Rosiglitazone Treatment in Zucker Diabetic Fatty Rats Is Associated With Ameliorated Cardiac Insulin Resistance and Protection From Ischemia/Reperfusion-Induced Myocardial Injury

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The mechanism responsible for the enhanced myocardial susceptibility to ischemic insult in patients with type 2 diabetes is not clear. The present study examines the effect of rosiglitazone treatment on cardiac insulin sensitization and its association with cardioprotection from ischemia/reperfusion injury in an animal model of diabetes. Male Zucker diabetic fatty (ZDF) rats were treated with rosiglitazone (3 mg·kg⁻¹·day⁻¹ orally) or vehicle for 8 days before undergoing 30 min of coronary artery ligation, followed by reperfusion for 4 h (apoptosis) or 24 h (infarction). Rosiglitazone reduced the blood levels of glucose, triglycerides, and free fatty acids; enhanced cardiac glucose oxidation; and increased Akt phosphorylation (Akt-pS473) 2.1-fold and Akt kinase activity 1.8-fold in the ischemic myocardium. The phosphorylation of two downstream targets of Akt, glycogen synthase kinase-3β and FKHR (forkhead transcription factor), was also enhanced by 2- and 2.9-fold, respectively. In rosiglitazone-treated rats, the number of apoptotic cardiomyocytes and the myocardial infarct size were decreased by 58 and 46%, respectively, and the myocardial contractile dysfunction was improved. Blockade of the insulin-Akt signaling pathway by wortmannin in the 8-day rosiglitazone-treated ZDF rats resulted in a markedly diminished cardioprotective effect of rosiglitazone. In addition, 8-day rosiglitazone treatment in Zucker lean rats or 2-day rosiglitazone treatment in ZDF rats, both of which showed no change in whole-body insulin sensitivity, resulted in a significant reduction in cardiac infarct size, but to a lesser degree when compared with that observed in 8-day rosiglitazone-treated ZDF rats. These results suggest that chronic treatment with rosiglitazone protects the heart against ischemia/reperfusion injury in ZDF rats, and that the enhanced cardiac protection observed after rosiglitazone treatment might be attributable in part to an improvement in cardiac insulin sensitivity. Diabetes 54:554–562, 2005

Heart disease is the leading cause of death among patients with diabetes (rev. in 1). Relative to nondiabetic individuals, patients with diabetes demonstrate impaired recruitment of contractile reserve in noninfarct segment (2), greater reduction in global left ventricular (LV) function (3), and increased incidence of congestive heart failure (4) and death (5) after an index myocardial infarction. A significant increase in the number of necrotic cardiomyocytes in ventricular myocardial biopsies obtained from diabetic patients was also reported, reflecting a primary impairment in myocardial ischemic tolerance (6,7). Therefore, type 2 diabetes has been recognized as an independent risk factor for ischemic cardiomyopathy (8).

Insulin resistance is known as a central pathophysiological feature of type 2 diabetes. Insulin resistance in the heart has been demonstrated in obese rats, associated with decreased GLUT4 protein content and impaired GLUT4 translocation to the sarcolemma (9,10). Cardiac insulin resistance was also demonstrated in diabetic patients with cardiac diseases (11–13). A recent study used direct techniques to measure the response of the arterial-coronary sinus glucose balance to an increase in local intracoronary insulin concentration, and it demonstrated that cardiac muscles of type 2 diabetic patients are insulin resistant during fasting relative to those of nondiabetic control subjects (14). These data suggest that a potential underlying mechanism related to the increased cardiac susceptibility to ischemic insult in type 2 diabetic patients may be caused in part by cardiac insulin resistance. However, the impact of type 2 diabetes on the insulin response system of the myocardium in vivo and the increased susceptibility to ischemic injury have not been fully examined.

In contrast to the overwhelming clinical data indicating that the diabetic heart is more sensitive to ischemic injury, experimental evidence from a number of studies using animal models of diabetes showed no change (15) or an...
increase (16) or a decrease (17,18) in susceptibility to ischemia. The reasons for such diverse effects may be due to the difference in the length or severity of the diabetic state, the severity of the ischemic insult, or the levels of exogenous substrates (16). Additionally, a number of in vivo studies in nondiabetic animals (19–22) and ex vivo studies in diabetic animals (23,24) have shown the cardioprotective effect of thiazolidinediones. Although there is a clear beneficial effect of thiazolidinediones in protecting insulin-sensitive hearts from ischemia-reperfusion injury, the mechanism for this protection is less clear. It has been postulated that thiazolidinediones may inhibit the inflammatory response after such injury in the heart (20–22,24).

However, the degree of in vivo cardioprotection from ischemia/reperfusion insult and the role of insulin sensitization in the heart after thiazolidinedione treatment in diabetic animals is not known.

The aim of this study was to determine whether rosiglitazone, a peroxisome proliferator–activated receptor (PPAR-γ) agonist, protects the heart against ischemia/reperfusion injury in the Zucker diabetic fatty (ZDF) rat, and, if so, whether this cardioprotection is associated with an improvement in cardiac insulin sensitivity.

**RESEARCH DESIGN AND METHODS**

This study was conducted in accordance with the guidelines for care and use of laboratory animals of the U.S. National Institutes of Health. Male ZDF rats (ZDF/Gmi-fa/fa) and lean Zucker (fa/−) rats (Genetic Models) at age 12–14 weeks were used (n = 245). Unless otherwise indicated, rosiglitazone maleate (GlaxoSmithKline) was administered at 3 mg·kg−1·day−1 via oral gavage for 7 days, and the eighth dose was given 1 h before ischemia. The animals were premedicated with atropine sulfate (0.04 mg/kg intramuscularly) and then anesthetized with Nembutal (60 mg/kg i.p.; Abbott Laboratories). Trachea were intubated and ventilated with a Harvard small animal respirator. The electrocardiogram and the body temperature of the rats were monitored throughout the experiment. Coronary artery (left anterior descending coronary) occlusion (30 min) and reperfusion (4 h for apoptosis and 24 h for infarct and functional assessment) were induced by inflating and then deflating a nontraumatic balloon occluder that was fixed on the left anterior descending artery. The successful performance of coronary occlusion and reperfusion was verified by visual inspection of color in the apex and typical electrocardiogram changes.

**Assessment of cardiac glucose versus fat oxidation.** In a separate group of rosiglitazone- and vehicle-treated ZDF rats, indwelling carotid artery and jugular vein catheters were surgically implanted. Upon recovery from surgery, rats were placed before baseline non–oxygen-enriched hyperoxic cardiac glucose oxidation measurements. A normal glycemic/hyperglycemic state, the circulating levels of glucose, triglycerides, and free fatty acids in plasma were determined, and 10,000 myocytes per slide were counted. We determined the index of apoptosis, i.e., the number of TUNEL-positive myocytes divided by the total number of myocytes that were stained with anti–α-sarcomeric actin (Sigma). After washing, the slides were incubated with biotinylated secondary antibody, and the DNA ladder was electromediated with a Texas red avidin. For detection of cell death, tissue sections after permeability treatment were incubated with TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL)) assay with a cell death detection kit (Roche), as described previously (26). Briefly, the tissue sections after permeability treatment were incubated with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescent-dUTP (deoxyuridine triphosphate). For double labeling, the tissue sections after incubation with TUNEL reaction mixture and blockade of endogenous peroxidase, and non specific binding sites were incubated with monoclonal antibody against α-sarcomeric actin (Sigma). After washing, the slides were incubated with biotinylated secondary antibody, and the cardiomyocytes were finally visualized with Texas red avidin. For detection of total nucleolus, the slides were covered with the mounting medium containing 4',6-diamidino-2-phenylindole (DAPI). At least six slides per block were evaluated. For each slide, 15 fields were randomly chosen, and a total of 2.5–3 × 104 myocytes per slide were counted. We determined the index of apoptosis, i.e., the number of TUNEL-positive myocytes divided by the total number of myocytes that were stained with anti–α-sarcomeric actin (see Fig. 4C) per field.

**DNA ladder.** DNA ladder experiments were performed as described previously (27). In brief, ischemic myocardial tissues collected 4 h after reperfusion were minced in lysis buffer on ice, and proteinase K (100 µg/ml) was added. After incubation at 55°C with shaking for 18 h, DNA was extracted with phenol/chloroform three times, precipitated in ethanol, treated with DNA-free RNase, re-extracted, and precipitated again. DNA concentration was determined, and 10 µg of DNA was used for electrophoresis on a 1.8% agarose gel.

**Determination of myocardial infarction.** The ischemic area (area at risk) was distinguished from the area not at risk by Evans blue dye staining, and the infarcted portion of the myocardium (neurotic area) was determined by the triphenyl tetrazolium chloride method as described in detail previously (29). The three portions (i.e., area at risk, area at risk, and necrotic area) of the left ventricle were quantified by use of Image-Pro software.

**Assessment of myocardial contractile function.** LV pressure and the arterial blood pressure were measured using two 1.4 F Millar Mikrotip catheter transducers that were inserted into the LV cavity through the right carotid artery and the right femoral artery, respectively. LV pressure and arterial blood pressure were digitally processed via a hemodynamic analyzing system (Gould 3P 6600). Mean arterial blood pressure, LV systolic pressure, LV end diastolic pressure, and LV dp/dt were derived by computer algorithms (20).

**Statistical analysis.** Data are expressed as the means ± SE and analyzed by one-way ANOVA with subsequent post hoc paired comparisons or by unpaired Student’s t test. Differences with a value of P < 0.05 were considered statistically significant.

**RESULTS**

Rosiglitazone improves whole-body and cardiac insulin sensitivity in ZDF rats. As shown in Table 1, treatment with rosiglitazone for 8 days significantly reduced the circulating levels of glucose, triglycerides, and free [3-13C]alanine pool enrichment) pool depends on the extent of label dilution from unlabeled fat oxidation present. Therefore, the relative glucose oxidation (%) is defined as [4-13C]glutamate/[3-13C]alanine] × 100, and relative fat/ketone oxidation (%) is calculated as (1 – relative glucose oxidation) × 100.
Phosphorylation (Akt-pS473) in the ischemic myocardium.

Rosiglitazone activates Akt signal pathway in ischemia/reperfusion.

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>Free fatty acids (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zucker lean</td>
<td>137 ± 4</td>
<td>44 ± 2</td>
<td>0.9 ± 0.05</td>
</tr>
<tr>
<td>ZDF</td>
<td>235 ± 12*</td>
<td>504 ± 41*</td>
<td>1.7 ± 0.12*</td>
</tr>
<tr>
<td>ZDF + rosiglitazone</td>
<td>179 ± 13‡</td>
<td>303 ± 39‡</td>
<td>1.1 ± 0.06‡</td>
</tr>
</tbody>
</table>

Data are means ± SD. Blood samples were collected from the animals that were fasted overnight (15 h). The glucose, triglycerides, and free fatty acid measurements were performed on an Olympus AU640 chemistry analyzer. *P < 0.01 vs. Zucker lean rats; †P < 0.05 vs. Zucker lean rats; ‡P < 0.01 vs. ZDF rats (n = 14–15).

Rosiglitazone attenuates ischemia/reperfusion-induced myocardial apoptosis. Myocardial tissue from sham-operated ZDF rats exhibited no DNA ladder formation (Fig. 4A, lane 2), but clear DNA ladder formation was observed in myocardial tissue from the hearts subjected to ischemia/reperfusion and receiving vehicle (Fig. 4A, lanes 3–8). In contrast, DNA ladder formation was less clear or almost not detectable in the ischemic myocardium from six ZDF rats treated with rosiglitazone (Fig. 4A, lane 9–14).

**FIG. 1.** Relative cardiac glucose oxidation at baseline or during a normoglycemic-hyperinsulinemic clamp. The relative cardiac glucose oxidation was expressed as the percent of acetyl-CoA units oxidized by glucose versus fatty acid oxidation. The perfusion experiments were performed in awake ZDF rats and the measurements were performed in whole heart in vehicle- and rosiglitazone (ROSI)-treated animals (n = 8).
between the two groups (Fig. 5, left side). In rosiglitazone-treated lean rats, the ischemia/reperfusion-induced cardiac infarction was reduced by 26.5% compared with the vehicle group (P < 0.05, n = 8).

**Rosiglitazone improves ischemia/reperfusion-induced myocardial dysfunction.** Treatment with rosiglitazone for 8 days significantly improved cardiac functional contraction in ZDF rats at 24 h after reperfusion, as shown in Fig. 5B. The values of LV systolic pressure, +dP/dt, and −dP/dt were significantly enhanced in the rosiglitazone-treated group compared with the vehicle group (all P < 0.05, n = 9). Additionally, the mean arterial blood pressure was also improved in the rosiglitazone-treated group versus the vehicle-treated group, but no difference in the heart rate between the two groups was observed (data not shown).

**Wortmannin attenuates the cardioprotective effect of rosiglitazone.** As shown in Fig. 6, pretreatment of the ZDF rats with wortmannin, a phosphatidylinositol (PI) 3-kinase inhibitor, 15 min before reperfusion (26) markedly attenuated the cardioprotective effect of 8-day rosiglitazone treatment (>90% reduction). However, wortmannin alone had no effect on ischemia/reperfusion-induced myocardial infarction.

**Rosiglitazone cardioprotection is independent of insulin sensitization.** To further differentiate the non-insulin-related cardioprotective effect of rosiglitazone, a separate group of ZDF rats were treated with rosiglitazone at 3 mg · kg⁻¹ · day⁻¹ for 2 days. There was no difference in glucose infusion rates between the rosiglitazone-treated and vehicle-treated groups (15.5 ± 1.2 and 15.4 ± 0.6 mg · kg⁻¹ · min⁻¹, respectively) during a normoglycemic-hyperinsulinemic clamp (n = 10) (Fig. 7A), indicating that 2-day rosiglitazone treatment did not change insulin resistance in the rats. The ischemia/reperfusion-induced cardiac infarct size (% ischemic area) was reduced by 22.1%, from 58.6 ± 1.8% (vehicle) to 46.2 ± 1.7% (rosiglitazone; n = 9, P < 0.05) (Fig. 7B). This reduction was significantly less when compared with the 8-day rosiglitazone-treated group (P < 0.05). Additionally, there was no activation in myocardial level of Akt-pS473 in rosiglitazone-treated animals, as shown in Fig. 7C. The apoptotic myocytes in the ischemic myocardium of ZDF rats was 16.7 ± 5.6% (reduced by 21.6% compared with the vehicle group).

**DISCUSSION**

Treatment with rosiglitazone for 8 days in ZDF rats resulted in a decrease in the in vivo ischemia/reperfusion-induced cardiac infarct size by 46% and apoptosis by 58%. This cardiac protection was associated with improved LV function as well as increased cardiac insulin sensitivity. There was a significant increase in insulin-sensitized cardiac glucose oxidation and a reduction in fat/ketone oxidation in rosiglitazone-treated ZDF rats versus the vehicle group. Concomitantly, a significantly enhanced level of Akt signaling in myocardium was observed in rosiglitazone-treated animals.

In ZDF rats, GLUT4 protein expression is decreased and insulin-stimulated cardiac glucose uptake is impaired (10,23). Previous studies have argued for a potential role of glucose in cardioprotection from ischemic injury (28–30). It was shown that cardiac myocytes in the presence of glucose and glycolytic inhibitors such as 2-deoxyglucose...
or iodoacetate reversed the protective effect of glucose on hypoxic injury (28). In isolated rat hearts, cardiac output after ischemia/reperfusion was increased when glucose was added to lactate in the absence of insulin (29). Conversely, if glucose is absent in the presence of hypoxia for 30 min followed by 60 min of reperfusion, LV function is impaired to a greater extent (30). A recent in vitro study has demonstrated that rosiglitazone treatment results in the normalization of insulin-stimulated glucose uptake in isolated perfused obese Zucker rat hearts (23). As a result, the increased insulin-sensitized cardiac glucose oxidation observed in the present study may in part contribute to the cardioprotection by rosiglitazone in the ZDF rats. However, it is also known that ischemia/reperfusion results in an increase in free fatty acid levels, which aggravates the severity of myocardial infarction (31). Therefore, the cardioprotection observed after the 8-day rosiglitazone treatment in the present study might be indirectly mediated by lowering systemic free fatty acid levels.

Akt signaling has been widely recognized as an important downstream modulator of the insulin receptor, and it plays a critical role in maintaining insulin sensitivity in humans (32). Akt2-deficient mice show resistance to insulin’s effects on glucose metabolism and develop frank diabetes (33,34). A recent study reported that a mutation in the gene encoding the Akt2 in a family resulted in severe insulin resistance and diabetes, providing an example of a monogenic inherited defect in postreceptor insulin signaling that leads to human insulin resistance and diabetes (32). An increasing body of evidence also suggests that Akt signaling plays an important cytoprotective role against ischemic injury in the heart. In cultured rat neonatal cardiomyocytes, activation of Akt significantly reduced H2O2-induced apoptosis, and blockade of Akt signaling by PI 3-kinase inhibitor or overexpression of a dominant-negative mutant of PI 3-kinase abolished the protective effect of insulin (35). In isolated perfused hearts or in vivo rat studies, insulin administration reduced ischemia/reperfusion-induced infarct size, and this protective effect was abolished if the upstream or downstream signaling in the Akt pathway was inhibited by pharmacological inhibitor, suggesting that the cardioprotective effect of insulin is mainly mediated via Akt signaling (26). It has been reported that the cytoprotective effect of Akt signaling is attributable to the inhibition of GSK-3 and FKHR, two downstream targets of Akt known to be involved in cell death. GSK-3 is inactivated by Akt via phosphorylation at serine 9 or 21 (36). Both the in vitro and in vivo studies have demonstrated that inactivation of GSK-3 enhanced cell survival (37,38), improved postischemic cardiac function, and reduced infarct size (39). Phosphorylation of FKHR by Akt keeps this transcriptional factor retention in the cytoplasm and away from the nucleus, where it triggers activation of genes critical for inducing cell death (40). The present study demonstrated a significant increase in the levels of GSK-3β-pS9 and FKHR-pS256 in the ischemic myocardium from rosiglitazone-treated ZDF rats, further suggesting a role of the Akt signaling pathway in PPAR-γ-mediated cardioprotection. It is interesting to note in the current study that treatment with rosiglitazone did not change the total Akt level in the heart, but only the active form of Akt (Fig. 2A), suggesting that PPAR-γ-mediated activation of Akt may serve as a protective mechanism when the heart is under an insulting stress.

Previous studies have shown that PPAR-γ agonists protected the heart against ischemia/reperfusion injury in isolated perfused hearts (24) as well as in vivo in the nondiabetic pig (19) and rat (20–22). The enhanced insulin sensitivity by chronic treatment with troglitazone was found in nondiabetic pigs, and it was assumed to contribute to the improved recovery of LV function after cardiac ischemia (19). However, other actions of the PPAR-γ agonist that do not appear to be linked to insulin sensiti-
zation, such as anti-inflammatory activities (20–22) and inhibition of c-Jun NH₂-terminal kinase/activating protein 1 pathway (24), have been suggested as potential mechanisms responsible for PPAR-γ-mediated cardioprotection.

Nevertheless, the present study provides several lines of evidence suggesting a potential association between cardiac insulin resistance and enhanced susceptibility to ischemic insult. First, the ischemia/reperfusion-induced...
myocardial injury (apoptosis and infarction) was greater in the ZDF rats than in the lean controls, suggesting a role for insulin resistance in the enhanced myocardial susceptibility to ischemia. Second, a greater degree of cardioprotection by rosiglitazone was observed in ZDF versus lean controls, suggesting that the improved cardiac insulin sensitivity provided the additional degree of cardioprotection. This finding is consistent with our previous study in nondiabetic Lewis rats (20). The cardioprotection by rosiglitazone against ischemia/reperfusion injury in Lewis rats was significant (24% reduction versus vehicle) but much less compared with that observed in ZDF rats in the present study (46%) while the animals were treated with the same dose regimen (3 mg · kg\(^{-1}\) · day\(^{-1}\) orally for 8 days) (20).

To further differentiate the insulin-sensitizing from the non–insulin-sensitizing mechanism in the cardioprotective role of rosiglitazone, a 2-day rosiglitazone dosing regimen was also used in the present study. The lack of insulin sensitization in the ZDF rats under this 2-day dosing regimen was associated with significantly less cardioprotection by rosiglitazone against ischemia/reperfusion-induced myocardial infarction in ZDF and lean Zucker rats. Rosiglitazone was administered at 3 mg · kg\(^{-1}\) · day\(^{-1}\) via oral gavage for 8 days, and the last dose was given 1 h before ischemia. Animals were subjected to ischemia for 30 min and killed 24 h after reperfusion. The left side of the figure shows the ischemic area, expressed as percent left ventricle. The right side is the infarct size, expressed as percent ischemic area (n = 8–10).

FIG. 5. A: Protection by rosiglitazone (ROSI) against ischemia/reperfusion-induced myocardial infarction in ZDF and lean Zucker rats. Rosiglitazone was administered at 3 mg · kg\(^{-1}\) · day\(^{-1}\) via oral gavage for 8 days, and the last dose was given 1 h before ischemia. Animals were subjected to ischemia for 30 min and killed 24 h after reperfusion. The left side of the figure shows the ischemic area, expressed as percent left ventricle. The right side is the infarct size, expressed as percent ischemic area (n = 8–10). B: Rosiglitazone improves ischemia/reperfusion-induced cardiac dysfunction. The ZDF rats were treated with rosiglitazone or vehicle, as described in A, and the myocardial contractile function was determined at 24 h after reperfusion (n = 9). LVSP, LV systolic pressure.

FIG. 6. Treatment with wortmannin (WN) attenuates the cardioprotection by rosiglitazone (ROSI). The ZDF rats received rosiglitazone for 8 days, as described in the legend for Fig. 5. Wortmannin (15 μg/kg) or vehicle was administered intravenously 15 min before reperfusion. The animals were killed 24 h later, and the ischemic area and infarct size were determined (n = 8–10).
clear at present to what extent insulin- and non–insulin-related mechanisms play a role in the cardioprotection observed in the diabetic and nondiabetic condition. Further studies are necessary to clearly define and differentiate the direct role for both insulin- and non–insulin-related cardioprotective mechanisms.

In conclusion, chronic treatment with rosiglitazone protects the heart from ischemia/reperfusion-induced cardiac injury and improves cardiac contractile dysfunction in ZDF rats. The improvement in cardiac insulin sensitivity after an 8-day rosiglitazone treatment, as reflected by increased Akt signaling and glucose oxidation, was associated with this cardioprotection.

REFERENCES


FIG. 7. Effect of 2-day rosiglitazone (ROSI) treatment on whole-body insulin sensitivity (A), ischemia/reperfusion-induced myocardial infarction size (B), and cardiac Akt phosphorylation (C). ZDF rats were treated with vehicle or rosiglitazone (3 mg·kg⁻¹·day⁻¹, orally) for 2 days, and whole-body glucose disposal was measured during a normoglycemic-hyperinsulinemic clamp (n = 10) (A), or rats were subjected to myocardial ischemia (30 min) followed by reperfusion for 24 h to determine infarct size (n = 9, P < 0.05 vs. vehicle) (B) or reperfusion for 4 h to measure cardiac Akt phosphorylation (n = 4, lanes 3–6) (C). Lanes 1–2 are samples from the ZDF rats treated with rosiglitazone for 8 days. *P < 0.05 vs. vehicle. GIR, glucose infusion rate; P-Akt, Akt phosphorylation (Akt-s473).


