Dipeptidyl Peptidase 4 Inhibition Alleviates Shortage of Circulating Glucagon-Like Peptide-1 in Heart Failure and Mitigates Myocardial Remodeling and Apoptosis via the Exchange Protein Directly Activated by Cyclic AMP 1/Ras-Related Protein 1 Axis

Morihiko Aoyama, MD*; Haruya Kawase, MD *; Yasuko K. Bando, MD, PhD; Akio Monji, MD; Toyoaki Murohara, MD, PhD

Background—Ample evidence demonstrates cardiovascular protection by incretin-based therapy using dipeptidyl peptidase 4 inhibitor (DPP4i) and glucagon-like peptide-1 (GLP-1) under either diabetic or nondiabetic condition. Their action on myocardium is mediated by the cyclic AMP (cAMP) signal; however, the pathway remains uncertain. This study was conducted to address the effect of DPP4i/GLP-1/cAMP axis on cardiac dysfunction and remodeling induced by pressure overload (thoracic aortic constriction [TAC]) independently of diabetes mellitus.

Methods and Results—DPP4i (alogliptin, 10 mg/kg per day for 4 weeks) prevented TAC-induced contractile dysfunction, remodeling, and apoptosis of myocardium in a GLP-1 receptor antagonist (exendin [9-39])–sensitive fashion. In TAC, circulating level of GLP-1 (in pmol/L; 0.86±0.10 for TAC versus 2.13±0.54 for sham control) unexpectedly declined and so did the myocardial cAMP concentration (in pmol/mg protein; 33.0±1.4 for TAC versus 42.2±1.5 for sham). Alogliptin restored the decline in the GLP-1/cAMP levels observed in TAC, thereby augmented cAMP signaling effectors (protein kinase A [PKA] and exchange protein directly activated by cAMP 1 [EPAC1]). In vitro assay revealed distinct roles of PKA and EPAC1 in cardiac apoptosis. EPAC1 promoted cardiomyocyte survival via concomitant increase in B cell lymphoma-2 (Bcl-2) expression and activation of small G protein Ras-related protein 1 (Rap1) in a cAMP dose–dependent and PKA–independent fashion.

Conclusions—DPP4i restores cardiac remodeling and apoptosis caused by the pathological decline in circulating GLP-1 in response to pressure overload. EPAC1 is essential for cardiomyocyte survival via the cAMP/Rap1 activation independently of PKA. (Circ Heart Fail. 2016;9:e002081. DOI: 10.1161/CIRCHEARTFAILURE.115.002081.)

Key Words: apoptosis ■ cyclic AMP-dependent protein kinases ■ heart failure ■ incretins ■ pharmacology ■ Rap1 GTP-binding proteins ■ signal transduction

Dipeptidyl peptidase 4 inhibitor (DPP4i) is among the most popular antidiabetic remedies worldwide, and its substrate glucagon-like peptide-1 (GLP-1), an incretin hormone, primarily mediates the drug class effects of DPP4i.1 To date, ample preclinical evidence has demonstrated the pleiotropic effects of DPP4i and GLP-1 analogs on the cardiovascular protection,2,3 including heart failure (HF).4–7 Recent clinical evidence exploring the effect of DPP4i on cardiovascular safety in patients with type 2 diabetes mellitus reported that saxagliptin increased the incidence of hospitalization for HF via unknown mechanisms.8 In contrast, the other trials for alogliptin (ALO)10 and sitagliptin 11 demonstrated that DPP4i did not worsen HF in type 2 diabetes mellitus.10–12 However, the underlying mechanisms remain uncertain.9

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See Clinical Perspective
This study was conducted to address the essential effect of the DPP4i on myocardium in terms of cardiac function and remodeling using nondiabetic mice with pressure overload (thoracic aortic constriction [TAC]). We unexpectedly found the pathological decline in casual GLP-1 level with concomitant reduction of its second messenger cyclic AMP (cAMP) concentration in myocardium of TAC, which was prevented by ALO. In the context of exploring the molecular mechanism(s) underlying the GLP-1/cAMP pathway, we found that the GLP-1–induced cAMP elevation diversely signals not only protein kinase A (PKA) but also exchange protein directly activated by cAMP 1 (EPAC1). Furthermore, we found that...
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EPAC1 was exclusively essential for cardiac survival via the small G protein Ras-related protein 1 (Rap1) activation. The present article, thus, dissected the neurohumoral significance of casual GLP-1 in response to cardiac stress independently of diabetes mellitus and the diverse roles of cAMP signaling in cardiac apoptosis.

Methods

Experimental Design
All the animal experimental procedures were approved by the Committee on the Use of Live Animals for Teaching and Research of Nagoya University and carried out in accordance with the Guideline for the Care and Use of Laboratory Animals published by the American Heart Association (1985) and the Guide for Care and Use of Laboratory Animals (1996). The experimental protocol is illustrated in Figure I in the Data Supplement. Male C57BL6 mice were allocated into 5 groups; pressure overload induced by TAC operation with and without ALO (10 mg/kg per day for 4 weeks; TAC-ALO and TAC-CON), sham-operated counterparts (sham-ALO and sham-CON), and with and without ALO (10 mg/kg per day for 4 weeks). The values are the mean±SEM. %GA indicates the percent of serum-glycated albumin in total albumin (a circulating surrogate for glycemic status because %GA value increases in proportion to plasma glucose level); ALO, alogliptin; BW, body weight; HW, heart weight; HW/BW, relative ratio of HW to BW; GLU, blood glucose; and TAC, thoracic aortic constriction.

Echocardiography
Cardiac function of each mouse was measured using echocardiography (ACUSON Sequoia 512 system with a 15-MHz high-frequency transducer [Micronson 15LS], Siemens, Pittsburgh, PA) at the time points of 4 weeks after the TAC operation under anesthesia using a combination of ketamine (75 mg/kg) and xylazine (10 mg/kg).

Analysis of Cardiac Remodeling
Cardiac capillary density and cardiomyocyte surface area were assessed by immunohistochemistry using anti-CD31 antibody (BD Biosciences, San Jose, CA) and antidystrophin antibody (Novus Biologicals, Littleton, CO). Cardiac fibrosis was assessed by using Picrosirius red staining. Each positive lesion was digitized and summarized using Image J software.

Analysis of Cardiac Apoptosis
The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using the DeadEnd Fluorometric TUNEL System (Promega, Fitchburg, WI), according to the manufacturer’s instructions. The number of TUNEL-positive cells was expressed as the percentage of total cell number.

Cardiac cAMP Measurement and Immunoblotting
Each heart tissue was subject to the frost shattering by using Cryopress (Microtech Nichion K.K., Chiba, Japan) to avoid any loss or degeneration during the sample processing. The cAMP concentration of each heart lysate was measured using commercially available kit (cAMP-Glo; Promega). For immunoblot analysis, tissue lysates (30 μg per lane) were incubated with Laemmli sample buffer. A list of antibodies used in this study is displayed in Table I in the Data Supplement.

RNA Silencing in Cultured Cardiomyocytes
Primary cultures of neonatal rat cardiomyocytes or H9C12 cells were plated onto an 8-well chamber slide coverslip precoated with collagen type 1 (BD Biosciences) at a density of 1×10⁶ cells per well. After 24 hours of serum starvation, cardiomyocytes were treated for 24 hours with the following reagents: exendin-4 (Ex4, 30 nmol/L; Sigma-Aldrich), 8-bromo-cAMP, 60 μmol/L; Sigma-Aldrich), Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (a PKA inhibitor, 100 μmol/L; Sigma-Aldrich), Rp-8-mercaptothienylthion-2′-O-methyl-cAMP (an EPAC inhibitor, 50 μmol/L; BioLog Life Science Institute, Bremen, Germany). Efficacy-validated siRNAs (Sigma-Aldrich) for silencing PKA (siPKA), silencing EPAC1, and a silencing negative control were introduced into cells using lipofectamine RNAiMAX (Life Technologies), according to the manufacturer’s instructions.

Analysis for Rap1 Activity
Rap1 activity as a surrogate for EPAC1 activation was assessed by 2 different types of pull-down system that detects specifically active Rap1; the recombinant glutathione S-transferase–fusion protein of the Rap1–binding domain from RafGDS (along with glutathione agarose resin [Thermofisher Scientific]) and immunoprecipitation using antiactive Rap1 antibody combined with protein G agarose (Abcam).

Analysis for PKA Activity
PKA activity was monitored to assess the effect of PKA siRNA by the use of nonradioisotope assay according to the manufacturer’s protocol (PepTag Non-Radioactive cAMP-Dependent Protein KinaseAssay; Promega).

Statistical Analysis
Statistical analysis was performed using JMP Pro (version 11; SAS Institute Inc, Cary, NC). Values are expressed as the mean±SEM.

Table 1. Baseline Characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham/CON (n=8)</th>
<th>Sham/ALO (n=8)</th>
<th>TAC/CON (n=10)</th>
<th>TAC/ALO (n=7)</th>
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<td>BW, g</td>
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<td>25.5±0.3</td>
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<td>HW, mg</td>
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<td>111±3</td>
<td>142±10</td>
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<td>HW/BW</td>
<td>4.2±0.1</td>
<td>4.2±0.1</td>
<td>5.6±0.4</td>
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<tr>
<td>GLU, mg/dL</td>
<td>153±8</td>
<td>139±5</td>
<td>214±15</td>
<td>158±20</td>
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<td>%GA</td>
<td>1.7±0.3</td>
<td>1.2±0.3</td>
<td>1.7±0.3</td>
<td>1.4±0.1</td>
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Data were obtained from 14-wk-old male mice that underwent TAC or sham operation with or without ALO (10 mg/kg per day for 4 weeks). The values are the mean±SEM. %GA indicates the percent of serum-glycated albumin in total albumin (a circulating surrogate for glycemic status because %GA value increases in proportion to plasma glucose level); ALO, alogliptin; BW, body weight; HW, heart weight; HW/BW, relative ratio of HW to BW; GLU, blood glucose; and TAC, thoracic aortic constriction.

"*P<0.05; statistically significant interaction between the effects of TAC and ALO on each parameter by 2-way ANOVA.

Table 2. Echocardiographic Findings

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham/CON (n=6)</th>
<th>Sham/ALO (n=8)</th>
<th>TAC/CON (n=8)</th>
<th>TAC/ALO (n=7)</th>
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</thead>
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<tr>
<td>LVd, mm</td>
<td>2.9±0.1</td>
<td>3.1±0.1</td>
<td>3.3±0.2</td>
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<td>LVs, mm</td>
<td>1.8±0.1</td>
<td>1.9±0.0</td>
<td>2.3±0.1</td>
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<tr>
<td>IVST, mm</td>
<td>0.8±0.0</td>
<td>0.9±0.0</td>
<td>1.1±0.1</td>
<td>0.9±0.0</td>
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<tr>
<td>PWT, mm</td>
<td>0.7±0.0</td>
<td>0.8±0.0</td>
<td>1.0±0.0</td>
<td>0.8±0.0</td>
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<tr>
<td>LAD, mm</td>
<td>1.8±0.1</td>
<td>1.7±0.0</td>
<td>1.9±0.1</td>
<td>1.7±0.0</td>
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<tr>
<td>FS, %</td>
<td>35.6±0.6</td>
<td>36.9±0.7</td>
<td>28.4±0.7</td>
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<td>Peak E, m/s</td>
<td>0.9±0.0</td>
<td>0.8±0.0</td>
<td>0.9±0.0</td>
<td>0.9±0.0</td>
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<td>E/A</td>
<td>2.7±0.2</td>
<td>2.2±0.2</td>
<td>2.6±0.3</td>
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<td>Dd, ms</td>
<td>35.3±0.3</td>
<td>31.0±0.4</td>
<td>43.8±0.7</td>
<td>36.6±0.5</td>
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</table>

Data were obtained from each anesthetized mouse that underwent TAC or sham operation with or without ALO treatment by transthoracic echocardiography. The values represent the mean±SEM. ALO indicates alogliptin; Dct, deceleration time; FS, fractional shortening; IVST, interventricular septal thickness; LAD, left atrial diameter; LVd, left ventricular diastolic dysfunction; LVs, left ventricular systolic dysfunction; PWT, posterior wall thickness; and TAC, thoracic aortic constriction.

"*P<0.05; statistically significant interaction between the effects of TAC and ALO on each parameter by 2-way ANOVA.
Group comparisons were performed by 1-way ANOVA (for parametric variables), Kruskal–Wallis ANOVA, and Mann–Whitney U test (for nonparametric) as appropriate. A 2-way ANOVA was conducted that examined the effect of TAC and ALO on baseline characteristics and cardiac function. Post hoc comparisons of considered pairs were performed using the Tukey–Kramer test for differences between means. Sample sizes and individual statistical results are provided in figure and table legends. Values of $P<0.05$ were considered to be statistically significant.

Results

ALO Ameliorates TAC-Induced Cardiac Dysfunction and Remodeling

We first examined the effects of ALO and TAC on the baseline characteristics (Table 1) and cardiac function by echocardiography (Table 2) in mice. TAC increased the heart weight/body weight ratio (Table 1) and promoted contractile dysfunction and myocardial hypertrophy (Table 2), which were restored by ALO. We next examined the molecular changes related to myocardial remodeling (Akt, mammalian target of rapamycin [mTOR], the ribosomal S6 kinase [S6K], AMP-activated protein kinase [AMPK], and extracellular signal-regulated kinase [ERK], Figure 1; Figure II in the Data Supplement) and sarcomeric proteins (SERCA, phospholamban, troponin T, troponin T-C, troponin I, myosin heavy chain [MYH] 6, and MYH 7; Figure 1). TAC upregulated the Akt/mTOR/S6K axis (Figure 1B–1D) and MYH 7 (Figure 1J), a thick filament protein that controls myofilament Ca$^{2+}$ sensitivity via PKA$^{14}$ signaling, and this effect was prevented by ALO. The level of phosphorylated phospholamban, which is regulated by PKA,$^{15}$ was decreased in TAC, and this effect was reversed by ALO (Figure 1H). In contrast, AMPK, ERK, MYH 6, troponin T-C, troponin I, and SERCA2a were upregulated in TAC but insensitive to ALO (Figure II in the Data Supplement; Figure 1E–1G and 1I).

ALO Restored TAC-Induced Decreases in Circulating GLP-1 and Myocardial cAMP

The cardioprotective action of DPP4i is primarily mediated by GLP-1.$^{7,16,17}$ On the other hand, it remains unclear whether endogenously secreted GLP-1 levels may be altered in response to cardiac stress likewise brain natriuretic peptide.$^{18}$ We, thus, measured the plasma GLP-1 levels in each mice (Figure 2). Notably, TAC reduced the casual GLP-1 levels in mice (in pmol/L; 0.86±0.10 for TAC-CON versus 2.13±0.54 for
ALO Reverses TAC-Induced Cardiac Apoptosis in a GLP-1–Dependent Fashion

We, thus, investigated the effect of ALO/GLP-1–mediated changes in myocardial cAMP on apoptosis (Figure 4). TAC increased the TUNEL-positive cell counts in heart (Figure 4A and 4B), which was partially attenuated by ALO in a manner dependent on Ex9-39. In noncardiac cells, cAMP-mediated apoptosis is largely blocked by B-cell lymphoma-2 (Bcl-2).23 Therefore, we measured changes of the Bcl-2 in each mouse heart (Figure 4C and 4D). We found that TAC decreased Bcl-2 levels in the heart with concomitant increase in cleaved caspase-3 levels, and these effects were completely reversed by Ex9-39 (light gray bars).

We next observed the changes in cAMP effectors in each myocardium (Figure 4E and 4F). ALO augmented the phosphorylation level of cAMP response element–binding protein,24 a surrogate for PKA activation, in TAC/CON hearts (dark gray bar, Figure 4E), which was completely reversed by Ex9-39 (light gray bar). EPAC1 is known as another effector of cAMP, and its activation contributes to cardiomyocyte remodeling.25,26 Contractility,27 and apoptosis21 in a PKA-independent fashion.21 Myocardial EPAC1 expression was significantly augmented by TAC (Figure 4F), and this effect was further enhanced by ALO. In contrast, EPAC2 levels remained unchanged by TAC and by ALO (Figure III in the Data Supplement).

ALO Ameliorates TAC-Induced Cardiac Remodeling

We further confirmed the effect of ALO on myocardial remodeling in TAC (Figure 3). TAC-CON exhibited increases in cardiomyocyte surface area (Figure 3A and 3B) and a concomitant decrease in capillary density (Figure 3A and 3C). ALO prevented the changes in cardiomyocyte surface area and capillary density that were partially but significantly reversed by Ex9-39, an inactive analog of GLP-1 (Figure 3A–3C). Picrosirius red staining revealed that TAC-induced cardiac interstitial fibrosis was attenuated by ALO, which was also reversed by Ex9-39 (Figure 3D and 3E).

ALO increases intracellular cAMP levels in various cell types,1 including cardiomyocytes, which contributes to its protective effects against HF.3,17,20 We, thus, measured changes in cAMP levels in each mouse heart (Figure 2B). We found a trend similar to that observed with circulating GLP-1 levels; specifically, the myocardial cAMP concentrations were reduced in TAC-CON, and this decline was prevented by ALO.

Figure 2. Heart failure suppresses the resident glucagon-like peptide-1 (GLP-1) level and the myocardial cAMP level in mice. A and B, Comparison of plasma GLP-1 (A) and cardiac cAMP levels (B) among thoracic aortic constriction (TAC)– and sham-operated mice with or without alogliptin (ALO; 10 mg/kg per day). The data are shown as the means±SEM. Data were compared by Kruskal–Wallis ANOVA followed by Tukey–Kramer test (P<0.0001, A; P<0.0006, B). *P<0.05 vs sham/CON (n=5) and #P<0.05 vs sham-CON; P<0.01; Figure 2A), and this effect was reversed by ALO. TAC/CON exhibited higher blood glucose levels (Table 1), which is consistent with the reduced GLP-1 level, a glucose-lowering hormone.1 No difference in body weight was observed between sham and TAC (Table 1). Because GLP-1 is known as an insulinotropic hormone under diabetic conditions,1 it is known as another effector of cAMP, and its activation contributes to cardiomyocyte remodeling.25,26 Contractility,27 and apoptosis in a PKA-independent fashion.21 Myocardial EPAC1 expression was significantly augmented by TAC (Figure 4F), and this effect was further enhanced by ALO. In contrast, EPAC2 levels remained unchanged by TAC and by ALO (Figure III in the Data Supplement).

ALO Reverses TAC-Induced Myocardial Apoptosis in a GLP-1–Dependent Fashion

cAMP is a unique second messenger that has diverse antiapoptotic and proapoptotic effects that are cell-type dependent.21 In cardiomyocytes, several reports have demonstrated that an increase in cAMP augments cell apoptosis, but there are contrary studies reporting its antiapoptotic effects.14,21,22
GLP-1 Exerts Antiapoptotic Action in Cardiomyocytes by PKA-Independent Pathway

To address the direct action of the GLP-1/cAMP activation on cardiomyocytes, we next examined the role of PKA in apoptosis by using cultured cardiomyocytes (Figure 5). In vitro TUNEL assay revealed that the topical application of the cleavage-resistant GLP-1 analog Ex4 attenuated cardiomyocyte apoptosis induced by serum depletion, which was unexpectedly insensitive to the PKA inhibitor Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (100 µmol/L; Figure 5A). We further confirmed the role of PKA in cardiomyocyte apoptosis in a more specific fashion by using cultured cardiomyocytes (Figure 5B). To test this hypothesis, we evaluated the effect of silencing PKA using PKA-silencing RNA (siPKA; Figure 5D). In contrast to siPKA (Figure 5B), silencing EPAC1 dramatically reversed the Ex4-mediated cardiomyocyte protection in vitro. We further measured Bcl-2 expression in cultured cardiomyocytes treated with the same assay condition to explore the relationship between Bcl-2 levels and the cAMP effectors PKA (Figure 5D and 5E) and EPAC1 (Figure 5F and 5G). Interestingly, the siPKA had no effects on Ex4-mediated Bcl-2 upregulation, which was contrarily abrogated by the EPAC1 silencing.

Role of Rap1 in Antiapoptotic Effect of GLP-1/EPAC Axis and Its cAMP Dependency in Cardiomyocytes

To address the effect of cAMP/EPAC1 axis on cardiomyocyte apoptosis, we tested the effect of Ex4 and a cell-permeable cAMP analog (8-bromo-cAMP) on small G protein Rap1 activity in cardiomyocytes because EPAC1 specifically activates Rap1 as its guanine nucleotide exchange factor in a cAMP-dependent fashion (Figure 6). We first compared the effect of 8-bromo-cAMP at serial concentration (in µmol/L; 0, 60, 6, and 0.06) on Rap1 activity (Figure 6A) and cardiomyocyte apoptosis (Figure 6B). 8-bromo-cAMP dose dependently activated Rap1, and the maximum response of Rap1 activation was obtained at 60 µmol/L (Figure 6A). We further compared

Figure 4. Effects of alogliptin (ALO) on cardiac apoptosis in pressure-overloaded heart. A, Representative images of the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (green) of each heart section. Nuclei were stained with DAPI (blue). Arrowheads: positivity for apoptosis. Scale bar, 50 µm. Original magnification was ×200 at high-power field. TUNEL-positive cells were counted, and the data are summarized in a bar graph (B). The data are shown as the means±SEM. C and D, Representative immunoblot analyses of cleaved-caspase3 (C) and B-cell lymphoma-2 (Bcl-2; D) in each heart. E and F, Changes in cAMP effectors in each mouse heart. E, Phosphorylated cAMP response element–binding protein (p-CREB) level as a surrogate for protein kinase A (PKA) activity. Each band was quantified by densitometry and is presented in the graph. The data are shown as the means±SEM. *P<0.05 vs sham/CON, #P<0.05 vs TAC/CON, and ¶P<0.05 vs TAC/ALO (n=4–9; Kruskal–Wallis ANOVA followed by Tukey–Kramer test). EPAC1 indicates exchange protein directly activated by cAMP 1; Ex4, exendin-4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and TAC, thoracic aortic constriction.
the effect of serial dose of 8-bromo-cAMP on cardiac muscle cell apoptosis (Figure 6B). TUNEL-positive cell counts induced by serum depletion were suppressed by 8-bromo-cAMP (24-hour exposure) in a dose-dependent fashion. Because the previous article reported that cAMP increase at nmol/L level induced by short-term exposure (<30 minutes) of cAMP (24-hour exposure) in a dose-dependent fashion. Induced by serum depletion were suppressed by 8-bromo-cAMP (100 μmol/L). Apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, and all nuclei were visualized with DAPI (blue). The data are summarized in the bar graph. Results are presented as a percentage of the total cell number. *P<0.05 vs compound-free control (n=4ea; Kruskal–Wallis ANOVA followed by Tukey–Kramer test). B and C, Effects of the RNA silencing of PKA (B) and EPAC1 (C). Cardiomyocytes were treated with Ex4 for 24-hours and subjected to the TUNEL assay. Scale bar, 100 μm. Original magnification: ×100. The data are presented as a percentage of the total cell number and are summarized in a bar graph. Middle, Effect of siPKA (D) and siEPAC1 (F) on B-cell lymphoma-2 (Bcl-2) expression. cAMP response element–binding protein (CREB) phosphorylation as a measure of PKA activity (D) and EPAC1 expression (F). Effects of Ex4 and each siRNA were assessed by Mann–Whitney U test (*P<0.05 vs compound-free control; n=4ea). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; siCON, silencing negative control; siEPAC1, silencing EPAC1; and siPKA, silencing PKA.

**Discussion**

The present study demonstrated 2 essential evidences as follows: (1) the nondiabetic cardiac stress promotes pathological decline in circulating GLP-1 that causes cardiac dysfunction and remodeling independently of diabetes mellitus. (2) DPP4i/GLP-1 axis ameliorates cardiomyocyte apoptosis via EPAC1/Rap1 activation in a PKA-independent manner.

One could assume that one of the reasons for this DPP4i/GLP-1–mediated protective action may result from their effect on blood glucose level. Indeed, we found that TAC/CON exhibited significant increase in casual blood glucose level (214±15 mg/dL), which was significantly reduced by ALO (158±20 mg/dL; Table 1). However, there were few studies that elucidate the effect of blood glucose on myocardium in nondiabetic model, and most evidences on the pathological effects of blood glucose on myocardium were observed in diabetic models that exhibit significantly higher level of blood glucose (for instance, >400 mg/dL).30,31 Even in the case of these diabetic models, the effect of high glucose on myocardium becomes obvious by

**Figure 5.** Glucagon-like peptide-1 activation ameliorates in vitro cardiomyocyte apoptosis in a protein kinase A (PKA)–independent but exchange protein directly activated by cAMP 1 (EPAC1)–dependent fashion. A, Cardiomyocytes were exposed to serum depletion for 24 hours and subjected to treatment with exendin-4 (Ex4; 30 nmol/L) with or without the PKA inhibitor Rp-cAMP (100 μmol/L). Apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, and all nuclei were visualized with DAPI (blue). The data are summarized in the bar graph. Results are presented as a percentage of the total cell number. *P<0.05 vs compound-free control (n=4ea; Kruskal–Wallis ANOVA followed by Tukey–Kramer test). B and C, Effects of the RNA silencing of PKA (B) and EPAC1 (C). Cardiomyocytes were treated with Ex4 for 24-hours and subjected to the TUNEL assay. Scale bar, 100 μm. Original magnification: ×100. The data are presented as a percentage of the total cell number and are summarized in a bar graph. Middle, Effect of siPKA (D) and siEPAC1 (F) on B-cell lymphoma-2 (Bcl-2) expression. cAMP response element–binding protein (CREB) phosphorylation as a measure of PKA activity (D) and EPAC1 expression (F). Effects of Ex4 and each siRNA were assessed by Mann–Whitney U test (*P<0.05 vs compound-free control; n=4ea). GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; siCON, silencing negative control; siEPAC1, silencing EPAC1; and siPKA, silencing PKA.
at least several months later, suggesting our experimental setting of 1-month exposure to pressure overload, although it leads to the mild elevation of blood glucose, presumably rules out the possible link between the effect of DPP4i but also those GLP-1 antagonist in blood glucose and those on myocardium.

GLP-1 has been shown to protect cardiomyocytes from apoptosis occurred in ischemia/reperfusion models. We found that the decline in the casual GLP-1 level contributes to the increased cardiac apoptosis in TAC/CON by demonstrating the possible link between the effect of DPP4i/GLP-1 axis and 8-bromo-cAMP dose dependently enhanced Rap1 activity and reduced the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–positive cell counts (*P<0.0005, 1-way ANOVA). Figure 6. Rap1 activation is a cAMP/exchange protein directly activated by cAMP 1 dependent but protein kinase A (PKA) independent. C. Representative images and densitometric summaries of the active Rap1 pull-down assay using active Rap1 antibody. A and E. Recombinant protein that bound to active Rap1. D. 8-bromo-cAMP dose dependently enhanced Rap1 activity and reduced the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–positive cell counts (*P<0.0005, 1-way ANOVA). C and E. Effect of exendin-4 (Ex4) and 60 μmol/L 8-bromo-cAMP on Rap1 activity for 24 hours. GTPγS-induced activated Rap1 (GTPγS-Rap1) as a positive control (*P<0.05 vs compound-free control by Mann–Whitney U test, n=4). F. PKA kinase activity was measured by fluorescent kemptide kinase assay. PKA activity in each cell lysate is expressed as ratio of phosphorylated and unphosphorylated PKA substrate (kemptide) that is separated in agarose gel electrophoresis. HPF indicates high-power field; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siCON, silencing negative control; and siPKA, silencing PKA.

Distinct Roles of Cyclic AMP in Heart Failure

Figure 6. Rap1 activation is a cAMP/exchange protein directly activated by cAMP 1 dependent but protein kinase A (PKA) independent. C. Representative images and densitometric summaries of the active Rap1 pull-down assay using active Rap1 antibody. A and E. Recombinant protein that bound to active Rap1. D. 8-bromo-cAMP dose dependently enhanced Rap1 activity and reduced the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–positive cell counts (*P<0.0005, 1-way ANOVA). C and E. Effect of exendin-4 (Ex4) and 60 μmol/L 8-bromo-cAMP on Rap1 activity for 24 hours. GTPγS-induced activated Rap1 (GTPγS-Rap1) as a positive control (*P<0.05 vs compound-free control by Mann–Whitney U test, n=4). F. PKA kinase activity was measured by fluorescent kemptide kinase assay. PKA activity in each cell lysate is expressed as ratio of phosphorylated and unphosphorylated PKA substrate (kemptide) that is separated in agarose gel electrophoresis. HPF indicates high-power field; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siCON, silencing negative control; and siPKA, silencing PKA.

In cardiac apoptosis (Figures 5 and 6). cAMP signaling is a versatile regulator of the cardiac pathophysiology that modulates apoptosis, hypertrophy, and contractile dysfunction. To date, ample evidence indicates that cAMP signaling uses distinct effectors, namely PKA and EPAC1, that impinge on the apoptosis regulatory molecules and Ca2+-handling molecules. Dodge-Kafka et al consistently demonstrated the diversity of cAMP effectors using the models for cardiac hypertrophy. They reasoned the cause of cAMP diversity to PKA-anchoring protein that forms a complex with PKA, EPAC1, and phosphodiesterase 4D3, of which activity is regulated by the PKA-anchoring protein–associated ERK5. Considering the differences in terms of models (hypertrophy versus apoptosis) and PKA dependency on EPAC1 activation, we examined whether the EPAC1/Rap1 activation may be PKA dependent in cardiac apoptosis (Figure 6). Surprisingly, we found that EPAC1/Rap1 activation is PKA independent (Figure 6E). The possible reasons about the gap observed in the PKA dependency of EPAC1/Rap1 pathway between the study by Dodge-Kafka et al and this study may account for the
distinct mechanism that exists in cAMP-dependent regulation of hypertrophy and apoptosis or the different experimental condition, such as differences in process to increase intracellular cAMP (indirectly via receptor activation or phosphodiesterase inhibition and directly by cAMP analog) and exposure time (Figure 4A). We further examined the possible role of ERK5 in the cAMP-dependent rescue of cardiac muscle cell from apoptosis (Figure IV in the Data Supplement). However, inhibition of ERK5 by its specific inhibitor BIX21088 had no effect on TUNEL-positive cell counts. Unexpectedly, the BIX compound reduced DAPI-positive total adhesive cell counts, suggesting that the ERK5 activity might affect cell adhesion of cardiomyocytes. Further study is awaited.

The small GTPase Rap1 is evolutionarily conserved and regulates cellular proliferation, differentiation, and apoptosis and Rap1 is specifically associated with cAMP and EPAC1, the Rap1 guanine nucleotide exchange factor. Interestingly, Rap1 modulates cell survival by interacting with CREB indicates cAMP response element–binding protein. In cardiomyocytes, phosphodiesterase 3, together with phosphodiesterase 4, accounts for ≈90% of the basal cAMP-hydrolyzing activity. In cardiomyocyte hypertrophy, Dodge-Kafka et al demonstrated the essential role of phosphodiesterase 4D3. In case of apoptosis, Ding et al demonstrated the primary roles of phosphodiesterase 3 in cardiac muscle cell survival. Another study demonstrated that distinct phosphodiesterase subtypes alternatively modulate the cell survival pathway. However, we did not elucidate the link between Rap1/EPAC1 axis and phosphodiesterase subtypes.

In conclusion, this study sheds light not only on the pleiotropic action of DPP4/GP1 axis but also on the role of another cAMP effector Rap1/EPAC1 axis in the restoration of cardiac apoptosis, revisiting the pathophysiological significance of cAMP signaling in both healthy and diseased myocardium.

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Disclosures

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References

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Distinct Roles of Cyclic AMP in Heart Failure


**CLINICAL PERSPECTIVE**

Substantial evidence demonstrates cardiovascular protection by incretin-based therapy using dipeptidyl peptidase inhibitors (DPP4i) and glucagon-like peptide-1 (GLP-1) under either diabetic or nondiabetic condition. Part of their action on myocardium is mediated by cAMP signaling; however, the pathway remains uncertain. This study was conducted to address the effect of DPP4i/glucagon-like peptide-1/cAMP axis on cardiac dysfunction and remodeling induced by pressure overload independently of diabetes mellitus, using a mouse model of pressure overload induced by transaortic constriction. This study elucidates that DPP4 protects the heart from the pathological changes induced by the lack of physiological level of glucagon-like peptide-1 independently of diabetic stress. The data suggest that GLP-1 may play a pivotal role in neurohumoral regulation in heart failure in terms of development of cardiac remodeling. Our preclinical data implicate an additional consideration for the administration of cAMP-elevating agents that might contribute to reducing myocardial apoptosis. This study sheds light on the pleiotropic action of the DPP4i/glucagon-like peptide-1 axis and on the role of another cAMP effector, the Rap1/exchange protein directly activated by cAMP 1 axis, in the restoration of cardiac apoptosis, revisiting the pathophysiological significance of cAMP signaling in both healthy and diseased myocardium.
Dipeptidyl Peptidase 4 Inhibition Alleviates Shortage of Circulating Glucagon-Like Peptide-1 in Heart Failure and Mitigates Myocardial Remodeling and Apoptosis via the Exchange Protein Directly Activated by Cyclic AMP 1/Ras-Related Protein 1 Axis
Morihiko Aoyama, Haruya Kawase, Yasuko K. Bando, Akio Monji and Toyoaki Murohara
Supplemental figure 1. Experimental protocol

UCG; echocardiography, TAC; thoracic aortic constriction surgery (pressure overload-induced heart failure), CON; vehicle, ALO; alogliptin (ALO; 10 mg/kg/day p.o.), Ex9-39; exendin fragment 9-39 (inactive form of GLP-1, 24nmol/kg/day, subcutaneously administrated by osmotic pump).

Male 9-week-old C57BL6 mice were randomly allocated into 5 groups. 1 week after each drug treatment, mice were subjected to surgery {TAC and sham}. All groups (sham/CON, sham/ALO, TAC/CON, TAC/ALO, and TAC/Ex9-39/ALO) were analyzed at 4 week after the surgery (14-week-old).
Supplemental figure 2  Alogliptin had no effect on AMPK and ERK signaling in TAC heart.

Representative immunoblot images of phosphorylated and total AMPK and levels in heart obtained from each group (A). Data obtained from densitometrical analysis were summarized in bar graph (B and C): White bar; sham/CON, light gray bar; sham/ALO, black bar; TAC/CON, dark gray bar; TAC/ALO, respectively. Data were shown as the mean±SEM. *P < 0.05 versus sham/CON (Mann-Whitney, n= 4-6).
Supplemental figure 3. Effects of alogliptin on myocardial expression of EPAC2 in mice.

A: Representative immunoblot images of EPAC2 in heart extract obtained from each group. Data were quantified by densitometrical analysis and summarized in the bar graph (B). White bar; sham/CON, light gray bar; sham/ALO, black bar; TAC/CON, and dark gray bar; TAC/ALO, respectively. Data were shown as the mean±SEM. (n=4-6).
Supplemental Figure 4.
ERK5 activity had no effect on cardiomyocyte apoptosis induced by serum depletion. Because Rap1/EPAC activation links to ERK5 axis in cultured cardiomyocyte hypertrophy (Nature 2005, 437, 574), we hypothesized whether the ERK5 activity might be involved in the Rap1/EPAC1-induced anti-apoptotic effect. We thus tested the effect of ERK5 inhibitor on cardiomyocyte apoptosis detected by TUNEL staining. The cAMP dose-dependently suppressed TUNEL-positive cell counts, which was unaffected by the ERK5 specific inhibitor BIX 02188 (10 μM; gray bar) (B; P=0.53, Mann-Whitney’s U test, n=4). P=0.005, Kruskal-Wallis ANOVA, n=4 ea, *P<0.05 versus 0 μM 8-bromo-cAMP group without BIX (white bar); #P<0.05 versus 0 μM 8-bromo-cAMP group with BIX compound (shaded bar), respectively. Unexpectedly, BIX21088 treatment reduced the total DAPI-positive attached cell counts (C; P=0.02, Mann-Whitney’s U test, n=4), suggesting ERK5 activation might be essential for cell adhesion of cardiomyocyte, independently of apoptosis. Further evaluation is awaited.
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**Supplemental Table 1. Antibody list in the present study.**
**Supplemental Table 2. Effect of time- and dose-dependent action of cyclic AMP on protein kinase A activity in cultured cardiomyocytes.**

Comparison of our results to previous papers demonstrating the impact of cAMP signaling cardiomyocytes (A) Short term (less than 30min) activation with PDE inhibitors activates PKA (reference # 29). Several critical differences in the experimental settings applied in the 3 papers are compared.

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Supplemental references
