An interaction between glucagon-like peptide-1 and adenosine contributes to cardioprotection of a dipeptidyl peptidase 4 inhibitor from myocardial ischemia-reperfusion injury

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GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is mostly known as a potent antihyperglycemic hormone secreted by intestinal cells to stimulate glucose-dependent insulin secretion from pancreatic beta cells. Once in circulation, GLP-1 is rapidly metabolized by dipeptidyl peptidase 4 (DPP4). Therefore, GLP-1 analogs and DPP4 inhibitors are commonly prescribed for the treatment of diabetes mellitus (6, 11). DPP4 inhibitors also exert extra-pancreatic effects in the brain, stomach, and heart (39). However, the mechanisms by which they mediate these effects remain largely unknown. Several reports showed that DPP4 inhibitors limit the infarct size after ischemia-reperfusion in the heart (15, 51), but the extent of their beneficial effects remains controversial (7, 52). Recently, several reports have indicated that DPP4 forms a complex with adenosine deaminase (ADA) in immune cells and that ADA regulates DPP4-mediated intracellular signaling (12, 19, 34, 44, 46). Adenosine is a well-established cardioprotective molecule that plays a major role in ischemic preconditioning (23, 25, 29), the most powerful cardioprotective mechanism (40). Therefore, any factor susceptible to enhancing adenosine accumulation in the heart during ischemia-reperfusion would reduce myocardial injury. Indeed, circulating adenosine is produced by ATP metabolism and eliminated by ADA activity. ADA inhibition-mediated cardioprotection occurs through an increase in adenosine receptor-mediated responses (1). However, there have been no studies that test whether a DPP4 inhibitor affects the catalytic properties of ADA or has chemical affinities for adenosine receptors. Therefore, we hypothesized that DPP4 inhibitors mediate cardioprotective effects during ischemia-reperfusion by interfering with the catalytic site of ADA nearby, resulting in enhanced adenosine-dependent limitation of infarct size.

The aim of this study was to elucidate the cardioprotective mechanisms of the DPP4 inhibitor alogliptin using a multidisciplinary approach. First, in vivo ischemia-reperfusion experiments were performed in dogs after a 4-day pretreatment with alogliptin to determine the effect on infarct size, ADA activity, and adenosine and GLP-1 accumulation. Second, in vivo experiments were performed to identify the signaling pathways that mediate the cardioprotective effects of GLP-1 in canine hearts.

Glycogen synthase kinase-3 (GSK-3) and Akt are key serine/threonine kinases that negatively regulate CREB phosphorylation. Indeed, circulating adenosine is produced by ATP metabolism and eliminated by ADA activity. ADA inhibition-mediated cardioprotection occurs through an increase in adenosine receptor-mediated responses (1). However, there have been no studies that test whether a DPP4 inhibitor affects the catalytic properties of ADA or has chemical affinities for adenosine receptors. Therefore, we hypothesized that DPP4 inhibitors mediate cardioprotective effects during ischemia-reperfusion by interfering with the catalytic site of ADA nearby, resulting in enhanced adenosine-dependent limitation of infarct size.

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MATERIALS AND METHODS

Reagents and antibodies. 8-(p-Sulfophenyl)-theophylline (8SPT), H2O2, 2,3,5-triphenyltetrazolium chloride (TTC), and GLP-1 (7–36) were purchased from Sigma-Aldrich (Tokyo, Japan). Evans blue was purchased from Wako-Chemical (Osaka, Japan). A protein kinase C (PKC) inhibitor, GF109203X, was purchased from Merck Millipore (Temecula, CA). The DPP4 inhibitor allogliptin was kindly provided by Takeda Pharmaceuticals Japan. The anti-DPP4 (1:1,000, ab28340) and ATP5F1 (F6F1–ATP synthase b subunit) antibodies (1:2,000, ab17991) were purchased from Abcam (Cambridge, UK), and anti-GLP-1 receptor antibody (1:1,000, NBPI-97308) was purchased from Novus Biologicals (Littleton, CO). The anti-adenosine A1 (1:1,000, sc-7500), Bcl-2 antibodies (1:500, sc-7382), and PKCo (1:500, sc-208) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The antibodies against Akt (1:1,000, 9272), phosho-Akt (1:1,000, 2921), glycogen synthase kinase-3β (GSK-3β) (1:1,000, 9135), phosho-GSK-3α/β (1:1,000, 9331), p84/p42 MAPK (Erk1/2) (1:1,000, 4692), p44/p42 MAPK (Erk2/1) (1:1,000, 4377), cAMP response element–binding protein (CREB) (1:1,000, 9197), phosho-CREB (1:1,000, 9189), Bax (1:1,000, 2772), phosho-PKCα/ß/II (1:1,000, 9375), phosho-Bad (Ser155) (1:1,000, 9292), and Bad (1:1,000, 9292) were purchased from Cell Signaling (Danvers, MA). Anti-α-tubulin antibody (1:1,000, PM054) was purchased from MBL (Tokyo, Japan). Cytochrome c antibody (1:1,000, 556433) was purchased from BD Pharmingen (Tokyo, Japan).

Animals and preparation for in vivo experiments. The animals were handled in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, approved by the Physiological Society of Japan. All animal procedures were approved by the National Cerebral and Cardiovascular Center Committee for Laboratory Animal Use.

Adult female beagle dogs (Kitayama Labs; Yoshiki Farm, Gifu, Japan) weighing 8–12 kg were anesthetized with pentobarbital sodium (15 mg/kg iv) and continuous isoflurane inhalation, intubated, and ventilated with room air mixed with oxygen (100% O2 at a flow rate of 1.0 to 1.5 l/min). Minimal thoracotomy was performed through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After administration of heparin (500 U/kg iv), the proximal left anterior descending coronary artery was cannulated and perfused with blood via the extracorporeal tube from the carotid artery. The left carotid artery was cannulated to monitor aortic blood pressure, and the cervical vein was cannulated to maintain fluid balance. Another cannula was placed in the atrium for microsphere injection to measure collateral blood flow during coronary occlusion. During all procedures, hemodynamic parameters were measured before the initiation of each protocol; 90 min after the onset of ischemia; and 1, 3, and 6 h after the onset of reperfusion (2).

Ischemia-reperfusion protocol. The beagle dogs were housed at least 1 wk before each experiment. They were then randomly assigned to one of four groups: control, allogliptin, allogliptin + 8SPT, or 8SPT. Encapsulated allogliptin (3 mg/kg) was administered orally once a day for 4 days. On the morning of day 4, postdye blood samples were collected to measure GLP-1, adenosine concentrations, and ADA activity. The dogs underwent coronary arterial occlusion (ischemia) for 90 min, followed by reperfusion for 6 h. 8SPT (25 mg/kg) was administered into the coronary artery at a rate of 0.0167 ml·kg⁻¹·min⁻¹ (1.5 mg/ml) 10 min before coronary occlusion and during the first hour of reperfusion. Hemodynamic parameters were measured at specific times, and infarct size was assessed 6 h after the reperfusion (Fig. 1A). Preliminary experiments showed that 3 mg/kg of allogliptin is required to effectively inhibit DPP4. Maximum inhibition (>98%) was maintained 15 min after administration, and effective inhibition (>65%) lasted at least 12 h (27).

Measurements of risk area, infarct size, and myocardial collateral blood flow. After the 6-h reperfusion, Evans blue dye was injected into a systemic vein to visualize the anatomical risk area and the nonischemic area of the heart. The heart then was immediately removed and sliced into serial transverse sections of 6 to 7 mm in width. The ischemic region was incubated (37°C, 20–30 min) in TTC solution containing 0.1 M phosphate buffer adjusted to pH 7.4. The infarct size was identified by dual staining with TTC and Evans blue. The heart sections were photographed, and magnified images (×10) were used to quantify the area at risk and area of necrosis in each slice. The data from all slices were compiled for each heart, and the infarct size was expressed as a percentage of total risk area.

Regional myocardial blood flow was determined by the microsphere technique with the remaining tissue. The nonradioactive microspheres (Sekisui Plastic, Tokyo, Japan) were made of inert plastic labeled with different stable heavy elements as previously described in detail (33). Microspheres labeled with bromine (Br) or niobium (Nb) were suspended in isotonic saline containing 0.01% Tween 80 to prevent aggregation and ultrasonicated for 5 min. The microspheres were administered 80 min after the onset of coronary occlusion. First, a reference blood sample was collected from the cervical artery at a constant rate of 8 ml/min for 2 min. Then, 4–8 × 10⁴ microspheres in 2 ml of solution were vortexed for 5 min and immediately injected into the left atrium, followed by several warm (37°C) saline flushes (5 ml). The X-ray fluorescence of Br and Nb was measured using a wavelength dispersive spectrometer (PW1480; Phillips, Almelo, The Netherlands). When the microspheres are irradiated by the primary X-ray beam, the electrons drop to a lower orbit and emit measurable energy with a characteristic X-ray fluorescence energy level for each element. Therefore, it is possible to quantify the X-ray fluorescence of several species of labeled microspheres in a single mixture. Myocardial blood flow (ml·min⁻¹·g wet wt⁻¹) was calculated using the formula time flow = (tissue counts) × (reference flow)/(reference counts).

Exclusion criteria. The following standards were used to exclude unsatisfactory dogs: 1) subendocardial collateral blood flow >15 ml·100 g⁻¹·min⁻¹, or 2) more than two consecutive attempts required to terminate ventricular fibrillation using low-energy direct current pulses applied directly to the heart.

GLP-1 ELISA. GLP-1 ELISA (Millipore) was performed according to the manufacturer’s instructions with minor modification. Canine blood was directly drawn into BD P700*, K2EDTA tubes (BD, Franklin Lakes, NJ) by venipuncture. Plasma samples were centrifuged immediately, and the resulting plasma from each tube was pooled into a new tube stored at −80°C. This ELISA is specific for the bioactive forms of GLP-1 ([GLP-1 (7–36) amide and GLP-1 (7–37)]) and will not detect other forms of GLP-1. There is no cross-reactivity with glucagon.

Adenosine concentrations. Blood samples were collected from the systemic vein before occlusion and from the coronary artery and vein at the end of the ischemic period. Plasma adenosine concentration was measured by adding 10 μl of [13C5]-labeled adenosine (internal standard) to 100 μl of plasma. The proteins were removed by adding trifluoroacetic acid to the samples, followed by a 5-min centrifugation (12,000 g, 4°C). The supernatant was analyzed by liquid chromatography tandem-mass spectrometry (LC-MS/MS) at Nemoto Science (Tsukuba, Japan) using an API 5000 quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Japan) coupled to a Shimadzu LC-20AT high-performance liquid chromatography system (Shimazu, Japan), which consisted of a degasser, two eluent pumps, a column oven, and an autosampler. Each sample was separated online with a reverse-phase column (Hypersil, 5 μm, 150 mm × 2.1 mm I.D.; ThermoFisher) maintained at 60°C. The sample injection volume was 5 μl at the final dilution and the mobile phase was 10 mmol/liter ammonium formate/water (60/40 vol/vol). The samples were eluted within 3 min at a flow rate of 250 μl/min. A mass spectrometer equipped with a lock electrospray ionization probe was operated in both positive and negative (ESI) modes. The selected reaction monitoring channels ranged from 268 to 136 m/z for adenosine, and from 273 to 136 m/z for the internal standard. Data processing was performed...
formed using Analyst 1.5 software (Applied Biosystems/MDS SCIEX).

ADA activity. ADA activity was measured using the Runpia liquid ADA assay kit (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) as per the manufacturer’s instructions, and a JCA-BM9030 automated analyzer (JEOL, Tokyo, Japan). Circulating adenosine is converted into inosine by ADA, and inosine is converted into hypoxanthine by purine nucleoside phosphorylase. Therefore, ADA activity was expressed as percent (inosine + hypoxanthine)/adenosine.

Protein extraction. Canine heart tissue samples were homogenized in TNE buffer, consisting of 10 mmol/l Tris·HCl (pH 7.2), 150 mmol/l NaCl, and 1 mmol/l EDTA, and containing a protease inhibitor cocktail (Nacalai Tesque) and containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The homogenate was centrifuged (1,000 g, 10 min) twice to eliminate nuclei and tissue debris. The final supernatant was gently solubilized in 10% Triton X-100 (1% final concentration). After an incubation of 15 min on ice, the sample was centrifuged for 60 min (100,000 g, 4°C), and the supernatant was collected as the soluble fraction.

Preparation of mitochondrial and cytosolic fractions. Canine heart tissue samples were homogenized in isotonic buffer, consisting of 25 mmol/l HEPES (pH 7.4), 250 mmol/l sucrose, and 2 mmol/l EDTA, and containing protease inhibitor cocktail (Nacalai Tesque) and PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science, Germany) using a Potter-Elvehjem tissue grinder. The homogenate was centrifuged (1,000 g, 10 min) to obtain postnuclear supernatant (PNS). The PNS was then centrifuged (8,000 g, 10 min) and the resulting pellet was used as the mitochondrial fraction, and the supernatant was further ultracentrifuged for 60 min (100,000 g, 4°C) to obtain the cytosolic fraction. The mitochondrial pellet was resuspended in isotonic buffer containing 0.5% NP-40. Equal amounts of proteins from different fractions were loaded on SDS-PAGE.

Western blot analysis. Protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL). Equal amounts of dog soluble fraction was fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane by electroblotting, and processed for Western blot analysis as previously described (48). The membrane was incubated with specific primary antibodies, followed by a secondary antibody-horseradish peroxidase conjugate, and developed with enhanced chemiluminescence reagent (GE Healthcare, UK). Band intensities were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Apoptotic cells were identified using a TUNEL-based in situ apoptosis detection kit (ST7100; Millipore) as described previously (53). After a 30-min treatment with 0.3% H2O2-methanol at room temperature, tissue sections were incubated with the TUNEL reaction mixture, rinsed, and counterstained with 1% methyl green solution. TUNEL-positive and counterstained (total number of nuclei) cells were observed using an all-in-one type, BZ-9000 microscope (Keyence, Osaka, Japan). The number of cells in 10 random fields was counted for each treatment. The percentage of TUNEL-positive cells was calculated as follows: (number of TUNEL positive cells/total number of nuclei) × 100.

Statistical analysis. All statistical analyses were performed using JMP 11 software (SAS Institute, Cary, NC). Data are expressed as
of the control group (Table 3). Therefore, the beneficial effect of alogliptin-treated group was significantly different from that of the control group. Regression analysis revealed an inverse relationship between infarct size and collateral blood flow in each group (Fig. 2A). The slope of the alogliptin-treated group was significantly different from that of the control group (43.1 ± 2.5%) whereas 8SPT completely prevented the effect of alogliptin (51.2 ± 8.2%) (Fig. 2B). However, 8SPT alone had no effect on infarct size compared with the control group. Regression analysis revealed an inverse relationship between infarct size and collateral blood flow in each group (Fig. 2C). The slope of the alogliptin-treated group was significantly different from that of the control group (Table 3). Therefore, the beneficial effect of alogliptin was independent of collateral flow. The efficiency of 4-day alogliptin pretreatment was tested by comparing the postprandial levels of plasma GLP-1 in alogliptin-treated dogs and untreated dogs on the fourth day, before the ischemia-reperfusion protocol. Figure 2D shows that alogliptin induced a sevenfold increase in plasma GLP-1 levels. Collectively, these data suggest that adenosine signaling is necessary for the cardioprotective effect of GLP-1.

Adenosine concentrations and expression of adenosine receptors, DPP4, and GLP-1 receptors in canine hearts. The involvement of ADA in alogliptin-mediated responses was tested by comparing ADA activity and plasma adenosine concentrations in control and alogliptin-treated animals. ADA activity was measured in the blood sampled from the systemic vein before ischemia, and adenosine levels were measured in blood samples collected from the coronary artery and coronary vein. Figure 3A (left) shows that compared with the control animals, alogliptin did not affect ADA activity. In addition, alogliptin did not affect adenosine levels measured in the blood of the systemic vein (Fig. 3A, middle) or the coronary arteriovenous difference in adenosine level (Fig. 3A, right). Western blot analysis indicated that 4-day alogliptin pretreatment did not alter the protein expression of DPP4, the GLP-1 receptor, or the adenosine receptor A1 (A1R), which is involved in ischemic preconditioning (Fig. 3B). These data rule out our original hypothesis that the cardioprotection mediated by DPP4 inhibitors was attributable to the increase in adenosine production or A1R upregulation, which is known to promote ischemic preconditioning.

Signaling pathways involved in alogliptin-mediated cardiac protection in canine hearts. That 4-day alogliptin pretreatment induced a sevenfold increase in circulating GLP-1 levels (Fig. 2D) suggested that this mediator may be responsible for the cardioprotective effect of the DPP4 inhibitor. Therefore, the role of GLP-1 receptor-mediated responses was tested using prosurvival elements involved in cardioprotection: Akt, GSK-3β, and Erk1/2 (36). We also tested whether adenosine-mediated signaling pathways that support ischemic preconditioning interact with GLP-1 receptor-mediated responses. We conducted Western blot analysis on myocardial tissue samples collected after ischemia-reperfusion under these conditions: control, alogliptin, and alogliptin + 8SPT. Figure 4A shows that alogliptin treatment did not alter expression levels of Akt or GSK-3β. In contrast, both proteins were highly phosphorylated in the alogliptin group compared with the control group, and these responses were not suppressed by the nonselective adenosine receptor antagonist 8SPT. Phosphorylation and protein expression of Erk1/2 did not change in any group. These data suggest that the DPP4 inhibitor mediates cardioprotection via the GLP-1–Akt–GSK-3β cascade without interference by adenosine signaling.

Table 1. Number of dogs assigned and excluded in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Dogs Originally Assigned</th>
<th>Dogs Used for Data Analysis</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Alo</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Alo + 8SPT</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>8SPT</td>
<td>9</td>
<td>6</td>
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</table>

Lethal Arrhythmia during Ischemia/Reperfusion

<table>
<thead>
<tr>
<th>Reason for Exclusion</th>
<th>Lethal Arrhythmia during Ischemia/Reperfusion</th>
<th>Death due to VF</th>
<th>High Collateral Flow (&gt;15 ml/100 g per min)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Alo</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Alo + 8SPT</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Data are means ± SE. There were no significant differences among protocols.

Table 2. Collateral blood flow and area at risk in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Collateral Blood Flow (ml/100 g per min)</th>
<th>Area at Risk (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8 ± 2</td>
<td>43 ± 4</td>
</tr>
<tr>
<td>Alo</td>
<td>10 ± 2</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>Alo + 8SPT</td>
<td>7 ± 2</td>
<td>47 ± 2</td>
</tr>
<tr>
<td>8SPT</td>
<td>10 ± 1</td>
<td>47 ± 4</td>
</tr>
</tbody>
</table>

Data are means ± SE. There were no significant differences among protocols.
Fig. 2. Alogliptin suppresses ischemia-reperfusion-induced infarction. Dogs were assigned to one of four groups (n = 6, unless stated otherwise): Con, Alo, 8SPT, and Alo + 8SPT. A: representative photographs of myocardial tissues stained with 2,3,5-triphenyltetrazolium chloride (TTC) from each group. B: effect of each treatment on infarct size (*P < 0.05; one-way ANOVA; Bonferroni post hoc test vs. Con group). C: regression analysis of the percent infarct size over the risk area vs. regional collateral flow. The results of the analysis of covariance test are presented in Table 3. D: comparison of plasma GLP-1 levels between the Con (n = 2) and Alo (n = 3) groups. Alogliptin induced a sevenfold increase in GLP-1 concentration.
Table 3. Linear regression model test in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$y = -5.096x + 86.739$</td>
</tr>
<tr>
<td>Alo</td>
<td>$y = -1.8117x + 56.692$</td>
</tr>
<tr>
<td>Alo + 8SPT</td>
<td>$y = -1.5223x + 53.209$</td>
</tr>
<tr>
<td>8SPT</td>
<td>$y = -2.9731x + 46.568$</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. Control group.

We next examined whether the signaling events activated by the A1R involve the phosphorylation of PKC and CREB. Alogliptin induced a robust CREB phosphorylation reaction, which was suppressed by 8SPT (Fig. 4B). In canine cardiomyocytes, adenosine induces ischemic preconditioning mainly via A1R-mediated PKCα activation (22). Therefore, we investigated the autophosphorylation of PKCα on the threonine at position 641, which triggers the release of PKCα in the cytosol (21). Figure 4B shows that PKCα was phosphorylated in the control and alogliptin groups, and this response was suppressed by 8SPT. These results indicate that cardioprotection mediated by alogliptin is attributable at least in part to GLP-1 receptor-dependent prosurvival pathways, which are amplified by the adenosine A1R-PKCα-CREB signaling pathway.

Alogliptin pretreatment prevents myocardial apoptosis. In the heart, ischemia-reperfusion provokes two forms of myocardial cell death: necrosis and apoptosis. Tissue analysis of the infarct area by using TTC staining demonstrated that alogliptin suppressed necrosis (Fig. 2). Several studies have demonstrated that pharmacological inhibition of the apoptotic signaling cascade during the reperfusion phase is able to attenuate not only apoptotic cell death but also necrotic cell death (16, 45, 57). Therefore, we tested the effect of alogliptin on myocardial apoptosis. Bcl2 family proteins including Bax and Bcl2 are key regulators of apoptosis, and the ratio of Bax-to-Bcl2 determines the susceptibility of cells to apoptosis (37). Figure 5A shows that alogliptin suppressed Bax expression but stimulated Bcl2 expression and that both responses were inhibited by 8SPT. Densitometric analysis indicated that the Bax-to-Bcl2 ratio of 1.2 induced by ischemia-reperfusion was reduced to 0.5 by alogliptin, which was inhibited by the addition of 8SPT (Fig. 5A). Bad, one of proapoptotic Bcl2 family proteins, is known to become activated and translocate from cytosol to mitochondria when cells are triggered by apoptotic stimuli (17, 49, 56). Ser155 phosphorylation of Bad by survival factors allows its confinement to the cytoplasm and inhibition of Bad-dependent death (10, 43). We then examined whether alogliptin or 8SPT affects Bad phosphorylation and translocation. As shown in Fig. 5B, Bad in the cytosolic fraction was phosphorylated at Ser155 upon treatment with alogliptin, in contrast to 8SPT-suppressed Ser155 phosphorylation. In parallel, Bad was not enriched in the mitochondrial fraction compared with the cytosolic fraction; therefore, we could not show differences in Bad phosphorylation in the mitochondrial fraction. We further examined the effect of alogliptin for cytochrome c localization because antiapoptotic reaction acts to prevent the release of cytochrome c from mitochondria. Cytochrome c levels in the cytosolic fraction were significantly increased in the control and alogliptin pretreated groups, and these effects were inhibited by 8SPT. Therefore, we could not show differences in Bad phosphorylation in the mitochondrial fraction. These results indicate that cardioprotection mediated by alogliptin is attributable at least in part to GLP-1 receptor-dependent prosurvival pathways, which are amplified by the adenosine A1R-PKCα-CREB signaling pathway.

Fig. 3. Adenosine regulation is not affected by alogliptin. A: adenosine deaminase (ADA) activity measured in the systemic vein (left, n = 5), adenosine concentration in the systemic vein (middle, n = 3), and coronary arteriovenous difference in adenosine concentration (right, Con group, n = 6; Alo group, n = 4) after ischemia. Neither ADA nor adenosine levels were affected by alogliptin. B: Western blot analysis of DPP4, GLP-1 receptor, and A1R adenosine receptor (A1R) using canine heart protein extracts collected after ischemia-reperfusion under these conditions: Con, Alo, 8SPT, and Alo + 8SPT. Tubulin expression was used as loading control (n = 3 for each treatment). Protein lysates (20 μg) were separated by SDS-PAGE followed by Western blot analysis and densitometric analysis of each protein over tubulin expression ratios.
The present study elucidated the cardioprotective mechanism of the DPP4 inhibitor alogliptin using a multidisciplinary approach. First, the increase in GLP-1 level induced by alogliptin pretreatment reduces myocardial damage during ischemia-reperfusion in vivo. Second, alogliptin induces the GLP-1R–Akt/GSK-3β signaling pathway in cardiomyocytes. Third, the protective effects of alogliptin require adenosine receptor-mediated CREB phosphorylation.

There is currently no consensus on the cardioprotective benefit of DPP4 inhibitors against ischemia-reperfusion injury. Ye et al. (51) showed that 3–14 days of pretreatment with sitagliptin limits the infarct size by >60% in mice. Hocher et al. (15) showed that 7-day pretreatment with linagliptin limits the infarct size by 28% in rats. In contrast, Yin et al. (52) showed that 2-day pretreatment with vildagliptin does not limit the infarct size, and Chinda et al. (7) showed that vildagliptin administration just before coronary occlusion limits the infarct size by only 17% in pigs. These studies suggest that the pretreatment period with DPP4 inhibitors should be at least 3 days for efficient cardioprotection. Therefore, we administered alogliptin 4 days before the coronary occlusion and reperfusion.

DISCUSSION

Contrary to DPP4 inhibitors, GLP-1 and GLP-1 analogs provide immediate cardioprotection because they interact directly with GLP-1 receptors. Such a difference in pharmacokinetics between DPP4 inhibitors and GLP-1 or GLP-1 analogs remains unclear. One possibility is that a certain time is required for circulating GLP-1 levels to increase significantly after DPP4 administration. Another possibility is that a certain period of time is necessary to prime the myocardium for cardioprotection. Ischemia-reperfusion induced an increase in GLP-1 receptor expression in the brain, reaching a maximum after 24 h (28). Thus the cardioprotective effect of sevenfold-higher GLP-1 levels detected after 4-day pretreatment with alogliptin may require priming of the myocardium by ischemia.

Adenosine is widely accepted as a major mediator of ischemic preconditioning (24, 29). As such, any factor susceptible to causing an increase in adenosine accumulation in the heart would be expected to reduce ischemia-reperfusion injury. Circulating adenosine is primarily produced by ATP metabolism and eliminated by ADA activity. DPP4 has been shown to form a functional complex with ADA in immune cells whereby ADA binding regulates DPP4-mediated intracellular signaling (38). Therefore, we hypothesized that DPP4 inhibitors may interfere with the nearby catalytic site of ADA. However, the
The present study showed that alogliptin does not affect ADA activity or concentrations of circulating adenosine in the heart.

The reperfusion injury salvage kinase (RISK) pathway confers cardioprotection against ischemia-reperfusion injury by antiapoptotic and antinecrotic responses, in particular the phosphatidylinositol 3-kinase (PI3K)-Akt and Erk1/2 pathways (14). The cardioprotective effect of GLP-1 against ischemia-reperfusion injury is also inferred to involve these pathways (14). Therefore, we tested the effect of alogliptin on Akt and Erk1/2 phosphorylation. Alogliptin did not affect Erk1/2 phosphorylation, but it stimulated the phosphorylation of Akt, GSK-3β, and CREB. These data differ from those reported in previous studies (18, 26, 51). Two studies have previously demonstrated Erk1/2 phosphorylation in cardioprotection. First, Ban et al. (3) showed that exendin-4 (a GLP-1 activator) increased phosphorylation of Erk1/2 in normoxic cardiomyocyte cultures. Second, however, Huisamen et al. (18) showed that GLP-1 could not directly activate Erk1/2 in hearts under normoxic conditions. Third, these researchers also showed that the significant increase in Erk1/2 phosphorylation during reperfusion after DPP4 inhibitor treatment occurred only in prediabetic rats, although it was decreased in control rats. As described above, Erk1/2 phosphorylation in cardioprotection against ischemia remains controversial. Therefore, we consider that our results on Erk1/2 phosphorylation are of some importance.

The nonselective adenosine receptor antagonist 8SPT suppressed CREB phosphorylation without affecting Akt, GSK-3β, or Erk1/2. 8SPT is a moderately selective adenosine A1R antagonist. Sulfophenyl residues and theophylline residues of 8SPT characterize its property of impermeability to plasma membrane (5). So how does adenosine activation modulate GLP-1 receptor signaling? Studies conducted in pancreatic β-cells showed that GLP-1 receptors are linked to Gs proteins, which activate adenylate cyclase (AC), leading to the production of cAMP and the activation of protein kinase A (PKA), PI3K-Akt, and MEK1/2-Erk1/2 (6). All these intermediates support cardioprotection. In contrast, the A1Rs are widely expressed in the myocardium, where they are coupled to Gi, Go, or Gq proteins (8). They inhibit AC-cAMP-PKA signaling and activate phospholipase C-diacyl glycerol-PKC signaling. The well-documented interactions between PKA and PKC signaling pathways (9, 13, 41, 50) indicate that A1R-PKC activation decreases the activation threshold of PKA by GLP-1. In addition, PKC directly activates AC (4, 54) and phosphorylates CREB in cultured rat neurons (31, 55). In canine cardiomyocytes, adenosine induces ischemic preconditioning via A1R-mediated PKCα activation (22). The data presented here suggest that the effect of DPP4 inhibitors on infarct size depends on A1R activation. If the amount of adenosine released during ischemia-reperfusion is not sufficient for GLP-1 receptors to mediate cardioprotection, the
effects of DPP4 inhibitors on the infarct size may be negligible. This fact may partially explain the controversy over the presence or absence of the size-limiting effects of DPP4 inhibitors in the previous studies, because the potency for adenosine production from ischemic hearts seems to be different.

Although we may not use DPP4 inhibitors to treat patients with acute myocardial infarction, DPP4 inhibitors could benefit patients with diabetes mellitus who are two to four times more at risk for developing cardiovascular diseases (CVD) (20, 32). The recent large-scale clinical trials EXAMINE and SAVOR showed that alogliptin does not reduce the rate of hospitalization or death due to CVD in patients with diabetes mellitus (42, 47). These clinical trials do not contradict the present study because a reduction in infarct size may not necessarily lead to a better clinical outcome. Thus GLP-1 is reported to be beneficial for patients with acute myocardial infarction (30, 35).

In conclusion, this study demonstrated that the cardioprotective effect of alogliptin involves GLP-1 receptor-mediated activation of the A1R-PKCα-CREB signaling cascade in ischemic cardiac tissue. These findings support the therapeutic potential of this interaction between GLP-1 and adenosine for disorders involving myocardial infarction, and thus heart failure.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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