Acute rosiglitazone treatment is cardioprotective against ischemia-reperfusion injury by modulating AMPK, Akt, and JNK signaling in nondiabetic mice

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Submitted 8 February 2011; accepted in final form 6 June 2011

Morrison A, Yan X, Tong C, Li J. Acute rosiglitazone treatment is cardioprotective against ischemia-reperfusion injury by modulating AMPK, Akt, and JNK signaling in nondiabetic mice. Am J Physiol Heart Circ Physiol 301: H895–H902, 2011. First published June 10, 2011; doi:10.1152/ajpheart.00137.2011.—Rosiglitazone (RGZ), a peroxisome proliferator-activated receptor (PPAR)-γ agonist, has been demonstrated to possess cardioprotective properties during ischemia-reperfusion. 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 nondiabetic mouse model. RGZ (1 μg/g) was injected intravenously via the tail vein 5 min before reperfusion. Myocardial infarction was significantly reduced in mice treated with RGZ compared with 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. Postischemic recovery was significantly improved with RGZ treatment administered at the onset of reperfusion compared with vehicle (P < 0.001). Immunoblot analysis data revealed that the levels of both phospho-AMP-activated protein kinase (Thr172) and phospho-Akt (Ser473) were significantly upregulated when RGZ was administered 5 min before reperfusion compared with vehicle. On the other hand, inflammatory signaling [phospho-JNK (Thr183/Tyr185)] was significantly downregulated as a result of RGZ treatment compared with vehicle (P < 0.05). Intriguingly, pretreatment with the selective PPAR-γ inhibitor GW-9662 (1 μg/g iv) 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 nondiabetic mouse heart via modulation of AMP-activated protein kinase, Akt, and JNK signaling pathways, which is dependent on PPAR-γ activation.

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thiazolidinedione; peroxisome proliferator-activated receptor-γ; myocardial infarction

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, thrombolitics, 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 (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 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 has suggested this thiazolidinedione (TZD) can modulate targets aimed at mitigating I/R injury. Previous studies have shown that RGZ and other TZDs can activate AMP-activated protein kinase (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 prosurvival and antiapoptotic protein, during I/R in the heart and in cardiomyocytes (16, 49, 51). Moreover, RGZ and PPAR-γ activation have been demonstrated to possess anti-inflammatory properties in patients (25), cardiomyocytes (34), and animal models of I/R injury by decreasing monocyte chemoattractant protein-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 nondiabetic patients (3, 30, 42). Such studies have reported 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 nondiabetic animal models of I/R (23, 36, 50). Intriguingly, other studies (2, 50) have reported that RGZ appears to have cardioprotective effects when administered acutely to non-diabetic animals. Furthermore, acute RGZ treatment of nondiabetic 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 nondiabetic mice against I/R injury and to investigate whether the mechanism is via a PPAR-γ-dependent process. We attempt to provide evi-
dence 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 mo of age) were used in all experiments. All animal procedures carried out in this study were approved by the Institutional Animal Care and Use Committee of University at Buffalo-State University of New York.

Experimental myocardial infarction. Mice were anesthetized with pentobarbital sodium (100 mg/kg ip) and placed on a ventilator (Harvard Rodent Ventilator, Harvard Apparatus, Holliston, MA), and core temperature was maintained at 37°C with a heating pad. After a left lateral thoracotomy, the left anterior descending coronary artery was occluded for 20 min with an 8-0 nylon suture and polyethylene tubing to prevent arterial injury and then reperfused for 4 h. The ECG (AD Instruments, Colorado Springs, CO) showing ST segment elevation and blanching of the left ventricle (LV) confirmed myocardial ischemia as a result of coronary occlusion. Vehicle (1:3 DMSO-saline) or RGZ [1 μg/g (50), Enzo Life Sciences] was injected via tail vein 5 min before reperfusion. Compound C [10 μg/g (20), Enzo Life Sciences] was injected intraperitoneally 30 min before coronary occlusion. Hearts were then excised, perfusion fixed, and stained to delineate the extent of myocardial necrosis as a percentage of the nonperfused ischemic area at risk (AAR) (22). Viable tissue in the ischemic region was stained red by 2,3,5-triphenyltetrazolium, and the nonischemic region was stained blue with Evans blue dye (22). Hearts were fixed, sectioned, photographed with a Leica microscope, and analyzed with National Institutes of Health ImageJ software.

Mouse heart perfusion and measurement of cardiac function. Mice were deeply anesthetized with pentobarbital sodium (100 mg/kg ip), and hearts were excised and placed in the Langendorff perfusion mode with Krebs-Henseleit 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 (AD Instruments) was inflated to achieve an end-diastolic pressure of 5 mmHg, which 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 min, and reperfusion was reinstated for 30 min at 4 ml/min. RGZ (5 μM) treatment or vehicle (DMSO) was administered at the onset of reperfusion. Cardiac function was measured as the heart rate-LV developed pressure product (in mmHg·beats·min⁻¹) (17, 24).

In vivo regional ischemia. Mice were anesthetized with pentobarbital sodium (100 mg/kg ip) and placed on a ventilator (Harvard Rodent Ventilator, Harvard Apparatus), and core temperature was maintained at 37°C with a heating pad. After a left lateral thoracotomy, the left anterior descending coronary artery was occluded for 20 min followed by 10 min of reperfusion. An ECG and blanching of the

Fig. 1. Rosiglitazone (RGZ) reduces myocardial infarct size after ischemia-reperfusion (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 the tail vein 5 min before reperfusion. The extent of myocardial necrosis was assessed as described in MATERIALS AND METHODS. A: representative sections of the extent of myocardial infarction. B: ratio of the area at risk (AAR) to the total myocardial area (left) and ratio of the infarcted area to the AAR (right). Values are means ± SE for 5 independent experiments. *P < 0.05 vs. vehicle.
LV confirmed ischemic repolarization changes (ST segment elevation) during coronary occlusion (AD Instruments). RGZ (1 μg/g) or vehicle (1:3 DMSO-saline) was injected via the tail vein 5 min before reperfusion. GW-9662 [1 μg/g (53), Enzo Life Sciences] was injected via the tail vein 10 min before reperfusion. All hearts were then rapidly excised, and the ischemic region of the LV was freeze clamped in liquid nitrogen for biochemical analysis.

**Immunoblot analysis.** Immunoblots were performed as previously described (21). Heart homogenates were resolved by SDS-PAGE, and 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 phosphorylated (p-)AMPK, total AMPK, p-Akt, p-ACC, p-eNOS, total eNOS, p-JNK, and total JNK were purchased from Cell Signaling. Rabbit polyclonal antibodies against total Akt were obtained from Santa Cruz Biotechnology. Anti-rabbit secondary antibodies were purchased from Cell Signaling.

**Statistical analysis.** Values are means ± SE. Data were analyzed by a two-tailed, unpaired Student’s t-test. P values of <0.05 were considered statistically significant.

**RESULTS**

**Acute RGZ treatment decreases myocardial infarction.** To determine the ability of RGZ to protect against myocardial infarction, nondiabetic mice were subjected to 20 min of ischemia followed by 4 h of reperfusion. A single dose of RGZ (1 μg/g) or vehicle was injected intravenously via the tail vein 5 min before reperfusion. Myocardial infarction was significantly reduced in mice treated with RGZ compared with vehicle (8.7 ± 1.1% vs. 20.2 ± 2.5%, respectively, P < 0.05). The infarct area was determined as a ratio of the ischemic AAR (Fig. 1).

**RGZ administered at the onset of reperfusion improves postischemic 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 min to get a stable baseline measurement followed by 20 min of global, no-flow ischemia followed by 30 min of reperfusion. Postischemic recovery was significantly improved with RGZ administered at the onset of reperfusion compared with vehicle (P < 0.001), as indicated by the dramatic increase in the heart rate-LV pressure product (in mmHg·beats·min⁻¹; Fig. 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. To investigate these mechanisms in vivo, mouse hearts were subjected to 20 min of ischemia and 10 min of reperfusion. RGZ (1 μg/g iv) or vehicle was injected 5 min before reperfusion (Fig. 3). The results demonstrated that phosphorylation of AMPK at Thr₁⁷² of the α-catalytic subunit was significantly upregulated when RGZ treatment was administered compared with vehicle after I/R (P < 0.01; Fig. 3). Confirmation of AMPK activation was determined by phosphorylation of downstream acetyl-CoA carboxylase (Fig. 3). Furthermore, another cardioprotective signaling protein, p-Akt (Ser⁴⁷³), was also significantly upregulated after I/R as a result of RGZ treatment compared with vehicle (P < 0.05; Fig. 4). To address whether these effects were dependent or independent on PPAR-γ activation, mice were pretreated with GW-9662, a selective PPAR-γ receptor antagonist (19), 10 min before reperfusion (Figs. 3–5).

RGZ was administered after GW-9662, AMPK and Akt activation were significantly blunted after I/R compared with RGZ alone (P < 0.05, Fig. 3, and P < 0.05, Fig. 4, 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 [P = not significant (NS); Fig. 3].

It has also been demonstrated that RGZ promotes cardioprotection through the activation of endothelial nitric oxide (NO) 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 Ser¹⁷⁷. 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 (P = NS; Fig. 5).

**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 (Thr¹⁸³/Tyr¹⁸⁵) was significantly down-regulated when RGZ was administered during ischemia 5 min before reperfusion compared with vehicle alone (P < 0.05; Fig. 6). Furthermore, when GW-9662 was administered before RGZ, the ability of RGZ to inhibit JNK phosphorylation was abolished (P < 0.01; Fig. 6).

**Inhibition of AMPK limits the ability of RGZ to reduce myocardial infarction.** To examine whether AMPK activation is necessary for RGZ-induced cardioprotection against myocardial infarction, we treated mice with compound C [10 μg/g ip (20)], an AMPK inhibitor (52), 30 min before myocardial ischemia. Interestingly, pretreatment with compound C followed by RGZ administration 5 min before the onset of reperfusion largely inhibited the reduction in infarct size seen...
compared with RGZ treatment alone (Fig. 7), suggesting that AMPK activation is significantly related to RGZ-induced cardioprotection.

DISCUSSION

Compared with the other clinically used antidiabetic 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-α, PPAR-β/δ, and PPAR-γ), PPAR-γ 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 demonstrated that a single dose of RGZ administered before the onset of reperfusion is efficient at limiting myocardial I/R injury and improving postischemic cardiac function in the nondiabetic mouse heart. The improvement in postischemic recovery seen with RGZ infusion at the onset of reperfusion is consistent with a previous report (38) suggesting that RGZ can strengthen cardiac hemodynamics. Taking this into consideration, experiments expressing dominant negative forms of AMPK and phosphoinositol 3-kinase in cardiomyocytes that were exposed to oxidative stress with H$_2$O$_2$ found complete abrogation of GLUT4 translocation to the

We have also further elucidated the molecular mechanisms whereby RGZ is able to modulate cardioprotective signaling pathways that appear to be dependent on PPAR-γ activation. First, our results suggest that one of the mechanisms contributing to cardioprotection is by activation of AMPK. This result was further confirmed by pretreatment with the AMPK inhibitor compound C, which 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 (GLUT)4 translocation (24), decreasing apoptosis, enhancing postischemic recovery, and limiting myocardial infarction (22, 29). Second, we 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 activation of 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 nondiabetic heart.
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 mitigate 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 C1q/TNF-related protein-13 has been shown to limit JNK signaling in response to metabolic stress (45).

Most interestingly, our results show that when AMPK phosphorylation is upregulated, JNK phosphorylation is downregulated when RGZ is given before 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 (31) has indicated that pharmacological AMPK activation was highly effective at reducing and protecting endothelial cells against ROS-induced activation of JNK. This is consistent with the results demonstrated by Qi et al. (27) showing that there is greater JNK activation in AMPK-deficient mouse hearts and that inhibiting the JNK pathway is cardioprotective during I/R. Furthermore, a recent report from our group (41) demonstrated that pharmacological activation of AMPK by activated protein C can attenuate JNK signaling and protect the heart against I/R injury. A study by Khandoudi et al. (15) has also suggested that inhibition of the JNK pathway is related to the improved posts ischemic hemodynamics seen with RGZ infusion ex vivo. 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 nondiabetic male patients (39). This led us to hypothesize that another cardioprotective signaling mechanism due to RGZ treatment would be through the phosphorylation of eNOS. A study (10) has shown that RGZ is able to upregulate the synthesis of NO, and another recent finding (4) suggests that RGZ's ability to stimulate NO is through an AMPK-dependent mechanism. In our study, we examined at p-eNOS (Ser1177) levels after acute RGZ treatment before 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 μg/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 ex vivo as RGZ has been suggested to improve Fig. 6. RGZ treatment inhibits the activation of JNK during in vivo I/R. RGZ treatment attenuated cardiac JNK phosphorylation during I/R in the heart, whereas pretreatment with the PPAR-γ inhibitor GW-9662, attenuated the inhibition of JNK phosphorylation by RGZ. Values are means ± SE; n = 3.

Fig. 5. Effect of RGZ on endothelial nitric oxide synthase (eNOS) phosphorylation during in vivo I/R. RGZ treatment during regional ischemia followed by reperfusion did not significantly affect eNOS phosphorylation during I/R in the heart. Pretreatment with the PPAR-γ inhibitor GW-9662, however, did modestly inhibit eNOS phosphorylation by RGZ. Values are means ± SE; n = 3.

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postischemic cardiac function by limiting ROS production (26) besides modulating the cardioprotective AMPK signaling pathway (28). A recent study (28) provided evidence that RGZ protects cardiomyocytes against oxidative stress by upregulating Bcl-2. Moreover, RGZ administration to rats reduced oxidative stress and increased the activity of superoxide dismutase in the vasculature (26). Findings by Tao et al. (35) demonstrated that RGZ possesses vasculoprotective properties by reducing superoxide, nitrotyrosine, and total NO levels. Interestingly, these studies administered RGZ to nondiabetic animal models of I/R, and pronounced effects were seen. A recent report (6) has also suggested that RGZ’s ability to act as an antioxidant is through its ability to activate AMPK, which, in turn, prevents hyperactivity of NADPH in endothelial cells. However, whether acute RGZ treatment in vivo can be antioxidative against I/R injury remains elusive.

Taken together, the results of this study demonstrate that by using RGZ acutely in the incidence of I/R, myocardial infarction is decreased and postischemic cardiac function is improved by modulating AMPK, Akt, and JNK signaling mechanisms in the nondiabetic mouse heart. Forms of therapy such as RGZ have recently become attractive in the case of a nondiabetic patient, as clinical studies (11, 13) 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. In parallel with our study, Calvert et al. (5) demonstrated that an acute low-dose of metformin, another AMPK activator, reduces myocardial infarction in nondiabetic mice. Although the use of RGZ pertains exclusively to patients with type 2 diabetes, the use of acute RGZ therapy to nondiabetic patients may be a potential beneficial treatment for I/R injury.

**GRANTS**

This work was supported by American Diabetes Association Grant 1-11-BS-92 and by American Heart Association Grant SDG 0835169N.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
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