Acute Rosiglitazone Treatment is Cardioprotective against Ischemia/Reperfusion Injury by Modulating AMPK, Akt, and JNK Signaling in Non-diabetic Mice

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Running title: Cardioprotective effects of acute rosiglitazone treatment

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ABSTRACT

Rosiglitazone (RGZ), a PPAR-γ agonist, has been demonstrated to possess cardioprotective properties during ischemia/reperfusion (I/R). However, this notion remains controversial as recent evidence has suggested an increased risk in cardiac events associated with long-term use of RGZ in patients with type 2 diabetes. In this study, we tested the hypothesis that acute RGZ treatment is beneficial during I/R by modulating cardioprotective signaling pathways in a non-diabetic mouse model. RGZ (1µg/g) was injected intravenously (i.v.) via tail vein 5 min before reperfusion. Myocardial infarction was significantly reduced in mice treated with RGZ compared to vehicle controls (8.7% ± 1.1% vs. 20.2% ± 2.5%, $P < 0.05$). Moreover, isolated hearts were subjected to 20 min of global, no-flow ischemia in an ex vivo heart perfusion system. Post-ischemic recovery was significantly improved with RGZ treatment administered at the onset of reperfusion compared to vehicle ($P < 0.001$). The immunoblotting data revealed that the levels of both p-AMPK (Thr$^{172}$) and p-Akt (Ser$^{473}$) were significantly up-regulated when RGZ was administered 5 min before reperfusion compared to vehicle. On the other hand, inflammatory signaling, p-JNK (Thr$^{183}$/Tyr$^{185}$), was significantly down-regulated as a result of RGZ treatment compared to vehicle ($P < 0.05$). Intriguingly, pre-treatment with the selective PPAR-γ inhibitor GW-9662 (1µg/g i.v.) 10 min before reperfusion significantly attenuated these beneficial effects of RGZ on the ischemic heart. Taken together, acute treatment with RGZ can reduce ischemic injury in a non-diabetic mouse heart via modulating AMPK, Akt, and JNK signaling pathways that is dependent upon PPAR-γ activation.

Key words: Thiazolidinedione, PPAR-γ, myocardial infarction
INTRODUCTION

Ischemic heart disease remains the number one leading cause of death for patients with and without diabetes (7). Current interventions rely on the rapid recanalization of an occluded coronary artery with percutaneous intervention, thrombolytics, or anticoagulants. However, these treatments are accompanied by an unfavorable consequence known as reperfusion injury. As this is recognized as a major clinical problem, potential therapies pertaining to cardiac metabolism, inflammation, oxidative stress, and apoptosis have yet to be implemented (43). Therefore, there is an increasing need for therapeutic strategies that limit myocardial ischemia/reperfusion (I/R) injury (37).

Rosiglitazone (RGZ), a Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) agonist indicated for the treatment of type 2 diabetes has been shown to possess cardioprotective properties in vivo during I/R (1, 23, 36) and in patients after undergoing percutaneous coronary intervention (40). However, the use of this drug remains controversial as recent meta-analyses and clinical trials have indicated that long-term use of RGZ in type 2 diabetic patients is associated with an increased risk of heart failure and myocardial infarction (54).

Despite the negative effects that have been associated with RGZ, recent evidence suggests this thiazolidinedione (TZD) can modulate targets aimed at mitigating I/R injury. Previous studies have shown that RGZ and other TZDs can activate AMPK (4, 9, 18, 48), a master metabolic regulator that has recently been demonstrated to be a cardioprotective stress activated protein kinase during I/R (22, 24, 29, 32). In addition, RGZ has been shown to activate Akt, a pro-survival and anti-apoptotic protein, during I/R in the heart and cardiomyocytes (16, 49, 51). Moreover, RGZ and PPAR-γ activation have been demonstrated to possess anti-
inflammatory properties in patients (25), cardiomyocytes (34), and in animal models of I/R injury, by decreasing MCP-1 and TNF-α levels, NF-κB activity, and leukocyte invasion (14, 50).

Interestingly, although the negative effects of RGZ have been seen with chronic use in type 2 diabetic patients, there is evidence that RGZ has beneficial cardiovascular effects in non-diabetic patients (3, 30, 42). Such studies report that RGZ increases plasma adiponectin concentrations, an adipocytokine that has been shown to have insulin sensitizing effects as well as protective effects against myocardial I/R injury (32, 36, 44). It has also been demonstrated that RGZ treatment possesses cardioprotective effects in non-diabetic animal models of I/R (23, 36, 50). Intriguingly, other studies report that RGZ appears to have cardioprotective effects when administered acutely to non-diabetic animals (2, 50). Furthermore, acute RGZ treatment to non-diabetic patients can result in rapid vasodilation, indicating the potential significance of its acute effects (39).

In this study we aimed to determine whether acute RGZ treatment is cardioprotective in non-diabetic mice against I/R injury and to investigate whether the mechanism is via a PPAR-γ-dependent process. We attempt to provide evidence that RGZ can be used as a cardioprotective drug for acute ischemic events in patients without diabetes.
MATERIALS AND METHODS

Experimental Animals

Male FVB/NJ mice, 4-6 months of age were used in all experiments. All animal procedures carried out in this study were approved by the University at Buffalo-SUNY Institutional Animal Care and Use Committee.

Experimental Myocardial Infarction

Mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.), placed on a ventilator (Harvard Rodent Ventilator, Harvard Apparatus, Holliston, Mass), and core temperature was maintained at 37°C with a heating pad. After left lateral thoracotomy, the left anterior descending coronary artery was occluded for 20 minutes with an 8–0 nylon suture and polyethylene tubing to prevent arterial injury and then reperfused for 4 hours. The ECG (ADInstruments Inc, Colorado Springs, CO) showing ST-segment elevation and blanching of the left ventricle confirmed myocardial ischemia as a result of coronary occlusion. Vehicle (1:3 DMSO:saline), or rosiglitazone (1 μg/g (50), Enzo Life Sciences) was injected via tail vein 5 minutes before reperfusion. Compound C (10 μg/g (20), Enzo Life Sciences) was injected i.p. 30 minutes prior to coronary occlusion. The hearts were then excised, perfusion fixed, and stained to delineate the extent of myocardial necrosis as a percent of non-perfused ischemic area at risk (22). Viable tissue in the ischemic region was stained red by 2,3,5-triphenyltetrazolium, and the non-ischemic region was stained blue with Evans blue dye (22). Hearts were fixed and sectioned, and photographed with a Leica microscope, and analyzed with the National Institute of Health’s Image J Software.
Mouse Heart Perfusion and Measurement of Cardiac Function

Mice were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and hearts were excised and placed in the Langendorff perfusion mode with KHB buffer containing 7 mmol/L glucose, 0.4 mmol/L oleate, 1% BSA, and 10 µU/mL insulin. Hearts were perfused at a flow of 4 ml/min and a fluid-inflated balloon connected to the LabChart5 system (ADInstruments) was inflated to achieve an end diastolic pressure of 5 mmHg that was kept constant during the baseline measurement of cardiac function. Global, “no flow” ischemia was induced by the termination of flow to the heart for 20 minutes and reperfusion was reinstated for 30 minutes at 4 ml/min. RGZ (5 µM) treatment or vehicle (DMSO) was administered at the onset of reperfusion. Cardiac function was measured as a heart rate-left ventricular developed pressure product (mmHg·beats·min⁻¹) (17, 24).

In vivo Regional Ischemia

Mice were anesthetized with sodium pentobarbital (100 mg/kg i.p.), placed on a ventilator (Harvard Rodent Ventilator, Harvard Apparatus, Holliston, Mass), and core temperature was maintained at 37°C with a heating pad. After left lateral thoracotomy, the left anterior descending coronary artery was occluded for 20 minutes followed by 10 minutes of reperfusion. An ECG and blanching of the left ventricle confirmed ischemic repolarization changes (ST-segment elevation) during coronary occlusion (ADInstruments Inc, Colorado Springs, CO). Rosiglitazone (1 µg/g) or vehicle (1:3 DMSO:saline) was injected via tail vein 5 minutes before reperfusion. GW-9662 (1 µg/g (53), Enzo Life Sciences) was injected via tail vein 10 minutes before reperfusion. All hearts were then rapidly excised, and the ischemic region of the left ventricle (LV) was freeze-clamped in liquid nitrogen for biochemical analysis.

Immunoblotting
Immunoblots were performed as previously described (21). Heart homogenates were resolved by SDS-PAGE and the proteins were transferred onto polyvinylidene difluoride membranes. For reprobing, membranes were stripped with 50 mmol/L Tris-HCl, 2% SDS, and 0.1 mol/L β-mercaptoethanol (pH 6.8). Rabbit polyclonal antibodies against phospho-AMPK, total AMPK, phospho-Akt, phospho-JNK, and total JNK were purchased from Cell Signaling. Rabbit polyclonal antibodies against total Akt were obtained from Santa Cruz. Anti-rabbit secondary antibodies were purchased from Cell Signaling.

**Statistical Analysis**

Values are means ± S.E. Data were analyzed by two-tailed, unpaired Student’s t test. A value of $P < 0.05$ was considered statistically significant.
RESULTS

Acute RGZ treatment decreases myocardial infarction.

In order to determine the ability of RGZ to protect against myocardial infarction, non-diabetic mice were subjected to 20 minutes of ischemia followed by 4 hours of reperfusion. A single dose of RGZ (1 μg/g) or vehicle was injected intravenously (i.v.) via tail vein 5 minutes before reperfusion. Myocardial infarction was significantly reduced in mice treated with RGZ compared to vehicle (8.7% ± 1.1% vs. 20.2% ± 2.5%, respectively, $P < 0.05$). Infarct area was determined as a ratio of the ischemic area at risk (AAR) (Figure 1).

RGZ administered at the onset of reperfusion improves post-ischemic recovery

To further confirm that RGZ exhibits protective effects against I/R injury, mouse hearts were subjected to Langendorff heart perfusion. Hearts were first perfused for 20 minutes in order to get a stable baseline measurement, followed by 20 minutes of global, no-flow ischemia, followed by 30 minutes of reperfusion. Post-ischemic recovery was significantly improved with RGZ administered at the onset of reperfusion compared to vehicle ($P < 0.001$), as indicated by the dramatic increase in the heart rate-LV pressure product (mmHg·beats·min$^{-1}$) (Figure 2).

Cardioprotective signaling activated by acute RGZ treatment

Since RGZ has previously been shown to activate AMPK and Akt (18, 51), we wanted to investigate whether acute treatment would activate such cardioprotective pathways. In order to investigate these mechanisms in vivo, mouse hearts were subjected to 20 minutes of ischemia and 10 minutes of reperfusion. RGZ (1 μg/g i.v.) or vehicle was injected 5 minutes before reperfusion (Figure 3). The results demonstrated that phosphorylation of AMPK at Thr$^{172}$ of the alpha catalytic subunit was significantly up-regulated when RGZ treatment was administered compared to vehicle after I/R (Figure 3, $P < 0.01$). Confirmation of AMPK activation was
determined by the phosphorylation of downstream acetyl-coA carboxylase (p-ACC) (Figure 3). Furthermore, another cardioprotective signaling protein, p-Akt (Ser<sup>473</sup>) was also significantly up-regulated after I/R as a result of RGZ treatment compared to vehicle (Figure 4, \( P < 0.05 \)). In order to address whether these effects were dependent or independent on PPAR-\( \gamma \) activation, mice were pre-treated with GW-9662 (GW), a selective PPAR-\( \gamma \) receptor antagonist (19), 10 minutes before reperfusion (Figures 3, 4 and 5). When RGZ was administered following GW-9662, AMPK and Akt activation was significantly blunted after I/R compared to RGZ alone (Figure 3, \( P < 0.05 \); Figure 4, \( P < 0.05 \), respectively). There appeared to be no effect of RGZ or vehicle during sham operation. However, there was a slight increase in p-AMPK when RGZ was administered during sham operation (Figure 3, \( P = \text{NS} \)).

It has also been demonstrated that RGZ promotes cardioprotection through the activation of endothelial nitric oxide synthase (eNOS) (10). Therefore we sought to determine whether acute RGZ treatment at this dose would be able to enhance the phosphorylation of eNOS at Ser<sup>1177</sup>. RGZ administered at the onset of reperfusion appeared to have no significant effect, although there was a modest increase in p-eNOS seen when RGZ was given during the sham condition (Figure 5, \( P = \text{NS} \)).

**Acute RGZ treatment inhibits JNK activation**

We next wanted to determine whether acute RGZ treatment would be able to attenuate JNK activation due to its role in inflammation and apoptosis as a result of I/R in the heart (15, 27). The results revealed that p-JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>) was significantly down-regulated when RGZ was administered during ischemia 5 minutes before reperfusion compared to vehicle alone (Figure 6, \( P < 0.05 \)). Furthermore, when GW-9662 was administered prior to RGZ, the ability of RGZ to inhibit JNK phosphorylation was abolished (Figure 6, \( P < 0.01 \)).
Inhibiting AMPK limits the ability of RGZ to reduce myocardial infarction

In order to examine whether AMPK activation is necessary for RGZ-induced cardioprotection against myocardial infarction, we treated mice with compound c (10 μg/g i.p. (20)), an AMPK inhibitor (52), 30 minutes prior to myocardial ischemia. Interestingly, pre-treatment with compound C followed by RGZ administration 5 minutes before the onset of reperfusion largely inhibited the reduction in infarct size seen compared to RGZ treatment alone (Figure 7), suggesting that AMPK activation is significantly related to RGZ-induced cardioprotection.
DISCUSSION

Compared to the other clinically used anti-diabetic TZD compounds, RGZ is the most potent which makes it an ideal candidate for studying its effects against I/R injury. Of the three PPARs expressed in the heart (PPAR-\(\alpha\), \(\beta/\delta\), \(\gamma\)), PPAR-\(\gamma\) is expressed at the lowest abundance (33) and our study reveals the potential importance of activating this receptor during acute ischemic events. In this study, we have demonstrated that a single dose of RGZ administered before the onset of reperfusion is efficient at limiting myocardial I/R injury, and improving post-ischemic cardiac function in the non-diabetic mouse heart. The improvement in post-ischemic recovery seen with RGZ infusion at the onset of reperfusion is consistent with previous reports suggesting that RGZ can strengthen cardiac hemodynamics (38). Intriguingly, the dose of 1\(\mu\)g/g (~1.4 mM) used to achieve cardioprotection in mice by RGZ in this study is highly relevant and is lower than the dose used clinically to reduce hyperglycemia in type 2 diabetic patients (4-8 mg/70kg man/day, ~2.8 mM).

We have also further elucidated the molecular mechanisms whereby RGZ is able to modulate cardioprotective signaling pathways that appears to be dependent on PPAR-\(\gamma\) receptor activation. First, our results suggest that one of the mechanisms contributing to cardioprotection is by activating AMPK. This result was further confirmed by pre-treatment with the AMPK inhibitor compound C that largely attenuated the cardioprotective effects seen with RGZ, implying that AMPK activation is causatively related to the beneficial acute actions of RGZ. Our group and others have recently shown that AMPK is cardioprotective during ischemia by enhancing glucose uptake and glucose transporter-4 (GLUT4) translocation (24), decreasing apoptosis, enhancing post-ischemic recovery, and limiting myocardial infarction (22, 29).

Secondly, we have demonstrated that acute RGZ treatment can activate Akt, which has been
previously shown to reduce ischemic injury (49, 51). Other TZDs such as pioglitazone have also 
been shown to protect against myocardial infarction by activating Akt (47). Both of these 
pathways are known to stimulate GLUT4 translocation to the sarcolemmal membrane, thus 
further studies are needed to determine the effects of RGZ eliciting a potential synergistic effect 
on myocardial glucose metabolism during I/R in the non-diabetic heart. Taking this into 
consideration, studies expressing dominant negative forms of AMPK and PI3K in 
cardiomyocytes that were exposed to oxidative stress with hydrogen peroxide found complete 
abrogation of GLUT4 translocation to the membrane, suggesting the significance of both 
pathways in response to metabolic stress (12).

Acute RGZ treatment also significantly decreased JNK phosphorylation suggesting that 
RGZ appears to be mitigating the inflammatory response against I/R injury. In parallel with 
these results, the inhibition of JNK signaling has been demonstrated to be protective against I/R 
by limiting apoptosis in endothelial cells (46). There is also evidence suggesting that RGZ can 
inhibit JNK activation in adipocytes through a PPAR-γ-dependent mechanism (8). In addition, 
AMPK activation by the adipokine CTRP13 has been shown to limit JNK signaling in response 
to metabolic stress (45).

Most interestingly, our results show that when AMPK phosphorylation is up-regulated, 
JNK phosphorylation is down-regulated when RGZ is given prior to reperfusion. These findings 
are in agreement with previous studies as the relationship between AMPK and JNK signaling in 
the heart is beginning to be understood. For instance, a recent study has indicated that 
pharmacological AMPK activation was highly effective at reducing and protecting endothelial 
cells against ROS-induced activation of JNK (31). This is consistent with the results 
demonstrated by Qi et al. showing that there is greater JNK activation in AMPK deficient mouse
hearts and that inhibiting the JNK pathway is cardioprotective during I/R (27). Furthermore, a recent paper from our group demonstrated that pharmacological activation of AMPK by activated protein C can attenuate JNK signaling and protect the heart against I/R injury (41). A study by Khandoudi et al. also suggests that inhibiting the JNK pathway is related to the improved post-ischemic hemodynamics seen with RGZ infusion \textit{ex vivo} (15). The inhibition of JNK signaling by RGZ in this case may also be a contributing factor toward improved cardiac function.

As mentioned previously, acute RGZ treatment was shown to rapidly induce vasodilation in a small population of healthy non-diabetic male patients (39). This led us to hypothesize that another cardioprotective signaling mechanism due to RGZ treatment would be through the phosphorylation of eNOS. Studies have shown that RGZ has been able to up-regulate the synthesis of nitric oxide (NO) (10) and another recent finding suggests that RGZ’s ability to stimulate NO is through an AMPK-dependent mechanism (4). In our study, we looked at p-eNOS (Ser$^{1177}$) levels after acute RGZ treatment prior to reperfusion and there appeared to be no significant increase in eNOS phosphorylation. However, there was a slight increase in p-eNOS during the sham condition that did not reach significance at the dose of 1$\mu$g/g.

It is possible, however, that RGZ may be exerting its cardioprotective functions through its ability to act as an antioxidant. This may be particularly true for our results obtained \textit{ex vivo} as RGZ is suggested to improve post-ischemic cardiac function by limiting ROS production (26) besides modulating the cardioprotective AMPK signaling pathway (28). Recent studies have provided evidence for which RGZ protects cardiomyocytes against oxidative stress by up-regulating Bcl-2 (28). Moreover, RGZ administration to rats reduced oxidative stress and increased the activity of superoxide dismutase in the vasculature (26). Findings by Tao et al.
demonstrated that RGZ possesses vasculoprotective properties by reducing superoxide,
nitrotyrosine, and total NO levels (35). Interestingly, these studies administer RGZ to non-
diabetic animal models of I/R and pronounced effects were seen. A recent paper also suggests
that RGZ’s ability to act as an antioxidant is through its ability to activate AMPK that will in turn
prevent hyperactivity of NADPH in endothelial cells (6). However, whether acute RGZ
treatment in vivo can be antioxidative against I/R injury remains elusive.

Taken together, this study demonstrates that by using RGZ acutely in the incidence of I/R,
myocardial infarction is decreased, and post-ischemic cardiac function is improved by
modulating AMPK, Akt, and JNK signaling mechanisms in a non-diabetic mouse heart. Forms
of therapy such as RGZ have recently become attractive in the case of a non-diabetic patient as
clinical studies have indicated that developed hyperglycemia as a result of acute myocardial
infarction is a strong predictor of morbidity and mortality in patients with and without diabetes
(11, 13). In parallel with our study, Calvert et al. have demonstrated that an acute low-dose of
metformin, another AMPK activator, reduces myocardial infarction in non-diabetic mice (5).
Although the use of RGZ pertains exclusively to patients with type 2 diabetes, the use of acute
RGZ therapy to non-diabetic patients may be a potential beneficial treatment for I/R injury.
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prostaglandin J2 and ciglitazone, in reperfusion injury: role of nuclear factor-kappaB, heat

Figure legends

**Figure 1.** RGZ reduces myocardial infarct size after I/R. *In vivo* hearts were subjected to 20 min of ischemia followed by 4 h reperfusion. RGZ (1 μg/g) or vehicle was administered via tail vein 5 min before reperfusion. The extent of myocardial necrosis was assessed as described under “Methods”. (A) Representative sections of the extent of myocardial infarction. (B) The ratio of area at risk (AAR) to the total myocardial area (left panel) and the ratio of infarct area to AAR (right panel). Values are means ± S.E. for 5 independent experiments. *P < 0.05 vs. vehicle.

**Figure 2.** RGZ ameliorates post-ischemic cardiac dysfunction. The heart rate and left ventricular pressure during baseline perfusion and post-ischemic reperfusion with or without administration of RGZ were assessed as described under “Methods”. RGZ (5 μM) or vehicle treatment (DMSO) was initiated at the onset of reperfusion. Cardiac function is presented as a heart rate-left ventricular developed pressure product (mmHg·beats·min⁻¹). Values are means ± S.E., n=4-6 hearts for each group. *P < 0.001 vs. vehicle during reperfusion.

**Figure 3.** RGZ stimulates AMPK activation during *in vivo* I/R. RGZ treatment during regional ischemia followed by reperfusion stimulates phosphorylation of both AMPK and downstream acetyl CoA carboxylase (ACC) as shown by the immunoblots. Pre-treatment with the PPARγ receptor inhibitor, GW-9662, can block the induction of both AMPK and ACC phosphorylation by RGZ. Values are means ± S.E., n=3. *P < 0.05 vs. I/R+vehicle; †P < 0.05 vs. I/R+RGZ.

**Figure 4.** RGZ stimulates Akt phosphorylation during *in vivo* I/R. RGZ treatment during regional ischemia followed by reperfusion stimulates Akt phosphorylation during I/R in the heart. Pre-treatment with the PPARγ receptor inhibitor, GW-9662, can block the induction of Akt phosphorylation.
phosphorylation by RGZ. Values are means ± S.E., n=3. *\(P<0.05\) vs. I/R+vehicle; †\(P<0.05\) vs. I/R+RGZ.

Figure 5. Effect of RGZ on eNOS phosphorylation during in vivo I/R. RGZ treatment during regional ischemia followed by reperfusion doesn’t significantly affect eNOS phosphorylation during I/R in the heart. Pre-treatment with the PPAR\(\gamma\) receptor inhibitor, GW-9662, however did modestly inhibit eNOS phosphorylation by RGZ. Values are means ± S.E., n=3.

Figure 6. RGZ treatment inhibits the activation of c-Jun N-terminal protein kinase (JNK) during in vivo I/R. RGZ treatment attenuates cardiac JNK phosphorylation during I/R in the heart, while pre-treatment with the PPAR\(\gamma\) receptor inhibitor, GW-9662, can attenuate the inhibition of JNK phosphorylation by RGZ. Values are means ± S.E., n=3. *\(P<0.01\) vs. Sham; †\(P<0.05\) vs. I/R+ vehicle; #\(P<0.01\) vs. I/R+RGZ.

Figure 7. AMPK signaling mediates RGZ-induced cardioprotection against myocardial infarction. In vivo hearts were subjected to 20 min of ischemia followed by 4 h reperfusion. Compound c (10 \(\mu\)g/g i.p.) or vehicle was administered 30 minutes prior to myocardial ischemia. RGZ (1 \(\mu\)g/g) or vehicle was administered via tail vein 5 min before reperfusion. The extent of myocardial necrosis was assessed as described under “Methods”. (A) Representative sections of the extent of myocardial infarction. (B) The ratio of area at risk (AAR) to the total myocardial area (left panel) and the ratio of infarct area to AAR (right panel). Values are means ± S.E. for 3-5 independent experiments. *\(P<0.05\) vs. vehicle without Compound C.
Ischemia
Vehicle or RGZ
Reperfusion
20 min
4 hours

A

1.00 mm
Vehicle
RGZ

B

AAR/Total Myocardium

Infarct size/AAR

Vehicle
RGZ

Figure 1
Figure 3

Sham I/R (20 min/10 min)

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- p-AMPK (Thr^{172})
- p-ACC (Ser^{79})
- AMPKα

Figure 3
Figure 4
Reperfusion

Vehicle or RGZ

GW-9662

Ischemia

20 min

Reperfusion

10 min

Sham I/R (20 min/10 min)

GW-9662

RGZ

Vehicle

-p-eNOS (Ser\textsuperscript{1177})

-eNOS

-p-eNOS

(relative units)

-  +  -  -  +  +  -  -  -  -

Vehicle

RGZ

GW-9662

Sham

I/R (20 min/10 min)

Figure 5
Figure 6
Vehicle or Compound C  

Vehicle or RGZ

**Ischemia**

30 min  

20 min  

4 hours

**Reperfusion**

A

![Images of tissue samples comparing Vehicle and Compound C treatment](Figure 7)

B

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**AAR/Total Myocardium**

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**Infarct size/AAR**

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* indicates significance.