFα is also achieved via activation of the NFκB transcription factor.3 In macrophages, GRK2 has been shown to intersect with NFκB signaling through phosphorylation of nontraditional sites of the inhibitor of NFκB, inhibitor of κB, inducing degradation and subsequent release, and nuclear localization of NFκB.36 This is notable in light of our data showing a reduction in TNFα mRNA after in vivo I/R in our GRK2 fKO mice. We also see in neonatal rat cardiac fibroblasts that postischemic nuclear localization of NFκB is prevented when GRK2 is ablated, which speaks to another nonclassical role for GRK2 in an injury setting.

βARs have been documented to be anti-inflammatory. In the setting of murine acute lung injury, βAR agonists reduced infiltrating neutrophils and TNFα levels in the supernatant of lipopolysaccharide-treated isolated macrophages.37 Additionally, neutrophil infiltration in rat venule endothelial cells after substance P treatment was inhibited using the β2AR agonist, formoterol.38 Cardiac fibroblasts in mice and humans are predominantly populated with the β2AR subtype and a major target of GRK2. Loss of GRK2 may maintain or even enhance βAR-mediated anti-inflammatory signaling in the setting of ischemic injury, which could have translational significance in cardiac patients.

Additionally, there is evidence that cAMP inhibits TNFα production through the βAR.41 After lipopolysaccharide treatment, the selective cAMP–phosphodiesterase IV inhibitor rolipram inhibited TNFα production.42 Conversely, treating murine macrophages with agents that elevate intracellular cAMP suppressed lipopolysaccharide-induced inflammatory gene expression, including TNFα.43 Mechanistically, elevated cAMP leads to activated protein kinase A, which phosphorylates and facilitates the degradation of p105, the precursor of p50.44 These data hint at another potential mechanism for how decreased GRK2 can result in attenuated TNFα levels: by maintaining cAMP levels formed by preserved or enhanced βAR signaling. In line with this reasoning, we see that loss of GRK2 in the cardiac fibroblasts resulted in potentiated levels of cAMP compared with WT fibroblasts after isoproterenol treatment.

Our results suggesting proinflammatory actions of GRK2 conflict with other reports concluding GRK2 is...
anti-inflammatory. Myeloid cell-specific GRK2 knockdown increased tissue injury after lipopolysaccharide injection by enhancing NFκB1p105–ERK signaling.45 Lymphocytes with reduced GRK2 levels show increased chemotaxis to CCL3, CCL4, and CCL5.46 However, although GRK2 hemizygous mice afflicted with experimental autoimmune encephalomyelitis have a quicker disease onset than wild-type experimental autoimmune encephalomyelitis counterparts, GRK2+/− mice do not have relapses like wild-type mice. The lack of relapses was correlated with attenuated inflammatory central nervous system infiltrates.47 In addition, high levels of GRK2 directly correlate with the severity of Alzheimer’s disease in human patients.48 These conflicting reports on the inflammatory role of GRK2 indicate that there is no definite answer, and conclusions may be dependent on cell type and disease model.

Cardiac fibroblasts secrete collagen to maintain structural integrity after I/R-induced cardiomyocyte death. Initially, this is a critical reparative process, but eventually excessive collagen deposition leads to myocardial stiffening and impedes contractility. Our present study demonstrates that GRK2 loss in fibroblasts decreases fibrosis after I/R. cAMP, in addition to its anti-inflammatory capabilities, also possesses antifibrotic qualities. Increased intracellular cAMP can attenuate the profibrotic phenotype of in vitro cardiac myofibroblasts.50 However, it is likely in our case that decreased fibrosis is a reaction to limiting myocyte death in GRK2 fKO hearts after I/R. Regardless of the underlying cause, limiting myocardial apoptosis and fibrosis is a desirable outcome caused by decreased levels of fibroblast GRK2, noted previously in myocyte-specific GRK2 KO mice.51 Our conditioned media experiments suggest that fibroblasts secrete factors that influence cardiomyocyte survival after in vitro I/R and that GRK2 knockdown augments this effect. Interestingly, conditioned media from fibroblasts has been shown to improve in vitro cardiomyocyte viability as measured by mitochondrial dehydrogenase activity and troponin I levels. In this case, augmented cardiomyocyte survival was linked to TIMP1 (tissue inhibitor of metalloproteinase-1) activity; inhibiting TIMP1 abolished the effect.52 Fibroblast growth factor-2, a protein secreted by cardiac fibroblasts, can improve contractility and tissue preservation in isolated perfused rat hearts; these cardioprotective qualities were attributed to increased AKT signaling.53 This is interesting in light of our findings that conditioned media from ischemic neonatal rat cardiac fibroblasts increased AKT signaling in neonatal rat myocytes. Cardiac fibroblasts are also the main source of IL33, the functional ligand of the ST2 receptor expressed basally on cardiomyocytes.54 IL33 is upregulated by angiotensin II, the known activator of the ATII receptor (angiotensin II receptor type 1), a GPCR desensitized by GRK2.55 IL33 is cardioprotective; this molecule prevented cardiomyocyte apoptosis, decreased infarct size, and improved systolic function when administered to mice after I/R injury.56 Loss of GRK2 in the cardiac fibroblast could augment the production of angiotensin II–induced IL33 to increase cardiomyocyte survival and preserve inotropy. Reduction in TNFα levels could also be playing a significant role in decreased myocyte apoptosis in the GRK2 fKO mice because cardiac-restricted overexpression of TNFα in mice has been shown to cause myocyte apoptosis and cardiac remodeling through the increased activation of cell death pathways.55

Another interesting aspect of our findings is that despite the reduced levels of fibrosis and fibrotic gene expression seen in the GRK2 fKO group, myofibroblast activation and α-SMA expression seems to be similar in both the WT and fKO mice. The events governing myofibroblast transformation and fibrogenic genes are multifactorial; with regards to GRK2, the cAMP axis is one signaling pathway important for regulating these events. Adenylate cyclase activation after GPCR activation leads to the formation of cAMP. cAMP can modulate responses through 2 signaling platforms, protein kinase A and exchange factor directly activated by cAMP. Swaney et al56 demonstrated that both cAMP and exchange factor directly activated by cAMP activation by exogenous agents can blunt collagen synthesis, α-SMA expression, and myofibroblast transformation in rat cardiac fibroblasts, indicating parallel roles. However, cAMP and exchange factor directly activated by cAMP have opposing effects on fibroblast migration.57 These contrasts are most likely because of differences in protocols (ex vivo versus in vivo), species (rat versus mouse), and injury model (I/R versus exogenous fibrotic agents) but dysregulation of this dual signaling axis after I/R could explain these differences.

From a therapeutic standpoint, our results are significant because they reinforce the idea that inhibition of GRK2 in both myocytes and fibroblasts is beneficial for maintaining inotropic reserve. There is a vast body of literature demonstrating the protective effects of GRK2 ablation in the cardiac myocyte after ischemia, but limited data showing the benefits of GRK2 deletion in the cardiac fibroblast. The goal of this study was to investigate the acute and chronic effects of GRK2 ablation in the cardiac fibroblast; we have seen that fibroblast GRK2 knockdown prevents myocyte death and excessive inflammation and confers long-term benefits to cardiac function. A detailed investigation into the role of GRK2 during posts ischemic inflammation in the heart is important for future analysis. However, our current findings add depth to the various beneficial roles previously attributed to loss of GRK2 through classical or nonclassical pathways.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- G protein–coupled receptor kinase 2 (GRK2) is the major GRK in the heart, and it is upregulated after cardiac injury and during heart failure.
- The classical role of GRK2 in the heart is to phosphorylate and desensitize β-adrenergic receptors controlling myocyte contractility; nonclassical roles for GRK2 have also been delineated in metabolism and insulin signaling.
- Myocyte-specific inhibition of GRK2 before or after myocardial infarction can rescue heart function and prevent adverse remodeling.

**What New Information Does This Article Contribute?**

- Inhibition of GRK2 via genetic ablation in the cardiac fibroblast, a major cell type in the heart, preserves cardiac contractility after ischemic injury in a mouse model.
- Excessive fibrosis, increased myocyte death, and inflammatory cell infiltration are also prevented.
- The above beneficial effects are linked to decreased tumor necrosis factor-α production and secretion caused by the attenuated ability of nuclear factor κ-light-chain-enhancer of activated B cells to translocate to the nucleus in the cardiac fibroblast.

The beneficial effects of GRK2 inhibition in the myocyte after cardiac injury are well documented; however, the role of GRK2 in the cardiac fibroblast after ischemic injury has yet to be investigated. In this study, we show that in a mouse model, fibroblast-specific GRK2 deletion decreased infarct size and partially rescued the drop in contractility. Myocyte apoptosis and neutrophil extravasation were also reduced. Furthermore, we found that cardiac fibroblasts lacking GRK2 express and secrete diminished levels of tumor necrosis factor-α, a critical modulator of inflammation. This correlates with the inability of nuclear factor κ-light-chain-enhancer of activated B cells, a major regulator of tumor necrosis factor-α transcription, to translocate to the nucleus after ischemia-reperfusion injury when GRK2 is not present in the cardiac fibroblast. These data lend further credence to the idea that inhibition of GRK2 in the heart could be a potential therapy to treat ischemia heart disease.
Cardiac Fibroblast GRK2 Deletion Enhances Contractility and Remodeling Following Ischemia/Reperfusion Injury
Meryl C. Woodall, Benjamin P. Woodall, Erhe Gao, Ancai Yuan and Walter J. Koch

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Supplemental Material

Detailed Methods

Experimental Animals
To obtain inducible, fibroblast-specific GRK2-KO mice, Collagen1α2-CreER(T) mice (Jackson Labs Stock #029235) were crossed with GRK2fl/fl mice (Jackson Labs Stock #012458). Pups from this cross were then backcrossed to generate Collagen1α2-CreER(T)/GRK2fl/fl mice (referred to as GRK2 fKO in text, figures and legends). GRK2fl/fl mice were used as wild-type (WT) littermate controls for all assays. Tamoxifen (Sigma T5648) was injected intraperitoneally (IP) daily for 10 days at a dose of 40mg/kg/day to induce Cre-mediated recombination and the loss of the GRK2 allele. Adult male mice (>2-3 months of age) that were at least 14 days out from the tamoxifen injections were subjected to I/R injury as we have described previously. Mice of this age, background and gender are commonly used for cardiovascular studies and represent a viable option for the purpose of our research study. All animal studies were conducted with the approval of the Animal Care and Use Committee at Temple University.

Isolation of Adult Mouse Cardiac Fibroblasts and Myocytes
To obtain adult fibroblasts, hearts were removed from >2-3 month old mice and minced in Hank’s buffered salt solution containing collagenase II (150 U/ml) and trypsin (0.6 mg/ml). The tissue was digested in a shaking incubator for five 15 minute intervals; between each digestion the solution was removed and stored at 37° degrees in DMEM supplemented with FBS and new digestion solution was added to the minced tissue. Samples were centrifuged to collect fibroblasts, strained through a 70 μm filter and plated on cell culture dishes for one hour. Weakly adherent cells, including myocytes and endothelial cells were removed, plates were washed with 1X PBS and then incubated in DMEM with 10% FBS. Isolation of adult mouse cardiac fibroblasts results in low yields with limited potential for passage. Therefore, adult fibroblasts were used only for experiments to investigate tamoxifen-induced murine GRK2 knockdown and for experiments with two experimental groups only (e.g. Figure 7C,D).

Isolation of Bone Marrow Cells and Lysate Preparation
Femurs were removed from >2-3 month old mice and extra tissue was removed. The ends of the femurs were trimmed off with scissors to expose the interior marrow. Contents were flushed using Hank’s Buffered Salt Solution (Corning 21-023-CV), a syringe and a 27 gauge needle. Bone marrow cells were then centrifuged to collect cells and washed with 1X PBS. Cells were then resuspended in RIPA buffer supplemented with protease inhibitors, sonicated and centrifuged to remove cell debris.

Isolation of Vascular Smooth Muscle and Lysate Preparation
Thoracic aortas were isolated from >2-3 month old mice by opening the chest cavity and removing the heart and lungs. The aorta was separated from the spine and removed using scissors. Care was taken to remove the layer of perivascular fat to prevent fibroblast contamination. Endothelial cells were also removed by gently rubbing the lumen with fine forceps. After washing in 1X PBS, aortas were responded in RIPA buffer supplemented with protease inhibitors, sonicated and centrifuged to remove cell debris.

Isolation of Neonatal Rat Cardiac Fibroblasts
Neonatal rat cardiac fibroblasts are isolated as a byproduct of neonatal rat cardiac myocyte isolation, which was performed as previously described. Fibroblast-containing plates from the preplating step are saved, washed and cultured in DMEM supplemented with 10% FBS. Neonatal rat cardiac fibroblasts are easily harvested, abundant and passage readily. Therefore these cells were used for all experiments requiring four experimental groups.

Immunoblotting
After SDS-PAGE and transfer to nitrocellulose membranes, primary antibody incubations were performed overnight at 4°C. Fluorescent secondary antibodies were obtained from either
Molecular Probes or Li-Cor. Membranes were scanned with the Odyssey infrared imaging system (LI-COR). Both GRK2 and GAPDH primary antibodies used were sourced from Santa Cruz. Phospho-AKT and total AKT antibodies were sourced from Cell Signaling.

**In Vivo Model of Ischemia/Reperfusion Injury**

Ischemia was induced by ligation of the coronary artery for 30 minutes following by release and reperfusion as described previously. All surgical procedures were performed on 8-10 week old GRK2fl/fl (WT) and GRK2 fKO male mice and all I/R was controlled with Sham operated mice where the suture was passed under the coronary artery but not tied. Animals were anesthetized with isoflurane using a vaporizer at a dose of 3% and maintained throughout the surgery at 1.5%. Infarct size was measured as the percentage of the infarcted area of the left ventricle (LV) within the ischemic area at risk (AAR) as described. All mouse surgeries and measurement of infarct size were performed in a blinded manner.

**Transthoracic Echocardiographic Analysis**

Transthoracic two-dimensional echocardiography was performed in a blinded manner in mice anesthetized with isoflurane (3% induction/1.5% maintenance) with a 12-MHz probe as described. M-mode echocardiography was carried out in the parasternal short axis in mice 24 hours, 1 week and 4 weeks after reperfusion to assess heart rate, LV ejection fraction and fractional shortening.

**Terminal Hemodynamic analysis of cardiac function**

Hemodynamic analysis was conducted in a blinded manner as described previously. Briefly, a 1.4-French micromanometer-tipped catheter (Millar Instruments) was inserted into the right carotid artery and then advanced into the LV. A polyethylene-50 catheter was placed in the left external jugular vein for infusion of increasing doses of isoproterenol (0.1ng, 0.5ng, 1ng, 5ng). Steady-state LV maximum (dP/dt max) and minimum (dP/dT min) was recorded in closed-chest mode throughout the experiment with a PowerLab DAQ System (Millar Instrument).

**Measurement of myocardial apoptosis**

Myocardial apoptosis was assessed by TUNEL staining. Mice were euthanized after 6 hours of reperfusion, and hearts were fixed in 4% paraformaldehyde. The hearts were embedded in paraffin and cut into long axis sections 6μm in thickness. TUNEL staining on the sections was carried out with the In Situ Cell Death Detection Kit TMR Red (Roche). α-sarcomeric actin primary antibody was obtained from Sigma. Slides were counterstained with DAPI-containing mounting medium. The border zone of the LV wall was visualized under a Nikon-Ti fluorescence microscope with a DAPI filter and a Rhodamine filter and digital images were collected and merged. Images for at least four sections per animal were taken. Apoptotic cells with red fluorescence were counted in a blinded fashion using NIS-Elements Software (Nikon, Japan) to assess the apoptotic index (number of TUNEL positive cardiomyocytes). For each heart, more than 1000 cells were counted.

**Assessment of myocardial fibrosis**

Collagen levels were measured using the Masson’s Trichrome staining kit (Sigma HT15) and the Hydroxyproline assay kit (Sigma MAK008). Mice were euthanized 72 hours or 4 weeks following reperfusion. For Masson’s Trichrome staining, hearts were removed and fixed in 4% paraformaldehyde. The hearts were embedded in paraffin and cut into long axis sections 6μm in thickness. Before treatment with the Trichrome staining kit, sections were incubated at room temperature in Bouin’s solution (HT101128) overnight. Images of the infarct area of the LV wall were taken on a Nikon DS-R1 and quantified in a blind manner using ImageJ. For each heart, at least five random fields were measured. For the Hydroxyproline assay, 10 mg pieces of tissue were taken from the infarct area of the LV wall and processed according to the kit directions.

**Immunofluorescence**

For myofibroblast staining, hearts were harvested 72 hours post reperfusion, fixed overnight in
4% paraformaldehyde, embedded in paraffin and cut into long axis sections 6 μm in thickness. After deparaffinization and rehydration, antigen retrieval was performed using Vector Antigen Unmasking Solution (Vector Labs H3300) and sections were blocked in 10% FBS in 1X PBS. Primary antibody (α-SMA, Sigma A5228) was diluted 1:2000 and incubated with sections overnight at 4°C. Secondary antibody (Life Technologies A21463) was applied for 1 hour at room temperature. Sections were treated with mounting media containing DAPI and coverslipped. Fluorescence intensity was measured using ImageJ. For staining of neonatal rat cardiac fibroblasts, cells were treated with methanol for 15 minutes at -20°, washed and blocked for 1 hour at room temperature in 5% BSA in PBS. Primary antibody for p65 (Cell Signaling 6956) was incubated overnight at 4°C and secondary antibody (Life Technologies ) was applied for 1 hour at room temperature. Sections were treated with mounting media containing DAPI and coverslipped. Cells were counted using Image J over at least five fields per group. All immunofluorescent images were taken with a Nikon Ti microscope.

Myeloperoxidase (MPO) Staining
To identify neutrophils using myeloperoxidase staining, mice were euthanized after 6 and 24 hours of reperfusion and hearts were fixed in 4% paraformaldehyde. The hearts were embedded in paraffin and cut into long axis sections 6μm in thickness. Immunohistochemistry was carried out on sections, including antigen retrieval with Vector Antigen Unmasking Solution. MPO primary antibody and secondary antibody were sourced from Santa Cruz (sc16129, sc2354, respectively). Images of the border and infarct area of the LV wall were taken from a least two randomly chosen fields on a Nikon DS-Ri1 and quantified in a blind manner using ImageJ.

Cytokine Microarray
Adult mouse cardiac fibroblasts were isolated as described above. Cells were subjected to in vitro ischemia buffer and supernatant was harvested to analyze for the presence of cytokines using the Proteome Profiler Mouse Cytokine Array Panel A (R&D ARY006). The kit was used according to directions with one change: IRDye 800CW Streptavidin (Rockland S000-31) was used in place of Streptavidin-HRP to visualize on the LI-COR.

Conditioned Media Experiment
Neonatal cardiac fibroblasts were treated with LacZ or shGRK2 containing adenovirus. After 24 hours fibroblasts were serum starved overnight, treated for 30 minutes with in vitro ischemia buffer and then reperfused in 0.1% DMEM for 3 hours. Supernatant was collected and placed on neonatal cardiac myocytes for 10 minutes. Fibroblasts were collected to ensure GRK2 loss and myocytes were collected for whole cell lysate.

In vitro Ischemia Reperfusion Buffer
This buffer is used to simulate the extracellular milieu of an ischemic environment because it is glucose deficient, hyperkalemic and acidic/lactate rich. It is composed of 137mM NaCl, 3.8mM KCl, 0.49mM MgCl2, 0.9mM CaCl2, 4.0mM Hepes supplemented with 10mM 2-deoxyglucose, 20mM sodium lactate, 1mM sodiumdithionite and 12mM KCL. The pH is then adjusted to 6.5.

Statistical Analysis
Data are expressed as mean±SE. Statistical significance was determined by unpaired t test on experiments comparing two groups or ANOVA and Tukey test for experiments involving four comparisons using the online site vassarstats.net. P values <0.05 were considered significant.
Online Figure I

2 months old | 10 days | 2 weeks | 3 months old
Male WT and IP Tamoxifen | wash out | I/R surgery
GRK2 fKO mice daily 40mg/kg

Online Figure I. Timeline of age, tamoxifen injection schedule and surgery for WT and GRK2 fibroblast knockout (GRK2 fKO) male mice. Two month old male WT and GRK2 fKO mice were injected intraperitoneally (IP) with 40 mg/kg of tamoxifen daily for 10 consecutive days. Following the conclusion of the tamoxifen regimen, mice were allowed to recover for 2 weeks before undergoing ischemia/reperfusion (I/R) surgery.
**Online Figure II.** Representative images of the infarct zone of myeloperoxidase (MPO) staining on heart sections of Sham, 6 and 24 hours post I/R, corresponding to the quantification data in Figure 6c. Scale bar, 50 μm.
Online Figure III. Neutrophil invasion following I/R injury in WT and GRK2 myocyte specific knockout mice. A) Representative images of myeloperoxidase (MPO) staining on heart sections of Sham, Border Zone and Infarct Zone 6 hours post I/R. Scale bar, 50 μm. B) Quantification of MPO positive cells per field in the border zone and infarct area 6 hours following ischemic injury. *P<0.05 vs. Sham, **P<0.01 vs. Sham. N=3-8 per group.
Online Figure IV. CXCL chemokine mRNA levels in WT and GRK2 fKO hearts 6 hours post I/R. mRNA levels of A) CXCL1, B) CXCL2 and C) CXCL5 levels measured by RT-PCR normalized to 18S, fold change vs WT Sham. *p<0.05 vs. Sham, **p<0.01 vs. Sham, ***p<0.001 vs. Sham. N=3-6 per group.
Online Figure V. Results from the Mouse Cytokine Microarray Kit. A) Licor scans of nitrocellulose membranes incubated with conditioned media from freshly isolated WT and GRK2fKO cardiac fibroblasts that were subjected to in vitro I/R. B) Signal average from panel A for the coordinates corresponding to each cytokine spotted in duplicate on the membrane.
Supplemental References


