รายงานวิจัยฉบับสมบูรณ์

โครงการ

การกระตุ้นจีแอลพี-หนึ่งรีเซปเตอร์ยับยั้งภาวะเครียดออกซิเดชันและอะพอพโตซิสที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ในเซลล์กล้ามเนื้อหัวใจของหนูแรกเกิด

Activation of GLP-1 receptor inhibits H_2O_2-induced oxidative stress and apoptosis in rat neonatal cardiomyocytes

โดย

ผศ. ดร. ภก. ศุภโชค มั่งมูล และคณะ

กรกฎาคม 2557

สัญญาเลขที่ MRG5580037

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Activation of GLP-1 receptor inhibits H_2O_2-induced oxidative stress and apoptosis in rat neonatal cardiomyocytes

คณะผู้วิจัย	สังกัด
1. ผศ. ดร. ภก. ศุภโชค มั่งมูล	คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล
2. ภกญ. พิริยา เหมพฤกษา	คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล
2. ศ. ดร. นพ. นิพนธ์ ฉัตรทิพากร	คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา สำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

EXECUTIVE SUMMARY

Project Title:

Activation of GLP-1 receptor inhibits H_2O_2-induced oxidative stress and apoptosis in rat neonatal cardiomyocytes

การกระตุ้นจีแอลพี-หนึ่งรีเซปเตอร์ยับยั้งภาวะเครียดออกซิเดชันและอะพอพโตซิสที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ในเซลล์กล้ามเนื้อหัวใจของหนูแรกเกิด

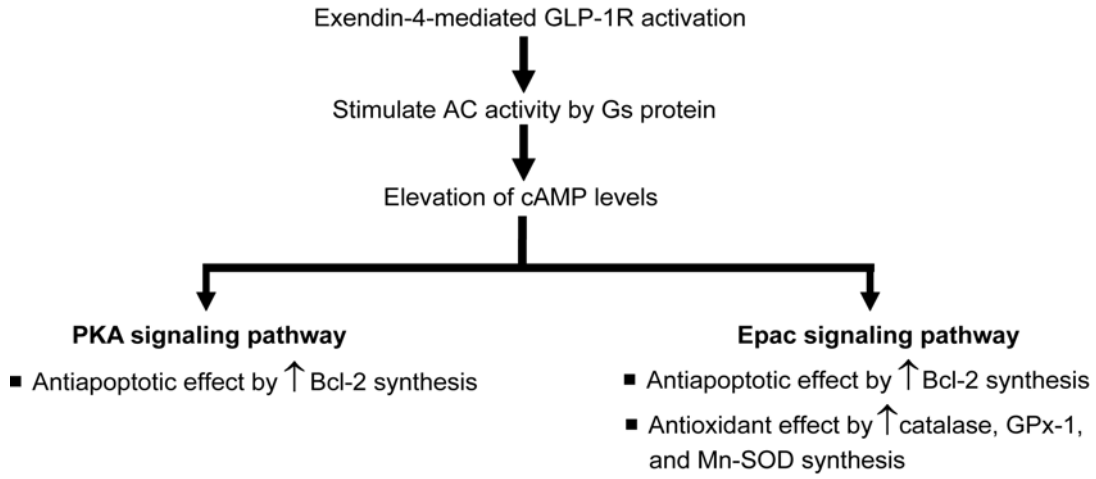
Increased oxidative stress is associated with cardiac injury, apoptosis, cardiac remodeling, and cell death. These conditions accelerate the progression of cardiovascular diseases and increase morbidity and mortality in patients.

Glucagon-like peptide-1 (GLP-1) is the incretin hormone released from intestine that enhances insulin secretion and inhibits glucagon secretion in pancreatic β-cells. GLP-1 also actions as antioxidant and anti-apoptotic effects in β-cells. In addition, several studies have been demonstrated the cardioprotective effects of GLP-1 and its analogues in heart. However, the effects of GLP-1 and its analogues on prevention of oxidative stress and apoptosis during oxidative stress in heart remain unclear.

After GLP-1 receptor agonist binding, GLP-1 receptor can couple with $G_{\alpha s}$ protein, which enhances the activity of adenylyl cyclase and then robust increasing of cAMP levels. cAMP regulates many cell functions through PKA-dependent and Epac-dependent pathways. In pancreatic β-cells, activation of GLP-1 receptor stimulates cAMP formation and provides the anti-apoptotic effects via both PKA-dependent and Epac-dependent manner. Moreover, GLP-1 can induce the oxidative defense gene heme oxygenase-1 (HO-1) in a PKA-dependent way in β-cells. Exendin-4 (GLP-1 - analogues) also inhibits ROS production in an Epac-dependent way in β-cells. Although the mechanisms of action of GLP-1 and its analogues in β-cells are established and well known, the role of GLP-1 cardioprotective signaling in heart is not fully elucidated. We hypothesized that activation of GLP-1 receptor could be involved in reducing of ROS production and inhibiting apoptosis in heart. Whether PKA-dependent pathway, Epac-dependent-pathway, or both, play a role in GLP-1-mediated antioxidant and anti-apoptosis effects in heart remain unknown.

Therefore, in this study, we will investigate the effects of exendin-4 on H_2O_2 induced oxidative stress and apoptosis in cardiac myocytes. We next identify the molecular mechanisms of GLP-1 receptor signaling on antioxidant and anti-apoptotic effects through PKA-dependent and Epac-dependent manner.

In this study, we have identified a new signaling mechanism for GLP1-R-mediated inhibition of oxidative stress and apoptosis in cardiomyocytes (figure below). Stimulation of GLP-1R provides an antioxidation via receptor-dependent Epac signaling pathway, and exerts an antiapoptosis through both PKA- and Epac-dependent manners.



Schematic diagram representing the cardioprotective effects of exendin-4

Exendin-4 binding to GLP-1Rs leads to G_s protein coupling, subsequently stimulate AC activity and generate cAMP, the second messenger. cAMP can binds to and activates both PKA and Epac signaling. Activation of GLP-1R results in the antioxidation by inducing mRNA expression of catalase, GPx-1 and Mn-SOD through Epac-dependent pathway and elicits the antiapoptosis by inducing mRNA expression of Bcl-2 through PKA- and Epac-dependent pathways.

The identification of molecular mechanism of GLP-1 receptor signaling in heart will advance our understanding the cardioprotective effects and these data will be served as scientific and pre-clinical data for further application to use GLP-1 receptor agonists as a therapeutic target for treatment of cardiovascular diseases.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to Prof. Niphon Chattipakorn, my mentor for his meaningful guidance, continuous interest, supervision and encouragement throughout this study.

I am indebted to the Thailand Research Fund (TRF), Office of the Higher Education Commission and Mahidol University for the grant.

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My special thanks are extended to Miss Piriya Hempleuksa for her great assistance in my laboratory.

Supachoke Mangmool

ABSTRACT

Project Code: MRG5580037

Project Title: Activation of GLP-1 receptor inhibits H_2O_2-induced oxidative stress and apoptosis in rat neonatal cardiomyocytes

Investigator: Supachoke Mangmool, Department of Pharmacology, Faculty of Pharmacy, Mahidol University

E-mail Address: supachoke.man@mahidol.ac.th

Project Period: July 2, 2012 to July 1, 2014

Background and purpose:

Stimulation of glucagon-like peptide-1 receptors (GLP-1Rs) has been shown to increase cAMP levels, thus eliciting PKA- and Epac-dependent signal transductions to evoke the cardioprotective actions in the heart. In this study, we focus on the antioxidant and antiapoptotic effects and the underlying mechanisms of exendin-4 against oxidative stress.

Experimental approach:

Cardiomyocytes were isolated from neonatal Sprague Dawley rats (1-2 day old). Intracellular reactive oxygen species (ROS) level was measured using 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA). The mRNA expressions of antioxidant and antiapoptotic genes were assessed by real-time quantitative PCR. Apoptotic cells were detected by TUNEL.

Results:

Exendin-4 attenuated H_2O_2-induced ROS production and increased the mRNA expression of antioxidant enzymes catalase, GPx-1 and Mn-SOD that is dependent on Epac. Additionally, exendin-4 has an antiapoptotic effect by decreasing a number of apoptotic cells and enhancing the production of antiapoptotic protein Bcl-2 which mediated through both PKA- and Epac-dependent pathways.

Conclusion and Implications:

The antioxidant and antiapoptotic effects of exendin-4 play an important role in myocardial protection against oxidative stress. Epac is required for exendin-4-mediated induction of catalase, GPx-1, Mn-SOD and Bcl-2 in neonatal rat cardiomyocytes.

Keywords:

Apoptosis, Epac, Exendin-4, GLP-1 receptor, Oxidative stress, PKA

บทคัดย่อ

รหัสโครงการ: MRG5580037

ชื่อโครงการ: การกระตุ้นจีแอลพี-หนึ่งรีเซปเตอร์ยับยั้งภาวะเครียดออกซิเดชันและอะพอพโตซิสที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ในเซลล์กล้ามเนื้อหัวใจของหนูแรกเกิด

ชื่อนักวิจัยและสถาบัน: ผศ. ดร. ภก. ศุภโชค มั่งมูล ภาควิชาเภสัชวิทยา คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล

E-mail Address: supachoke.man@mahidol.ac.th

ระยะเวลาโครงการ: 2 กรกฎาคม พ.ศ. 2555 ถึง 1 กรกฎาคม พ.ศ. 2557

ความเป็นมาและวัตถุประสงค์:

การกระตุ้นตัวรับชนิดจีแอลพี-หนึ่งส่งผลเพิ่มระดับของไซคลิกเอเอ็มพีและนำไปสู่ฤทธิ์ปกป้องหัวใจโดยส่งสัญญาณภายในเซลล์ผ่านทางโปรตีนพีเคเอและโปรตีนอีแพ็ค การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านอนุมูลอิสระและยับยั้งกระบวนการตายของเซลล์ รวมถึงกลไกการออกฤทธิ์ยับยั้งภาวะเครียดออกซิเดชันของเอ็กเซนดิน-สี่ที่หัวใจ

วิธีการทดลอง:

เซลล์กล้ามเนื้อหัวใจถูกแยกมาจากหัวใจของหนูแรทแรกเกิดที่มีอายุประมาณ 1-2 วัน ระดับของอนุมูลอิสระของสารประกอบออกซิเจนภายในเซลล์วัดได้โดยใช้สารเรืองแสงฟลูออเรสเซ้นต์ชนิดซีเอฟเอช-ดีเอ ระดับของเอ็มอาร์เอนเอของยีนต้านอนุมูลอิสระและยีนต้านกระบวนการตายของเซลล์วัดได้ด้วยวิธีพีซีอาร์ เซลล์ที่มีกระบวนการตายของเซลล์วัดได้ด้วยวิธีทันเนล

ผลการทดลอง:

เอ็กเซนดิน-สี่ออกฤทธิ์ลดการสร้างอนุมูลอิสระของสารประกอบออกซิเจนที่ถูกกระตุ้นด้วยไฮโดรเจนเปอร์ออกไซด์ และยังมีฤทธิ์เพิ่มการสร้างของเอ็มอาร์เอนเอของยีนต้านอนุมูลอิสระ เช่น แคทาเลส จีพีเอ็ก-หนึ่ง และ แมงกานิส-ซุปเปอร์ออกไซด์ดิสมิวเทส ผ่านทางโปรตีนอีแพ็ค ในขณะที่ เอ็กเซนดิน-สี่ออกฤทธิ์ยับยั้งการเพิ่มขึ้นของเซลล์ที่พบภาวะอะพอพโตซิส และยังไปมีฤทธิ์เพิ่มสร้างของเอ็มอาร์เอนเอของยีนต้านอะพอพโตซิส เช่น บีซีแอล-สอง ผ่านทางโปรตีนพีเคเอและโปรตีนอีแพ็ค

สรุปและวิจารณ์ผลการทดลอง:

ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านอะพอพโตซินของเอ็กเซนดิน-สี่มีความสำคัญในการปกป้องหัวใจจากภาวะเครียดออกซิเดชั่น โปรตีนอีแพ็คจำเป็นสำหรับฤทธิ์ของเอ็กเซนดิน-สี่ในการเพิ่มการสร้างของ แคทาเลส จีพีเอ็ก-หนึ่ง แมงกานิส-ซุปเปอร์ออกไซด์ดิสมิวเทส และ บีซีแอล-สอง ในเซลล์กล้ามเนื้อหัวใจของหนูแรกเกิด

คำหลัก: อะพอพโตซิส อีแพ็ค เอ็กเซนดิน-สี่ ตัวรับชนิดจีแอลพี-หนึ่ง ภาวะเครียดออกซิเดชั่น พีเคเอ

CONTENTS

OBJECTIVES

1. To determine the effect of GLP-1 receptor agonist (exendin-4) on inhibition of H_2O_2-induced oxidative stress
2. To determine the effect of exendin-4 on inhibition of H_2O_2-induced apoptosis
3. To identify the molecular mechanism of antioxidant and anti-apoptotic effects of exendin-4 through PKA-dependent and Epac-dependent pathway

INTRODUCTION

Oxidative stress is defined as an imbalance between the antioxidant defenses and the production of reactive oxygen species (ROS), which at high levels can cause cell injury and damage. ROS levels increase in heart failure patients (Maack et al., 2003) and in animal models after myocardial infarction (MI) (Fukui et al., 2001) or dilated cardiomyopathy (Cesselli et al., 2001). ROS also cause myocardial apoptosis which results in cardiac remodeling.

GLP-1R is the G protein-coupled receptor that stimulates insulin secretion and pro-insulin gene transcription (Yu and Jin, 2010). GLP-1Rs are widely expressed in many organs such as pancreas, brain, and heart (Wei and Mojsov, 1995). Several studies demonstrated GLP-1 and its analogues have cardioprotective benefits. For example, infusion with GLP-1 enhanced glucose uptake and preserved left ventricular (LV) function in dogs with dilated cardiomyopathy (Nikolaidis et al., 2004; Nikolaidis et al., 2005). GLP-1 also prolonged survival, associated with improved LV performance when infused in spontaneously hypertensive rats (Poornima et al., 2008). Moreover, GLP-1 was shown to have an anti-apoptotic action mediated by a cAMP/PKA-dependent signaling pathway in pancreatic β-cells (Hui et al., 2003) and endothelial cells (Oeseburg et al., 2010). However, the exact mechanisms underlying cardioprotective effects following GLP-1R stimulation have not fully elucidated in the heart. We hypothesize that the cardioprotective effect might be due to antioxidant and antiapoptotic properties.

After agonist binding, the GLP-1R can couple with α subunit of heterotrimeric $G\alpha_s$ protein, which results in activation of adenylyl cyclase (AC), followed by elevation of cAMP levels (Doyle and Egan, 2007; Drucker et al., 1987). There are at least two pathways induced by cAMP, the PKA-dependent pathway and the Epac (a cAMP-regulated guanine nucleotide exchange factor)-dependent pathway (Holz, 2004; Roscioni et al., 2008). In pancreatic β-cells, the binding of GLP-1 to its receptor stimulates cAMP formation, activation of PKA-dependent and -independent pathways, and a rise in intracellular Ca^{2+} concentration (Fehmann et al., 1995; Holz et al., 1995). Activation of GLP-1R by exendin-4 decreases endogenous ROS production and increase ATP production in diabetic Goto-Kakizaki (GK) rat islets through suppression of Src activation, dependently on Epac (Mukai et al., 2011). Interestingly, GLP-1 and exenatide provide the protection of apoptosis in both a PKA- and Epac-dependent

manner in β-cells (Kwon et al., 2004). Moreover, GLP-1 activates the cAMP response element-binding protein (CREB) transcription factor in a cAMP/protein kinase A (PKA)-dependent manner, and inhibition of the cAMP/PKA pathway abolished the GLP-1 protective effects in endothelial cells (Oeseburg et al., 2010). These data suggested that both PKA- and Epac-dependent pathways play a role on inhibition of oxidative stress and apoptosis in several cells type. However, the specific downstream signaling mediators of PKA- and Epac-dependent pathways those are responsible for cardioprotective effects in the heart are currently unknown. We hypothesized that GLP-1R stimulation involves in reducing the detrimental effects of oxidative stress and inhibit apoptosis in cardiomyocytes. We show that stimulation of GLP-1R provides the cardioprotective effects from oxidative stress through Epac-dependent way by increasing the production of antioxidant enzymes such as glutathione peroxidase-1 (GPx-1), Catalase, and manganese superoxide dismutase (MnSOD). Stimulation of GLP-1R also elicits the inhibitory effect on apoptosis through either PKA- or Epac-dependent manners by enhancing the production of B-cell lymphoma 2 (Bcl-2), an antiapoptotic protein.

MATERIALS AND METHODS

Neonatal rat cardiomyocytes isolation and culture

Animal studies were approved by the Committee for Animal Care and Use of the Faculty of Pharmacy, Mahidol University and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (8^{th} Edition, 2011). Primary cultures of cardiomyocytes were prepared as previously described (Yamashita et al., 1997). Briefly, neonatal Sprague Dawley rats (1-2 day old) were euthanized by decapitation and the hearts were removed. Cardiomyocytes were dispersed from the ventricles by enzymatic digestion using collagenase type A (Roche Applied Science). The cells were preplated at 37 °C for 2-3 h to discard non-myocyte cells. Cardiomyocytes were plated on gelatin-coated 6-well plate at $1x10^6$ cells/well or 12-well plate at $5x10^5$ cells/well. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 5 mM taurine, 1 μg/ml insulin, 1 μg/ml transferring, 10 ng/ml selenium and 1% (v/v) penicillin-streptomycin solution (Gibco). The medium was changed to serum-free DMEM for 24 h before stimulation.

Measurement of intracellular reactive oxygen species (ROS) level

Intracellular ROS level was quantified using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) to estimate intracellular ROS production. Oxidative stress in cardiomyocytes was induced by incubating cells with 100 μM H_2O_2 for 30 min. Oxidative stress should be confirmed by the increasing in ROS production compared with control. Cardiomyocytes were seeded in 12-well plate ($1x10^5$ cells/well) or 35-mm glass bottomed dishes ($1x10^5$ cells/dish) and treated with H_2O_2 with or without prior incubation with 20 nM exendin-4, a GLP-1 receptor agonist (Sigma-Aldrich). The cells were washed once with phosphate-buffered saline (PBS). Thereafter, 10 μM DCFH-DA was added and incubated with the cells at 37 °C in the dark for 30 min. The fluorescence intensity of DCF was quantified by Multi-Detection microplate reader (BioTek Instruments) with λ485 nm excitation wavelength and λ530 nm emission wavelengths. In order to monitor the ROS production in living cells, cardiomyocytes were visualized using single line excitation (488 nm) of a fluorescence microscope (IX 81, Olympus) with a 40X (NA 1.4) objective lens (Olympus).

mRNA expression analysis

The extraction of RNA from cardiomyocytes was performed by using the RNeasy kits (Qiagen). Quantitative mRNA analysis was perform on an Mx 3005p Real Time PCR system (Stratagene) using the KAPA SYBR FAST One-step RT-qPCR kits (KAPA biosystems) following the manufacturer's instructions. The temperature cycle profile for the qPCR reactions was 42 °C for 5 min, then of 95 °C for 5 min and 40 cycles of 95 °C for 3 s, 60 °C for 30 s, and 72 °C for 30 s. Melting curve analysis was also included at one cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s to verify the specificity of the amplified PCR products. Primers sequences are shown in Table 1. Relative mRNA expression levels were evaluated by the comparative cycle threshold (CT) method and normalized to an endogenous reference (GAPDH). The fold increase in mRNA expression of target gene are calculated from $2^{-\Delta\Delta CT}$ as recommended by the manufacturer.

Table 1. Specific primers for PCR amplifications

Gene specific primer		Sequences
GPx-1	Sense	5′-CTCTCCGCGGTGGCACAGT-3′
	Antisense	5′-CCACCACCGGGTCGGACATAC-3′
HO-1	Sense	5′-AGAGTTTCCGCCTCCAACCA-3′
	Antisense	5′-CGGGACTGGGCTAGTTCAGG-3′
Catalase	Sense	5′-GGCAGCTATGTGAGAGCC-3′
	Antisense	5′-CTGACGTCCACCCTGACT-3′
Cu/Zn-SOD	Sense	5′-TCTAAGAAACATGGCGGTCC-3′
	Antisense	5′-CAGTTAGCAGGCCAGCAGAT-3′
Mn-SOD	Sense	5′-CTGAGGAGAGCAGCGGTGGT-3′
	Antisense	5′-CTTGGCCAGCGCCTCGTGGT-3′
Bcl-2	Sense	5′-GCTACGAGTGGGATACTGG-3′
	Antisense	5′-GTGTGCAGATGCCGGTTCA-3′
Bcl-xL	Sense	5′-AGGATACAGCTGGAGTCAG-3′
	Antisense	5′-TCTCCTTGTCTACGCTTTCC-3′
Bax	Sense	5′-CTGCAGAGGATGATTGCTGA-3′
	Antisense	5′-GATCAGCTCGGGCACTTTAG-3′

Epac1	Sense	5′-GGCCC GGAATGCACCTGTTTG-3′
	Antisense	5′-CTGGCCATCATTCGCATCTTCTCA-3′
GAPDH	Sense	5′-CAGTCAAGGCTGAGAATG-3′
	Antisense	5′-CGACATACTCAGCACCAGC-3′

TUNEL Assay

Terminal deoxynucleotidyl transferase (TdT) -mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay was performed utilizing the *in situ* Cell Death Detection Kit (fluorescein), from Roche Applied Sciences according to the manufacturer's instructions. Briefly, cardiac myocytes plated in 35-mm dish were treated with various agents, fixed in freshly prepared 4% paraformaldehyde, and then permeabilized in 0.1% Triton X-100. Cells were then washed twice with phosphate-buffered saline and subjected to the TUNEL reaction at 37 °C in a humidified atmosphere in the dark for 60 min. At the end of the incubation cells were counterstained with DAPI. The fluorescent signal, emitted by fluorescein-labeled dUTP incorporated into fragmented DNA, was visualized using single line excitation (488 nm) of a fluorescence microscope (IX 81, Olympus) with a 20X (NA 1.4) objective lens (Olympus).

siRNA experiments targeting Epac

Three stealth siRNA duplex oligoribonucleotides against rat Epac-1 (NCBI Reference Sequence: NM_021690.1) were synthesized by Invitrogen. The sequences were as follows: (i) 5′-UCACAGAGCAUGACUGGAACCUCUU-3′, position 1892-1916; and (ii) 5′- CAGGCAUCAAGAAUCUCCACAUGUU-3′, position 2515-2539 relative to the start codon. Cardiomyocytes were transfected with 100 nM Epac-1 siRNA or control siRNA using Lipofactamine 2000 reagent (Invitrogen) according to the manufacturer' s instructions. Briefly, 5 μl of transfection reagent was added to 45 μl of Opti-MEM I (Invitrogen), whereas 100 nM of siRNA duplex was mixed with 45 μl of Opti-MEM I. Both solutions were allowed to stand for 5-10 min at room temperature and were then mixed by inversion. After 20 min of incubation, the entire transfection mixture was added to cells in a 6-well plate containing 2 ml of fresh, serum-free DMEM. After cells were incubated for 5 hr at 37 °C, 5% CO_2, transfection media was replaced with the original conditioned medium. All assays were performed at least 2 days after siRNA transfection.

Statistical analysis

Data are presented as mean ± SEM. The statistical analysis was determined using one-way analysis of variance (ANOVA) and Student's t-test, and values of $P < 0.05$ were considered to be significant.

RESULTS

Exendin-4 inhibits on H_2O_2-induced ROS production in rat neonatal cardiomyocyte

In our study, we investigated whether exendin-4 ameliorates H_2O_2-induced ROS production. The levels of intracellular ROS in cardiomyocytes were measured by using a fluorescenct probe, DCFH-DA. After incubation with H_2O_2, the levels of intracellular ROS markedly increased compared to that of control (vehicle) group (Figure 1, left panel). Exposure of 20 nM exendin-4 significantly inhibited H_2O_2-induced ROS production which had effects similar to those of vitamin C (a potent antioxidant) (Figure 1, left panel). Exendin-4 did not decrease ROS production in the presence of exendin-(9-39), GLP-1 receptor antagonist, suggesting that the antioxidant effect of exendin-4 is mediated through GLP-1R-dependent pathway.

We next quantified ROS production in intact cardiomyocytes using the fluorescent microscope. Incubation of cardiomyocytes with H_2O_2 induced a significant increase in the intensity of DCF fluorescence in intact cells of control group, as shown in green color (Figure 1, right panel). Treatment with exendin-4 decreased the intensity of DCF fluorescence suggesting that exendin-4 suppressed H_2O_2-induced ROS production in intact cells (Figure 1, right panel). Taken together, these data demonstrated that exendin-4 has the antioxidant effect by preventing the production of ROS induced by H_2O_2. This effect appears to be predominantly mediated by GLP-1 receptor.

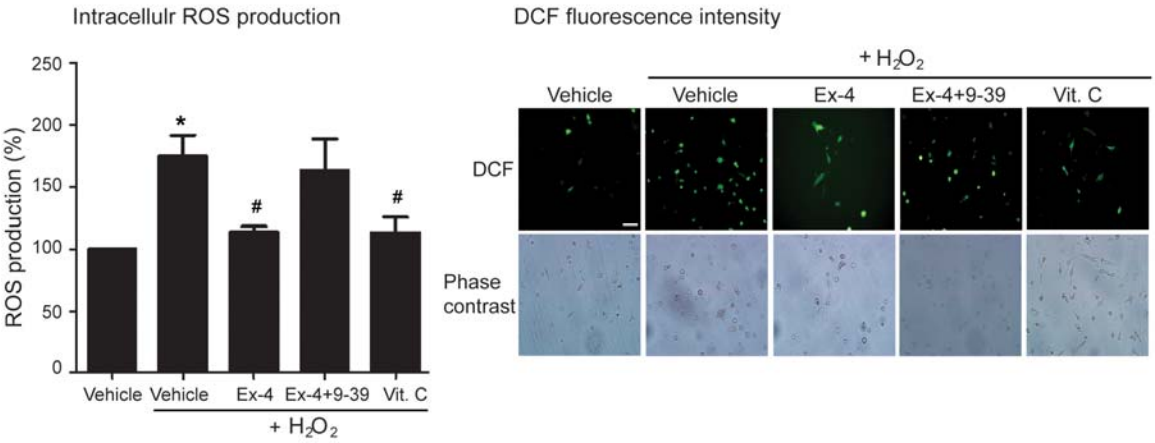

Figure 1. Exendin-4 inhibits H_2O_2-induced ROS production in rat neonatal cardiomyocytes.

Serum-starved cells were pretreated without or with exendin-(9-39) (9-39) before treatment with vehicle (control), 20 nM exendin-4 (Ex-4), or 100 μM vitamin C at 37 °C. Then, cells were incubated with 100 μM H_2O_2. (For intracellular ROS production) The ROS production was quantified, expressed as a percentage of the control activity, and shown as the mean ± SEM (n=4). *P < 0.05 versus the control group; $^{\#}P$ < 0.05 versus the H_2O_2-treated group. (For DCF fluorescence intensity) Cells were washed with PBS and incubated with 10 μM DCFH-DA at 37 °C in the dark for 30 min. Cells were changed with phenol red-free medium and visualized by fluorescence microscopy (n=4).

Antioxidant effect of exendin-4 is cAMP dependent

Stimulation of GLP-1R which couples with $G\alpha_s$ protein leads to the activation of AC, followed by elevation of cAMP levels. GLP-1 was also shown to increase cAMP levels in adult rat cardiomyocytes (Vila Petroff et al., 2001). Therefore, we investigated whether the reduction in ROS production by exendin-4 is dependent on cAMP. The antioxidant effect of exendin-4 can be inhibited by a specific AC inhibitor, ddA (Figure 2), confirming that the antioxidant effect of exendin-4 is cAMP dependent.

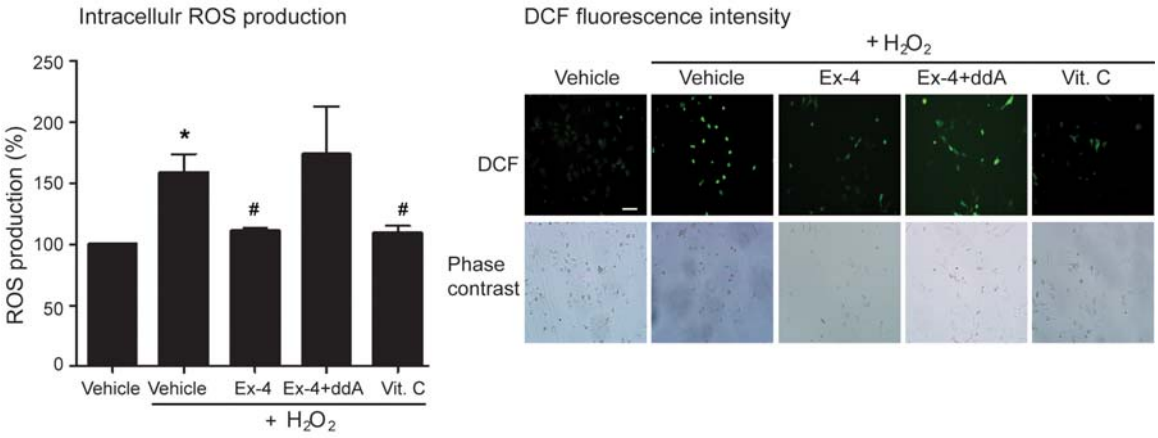

Figure 2. Exendin-4 inhibits H_2O_2-induced ROS production is dependent of cAMP

Serum-starved cells were pretreated without or with 1 μM ddA before treatment with vehicle (control), 20 nM exendin-4 (Ex-4), or 100 μM vitamin C at 37 °C. Then, cells were incubated with 100 μM H_2O_2. (For intracellular ROS production) The ROS production was quantified, expressed as a percentage of the control activity, and shown as the mean ± SEM (n=4). *P < 0.05 versus the control group; $^{\#}P$ < 0.05 versus the H_2O_2-treated group. (For DCF fluorescence intensity) Cells were washed with PBS and incubated with 10 μM DCFH-DA at 37 °C in the dark for 30 min. Cells were changed with phenol red-free medium and visualized by fluorescence microscopy (n=4).

Exendin-4-mediated inhibition of oxidative stress in an Epac-dependent, PKA-independent manner

There are at least two pathways induced by cAMP, the PKA-dependent pathway and the Epac-dependent pathway (Drucker, 2006; Holz, 2004). We next determine whether PKA-dependent pathway, Epac-dependent pathway, or both, play a role in exendin-4-mediated inhibition of oxidative stress. As shown in Figure 3, a specific PKA inhibitor (PKI) has no effect on exendin-4 mediated inhibition of ROS production, indicating that the antioxidant effect of exendin-4 is PKA independent.

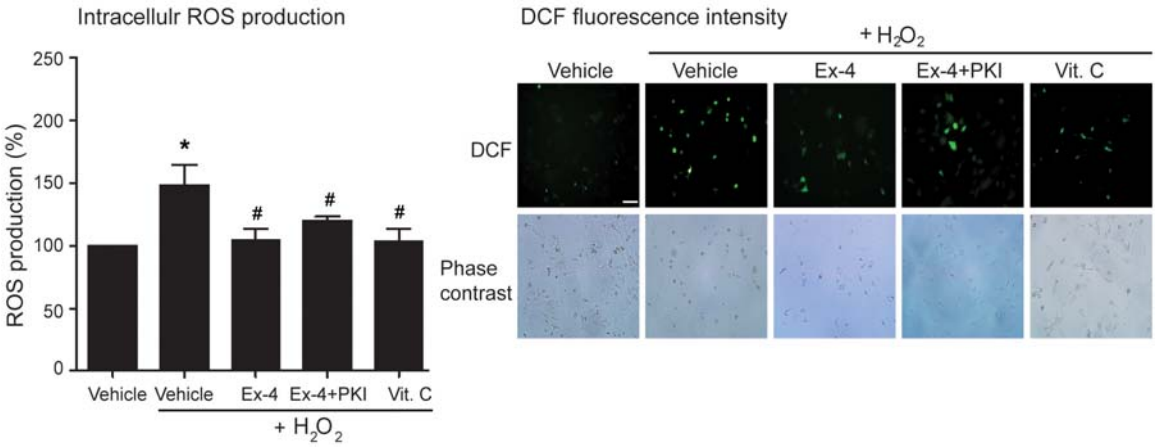

Figure 3. Exendin-4 inhibits H_2O_2-induced ROS production is independent of PKA

Serum-starved cells were pretreated without or with 10 μM PKI before treatment with vehicle (control), 20 nM exendin-4 (Ex-4), or 100 μM vitamin C at 37 °C. Then, cells were incubated with 100 μM H_2O_2. (For intracellular ROS production) The ROS production was quantified, expressed as a percentage of the control activity, and shown as the mean ± SEM (n=4). *P < 0.05 versus the control group; #P < 0.05 versus the H_2O_2-treated group. (For DCF fluorescence intensity) Cells were washed with PBS and incubated with 10 μM DCFH-DA at 37 °C in the dark for 30 min. Cells were changed with phenol red-free medium and visualized by fluorescence microscopy (n=4).

Epac serves as an important downstream effector of cAMP. Therefore, we next tested whether Epac is required for exendin-4-mediated antioxidant effect in cardiomyocytes. We used siRNA to specifically target Epac and examined the contribution of Epac on the antioxidant effect of exendin-4. Epac siRNAs corresponding to the rat Epac1 gene were synthesized and transfected into cardiomyocytes to test their ability to deplete Epac1. The Epac 1 mRNA expression levels in siRNA-transfected cells were analyzed by RTq-PCR. As shown in figure 4, two unique siRNAs suppressed

the expression of endogenous Epac1 compared with nonsilencing, control siRNA-transfected cells. The degrees of depletion by siRNAs were from 40 to 50% (Figure 4).

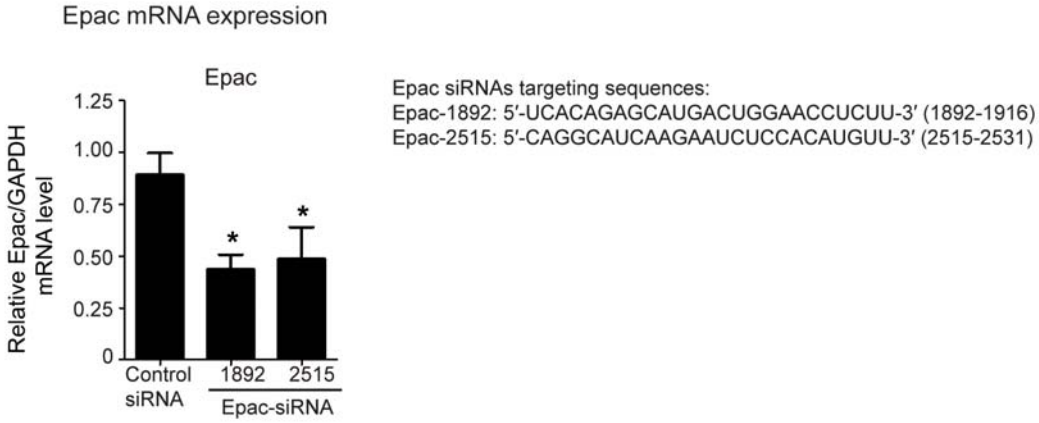

Figure 4. siRNA-mediated depletion of Epac endogenously expressed in cardiomyocytes

Cardiomyocytes were transfected with 100 nM Epac siRNA or control siRNA. The fold increase in mRNA expression of target gene are calculated from $2^{-\Delta\Delta CT}$. The mRNA levels were quantified and shown as the mean ± SEM (n=4). * $P < 0.05$ versus the control siRNA group.

After GLP-1R stimulation with exendin-4, the level of ROS production significantly decreased in control-siRNA transfected cells compared to that of H_2O_2-treated group (Figure 5, left panel). In contrast, exendin-4 did not decrease ROS production in Epac-siRNA transfected cells (Figure 5, left panel). Similar results were obtained from intact cardiomyocytes using the fluorescent microscope (Figure 5, right panel). Collectively, these results demonstrated that Epac is required for exendin-4-mediated inhibition of oxidative stress.

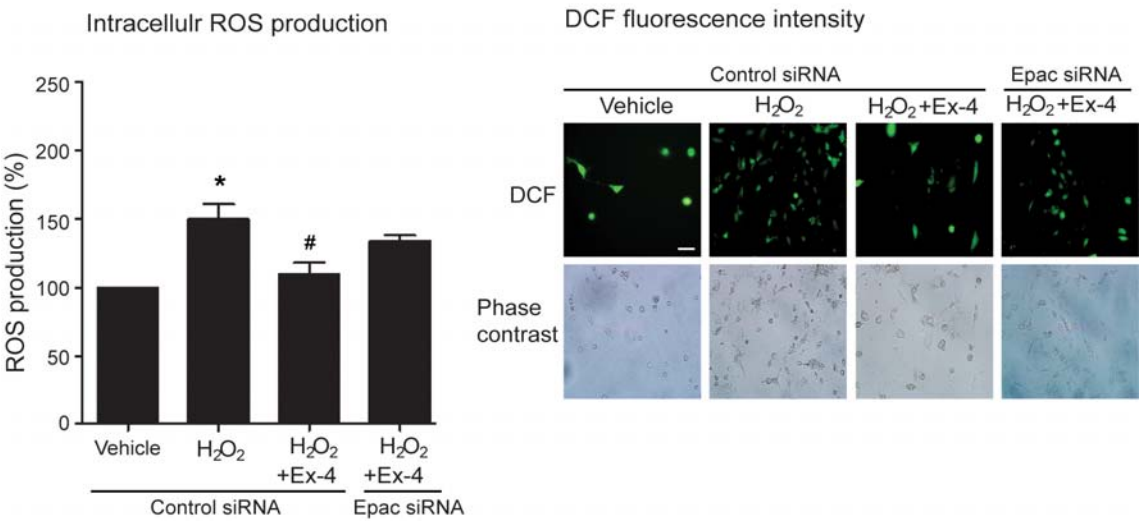

Figure 5. Antioxidant effects of exendin-4 are required Epac

Cardiomyocytes were transfected with either Epac siRNA or control siRNA 24 hr before experiment. The siRNA-transfected cells were treated with vehicle (control), 20 nM exendin-4, or 100 μM vitamin C at 37 °C. Then, cells were incubated with 100 μM H_2O_2. (For intracellular ROS production) The ROS production was quantified, expressed as a percentage of the control activity, and shown as the mean ± SEM (n=4). *P < 0.05 versus the control group; $^{\#}P$ < 0.05 versus the H_2O_2-treated group. (For DCF fluorescence intensity) Cells were washed with PBS and incubated with 10 μM DCFH-DA at 37 °C in the dark for 30 min. Cells were changed with phenol red-free medium and visualized by fluorescence microscopy (n=4).

Exendin-4-induced expression of antioxidant enzymes is Epac dependent

Antioxidant enzymes (e.g., GPx-1, Catalase, Mn-SOD, CuZn-SOD and heme oxygenase-1, HO-1) are the major components of antioxidant signaling cascade in many cell types. It has been shown that treatment with exendin-4 suppressed hepatic oxidative stress by activating many antioxidant enzymes such as catalase, SOD and GPx in the liver of diabetic mice (Gezginci-Oktayoglu et al., 2011). Moreover, stimulation of GLP-1 receptor also induced the production of HO-1 and NQO1, antioxidant genes, in endothelial cells (Oeseburg et al., 2010). Consistent with these studies, we showed that treatment with exendin-4 for 24 hr significantly elevated the mRNA levels of GPx-1, catalase, and Mn-SOD (Figure 6, upper panel). In addition, the mRNA levels of HO-1 and CuZn-SOD tend to increase when treated with exendin-4 (Figure 6, lower panel), indicating that stimulation of GLP-1R appears to play an important role in the prevention of oxidative stress by enhancing the production of antioxidant enzymes.

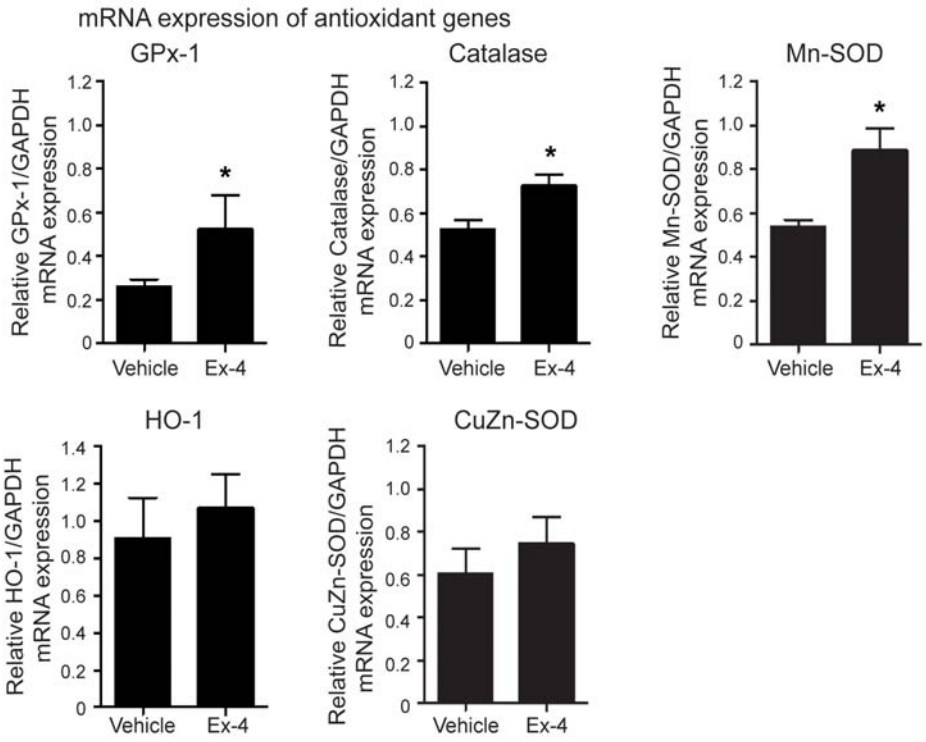

Figure 6. Exendin-4 induces mRNA expression of antioxidant genes

GPx-1, Catalase, HO-1, CuZn-SOD, and MnSOD mRNA expression levels in cardiomyocytes were assayed by RT-qPCR. Serum-starved cells were stimulated with 20 nM exendin-4 for 24 h at 37 °C. Serum-starved cells were pretreated without or with 10 μM PKI before treatment with vehicle (control) or 20 nM exendin-4 for 24 h at 37 °C. After treatment, the total RNA was extracted from cells and the mRNA expression was analyzed using specific primers for antioxidant genes. The fold increase in mRNA expression of target gene are calculated from $2^{-\Delta\Delta CT}$. The mRNA levels were quantified, expressed as fold increase over vehicle (non-treated group) and shown as the mean ± SEM (n=4). **P* < 0.05 versus vehicle.

As we showed that exendin-4-mediated inhibition of ROS production in an Epac-dependent, PKA-independent manner, we further investigated whether the increase in mRNA expression of antioxidant enzymes by exendin-4 is also dependent on Epac, but not PKA. The increased mRNA expressions of GPx-1, catalase, and Mn-SOD by exendin-4 were not affected by pretreatment with PKI, specific PKA inhibitor (Figure 7). Moreover, exendin-4-mediated induction of GPx-1, catalase, and Mn-SOD mRNA expression was significantly enhanced in control siRNA-transfected cells, but was unable to increase mRNA levels of these antioxidant genes in the presence of siRNA targeting Epac1 (Figure 7). Taken together, these results confirm that stimulation of GLP-1R with exendin-4 increases expression of GPx-1, catalase, and Mn-SOD in an Epac-dependent, PKA-independent manner.

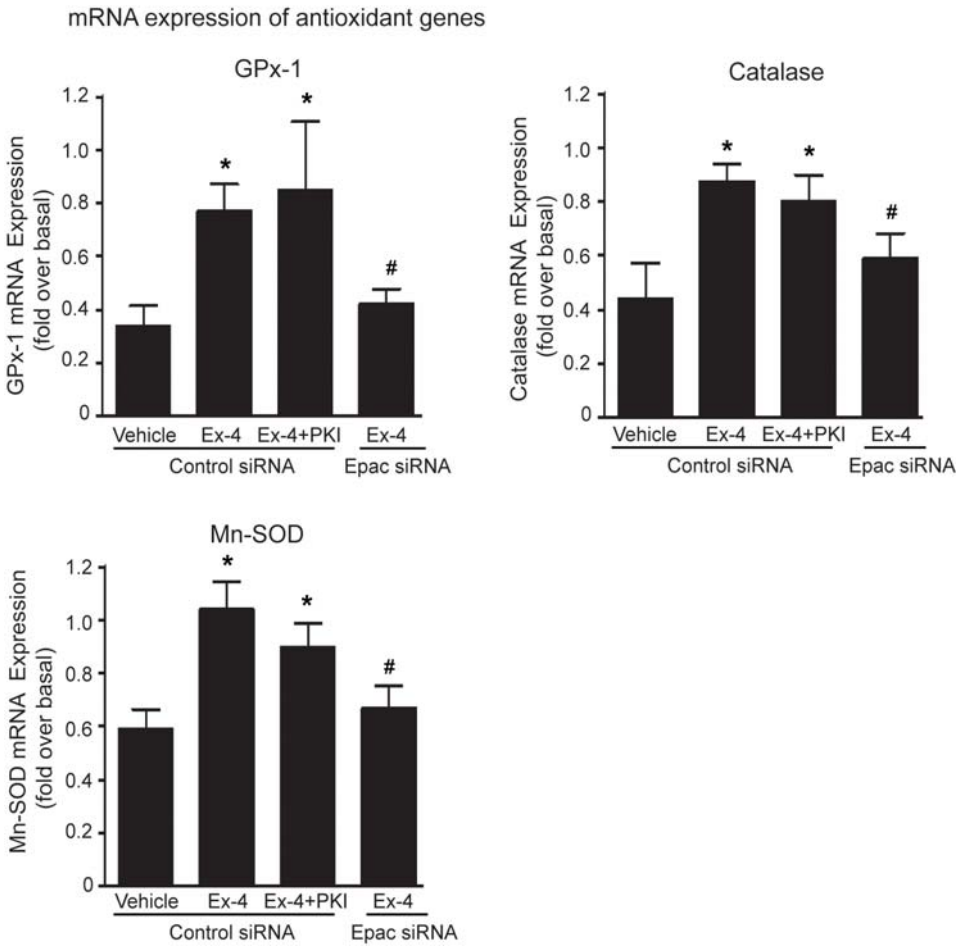

Figure 7. Exendin-4-induced expression of antioxidant enzymes is dependent of Epac

Cardiomyocytes were transfected with either Epac siRNA or control siRNA. Serum-starved cells were pretreated without or with PKI before treatment with vehicle (control) or 20 nM exendin-4 for 24 h at 37 °C. After treatment, the total RNA was extracted from cells and the mRNA expression was analyzed using specific primers for antioxidant genes. The fold increase in mRNA expression of target gene are calculated from $2^{-\Delta\Delta CT}$. The mRNA levels were quantified, expressed as fold increase over vehicle (non-treated group) and shown as the mean ± SEM (n=4). $^{*}P < 0.05$ versus vehicle; $^{\#}P < 0.05$ versus the Ex-4-treated control siRNA group.

Exendin-4 inhibits on H_2O_2-induced apoptosis in rat neonatal cardiomyocytes

We next investigated whether stimulation of GLP-1R prevents H_2O_2-induced apoptosis in cardiomyocytes. We used a TUNEL assay that detects apoptotic cells. Exposure to H_2O_2 for 24 h leads to an increase in a number of apoptotic cells as shown in the green color (Figure 8). Interestingly, stimulation of GLP-1R by exendin-4 protected cardiomyocytes from H_2O_2-induced apoptosis compared to that of control group. Exendin-4 did not exert the antiapoptotic effects in the presence of exendin-(9-39), GLP-1R antagonist (Figure 8). These results demonstrated the antiapoptotic effect of exendin-4 is mediated by receptor activation.

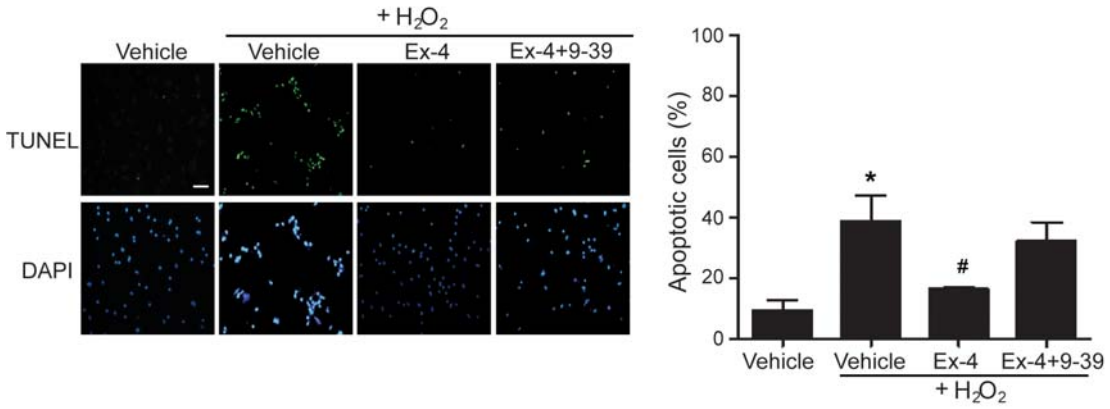

Figure 8. Exendin-4 inhibits H_2O_2-induced apoptosis in rat neonatal cardiomyocytes

Serum-starved cells were pretreated without or with exendin-(9-39) (9-39) before treatment with vehicle (control) or 20 nM exendin-4 (Ex-4) at 37 °C. Then, cells were incubated with 200 nM H_2O_2 for 24 h. Cells were washed with PBS, incubated with TUNEL solution at 37 °C in the dark for 1 h, and then incubated with DAPI for 10 min. The medium was changed with PBS and visualized the cells by fluorescence microscopy. The values are expressed as the percentage of apoptotic cells over non-stimulated cells (vehicle) and shown as the mean ± SEM (n=4). $^{*}P < 0.05$ versus the control (vehicle) group; $^{\#}P < 0.05$ versus the H_2O_2-treated group.

Antiapoptotic effects of exendin-4 is cAMP dependent

Most of the activities of exendin-4 initiated by binding of exendin-4 to its receptor are mediated by an elevation of cAMP levels. We therefore determined whether cAMP is involved in the regulation of the antiapoptotic action of exendin-4. The antiapoptotic activity of exendin-4 can be inhibited by blocking cAMP synthesis with ddA (AC inhibitor) (Figure 9). Thus, GLP-1R activation delivers a potent antiapoptotic effect through cAMP signaling.

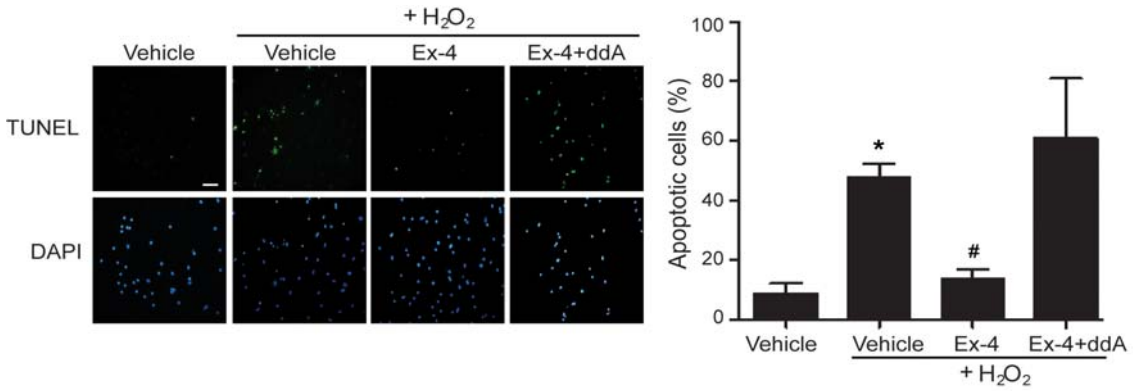

Figure 9. Exendin-4 inhibits H_2O_2-induced apoptosis is dependent of cAMP

Serum-starved cells were pretreated without or with 1 μM ddA before treatment with vehicle (control) or 20 nM exendin-4 (Ex-4) at 37 °C. Then, cells were incubated with 200 nM H_2O_2 for 24 h. Cells were washed with PBS, incubated with TUNEL solution at 37 °C in the dark for 1 h, and then incubated with DAPI for 10 min. The medium was changed with PBS and visualized the cells by fluorescence microscopy. The values are expressed as the percentage of apoptotic cells over non-stimulated cells (vehicle) and shown as the mean ± SEM (n=4). $^{*}P < 0.05$ versus the control (vehicle) group; $^{\#}P < 0.05$ versus the H_2O_2-treated group.

Exendin-4-mediated inhibition of apoptosis is both PKA and Epac dependent

There are at least two pathways induced by cAMP, the PKA-dependent pathway and the Epac-dependent pathway (Drucker, 2006; Holz, 2004). To investigate the potential role of PKA and Epac in exendin-4-mediated antiapoptotic effect, we used PKI, a specific PKA inhibitor and Epac-siRNA to deplete the expression of Epac1. Exendin-4-mediated antiapoptosis can be inhibited by PKI (Figure 10), confirming that stimulation of GLP-1R inhibits H_2O_2-induced apoptosis in a PKA-dependent manner.

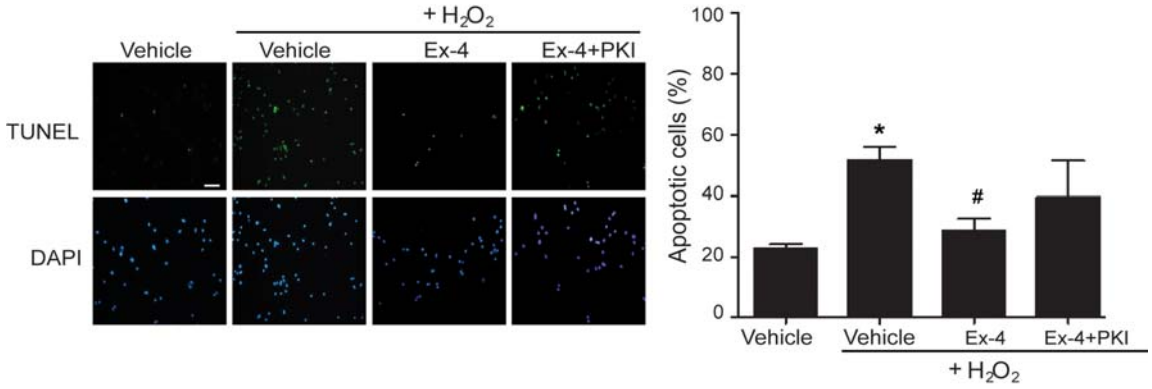

Figure 10. PKA is required for the antiapoptotic effect of exendin-4

Serum-starved cells were pretreated without or with 10 μM PKI before treatment with vehicle (control) or 20 nM exendin-4 (Ex-4) at 37 °C. Then, cells were incubated with 200 nM H_2O_2 for 24 h. Cells were washed with PBS, incubated with TUNEL solution at 37 °C in the dark for 1 h, and then incubated with DAPI for 10 min. The medium was changed with PBS and visualized the cells by fluorescence microscopy. The values are expressed as the percentage of apoptotic cells over non-stimulated cells (vehicle) and shown as the mean ± SEM (n=4). $^{*}P < 0.05$ versus the control (vehicle) group; $^{\#}P < 0.05$ versus the H_2O_2-treated group.

Stimulation of GLP-1R by exendin-4 markedly decreased a number of apoptotic cells in control siRNA-transfected cells (Figure 11). In contrast, apoptotic cells were significantly increased in the presence of siRNA targeting Epac. Treatment with exendin-4 had no inhibitory effect on H_2O_2-induced apoptosis when blocking both Epac and PKA activities (Figure 11). Collectively, these results showed that both Epac and PKA are required for the antiapoptotic effect of exendin-4.

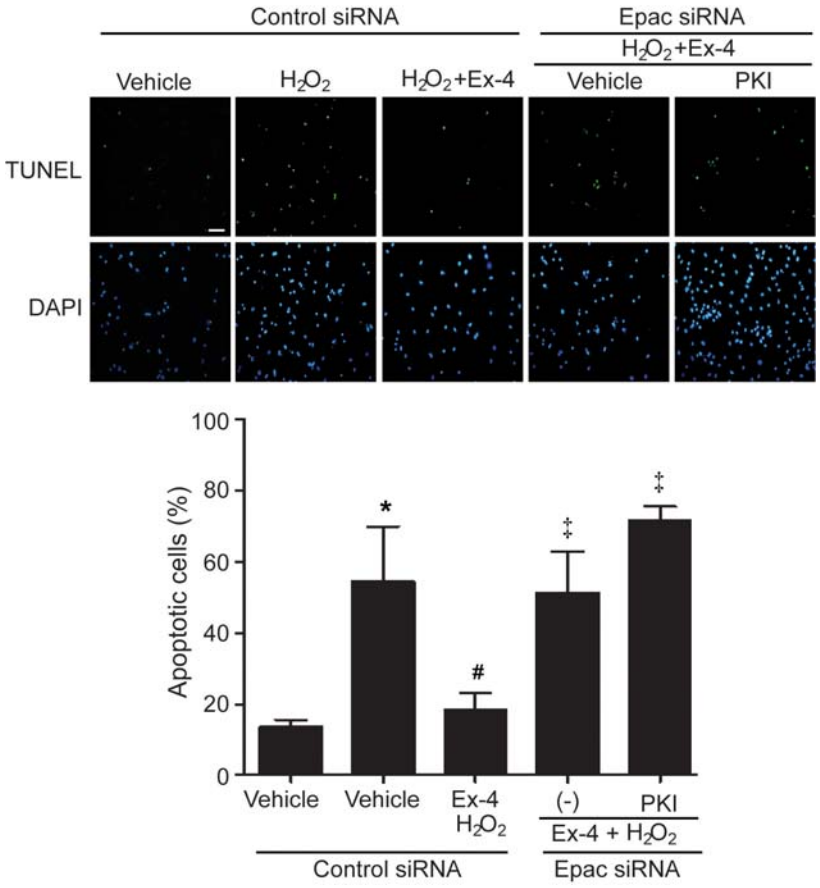

Figure 11. PKA is required for the antiapoptotic effect of exendin-4

Cells were transfected with either Epac siRNA or control siRNA before experiment. The siRNA-transfected cells were pretreated without or with 10 μM PKI, then treated with vehicle (control) or 20 nM exendin-4 at 37 °C. Next, cells were incubated with 200 nM H_2O_2 for 24 h. Cells were washed with PBS and incubated with TUNEL solution at 37 °C in the dark for 1 h then incubated with DAPI for 10 min. The medium was changed with PBS and visualized the cells by fluorescence microscopy. The values are expressed as the percentage of apoptotic cells over non-stimulated cells (vehicle) and shown as the mean ± SEM (n=4). $^{*}P < 0.05$ versus the control (vehicle) group; $^{\#}P < 0.05$ versus the H_2O_2-treated group. $^{‡}P < 0.05$ versus the Ex-4+H_2O_2-treated group

Exendin-4 induces mRNA expression of antiapoptotic proteins is dependent of PKA- and Epac- signaling

Several antiapoptotic proteins such as Bcl-2 and Bcl-xL and pro-apoptotic proteins such as Bax and Bad are the major components that play a role on apoptotic signaling cascade (Lee and Gustafsson, 2009). To examine the effects of exendin-4 on the production of these proteins, we measure the mRNA expression levels of them after treatment with exendin-4 in neonatal rat cardiomyocytes. Treatment with exendin-4 for

24 hr significantly elevated the mRNA levels of Bcl-2, whereas the Bcl-xL mRNA expression tended to increase compared to that of vehicle (control) (Figure 12). These data are in concordance with a recent study reporting the upregulation of Bcl-2 expression after treatment with exendin-4 in mouse pancreatic β-cells (MIN6) (Wei et al., 2012). However, exendin-4 did not affect on the induction of pro-apoptotic genes, Bax and Bad in cardiomyocytes (Figure 12). These results reported that activation of GLP-1R plays an important role in the prevention of apoptosis by enhancing the production of antiapoptotic proteins.

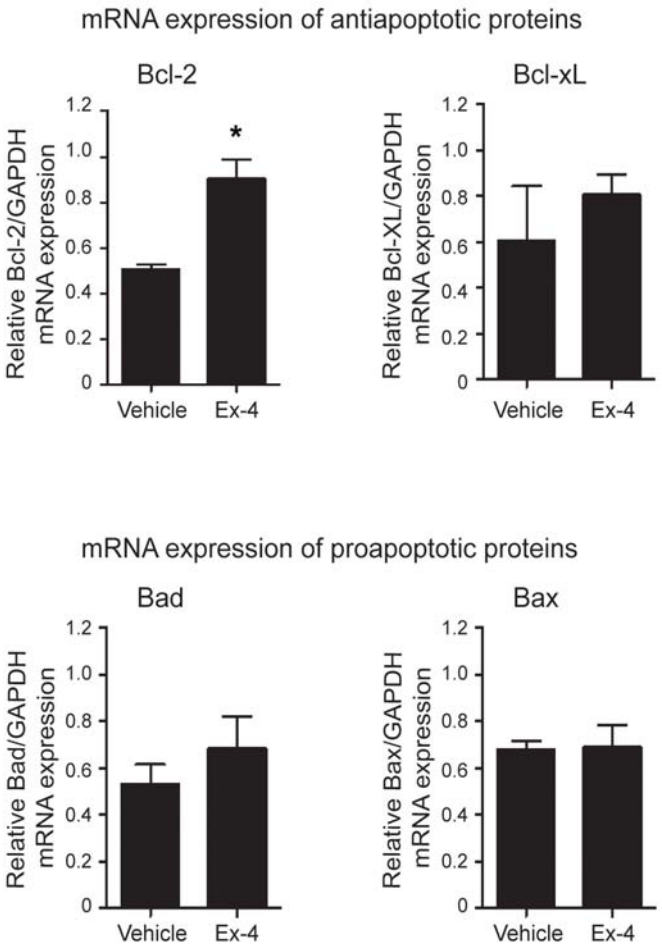

Figure 12. Exendin-4 increases the mRNA expression of anti-apoptotic genes

Serum-starved cells were stimulated with 20 nM exendin-4 for 24 h at 37 °C. After treatment, the total RNA was extracted from cells and the mRNA expression was analyzed using specific primers for Bcl-2, Bcl-xL, Bax and Bad genes. The fold increase in mRNA expression of target gene are calculated from $2^{-\Delta\Delta CT}$. The mRNA levels were quantified, expressed as fold increase over vehicle (non-treated group) and shown as the mean ± SEM (n=4). *P < 0.05 versus vehicle.

It has been shown that GLP-1 and exenatide prevent plamitate-mediated apoptosis in both PKA- and Epac-dependent pathways in β-cells (Kwon et al., 2004). Moreover, GLP-1 also inhibits apoptosis via cAMP/PKA-dependent pathway in insulin-secretion cells (Hui et al., 2003). However, the mechanism by which signaling pathway mediates exendin-4-induced mRNA expression of Bcl-2 in cardiomyocytes is unknown. Blockade of PKA activity by using PKI attenuated the exendin-4-induced Bcl-2 synthesis (Figure 13). Similarly, depletion of endogenous Epac1 by Epac siRNA also decreased Bcl-2 mRNA expression inducing by exendin-4. In addition, blockade of both PKA and Epac completely inhibited exednin-4-induced Bcl-2 mRNA expression (Figure 13). These results suggested that exendin-4 induced Bcl-2 mRNA production via both PKA- and Epac-dependent pathways in cardiomyocytes.

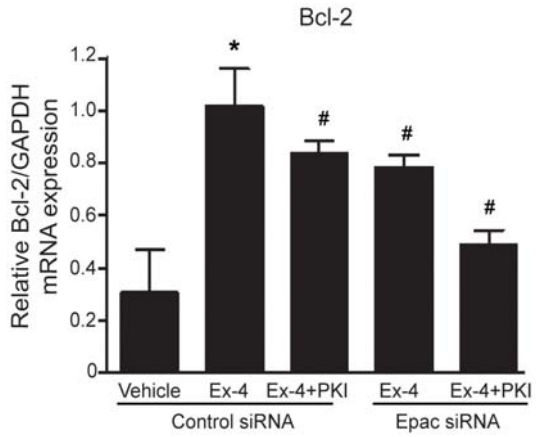

Figure 13. Exendin-4 increases Bcl-2 mRNA expression through PKA- and Epac-dependent manners

Cardiomyocytes were transfected with either Epac siRNA or control siRNA. Serum-starved cells were pretreated without or with 10 μM PKI before treatment with vehicle (control) or 20 nM exendin-4 for 24 h at 37 °C. After treatment, the total RNA was extracted from cells and the mRNA expression was analyzed using specific primers for Bcl-2 gene. The fold increase in mRNA expression of Bcl-2 are calculated from $2^{-\Delta\Delta CT}$. The mRNA levels were quantified, expressed as fold increase over vehicle (non-treated group) and shown as the mean ± SEM (n=4). $^{*}P < 0.05$ versus vehicle; $^{\#}P < 0.05$ versus Ex-4-treated Epac siRNA group.

DISCUSSION

In this study, we provide a new molecular mechanism of GLP-1R signaling on inhibition of oxidative stress and apoptosis in cardiomyocytes. We demonstrate that exendin-4, a GLP-1R agonist, has an antioxidant effect by suppressing ROS production and inducing the mRNA expression of antioxidant enzymes such as GPx-1, catalase and Mn-SOD. The antioxidant effect of exendin-4 is dependent on Epac. Furthermore, exendin-4 exerts an antiapoptotic effect by decreasing a number of apoptotic cells and enhancing the mRNA expression of Bcl-2, antiapoptotic protein, which mediated through both PKA- and Epac-dependent pathways.

Oxidative stress has long been implicated in clinical and experimental heart diseases. Oxidative stress refers to an imbalance between the production of ROS (e.g., superoxide and hydrogen peroxide) and endogenous antioxidant defense mechanisms, which high level of ROS cause cell damage and death through modifications of proteins, lipids, and DNA/RNA . Cardiomyocyte apoptosis occurs in hypertrophied, ischemic, and failing hearts and may contribute to the development and progression of cardiac dysfunction and heart failure (Cesselli et al., 2001; Lee and Gustafsson, 2009). In cardiomyocytes, a higher level of H_2O_2 activates the JNK and p38 MAPKs and induces apoptosis (Kwon et al., 2003). These data implicated that increasing in ROS level during oxidative stress can cause apoptosis and cell damage in the heart.

GLP-1 is one of the incretin hormones released from the intestine in response to food ingestion that enhances glucose-induced insulin secretion from β-cells (Baggio and Drucker, 2007; Holst, 2007). Therapeutic use of native GLP-1 is limited by its rapid degradation by dipeptidyl peptidase-4 (DPP-4) into inactive metabolite. Several GLP-1 analogues (e.g., exendin-4, exenatide, and liraglutide) are resistant to cleavage by DPP-4 and have similar properties with native GLP-1 that can bind to and activate GLP-1R (Nielsen et al., 2004). The binding of GLP-1 with its receptor has been shown the protective effects on pancreatic β-cells which promote β-cell proliferation and inhibit β-cell apoptosis (Doyle and Egan, 2007). GLP-1Rs are also expressed in the heart (Okerson and Chilton, 2010), and several studies suggested GLP-1 and its analogues have cardioprotective actions and play an important role in regulation of heart functions (Fields et al., 2009). For example, *in vivo* experiments, infusion with GLP-1 resulted in an elevation of blood pressure and heart rate (Barragan et al., 1994; Yamamoto et al., 2002). Studies using GLP-1R knockout mice have also suggested a role for the GLP-1R

in the control of cardiac structure and function (Gros et al., 2003). Furthermore, in patients admitted due to acute myocardial infarction for primary coronary angioplasty, ejection fraction and other functional indices improved following a 72 hour-infusion of GLP-1 (Nikolaidis et al., 2004). Administration of exendin-4 reduced infarct size and improved mechanical performance in an isolated rat heart (Sonne et al., 2008). Although many studies have shown the cardioproctive effects of GLP-1 in many conditions of *in vitro* and *in vivo* animal experiments and in clinical trials, however, the exact molecular mechanisms of cardioprotection of GLP-1 and its analogues are unclear.

Agonist binding to the GLP-1R, a G_s protein–coupled receptor, induces the activation of AC and elevation of intracellular cAMP levels, which elicits PKA-dependent signal transduction (Doyle and Egan, 2007; Drucker, 2006). Moreover, Epac has been shown to be a novel cAMP sensor in the PKA-independent pathway (Roscioni et al., 2008). Epac exhibits guanine nucleotide exchange factor activity toward Rap1 (de Rooij et al., 1998). GLP-1 had a direct protective effect on oxidative stress (H_2O_2)-induced senescence and was able to attenuate oxidative stress-induced DNA damage and cellular senescence in human umbilical vein endothelial cells (HUVECs) (Oeseburg et al., 2010). The previous studies in rodent cardiomyocytes showed that GLP-1 or liraglutide (GLP-1R agonist) significantly increased intracellular cAMP, which was abolished by the GLP-1R antagonist exendin(9-39) (Noyan-Ashraf et al., 2009; Vila Petroff et al., 2001). Similar to GLP-1 treatment, our study showed that exendin-4 strongly attenuated ROS production in cardiomyocytes. Pretreatment with exendin(9-39) completely abolished the protective effect of exendin-4. These data provide evidence that antioxidative effects of both GLP-1 and exendin-4 are GLP-1 receptor dependent.

In pancreatic β-cells, GLP-1R activation stimulates AC activity which leads to elevation of cAMP, activation of PKA-dependent and –independent pathways, and a rise in intracellular Ca^{2+} concentration (Fehmann et al., 1995; Holz et al., 1995). A few observations suggested that GLP-1 and its analogues may also exert their effects in PKA-independent manners. For example, GLP-1-induced cytosolic free Ca^{2+} elevation was shown to be mediated independently of PKA (Bode et al., 1999). Furthermore, GLP-1 stimulated pro-insulin I gene transcription was also shown to be mediated via a PKA-independent manner (Skoglund et al., 2000). Exendin-4 also inhibits ROS production in an Epac-dependent way and the effect of GLP-1 signaling, which suppresses ROS production, was found to be independent of PKA in diabetic Goto-

Kakizaki rat iselts (Mukai et al., 2011). Consistent with these previous studies, we show in this study that stimulation of GLP-1R with exendin-4 inhibits ROS production through an Epac-dependent, PKA-independent pathway in cardiomyocytes. Epac has been identified as a new target of cAMP signal. Epac is a guanine nucleotide exchange protein and may be involved the Ras-realated small GTPase Rap1 and Rap2 pathway.

There are two subtypes of Epac, Epac1 and Epac2. The different of each subtypes depend on the cAMP domain binding (Bos, 2006). Epac 2 is mainly found in pancreas and activation of Epac pathway can involved insulin secretion (Holz, 2004). Epac1 is mainly expressed in human heart and is increased in heart failure (Metrich et al., 2008). Previous studies showed that activation Epac1 protects cardio hypertrophy by inhibiting ERK5 pathway (Dodge-Kafka et al., 2005). Epac1 regulates the contraction of heart muscle cells by controlling gap junction in cardiomyocytes (Somekawa et al., 2005). Thus, these data simplify that the activation of Epac1 signaling seems to play several roles in the heart. Our data also demonstrated that Epac1 is necessary for exendin-4-mediated antioxidation in cardiomyocytes. It has been reported that activation of β_2-ARs can stimulate a cAMP-Epac-RapGTP-PLCε pathway (Schmidt et al., 2001). Nonetheless, it remains an unsolved question whether the mechanism by which Epac1 mediates antioxidation after GLP-1R activation.

Interestingly, it has been reported that the antioxidant effect of GLP-1 might be due to the induction of antioxidant genes, HO-1 and NQO1 (NADPH quinone oxidoreductase 1) in endothelial cells (Oeseburg et al., 2010). In addition, liraglutide also induced mRNA and protein expression of HO-1 in mice heart (Noyan-Ashraf et al., 2009). The activation of HO-1 through GLP-1R (Yin et al., 2010) reduces fibrosis and LV remodeling and restores LV function after MI (Liu et al., 2006). HO-1 acts via induction of nuclear factor-E2-related factor 2 (Nrf2) gene expression and nuclear translocation and subsequent stimulation of Akt (Piantadosi et al., 2008). In addition, our study showed that stimulation of GLP-1R with exendin-4 tends to induced mRNA expression of HO-1 in cardiomyocytes. We also demonstrated that exendin-4 is able to induce the mRNA expression of other antioxidant enzymes such as Mn-SOD, but not CuZn-SOD. However, unlike the study by Mukai et al. (Mukai et al., 2011) in which Mn-SOD activity was not increased after exendin-4 treatment in islet β-cells. An enzymatic assay revealed that Mn-SOD activity in GK islets was similar to that in Wistar islets and that it was not affected by exendin-4, indicating that regulation of MnSOD activity does not play a role in the suppressive effects of ROS production by exendin-4 (Mukai et al.,

2011). Further evaluation of the exendin-4-induced Mn-SOD activity in cardiomyocytes is required. Previous study has shown that treatment with exendin-4 suppressed hepatic oxidative stress by activating many antioxidant enzymes such as catalase, SOD and GPx in the liver of diabetic mice (Gezginci-Oktayoglu et al., 2011). Our data are consistent with this recent study showing that cardiomyocyte catalase and GPx-1 mRNA levels were increased after treatment with exendin-4. These data demonstrate that stimulation of GLP-1R might exert the cardioprotective effect through stimulating oxidative defense system in cardiomyocytes. Our results also showed that exendin-4-mediated induction of mRNA expressions of catalase and Mn-SOD, antioxidant enzymes, was not affected by PKA inhibitor whereas blockade of Epac strongly inhibited this effect. These results demonstrated that the induction of these antioxidant enzymes by exendin-4 is mediated through GLP-1R/cAMP/Epac pathway.

To date, the antiapoptotic effects of GLP-1 have been examined most extensively in pancreas. GLP-1 has an antiapoptotic effect on β-cells during oxidative stress probably via blocking the c-Jun-N-terminal kinase (JNK) and glycogen synthase kinase 3β (GSK3β) mediated apoptotic pathway (Kim et al., 2010). Liraglutide has also been shown to inhibit apoptosis in primary neonatal rat islets (Bregenholt et al., 2005). In addition to pancreatic cells, activation of GLP-1R enhanced neuronal survival in cellular and animal models of neuronal toxicity (During et al., 2003; Perry et al., 2002). Several studies also showed the antiapoptotic effect of GLP-1 in the heart. For example, treatment of mouse neonatal cardiomyocytes with exendin-4 resulted in improved cell viability in response to ischemia-reperfusion (I/R) injury (Ban et al., 2010). They suggested that GLP-1R-dependent antiapoptotic effects were described for exendin-4. Moreover, infusion with GLP-1 reduced infarct size in isolated rat heart and this effect was abolished by exendin(9-39) (Ossum et al., 2009). Consistent with these previous studies, our study also showed that exendin-4 had the antiapoptotic effects while exendin(9-39), GLP-1R antagonist, inhibited the exendin-4-dependent protection against apoptosis in cardiomyocytes. Collectively, we concluded that antiapoptotic effect of exendin-4 can be mediated through the GLP-1R-dependent fashion.

GLP-1 exerts cAMP/PKA-mediated insulinotropic actions in endocrine tissues (Holz et al., 1995; Ramos et al., 2008) and stimulates AC activity to cause an increase in cAMP level in pancreatic islet cells (Ramos et al., 2008). An increase in cellular cAMP levels has been shown to regulate apoptosis in several cell types. Agents capable of increasing intracellular cAMP protect against apoptosis induced by palmitate

of the β-cell line RINm5F (Kwon et al., 2004). In addition, the apoptotic prevention exerted by GLP-1 in MIN6 cells challenged with H_2O_2 was abolished by Rp-cAMP (a cAMP-dependent PKA inhibitor) (Hui et al., 2003). Interestingly, stimulation of GLP-1R inhibits H_2O_2-induced apoptosis. This effect was mediated by an increased expression of the anti-apoptotic protein Bcl-2 and Bcl-xL in insulinoma cells (Hui et al., 2003). Liraglutide significantly increased the level of intracellular cAMP in mouse cardiomyocytes in a GLP-1R-dependent manner, because cAMP stimulation was abolished by the GLP-1R antagonist Exendin(9-39) (Noyan-Ashraf et al., 2009). These results strongly suggested that the antiapoptotic effects of GLP-1 and its analogues involve the GLP-1R/cAMP pathway in many cell types. In the present study, we showed that the apoptotic protection exhibited by exendin-4 was abolished by ddA (AC inhibitor), suggesting that cAMP is an important mediator in the prevention of apoptosis of cardiomyocytes.

As we knew that liraglutide inhibited apoptosis in mouse cardiomyocytes, it also reduced TNF-α-induced activation of caspase-3 (Noyan-Ashraf et al., 2009). Furthermore, treatment with exenatide increased myocardial phosphorylated Akt and Bcl-2 expression level and inhibited expression of active caspase-3 (Sonne et al., 2008). Taken together, these studies have been shown that GLP-1 analogues have the antiapoptotic effect. The antiapoptotic effect of GLP-1 is PKA-dependent, since the improvement of cell viability of GLP-1 after H_2O_2 exposure in cardiomyocytes was blocked by H89, a PKA inhibitor (Ku et al., 2013). In correlated with other studies, GLP-1 was shown to have an antiapoptotic action mediated by a cAMP/PKA-dependent signaling pathway in pancreatic β-cells (Hui et al., 2003) and endothelial cells (Oeseburg et al., 2010). In addition, activation of PK3K/Akt can be achieved by activation of PKA (Bellis et al., 2009), and a direct interaction of PKA and Akt was also found in GLP-1 action (Hui et al., 2003). GLP-1R antagonist was shown to abolish the elevation of Akt phosphorylation in response to H_2O_2 in cardiomyocytes (Ku et al., 2013). Although the activation of Akt by PKA was not completely understood, these data imply the idea that GLP-1R/PKA/Akt pathway is required in the protective signaling of GLP-1 and its analogues in the heart.

cAMP causes an even broader range of cellular responses by activating signaling pathways that are independent of PKA. cAMP has been shown to regulate gene transcription, cellular proliferation, cell adhesion, cell secretion, and cytokine signaling through both PKA-dependent and -independent pathways (Bos, 2006).

Interestingly, GLP-1 and exenatide provide the protection of apoptosis in both a PKA- and Epac-dependent manner in β-cells (Kwon et al., 2004). In this present study, we showed that blockade of either PKA or Epac pathway inhibited exendin-4-mediated antiapoptosis in cardiomyocytes. Interestingly, dual inhibition of these two pathways completely abolished the antiapoptotic effect of exendin-4, indicating that both PKA- and Epac-dependent pathways play a role on inhibition of apoptosis.

Stimulation of GLP-1R with GLP-1 inhibits H_2O_2-induced apoptosis in mouse insulinoma (MIN6) cells and this effect was mediated by an increased the expression of the antiapoptotic proteins Bcl-2 and Bcl-xL (Hui et al., 2003). Consistent with this study, we show that stimulation of GLP-1R with exedin-4 also inhibits apoptosis by inducing the Bcl-2 mRNA expression in cardiomyocytes. These results led to the hypothesis that GLP-1R signaling can inhibit apoptosis by increasing antiapoptotic proteins production in the heart, leading to exhibit the cardioprotective effects.

Bcl-2 family is a group of apoptosis-regulating proteins that can be divided into two subgroups; antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, and Bcl-W) and proapoptotic proteins (e.g., Bax, Bak, Bad, and Bid) (Cory et al., 2003; Gross et al., 1999). The antiapoptotic members such as Bcl-2 and Bcl-xL are important in cell survival and protect cardiac myocytes against various stressors. For instance, overexpression of Bcl-xL in H9c2 cardiac cells protected against doxorubicin- and hypoxia-mediated apoptosis by preserving mitochondrial integrity (Reeve et al., 2007), and Bcl-2 was shown to prevent p53-mediated apoptosis in cardiomyocytes (Kirshenbaum and de Moissac, 1997). Transgenic mice overexpressing Bcl-2 in the heart have reduced I/R injury and fewer apoptotic cells compared to wild type mice, suggesting that the cardioprotective effect of Bcl-2 is via inhibition of the mitochondrial death pathway (Brocheriou et al., 2000). Our results showed that exendin-4 increased the expression of Bcl-2 and tended to increase Bcl-xL expression, whereas the expression of Bax and Bad were not changed. In rodent islets, free fatty acid (FFA)-induced apoptosis may be attributed to the dysfunction of mitochondria, which is characterized by a decrease of Bcl-2 mRNA and an increase of Bax mRNA expression (Lupi et al., 2002). Similar to rodent results, apoptosis of pancreatic β-cells exposed to FFAs was accompanied by a marked increase of Bax mRNA, and decrease of Bcl-2 mRNA, in keeping with increased activity of caspase-3 (Wei et al., 2012). After treatment with exendin-4 in a lipotoxic enviroment, Wei et al. observed a decrease of caspase-3 activity, significant down-regulation of Bax mRNA, and up-regulation of Bcl-2 mRNA (Wei et al., 2012). These results imply that the

protective effect of exendin-4 is associated with the mitochondrial pathway accompanied by inhibition of Bax mRNA expression and promotion of Bcl-2 mRNA expression. However, the detailed mechanism by which the other Bcl-2 family members regulate caspase activity and the effect of exendin-4 on mitochondrial pathway in cardiomyocytes remain to be determined.

In conclusion, we have identified a new signaling mechanism for GLP-1R-mediated inhibition of oxidative stress and apoptosis in cardiomyocytes (Figure 14). Stimulation of GLP-1R provides an antioxidation via receptor-dependent Epac signaling pathway, and exerts an antiapoptosis through both PKA- and Epac-dependent manners.

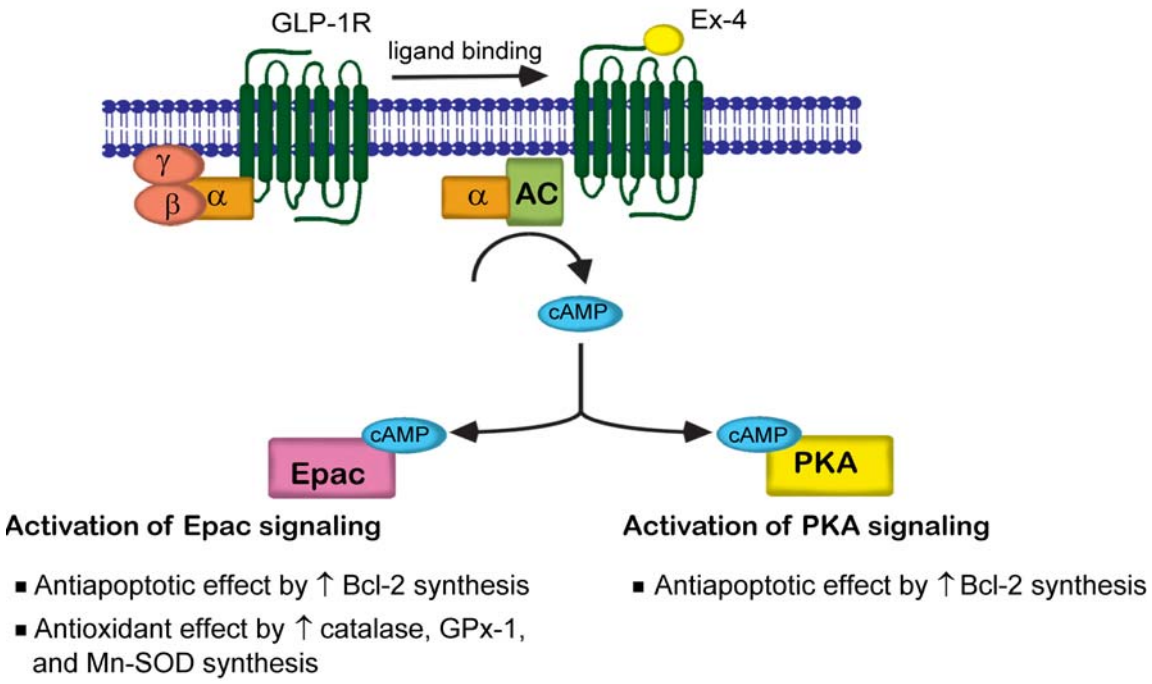

Figure 14. Schematic diagram representing cardioprotective effects of exendin-4

Exendin-4 (Ex-4) binding to GLP-1R leads to G_s protein coupling, subsequently stimulate AC activity and generate cAMP, the second messenger. cAMP can binds to and activates both PKA and Epac signaling. Activation of GLP-1R results in the antioxidation by inducing mRNA expression of catalase, GPx-1 and Mn-SOD through Epac-dependent pathway and elicits the antiapoptosis by inducing mRNA expression of Bcl-2 through PKA- and Epac-dependent pathways.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกอ. และ สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ:

อยู่ในระหว่างการตีพิมพ์

2. การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป):

ไม่มี

2.2 เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน):

ไม่มี

2.3 เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง):

1. เข้าร่วมประชุมวิชาการ “เมธีวิจัยอาวุโส สกว. ศ. ดร. นพ. นิพนธ์ ฉัตรทิพากร” ประจำปี 2556 “Pathophysiologic effects of metabolic syndromes: From heart to brain dysfunction” บรรยายในหัวข้อ “Effects of exendin-4 on inhibition of oxidative stress and apoptosis” โดยมีการแลกเปลี่ยนและแสดงความคิดเห็นระหว่างนักวิจัยและผู้เข้าร่วมประชุม

2.4 เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

1. นำผลงานวิจัยมาสอนให้นักศึกษาระดับปริญญาโท หลักสูตรเภสัชศาสตรมหาบัณฑิต สาขาวิชาเภสัชวิทยาและวิทยาศาสตร์ชีวโมเลกุล วิชา Molecular Pharmacology and Biomolecular Science (PYID696) ในหัวข้อ “Cardiovascular effects of GLP-1 analogue”

2. สร้างนักวิจัยใหม่ น.ส. พิริยา เหมพฤกษา นักศึกษาระดับปริญญาโท หลักสูตรเภสัชศาสตรมหาบัณฑิต สาขาวิชาเภสัชวิทยา วิทยานิพนธ์เรื่อง “Effects of exendin-4 on the inhibition of hydrogen peroxide-induced oxidative stress and apoptosis in neonatal rat cardiomyocytes”

3. อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

3.1 การเสนอผลงานในที่ประชุมระดับนานาชาติ

Mangmool S, Hemplueksa P, and Chattipakorn N. Stimulation of glucagon-like peptide-1 (GLP-1) receptor inhibits oxidative stress and apoptosis in an Epac-dependent manner. European Society of Cardiology (ESC) congress 2013. Amsterdam, Netherlands, Aug 31 – Sep 14, 2013

3.2 การเสนอผลงานในที่ประชุมระดับชาติ

Hemplueksa P, Pornchirasilp S, Nakornchai S, and Mangmool S. Activation of glucagon-like peptide-1 (GLP-1) receptor inhibits oxidative stress and apoptosis in neonatal rat cardiomyocytes. The 35th Pharmacology Conference 2013. Pitsanulok, THAILAND, March 20-22, 2013