

Essential Role Of High Glucose-Induced Overexpression Of PKC β And PKC δ In GLP-1 Resistance In Rodent Cardiomyocytes

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Purpose: Myocardia in diabetic patients exhibit increased vulnerability after ischemia/reperfusion injury (IRI). It has been demonstrated that glucagon-like peptide-1 (GLP-1) has a protective effect on cardiomyocytes. Protein kinase C (PKC) acts as a key regulator of many signaling pathways including oxidative stress and apoptosis. Our hypothesis is that increased vulnerability of myocardia in diabetic patients is partly due to GLP-1 resistance. The aim of this study was to explore the role of PKC in GLP-1 resistance in diabetic cardiomyocytes.

Methods: Cardiac function of diabetic or non-diabetic mice after myocardial IRI was detected with or without administration of GLP-1 analog exendin-4. Impacts of diabetes mellitus on GLP-1R expression in myocardia after IRI were accessed by Western blot. By transfecting PKC isoforms siRNA, in vitro study helped to identify the exact PKC isoforms which contributed to the downregulation of GLP-1R or impaired post-receptor signaling pathways in rodent cardiomyocytes (H9C2 cells) cultured by high glucose.

Results: The cardioprotective effects of endogenous GLP-1 were impaired in diabetic mice after myocardial IRI and administration of exendin-4 had no significant effects in restoring cardiac function. GLP-1 receptor (GLP-1R) expression decreased in H9C2 cells cultured by high glucose and knockdown of PKC β partly restored GLP-1R expression. Overexpression of PKC δ induced by high glucose in H9C2 cells impaired GLP-1 post-receptor anti-apoptotic signaling pathways by inhibition of Akt phosphorylation. Knockdown of both PKC β and PKC δ significantly restored cardioprotective effects of GLP-1 in H9C2 cells cultured by high glucose.

Conclusion: Our study found out a new mechanism of GLP-1 resistance that high glucose-induced overexpression of PKC β and PKC δ impaired cardioprotective effects of GLP-1 by downregulation of GLP-1R and inhibition of GLP-1 post-receptor anti-apoptotic signaling pathways, thus provided a new perspective in treating myocardial IRI in diabetic patients.

Keywords: ischemia/reperfusion injury, protein kinase C, GLP-1 resistance, diabetic cardiomyocytes

Introduction

People with diabetes are two to three times more likely to have cardiovascular disease than people without diabetes.^{1,2} Cardiovascular complications are the major causes of morbidity and mortality in diabetic patients, among which ischemic heart disease is the leading cause of death.³ It has been proven that myocardial infarction causes much more cell death in diabetic cardiomyocytes than in non-diabetic cardiomyocytes.⁴ Upon acute myocardial ischemia due to coronary occlusion, restoring myocardial perfusion through either percutaneous coronary intervention

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or thrombolytic therapy is the most effective treatment. However, reperfusion introduces a new problem that aggravates myocardial damage: myocardial IRI.^{5,6} Studies also showed that myocardial IRI brings severer damage to diabetic patients.^{7,8} Increased vulnerability of myocardia in diabetic patients has been extensively studied; however, the underlying mechanisms remained ambiguous.

GLP-1, an incretin hormone secreted from intestinal L cells, is now clinically used as an anti-diabetic drug.^{9,10} GLP-1 exerts physiological function upon binding to its receptor, which belongs to the class B family of heterotrimeric G-protein-coupled receptors.¹¹ GLP-1R has been proved to be expressed in many tissues, including heart. Research found that GLP-1 improves cardiac function, decreases infarct size after myocardial IRI.^{12,13} However, studies have found that in diabetic mice there exists GLP-1 resistance in several cell types such as pancreatic β cells and endothelial cells.^{14,15} Thus, raises questions about whether increased vulnerability of diabetic myocardia is partly due to GLP-1 resistance.

PKC isoforms are a family of serine/threonine kinase which play a crucial role in mediating cell signal pathways as diverse as cell growth, differentiation, apoptosis, oxidation stress and others.^{16,17} PKC isoforms are ubiquitously expressed in all tissues at all times of development.¹⁸ Interestingly, PKCs have demonstrated to have sometimes opposing roles in both normal and diseased states.¹⁹ In diabetic state, it has been proved that activation of PKCs is responsible for GLP-1 resistance in endothelial cells.²⁰ However, to our knowledge, the possible role of PKC in GLP-1 resistance of diabetic cardiomyocytes remains unclear.

In the present study, we provide evidence that in diabetic myocardia there exists GLP-1 resistance. We associate this phenomenon to a decrease in GLP-1R expression induced by PKC β overexpression and an impairment of GLP-1 post-receptor anti-apoptotic signaling pathways induced by PKC δ overexpression. When PKC β and PKC δ are specifically knockdown, GLP-1 actions are partially recovered.

Materials And Methods

Animal Models Of Diabetes And Myocardial Ischemia/Reperfusion Injury

All animal studies were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the ethical committee of Air Force Medical University. All commercially available kits and antibodies used in the present

study are listed in Table 2. Spontaneous diabetes model (random blood glucose ≥ 16.6 mmol/L) and non-diabetes model were achieved using db/db C57BL/6 mice (male, 8 weeks old) and C57BL/6 mice (male, 8 weeks old), respectively. Mice were randomly divided into 6 groups: 1) Diabetes Mellitus (DM); 2) Non-Diabetes Mellitus (Non-DM); 3) Diabetes Mellitus + Ischemia-Reperfusion Injury (DM + IRI); 4) Non-Diabetes Mellitus + Ischemia-Reperfusion Injury (Non-DM + IRI); 5) Diabetes Mellitus + Ischemia-Reperfusion Injury + exendin-4 (DM + IRI + exendin-4); and 6) Non-Diabetes Mellitus + Ischemia-Reperfusion Injury + exendin-4 (Non-DM + IRI + exendin-4) (n=6 for each group). Myocardial IRI models were established by ligation of the left anterior descending coronary artery 1–2 mm from the tip of the auricle with a 6–0 polypropylene suture for 30 mins. GLP-1 analog exendin-4 pretreatment (1 nmol/kg) was conducted by intraperitoneal injection 1 hr before the operation and the control group injected saline instead.

Measurement Of Cardiac Function By Echocardiograms

One day after IRI, echocardiography (Sequoia Acuson, Siemens, 15-MHz linear transducer, Erlangen, Germany) were performed. Gaseous anesthesia was conducted and maintained using 3% isoflurane, and the mice were placed on the scanning table. Echocardiographic images were obtained using a dedicated small-animal high-resolution imaging unit and a 30-MHz linear transducer. Left ventricular end-diastolic diameters (LVEDD) and left ventricular end-systolic diameters (LVESD) were measured using a parasternal short-axis view, and LV fractional shortening was calculated as (LVEDD-LVESD)/LVEDD*100. All measurements were averaged over 3 successive cardiac cycles.

Measurement Of Serum GLP-1 Level

The concentrations of GLP-1 were accessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Abcam, USA). Serum samples were collected at different time points (0 hr, 1 hr, 2 hrs, 3 hrs, 4 hrs, 5 hrs) after the surgery in group (1)-(4) and DPP-4 inhibitor was added to the samples immediately to prevent degradation of GLP-1.

Cell Culture And Hypoxia/Re-Oxygenation Injury

Cardiac myoblast cell lines H9C2 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai,

China). H9C2 cells were cultured in DMEM containing 10% neonatal bovine serum and 1% penicillin/streptomycin and incubated in a humidified chamber with 95% ambient air and 5% CO₂ at 37°C. Cells were grown at 1.2×10^5 in 6-well plates. H9C2 cells were cultured in high glucose (HG, 33 mM) or normal glucose (NG, 5 mM) during 21 days, changing the media each 48 hrs without passaging the cells. Exendin-4(50nM), Phorbol 12-myristate 13-acetate (PMA, 10 μM), Go 6983(10 μM), Ruboxistaurin (RBX, 0.5 μM), Wortmannin (1 μM) and insulin-like growth factor-1 (IGF-1, 10 nM) were added according to experimental need alone or in combination 1 hr before cell harvesting. Hypoxia/re-oxygenation injury of H9C2 cells was obtained by exposure to hypoxia (95% N₂, 5%CO₂) in an anaerobic system (Thermo Forma) at 37°C for 6 hrs followed by re-oxygenation in normoxia (95% ambient air, 5%CO₂) for 10 hrs. In the control group, H9C2 cells were maintained at normoxia for equivalent periods.

siRNAs Transient Transfection And Real-Time Quantitative PCR

Small interfering (si)RNA against PKC α , β , γ , and δ was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Universal negative control (UNC) siRNA (Sigma, USA) was used as a negative control. H9C2 cells were transfected with the siRNAs using Lipofectamine RNAiMAX Reagent (Life Invitrogen, USA) according to the manufacturer's instruction. Co-transfection of RKC β siRNA and PKC δ siRNA was established at a proportion of 1:1.

Total RNA was isolated from H9C2 cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Real-time quantitative PCR was conducted following standard methods. Primer sequences are provided in Table 1.

Assessment Of Apoptosis And Measurement Of ROS

TUNEL staining was performed on H9C2 cells according to the manufacturer's instructions (MEBSTAIN Apoptosis TUNEL kit, Takara). For detection of total nuclei, the slides were covered with the mounting medium containing DAPI (Abcam, UK). Digital photographs were taken at high magnification ($\times 400$) using a fluorescent microscopy (Olympus). Cells in which the nucleus was stained were

Table 1 Premier Sequences

Gene Symbol	Sequence
PKC α	5'- GTTTACCCGGCCAACGACT 3'- GGGCGATGAATTTGTGGTCTT
PKC β	5'- GTGTCAAGTCTGCTGCTTTGT 3'- GTAGGACTGGAGTACGTGTGG
PKC γ	5'-CTCGTTTCTTCAAGCAGCCAA 3'- GTGAACCACAAAGCTACAGACT
PKC δ	5'-CCTCTGTACGAAATGCTCATC 3'- GTTTCCTGTTACTCCCAGCCT
NOX4	5'-GAAGGGGTTAAACACCTCTGC, 3'-ATGCTCTGCTTAAACACAATCCT
p22-phox	5'- TGCCAGTGTGATCTATCTGCT, 3'- TCGGCTTCTTCGGACCTCT
HMOX-1	5'-AAGCCGAGAATGCTGAGTTCA 3'-GCCGTGTAGATATGGTACAAGGA
NQO-1	5'-AGGATGGGAGGTAATCGAATC 3'-TGCTAGAGATGACTCGGAAGG
TXNIP	5'- GGCCGGACGGGTAATAGTG 3'- AGCGCAAGTAGTCCAAAGTCT
Bcl-2	5'-GTCGCTACCGTCGTGACTTC 3'-CAGACATGCACCTACCCAGC
BAX	5'-TGAAGACAGGGGCTTTTTG 3'-AATTCCGCCGGAGACTCTCG
GAPDH	5'-AGGTCGGTGTGAACGGATTTG 3'-TGTAGACCATGTAGTTGAGGTCA

defined as TUNEL positive. Apoptosis index (AI) is defined as cell numbers of apoptotic cells in 100 cells averagely. Each AI was accessed in 20 randomly selected fields. Caspase-3 activity was measured using a caspase-3 assay kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. ROS level was measured with the dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) according to the manufacturer's instructions.

Western Blot Analysis

Both H9C2 cells and myocardial tissues were harvested for Western blot analysis (Western Blot Detection Kit, Elabscience) following standard protocol according to the manufacturer's instructions and Towbin system buffer was used. Primary antibodies used in Western blot were as follows: anti-GLP-1R (1:200, Abcam, UK), anti-Akt

Table 2 Catalog Numbers Of Commercially Available Kits And Antibodies

Product Name	Company	Catalog No.
Exendin-4	Abcam, UK	ab120214
Isoflurane	Santa Cruz, USA	Sc-470926
GLP-1 ELISA Kit	Abcam, UK	ab184857
DPP-4 inhibitor	Abcam, UK	ab235551
PMA	MCE, USA	HY-18739
Go 6983	MCE, USA	HY-13689
RBX	MCE, USA	HY-10195B
Wortmannin	MCE, USA	HY-10197
IGF-1	MCE, USA	HY-P7070
TRizol reagent	Invitrogen, USA	15596018
TUNEL kit (green)	Takara, Japan	MK500
TUNEL kit (red)	Beyotime, China	C1090
DAPI	Abcam, UK	ab228549
Caspase-3 assay kit	Clontech, CA	630217
ROS assay kit	Abcam, UK	ab113851
Western Blot Detection Kit	Elabscience, China	E-IR-R304
Anti-GLP-1R antibody	Abcam, UK	ab218532
Anti-Akt antibody	Abcam, UK	ab179463
Anti-p-Akt antibody	Abcam, UK	ab192623
Anti-PI3K antibody	Abcam, UK	ab32089
Anti-PKC antibody	Abcam, UK	ab179522
Anti-PKC α antibody	Abcam, UK	ab32376
Anti-PKC β antibody	Abcam, UK	ab71123
Anti-PKC γ antibody	Abcam, UK	ab71558
Anti-PKC δ antibody	Abcam, UK	ab182126
Anti- β -actin antibody	Abcam, UK	ab8227
Goat anti-rabbit IgG	Abcam, UK	ab97052

(1:200, Abcam, UK), anti-p-Akt (1:200, Abcam, UK), anti-PI3K (1:500, Santa Cruz, USA), anti-PKC (1:500, Abcam, UK), anti-PKC α (1:500, Abcam, UK), anti-PKC β (1:500, Abcam, UK), anti-PKC γ (1:500, Abcam, UK), anti-PKC δ (1:500, Abcam, UK) and anti- β -actin (1:500, Santa Cruz, USA). Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, USA) at 1:5000 dilution. The results were visualized using an enhanced chemiluminescence system (ECL, Amersham). Densitometric analysis was conducted using ImageJ software (NIH, USA).

Statistical Analysis

Results are represented as mean \pm SD. Statistical analyses were performed by one-way analysis of variance (AVONA) and a Student–Newman–Keuls multiple comparison test was performed to identify differences between groups. A P value of less than 0.05 was considered statistically significant.

Results

Impaired Cardioprotective Effects In Response To Increased Endogenous GLP-1 In Diabetic Mice After Myocardial IRI

First, we examined whether endogenous GLP-1 and GLP-1 analog exendin-4 exhibit intact cardioprotective effects in diabetic mice after myocardial IRI. As shown in [Figure 1A](#), diabetic mice showed higher circulating GLP-1 level than non-diabetic mice before (0 hr: 5.10 \pm 0.21pM vs 7.42 \pm 0.32pM) and after (5 hrs: 6.82 \pm 0.14pM vs 14.41 \pm 0.45pM) myocardial IRI. However, corresponding left ventricle (LV) fractional shortening of diabetic mice remained lower than non-diabetic mice. Furthermore, addition of exendin-4 brought no significant change in LV fractional shortening of diabetic mice after myocardial IRI, while in contrast, LV fractional shortening of non-diabetic mice significantly increased with administration of exendin-4 after myocardial IRI ([Figure 1B](#)).

Anti-apoptotic factor B-cell lymphoma-2 (Bcl-2) and pro-apoptotic factor Bcl-2 associated X protein (BAX) were accessed by qRT-PCR, the results showed that exendin-4 exerted anti-apoptotic effects in non-diabetic mice after myocardial IRI while in diabetic mice no significant changes in Bcl-2 and BAX were detected ([Figure 1C](#)). Same conclusion can be made according to caspase-3 activity change ([Figure 1D](#)).

Essential subunits of NAD(P)H oxidase, NADPH oxidase 4 (NOX4) and p22-phox were analyzed by qRT-PCR. The results indicated that exendin-4 effectively reduced oxidative stress in non-diabetic mice after myocardial IRI while no such effect can be seen in diabetic mice ([Figure 1E](#)). Results of reactive oxygen species (ROS) measurement showed a similar tendency with NOX4 and p22-phox ([Figure 1F](#)). Thus indicated that the cardioprotective effects of GLP-1 were impaired in diabetic mice.

Overexpression Of PKC β Induced By High Glucose Downregulated GLP-1R Expression In H9C2 Cells

Then, we examined whether impaired cardioprotective effects of GLP-1 in diabetic mice were due to decreased GLP-1R expression in cardiomyocyte. As shown in [Figure 2A](#), compare to non-diabetic mice, GLP-1R expression was lower in diabetic mice before and after myocardial IRI. Since PKC is activated by high glucose in various cell types,^{21,22} we hypothesized that PKC activation is involved in the reduction

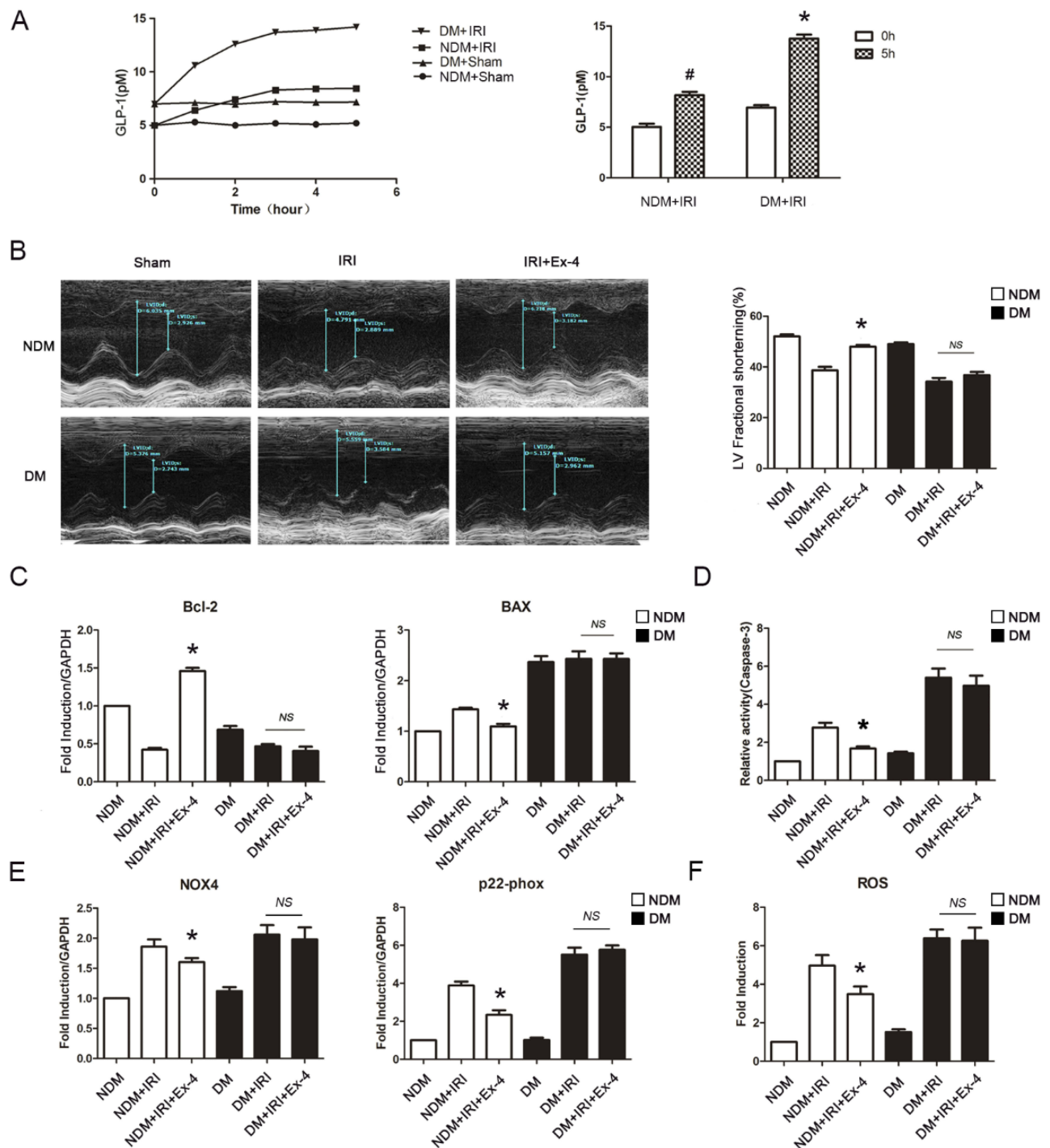


Figure 1 Impaired cardioprotective effects in response to GLP-1 in diabetic mice after myocardial IRI. **(A)** Elevated circulating GLP-1 level induced by myocardial IRI. * $P < 0.01$ vs 0 hr+NDM+IRI group and 0 hr + DM + IRI group; # $P < 0.05$ vs 0 hr + NDM + IRI group. **(B)** Representative echocardiography and LV fractional shortening change of each group. * $P < 0.05$ vs NDM + IRI group. **(C)** QRT-PCR analysis of Bcl-2 and BAX. * $P < 0.05$ vs NDM + IRI group. **(D)** Caspase-3 activity assay. * $P < 0.05$ vs NDM + IRI group. **(E)** QRT-PCR analysis of NOX4 and p22-phox. * $P < 0.05$ vs NDM + IRI group. **(F)** ROS measurement. * $P < 0.05$ vs NDM + IRI group.

Abbreviations: DM, diabetes mellitus; NDM, non-diabetes mellitus; IRI, ischemia/reperfusion injury; LV, left ventricle; ROS, reactive oxygen species.

of GLP-1R expression by high glucose. H9C2 cell line was originated from rat heart myoblast. In the present study, H9C2 cells were cultured for in vitro experiment. As shown in Figure 3B, PKC expression increased remarkably in H9C2

cells cultured by high glucose. Addition of PKC activator phorbol-12-myristate-13-acetate (PMA) in H9C2 cells cultured by high glucose resulted in further reduction of GLP-1R expression, while in contrast Go 6983 (PKC inhibitor)

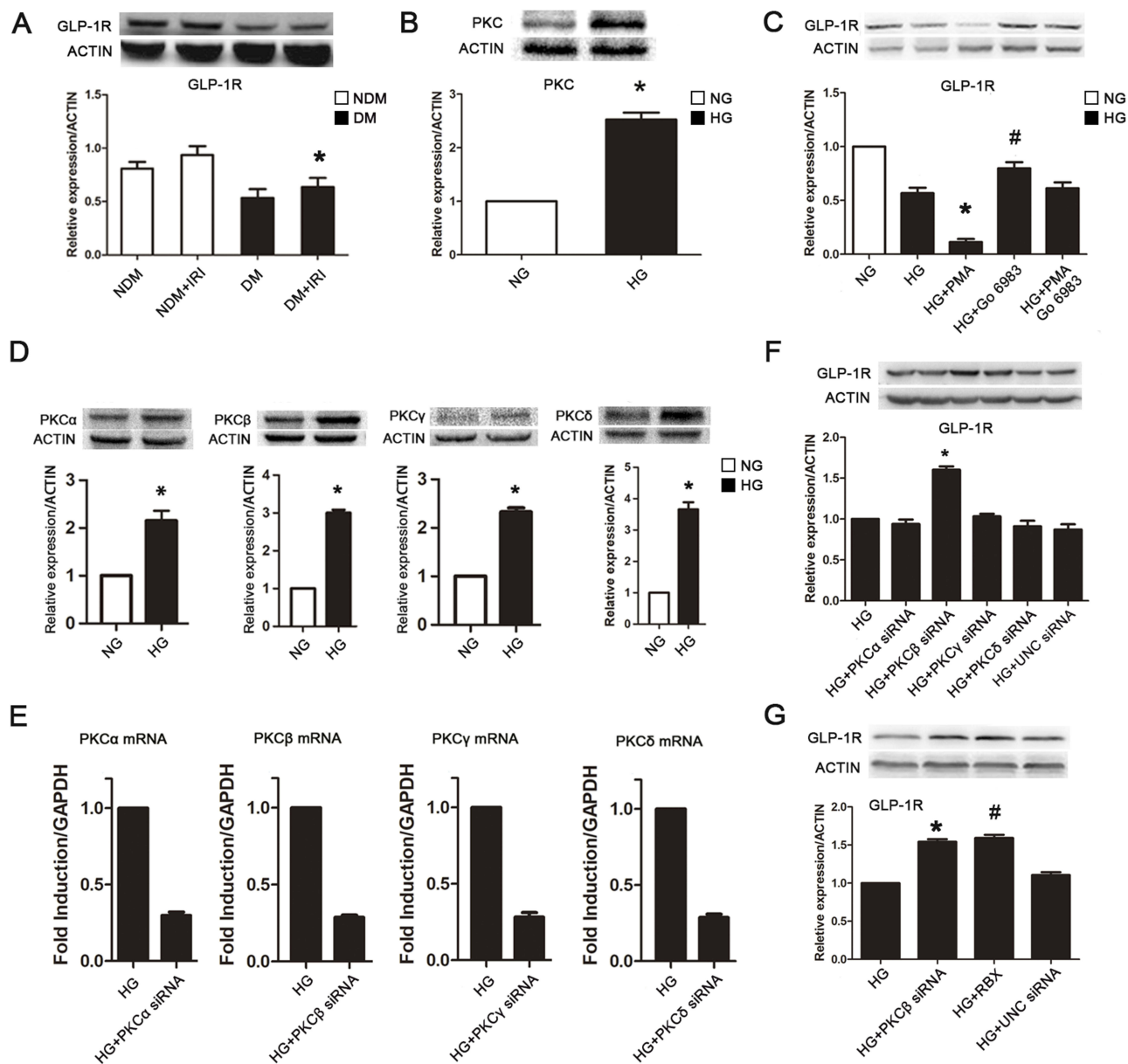


Figure 2 Overexpression of PKC β induced by high glucose downregulated GLP-1R expression in H9C2 cells. **(A)** High glucose-induced downregulation of GLP-1R and impact of myocardial IRI on GLP-1R expression. * $P < 0.05$ vs NDM group and NDM + IRI group; **(B)** high glucose-induced overexpression of PKC. * $P < 0.01$ vs NG group; **(C)** impact of PMA and Go 6983 on GLP-1R expression in H9C2 cells. * $P < 0.01$ vs HG group, # $P < 0.05$ vs HG group and HG + PMA + Go6983 group; **(D)** high glucose-induced overexpression of PKC α , β , γ and δ . * $P < 0.01$ vs NG group; **(E)** transfection efficiency of PKC α (70.3 \pm 2.1%), β (71.5 \pm 3.2%), γ (68.3 \pm 2.6%) and δ (66.4 \pm 3.1%) siRNA; **(F)** change of GLP-1R expression by knockdown of PKC α , β , γ and δ . * $P < 0.01$ vs HG group and HG + UNC siRNA group; **(G)** impact of RBX and PKC β siRNA on GLP-1R expression in H9C2 cells, * $P < 0.05$ vs HG group and HG + UNC siRNA group, # $P < 0.05$ vs HG + RBX group.

Abbreviations: NDM, non-diabetes mellitus; DM, diabetes mellitus; NG, normal glucose; HG, high glucose; PMA, phorbol-12-myristate-13-acetate; RBX, ruboxistaurin; UNC, universal negative control.

partially restored the downregulation of GLP-1R (Figure 3C). It has been proven that PKC α , β , γ , and δ were expressed in cardiomyocytes,^{23–26} so we examined the effects of each PKC isoforms on GLP-1R expression in H9C2 cells cultured by high glucose. Western blot analysis showed that PKC α , β , γ , and δ expression significantly increased in high glucose-cultured H9C2 cells (Figure 2D). To figure out the exact isoform of PKC that was responsible for the downregulation of

GLP-1R, we knockdown PKC α , β , γ , and δ , respectively, in H9C2 cells. As shown in Figure 2E, PKC α , β , γ , and δ were successfully knockdown. In H9C2 cells cultured by high glucose, knockdown of PKC β resulted in significantly increased GLP-1R expression, while in contrast, knockdown of PKC α , γ , and δ did not result in significant change of GLP-1R expression (Figure 2F). Addition of PKC β -specific inhibitor Ruboxistaurin (RBX) in H9C2 cells

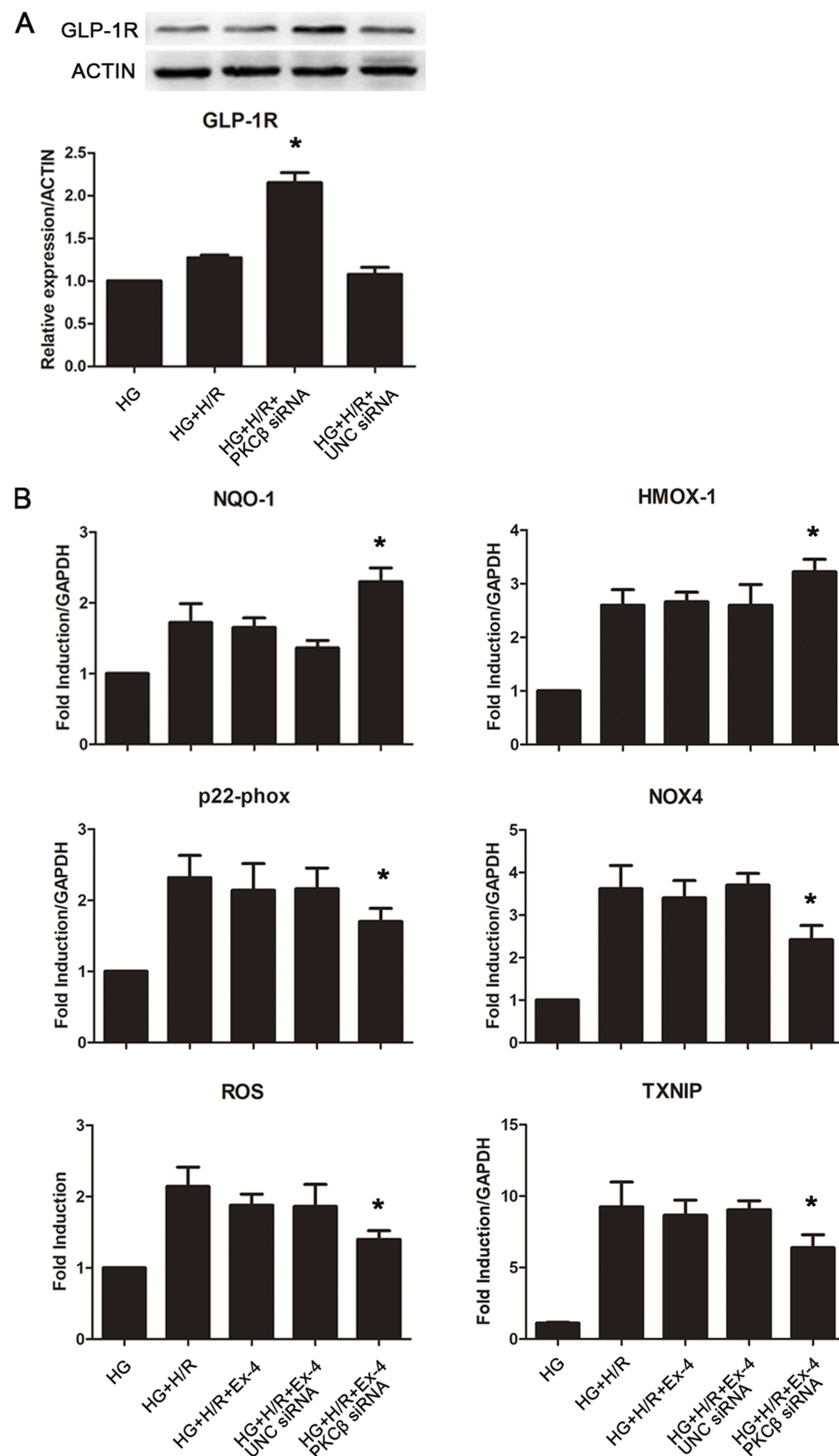


Figure 3 Restoration of GLP-1R expression by knockdown of PKC β recovered anti-oxidative stress effects of GLP-1R in H9C2 cells cultured by high glucose. **(A)** Western blot analysis confirmed the restoration of GLP-1R expression by knockdown of PKC β in H9C2 cells cultured by high glucose. * $P < 0.01$ vs HG, HG + H/R and HG + H/R + UNC siRNA group. **(B)** Restored anti-oxidative stress effects of GLP-1R. * $P < 0.05$ vs HG + H/R + Ex-4 group.

Abbreviations: HG, high glucose; Ex-4, exendin-4. H/R, hypoxia/re-oxygenation; UNC, universal negative control.

increased GLP-1R expression in a similar way as PKC β siRNA did, and the upregulation effect of PKC β siRNA on GLP-1R expression can be blocked by PMA (Figure 2G).

Thus indicated that downregulation of GLP-1R expression in H9C2 cells was mediated by PKC β overexpression induced by high glucose.

Restoration Of GLP-1R Expression By Knockdown Of PKC β Recover Anti-Oxidative Stress Effects Of GLP-1 Analog In H9C2 Cells Cultured By High Glucose While Anti-Apoptotic Effects Remain Suppressed

To access the possible functional change of restored GLP-1R expression, H9C2 cells cultured by high glucose underwent hypoxia/re-oxygenation (H/R) procedure. Restored GLP-1R expression by knockdown of PKC β was confirmed by Western blot in H9C2 cells cultured by high glucose (Figure 3A). Markers that can reflect anti-oxidative stress functions such as NAD(P)H quinone dehydrogenase 1 (NQO-1), heme oxygenase 1 (HMOX-1), NOX4, p22-phox and thioredoxin-interacting protein (TXNIP) were analyzed by qRT-PCR, results showed that restored GLP-1R expression by knockdown of PKC β correlated with a partial recovery of the anti-oxidative stress effects of GLP-1 analog in H9C2 cells cultured in high glucose. Results of reactive oxygen species (ROS) measurement consistent with the findings above (Figure 3B).

Anti-apoptotic effects of GLP-1R were also evaluated. As seen in representative TUNEL images and corresponding apoptosis index (AI) (Figure 4C), a significantly higher AI was observed in H/R group compared with control group ($5.2\pm 0.3\%$ vs $43.4\pm 2.5\%$). In H9C2 cells cultured by high glucose, addition of exendin-4 alone partially reversed AI ($32.0\pm 1.3\%$) that elevated by H/R. However, knockdown of PKC β brought no significant change in AI ($28.6\pm 0.7\%$) compared with exendin-4. Similar conclusion can be deduced by results of qRT-PCR analyzed anti-apoptotic markers (Figure 4A and B). Taken together, increased GLP-1R expression hardly intensifies anti-apoptotic effects of exendin-4 in H9C2 cells cultured by high glucose. Thus arises questions about whether GLP-1 resistance in the diabetic condition is in part due to impaired GLP-1 post-receptor anti-apoptotic signaling pathways.

Overexpression Of PKC δ Induced By High Glucose Impaired Post-Receptor Anti-Apoptotic Signaling Pathways Of GLP-1R In H9C2 Cells By Inhibition Of Akt Phosphorylation

Next, we examined whether and why post-receptor apoptosis-related signal pathways of GLP-1R were impaired.

As shown in Figure 5A and B, caspase-3 activity and BAX decreased significantly in response to knockdown of PKC δ in H9C2 cells while Bcl-2 increased in the meantime. Thus implicated that high glucose-induced overexpression of PKC δ is involved in impaired anti-apoptotic effects of GLP-1 in H9C2 cells. It has been reported that GLP-1 exerts its anti-apoptotic effects through activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and phosphorylation of Akt results in downstream activation of anti-apoptotic factors such as Bcl-2.²⁷ Thus, we examined whether high glucose-induced overexpression of PKC δ impairs post-receptor anti-apoptotic signaling pathways of GLP-1 by blocking PI3K/Akt pathway.

Figure 5C shows that knockdown of PKC δ in H9C2 cells cultured by high glucose increased phosphorylation level of Akt, addition of exendin-4 on the basis of PKC δ knockdown further increased phosphorylation level of Akt. Thus indicating that high glucose-induced overexpression of PKC δ impairs anti-apoptotic effects of GLP-1 by inhibition of Akt phosphorylation. Further studies showed that neither inhibition nor activation of PI3K by wortmannin and insulin-like growth factor 1 (IGF-1) respectively influenced PKC δ level, and change in PKC δ level did not influence PI3K level either (Figure 5D). Thus indicating that PKC δ and PI3K are mutually independent and both of them affect Akt phosphorylation.

Knockdown Of Both PKC β And PKC δ Significantly Restored Anti-Apoptotic Effects Of GLP-1 Analog On H9C2 Cells

To further confirm these results, H9C2 cells were co-transfected with PKC β and PKC δ siRNA. QRT-PCR analysis of HMOX-1, NQO-1, NOX4, p22-phox and TXNIP showed that knockdown of PKC β restored anti-oxidative stress effects of GLP-1 analog in H9C2 cultured by high glucose, and knockdown of PKC δ exhibited no significant contribution in restoration of anti-oxidative stress effects of GLP-1 analog. Results of ROS measurement arrived at the same conclusion (Figure 6).

Representative TUNEL images and corresponding apoptosis index showed that, on the basis of HG + H/R + Ex-4 group ($40.4\pm 2.1\%$), addition of PKC β knockdown hardly changed apoptosis index ($38.3\pm 1.9\%$) while addition of PKC δ knockdown decreased apoptosis index ($33.5\pm 1.3\%$). Notably, knockdown of both PKC β and PKC δ caused a significant reduction in AI ($14.5\pm 1.2\%$) (Figure 7C). Analysis of apoptosis-related markers such as Bcl-2, BAX

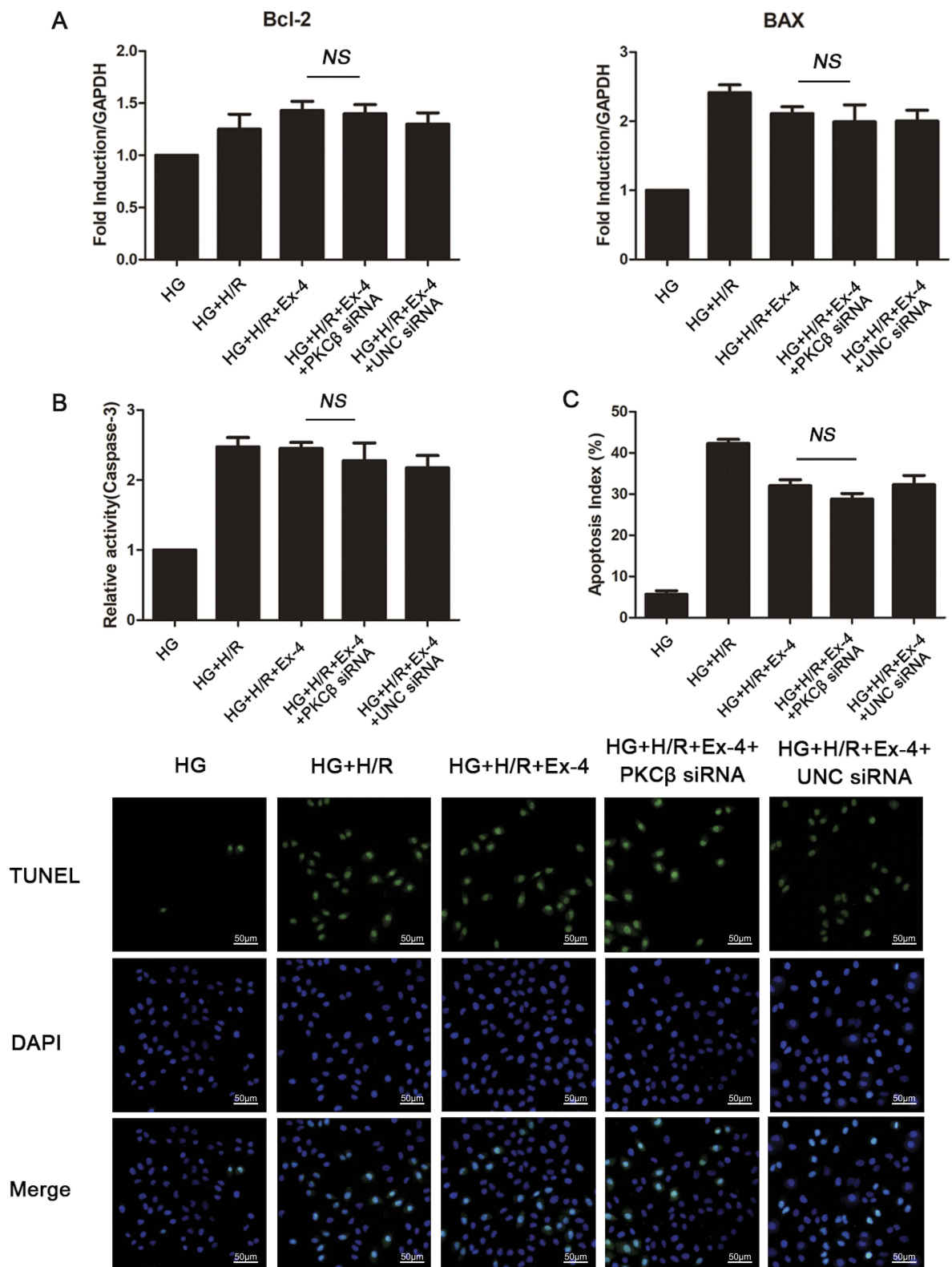


Figure 4 Restoration of GLP-1R expression by knockdown of PKCβ insignificantly recovers anti-apoptotic effects of GLP-1R in H9C2 cells cultured by high glucose. **(A)** QRT-PCR analysis of Bcl-2 and BAX. **(B)** Caspase-3 activity. **(C)** Representative TUNEL images and quantitative analysis of the TUNEL results (shown as apoptosis index). **Abbreviations:** HG, high glucose; Ex-4, exendin-4; H/R, hypoxia/re-oxygenation; UNC, universal negative control.

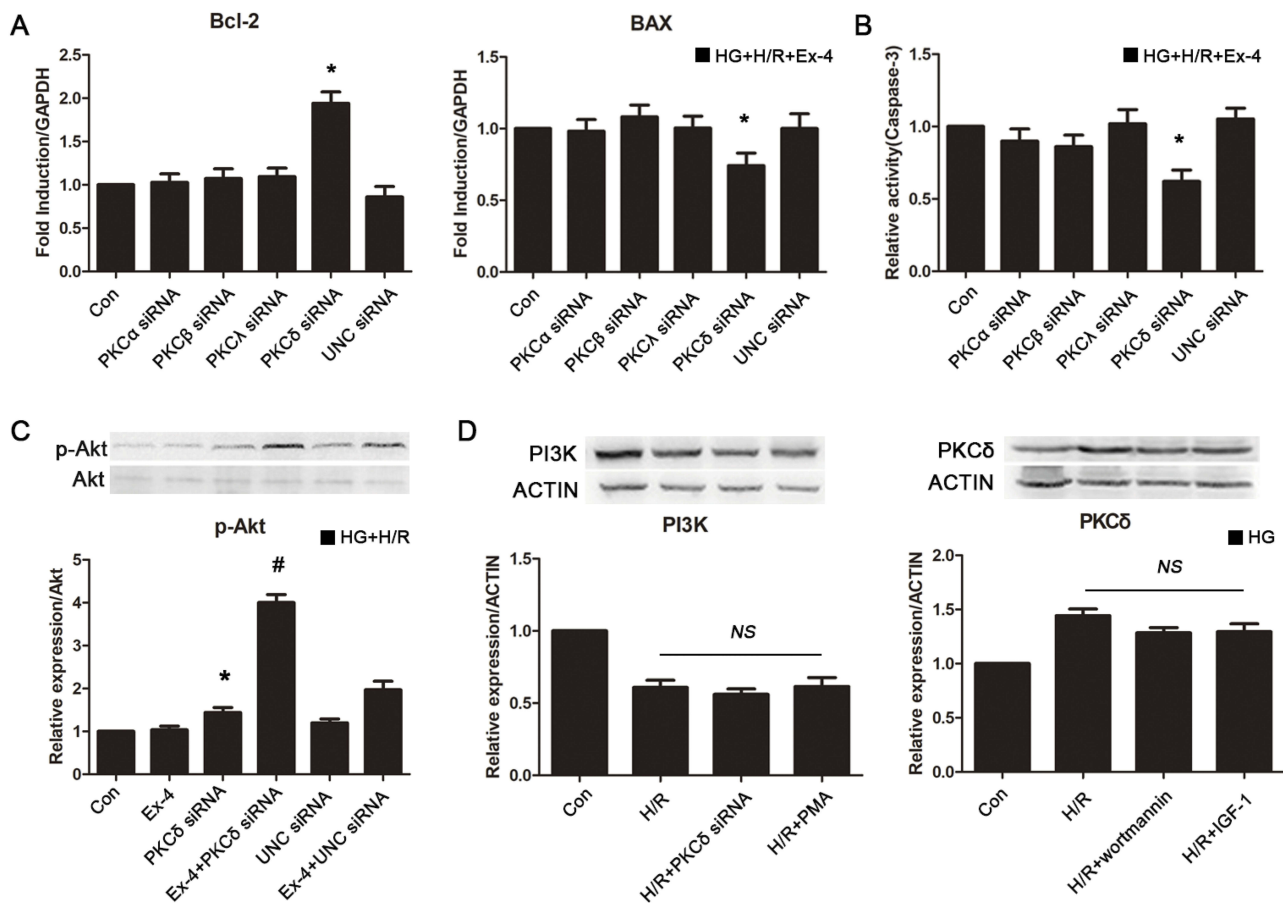


Figure 5 Overexpression of PKC δ induced by high glucose-impaired post-receptor anti-apoptotic signaling pathways of GLP-1 in H9C2 cells by inhibition of Akt phosphorylation. **(A)** Impact of PKC α , β , γ , δ siRNA and UNC siRNA on Bcl-2 and BAX mRNA level after H/R in H9C2 cells cultured by high glucose. * $P < 0.01$ vs HG + H/R + Ex-4 group and HG + H/R + Ex-4 + UNC siRNA group; **(B)** impact of PKC α , β , γ , δ siRNA and UNC siRNA on caspase-3 activity level after H/R in H9C2 cells cultured by high glucose. * $P < 0.01$ vs HG + H/R + Ex-4 group and HG + H/R + Ex-4 + UNC siRNA group; **(C)** knockdown of PKC δ increased phosphorylation level of Akt in H9C2 cells. * $P < 0.05$ vs HG + H/R group; # $P < 0.01$ vs HG + H/R + Ex-4 group and HG + H/R + Ex-4 + UNC siRNA group; **(D)** impact of activation and inhibition of both PKC δ and PI3K on each other.

Abbreviations: Con, control; HG, high glucose; H/R, hypoxia/re-oxygenation; IGF-1, insulin growth factor-1; UNC, universal negative control; Ex-4, exendin-4.

and Caspase-3 activity pointed to the same conclusion (Figure 7A, B). Thus indicating an ideal method to restore the anti-apoptotic effects of GLP-1 in diabetic condition.

Discussion

The current study provides a new perspective that increased vulnerability in diabetic myocardium is in part due to GLP-1 resistance. These data demonstrated that hyperglycemia via overexpression of PKC β and PKC δ can increase cardiomyocyte vulnerability through a dual pathway including downregulation of GLP-1R expression and impairment of GLP-1 post-receptor anti-apoptosis signaling pathway.

Previously, downregulation of GLP-1R has been proven to exist in several cell types in the diabetic state. Gang Xu et al, reported that downregulation of GLP-1R in pancreatic β cells is due to overexpression of PKC α in

diabetes.¹⁵ Akira Mima et al, reported that overexpression of PKC β is responsible for reduced GLP-1R in glomerular endothelium in diabetes.²⁸ Thus implicated a pivotal role of PKC isoform in the regulation of GLP-1R in diabetes. Our results demonstrated that in high glucose-cultured cardiomyocyte, it was PKC β which downregulate GLP-1R expression. Knockdown of PKC β restored GLP-1R expression in high glucose-cultured cardiomyocyte. These findings are consistent with the hypothesis that the unresponsiveness of myocardium to GLP-1 is in part due to the receptor change. However, with regard to anti-apoptotic effects of GLP-1, differs from observations in human endothelial cells, restored GLP-1R expression insignificantly reverse the anti-apoptotic effects of GLP-1 that lost in high glucose condition. Thus implicated an impairment in GLP-1 post-receptor signaling pathways in diabetic myocardium.

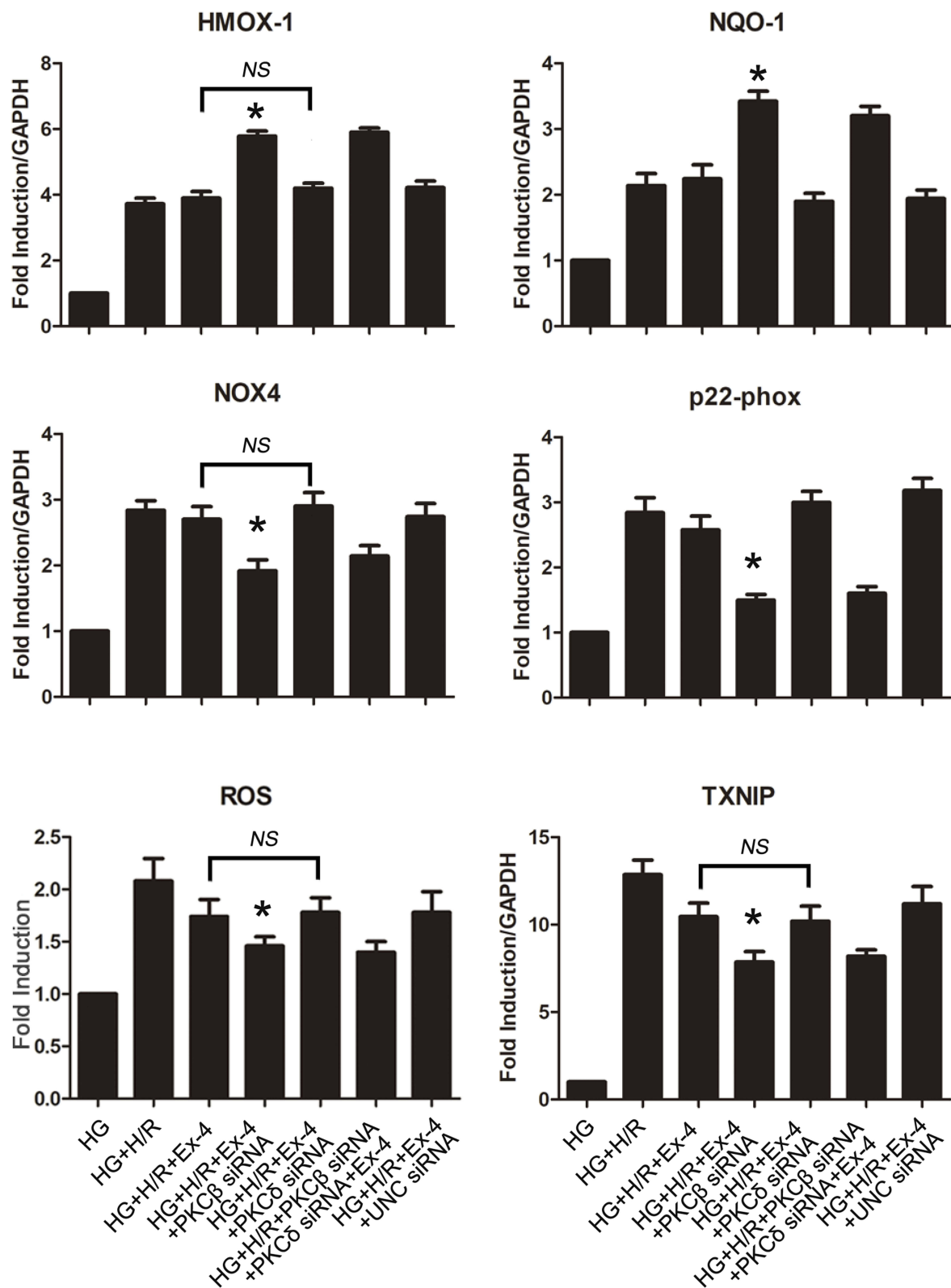


Figure 6 Knockdown of PKC β but not PKC δ restored anti-oxidative stress effects of GLP-1R in H9C2 cells cultured by high glucose. *P<0.05 vs HG + H/R + Ex-4 group and HG + H/R + Ex-4 + UNC siRNA group.

Abbreviations: HG, high glucose; H/R, hypoxia/re-oxygenation; Ex-4, exendin-4; UNC, universal negative control.

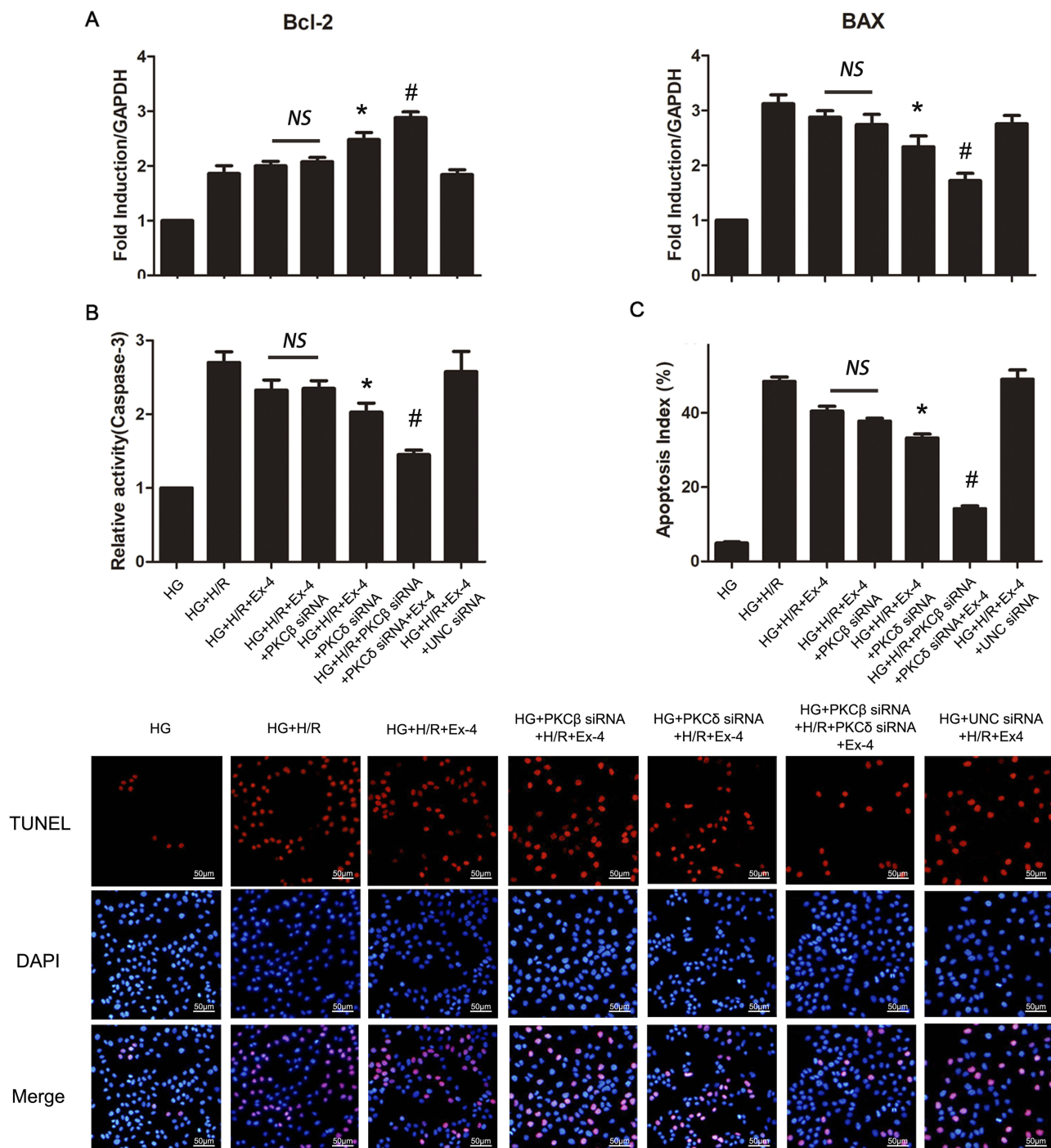


Figure 7 Knockdown of both PKC β and PKC δ restored anti-apoptotic effects of GLP-1 analog significantly in H9C2 cells cultured by high glucose. **(A)** QRT-PCR analysis of Bcl-2 and BAX mRNA. **(B)** Caspase-3 activity analysis. **(C)** Representative images of TUNEL staining and corresponding apoptosis index. * $P < 0.05$ vs HG + H/R + Ex-4 group, HG + H/R + Ex-4 + PKC β siRNA + PKC δ siRNA group and HG + H/R + Ex-4 + UNC siRNA group. # $P < 0.01$ vs HG + H/R + Ex-4 group and HG + H/R + Ex-4 + UNC siRNA group.

Abbreviations: HG, high glucose; H/R, hypoxia/re-oxygenation; Ex-4, exendin-4; UNC, universal negative control.

The relationship between reduced responsiveness to GLP-1 and changes in GLP-1 post-receptor signaling pathways is needed to be examined. GLP-1 exerts anti-apoptotic effects by inhibition of apoptosis through the

PI3K-Akt pathway.¹² PKCs are known to be major mediators of signal transduction pathways and have been shown to regulate sets of biological functions including apoptosis.¹⁶ Significant effort has been made

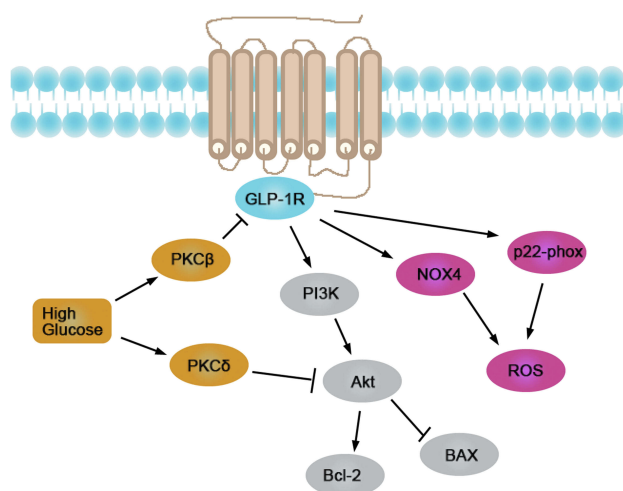


Figure 8 Essential role of PKC β and PKC δ in GLP-1 resistance in diabetic cardiomyocytes.

in understanding the role of specific PKCs in myocardium ischemia/reperfusion injury over the past two decades, but it is still a subject of debates.^{29,30} One hypothesis suggests that post G-protein-coupled receptor signaling pathways lead to the activation of PKC via diacylglycerol, after which, activated PKC then phosphorylates a secondary effector protein, which is thought to be induce protection.³¹ However, numerous research have shown that PKC inhibition at reperfusion is protective.³² Thus indicate a variability of actions that PKC isoform exhibited in myocardial ischemia/reperfusion condition. However, the above-mentioned researches did not take diabetes into consideration. In the present study, our results demonstrated that in diabetic cardiomyocyte, overexpression of PKC δ impaired GLP-1 post-receptor anti-apoptosis signaling pathways by inhibition of Akt phosphorylation. Knockdown of PKC δ partly restored the anti-apoptotic effects of GLP-1R. Overexpression of PKC δ inhibited downstream signaling pathways of GLP-1R, and that explains why knockdown of PKC β alone hardly restored the anti-apoptotic effects of GLP-1R. By knockdown of both PKC β and PKC δ , we restored GLP-1 actions significantly in diabetic condition, thus implicated that PKC β and PKC δ contributed to GLP-1 resistance through different mechanisms. Further studies explored the relationship between PI3K and PKC δ , the results showed that they exert no influences to each other (Figure 8). To further explore the anti-apoptotic effects of GLP-1 in diabetic cardiomyocytes, signaling pathways involve PKC δ and Akt are need to be elucidated.

Conclusion

In the present study, we found that increased myocardial vulnerability is in part due to GLP-1 resistance in diabetic condition. We suggest that downregulation of the GLP-1R induced by PKC β overexpression and impaired GLP-1 post-receptor anti-apoptotic signaling pathways induced by PKC δ overexpression are responsible for GLP-1 resistance in diabetic condition. This finding provides a new perspective in developing novel therapeutic strategy in treating myocardial IRI in diabetic condition based on activation of the GLP-1R.

Abbreviations

IRI, Ischemia/reperfusion injury; GLP-1, Glucagon-like peptide-1; PKC, Protein kinase C; GLP-1R, Glucagon-like peptide-1 receptor; Bcl-2, B-cell lymphoma-2; BAX, Bcl-2 associated X protein; NOX4, NAD(P)H oxidase 4; ROS, Reactive oxygen species; PMA, Phorbol-12-myristate-13-acetate; RBX, Ruboxistaurin; H/R, Hypoxia/re-oxygenation; NQO-1, NAD(P)H quinone dehydrogenase 1; HMOX-1, Heme oxygenase 1; TXNIP, Thioredoxin-interacting protein; AI, Apoptosis index; PI3K, Phosphatidylinositol 3-kinase; IGF-1, Insulin-like growth factor 1.

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Disclosure

The authors report no conflicts of interest in this work.

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