Phosphodiesterase-3 inhibition augments the myocardial infarct size-limiting effects of exenatide in mice with type 2 diabetes

Yumei Ye,1* Jinqiao Qian,1* Alexander C. Castillo,1 Shukuan Ling,1 Hongmei Ye,2 Jose R. Perez-Polo,1 Mandeep Bajaj,3,5 and Yochai Birnbaum1,4,5

1Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas; 2Space Medicine Fundamentals and Application, China Astronaut Research and Training Center, Beijing, China; 3Section of Endocrinology, Baylor College of Medicine, Houston, Texas; 4Section of Cardiology, Baylor College of Medicine, Houston, Texas; and 5Texas Heart Institute, St. Luke Episcopal Hospital, Houston, Texas

Submitted 13 August 2012; accepted in final form 21 October 2012

Ye Y, Qian J, Castillo AC, Ling S, Ye H, Perez-Polo JR, Bajaj M, Birnbaum Y. Phosphodiesterase-3 inhibition augments the myocardial infarct size-limiting effects of exenatide in mice with type 2 diabetes. Am J Physiol Heart Circ Physiol 304: H131–H141, 2013. First published October 26, 2012; doi:10.1152/ajpheart.00609.2012.—Glucagon-like peptide (GLP)-1 receptor activation increases intracellular cAMP with upstream activation of PKA. Cilostazol (CIL), a phosphodiesterase-3 inhibitor, prevents cAMP degradation. We assessed whether CIL amplifies the exenatide (EX)-induced increase in myocardial cAMP levels and PKA activity and augments the infarct size (IS)-limiting effects of EX in db/db mice. Mice fed a Western diet received oral CIL (10 mg/kg) or vehicle by oral gavage 24 h before surgery. One hour before surgery, mice received EX (1 μg/kg sc) or vehicle. Additional mice received H-89, a PKA inhibitor, alone or with CIL + EX. Mice underwent 30 min of coronary artery occlusion and 24 h of reperfusion. Both EX and CIL increased myocardial cAMP levels and PKA activity. Levels were significantly higher in the EX + CIL group. Both EX and CIL reduced IS. IS was the smallest in the CIL + EX group. H-89 completely blocked the IS-limiting effects of EX + CIL. EX + CIL decreased phosphatase and tensin homolog on chromosome 10 upregulation and increased Akt and ERK1/2 phosphorylation after ischemia-reperfusion. These effects were blocked by H-89. In conclusion, EX and CIL have additive effects on IS limitation in diabetic mice. The additive effects are related to cAMP-induced PKA activation, as H-89 blocked the protective effect of CIL + EX.

Increased myocardial salvage in patients that presented with myocardial infarction with ST elevation and were treated with primary percutaneous coronary intervention (42). However, it has remained unclear whether GLP-1R activation can limit IS in diabetic animals, as myocardial protection by preconditioning and postconditioning is hampered in diabetic animals (23, 55, 69).

Activation of GLP-1Rs causes a rapid increase in intracellular cAMP and Ca2+ levels (19), with downstream activation of PKA and phosphorylation of cAMP response element-binding protein (CREB) (69). PKA is involved in myocardial protection against I/R injury (58, 67, 69). We hypothesized that a further increase in intracellular cAMP levels by preventing its degradation would augment the protective effect of GLP-1R activation. Cilostazol (CIL) is a phosphodiesterase-3 inhibitor that increases intracellular cAMP levels by preventing its degradation (35). CIL has antiplatelet and vasodilatory properties (35) and is approved in the United States for the treatment of patients with intermittent claudication symptoms related to peripheral arterial disease. We have previously shown that oral CIL (20 mg·kg−1·day−1) for 3 days significantly limited myocardial IS in nondiabetic rats. Furthermore, IS was significantly smaller when atorvastatin and CIL were coadministered, and this was associated with higher PKA activity in the combination group (43).

We (68) have also shown that PKA phosphorylates 5-lipoxigenase at Ser232, leading to increased production of 15-epi-lipoxin A4, an arachidonic acid-derived eicosanoid with potent anti-inflammatory and inflammation resolution properties.

Myocardial levels of phosphatase and tensin homolog on chromosome 10 (PTEN) are increased in diabetic rats compared with nondiabetic rats, leading to a reduced ability to phosphorylate Akt and, hence, blunting the protective effects of ischemic preconditioning (47, 65). PTEN inhibition reduces myocardial IS (36). It has also been suggested that PKA modulates PTEN by phosphorylation (28, 38, 40).

In the present study, we sought to assess the following: 1) whether EX limits myocardial IS in mice with type 2 diabetes mellitus, 2) whether the myocardial protective effect is augmented with CIL, 3) whether the augmented protection is associated with higher levels of cAMP and PKA activation, 4) whether EX + CIL deactivates PTEN in a PKA-dependent manner, and 5) whether the combination...
of EX + CIL is associated with increased myocardial levels of 15-epi-lipoxin A4.

We chose the leptin receptor-deficient [B6.BKS(D)-Leprdb/hdb] db/db mouse as a model for type 2 diabetes mellitus. These mice, which express a homozygotic diabetic spontaneous mutation (Leprdb; db/db), are reported to become obese at the age of 3–4 wk. Plasma insulin levels are reported to increase at the age of 10–14 days, and blood sugar levels increase at the age of 4–8 wk. The course of the disease is markedly influenced by the genetic background of the mice. db/db mice fed with a Western diet have a plasma lipoprotein phenotype that shows some similarities to that in patients with type 2 diabetes mellitus (especially high LDL-cholesterol levels). db/db mice are a useful model in which to study the pathogenesis and treatment of diabetic dyslipidemia (39). Several studies (1, 2, 13, 14, 23, 29, 34, 63, 71) have used the db/db mouse model as a model of experimental myocardial infarction. IS in db/db mice is reported to be larger than in nondiabetic mice (13, 34, 71).

MATERIALS AND METHODS

Male db/db mice (stock no. 000697) and their wild-type nondiabetic controls (C57BL/6J mice, stock no. 00664) were purchased from Jackson Laboratories (Bar Harbor, MA) and received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). This protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch.

Materials

Western diet (41% of calories from fat, 43% from carbohydrates, 17% from protein, and 1.5% g cholesterol) was purchased from Research Diets (New Brunswick, NJ). Anti-Akt, anti-phospho-Akt (Thr308), and anti-phospho-Akt (Ser473) antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-CREB, anti-phospho-CREB (Ser133), anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-PTEN, and anti-activated caspase-3 antibodies were purchased from Cell Signaling (Beverly, MA). Anti-β-actin antibodies were purchased from Sigma (St Louis, MO). The cAMP-dependent PKA inhibitor, KT-5720 and adenosine-3′,5′-cyclic phosphorothioate (Rp-cAMPS) (both PKA inhibitors) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Treatment

All experiments were conducted with 6-wk-old db/db mice that were fed a Western diet for 2 wk.

Experiment 1. At the age of 8 wk, animals received EX (1, 2.5, or 5 μg/kg sc) or vehicle (control) 1 h before surgery.

Experiment 2. At the age of 8 wk, animals received vehicle (control) or CIL (10 mg/kg) by oral gavage 24 h before surgery. One hour before surgery, mice received EX (1 μg/kg sc) or vehicle. The dose of CIL was based on our previous studies showing that 3-day pretreatment with CIL at 20 mg·kg⁻¹·day⁻¹ (43) or 15 mg·kg⁻¹·day⁻¹ (7) significantly reduced IS. We wanted to try a lower dose to determine any synergistic effects.

Experiment 3. At the age of 8 wk, animals received H-89 (20 mg/kg iv), a PKA inhibitor, or vehicle (5% DMSO) 1 h before surgery, alone or with CIL (10 mg/kg) + EX (1 μg/kg), as described above.

IS

Mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg), intubated, and ventilated (fraction of inspired O₂: 30%). 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 a change in the color of the myocardium. Subcutaneous 0.1 mg/kg buprenorphine was administered, the chest was closed, and mice were allowed to recover from anesthesia. Twenty-four hours after reperfusion, mice were reanesthetized, the coronary artery was reoccluded, Evan’s blue dye (3%) was injected into the right ventricle, and mice were euthanized under deep anesthesia (70).

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

Determination of the AAR and IS

Hearts were excised, and the LV 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 AAR (uncolored by the blue dye), the IS (unstained by TTC), and the nonischemic zone (colored by blue dye). Areas of the AAR and IS in each slice were 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 AAR and IS (70).

Myocardial cAMP Levels and PKA Activity

Mice received treatment as in experiments 2 and 3. Mice were exposed to 30 min of ischemia and 24 h of reperfusion. Twenty-four hours after reperfusion, mice were reanesthetized, the coronary artery was reoccluded, Evan’s blue dye (3%) was injected into the right ventricle for delineation of the ischemic AAR, and mice were euthanized under deep anesthesia. Myocardial samples from the postischemic zone of the LV were homogenized in 1 ml of cold extraction buffer [20 mM Tris·HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 0.1 mM AP, 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 (67).

Immunoblot Analysis

Myocardial samples from the risk zone of the anterior wall of the LV wall exposed to IR, the nonischemic zone, or from the anterior wall of control hearts not exposed to ischemia (sham-operated mice pretreated as in the vehicle-sham group) were homogenized in lysis buffer containing 25 mM Tris·HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1 mM DT, 25 mM NaF, 1 mM 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-
Table 1. *Experiment 1*: body weight, LV weight, size of the ischemic AAR, and IS with three different doses of EX

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>LV Weight, mg</th>
<th>AAR, % of the LV</th>
<th>IS, % of the LV</th>
<th>IS, % of the AAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.0 ± 0.7</td>
<td>131.8 ± 1.2</td>
<td>37.6 ± 1.3</td>
<td>17.5 ± 1.2</td>
<td>46.8 ± 3.8</td>
</tr>
<tr>
<td>1 μg/kg EX</td>
<td>32.5 ± 1.7</td>
<td>130.3 ± 2.1</td>
<td>36.4 ± 2.0</td>
<td>9.0 ± 1.5*</td>
<td>24.4 ± 3.4*</td>
</tr>
<tr>
<td>2.5 μg/kg EX</td>
<td>30.3 ± 1.2</td>
<td>129.5 ± 1.8</td>
<td>38.1 ± 1.1</td>
<td>9.2 ± 0.9*</td>
<td>24.4 ± 2.9*</td>
</tr>
<tr>
<td>5 μg/kg EX</td>
<td>30.7 ± 1.2</td>
<td>128.8 ± 2.6</td>
<td>41.4 ± 0.8</td>
<td>9.4 ± 1.5*</td>
<td>22.5 ± 3.5*</td>
</tr>
<tr>
<td>P value</td>
<td>0.166</td>
<td>0.744</td>
<td>0.095</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals/group. LV, left ventricular; AAR, area at risk; IS, infarct size; EX, exenatide. *P < 0.003 vs. the control group.

RESULTS

IS

*Experiment 1*. A total of 29 animals were studied. Two mice in the control group, one mouse in the 1 μg/kg EX-treated group, and two mice in 5 μg/kg EX-treated group died during surgery. A total of 24 animals were included in the analysis. Body weight, LV weight, and the size of AAR were comparable among groups. IS was smaller in EX-treated animals, without significant differences among the three different doses (1, 2.5, or 5 μg/kg; Table 1).

*Experiments 2 and 3*. A total of 51 animals were studied. For each group, four animals died during surgery: one mouse in the control group, one mouse in the 1 μg/kg EX-treated group, one mouse in the CIL-treated group, and one mouse in the H-89-treated group. A total of 47 animals were included. Body weight, LV weight, and the size of AAR were comparable among groups in experiment 2 and experiment 3 (Table 2). Both EX and CIL reduced IS. IS was significantly smaller in the EX + CIL group than in the control, EX alone, or CIL alone groups (Table 2 and Fig. 1). H-89 alone had no effect on IS; however, H-89 completely blocked the IS-limiting effects of EX + CIL (Table 3 and Fig. 1).

Blood glucose, HbA1c, Total cholesterol, and Triglyceride Levels

There were five animals in each group. Fasting serum glucose was significantly elevated in db/db mice compared with control normal nondiabetic mice (Table 4). One hour after EX (1 μg/kg sc) injection, serum glucose was comparable with that of nondiabetic animals. Twenty-four hours after CIL (10 mg/kg) was administered by oral gavage, serum glucose was significantly reduced in db/db mice. Serum glucose in the EX + CIL group was comparable with that of nondiabetic mice or EX alone-treated db/db mice. As expected, glycated HbA1c levels were significantly higher in db/db mice, without significant differences among the four treatment groups.

Serum total cholesterol and triglyceride levels were higher in db/db mice than in control nondiabetic mice (Table 4). EX,
Myocardial 15-Epi-Lipoxin A4 Levels

induced increase in myocardial PKA activity (Fig. 2).

EX and CIL increased PKA activity. PKA activity was significantly higher in the EX and CIL group than in the EX alone or CIL alone groups. H-89 had no effect on myocardial cAMP levels, and it did not block the EX + CIL-induced increase in cAMP levels (Fig. 2A).

Myocardial PKA Activity

I/R did not have a significant effect on PKA activity. Both EX and CIL increased PKA activity. PKA activity was significantly higher in the EX + CIL group than in the EX alone or CIL alone groups. H-89 completely blocked the EX + CIL-induced increase in myocardial PKA activity (Fig. 2B).

Myocardial 15-Epi-Lipoxin A4 Levels

I/R did not have a significant effect on myocardial 15-epi-lipoxin A4 levels. Myocardial 15-epi-lipoxin A4 levels were significantly higher in the EX + CIL group than in the EX alone or CIL alone groups. H-89 completely blocked the EX + CIL-induced increase in myocardial 15-epi-lipoxin A4 levels, suggesting that 15-epi-lipoxin A4 production depends on PKA (Fig. 2C).

Immunoblot Analysis

I/R caused an insignificant increase in myocardial levels of Ser473- and Thr308-phosphorylated Akt compared with sham-operated mice (Fig. 3, A, C, and D). EX + CIL significantly increased Akt phosphorylation of both Ser473 and Thr308 without affecting total Akt levels (Fig. 3, A and E). H-89 alone had no significant effect on myocardial phospho-Akt levels; however, it blocked the EX + CIL-induced augmentation of these levels (Fig. 3, A, C, and D).

I/R induced a small, insignificant increase in phospho-ERK1/2 levels without affecting total ERK1/2 levels (Fig. 3, B, F, and G). EX + CIL significantly increased phospho-ERK1/2 levels without affecting total ERK1/2 levels (Fig. 3, B, F, and G). H-89 alone had no significant effect on phospho-ERK1/2 levels; however, it completely blocked the EX + CIL-induced increase in phospho-ERK1/2 levels.

Likewise, I/R caused an insignificant increase in myocardial phospho-CREB levels without affecting total CREB levels (Fig. 3, B, H, and I). EX + CIL significantly augmented phospho-CREB levels. H-89 alone had no significant effect, yet it completely blocked the EX + CIL-induced increase in phospho-CREB levels.

I/R had no significant effect on PTEN levels (Fig. 4, A and B). EX + CIL induced a significant decrease in PTEN levels. H-89 alone had no effect on PTEN levels. However, H-89 completely blocked the decrease in PTEN levels, suggesting that the decrease in PTEN expression is PKA dependent.

I/R increased activated caspase-3 levels (Fig. 4, A and C). EX + CIL prevented this increase. H-89 had no effect on activated caspase-3 levels; however, it completely blocked the EX + CIL-induced effect. Similar effects on Akt, ERK1/2, CREB, and PTEN were seen in the nonischemic zone (data not shown).

In Vitro Experiment

As H-89 is not specific for PKA, we designed an in vitro experiment to examine whether blockade of PKA with two structurally unrelated specific PKA inhibitors could block the effects of EX on PTEN expression and Akt phosphorylation. SIR increased PKA activity compared with the NSIR group (Fig. 5A). EX further increased PKA activity. H-89, KT-5720, and Rp-cAMPS equally blocked the EX-induced increase in PKA activity.

SIR did not affect PTEN levels. Pretreatment with EX significantly decreased PTEN expression after exposure to SIR. H-89, KT-5720, and Rp-cAMPS prevented the EX-induced decrease in PTEN expression (Fig. 5, B and C).

Table 4. Serum glucose, HbA1C, total cholesterol, and triglyceride levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Nondiabetic</th>
<th>Control</th>
<th>EX</th>
<th>CIL</th>
<th>EX + CIL</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/l</td>
<td>8.23 ± 0.80†</td>
<td>23.69 ± 1.57*</td>
<td>8.34 ± 1.02*</td>
<td>14.30 ± 0.95†</td>
<td>8.56 ± 0.81†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1C, mg/ml</td>
<td>10.4 ± 0.6†</td>
<td>25.3 ± 4.5*</td>
<td>23.7 ± 1.5*</td>
<td>22.1 ± 1.9*</td>
<td>19.7 ± 0.9</td>
<td>0.002</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>1.81 ± 0.05†</td>
<td>3.10 ± 0.46*</td>
<td>1.75 ± 0.07†</td>
<td>1.83 ± 0.07†</td>
<td>1.68 ± 0.10†</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>2.09 ± 0.28†</td>
<td>5.26 ± 0.58*</td>
<td>2.68 ± 0.38†</td>
<td>2.45 ± 0.23†</td>
<td>1.79 ± 0.33†</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

HbA1C, glycated hemoglobin. *P < 0.05 versus nondiabetic mice; †P < 0.006 vs. control db/db mice.
SIR increased Akt phosphorylation at both Ser\(^{773}\) and Thr\(^{308}\) without affecting total Akt levels (Fig. 5, B and D–F). Pre-treatment with EX further increased Akt phosphorylation, without affecting total Akt concentration, after exposure to SIR. H-89, KT-5720, and Rp-cAMPS completely blocked the EX-induced increase in Akt phosphorylation.

DISCUSSION

It is well known that IS in diabetic animals is larger than in nondiabetic animals (13, 22, 34, 71). Moreover, myocardial protection by preconditioning and postconditioning is hampered in diabetic animals (23, 55, 69). Here, we show, for the first time, that EX limits IS in hearts of animals with type 2 diabetes, as has been previously shown in nondiabetic animals (3, 5, 10–12, 18, 31, 33, 45, 51, 61, 64). The protective effect was maximal at a dose of \(1/2426\) g/kg, as the IS at doses of 2.5 and 5 \(1/2426\) g/kg was not smaller than that at 1 \(1/2426\) g/kg. Our findings confirm the results of Sonne et al. (61), who found that in an isolated rat heart model, an exendin-4 concentration higher than 0.03 nM did not achieve greater myocardial protection than 0.03 nM, suggesting that GLP-1Rs are saturated at the lower dose. We also showed that CIL, a phosphodiesterase-3 inhibitor that prevents cAMP degradation, at 10 mg/kg, reduced myocardial IS. When CIL was combined with EX, IS was significantly smaller than with EX alone and CIL alone. The combination of EX (which increases cAMP production by stimulating the GLP-1R) and CIL (which prevents cAMP degradation) resulted in significantly higher myocardial cAMP levels and PKA activation than each drug alone, explaining the additive IS-limiting effect. The IS-limiting effect was PKA dependent, as H-89, a PKA inhibitor, completely blocked the protective effect of EX/CIL. Moreover, both EX and CIL increased myocardial levels of 15-epi-lipoxin A\(_4\), an eicosanoid with potent anti-inflammatory and inflammation resolution properties (68). This effect was also PKA dependent, as H-89 prevented the EX/CIL-induced increase in 15-epi-lipoxin A\(_4\) levels.

Studies (10, 51, 61) in nondiabetic rodents have shown that blockade of the GLP-1R with exendin(9–39) abolishes the IS-limiting effects of GLP-1R activation. One group (4) reported that GLP-1R agonists may have some protective effects in GLP-1R\(^{-/-}\) mice, suggesting that some of the protective effects may be GLP-1R independent. However, liraglutide does not increase cAMP levels and Akt and glycogen synthase kinase-\(\beta\) phosphorylation in GLP-1R\(^{-/-}\) mice, as it does in wild-type mice (50). On the other hand, the GLP-1 analog exendin-4 does not protect cardiomyocytes treated concomitantly with the GLP-1R inhibitor exendin(9–39) and cardiomyocytes derived from GLP-1R\(^{-/-}\) mice, although exendin(9–39) blocks the protective effect (3). These results suggest that receptors other than GLP-1 may be involved or that there are significant differences between GLP-1 analogs and native GLP-1 or the metabolite GLP-1(9–36) (69).

PKA

Activation of adenyl cyclase by extracellular ligands increases intracellular concentrations of the second messenger cAMP. Binding of cAMP to the regulatory subunit of PKA induces dissociation of the holoenzyme and the subsequent phos-
phorylation of key substrates by the catalytic subunit (21). Cytoplasmic and nuclear substrate phosphorylation mediated by PKA are critical for multiple cell functions, including metabolism, differentiation, synaptic transmission, ion channel activity, growth, and development (21). Sanada et al. (58) 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 (58). They suggested that PKA activation leads to Rho kinase and actin cytoskeletal deactivation, mediating cardioprotection (58). On the other hand, Inserte et al. (32) suggested that the protective effect of ischemic preconditioning is caused by PKA-dependent attenuation of calpain-mediated degradation of structural proteins. Sanada et al. (59) suggested that PKA activation mediates myocardial protection by upregulating p38 MAPK. It has been suggested that PKA leads to Akt activation. Two different pathways have been described: 1) PKA activates the p85 regulatory α-subunit of phosphoinositol 3-kinase (PI3K) and, thus, increases Akt phosphorylation (15, 17, 66) or 2) PKA physically interacts with Akt and phosphorylates it (6). Here, we showed a different mechanism by which PKA may contribute to Akt activation. We found that in the heart, PKA activation leads
to decreased expression of total PTEN levels. PTEN degrades phosphatidylinositol 3-phosphate (PI3P). As expression of PTEN decreases, PI3P levels are expected to increase, leading to enhanced Akt phosphorylation. Akt is part of the prosurvival pathway that mediates protection against I/R injury (25). Furthermore, it has been reported that PKA directly phosphorylates endothelial nitric oxide synthase (eNOS) at Ser^{1177} and Ser^{633} (24). eNOS is also involved in ischemic preconditioning and various forms of pharmacological preconditioning (25). Moreover, phosphorylation of 5-lipoxygenase at Ser^{523} by PKA augments the production of 15-epi-lipoxin A4, an eicosanoid with potent anti-inflammatory properties that potentially also favorably attenuate I/R injury (68).

We (43) have previously shown that oral CIL limits IS and increases myocardial PKA activity, Akt phosphorylation at Ser^{473}, and eNOS phosphorylation at both Ser^{633} and Ser^{1177} in nondiabetic rats.

---

**Fig. 4.** Representative immunoblots (A) and densitometric analyses of phosphatase and tensin homolog on chromosome 10 (PTEN; B) and activated caspase-3 (C) in the border zone of hearts exposed to I/R. The myocardium of db/db mice that had not been exposed to I/R and received vehicle served as the control (sham) (n = 4 animals/group). β-Actin was used as the loading control. *P < 0.001 vs. sham; †P < 0.001 vs. EX + CIL.

---

**Fig. 5.** In vitro experiment. A: PKA activity in cardiomyocytes pretreated with vehicle (Cont), EX alone, EX + H-89, EX + KT-5720, or EX + Rp-cAMPS for 12 h and exposed to 2 h of simulated ischemia followed by 2 h of reoxygenation (SIR). Cells not exposed to SIR (NSIR) served as controls. The experiment was repeated five times. B–F: representative immunoblots (B) and densitometric analyses of PTEN (C), Ser^{473} P-Akt (D), Thr^{308} P-Akt (E), and total Akt (F). β-Actin was used as the loading control. Each experiment was repeated four times. *P < 0.006 vs. Cont (vehicle + SIR); †P < 0.001 vs. EX.
PTEN Expression

As mentioned above, PTEN has a major role in controlling PI3P levels and, hence, activation of proapoptotic and prosurvival pathways. PTEN inhibition decreases myocardial IS (36, 56). Myocardial levels of PTEN are increased in diabetic rats compared with nondiabetic rats, leading to a reduced ability to phosphorylate Akt and, hence, blunting the protective effects of ischemic preconditioning (7, 47, 65). It has been suggested that PTEN upregulation attenuates the myocardial protective effects of statins over time (46). Here, we showed that PKA activation by EX + CIL leads to a downregulation of PTEN expression with downstream upregulation of phosphorylation of two pivotal prosurvival mediators, Akt and ERK1/2. Our in vitro experiment confirmed that EX increased PKA activity, decreased PTEN levels, and augmented Akt phosphorylation. All three PKA inhibitors completely blocked the EX-induced decrease in PTEN expression and the EX-induced increase in Akt phosphorylation (Fig. 5). Thus, activation of PKA by GLP-1R activation and/or phosphodiesterase-3 inhibition may prevent tolerance to the protective effects of statins. Moreover, as PTEN upregulation may be involved in insulin intolerance, this approach may prevent the association between statins and diabetes (54).

CREB Phosphorylation and Protection Against I/R Injury

CREB is a major nuclear transcription factor that transduces cAMP activation of gene transcription (60). CREB is activated by phosphorylation at Ser133 by PKA (60). CREB phosphorylation induces the translocation of cytoplasmic CREB to the nucleus (60). CREB is involved in ischemic (44, 48) and pharmacological (16, 20) preconditioning. The promoter region of genes encoding cytochrome c and Bcl-xL carries a cAMP response element site, and CREB has been recognized as a positive regulator of these genes (20). Das et al. (16) reported that adenosine type 3 receptor activation directly augments CREB Ser133 phosphorylation followed by Bcl-2 phosphorylation. We (67) have previously shown that sitagliptin, an oral form of dipeptidylpeptidase IV inhibitor, increases endogenous levels of GLP-1 and augments CREB phosphorylation in nondiabetic mice hearts. Here, we showed that EX + CIL increases CREB phosphorylation and that H-89 blocks this augmentation, confirming that PKA phosphorylates CREB (67).

Akt Phosphorylation

Akt is part of the prosurvival pathway that mediates protection against I/R injury (25). Here, we showed that the combination of EX + CIL resulted in augmented Akt phosphorylation at both Ser473 and Thr308. In vivo, we showed H-89, a PKA inhibitor, blunted this increase, suggesting that Akt activation is dependent on PKA. Our in vitro experiment confirmed that two additional unrelated PKA inhibitors blocked the EX-induced increase in Akt phosphorylation. Others (3, 10, 64, 67) have also shown that GLP-1R activation leads to enhanced Akt phosphorylation. PI3K inhibitors blocked the protective effects of GLP-1 in mouse cardiomyocytes exposed to hypoxia-reoxygenation (3, 67) and in isolated hearts (10). As mentioned above, it has been reported that PKA increases Akt phosphorylation by increasing intracellular PI3P levels via activation of the p85 regulatory α-subunit of PI3K, leading to increased PI3P generation (15, 17, 66) or by direct phosphorylation of Akt (6). Here, we showed that the augmentation of Akt phosphorylation is related to the PKA-dependent decrease in PTEN expression (resulting in decreased degradation of PI3P).

ERK1/2 Phosphorylation

ERK1/2 is also involved in the protection against I/R injury (25). Here, we showed that the combination of EX + CIL resulted in augmented ERK1/2 phosphorylation, confirming the results of previous studies (3, 10) showing that GLP-1 activation augments ERK1/2 phosphorylation. ERK1/2 inhibition abrogates the protective effects of GLP-1 in isolated cardiomyocytes (67) and in isolated hearts (10) exposed to hypoxia-reperfusion. Interestingly, in the present study, we showed that PKA inhibition by H-89 blocked the augmentation of ERK1/2 phosphorylation by EX + CIL, suggesting that PKA is also involved in ERK1/2 activation in our model.

Caspase-3

Caspase-3 activation has a major role in mediating apoptotic cell death after I/R injury (9). We (36) have previously shown that PTEN inhibition attenuates caspase-3 activation after I/R injury. Here, we showed that EX + CIL blocked the upregulation of caspase-3 after I/R injury. This effect was blocked by H-89, a PKA inhibitor. Thus, PKA downregulation of PTEN leads to inhibition of caspase-3 activation after I/R injury.

15-Epi-Lipoxin A₄

15-Epi-lipoxin A₄ is produced by cyclooxygenase-2 (which converts arachidonic acid to 15-R-HETE) and 5-lipoxygenase (which converts 15-R-HETE to 15-epi-lipoxin A₄) (8, 68). 15-Epi-lipoxin A₄ has potent anti-inflammatory effects that may be beneficial for patients with type 2 diabetes and/or atherosclerosis. We (68) have shown that phosphorylation of 5-lipoxygenase at Ser232 by PKA is essential to prevent 5-lipoxygenase from interacting with cytosolic phospholipase A₂ to generate proinflammatory leukotrienes and enhances interactions with cyclooxygenase-2 to generate 15-epi-lipoxin A₄. Here, we showed that augmentation of PKA activity with EX + CIL increased myocardial levels of 15-epi-lipoxin A₄. It is unclear whether 15-epi-lipoxin A₄ mediates (at least part of) the IS-limiting effects of EX and CIL; however, a similar eicosanoid, resolving E₁ (derived by cyclooxygenase-2 and 5-lipoxygenase from eicosapentaenoic acid) limits IS in nondiabetic rats (37).

Effects of Treatment on Biochemical Parameters

As expected, EX alone or in combination with CIL decreased serum glucose levels. Interestingly, CIL alone significantly reduced serum glucose (Table 4). Although in humans such an effect has not been reported, several other investigators have reported that CIL improved insulin sensitivity in rats with type 2 diabetes (49, 52) and decreased serum glucose in db/db mice (53). As much higher doses of CIL are used in rodents, it is probable that the effect is dose dependent and is not seen at doses used in the clinical setting (50–100 mg bid). Nevertheless, CIL may augment the effects of GLP-1R activator in the clinical setting.

Decreasing serum glucose or increasing insulin levels could explain some of the beneficial effects of EX and/or CIL seen in our model; yet, GLP-1R activation has been reported to reduce IS in nondiabetic models (69). Moreover, we have
The combination of GLP-1R activation with phosphodies- 
terase-3 inhibition may have clinical implications. As men- 
tioned above, EX has been shown to reduce IS in patients with 
acute myocardial infarction undergoing reperfusion therapy 
with primary percutaneous coronary intervention (42). CIL 
has antiplatelet and vasodilatatory properties (35) and is approved 
with primary percutaneous coronary intervention (42). CIL has 
antiplatelet and vasodilatatory properties (35) and is approved 

In conclusion, in the present study, we have shown that the 
combination of EX + CIL has additive effects on myocardial 
protection. EX activates GLP-1Rs, leading to the activation of 
adenylate cyclase and generation of cAMP, whereas CIL 
prevents cAMP degradation. Indeed, the combination of EX + 
CIL resulted in higher levels of cAMP and augmented PKA 
activity. It seems that PKA is upstream of PTEN inhibition and 
Akt and ERK1/2 phosphorylation, as PKA inhibition with 
H-89 blocked PTEN upregulation and Akt and ERK1/2 phos- 
phorylation and abrogated the IS-limiting effects of EX + CIL. 

CIL has antiplatelet properties and has been used for patients 
undergoing percutaneous coronary intervention in addition to 
aspirin and clopidogrel. Further studies should be conducted 
to assess the efficacy and safety of EX + CIL in patients with 
acute coronary syndromes.

ACKNOWLEDGMENTS

Present address of J. Qian: Department of Anesthesiology, First Affiliated 
Hospital of Kunming Medical College, Kunming, Yunnan, China.

GRANTS

Y. Ye was supported by research grants from Amylin, Merck, Takeda, Roche, 
and Boehringer Ingelheim. M. Bajaj was supported by research grants 
from American Diabetes Association, Roderick MacDonald Research Fund at 
St. Luke’s Episcopal Hospital, Takeda, Amylin, Eli Lilly, Bristol-Myers 
Squibb, and Astra Zenica and was honoraria for speaking from Takeda, Eli 
Lilly, Boehringer Ingelheim, and Sanofi-Aventis. Y. Birnbaum was supported 
by research grants from Amylin, Merck, Takeda, Roche, and Boehringer 
Ingelheim.

DISCLOSURES

M. Bajaj has served as a consultant to Takeda and Sanofi-Aventis.

AUTHOR CONTRIBUTIONS

Author contributions: Y.Y., J.Q., and Y.B. conception and design of 
research; Y.Y., J.Q., A.C.C., and S.L. performed experiments; Y.Y., J.Q., 
interpreted results of experiments; Y.Y. and Y.B. drafted manuscript; Y.Y., 
J.Q., A.C.C., S.L., H.Y., J.R.P.-P., M.B., and Y.B. edited and revised manu-
version of manuscript; Y.B. prepared figures.

REFERENCES

1. Aasum E, Hafstad AD, Severson DL, Larsen TS. Age-dependent 
changes in metabolism, contractile function, and ischemic sensitivity in 


