The myocardial infarct size-limiting effect of sitagliptin is PKA-dependent, whereas the protective effect of pioglitazone is partially dependent on PKA.
leading to activation of PKA and cAMP response element-binding protein (CREB) phosphorylation (13). GLP-1 receptor agonists also regulate CREB activity through glucose-dependent stimulation of the cytoplasmic to nuclear translocation of target of rapamycin complex 2 (TORC2), a CREB coactivator (13). GLP-1 receptor activation leads to ERK1/2, protein kinase C, and phosphatidylinositol 3-kinase (PI 3-kinase) activation, all involved in protection against ischemia-reperfusion injury.

Furthermore, several studies have shown that GLP-1 or GLP-1 analogs limit myocardial IS in various animal models (1, 5–7, 30, 36, 45, 47). However, it is unknown whether oral DPP-IV inhibitors have the same cardioprotective effects. We assessed whether oral sitagliptin (SIT), a DPP-IV inhibitor that has been introduced for the treatment of type 2 DM, limits myocardial IS by PKA activation in the mouse and whether PIO and SIT have additive effects on cardioprotection by activating PKA.

**METHODS**

Male CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA) and received humane care in compliance with The Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

**Materials**

cAMP-dependent PKA assay kit was purchased from Promega (Madison, WI). ELISA kits for 6-keto-PGF1α, and cPLA2 and COX activity were purchased from Cayman Chemicals (Ann Arbor, MI); ELISA kit for 15-epi-lipoxin A4 was from Oxford Biomedical Research (Oxford, MI); ELISA kit for 15-deoxy-PGJ2 and enzyme immunoassay for cAMP levels were from Assay Designs (Ann Arbor, MI); and ELISA kit for GLP-1-(7–36) from was Alpcor Diagnostics (Salen, NH). PIO was provided by Takeda Pharmaceuticals North America (Lincolnshire, IL) and SIT by Merck. H-89, monoclonal (Salem, NH). PIO was provided by Takeda Pharmaceuticals North America (Lincolnshire, IL) and SIT by Merck. H-89, monoclonal anti-β-actin antibodies, and monoclonal anti-myoxy antibodies were purchased from Sigma (St. Louis, MO). Anti-protein kinase B (Akt), serine-473 P-Akt, threonine-308 P-Akt, eNOS, serine-633 P-eNOS, serine-1177 P-eNOS, CREB, and serine-133 P-CREB antibodies were from Cell Signaling (Beverly, MA).

**Treatment**

**Protocol 1.** Mice received 3-day pretreatment with: 1) SIT (300 mg·kg−1·day−1); 2) PIO (5 mg·kg−1·day−1); 3) SIT + PIO; or 4) water alone (control). Drugs were suspended in water and administered by oral gavage once daily. On the 4th day, mice received intravenous H-89 (20 mg/kg) or vehicle (5% DMSO) (54). After injection (1 h), mice underwent coronary artery ligation for 30 min followed by 4 h reperfusion, or mice were killed under anesthesia, and hearts were explanted without being subjected to ischemia.

For immunoblotting and enzyme activity, hearts were 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 for further analyses.

**Protocol 2.** Mice received 14-day pretreatment with: 1) SIT (300 mg·kg−1·day−1); 2) PIO (5 mg·kg−1·day−1); 3) SIT + PIO; or 4) water alone (control), as above. Mice underwent coronary artery ligation for 30 min followed by 4 h reperfusion.

**Infarct Size**

On the 4th (protocol 1) or 15th (protocol 2) day, mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated, and ventilated (FiO2 = 30%). The rectal temperature was monitored, and body temperature was maintained between 36.7 and 37.3°C throughout the experiment. The chest was opened, and the left coronary artery was encircled with a suture and ligated for 30 min. Ischemia was verified by regional dysfunction and discoloration of the ischemic zone. Isoflurane (1–2.0% titrated to effect) was added after the beginning of ischemia to maintain anesthesia. At 30 min of ischemia, the snare was 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 mice recovered from anesthesia. After reperfusion (4 h), the mice were reanesthetized, the coronary artery was reoccluded, 3% Evan’s blue dye was injected in the right ventricle, and the mice were killed under deep anesthesia (52, 53).

The prespecified exclusion criteria were lack of signs of ischemia during coronary artery ligation, lack of signs of reperfusion after release of the snare, prolonged ventricular arrhythmia with hypertension, and area at risk (AR) ≤10% of the left ventricular weight.

**Determination of AR 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-triphenyl-tetrazolium-chloride (TTC), fixed in 10% formaldehyde, and photographed to identify the AR (uncolored by the blue dye), the IS (unstained 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. The results were summed to obtain the weight of the myocardial AR and IS (52, 53).

**cAMP Levels and PKA Activity**

Myocardial samples from the anterior wall of the left ventricle of hearts that were not subjected to ischemia were homogenized in 1 ml cold extraction buffer (20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.1% Triton X-100) and centrifuged at 14,000 g for 15 min at 4°C, and the supernatants were collected. cAMP levels and PKA activity were measured using assay kits according to the manufacturer’s instructions.

**6-Keto-PGF1α, 15-Deoxy-PGJ2, 15-Epi-Lipoxin A4 Levels, and cPLA2 and COX2 activity**

Myocardial samples of the anterior wall of the left ventricle were homogenized in cold PBS (pH 7.4) and centrifuged. The supernatants were collected and stored on ice. Measurements of 6-keto-PGF1α, the stable metabolite of prostacyclin, 15-deoxy-PGJ2, 15-epi-lipoxin A4, and cPLA2 activity were made using immunoassay assay kits. The COX activity assay kit measures the peroxidase activity of COX, assayed colorimetrically 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 COX2 inhibitor; and the third with Sc-560, a specific COX1 inhibitor). COX1 activity was calculated as the difference between total COX activity in the sample without an inhibitor and the sample with Sc-560, and COX2 activity was calculated as the difference between total COX activity in the sample without an inhibitor and the sample with DuP-697.

**GLP-1-(7–36) Levels**

Mice received 3 day pretreatment with water alone (control) or SIT for 3 days (6 animals in each group). On the 4th day, whole blood samples were collected from the tail vein of nonfasting animals in EDTA-plasma tubes containing 50 μM of a DPP-IV inhibitor (Milipore, Billerica, MA). Samples were mixed and centrifuged at 1,000
Table 1. Body weight, LV weight, AR, and IS of mice in protocol 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SIT</th>
<th>PIO</th>
<th>SIT + PIO</th>
<th>H-89</th>
<th>SIT + H-89</th>
<th>PIO + H-89</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>25.3 ± 0.3</td>
<td>24.7 ± 0.3</td>
<td>24.9 ± 0.3</td>
<td>24.9 ± 0.4</td>
<td>25.5 ± 0.3</td>
<td>25.7 ± 0.5</td>
<td>25.7 ± 0.9</td>
<td>0.455</td>
</tr>
<tr>
<td>LV, mg</td>
<td>121 ± 2</td>
<td>125 ± 2</td>
<td>126 ± 3</td>
<td>126 ± 2</td>
<td>118 ± 1</td>
<td>119 ± 1</td>
<td>121 ± 1</td>
<td>0.183</td>
</tr>
<tr>
<td>AR, %LV</td>
<td>47.4 ± 1.0</td>
<td>46.1 ± 1.7</td>
<td>44.3 ± 1.7</td>
<td>43.9 ± 1.6</td>
<td>44.1 ± 1.9</td>
<td>45.1 ± 1.9</td>
<td>40.7 ± 1.2</td>
<td>0.267</td>
</tr>
<tr>
<td>IS, %LV</td>
<td>21.2 ± 1.8</td>
<td>11.3 ± 1.4</td>
<td>9.8 ± 0.6</td>
<td>6.5 ± 0.4</td>
<td>20.0 ± 0.2</td>
<td>20.5 ± 2.5</td>
<td>13.7 ± 1.2</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. or mice. SIT, sitagliptin; PIO, pioglitazone; LV, left ventricular; AR, area at risk (%LV weight); IS, infarct size (%LV weight).

Results

Infarct Size

Protocol 1. A total of 70 mice were included, 3 died (1 in the PIO group died during ischemia and 2 in the SIT + H-89 died before coronary artery occlusion). Body weight, left ventricular weight, and the size of the AR were comparable among groups (Table 1). IS, expressed as a percent of the left ventricle (Table 1) or a percent of the AR (Fig. 1A) was significantly smaller in the SIT and PIO group than in the control group. IS was the smallest in the SIT + PIO group (P < 0.001 vs. the control group; P = 0.053 vs. SIT; P = 0.288 vs. PIO). H-89 alone had no effect on IS; however, it completely blocked the effect of SIT, whereas it only partially blocked the effect of PIO.

Protocol 2. A total of 32 mice were included; none were excluded or died. Body weight, left ventricular weight, and the size of the ischemic AR were comparable among groups (Table 2). IS, expressed as a percent of the left ventricle (Table 2) or a percent of the AR (Fig. 2), was significantly smaller in the SIT and PIO groups than in the control group. IS was the smallest in the SIT + PIO group (P < 0.001 vs. the control and PIO groups; P = 0.014 vs. SIT group).

Blood Glucose and GLP-1 Levels

Mice received water alone (control), SIT, or PIO for 3 days. On the 4th day, blood samples from the tail vein of nonfasting mice were collected. Plasma was separated and stored at −70°C until assayed. The concentrations (pmol/L) of active GLP-1 were determined using an active GLP-1-(7–36) ELISA kit according to the manufacturer’s instructions.

Immunoblotting

Myocardial samples from the risk zone of the anterior wall of the left ventricular wall exposed to ischemia-reperfusion, or from the anterior wall of control hearts not exposed to ischemia, were homogenized in lysis buffer (in mmol/L): 25 Tris·HCl (pH 7.4), 0.5 EDTA, 0.5 EGTA, 1 phenylmethylsulfonyl fluoride, 1 dithiothreitol, 25 NaF, 1 Na3VO4, 1% Triton X-100, 2% SDS, and 1% protease inhibitor cocktail. The lysate was centrifuged at 10,000 g for 15 min at 4°C. The resulting supernatants were collected. Protein (50 μg) was fractionated by SDS-PAGE (4–20% polyacrylamide gels) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated overnight at 4°C with primary antibodies. Bound antibodies were detected using the chemiluminescent substrate (NEL Life Science Products, Boston, MA). The protein signals were quantified with an image-scanning densitometer, and the strength of each protein signal was normalized to the corresponding β-actin signal. Data are expressed as percent of the expression in the control group.

Statistical Analysis

Data are presented as means ± SE. The significance level α is 0.05. Body weight, left ventricular weight, the size of the AR and IS, enzyme activity, eicosanoid levels, and protein expression were compared using ANOVA with Sidak corrections for multiple comparisons. Values of P < 0.05 were considered statistically significant.
animals were assessed for blood glucose levels ($n = 4$ in each group). Glucose levels in the control group were $137 \pm 12$ mg/dl; in the SIT group $135 \pm 23$ mg/dl; and in the PIO group $132 \pm 11$ mg/dl ($P = 0.975$).

GLP-1 levels were significantly higher than in the control no ischemia-reperfusion group. Glucose levels in the control group were 137 mg/dl, and in the PIO group 135 mg/dl. SIT did not block the effect of PIO. In contrast, SIT had no effect on COX2 activity, and it did not block the PIO-induced increase in COX2 activity.

**Eicosanoid Levels**

SIT had no effect on 6-keto-PGF$_{1\alpha}$ (Fig. 5A) or 15-deoxy-PGF$_2$ (Fig. 5B) levels. On the other hand, PIO increased these levels. Levels of 6-keto-PGF$_{1\alpha}$ and 15-deoxy-PGF$_2$ were comparable in the PIO alone group and the SIT + PIO group. H-89 did not block the effect of PIO. In contrast, SIT significantly increased 15-epi-lipoxin A$_4$ levels. PIO caused a small insignificant increase in 15-epi-lipoxin A$_4$ levels (Fig. 5C). 15-Epi-lipoxin A$_4$ levels were the highest in the SIT + PIO group ($P < 0.001$ vs. control and PIO; $P = 0.007$ vs. SIT). H-89 completely blocked the effect of both PIO and SIT.

**Immunoblotting**

For control, we used myocardial samples from mice treated with oral saline for 3 days and not exposed to ischemia-reperfusion. Ischemia-reperfusion did not affect total Akt levels (Fig. 6, A and B); however, it increased the levels of serine-473 P-Akt (Fig. 6, A and C) and threonine-308 P-Akt (Fig. 6, A and D). Both SIT and PIO further augmented this increase. Interestingly, H-89 did not affect the effect of SIT on PIO on Akt phosphorylation at either site.

Ischemia-reperfusion did not affect total eNOS levels. PIO and SIT had no effect on total eNOS levels (Fig. 7, A and B). Ischemia-reperfusion induced an increase in serine-1177 P-eNOS levels (Fig. 6, A and C). PIO and SIT augmented this increase, and H-89 attenuated the effects of both SIT and PIO, suggesting that PKA is involved in SIT- and PIO-induced eNOS phosphorylation at serine-1177. Similarly, ischemia-reperfusion increased myocardial levels of serine-633 P-eNOS. Both PIO and SIT augmented this increase. H-89 attenuated this increase, suggesting that PKA is involved also in the augmented phosphorylation of eNOS at serine-633 by both SIT and PIO.

Ischemia-reperfusion without or with PIO or SIT did not affect total CREB levels (Fig. 8, A and B). Ischemia-reperfusion induced a small insignificant increase in P-CREB. However, in mice pretreated with PIO or SIT, levels of P-CREB were significantly higher than in the control no ischemia-
reperfusion or control ischemia-reperfusion groups (Fig. 8, A and C). H-89 blocked this effect of SIT and PIO.

Simulated Ischemia-Reperfusion Injury in Adult Rat Cardiomyocytes

In cells not exposed to SIR (NSIR), DMSO, GLP-1, SIT, and SIT + GLP-1 (alone or in combination with wortmannin, U-0126, Sc-58125, or H-89) had no effect on cell death, as assessed by trypan blue exclusion assay (P = 0.997) (data not shown). DMSO had no significant effect on cell death in cells exposed to SIR (Fig. 9A). GLP-1 alone and SIT alone had no effect on cell death; however, SIT + GLP-1 significantly reduced cell death in cells exposed to SIR. Wortmannin (a PI 3-kinase inhibitor), U-0126 (an ERK1/2 inhibitor), and H-89 (a PKA inhibitor) completely blocked the protective effect of SIT + GLP-1, whereas Sc-58125 (a COX2 inhibitor) had no effect (Fig. 9A), suggesting that COX2 is not essential for the protective effect of SIT.

In cells not exposed to SIR (NSIR), DMSO and PIO (alone or in combination with wortmannin, U-0126, Sc-58125, or H-89) had no effect on cell death (P = 0.999) (data not shown). DMSO had no significant effect on cell death in cells exposed to SIR (Fig. 9B). PIO significantly reduced cell death. Wortmannin and Sc-58125 completely inhibited the protective effect, suggesting an essential role of PI 3-kinase and COX2 in the protective effect of PIO. In contrast, U-0126 and H-89 did not alter the protective effect of PIO (Fig. 9B), suggesting that the protective effect of PIO is ERK1/2 and PKA independent.

GLP-1 levels in the supernatant of the cultures after 4 h incubation are presented in Fig. 9C. GLP-1 was undetectable in the control, DMSO, and SIT alone groups. GLP-1 levels were increased in the GLP-1 alone group and were significantly much higher in the SIT + GLP-1-treated cells.

DISCUSSION

The present study shows that pretreatment with oral SIT, a DPP-IV inhibitor, limited myocardial IS. The magnitude of the protective effect was comparable to that of oral PIO, a PPAR-γ agonist, and the combination of SIT and PIO resulted in an additive protective effect. The protective effect of both SIT and PIO was sustained after 14 days of treatment, suggesting that “tolerance” over long treatment periods may not occur. However, the signal pathways mediating protection are different for SIT and PIO. SIT increased myocardial levels of cAMP with subsequent increase in PKA activity, but no change was seen in cPLA2 and COX2 activity. On the other hand, PIO induced an insignificant increase in cAMP levels, lower magnitude of PKA activation than SIT, and significant increase in cPLA2 and COX2 activity. Accordingly, PIO augmented myocardial levels of 6-keto-PGF1α and 15-deoxy-PGJ2, whereas its effect on myocardial 15-epi-lipoxin A4 levels was not statistically significant. SIT, on the other hand, did not affect 6-keto-PGF1α and 15-deoxy-PGJ2 levels but significantly increased 15-epi-lipoxin A4 levels. PIO, and to a greater extent SIT, increased eNOS phosphorylation at serine-1177 and serine-633, an effect that was attenuated by PKA inhibition. Both SIT and PIO augmented CREB phosphorylation by a PKA-dependent manner. The IS-limiting effects of SIT were completely abrogated by PKA inhibition, whereas that of PIO was only partially attenuated by H-89.

In vitro experiments showed that the protective effects of both SIT and PIO are dependent on PI 3-kinase activation. However, ERK1/2 is essential only for the protective effect of SIT, whereas inhibiting ERK1/2 did not affect the protective effect of PIO. The protective effect of SIT is PKA dependent and not affected by COX2 inhibition. In contrast, the protective
effect of PIO is independent of PKA but completely abrogated by COX2 inhibition.

GLP-1 and DPP-IV Inhibitors and Protection Against Ischemia-Reperfusion Injury

GLP-1 increases intracellular cAMP levels, leading to activation of PKA and CREB phosphorylation (Fig. 10) (13). GLP-1 receptor agonists also upregulate CREB activity through glucose-dependent stimulation of the cytoplasmic to nuclear translocation of TORC2, a CREB coactivator (Fig. 10) (13). It has been reported that GLP-1 receptor activation leads to ERK1/2, protein kinase C, and PI 3-kinase activation. All are involved in protection against ischemia-reperfusion injury. Several studies have shown that GLP-1 (1, 5–7, 37) or GLP-1 analogs (36, 45) limit myocardial IS in the rat and the mouse. GLP-1 analogs also reduce myocardial IS in the rabbit when given either before ischemia or immediately after reperfusion (30) and, in the pig, when given at reperfusion (47). In contrast, GLP-1-(9–36), the product of DPP-IV, does not reduce IS in the rat (37, 45), although it also augments (45) or worsens (37) postreperfusion left ventricular systolic function. Ban et al. (1) found that, in the mouse isolated heart model, GLP-1-(9–36) enhances postischemia recovery of function through NOS activation and cGMP release that are independent of the GLP-1 receptor. However, administration of GLP-1-(9–36) before ischemia has no effect on lactic dehydrogenase (LDH) release, and administration of GLP-1-(9–36) postischemia has only a small effect on LDH release (1), suggesting that the augmentation of function is explained by inotropic effects and not protection against ischemia-reperfusion injury. The protective effect of GLP-1 could be blocked by GLP-1 receptor antagonist, cAMP inhibitor, PI 3-kinase inhibitor, and ERK1/2 inhibitor and is associated with increased phosphorylation of BCL2-associated agonist of cell death at serine-136, leading to inactivation of this proapoptotic protein (5). The protective effect is also blocked by rapamycin (7). The authors suggested that p70s6K may be involved; however, they could not demonstrate an effect of GLP-1 on p70s6K phosphorylation (7). Because rapamycin has additional targets, other proteins may be responsible for the blocking effect of rapamycin. In mice, liraglutide, a GLP-1 analog, increases myocardial levels of P-Akt, P-glycogen synthase kinase-3β, PPAR-β/δ, heme oxygenase-1, and nuclear factor erythroid 2-related factor 2; reduces the levels of activated caspase-3; and reduces myocardial IS (36).

Xie et al. (49) reported that both LY-294002 (a PI 3-kinase inhibitor) and U-0126 blocked the protective effects of GLP-1 against hypoxia-reoxygenation injury in neonatal rat cardiomyocytes. Bose et al. (5) found that U-0126 blocked the protective effect of GLP-1 in isolated rat hearts. Our in vitro study supports the previous studies showing that the protective effect of SIT + GLP-1 was blocked by ERK1/2 and PI 3-kinase inhibition.

Interestingly, valine pyrolididine (VP), a GLP-1 breakdown inhibitor, administered either before ischemia or at reperfusion, does not limit IS in the rat either in the isolated heart model (5–7) or in vivo (5). If the gut is the sole source of GLP-1, it is reasonable that DPP-IV inhibition will not be effective in isolated heart models. Concerning the in vivo experiment, VP was given subcutaneously only 30 min before anesthesia, suggesting that short-term inhibition of DPP-IV may not be enough to increase GLP-1 above a certain threshold. In contrast, in the present study, SIT was given for 3 or 14 days, allowing time for GLP-1 buildup (Fig. 3).

In our in vitro experiment, SIT alone had no protective effect. In cells incubated with SIT alone, GLP-1 was undetected, confirming that cardiomyocytes do not secrete GLP-1. It has been reported that DPP-IV is involved in degradation of bradykinins and substance P, both implicated in attenuation of ischemia-reperfusion injury (8, 24). The fact that SIT did not affect SIR injury in the in vitro experiment does not support a...
role for these mediators in the protection afforded by DPP-IV inhibition with SIT.

Interestingly, GLP-1 alone had no significant protective effect in vitro. However, we found that GLP-1 levels were significantly lower in cell cultures incubated with GLP-1 alone than in those exposed to SIT + GLP-1, suggesting that degradation of GLP-1 by DPP-IV occurs in the adult cardiomyocyte culture.

Fig. 6. Samples of immunoblots (A) and densitometric analysis of total protein kinase B (Akt; B), serine (Ser)-473 phosphorylated (P)-Akt (C), and threonine (Thr)-308 P-Akt (D). P < 0.001 vs. control no ischemia-reperfusion (*) and control ischemia-reperfusion (#).

Fig. 7. Samples of immunoblots (A) and densitometric analysis of total endothelial nitric oxide synthase (eNOS) (B), serine-1177 P-eNOS (C), and serine-633 P-eNOS (D). P < 0.001 vs. control no ischemia-reperfusion (*), control ischemia-reperfusion (#), and H-89 vs. no H-89 ($).
PKA and Protection Against Ischemia-Reperfusion Injury

We found that only SIT increased myocardial levels of cAMP; however, both SIT and PIO increased PKA activity. H-89, a PKA inhibitor, completely abrogated the IS-limiting effects of SIT and partially blocked those of PIO. Sanada et al. (41) reported that ischemic preconditioning activates PKA in the canine myocardium. Dibutyryl-cAMP, a PKA activator, reduced myocardial IS, whereas PKA inhibition abrogated the effects of ischemic preconditioning. PKA activation decreases Rho kinase activation during sustained ischemia. Sanada et al. (41) suggested that PKA activation leads to Rho kinase and actin cystoskeletal deactivation, mediating cardioprotection (Fig. 10). Inserte et al. (21) suggested that the protective effect of PKA, induced by ischemic preconditioning, is mediated by attenuation of calpain-mediated degradation of structural proteins (Fig. 10). Phosphodiesterase III inhibitors that increase cAMP levels reduce IS and increase myocardial p38 MAPK activity during the preischemic period (Fig. 10). Both effects are blocked by H-89 (42). In addition, PKA activates eNOS by...
phosphorylation at both serine-633 and serine-1177 (Fig. 10) (3, 4, 17). eNOS is involved in ischemic preconditioning and various forms of pharmacological preconditioning (18). Oral cilostazol, a phosphodiesterase III inhibitor, limits IS and increases myocardial PKA activity, Akt phosphorylation at serine-473, and eNOS phosphorylation at both serine-633 and serine-1177 (27). Moreover, phosphorylation of 5-lipoxygenase at serine-523 by PKA augments the production of 15-epi-lipoxin A4, an eicosanoid with potent anti-inflammatory properties that potentially also favorably attenuates ischemia-reperfusion injury (Fig. 10) (54).

Interestingly, despite the fact that H-89 completely blocked the IS-limiting effect of SIT (Fig. 1), it did not block Akt phosphorylation following ischemia-reperfusion in vivo (Fig. 6). However, it attenuated the increase in serine-1177 and serine-633 P-eNOS (Fig. 7). On the other hand, the in vitro experiments showed that blocking PI 3-kinase by wortmannin completely blocked the protective effect of SIT, indicating that Akt activation is essential for mediating the protective effects of SIT. We cannot exclude that PKA was responsible for Akt phosphorylation during the 3-day pretreatment with SIT before it was inhibited by H-89. However, Akt phosphorylation is tightly controlled, and the effect of PKA should have disappeared 5.5 h after H-89 administration. These results suggest that Akt phosphorylation alone is insufficient for myocardial protection and that PKA is probably needed to activate mediators downstream of Akt. It seems that Akt phosphorylation is independent of PKA, whereas PKA is (at least partially) needed for eNOS phosphorylation at both serine-1177 and serine-633.

**CREB and Protection Against Ischemia-Reperfusion Injury**

CREB is a major nuclear transcription factor that transduces cAMP activation of gene transcription (43). CREB is activated by phosphorylation at serine-133 by PKA (23, 43). CREB phosphorylation at serine-133 may also be mediated by Akt (10, 43), the MEK/MAPK/p90rsk pathway (16), Ca²⁺/calmodulin-dependent protein kinase II (46), protein kinase C-ε (25), protein kinase D (38), ERK1/2, and mitogen and stress activated protein kinase-1 (29). CREB phosphorylation induces translocation of cytoplasmic CREB to the nucleus (Fig. 10) (43). CREB is involved in ischemic preconditioning (29, 34). The promoter region of genes encoding cytochrome c and Bcl-xl carries a cAMP-response element site, and the transcription factor CREB has been recognized as a positive regulator of these genes (15). Das et al. (9) found that adenosine receptor type 3 activation directly augments CREB serine-133 phosphorylation followed by Bcl-2 phosphorylation.

In our model, SIT- and PIO-induced CREB phosphorylation was completely blocked by H-89 (Fig. 8), suggesting that PKA mediates CREB phosphorylation induced by both agents. In the present study, we have not established a direct “cause and effect” relationship between CREB and myocardial protection. However, PKA inhibition by H-89 blocked the IS-limiting effects along with complete inhibition of the increase in CREB phosphorylation by SIT. On the other hand, H-89 completely blocked PIO induction of P-CREB with only partial attenuation of the IS-limiting effect of PIO. Thus CREB phosphorylation may contribute but is not essential for pharmacological preconditioning by PIO.

**Role of eNOS in Myocardial Protection**

Activation of eNOS is essential for the IS-limiting effects of late ischemic preconditioning (50) and statins (55). We have shown before that PIO limits IS in eNOS−/− and iNOS−/− knockout mice, as well as in wild-type mice treated with l-NAME, a nonspecific NOS inhibitor (53). However, the magnitude of protection was less in eNOS−/− mice, suggesting that the protective effect of PIO is partially dependent on eNOS. In the present study, PIO, and to a greater extent SIT, increased myocardial levels of serine-633 and serine-1177 P-eNOS, confirming our previous report that PIO increases phosphorylation of eNOS at both sites (53). Interestingly, the increase in serine-1177 and serine-633 P-eNOS by both PIO and SIT was attenuated by H-89, suggesting that PKA contributes to eNOS phosphorylation at these sites in our model. It is
yet unclear whether eNOS phosphorylation is essential for the myocardial protective effects of SIT.

**Eicosanoids and Myocardial Protection**

PIO increases myocardial levels of 6-keto-PGF₁α (the stable metabolite of prostacyclin) (52), 15-epi-lipoxin A₄ (a potent anti-inflammatory mediator)(2), and 15-deoxy-PGJ₂ (the natural ligand of PPAR-γ) (56). These mediators have been reported to have anti-inflammatory, anti-atherosclerotic, and myocardial protective effects. The IS-limiting effects of PIO are abrogated with COX2 inhibition (52). The results of the present study confirm our previous findings (2) showing that PIO (5 mg·kg⁻¹·day⁻¹ for 3 days) increased myocardial activity of cPLA₂ and COX2 (Fig. 4), and levels of 6-keto-PGF₁α and 15-deoxy-PGJ₂ (Fig. 5). As in our previous study, the effect of PIO at 5 mg·kg⁻¹·day⁻¹ on 15-epi-lipoxin A₄ was relatively small. However, with 10 mg·kg⁻¹·day⁻¹ for 3 days, PIO caused a significant increase in 15-epi-lipoxin A₄ levels (2).

In contrast, SIT had no effect on cPLA₂ and COX2 activity (Fig. 4), or on 6-keto-PGF₁α and 5-deoxy-PGJ₂ levels (Fig. 5), but caused a significant increase in 15-epi-lipoxin A₄ levels. 15-Epi-lipoxin A₄ has potent anti-inflammatory effects that may be beneficial for patients with type 2 diabetes. However, it has to be further studied whether 15-epi-lipoxin A₄ mediates (at least part of) the IS-limiting effects of SIT and PIO.

Of note, SIT and PIO had additive effects on IS limitation and on 15-epi-lipoxin A₄ levels.

**PIO and Myocardial Protection**

PIO reduces myocardial IS. The mechanism of protection involves upregulation of prostaglandin production through cPLA₂ and COX2 (52, 53). We have previously shown that the protective effect is eNOS and iNOS independent (53) but is COX2 dependent (52). The results of the present study confirm our previous studies that PIO increases myocardial levels of 6-keto-PGF₁α (the stable metabolite of prostacyclin) (52) and 15-deoxy-PGJ₂ (the natural ligand of PPAR-γ) (56). These mediators have been reported to have anti-inflammatory, anti-atherosclerotic, and myocardial protective effects. The increase in 15-epi-lipoxin A₄ was smaller, and it did not reach statistical significance. We have previously shown that this increase is dose dependent and is seen mainly with higher doses (10 mg·kg⁻¹·day⁻¹) (2). In addition, PIO activates PKA, which may be involved in protection against ischemia-reperfusion injury. (54).

The results of the in vivo study confirm reports of others that PIO increases Akt phosphorylation in the heart (48, 51). As reported in the previous studies, our in vitro experiment shows that the PIO effect is PI 3-kinase dependent. Our results support the findings of Wynne et al. (48) that the protective effect of PIO is ERK1/2 independent. We rule out insufficient inhibition by U-0126, since the same concentration blocked the protective effect of SIT in a parallel experiment (Fig. 9).

An encouraging finding is that the protective effect of SIT, PIO, and their combination did not decay over time. Mensah et al. (32) reported that the IS-limiting effects of atorvastatin disappeared after 1 and 2 wk of pretreatment, suggesting that upregulation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) leads to inactivation of Akt. This questions the potential benefits of long-term treatment with statins on myocardial protection. Here we are showing that both PIO and SIT significantly reduced IS after 14 day treatment. Moreover, we have previously shown that PIO (10 mg·kg⁻¹·day⁻¹) for 3 days reduces IS despite upregulating PTEN levels (52). Hence, in contrast to the protective effect of statins that may be transient, the protective effects of SIT and PIO are probably long lasting. It has been reported that the protective effect of morphine does not decay over time and is also dependent of PKA activation (40).

Several multicenter clinical trials and meta-analyses have shown that PIO decreases cardiovascular events in diabetic patients; however, PIO increases the incidence of heart failure admissions (12, 26, 28, 31). However, it seems that rosiglitazone, another thiazolidinedione drug that is used to treat type 2 DM, does not share the same clinical benefits (35), despite reports that it protects against ischemia-reperfusion injury in animal models (33, 57).

Further studies are needed to explore the mechanism of myocardial protection by both DPP-IV inhibitors and PPAR-γ agonists and their potential benefits in the clinical setting.

**Limitations**

In the present study, we assessed the effects of two different anti-diabetic drugs on myocardial IS in nondiabetic animals. Based on the encouraging results of the present study, we will continue to assess the effects of these drugs in diabetic animal models.

In the present study, we assessed the effects of 14- and 3-day pretreatment on IS. In the clinical setting, we cannot predict when myocardial infarction will occur; therefore, in the setting of ST elevation myocardial infarction, therapies should be initiated during ischemia or just before reperfusion. However, pharmacological preconditioning of the heart can be used in preparation for scheduled surgery or percutaneous coronary interventions that may increase the risks for ischemia and infarction.

We studied two different anti-diabetic drugs and have shown that these agents activate different survival pathways. The combination of the two drugs resulted in an additive protective effect. In the clinical setting, combination therapy is very common. We have previously shown that coadministration of various drug combinations may affect myocardial protection by statins, etc. Therefore, showing the additive protective effect of the two agents may have clinical relevance.

We used H-89 to inhibit PKA. However, H-89 may possess additional nonspecific actions. Results of the present study should be confirmed with the use of different PKA antagonists.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.

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