In Vivo Myocardial Protection From Ischemia/Reperfusion Injury by the Peroxisome Proliferator–Activated Receptor-γ Agonist Rosiglitazone

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Background—Diabetes is associated with increased risk of mortality as a consequence of acute myocardial infarction. This study determined whether rosiglitazone (ROSI) could reduce myocardial infarction after ischemia/reperfusion injury.

Methods and Results—Male Lewis rats were anesthetized, and the left anterior descending coronary artery was ligated for 30 minutes. After reperfusion for 24 hours, the ischemic and infarct sizes were determined. ROSI at 1 and 3 mg/kg IV reduced infarct size by 30% and 37%, respectively (P < 0.01 versus vehicle). Pretreatment with ROSI (3 mg · kg⁻¹ · d⁻¹ PO) for 7 days also reduced infarct size by 24% (P < 0.01). ROSI also improved ischemia/reperfusion-induced myocardial contractile dysfunction. Left ventricular systolic pressure and positive and negative maximal values of the first derivative of left ventricular pressure (dP/dt) were significantly improved in ROSI-treated rats. ROSI reduced the accumulation of neutrophils and macrophages in the ischemic heart by 40% and 43%, respectively (P < 0.01). Ischemia/reperfusion induced upregulation of CD11b/CD18 and downregulation of L-selectin on neutrophils and monocytes; these effects were significantly attenuated in ROSI-treated animals. Likewise, intercellular adhesion molecule-1 expression in ischemic hearts was markedly diminished by ROSI, as was the ischemia/reperfusion-stimulated upregulation of monocyte chemoattractant protein-1.

Conclusions—ROSI reduced myocardial infarction and improved contractile dysfunction caused by ischemia/reperfusion injury. The cardioprotective effect of ROSI was most likely due to inhibition of the inflammatory response. (Circulation. 2001;104:2588-2594.)

Key Words: ischemia ■ myocardial infarction ■ diabetes ■ PPAR-γ ■ rosiglitazone

Cardiovascular disease is the leading cause of death in diabetic patients.¹ It has been reported that diabetic patients develop congestive heart failure more readily and generally have a greater indication for adverse clinical prognosis and higher mortality. One of the potential mechanisms responsible for the excess mortality in diabetic patients is the greater myocardial injury in response to ischemia/reperfusion.² The increased mortality in patients with diabetes in the setting of acute myocardial infarction has been demonstrated in numerous studies, and the mortality after acute myocardial infarction in patients with diabetes is approximately twice that of nondiabetic patients.²⁻⁴ A significant increase in the number of necrotic cardiomyocytes in ventricular myocardial biopsies obtained from diabetic patients was reported recently.⁵

Rosiglitazone (ROSI, Avandia) is a peroxisome proliferator–activated receptor-γ (PPAR-γ) agonist and the most potent member of the thiazolidinedione antidiabetic agents and was recently approved by the FDA for treatment of type II diabetes mellitus. In animal models of insulin resistance, ROSI decreased plasma glucose, insulin, and triglycerides and also attenuated or prevented diabetic nephropathy and pancreatic islet cell degeneration. In patients with type 2 diabetes mellitus, ROSI acts as an insulin sensitizer, increasing insulin-mediated glucose disposal, as shown by decreases in fasting plasma glucose and glycosylated hemoglobin. Addition of ROSI to existing sulfonylurea, metformin, or insulin therapy achieved further reductions in fasting plasma glucose and glycosylated hemoglobin.⁶

The effect of ROSI or other thiazolidinedione members on severe ischemia/reperfusion myocardial injury in vivo is thus far unknown. Knowledge of this information would be very important, considering that the drug is used for a population with a high risk of developing acute myocardial infarction.

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The goal of the present study was to determine whether ROSI protects the heart from severe ischemia/reperfusion-induced myocardial injury in a well-established myocardial infarction model in the rat and if so, to explore underlying mechanisms.

Methods

Experimental Preparation
Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health. Male Lewis rats were anesthetized with isoflurane before surgery. Myocardial ischemia was produced by occlusion of the left anterior descending coronary artery (LAD) for 30 minutes, followed by reperfusion for 24 hours. The ischemic area (area at risk, AAR) was distinguished from the area not at risk (ANAR) by Evans blue dye staining, and the infarcted portion of the myocardium (necrotic area, NEC) was determined by the triphenyl tetrazolium chloride method as described previously. The 3 portions (ie, ANAR, AAR, and NEC) of the left ventricle (LV) were quantified by use of Image-Pro software. Unless otherwise indicated, ROSI was administered intravenously; half the dosage before occlusion and half immediately after reperfusion.

Assessment of Myocardial Contractile Function
A micropipet catheter transducer (Millar) was passed through the right carotid artery into the LV. The arterial blood pressure (ABP) was measured via a polyethylene catheter cannulated to the right femoral artery. LV pressure and ABP were digitally processed via a hemodynamic analyzing system (Gould 3P 6600). Mean ABP, LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), positive and negative maximal values of the first derivative of LVP (+dP/dt and −dP/dt), and heart rate were derived by computer algorithms. The cardiac contractile function was monitored continuously during the entire ischemic and early reperfusion period up to 4 hours. In a separate study, cardiac function was evaluated at 24 hours after reperfusion.

Determination of Myeloperoxidase Activity
Neutrophil accumulation in myocardium was investigated by measurement of myeloperoxidase (MPO) (a marker enzyme) as described previously.

Immunohistochemical Analysis for ED-1 (Macrophage Marker) and Intercellular Adhesion Molecule-1
Indirect immunohistochemical staining for macrophages was performed with a mouse monoclonal anti-rat ED-1 antibody (1:50) (Serotec), and staining for intercellular adhesion molecule-1 (ICAM-1) was performed with a mouse monoclonal anti-rat ICAM-1 antibody (0.5 μg/mL) (BD PharMingen). The sections were then incubated with a biotinylated anti-mouse IgG secondary antibody, and streptavidin-biotin-peroxidase for ED-1 or streptavidin-biotin, peroxidase for ICAM-1. Detection of antibody binding was with 3,3′-diaminobenzidine. The slides were counterstained with hematoxylin. The total number of macrophages (ED-1-positive cells) was quantified with Image-Pro software and expressed as percent of area of the field examined.

Flow Cytometric Analysis of Surface Expression of CD11b, CD18, and L-Selectin (CD62L) on Leukocytes
Fresh blood samples were collected from rats before ischemia (time 0) and 2, 4, 6, and 24 hours after reperfusion. Rat leukocyte populations isolated from the whole blood by erythrocyte lysis were incubated with FITC-conjugated monoclonal antibody against rat CD11b (1:8 dilution), rat CD18 (1:50 dilution), and a phycoerythrin-labeled monoclonal antibody against rat L-selectin (1:100 dilution) (BD PharMingen). Neutrophils were distinguished from monocytes as described previously.

Northern Blot Analysis of ICAM-1 and Monocyte Chemoattractant Protein-1
Standard Northern blotting was used to investigate monocyte chemoattractant protein-1 (MCP-1) and ICAM-1 mRNA expression, and ribosomal protein L32 (rpL32) was used as a housekeeping gene for normalization. Primers were designed according to published sequences.

Statistical Analysis
Data are expressed as mean±SEM of n independent experiments. Statistical evaluation was performed by 1-way ANOVA with subsequent post hoc paired comparisons. Differences with a value of P<0.05 were considered statistically significant.

Results
Effect of ROSI on Ischemia/Reperfusion-Induced Myocardial Infarction
The AAR, determined by negative staining after reperfusion with Evans blue dye and expressed as percent of LV, showed no difference between vehicle- and ROSI-treated groups (see Figure 1, AAR/LV), indicating that a comparable degree of ischemic jeopardy existed between vehicle- and ROSI-treated groups after occlusion of the LAD.

ROSI, administered intravenously, reduced ischemia/reperfusion-induced cardiac infarct size dose-dependently (Figure 1, top). At 1 and 3 mg/kg, the ratio of NEC versus...
AAR was decreased by 30% and 37% compared with vehicle, respectively (both \(P<0.01\)). Similar results were obtained with data calculated as NEC versus LV.

Meanwhile, ROSI significantly prevented creatine kinase loss from ischemic myocardium (data not shown).

Figure 1, bottom, shows the protective effect of ROSI when the drug was given orally at 3 mg·kg\(^{-1}\)·d\(^{-1}\) for 7 days before ischemic injury. The infarct size (NEC), expressed either as percent of AAR or percent of LV, was determined at 24 hours after reperfusion. ○, Vehicle; ●, ROSI; *\(P<0.05\) vs vehicle (\(n=8\)). OCC indicates occlusion.

There was no difference between vehicle- and drug-treated groups in the plasma levels of glucose, triglycerides, and insulin at 30 minutes after ischemia or 2, 4, and 24 hours after reperfusion.

**ROSI Improves Myocardial Contractile Function After Ischemia/Reperfusion Injury**

LAD occlusion caused a marked decrease in LVSP, \(+dP/dt\) and \(-dP/dt\) (Figure 2). When reperfusion was restored, functional contraction was further reduced, rebounded, and then gradually decreased and remained at a relatively stable level for up to 4 hours. In the ROSI-treated group, the recovery in cardiac contractile function was enhanced (1.5 to 4 hours after reperfusion). The values of \(+dP/dt\), \(-dP/dt\), and LVSP at 4 hours after reperfusion were enhanced from (% basal) 41.7±5.1, 37.9±6.2, and 63.8±6.2 in the vehicle group to 59±3.9, 55.9±3.2, and 77.2±1.3 in the ROSI-treated group, respectively (\(P<0.05\); \(n=8\)). The mean ABP in the ROSI-treated group was also improved from (% basal) 49.7±6.9 to 61.3±3.3 (\(P<0.05\)) at 4 hours after reperfusion.

The LVEDP was reduced (% basal) from 82.1±26 in vehicle to 58.3±19.3 in the ROSI-treated group, but the difference was not significant (\(P>0.05\)). There was no difference in the heart rate between the 2 groups (data not shown). A similar improvement in cardiac contractile function was also observed at 24 hours after reperfusion in a separate study (Figure 2). In that study, the myocardial infarct size was reduced by 34% in the ROSI-treated group compared with the vehicle-treated group (\(P<0.01\); \(n=8\)).

**Effect of ROSI on Neutrophil Accumulation in the Ischemic/Reperfused Myocardium**

MPO activity was very low in the ANAR but markedly increased in the AAR, as shown in Figure 3. Treatment with ROSI, however, resulted in a significant 40% reduction (\(P<0.01, n=12\) to 14) of MPO activity in the AAR.

**Effect of ROSI on Macrophage Accumulation in the Ischemic/Reperfused Myocardium**

The number of ED-1-positive cells in sham ischemic myocardium was very low (Figure 4A). In the hearts subjected to ischemia/reperfusion, however, the ED-1-positive infiltrates were markedly increased, frequently concentrated in the subepicardial regions, and extended into the area of injured myocardium of the LV (Figure 4B). As shown in Figure 4C, the number of ED-1-positive cells was reduced in ROSI-treated rats. Figure 4D gives the quantitative data showing that the extent of ED-1-positive infiltrates in the myocardium 24 hours after reperfusion was reduced in ROSI-treated animals by 43% compared with the vehicle (\(P<0.01, n=6\)).

**Effect of ROSI on Ischemia/Reperfusion-Stimulated Upregulation of CD11b/CD18 and Downregulation of L-Selectin on Leukocytes**

As shown in Figure 5, surface expression of CD11b/CD18 on neutrophils from sham animals remained at a constant level during the entire experimental period. The level of CD11b/CD18 on rat neutrophils was not markedly changed during the ischemic period but was significantly upregulated after reperfusion. The peak levels of CD11b and CD18 appeared at 2 to 4 hours after reperfusion and were enhanced by 2- to 3-fold in the vehicle-treated group over the basal level. In contrast, this upregulation in CD11b/CD18 expression on neutrophils was significantly attenuated in ROSI-treated an-
inal. Ischemia/reperfusion induced a significant downregulation in L-selectin (shedding) on neutrophils, and this change was also remarkably diminished in ROSI-treated rats (Figure 5).

Effect of ROSI on ICAM-1 mRNA and Protein Expression in the Ischemic-Reperfused Myocardium

Figure 6, top, is the representative Northern blot, and Figure 6, bottom, shows the quantitative results. The basal level of ICAM-1 mRNA was low in the heart and was significantly lower in ROSI-treated group (Figure 6). *P<0.05; **P<0.01 vs basal.

Effect of ROSI on Ischemia/Reperfusion-Induced Changes in Cell Surface Expression of CD11b/CD18 and L-Selectin on Monocytes

<table>
<thead>
<tr>
<th></th>
<th>% Basal</th>
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<tr>
<td>CD11b</td>
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<tr>
<td>Vehicle</td>
<td>345±67</td>
</tr>
<tr>
<td>ROSI</td>
<td>177±16*</td>
</tr>
<tr>
<td>CD18</td>
<td>313±18</td>
</tr>
<tr>
<td>Vehicle</td>
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<tr>
<td>ROSI</td>
<td>114±11†</td>
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<tr>
<td>L-Selectin</td>
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Monocyte surface expression of CD11b, CD18, and L-selectin was measured before ischemia (basal) and 4 hours after reperfusion.

*P<0.05, †P<0.01 vs vehicle group (n=5).
upregulated by ischemia/reperfusion. Treatment of animals with ROSI resulted in a significant reduction in ICAM-1 mRNA expression, by 49.4%, compared with the vehicle group ($P < 0.05, n = 11$). The results of immunohistochemical analysis were consistent with Northern analysis. The ICAM-1 immunoreactivity, expressed mainly in thin-walled vessels, was upregulated by ischemia/reperfusion and attenuated by ROSI treatment (data not shown).

**Effect of ROSI on MCP-1 mRNA Expression in Ischemic/Reperfused Myocardium**

Northern blot analysis demonstrated that MCP-1 mRNA expression was significantly upregulated in ischemic myocardium and that the peak appeared at 12 to 24 hours after reperfusion (Figure 7A). Figure 7B is a representative Northern blot from one of the studies. Figure 7C shows the quantitative results indicating that MCP-1 mRNA levels in ischemic myocardium in ROSI-treated rats were reduced by 43.5% compared with the vehicle-treated group ($P < 0.01, n = 11$ to 12).

**Discussion**

Expression of PPAR-γ in the heart and cardiomyocytes has been reported, but the function of PPAR-γ in the heart is little known. A previous in vitro study using isolated perfused hearts from streptozotocin-induced diabetic rats found that pretreatment of the rats with troglitazone, a PPAR-γ agonist, for 6 weeks improved the postischemic heart rate and cardiac work. In a recent study of nondiabetic pigs, long-term treatment with troglitazone for 8 weeks improved a moderate ischemia-induced cardiac dysfunction and increased net myocardial lactate uptake, suggesting an enhanced myocardial carbohydrate oxidation. The present study has demonstrated a dramatic protection by ROSI, a new PPAR-γ agonist, against myocardial infarction after severe ischemia (no-flow) and reperfusion injury. Short- or long-term treatment with ROSI resulted in a significant reduction in infarct size in rats subjected to ischemia/reperfusion. ROSI also significantly improved cardiac contractile function. There was no difference in AAR versus LV between vehicle- and ROSI-treated animals, indicating that the animals in the vehicle- or drug-treated groups received a comparable degree of ischemic jeopardy. Therefore, the reduction of infarct size and enhancement of myocardial function in ROSI-treated animals was due to the effect of the drug. To the best of our knowledge, this is the first study showing that a PPAR-γ agonist has an in vivo protective effect against myocardial infarction induced by severe ischemia.

It has been reported that the process of ischemia/reperfusion injury is characterized by an inflammatory response in which neutrophils and monocytes/macrophages play an important role. Activated neutrophils release a variety of cytotoxic substances, such as oxygen-derived free radicals and proteases, and activated monocytes/macrophages are a main source of production of inflammatory cytokines in ischemic heart. These substances released from activated leukocytes directly mediate vascular endothelial dysfunction as well as myocardial injury. Recent studies have demonstrated the expression of PPAR-γ in monocytes/macrophages and neutrophils and suggested a role of PPAR-γ in negatively regulating expression of proinflammatory genes. Our data demonstrated a significant reduction in infiltration of neutrophils and monocytes/macrophages into the ischemic myocardium in ROSI-treated animals, suggesting a potential mechanism by which ROSI protects the heart from ischemia/reperfusion-induced myocardial infarction.
To further examine the mechanism by which ROSI inhibited recruitment of neutrophils and monocytes into myocardium, we studied the effect of ROSI on surface expression of CD11b/CD18 and L-selectin on neutrophils and monocytes. Leukocytes activated by ischemia/reperfusion provoke translocation of CD11b/CD18 from intracellular granules to the plasma membrane as a prerequisite for subsequent adhesion. Meanwhile, a concomitant decrease in surface L-selectin (shedding) facilitates leukocyte rolling and extravasation. Our results clearly indicate that ischemia/reperfusion stimulated a significant upregulation of CD11b/CD18 and downregulation of L-selectin on neutrophils and monocytes and that these changes happened after reperfusion. Our data are consistent with the recent reports demonstrating an increased expression of neutrophil and monocyte CD11b/CD18 throughout the acute phase of myocardial infarction and a negative correlation between the myocardial infarct size and L-selectin levels on neutrophils in patients with myocardial infarction. Our data have demonstrated that ischemia/reperfusion-induced upregulation of CD11b/CD18 and shedding of L-selectin on leukocytes were markedly attenuated in ROSI-treated animals. Previous studies have shown that anti-CD11b or anti-CD18 monoclonal antibody significantly decreased infarct size in rat and dog models of ischemia/reperfusion. Therefore, it is conceivable that attenuating ischemia/reperfusion-induced changes in CD11b/CD18 and L-selectin expression on leukocytes by ROSI could be an important mechanism for its cardioprotection.

Previous studies also demonstrated that leukocytes were capable of infiltration into myocardium under circumstances in which ICAM-1 was induced. ICAM-1 serves as a ligand for CD11b/CD18, is expressed mainly on endothelium, and plays a pivotal role in leukocyte adhesion and infiltration into myocardium. As shown in Figure 6, the expression of ICAM-1 in the ischemic hearts was upregulated by ischemia/reperfusion and markedly attenuated in ROSI-treated animals, consistent with a recent observation in which activation of PPAR-γ resulted in downregulation of ICAM-1 expression in intestinal endothelium subjected to ischemia/reperfusion. The data further support our hypothesis that ROSI reduces leukocyte accumulation in the heart through its combined effects on expression of CD11b/CD18, L-selectin, and the ligand ICAM-1, thereby attenuating leukocyte adhesion on coronary microvasculature, reducing cell rolling and migration across endothelium, and inhibiting inflammatory cell infiltration into myocardium.

MCP-1 is a C-C chemokine produced in a variety of cells, including cardiovascular cells, in response to ischemic injury or exposure to other cytokines. MCP-1 has been documented not only to recruit monocytes to inflammatory sites but also to stimulate them to release lysosomal enzymes and to produce superoxide anion. The role of MCP-1 in ischemic injury was further supported by a study demonstrating that treatment with an antibody against MCP-1 reduced the infiltration of monocytes into heart and prevented myocardial reperfusion injury. Moreover, blockade of MCP-1 markedly decreased the expression of ICAM-1 in the ischemic heart.

Two recent in vitro studies have shown that troglitazone decreased MCP-1 production in human aortic endothelial cells and in colonic epithelial cells. Our in vivo data are the first to demonstrate that treatment with a PPAR-γ agonist, such as ROSI, significantly reduced expression of MCP-1 mRNA in ischemic myocardium. The data provide another mechanism by which ROSI protects against ischemia/reperfusion-induced myocardial infarction. This could also be the mechanism by which ROSI diminished ICAM-1 expression in the ischemic heart.

The present study demonstrates the cardioprotective effect of ROSI and its potential mechanism. Whether this in vivo cardioprotection of ROSI is shared by other PPAR-γ agonists, however, is not clear. We recently tested a nonthiazolidinedione PPAR-γ agonist, SB219994 (S enantiomer) and its R enantiomer, SB219993, which is 100-fold less potent than SB219994 as a PPAR-γ agonist for their in vivo cardioprotection. SB219994 significantly reduced ischemia/reperfusion-induced myocardial infarction, but SB219993, under the same dose, showed no effect, indicating a PPAR-γ-mediated effect (data not shown). To further confirm this conclusion, more PPAR-γ agonists with different structures should be studied. There is controversy regarding the role of PPAR-γ in macrophage function. Both the previous studies demonstrating the inhibition by PPAR-γ agonists of cytokine release from macrophages and more recent studies demonstrating PPAR-γ–independent anti-inflammatory effects were performed in vitro with extremely high concentrations of PPAR-γ agonists or a nonselective PPAR-γ agonist. It is hard to correlate these data with our in vivo data, which were obtained with a clinically relevant dose of ROSI. Further investigations are necessary to clarify how PPAR-γ agonists inhibit adhesion molecule expression. It also needs to be explored whether other mechanisms or molecular targets of PPAR-γ are involved in the protection of myocardial infarction by ROSI. The findings of this study, however, offer a new possibility for ROSI therapy in acute ischemic myocardial injury. The cardioprotective effect of ROSI would also benefit the diabetic patients for whom myocardial dysfunction is a common life-threatening complication.


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