Antidiabetic drug pioglitazone protects the heart via activation of PPAR-γ receptors, PI3-kinase, Akt, and eNOS pathway in a rabbit model of myocardial infarction

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PATIENTS WITH TYPE 2 DIABETES mellitus are at substantial risk of coronary artery disease (16). The insulin-sensitizing drug pioglitazone is a ligand for peroxisome proliferator activated receptor (PPAR)-γ, which ameliorates the basic problem of insulin resistance and has therefore been thought to reduce the risk of cardiovascular disease in patients with type 2 diabetes mellitus (26A). As a matter of fact, it has recently been reported that treatment with pioglitazone significantly reduces the risk of major cardiovascular events in patients with type 2 diabetes mellitus (7). This may be due to the improvement of glucose metabolism and thereafter a vasoprotective effect and due to a direct antiatherosclerotic effect because it has been reported that activation of PPAR-γ shows antiatherosclerotic effect (5, 6). In addition, it has been reported that activation of PPAR-γ is protective against ischemia-reperfusion injury (24). However, the precise mechanism of pioglitazone for its cardioprotection is still not fully clarified. Therefore, in the present study, we investigated whether pioglitazone reduces the myocardial infarct size and investigated its precise mechanisms in a rabbit model of myocardial infarction without collateral circulation (11).

METHODS

In this study, all rabbits received humane care in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, Revised 1996). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine (Gifu, Japan).

Surgical preparation. Male Japanese white rabbits weighing 1.9–2.5 kg were anesthetized with 30 mg/kg pentobarbital sodium, and additional doses were given when required throughout the experiment so that pain relief was provided. They were orally intubated and mechanically ventilated with room air supplemented with a low-flow oxygen by mechanical ventilation (tidal volume 25–35 ml/min; model SN-480-5, Shimano, Tokyo, Japan). Serial blood gas analysis was performed, and ventilatory conditions were adjusted to keep the arterial blood gas within the physiological range. For rabbits with 48 h reperfusion, all surgical procedures were performed aseptically. The left carotid artery and jugular vein were cannulated to monitor arterial blood pressure and to administer drugs or saline, respectively. The rabbits were given heparin (500 U/kg). After a left thoracotomy was performed at the third intercostal space, the heart was exposed and 4-0 silk ligature was placed beneath the large coronary arterial branch coursing down the middle of the anterolateral surface of the left ventricle (LV). A small vinyl tube was passed into both ends of the suture, and the coronary branch was occluded by pulling the snare, which was then fixed by clamping the tube with a mosquito hemostat. Myocardial ischemia was confirmed by regional cyanosis and electrocardiographic change. Reperfusion was confirmed by myocardial blush over the risk area after releasing the snare. After the initial...
preparation but after coronary occlusion, the animals were assigned randomly to each group. All rabbits were allowed to rest for 20 min after the completion of the surgical preparation before the start of the protocol. After the experiment, the chest was closed and the rabbits were allowed to recover from anesthesia for survival of 2 days. At the end of the study 48 h after myocardial infarction, the rabbits were anesthetized with 30 mg/kg pentobarbital and the hemodynamic parameters were measured and echocardiography was performed. The rabbits were then heparinized (500 U/kg) and euthanized by an intravenous overdose of pentobarbital. The rabbit hearts in which the coronary vein was ligated along with the artery were excluded, because this may increase the magnitude of ischemic lactic acidosis. Plasma glucose level. Twenty rabbits were used for the measurement of plasma glucose levels. The pioglitazone group (n = 10) was fed diets containing 1 mg·kg⁻¹·day⁻¹ pioglitazone for 7 days, and the control group (n = 10) was fed normal diets for 7 days. Arterial blood samples were taken slowly from the ear artery at time points before diet and 1 h after diet in the morning. The diet was not given to the rabbits from day 6 in the evening until day 7 early in the morning after the diet was started. The blood samples were taken on day 7 in the morning before and 1 h after the restart of the diet.

The samples were immediately put into heparinized ice-cold centrifuge tubes and stored at −83°C until assays were performed to measure plasma glucose levels. Pioglitazone was provided from Takeda Pharmaceutical (Osaka, Japan). First, pioglitazone was measured in the same blood samples that were used to measure the plasma glucose concentration, which was taken from the ear artery after the diet with pioglitazone containing chou was stopped for 12 h (on day 6) and then again 1 h after refeeding was initiated (on day 7). Finally, to examine the steady-state plasma levels of pioglitazone, the blood samples were taken on day 7 immediately before the start of the experiment under the condition with pioglitazone containing chou. Plasma pioglitazone levels were measured by using high-performance liquid chromatography.

Study protocol. To investigate the infarct size-reducing effect of pioglitazone, 90 Japanese white rabbits underwent 30 min of coronary occlusion followed by 48 h of reperfusion and were assigned randomly to nine groups as shown in Fig. 1 (n = 10 in each): the control group, pioglitazone group (fed diets containing 1 mg·kg⁻¹·day⁻¹ pioglitazone for 7 days), pioglitazone + 5-hydroxydecanoate (HD) group [fed the same diet as the pioglitazone group + 5 mg/kg iv 5-HD, a mitochondrial ATP-sensitive K⁺ (K₅ATP) channel blocker, 10 min before ischemia], pioglitazone + GW9662 group [fed the pioglitazone diet + 2 mg/kg iv GW9662, a PPAR-γ antagonist, 10 min before ischemia], GW9662 group [fed a normal diet + iv administered GW9662], pioglitazone + wortmannin group [fed the same diet as the pioglitazone group + 0.6 mg/kg iv wortmannin, a phosphatidylinositol (PI)-3 kinase inhibitor], wortmannin group (fed a normal diet + iv wortmannin), pioglitazone + nitro-L-arginine methyl ester (L-NAME) group [fed the same diet as the pioglitazone group + 10 mg/kg iv L-NAME, a nitric oxide (NO) synthase (NOS) inhibitor], and L-NAME group [fed a normal diet + iv L-NAME]. For all treatments, the injected volume was <1 ml. In the control group, an equivalent dose of pentobarbital. The heart was excised and mounted on a Langendorff apparatus. The coronary branch was reoxygenated, and Evans blue dye (4%; Sigma Chemical, St. Louis, MO) was injected from the aorta at 80 mmHg to determine the area at risk. The LV was sectioned into seven slices parallel to the atrioventricular ring. Each slice was weighed, incubated in a 1% solution of triphenyltetrazolium chloride (TTC) at 37°C to visualize the infarct area (Fishbein et al., 1981), and photographed. The areas of the ischemic region and the infarcted myocardium were traced on each LV slice and multiplied by the slice weight and then expressed as a fraction of the risk region or LV for each heart. Physiological studies. Both before ischemia at baseline and 48 h after reperfusion, echocardiographic studies (SSD2000; Aloka), and measurement of arterial blood pressure, heart rate and cardiac function such as maximal or minimal change in pressure over time (±dP/dt) using a micromanometer-tipped catheter were performed under light anesthesia with 10 mg/kg pentobarbital sodium and spontaneous respiration. A two-dimensional parasternal long-axis view of the LV was obtained. In general, the best views were obtained with the transducer lightly applied to the midupper left anterior chest wall. The transducer was then gently moved, cephalad or caudal, and angulated until desirable images were obtained. Ejection fraction and LV end-diastolic dimensions were obtained. Ejection fraction was measured using Teichholz method from M-mode images by echocardiography.

Arterial blood pressure and heart rate were also measured via a catheter introduced in the carotid artery. A micromanometer-tipped catheter (SPR 407; Millar Instruments) was inserted into the LV to record +dP/dtmax, representing the cardiac systolic function, as well as −dP/dtmax, the indicator of cardiac diastolic function. All measurements were made by two persons blinded to the treatment.

Western blot analysis. Western blotting was performed to assess levels of Akt and phospho-Akt (ERK) and phospho-ERK and phospho-eNOS in the myocardium following reperfusion. Hearts were excised, and transmural samples, each weighing ∼200 mg, were taken from the center of the LV ischemic region and the opposite nonischemic region at the end of the 2-day reperfusion. The border of the ischemic region was defined by the distribution of cyanosis and marked on the epicardium in ink. The samples were frozen immediately and stored at −83°C until the assays were performed. Samples were weighed, homogenized, and used for the following measurements. Phosphorylation states of Akt (phosphor-Akt, serine 473; Cell Signaling), ERK (Cell Signaling), and eNOS (BD Biosciences Pharmingen) and total levels of Akt and ERK were analyzed by SDS-PAGE immunoelectrophoresis using antibodies obtained from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. The phosphorylation (activation) of Akt, ERK, and eNOS was assessed using antibodies against phosphorylated (p)-Akt, p-ERK (Santa Cruz Biotechnology), and p-eNOS (BD Biosciences Pharmingen).

Statistical analysis. All values are presented as mean ± SE. Risk and infarct sizes were compared among the groups by one-way ANOVA combined with Bonferroni’s post hoc test for multiple comparisons. The difference in hemodynamics over the time course between the control and the drug-treated groups was assessed by two-way repeated-measures ANOVA. Differences with P < 0.05 were considered statistically significant.

RESULTS

Plasma glucose level. Figure 2A shows the comparison of plasma glucose levels. Although blood glucose levels significantly increased at 1 h after diets compared with those at before diets in both groups, there were no significant differences between the control group and the pioglitazone group in the blood glucose levels at before and 1 h after diets, respectively.

Plasma pioglitazone level. Figure 2B shows the comparison of plasma pioglitazone levels. Plasma pioglitazone levels significantly increased at 1 h after diets from 1.0 ± 0.1 to 103 ±
6.5 ng/ml in the pioglitazone group. The steady-state plasma level of pioglitazone was 78 ± 4 ng/ml. Pioglitazone was not detected in plasma from rabbits under normal chow.

**Physiological findings.** Table 1 shows hemodynamic parameters that might influence the infarct size. Among the nine groups, there were no significant differences in blood pressure or heart rate. Figure 3 shows echocardiographic data and dP/dt. There was no significant difference in LV ejection fraction at before ischemia among the nine groups. However, at 48 h after reperfusion, dP/dt was significantly improved in the pioglitazone group compared with the control group.

**Infarct size.** The mean percentages of the area at risk (percentage of LV) were 27.2 ± 2.7%, 31.5 ± 1.6%, 32.2 ± 2.4%, 30.8 ± 4.6%, 28.2 ± 3.5%, 31.6 ± 2.2%, 27.5 ± 1.3%, 31.1 ± 2.8%, and 28.3 ± 2.1% in the control, pioglitazone, pioglitazone + 5-HD, pioglitazone + GW9662, pioglitazone + wortmannin, wortmannin, pioglitazone + L-NAME, and L-NAME groups, respectively (Fig. 4A). No significant difference in the mean area at risk as a percentage of LV was seen among these groups. As shown in Fig. 4B, the infarct size as a percentage of the area at risk was significantly reduced in the
The present study demonstrated that pioglitazone reduces myocardial infarct size and improves LV function. The infarct size-reducing effect and LV function-improving effect of pioglitazone were both abolished by pretreatment with GW9662, a PPAR-γ antagonist, wortmannin, a PI3-kinase inhibitor, and L-NAME, a NOS inhibitor, but not by 5-HD, a mitochondrial K_{ATP} channel blocker. Western blot analysis revealed that pioglitazone activates Akt and eNOS but not ERK in the myocardium.

In the present study, there was no significant difference in plasma glucose levels at before eating and 1 h after diet between the control and pioglitazone groups, respectively. This suggests that changes in plasma glucose levels are not involved in the infarct size-reducing effect of pioglitazone in the present study. The impact of the study could have been increased if the studies were conducted in a model of diabetes. However, in the present study, we performed the experiment in a normal rabbit model of myocardial infarction in which 7 days treatment with pioglitazone-containing diet did not affect the plasma glucose levels before and 1 h after diet. We and other investigators previously reported that another antidiabetic treatment such as acarbose, an oral hypoglycemic agent, also reduced the myocardial infarct size, and this effect was related to the improvement of postprandial hyperglycemia by acarbose (8, 18). Therefore, the mechanism by which pioglitazone reduces the myocardial infarct size seems to be different from that of hypoglycemic agent such as acarbose.

As for the plasma pioglitazone levels, the steady-state plasma level of pioglitazone was 78 ± 4 ng/ml and that of 1 h after diets with pioglitazone was 103 ± 6.5 ng/ml. These data suggest that orally taken pioglitazone was well absorbed from the intestine into systemic circulation.

Among the nine groups, there were no significant differences in systolic and diastolic blood pressure or heart rate that might influence the infarct size (19). Therefore, the infarct size-reducing effect of pioglitazone was not caused by the decrease in oxygen consumption.

The infarct size was significantly smaller in the pioglitazone group (21 ± 2%) than in the control group (43 ± 3%). The infarct size-reducing effect of pioglitazone was completely abolished by pretreatment with GW9662 (42 ± 3%), a PPAR-γ receptor blocker, wortmannin (40 ± 3%), a PI3-kinase inhibitor, and L-NAME (42 ± 7%), a NOS inhibitor, but not by 5-HD (24 ± 5%), a mitochondrial K_{ATP} channel blocker, suggesting that pioglitazone reduced the myocardial infarct size via the activation of PPAR-γ receptors, PI3-kinase, and the production of NO but not via the opening of mitochondrial K_{ATP} channel. The LV ejection fraction at 48 h of reperfusion assessed by echocardiography was improved in the pioglitazone compared with the control group. The ±dP/dt at 48 h of reperfusion was also improved in the pioglitazone compared with the control group. The improvement of LV function in the pioglitazone group may be due to the reduction in the infarct size since the improvement of LV ejection fraction and ±dP/dt was correlated with the reduction in the infarct size.

With the consideration of mitochondrial K_{ATP} channels, it has been reported that ischemic preconditioning, a brief episode of ischemia and reperfusion, opens the mitochondrial K_{ATP} channels and reduces the myocardial infarct size (10, 13).
ences between the present study and previous works are that an
tazone reduces the myocardial infarct size. However, differ-
reported that pioglitazone mimics preconditioning in the iso-
and improved cardiac function via reducing cardiomyocyte
molecule-1 and a smaller number of infiltrating macrophages
monocyte chemoattractant protein-1 and intercellular adhesion
cardial infarct size in a rat model of 30 min of ischemia and
duce myocardial infarct size (24). Ito et al. (15) has reported
peroxisome proliferated receptors (PPAR-
tazone was abolished by the pretreatment with GW9662,
preconditioning, both of which are the prosurvival signals (12).
both involved in the infarct size-reducing effect of ischemic
Akt pathway and activation of MEK-1/2-ERK-1/2 pathway are
5-HD, a mitochondrial KATP channel blocker, suggesting that
effect of pioglitazone was not affected by the pretreatment with
It has also been reported that the activation of PI3-kinase and
antagonist, suggesting that the infarct size-reducing
collateral circulation (11) was used in the present study and
the myocardial infarct size via opening the mito-
chondrial KATP channels. As a result, the infarct size-reducing
pioglitazone was abolished by the pretreatment with wortman-
nin, a PI3-kinase inhibitor, suggesting that the infarct size-
pioglitazone was abolished by the pretreatment with L-NAME, a NOS inhibitor, suggesting an involve-
m of NO production in the infarct size-reducing effect.
Furthermore, there has been only one report investigating an
volvement of NO production in the infarct size-reducing effect.
Since many pharmacological agents protect the heart via open-
ing of the mitochondrial K_{ATP} channels (1, 4, 13, 20, 22, 23),
we first examined in the present study whether pioglitazone
and PPAR-
receptors. It has previously been reported that ligands of
effect of pioglitazone was due to the activation of PPAR-
5-HD, hydroxydecanoic acid; l-NAME, nitro-l-arginine methyl ester.
Mean blood pressure, mmHg

<table>
<thead>
<tr>
<th></th>
<th>Before Ischemia</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>After 10-min Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83.000 ± 2.984</td>
<td>72.209 ± 3.681</td>
<td>69.782 ± 3.941</td>
<td>68.182 ± 2.611</td>
<td>71.282 ± 3.448</td>
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<tr>
<td>Pioglitazone</td>
<td>76.883 ± 4.274</td>
<td>70.275 ± 3.498</td>
<td>69.633 ± 2.411</td>
<td>68.458 ± 2.461</td>
<td>72.717 ± 4.294</td>
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<tr>
<td>Pioglitazone + 5-HD</td>
<td>78.178 ± 3.589</td>
<td>71.500 ± 4.414</td>
<td>70.656 ± 3.400</td>
<td>68.178 ± 3.183</td>
<td>76.389 ± 2.607</td>
</tr>
<tr>
<td>Pioglitazone + GW9662</td>
<td>84.017 ± 4.991</td>
<td>70.217 ± 6.631</td>
<td>70.283 ± 4.314</td>
<td>68.967 ± 3.600</td>
<td>71.367 ± 4.914</td>
</tr>
<tr>
<td>GW9662</td>
<td>82.000 ± 3.033</td>
<td>72.917 ± 3.466</td>
<td>70.500 ± 3.469</td>
<td>69.350 ± 3.314</td>
<td>73.300 ± 2.831</td>
</tr>
<tr>
<td>Pioglitazone + wortmannin</td>
<td>86.643 ± 2.342</td>
<td>79.271 ± 2.325</td>
<td>76.100 ± 2.020</td>
<td>72.500 ± 1.004</td>
<td>80.358 ± 1.228</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>83.520 ± 1.329</td>
<td>80.180 ± 1.223</td>
<td>75.800 ± 1.423</td>
<td>74.280 ± 1.233</td>
<td>76.080 ± 2.488</td>
</tr>
<tr>
<td>Pioglitazone + l-NAME</td>
<td>78.214 ± 2.364</td>
<td>64.271 ± 3.466</td>
<td>67.443 ± 1.480</td>
<td>67.457 ± 3.126</td>
<td>71.043 ± 3.626</td>
</tr>
<tr>
<td>l-NAME</td>
<td>77.543 ± 3.844</td>
<td>66.071 ± 4.750</td>
<td>69.114 ± 3.053</td>
<td>67.143 ± 3.767</td>
<td>71.786 ± 3.119</td>
</tr>
</tbody>
</table>

Values are means ± SE. 5-HD, hydroxydecanoic acid; l-NAME, nitro-l-arginine methyl ester.
On the other hand, there was no significant difference in the expression of p-ERK between the control and pioglitazone groups, suggesting that pioglitazone does not activate ERK in the myocardium and ERK is not involved in the infarct size-reducing effect of pioglitazone.

In the present study, wortmannin alone or L-NAME alone did not affect the infarct size, and this is also true in other reports (2, 9). Indeed, there was some Akt phosphorylation and some eNOS phosphorylation even in the controls with ischemia and reperfusion in the present study, and another report also demonstrated that there is some Akt phosphorylation and some eNOS phosphorylation even in the controls with ischemia and reperfusion (2). However, the level of Akt phosphorylation or eNOS phosphorylation was significantly smaller in the control than in the pioglitazone group in the present study. Therefore, sufficient Akt phosphorylation or sufficient eNOS phosphorylation may be required to exceed the threshold level to reduce the myocardial infarct size. This may be similar since the concept of ischemic preconditioning that a threshold level of PKC stimulation must be reached before protection can be triggered (9).

Therefore, our results suggest that treatment with pioglitazone activates PPAR-γ receptors and then activates PI3-kinase, Akt, and eNOS pathway and reduces the myocardial infarct size.

**Clinical implications.** Patients with diabetes mellitus have an increased risk of coronary artery diseases such as myocardial infarction. Sulfonylureas have ever frequently been used in the treatment of diabetes mellitus and have been linked with adverse cardiovascular effects due to an effect on myocardial ischemic preconditioning; that is, since ischemic preconditioning effect has been reported to be related to the opening of mitochondrial K_{ATP} channels (10, 13), sulfonylureas may pharmacologically block the opening of cardiac mitochondrial K_{ATP} channels in patients with both diabetes and coronary artery disease. In the present study, the infarct size-reducing effect of pioglitazone was not affected by the pretreatment with 5-HD, a mitochondrial K_{ATP} channel blocker, suggesting that pioglitazone reduces the infarct size without affecting cardiac mitochondrial K_{ATP} channels. Pioglitazone has an ischemic...
Fig. 5. Western blot analysis of myocardial Akt, phosphorylated (p) Akt (p-Akt), and p-endothelial NOS (eNOS) expression in the sham, control, and pioglitazone groups at 48 h after 30 min of ischemia and reperfusion. There were no significant differences in the expression of Akt protein between the normal area and ischemic area among each groups. However, in the pioglitazone group, the expression of phospho-Akt and of phospho-eNOS were significantly upregulated in both the normal and infarct areas compared with those of the control group. Western blotting showed higher levels of phospho-Akt and phospho-eNOS in the pioglitazone group. On the other hand, there was no significant difference in the levels of ERK or phospho-ERK between the control and pioglitazone groups. * P < 0.05 vs. sham, control normal area, and control infarct area. ns, Not significant.
pioglitazone without involving the mitochondrial KATP channels, a main pathway of ischemic preconditioning, but via the activation of PPAR-γ receptors, PI3-kinase, Akt, and the eNOS pathway, another pathway of ischemic preconditioning. This cardioprotective mechanism of pioglitazone will not be affected by the mitochondrial KATP channels that would be influenced by sulfonylureas, which will have to be used unwillingly in cases of moderate to severe patients with diabetes.

In conclusion, the findings in the present study may provide new insight into therapeutic strategies for the treatment of patients with both diabetes and coronary artery disease.

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GRANTS

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