Myocardial protection by pioglitazone, atorvastatin, and their combination: mechanisms and possible interactions

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A recent clinical trial has shown that pioglitazone (Pio), a thiazolidinedione with peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist activity, reduces the composite endpoint of all-cause mortality, nonfatal myocardial infarction, and stroke in patients with Type 2 diabetes mellitus who have a high risk for macrovascular events (21). In addition to decreasing insulin resistance and β-cell dysfunction (47), increasing serum HDL cholesterol and decreasing serum triglycerides levels (25), improving the procoagulant state and endothelial dysfunction in diabetes, and reducing “nontraditional” inflammatory cytokines (12), thiazolidinediones have been shown to reduce myocardial infarct size (IS) in the rat (35, 61, 66, 67). However, the specific underlying mechanisms for myocardial protection by thiazolidinediones are still unknown. Knowledge of the exact cardioprotective mechanisms is particularly important because another PPAR agonist, muraglitazar, which targets both PPAR-γ and PPAR-α, has been shown to increase the incidence of the composite end point of death, major adverse cardiovascular events (myocardial infarction, stroke, and transient ischemic attack), and heart failure (43).

Other studies have shown that pretreatment with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) reduces myocardial IS in the rat (6, 7, 22, 41, 48, 58). Currently, many patients with diabetes are receiving concomitant therapy with statins and hypoglycemic agents. We have shown that, in rats, glyburide, a sulfonylurea agent that is commonly used by diabetic patients, abrogates the IS-limiting effect of statins by blocking ATP-sensitive K+ channels (58). It is yet unknown whether there is an interaction between thiazolidinediones and statins that affects myocardial protection. The combination may have additive, or even synergistic effects, as have been shown for sildenafil and atorvastatin (ATV) (48). However, some of the described effects of thiazolidinediones may potentially interfere with the IS-limiting effects of statins, and thus the combination of thiazolidinediones with statins may result in attenuation of the protective effect. Pio and rosiglitazone increase the expression of phosphatase and tensin homologue deleted on chromosome 10, anti-Src homology 2-containing inositol phosphatase-2 (PI3K) activity and reduced phosphorylation of protein kinase Akt (PTEN), leading to inactivation of phosphatidylinositol 3-kinase (PI3K) activity and reduced phosphorylation of protein kinase Akt (38). Phosphorylation of Akt is a crucial step in mediating the protective effect of statins (22, 62). Mensah et al. (41) have recently reported that, after 1 wk of treatment with ATV, PTEN expression is increased and myocardial protection is no longer apparent (41). Early activation of PTEN may blunt the protective effect of statins. In addition, thiazolidinediones have been shown to inhibit inducible nitric oxide (NO) synthase (iNOS) (17, 19, 40) and cyclooxygenase-2 (COX-2) (19, 40) expression, both of which are crucial for the myocardial IS-limiting effects of statins (1, 7). Alternatively, both thiazolidinediones (11, 15, 31) and statins (1, 22, 37, 48, 51) increase the activity of calcium-dependent NOS (cNOS), which also participates in mediating the protective effect of statins by blocking ATP-sensitive K+ channels (58).
NOS (eNOS), iNOS, and/or COX-2 upregulation; and 3) whether there are interactions between Pio and ATV that affect myocardial protection, leading to either augmentation or diminution of the protective effect.

METHODS

Animal Care

All animals received humane care in compliance with The Guide for the Care and Use of Laboratory Animals, published by National Institutes of Health (NIH Publication No. 85-23, Revised 1996). Experiments were conducted on male Sprague-Dawley rats. The study was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Materials

SC-58125, SC-560, NOS-activity kit, and ELISA kits for 6-keto-PGF1α, COX-2 and phospholipase A2 (PLA2) activity were purchased from Cayman Chemical (Ann Arbor, MI). Monoclonal anti-c-Src homology (SH) 2-containing polyclonal anti-Src homology (SH) 2-containing inositol phosphatase-2 (SHIP2) antibodies were purchased from Santa Cruz Biototechnology (Santa Cruz, CA); and monoclonal anti-COX-2 and monoclonal anti-iNOS antibodies from Cayman Chemical; monoclonal anti Ser1177 phosphorylated-eNOS (P-eNOS) antibodies from Cell Signaling (Beverly, MA); monoclonal anti-Ser473 phosphorylated Akt (P-Akt) and monoclonal anti-PTEN antibodies from R & D (Minneapolis, MN); and monoclonal anti-β-actin antibody from Sigma (St. Louis, MO). N-(3-maleimidylpropionyl) biocytin was purchased from Molecular Probes (Eugene, OR).

Drugs and Pretreatment

Protocol 1. Rats received 3-day pretreatment with 1) Pio 10 mg·kg−1·day−1; 2) ATV 10 mg·kg−1·day−1; or 3) ATV 10 mg·kg−1·day−1 and Pio 10 mg·kg−1·day−1; or 4) water (control). Medications were suspended in 1 ml of water and given once a day by oral gavage. Rats underwent either coronary artery ligation (IS protocol), or the hearts were explanted without being subjected to ischemia for enzyme activity determination and for immunoblotting.

Protocol 2. An additional group of rats received oral 10 mg Pio·kg−1·day−1 for 3 days and then intravenous SC-58125 (COX-2 inhibitor, 5 mg/kg) or SC-560 (COX-1 inhibitor, 2.5 mg/kg). Fifteen minutes after the intravenous administration of the inhibitors, the rats underwent coronary artery ligation (IS protocol).

IS Surgical Protocol

The rat model of myocardial ischemia-reperfusion injury has been described in detail (6, 7, 48, 58). On the 4th day, the rats were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated, and ventilated (fractionated inspired O2 content = 30%). The rectal temperature was monitored, and body temperature was maintained between 36.7° and 37.3°C throughout the experiment. The left carotid artery was exposed and cannulated with a pressure wire (Rat Mikro-Tip BP System) connected to PowerLab data acquisition system (ADInstruments, Colorado Springs, CO). The chest was opened, and the left coronary artery was encircled with a suture and ligated for 30 min. Isoflurane (1–2.5% titrated to effect) was added after the beginning of ischemia to maintain anesthesia. The snare was then released, and myocardial reperfusion was verified by change in the color of the myocardium. Subcutaneous 0.1 mg/kg buprenorphine was administered, the chest was closed, and the rats were recovered from anesthesia. Four hours after reperfusion, the rats were reanesthetized, the coronary artery was reocluded, 1.5 ml of Evan’s blue dye 3% was injected into the right ventricle, and the rats were killed under deep anesthesia.

Heart rate and mean blood pressure were noted at baseline (10 min after completion of surgery), just before coronary artery occlusion; at 25 min of ischemia; and at 20 min of reperfusion.

Determination of Area at Risk and IS

Hearts were excised, and the left ventricle was sliced transversely into six sections. Slices were incubated for 10 min at 37°C in 1% buffered (pH = 7.4) 2,3,5-triphenyltetrazolium chloride (TTC), fixed in a 10% formaldehyde, and photographed to identify the ischemic area at risk (AR) (uncolored by the blue dye), the IS (stained by TTC), and the nonischemic zones (colored by blue dye). The area of AR and IS in each slice was determined by planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice, and the results were summed to obtain the weight of the myocardial AR and IS (6, 7, 48, 58).

NOS Activity

Myocardial samples were homogenized in a buffer [25 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 1 mM EGTA] and centrifuged at 10,000 g for 15 min. The supernatant, containing the soluble enzyme iNOS, and the pellet, containing the membrane-bound eNOS and neuronal NOS (eNOS), were separated. The pellet was resuspended in homogenization buffer. NOS activity was determined by measuring the conversion of L-[14C]arginine to L-[14C]citrulline using a commercial kit (Cayman Chemical). For assessing eNOS activity, CaCl2 was added to the samples. For assessing calcium-independent (cNOS) activity, CaCl2 was omitted from the solution. NOS activity was defined as counts per minute (48).

6-keto-PGF1α and PLA2 Activity

Myocardial samples of the anterior wall of the left ventricle were rinsed in PBS solution (pH 7.4) containing 0.16 mg/ml heparin to remove red blood cells and clots, homogenized in cold PBS (pH 7.4), and centrifuged at 10,000 g for 10 min at 4°C. The supernatants were collected and stored on ice. Measurement of 6-keto-PGF1α, the stable metabolite of prostacyclin, and PLA2 activity were made using immunoassay assay kits (Cayman Chemical).

COX-2 Activity

Myocardial samples of the anterior wall of the left ventricle were perfused and rinsed with 0.05 M Tris buffer, pH 7.4, containing 0.16 mg/ml heparin to remove any red blood cells and clots. Samples were homogenized in 5–10 ml of cold buffer (0.1 M Tris·HCl, pH 7.8, containing 1 mM EDTA) per gram tissue and centrifuged at 10,000 g for 15 min at 4°C. The supernatant was collected and stored on ice. The COX activity assay kit (Cayman Chemical) measures the peroxidase activity of COX, assayed colorometrically by monitoring the appearance of oxidized N,N,N′,N′-tetramethyl-p-phenylenediamine at 590 nm. Each myocardial sample was tested in triplicate (the first without an inhibitor; the second with DuP-697, a specific COX-2 inhibitor; and the third with SC-560, a specific COX-1 inhibitor). Per the manufacturer instructions, COX-1 activity was calculated as the difference between total COX activity in the first sample and the activity in the sample with SC-560 (the COX-1 inhibitor). COX-2 activity was calculated as the difference between total COX activity in the first sample and the activity in the sample with DuP-697 (the specific COX-2 inhibitor).

Western Immunoblotting

Determinations of PTEN, SHIP2, P-Akt, eNOS, P-eNOS, iNOS, cPLA2, and COX-2 expression were performed in samples taken from the left ventricles of rats (6 rats in each group). The hearts were rapidly explanted, rinsed in cold PBS (pH 7.4) containing 0.16 mg/ml heparin to remove red blood cells and clots, frozen in liquid nitrogen, and stored at −80°C.
Tissue samples were homogenized in buffer A [25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 25 mM NaF, 1 mM Na2VO4, and 1% protease inhibitor cocktail (P8340; Sigma)] and centrifuged at 10,000 g for 15 min at 4°C. The supernatants were collected as cytosolic fraction. The pellets were then incubated on ice in buffer B (buffer A plus 1% Triton X-100) for 2 h and centrifuged for 12 min at 4°C. The resulting supernatants were collected as membranous fractions and combined with the cytosolic fraction. The expression of the proteins (cytosolic + membranous protein) was assessed by standard SDS/PAGE Western immunoblotting (7). Protein (50 μg) was loaded for each sample, except that, for immunoblotting of COX-2 in the control group (Fig. 1). IS in the Pio group (one was excluded because of lack of reperfusion). There were 13 rats in the ATV group (one was excluded because of lack of reperfusion). There were 13 rats in the Pio + ATV group (one was excluded because of lack of reperfusion). There were significant differences in body weight, left ventricular weight, and AR among the groups (Table 1). IS (percentage of the AR) was significantly smaller in the Pio + SC-560 group than in the Pio + SC-56125 group.

Protocol 2. Four rats were included in the SC-58125 and four in the SC-560 group. None of the rats were excluded. There were no differences in body weight, left ventricular weight, and AR between the groups (Table 4). IS was significantly smaller in the Pio + SC-560 group.

Protein Expression

Ser473-phosphorylated Akt. There were significant differences in P-Akt expression among groups (P < 0.001). The expression of P-Akt in the Pio group was 142% of sham-treated group (P = not significant). In contrast, P-Akt expression in the ATV group was 440% compared with the sham-treated group. In the combination group, P-Akt was increased by 374% (Fig. 2). These data suggest that there is no correlation between P-Akt levels and the magnitude of myocardial protection. The magnitude of IS limitation was comparable between the Pio and ATV groups, despite a significant difference in P-Akt augmentation. Moreover, in the Pio + ATV group, there was less augmentation of P-Akt than in the ATV-alone group, despite significantly smaller IS. Thus it is plausible that Akt activation could not entirely explain the protective effect, especially of Pio. Alternatively, only a small increase in P-Akt may be needed to confer protection; further increase in P-Akt does not increase the protective effect.

Table 1. Protocol 1: body weight, left ventricular weight, area at risk, and infarct size

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pio</th>
<th>ATV</th>
<th>Pio + ATV</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>0.998</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>254 (SD 26)</td>
<td>252 (SD 35)</td>
<td>253 (SD 2)</td>
<td>254 (SD 13)</td>
<td>0.001</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td>839 (SD 18)</td>
<td>838 (SD 11)</td>
<td>841 (SD 3)</td>
<td>839 (SD 2)</td>
<td>0.953</td>
</tr>
<tr>
<td>AR, % of LV</td>
<td>54.8 (SD 6.9)</td>
<td>54.7 (SD 6.4)</td>
<td>49.9 (SD 6.9)</td>
<td>52.0 (SD 4.1)</td>
<td>0.180</td>
</tr>
<tr>
<td>IS, % of LV</td>
<td>16.2 (SD 4.4)</td>
<td>7.3 (SD 3.0)</td>
<td>6.2 (SD 2.0)</td>
<td>2.8 (SD 1.4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means (SD); n, no. of rats. Pio, pioglitazone; ATV, atorvastatin; LV, left ventricle; AR, ischemic area at risk; IS, infarct size. *P < 0.001 vs. control; †P < 0.05 vs. Pio + ATV; ‡P < 0.01 vs. Pio + ATV.

Fig. 1. Infarct size (IS; percentage of ischemic area at risk). Overall, there were significant differences among groups (P < 0.001). Each treatment group was significantly different from sham-treated group (P < 0.001 for each comparison). There was no difference between pioglitazone (Pio) and atorvastatin (ATV) groups. However, IS in Pio + ATV group was significantly smaller than in sham (P < 0.001), Pio (P = 0.004), and ATV (P = 0.01) groups.

Statistical Analysis

Data are presented as means (SD). The significance level α is 0.05. Body weight, left ventricular weight, the size of the AR and IS, enzyme activity, and protein expression were compared by using analysis of variance (ANOVA). The differences in heart rate and mean blood pressure were compared using two-way repeated-measures ANOVA with Holm-Sidak multiple comparison procedures. Values of P < 0.05 were considered statistically significant.

Results

Infarct Size

Protocol 1. Fifty-five rats were included in the protocol. Overall, there were 16 rats in the sham-treated group (four rats were excluded: three died during the reperfusion period, and one did not exhibit evidence of ischemia). There were 13 rats in the Pio group (one died during ischemia). There were 13 rats in the ATV group (one was excluded because of lack of reperfusion). There were 13 rats in the Pio + ATV group (one was excluded because of lack of reperfusion). There were no differences in body weight, left ventricular weight, and AR among the groups (Table 1). IS (percentage of the AR) was significantly reduced in the Pio (by 56%), ATV (by 58%), and Pio + ATV (by 81%) groups compared with the sham-treated group (Fig. 1). IS in the Pio + ATV group was significantly smaller than in the sham-treated group. In the combination group, IS was smaller than in the sham-treated group (P < 0.001), Pio (P = 0.004), and ATV (P = 0.01) groups.
Table 2. Average heart rate during IS experiment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pio</th>
<th>ATV</th>
<th>Pio + ATV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>216 (SD 6)</td>
<td>219 (SD 6)*</td>
<td>216 (SD 8)</td>
<td>225 (SD 2)†</td>
</tr>
<tr>
<td>Preocclusion</td>
<td>218 (SD 7)</td>
<td>220 (SD 5)*</td>
<td>207 (SD 17)</td>
<td>224 (SD 3)*</td>
</tr>
<tr>
<td>25-Min occlusion</td>
<td>229 (SD 8)</td>
<td>226 (SD 10)</td>
<td>218 (SD 6)</td>
<td>225 (SD 7)</td>
</tr>
<tr>
<td>20-Min reperfusion</td>
<td>216 (SD 6)</td>
<td>217 (SD 8)</td>
<td>206 (SD 23)</td>
<td>199 (SD 56)‡</td>
</tr>
</tbody>
</table>

Values (in beats/min) are mean (SD) heart rate. Overall, there were significant differences among groups (P = 0.019 for group effect). There was also a significant effect over time (P < 0.001). At baseline and during coronary occlusion, there were no differences among groups. However, at preocclusion, heart rate was higher in Pio + ATV group than in ATV group († P = 0.002 vs. ATV). At reperfusion, heart rate was lower in Pio + ATV group than in control group (‡ P = 0.004 vs. control) and Pio group († P = 0.004 vs. Pio).

Table 3. Mean blood pressure during IS experiment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pio</th>
<th>ATV</th>
<th>Pio + ATV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>119 (SD 5)</td>
<td>123 (SD 2)*</td>
<td>121 (SD 4)</td>
<td>124 (SD 2)*†</td>
</tr>
<tr>
<td>Preocclusion</td>
<td>114 (SD 7)</td>
<td>121 (SD 4)*</td>
<td>118 (SD 6)</td>
<td>122 (SD 2)*‡</td>
</tr>
<tr>
<td>25-Min occlusion</td>
<td>95 (SD 2)</td>
<td>97 (SD 1)</td>
<td>96 (SD 1)</td>
<td>97 (SD 1)</td>
</tr>
<tr>
<td>20-Min reperfusion</td>
<td>96 (SD 2)</td>
<td>95 (SD 1)</td>
<td>95 (SD 1)</td>
<td>95 (SD 1)</td>
</tr>
</tbody>
</table>

Values (in mmHg) are mean (SD) blood pressure (MBP). Overall, there were significant differences among groups (P = 0.008 for group effect). There was also a significant effect over time (P < 0.001). At baseline and preocclusion, MBP in control group was lower than in Pio and Pio + ATV group (P < 0.007). MBP at baseline and preocclusion was lower in ATV group than in Pio + ATV group († P < 0.01 vs. ATV). During occlusion and postreperfusion, there were no differences in MBP among groups.

However, if this is true, it is unclear why IS was smaller in the Pio + ATV group than in the Pio and ATV groups.

Ser1177-phosphorylated eNOS. There were significant differences in P-eNOS expression among groups (P < 0.001). Pio did not affect the expression of P-eNOS. In contrast, P-eNOS expression in the ATV group was 300% compared with the sham-treated group. P-eNOS expression in the Pio + ATV group was 295% of the sham-treated group (Fig. 3). These data suggest that the myocardial protection effect of Pio does not involve eNOS activation. Moreover, despite similar increases in P-eNOS levels in the Pio + ATV group and ATV-alone group, IS was significantly smaller in the combination group, which supports the hypothesis that the protective effect of Pio is independent of eNOS activation.

iNOS. There were significant differences in iNOS expression among groups (P < 0.001). Pio did not affect iNOS expression, whereas the expression of iNOS in the ATV group was 740% compared with the sham-treated group. The expression of iNOS in the Pio + ATV group was 729% compared with sham-treated group (Fig. 4). Again, these data suggest that the IS-limiting effect of Pio is independent of iNOS activation. Despite comparable increase in iNOS expression, IS was significantly smaller in the Pio + ATV group than in the ATV group, suggesting that Pio had an additive effect that was unrelated to eNOS and iNOS upregulation.

PTEN. There were significant differences in PTEN expression among groups (P < 0.001). ATV did not affect PTEN expression, whereas the expression of PTEN in the Pio group and Pio + ATV groups was 210% and 188% compared with sham-treated group, respectively. The expression of PTEN in the Pio group was significantly higher than in all other three groups (P < 0.001 vs. each group) (Fig. 5). PTEN expression in the Pio group was 321% compared with the sham and ATV groups. PTEN expression in the Pio group and ATV alone group was 320% (not different from the ATV-alone group). However, eNOS phosphorylation was comparable. Moreover, upregulation of PTEN by Pio did not attenuate the IS-limiting effect of ATV in the combination group.

SHIP2. There were significant differences in SHIP2 expression among groups (P < 0.001). Both ATV (338% compared with sham-treated group) and Pio (321% compared with sham-treated group) increased SHIP2 expression. The expression of SHIP2 in the Pio + ATV group was 320% (not different from the ATV or Pio groups) (Fig. 6). SHIP2 has also been reported to inhibit Akt phosphorylation (36). We cannot exclude that, without SHIP2 upregulation, Akt phosphorylation and myocardial protection were higher; however, in our model, upregulation of SHIP2 did not block Akt and eNOS phosphorylation by

Table 4. Protocol 2: body weight, LV weight, AR, and IS

<table>
<thead>
<tr>
<th></th>
<th>Pio + SC-58125</th>
<th>Pio + SC-560</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>252 (SD 1)</td>
<td>252 (SD 1)</td>
<td>1.00</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td>840 (SD 1)</td>
<td>840 (SD 2)</td>
<td>0.853</td>
</tr>
<tr>
<td>AR, % of LV</td>
<td>54.1 (SD 1.3)</td>
<td>54.3 (SD 3.9)</td>
<td>0.929</td>
</tr>
<tr>
<td>IS, % of LV</td>
<td>12.3 (SD 2.0)</td>
<td>5.1 (SD 0.3)</td>
<td>0.005</td>
</tr>
<tr>
<td>IS, % of AR</td>
<td>22.7 (SD 3.5)</td>
<td>9.5 (SD 0.8)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Values are means (SD). There were no differences in body weight, LV weight, and size of AR among groups. However, IS was larger in Pio + SC-58125 group than in Pio + SC-560 group, indicating that cyclooxygenase-2, but not cyclooxygenase-1, is involved in protective effect.
ATV. Moreover, upregulation of SHIP2 did not block the IS-limiting effect of ATV, Pio, and their combination. cPLA$_2$. cPLA$_2$ expression was increased by Pio (157%) and, to a greater extent, by ATV (370%; $P < 0.001$ vs. Pio). In the Pio + ATV group, cPLA$_2$ expression was increased to 408%. (Fig. 7). Thus it seems that ATV augments cPLA$_2$ expression to a greater extent than Pio; however, ATV and Pio have additive effects on cPLA$_2$ expression.

Fig. 4. Representative immunoblot (A) and densitometric analysis (B) of inducible NOS (iNOS) expression. Data are expressed as percentage of expression in sham group. Overall, there were significant differences among groups ($P < 0.001$). Pio did not affect NOS expression, whereas ATV alone or in combination with Pio significantly increased myocardial iNOS levels. +, Positive control [iNOS electrophoresis standard (catalog no. 360862, Cayman Chemical, Ann Arbor, MI)]. *$P < 0.001$ vs. sham.

PTEN. Overall, there were significant differences among groups ($P < 0.001$). ATV did not affect PTEN expression, whereas Pio alone or in combination with ATV caused a significant increase in myocardial PTEN levels. *$P < 0.001$ vs. sham. +, Positive control [recombinant human PTEN (catalog no. Alex-201–079; Alexis Bioscience, Alexis Biochemicals)].

Fig. 5. Representative immunoblot (A) and densitometric analysis (B) of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) expression. Data are expressed as percentage of expression in sham group. Overall, there were significant differences among groups ($P < 0.001$). ATV did not affect PTEN expression, whereas Pio alone or in combination with ATV caused a significant increase in myocardial PTEN levels. *$P < 0.001$ vs. sham. +, Positive control [recombinant human PTEN (catalog no. Alex-201–079; Alexis Bioscience, Alexis Biochemicals)].

Fig. 6. Representative immunoblot (A) and densitometric analysis (B) of Src homology (SH) 2-containing inositol phosphatase-2 (SHIP2) expression. Data are expressed as percentage of expression in sham group. Overall, there were significant differences among groups ($P < 0.001$). Both Pio and ATV increased myocardial SHIP2 levels by the same magnitude. There was no additive effect in Pio + ATV group. *$P < 0.001$ vs. sham.
ATV. cPLA2 expression. There was an additive effect, because myocardial cPLA2

exences among groups (P < 0.001). Both Pio and ATV significantly increased
cPLA2 expression. There was an additive effect, because myocardial cPLA2

levels were higher in Pio + ATV group than in Pio-alone and ATV-alone
groups. *P < 0.001 vs. sham; †P < 0.001 vs. Pio + ATV; ‡P < 0.001 vs.

ATV.

COX-2. COX-2 expression in the sham-treated group was

very low. ATV significantly increased COX-2 expression compared

with the sham-treated group (≈25-fold; P < 0.001 vs. sham). In the same blot, we were not able to detect increased

COX-2 expression in the Pio-alone group. Therefore, we

repeated the experiment for sham and Pio groups (n = 8 in each
group); with a larger protein load in each sample (see METHODS)

and longer film exposure, we were able to detect a significant

increase in the Pio-alone group (≈3.4-fold; P = 0.007 vs.

sham), although of a much smaller magnitude than seen with

ATV (Fig. 5). Pio did not blunt COX-2 expression by ATV in

the Pio + ATV group (≈25-fold) compared with the sham-
treated group (Fig. 8). Thus there is no linear correlation

between augmentation of COX-2 expression and IS limitation

by Pio, ATV, and their combination.

Enzyme Activity

cNOS activity significantly increased in the ATV and Pio +

ATV groups (Fig. 9). Pio alone caused a much smaller increase

in cNOS activity, although the difference was significant compared

with the sham-treated group (P = 0.012).

cNOS activity was significantly increased in the ATV and

Pio + ATV groups (Fig. 10). Pio alone did not affect cNOS

activity.

Thus, again, we found no linear correlation between cNOS

and cNOS activation and myocardial protection. The protec-
tive effect of Pio is independent of cNOS and cNOS activa-
tion.

Myocardial content of 6-keto-PGF1α was increased by Pio

and, to a greater extent, by ATV. Myocardial content of

6-keto-PGF1α in the Pio + ATV group was significantly higher

than in the sham-treated and the Pio-alone group, but the
difference between the Pio + ATV and ATV-alone groups was

not significant (Fig. 11). These results suggest that there is a

correlation between myocardial 6-keto-PGF1α levels and the

magnitude of IS limitation.

PLA2 activity was increased by Pio and, to a greater extent, by

ATV. PLA2 activity was significantly higher in the Pio +

ATV group than in the other three groups (Fig. 12). ATV

increased total COX activity and COX-2 activity. Pio aug-

mented total COX and COX-2 activity but to a lesser extent

than ATV. Total COX and COX-2 activity were further in-

creased in the Pio + ATV group (Fig. 13, A and B). Pio and

ATV, singly or in combination, did not alter COX-1 activity

(Fig. 13C). These results show that COX-2, and not COX-1,

increases myocardial prostacyclin levels in response to ATV,

Pio, and their combination.

DISCUSSION

Pretreatment with either Pio (10 mg·kg−1·day−1) or ATV

(10 mg·kg−1·day−1) reduced IS by 56% and 58%, respec-


tively. The protective effect was additive, because IS was

reduced by 81% in the Pio + ATV group. Although the IS

reduction by Pio is COX-2 dependent, as has also been shown

...
for ATV (1, 7), the pathways leading to enhanced PGI2 production are different. Activation of COX-2 by ATV is associated with Akt and eNOS phosphorylation and increased iNOS expression (1), whereas Pio did not affect eNOS and iNOS expression and caused only a marginal increase in P-Akt. Moreover, 3-day pretreatment with ATV resulted in a marked increase in activity and expression of both cPLA2 and COX-2 (1, 7), whereas pretreatment with Pio resulted in an increase in cPLA2 activity and expression but only a small increase in COX-2 expression and activity.

Thiazolidinediones and Myocardial Protection

Intravenous administration of rosiglitazone at 1 and 3 mg/kg reduces IS by 30% and 37%, respectively (67), whereas a 7-day pretreatment with oral rosiglitazone at 3 mg kg-1 day-1 reduces IS by 24% (67). Rosiglitazone reduces the accumulation of neutrophils and macrophages in the ischemic heart and blunts the ischemia-related induction of CD11b/CD18, intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) and the downregulation of L-selectin (67). Eight-day pretreatment with rosiglitazone at 3 mg kg-1 day-1 reduces both apoptosis and necrosis in Male Zucker diabetic fatty rats (66). This protective effect is diminished with wortmannin, suggesting that the phosphatidylinositol 3-kinase/Akt pathway is mediating the protective effect (66). In an in vivo rat model, using 25 min of ischemia and 2 h of reperfusion, the intravenous administration of rosiglitazone (0.3 or 1 mg/kg), ciglitazone (0.3 mg/kg), or Pio (0.3 or 1 mg/kg) 30 min before myocardial ischemia reduced IS. Of note is that ciglitazone at 1 mg/kg did not reduce IS (61). Pretreatment with Pio at 3 mg kg-1 day-1 for 7 days reduced IS and the number of infiltrating macrophages in the ischemic region in rats subjected to 30 min of ischemia followed by 24 h of reperfusion (35). The expression of ICAM-1 and MCP-1 mRNA in the ischemic-reperfused region after 24 h of reperfusion was significantly lower in the Pio-treated rats (35). Recently, Wynne et al. (65) used the Langendorff perfused rat...
heart model to show that pretreatment with PIO reduces IS and that adding LY-294002 or wortmannin, both PI3K inhibitors, before ischemia blocks the protective effect. Thus most published studies support an anti-inflammatory mechanism for myocardial protection by thiazolidinediones, although two studies did suggest that activation of PI3K/Akt may mediate the protective effect.

Akt Phosphorylation and Myocardial Protection

The PI3K pathway plays an important role in regulating numerous biological processes, including survival, proliferation, adhesion, migration, insulin activity, and cell activation, through the generation of the potent second messenger PI-3,4,5-trisphosphate (56). PI-3,4,5-trisphosphate promotes Akt phosphorylation; thus initiating a pro-survival pathway (4). Statins also induce Akt phosphorylation (22, 37, 41, 51). Blocking PI3K with wortmannin prevents Akt phosphorylation and blocks the IS-limiting effects of statins (22, 51) and PIO (64). Here we show that ATV increased Akt phosphorylation by 440%. In contrast, PIO caused a minor but not significant increase in myocardial P-Akt expression (142%) as compared with the sham-treated group. The small P-Akt increase present in the PIO group was not sufficient to induce a detectable change in P-eNOS levels, and the increase in eNOS activity in the PIO group was also less than that in the ATV group. Moreover, despite the significant difference in P-Akt expression in the PIO and ATV groups, the IS-limiting effects of these drugs were comparable. This suggests that the IS-limiting effect of PIO is probably independent of Akt phosphorylation.

There are conflicting reports on the effect of thiazolidinediones on Akt phosphorylation. Some studies (2, 15, 42) have shown that troglitazone increases P-Akt. However, there are reports that PIO (15, 32, 38), ciglitazone (24), and rosiglitazone (5, 38, 55) suppress Akt phosphorylation. Barthel et al. (3) showed that, at low concentrations, troglitazone enhances Akt phosphorylation, whereas at high concentrations, Akt phosphorylation is inhibited. Alternatively, PIO increases P-Akt levels in adipose biopsies of patients with insulin resistance (27). These observations are consistent with the biphasic nature of stress response mechanisms and tissue-specific nature of stress responses.

NOS Expression and Activity

Several studies (22, 37, 51) have suggested that statins increase the activity of eNOS by either increasing its expression or augmenting its phosphorylation at Ser1177. eNOS has a crucial role in mediating the IS-limiting effects of statins; for example, statins are not protective in eNOS knockout mice (36, 52). We have previously shown that 3-day pretreatment with ATV augments the activity of cNOS by increasing the expression of P-eNOS, but not total eNOS or neuronal NOS, in the rat (7, 48). In the present study, we confirmed that ATV increases P-eNOS expression and cNOS activity.

In contrast, PIO did not affect P-eNOS expression when given alone and did not change P-eNOS expression by ATV in the combination treatment group. cNOS activity was mildly increased by PIO; however, the magnitude of this increase was much smaller than with ATV. Previous studies (11, 31, 50) have suggested that PPAR-γ agonists increase NO production by eNOS, without affecting total eNOS expression. A recent study (31) has suggested that ciglitazone increases the bioavailability of NO, given its antioxidant effects, which are mediated by increased expression of Cu/Zn-superoxide dismutase and suppression of NADPH oxidase. However, in this study, P-eNOS expression was not measured. On the other hand, Cho et al. (15) demonstrated that troglitazone increased cNOS activity in bovine aortic endothelial cells by augmenting eNOS phos-
phorylation at Ser1179 by Akt, without altering total eNOS expression. Thus our results are in accordance with previous studies showing that Pio may cause a mild increase in eNOS activity; however, in contrast to Cho, we did not detect a change in P-eNOS levels in our in vivo model.

iNOS is essential for mediating the cardioprotective effects of late ischemic preconditioning (54, 68), opioid agonists (26), and sildenafil (48). Previously, we have shown that iNOS activation is mandatory for mediating the protective effect of ATV in the rat (1). In addition, simvastatin failed to reduce IS in iNOS knockout mice (52). In the present study, we confirmed that ATV increased iNOS expression and calcium-independent NOS activity. However, Pio did not affect iNOS expression and activity. In contrast, several investigators have suggested that PPAR-γ agonists, including ciglitazone (17), Pio (18), and rosiglitazone (18, 19), reduce iNOS expression and activity in various experimental models.

**PTEN and SHIP2**

PI-3,4,5-trisphosphate levels are negatively controlled by several phosphatases, including the ubiquitously expressed PTEN (which hydrolyzes PI-3,4,5-trisphosphate to PI-4,5-biphosphate) and SHIP2 [which hydrolyzes PI-3,4,5-trisphosphate to PI-3,4-bisphosphate (56)]. Mensah et al. (41) have shown that myocardial expression of PTEN was increased after 7 and 14 days of treatment with ATV at 20 mg·kg⁻¹·day⁻¹, although after a 3-day pretreatment, PTEN expression was unchanged. The IS-limiting effect of ATV at 20 mg·kg⁻¹·day⁻¹, apparent after 1 and 3 days, was lost after 7 and 14 days of treatment, suggesting that PTEN prevented activation of Akt and, hence, blunted the IS-limiting effect of ATV (41). Here, our finding that 3-day ATV treatment did not affect PTEN expression is in agreement with Mensah et al.’s (41) finding.

Previous studies (14, 23, 29, 38, 46, 63) have shown that thiazolidinediones increase the expression of PTEN and down-regulate PI3K activity. In our study, Pio increased myocardial PTEN expression. Both ATV and Pio increased the expression of SHIP2. Previous studies (30) have also shown that rosiglitazone decreases SHIP2 overexpression in skeletal muscles and fat tissue of diabetic db/db mice. Nevertheless, in the present study, the increased expression of SHIP2 and PTEN did not prevent the induction of Akt and Ser1177 eNOS phosphorylation by ATV and the IS-limiting effect of Pio alone, ATV alone, or Pio and ATV in combination. Because activation of Akt occurs rapidly after statins are introduced (37), we cannot exclude that Akt phosphorylation occurs before the increase in PTEN and SHIP2 expression and that, with longer pretreatment periods (i.e., 1–2 wk), PTEN and/or SHIP2 may eventually block Akt phosphorylation and thus the protective effect of ATV, Pio, or the combination. Alternatively, it might be that the activation of both SHIP2 and PTEN is reactive to the increase in PI3K activity and consequently, PI-3,4,5-trisphosphate levels, because it has recently been shown that prolonged activation of P-Akt may have deleterious effects (44). However, there is a net increase in PI-3,4,5-trisphosphate levels and, therefore, in Akt phosphorylation, as shown in the present study. The effect of Pio and ATV on SHIP2 and PTEN, especially with long-term treatment, should be further investigated.

**Myocardial Protection and PGI2 Production**

Late ischemic preconditioning causes an increase in myocardial concentrations of PGE₂ and 6-keto-PGF₁α, with only marginal increases in PGF₂α (53). Intravenous PGI₂, given before ischemia, reduces IS (59, 65). It has been reported that administration of COX-2 inhibitors before an infarction abrogates the IS-limiting effects of late ischemic preconditioning (10, 54).

**Statins and Prostaglandin Production**

There have been only sparse and conflicting data on the effects of statins on COX-2 regulation. Both mevastatin and lovastatin increase COX-2 content and 6-keto-PGF₁α concentration in human aortic smooth muscle cells (20). COX-2 levels also increase in interleukin-1β-stimulated mesangial cells after incubation with cerivastatin (9). On the other hand, three other studies have shown opposite results. Fluvastatin and simvastatin treatment have been reported to decrease the concentrations of COX-2 mRNA and protein levels in human umbilical vein endothelial cells (34). ATV also decreases the expression of COX-2 mRNA and protein in macrophages and smooth muscle cells of hypercholesterolemic rabbits (28). Finally, simvastatin decreases COX-2 expression in human carotid artery plaques (16). The present study confirms our previous findings that ATV increases the myocardial expression and activity of COX-2 in the rat myocardium (1, 7). Thus it seems that statins suppress COX-2 expression in inflammation models and atherosclerotic plaque but increase COX-2 expression in the normal myocardium.

We have previously reported that selective COX-2 inhibitors, given either orally together with ATV (7) or intravenously just before ischemia (1), abrogated the IS-limiting effect of ATV, suggesting that COX-2 products are also mediating the IS-limiting effects of statins. In both studies, when given alone, COX-2 inhibitors did not affect IS. In the same study, we have shown that SC-560, a selective COX-1 inhibitor, does not block the IS-limiting effect of ATV and does not affect IS when administered alone (1).

**Thiazolidinediones and Prostaglandin Production**

Here, we show that Pio increased myocardial 6-keto-PGF₁α levels. Pio increased cPLA2 expression and activity. Pio induced a mild increase in COX-2 activity, which was not associated with any detectable changes in COX-2 expression. There are conflicting data in the literature on the effect of thiazolidinedione on COX-2 expression. It has been reported that very high doses of rosiglitazone (30 µM) increase the expression of COX-2 in cultures of rat aortic vascular smooth muscle cells (8). However, at lower concentrations (10 µM), there was an increased release of prostaglandins without an increase in COX-2 expression (8). The authors have suggested that, at the lower concentrations, rosiglitazone induces PLA₂; although in their study, there was no induction of type II soluble PLA₂ by rosiglitazone (8). Toglitazone, but not 15d-PGJ₂, induces COX-2 expression and PGE₂ production in the human lung epithelial cell line A549 (45); however, this activation could not be blocked by PPAR-γ antagonists (45). Levine (39) has reported that ciglitazone increased the release of arachidonic acid from rat liver cells; however, the author did
not explore the mechanism of this effect. Others have reported that both rosiglitazone and Pio augment prostaglandin production by increasing the availability of arachidonic acid, without affecting COX-1, COX-2, and cPLA2 expression (60). They suggested that thiazolidinediones increase the availability of arachidonic acid by preventing the reuptake of arachidonic acid by cell membranes (60). On the other hand, others have suggested that PPAR-γ negatively controls COX-2 expression (33). The induction of COX-2 expression by mNF-κB in macrophages was blunted by both troglitazone and ciglitazone (13).

In the present study, we show that Pio alone caused a smaller (~3.4-fold) increase in COX-2 expression than ATV (~25-fold). However, total COX and COX-2 activity were both increased by Pio, and total COX and COX-2 activity were significantly higher in the Pio + ATV group than in the ATV-alone or Pio-alone group. Recently, we have shown that ATV activates COX-2 via S-nitrosylation mediated by iNOS (1). Although the increase in COX-2 expression and activity in the Pio-treated rats was smaller than in the ATV-treated rats, the IS-limiting effect of both agents was abrogated by SC-58125, a selective COX-2 inhibitor. In contrast, despite the fact that COX-1 activity is higher than COX-2 activity in the Pio group, selective COX-1 inhibition did not blunt the protective effect of Pio, as has been previously shown for ATV (1). Thus the Pio effect may be due in part to posttranslational modification of COX-2 and not only to its synthesis regulation.

cPLA2 and Myocardial Protection

A previous study (57) suggested that PLA2 is involved in ischemic preconditioning. We have reported that ATV induced the expression and activity of cPLA2 (1, 7). Coadministration of valdecoxib, a specific COX-2 inhibitor, together with ATV for 3 days blunts the ATV-induced increased expression and activity of cPLA2 (7). In contrast, acute intravenous administration of SC-58125, another specific COX-2 inhibitor, after a 3-day pretreatment with ATV, did not affect cPLA2 expression or activity (1), suggesting that COX-2 is needed to augment the expression, but not the immediate activation, of cPLA2.

In the present study, we show that both ATV and Pio increased the expression and activity of cPLA2 and COX-2. Pio and ATV have additive effects on cPLA2 expression and activity. However, as selective COX-2 inhibition abrogated the IS-limiting effects of both Pio and ATV (1, 7), it seems that cPLA2 does not have an independent effect, rather, the arachidonic acid, released by cPLA2, is converted to protective prostaglandins by COX-2.

In conclusion, the IS-limiting effects of both Pio and ATV involve prostaglandin production by COX-2. However, the upstream steps differ. ATV induces eNOS phosphorylation, iNOS, cPLA2, and COX-2 expression, whereas Pio induces mainly the expression and activity of cPLA2. Although the activity of COX-2 is increased in Pio-treated rats, there is a relatively small increase in COX-2 expression. The effect of Pio and ATV is additive. The efficacy of this combination in myocardial protection should be further investigated in the clinical setting.

GRANTS

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