Erythropoietin attenuates myocardial ischemia-reperfusion injury aggravated by hyperglycemia which is involved in the upregulation of Bcl-2 through caspase-3 inhibition and GATA-4 regulation

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Directed by Professor Young Lan Kwak

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ABSTRACT

Erythropoietin attenuates myocardial ischemia-reperfusion injury aggravated by hyperglycemia which is involved in the upregulation of Bcl-2 through caspase-3 inhibition and GATA-4 regulation

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Erythropoietin (EPO), an essential hormone for erythropoiesis, can provide protection against myocardial ischemia/reperfusion (I/R) injury. Hyperglycemia during acute myocardial infarction is common and associated with increased organ damage and attenuated efficacy of protective measures. This study aimed to investigate whether EPO could ameliorate the myocardial I/R injury in hyperglycemic rats as well as in normoglycemic rats.

Eighty-two Sprague-Dawley rats were randomly assigned to six groups: normoglycemia (NG)-Sham, NG-I/R–control (IRC), NG-I/R-EPO (IRE), hyperglycemia (HG)-Sham, HG-IRC, and HG-IRE. The rats received 1.2 g/kg dextrose or the same volume of normal saline depending on the group. I/R was induced by a 30 min period of ischemia followed by reperfusion for 4 h. For 1 h before I/R injury, intravenous 4000 IU/kg of EPO was administered. EPO

pretreatment significantly reduced the infarct size, number of apoptotic cells, and caspase-3 activity compared with untreated I/R rats in hyperglycemic condition. I/R-induced down-regulation of Bcl-2 in myocardium was remarkably attenuated in EPO pretreatment groups under hyperglycemic conditions. EPO administration significantly down-regulated the level of GATA-4 degradation, which was associated with increased stability caused by ERK-regulated GATA-4 post-translational modifications in the myocardium following I/R in hyperglycemic conditions.

These results indicate that the protective mechanism of EPO pretreatment before I/R injury involves up-regulation of Bcl-2 via not only down-regulation of caspase-3 activity but also increase of ERK-induced GATA-4 stability in hyperglycemic condition.

Key words : ischemia-reperfusion injury, erythropoietin, hyperglycemia, caspase-3 activity, cell signaling pathway, GATA-4, bcl-2

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I. INTRODUCTION

Hyperglycemia (HG) is a well known risk factor for cardiovascular events regardless the presence of diabetes mellitus (DM).[1, 2] Even transient HG, which is often present in cardiac surgery or myocardial infarction, could aggravate myocardial ischemia/reperfusion (I/R) injury incurring poor prognosis.[3] Although detailed mechanisms for the loss of cardioprotective effect by HG is still unclear, aggravation of reactive oxygen species (ROS) production and apoptosis, mitigation of protective signal pathways, down-regulation of endothelial nitric oxide synthase activities, and impairment of mitochondrial K_{ATP} channel function had been proposed as plausible mechanisms.[3-6]

Furthermore, HG has been reported to attenuate the cardioprotective effects of anesthetics or other pharmacologic measures against I/R injury in both experimental and clinical settings.[7-9] The degree of myocardial injury was

directly related with the severity and duration of HG.[10, 11] Of note, HG's negative influence could be reduced by increasing the dose of protective agents in moderate HG (blood glucose level < 300 mg/dl) but not in profound HG (blood glucose level 300-600 mg/dl).[11, 12] Since perioperative HG is mostly transient or moderate, evaluation of protective agents to avoid HG would be useful to reduce myocardial injury against I/R injury.

Apart from its primary function on erythropoiesis, erythropoietin (EPO) has been used for reducing organ damage caused by I/R injury thorough multidisciplinary ways.[13-15] EPO decreased cardiac myocyte apoptosis via diverse signal transduction pathways including phosphatidylinositol 3 kinase (PI3K)/AKT and extracellular signal-regulated kinase (ERK) 1/2.[16, 17] Under normoglycemic (NG) condition, EPO's cardioprotective effects has been shown to be related to enhanced anti-apoptotic effect like induction of Bcl-2 expression.[18, 19] However, the molecular mechanisms of EPO in HG are not fully understood.

Considering the multidisciplinary cardioprotective mechanisms of EPO including anti-apoptotic effect, we hypothesized that EPO may provide myocardial protection against I/R injury even under HG. The aim of this study was to investigate EPO's ability to provide myocardial protection against I/R injury under HG and its related mechanisms in terms of Bcl-2 regulation through caspase-3 and GATA-4.

II. MATERIALS AND METHODS

1. Animal preparation

All animal experiments were approved by the committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.[20] Male Sprague-Dawley rats (10-12 wk old, 250-300 g) were anesthetized with pentobarbital (50 mg/kg, intraperitoneally), which was given repeatedly (25 mg/kg) every 60-90 min as necessary. The rats were intubated with a 16-gauge (G) catheter and artificially ventilated (Harvard Apparatus 683, Holliston, MA) at 30-35 cycles/min. The right internal jugular vein was cannulated with a 24-G catheter for IV access and the carotid artery was cannulated with a 24-G catheter, which was connected to the power lab system (ML845 PowerLab with ML132; AD Instruments, Colorado Springs, CO) for monitoring the mean arterial pressure (MAP) and heart rate (HR). The body temperature was maintained around 37℃ using a heating pad and continuously monitored throughout the experiment.

2. Experimental models and study groups

We employed a rodent model of myocardial I/R injury as described previously.[21] Rats underwent 30 min of the left anterior descending coronary

artery (LAD) occlusion followed by 4 h of reperfusion. Ischemia and reperfusion were confirmed by the appearance of a regional cyanosis and a marked hyperemic response on the corresponding distal myocardium, respectively. MAP and HR were continuously monitored during the procedures (baseline, during ischemia, after reperfusion) and were recorded. All rats underwent a left thoracotomy and pericardiotomy to expose the heart. To examine the effect of EPO on cardioprotection, EPO (4000 IU/kg) was administered 1 h before ischemia, while the control groups received equivalent amount of normal saline via the IV catheter at the same time. The rats in the HG groups received dextrose (1.2 g/kg) 1 h before ischemia, while the normoglycemia (NG) groups received equivalent volume of normal saline via the IV catheter at the same time. A blood sugar concentration >11.1 mmol/L was considered HG.[22] Blood sugar concentrations were serially monitored.

The animals were randomly assigned to six groups: 1) NG- Sham group (N=6); normal saline only, 2) NG- I/R control (IRC) group (N=15); normal saline + I/R, 3) NG- I/R EPO (IRE) group (N=15); normal saline + EPO + I/R, 4) HG- Sham group (N=16); dextrose only, 5) HG- IRC group (N=15); dextrose + I/R, 6) HG- IRE group (N=15); dextrose + EPO + I/R.

Hearts were collected at the end of reperfusion and used for protein extraction for immunoblot analysis.

3. Area at risk and infarct size determination

At the end of the 4 h reperfusion, myocardial infarction was determined by Evans blue (1%) and 1% solution of phosphate-buffered 2,3,5-triphenyltetrazolium chloride (TTC) as described previously.[23] Hearts were cut into five to six slices from apex to base. Viable non-ischemic myocardium was stained blue with TTC. Ischemic myocardium was stained pale white, whereas the viable myocardium in ischemic zone was stained red. Area at risk and area of non at risk of heart images were measured using image J software. Area at risk (AAR) = sum of area at risk (RA)/ sum of the total left ventricular area (LV) X 100%. Infarct size (IS) = sum of infarct area (IA)/ sum of area at risk (RA) X 100%.

4. TUNEL assay

Detection of apoptosis from each group was examined using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method.[23] Following infarct size assessment, LV tissues from the ischemic zone and from the remote myocardium were fixed in formalin for 24 h and embedded in paraffin, and 5-μm sections were obtained. The sections were then deparaffinized and rehydrated with xylene and graded alcohol series. The sections were stained using the in situ DeadEndTM Colorimetric Apoptosis Detection System (Promega, Madison, WI) according to the manufacturer's instructions. In short, tissue sections were washed in PBS and then fixed in 4% paraformaldehyde solution prior to incubation in 20 μg/ml proteinase K for 10

min. Sections were washed in PBS and incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37℃ for 60 min to incorporate biotinylated nucleotides at the 30-OH DNA ends. The reaction was terminated by transferring the slices to 29 saline sodium citrate. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide. Finally, streptavidin horseradish peroxidase was bound to the biotinylated nucleotides, and peroxidase activity in each section was demonstrated by the application of a stable chromogen diaminobenzidine. When using this procedure, apoptotic nuclei were stained dark brown. The sections were counterstained with hemotoxylin for total nuclei counting. Four sections from each myocardial sample block were randomly selected and analyzed by a blind observer using an Olympus microscope with magnification X400.

5. Cell culture and transient transfection

Since the primary cardiac myocytes have low transfection efficiency, we used H9c2 rat embryonic cardiac cells. H9c2 rat embryonic cardiac cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 units/ml penicillin, and 100 μg/ml streptomycin. Culture media and supplements were purchased from WelGENE (Seoul, Korea). High-glucose (30 mM) DMEM were used. Cells were plated into 60 or 100 mm tissue culture dishes with each culture medium and incubated under normoxic

(5% CO_2 in air) or hypoxic (1% O_2, 5% CO_2, and 94% N_2) condition. EPO was purchased from calbiochem (La Jolla, CA). The MEK1/2 inhibitor U0126 was purchased from Cell Signaling Technology (Beverly, MA). The cells were transfected using Lipofectatmine 2000 reagent as recommended by the manufacturer (Invitrogen Life Technologies, CA, USA). Cells were cultured in low-glucose culture medium for 16 h, and the medium was changed with OptiMEM (Gibco, Life Technologies, CA, USA). After that, these cells were transfected with DNA constructs or with siRNAs. After 6 h, the medium was replaced with low-glucose culture medium for overnight. These cells were then exposed to culture mediums of high glucose concentration and incubated in hypoxia (1% O_2, 5% CO_2, and 94% N_2) for 2 h following reoxygenation for 4 h (5% CO_2 in air).

6. DNA constructs and gene knockdown by small interfering RNA (siRNA)

The His-GATA-4 expression plasmid was previously reported.[24] pcDNA and the constitutively active MEK1 plasmid (pFC-MEK1) were purchased from stratagene (La Jolla, CA). ON-TARGET*plus* SMARTpool siRNAs for rat GATA-4 and a non-targeting siRNA for siRNA-control were purchased from Dharmacon (Chicago, IL). Transfection of siRNA into H9c2 cells was performed according to the manufacturer's instructions. At 6 h post-transfection, cells were transiently transfected with His-GATA-4 and/or caMEK1 expression vectors for 6 h, and incubated for 24 h in culture medium. These cells were

washed and harvested, and immunoprecipitation and immunoblot analysis was performed.

7. Real-time PCR

Expression level of Bcl-2 mRNA in H9c2 cells was examined by real-time PCR. Total RNA isolation and real-time PCR analysis was performed as previously described.[25] RNeasy-mini kits were purchased from Qiagen (Valencia, CA, USA). *Maxime* RT PreMix kit was purchased from iNtRON Biotechnology (Sungnam, Korea). SYBR *premixExTaq*^{TM} was purchased from TaKaRa (Otsu, Japan). Each sample was examined in quadruplicate and Bcl-2 gene was normalized to the reference housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Fold differences were then calculated for each treatment group using normalized CT values for the control.

The primer sequences for real-time PCR were as follows: forward 5'-CATGCGACCTCTGTTTGA-3' / reverse 5'-GTTTCATGGTCCATCCTTG-3' for Bcl-2; forward 5'-CTGGAGAAACCTGCCAAGTA-3' and reverse 5'-AGACAACCTGGTCCTCAGTG-3' for GAPDH.

8. Ubiquitination, immunoprecipitation and immunoblot analysis

After appropriate treatments, the cells from each group underwent ubiquitination or immunoprecipitation assay as described previously.[24, 26] For

ubiquitination assay under each condition, the cells were pre-treated with 20 μM MG132 (a proteasome inhibitor, Sigma) and subjected to the administration of 20 μM U0126 or 20 IU/ml EPO as indicated, for additional 6 h of hypoxia-reoxygenation. Each cell lysate was immunoprecipitated with the appropriate primary antibodies and protein G-agarose beads, and then subjected to SDS-PAGE and immunoblot analysis. Anti-casepase-3, Anti-cleavage-caspase-3, Anti-acetyl lysine, anti-phospho ERK, anti-ERK, anti-ubiquitin antibody and cell lysis buffer were purchased from Cell Signaling Technology (Beverly, MA). Anti-GATA-4, Anti-Actin, and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho GATA-4 at Ser 105 was purchased from Abcam (Cambridge, MA). Each experiment was performed at least three times.

9. Caspase-3 activity

The activity of caspase-3, an enzyme that initiates apoptosis, was measured in heart tissue lysates using a colorimetric assay from the BioVision Research Products (Mountain View, CA) according to the manufacturer's instruction. When the provided substrate is cleaved by caspase-3, free DEVD-pNA chromophore is generated and quantified by light absorbance using a microplate reader at 405 nm. Cultured cardiomyocytes were harvested in lysis buffer (1 M DTT) after treatment, and cell extracts centrifuged to eliminate cellular debris. Protein concentration was determined with the Bradford assay (Bio-Rad, San

Diego, CA). Aliquots (50 mL) of the cell extracts were incubated at 37℃ for 2 h in the presence of the chromophore substrate. Fold-increase in caspase-3 activity was determined by comparing absorbance of pNA from apoptotic samples with uninduced controls.

10. Statistical Analysis

All results were expressed as mean ± standard deviation. The statistical analysis was performed using one-way analysis of variance (ANOVA) or Student's *t* test followed by Bonferroni correction. Values of $P < 0.05$ were considered significant.

III. RESULTS

1. Hemodynamic and glycemic parameters

Data for MAP and HR are indicated in Table 1. Baseline MAP and HR were similar among the groups. Compared to baseline values, MAP significantly decreased in the NG-IRE during ischemia and after reperfusion while it significantly decreased in the HG-IRC during ischemia. MAP was significantly lower in the NG-IRE group than in the NG-IRC during ischemia and lower in the HG-IRE than in the HG-IRC after reperfusion. In all groups, HR significantly reduced during ischemia compared to baseline (Table 1).

There were no differences in mean baseline blood sugar concentration among the groups. Blood sugar concentrations were significantly higher in the HG groups before ischemia and after reperfusion than in the NG groups (Table 2).

Table 1. Changes in hemodynamic parameters (mean ± standard deviation)

Parameter/group	Baseline	During ischemia	After reperfusion
MAP			
Normoglycemia			
Control	117±19	100±12	100±17
EPO	118±24	75±16$^{*,\#}$	86±16*
Hyperglycemia			
Control	109±16	88±19*	111±11
EPO	102±14	89±23	100±11$^{\#}$
HR			
Normoglycemia			
Control	521±98	288±187*	464±138
EPO	494±31	334±51*	531±50
Hyperglycemia			
Control	537±117	244±195*	486±152
EPO	499±90	240±180*	467±124

Baseline = MAP and HR monitored at 15 min after starting IV anesthetics; during ischemia = MAP and HR monitored at 30 min during myocardial ischemia; after reperfusion = MAP and HR monitored at 4 h of reperfusion, respectively. MAP = mean arterial blood pressure; HR = heart rate; EPO = erythropoietin. $^{*}P < 0.05$ compared with the baseline. $^{\#}P < 0.05$ compared with the nomoglycemia groups.

Table 2. Blood sugar concentrations (mmol/L and mean ± standard deviation)

Group	Baseline	Before ischemia	After reperfusion
Normoglycemia			
Control	5.7±0.4	5.6±0.5	6.8±0.5
EPO	5.8±0.4	5.7±0.5	6.6±0.5
Hyperglycemia			
Control	5.7±0.3	17.1±0.8$^{*,\#}$	18.4±0.7$^{*,\#}$
EPO	5.6±0.4	17.2±0.7$^{*,\#}$	17.9±0.6$^{*,\#}$

Baseline = blood collected before administering dextrose; before ischemia = blood collected before inducing ischemia; after reperfusion = blood collected after reperfusion 4 h, respectively. EPO = erythropoietin. $^{*}P < 0.05$ compared with the baseline. $^{\#}P < 0.05$ compared with the nomoglycemia groups.

2. EPO reduced infarct size under HG as well as NG

To investigate whether EPO provides cardioprotection against in myocardial I/R injury under HG as well as NG, we created a transient HG with intravenous injection of dextrose before EPO treatment and I/R injury. Myocardial infarct size was demarcated by TTC staining. The infarct size was significantly smaller in the NG-IRE group than in the NG-IRC group (30±8%.*vs* 62±8%, P=0.008) and also smaller in the HG-IRE group than in the HG-IRC group (38±5% *vs* 68±5%, P=0.0005) (Figure 1). The degree of decrease in infarction size with EPO treatment was 52% and 44% in the NG-IRE and HG-IRE group compared to the NG-IRC and HG-IRC group, respectively.

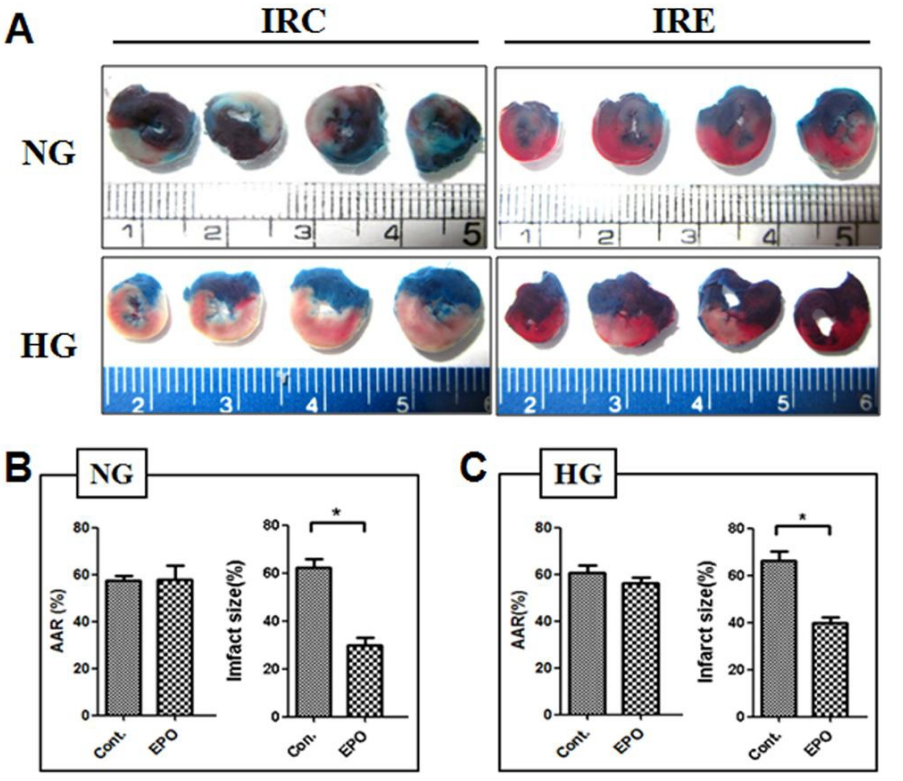

Figure 1. EPO reduced infarct size under hyperglycemia as well as normoglycemia. (A) Representative pictures of left ventricle sections after TTC staining in normoglycemic (NG) and hyperglycemic (HG) groups treated with or without EPO (4000 IU/kg). (B, C) Area at risk (AAR) and infarct size expressed as a percentage of left ventricular area and area at risk, respectively. *P < 0.05 compared to saline (control) treated rat hearts. AAR = area at risk, IRC = rats underwent ischemia (30 min) - reperfusion (4 h) only, IRE = rats treated with EPO (4000 IU/kg) before ischemia-reperfusion injury. N=6 hearts in each group.

3. EPO decreased apoptotic cardiomyocytes under HG following I/R

In HG, increase in apoptotic cardiomyocytes plays an important role in the aggravation of myocardial I/R injury compared with NG.[27] Heart tissues from sham-operated rats revealed low numbers of apoptotic cells in both NG and HG group (Figure 2). The number of TUNEL-positive myocytes in ischemic myocardial tissue was 25% greater in the HG-IRC group than in the NG-IRC group. EPO pretreatment significantly attenuated apoptotic cardiomyocytes by 47% in the NG-IRE group compared to the NG-IRC group (148±18 *vs* 277±41, P=0.002) and by 40% in the HG-IRE group compared to the HG-IRC group (225±13 *vs* 372±35, P=0.0004).

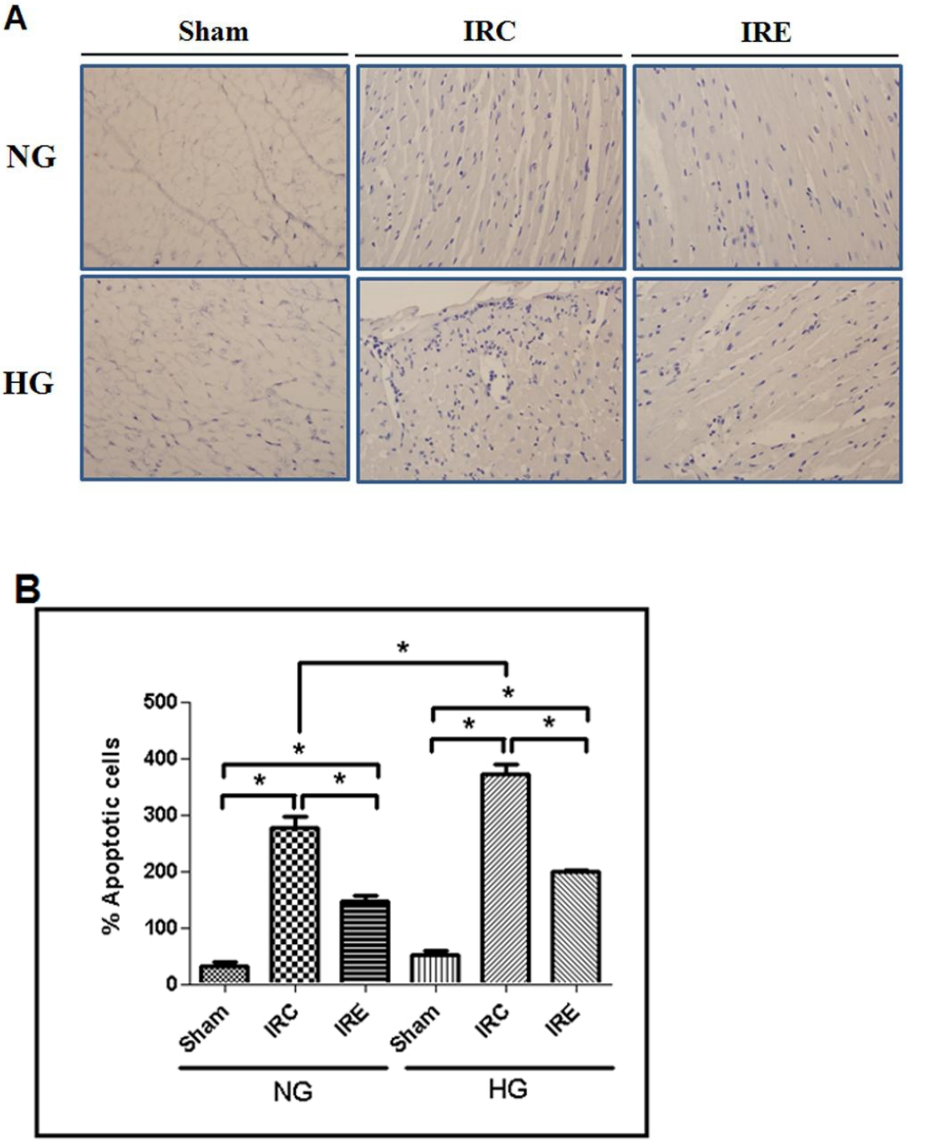

Figure 2. EPO decreased cardiac myocytes apoptosis under normoglycemia as well as hyperglycemia following I/R. (A) TUNEL assay shows the apoptotic cardiomyocytes after ischemia/reperfusion in normoglycemic (NG) and hyperglycemic (HG) rats. (B) TUNEL-positive nuclei of cardiomyocytes were

determined by random counting of four fields per section. . *P < 0.05 for intergroup comparisons. Sham = rats not underwent ischemia-reperfusion, IRC; rats underwent ischemia (30 min) - reperfusion (4 h) only, IRE; rats treated with EPO (4000 IU/kg) before ischemia-reperfusion injury. N = 4 hearts in each group.

4. EPO reduced caspase-3 activity

Our results have shown that the EPO-induced cardioprotection in HG as well as NG. In HG rats, cleaved capase-3 protein levels were downregulated by EPO treatment, which were increased with I/R injury (Figure 3A). The degree of decrease in caspase-3 activity with EPO treatment was 28% in the HG-IRE group compared to the HG-IRC group (Figure 3B). Those differences were statistically significant (P=0.0002). We further confirmed the regulation of caspase-3 activity, one of the initiate apoptotic signaling pathway.[28]

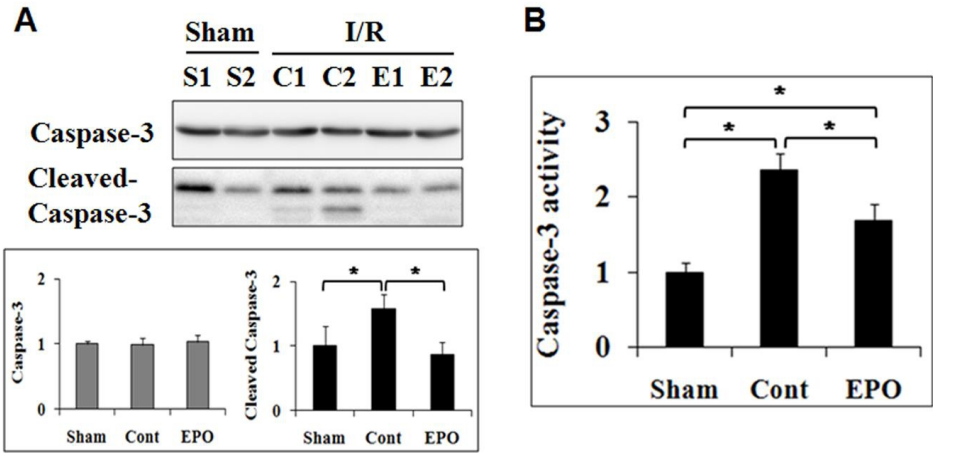

Figure 3. EPO reduced caspase-3 activity in hyperglycemia. (A) The hyperglycemic (HG) rats were subjected to ischemia (30 min) and reperfusion (4 h). The hearts were harvested for the use of protein preparation. The protein levels of caspase-3 and cleaved caspase-3 were determined by immunoblot analysis with specific antibodies. N = 5 in each group. Lower panels were quantified signal by scanning densitometry. (B) The heart tissue proteins were harvested for measuring caspase-3 activity as described in Methods section. Values are means ± standard deviation from 5 independent experiments. Bars with different superscript letters are significantly differentiated from each other (*P < 0.05). S=Sham, C=control, E=EPO group.

5. EPO upregulated I/R-induced Bcl-2 reduction was in the HG rat heart

In HG rats, Bcl-2 protein level was more prominent in EPO treatment group than the untreated group during I/R damage (Figure 4A). The upregulation of Bcl-2 might be one of the mechanisms by which EPO-downregulated caspase-3 activity in HG. Furthermore, Bcl-2 is a downstream molecule from GATA-4 mediated survival pathways for cardioprotection.[31-33] Thus, we examined how endogenous GATA-4 knock-down or exogenous GATA-4 overexpression affected Bcl-2 expression under HG in H9c2 cells. As shown Figure 4B, Bcl-2 mRNA expression decreased by *siGATA-4* and increased by GATA-4-overexpression, which demonstrated that GATA-4 could affect the expression of Bcl-2 in H9c2 cells in HG.

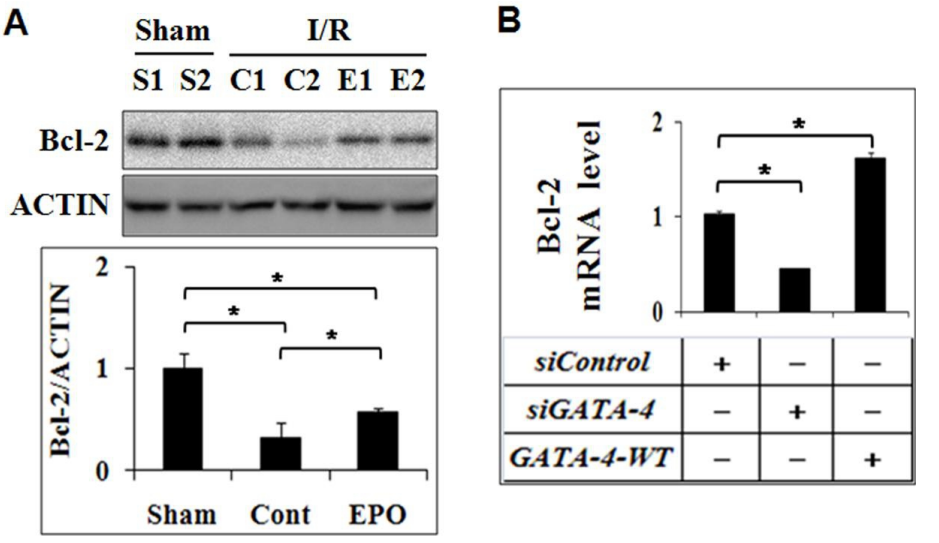

Figure 4. I/R-induced Bcl-2 reduction was upregulated by EPO in hyperglycemia. (A) The hyperglycemic (HG) rats were subjected to ischemia (30 min) and reperfusion (4 h). The hearts were harvested as described above. The levels of Bcl-2 and ACTIN were determined by immunoblot analysis with specific antibodies. N = 5 in each group. Lower panels were quantified signal by scanning densitometry. (B) Knock-down GATA-4 using siRNA suppressed Bcl-2 mRNA expression in HG medium. H9c2 cells were transfected with siRNAs for GATA-4 (*siGATA-4*) and a non-targeting control siRNA (*siControl*) and GATA-4 wild type (WT) plasmids. These cells were subjected to hypoxia for 2 h followed by reoxygenation for 4 h in HG medium and then real-time PCR were performed. Values are means ± standard deviation from 5 independent experiments. Bars with different superscript letters are significantly differentiated from each other (*P < 0.05)

6. EPO increased GATA-4 phosphorylation and acetylation via ERK in HG rat myocardium

As the MAP kinase pathway was previously shown to be activated by EPO and one of the major downstream signaling pathways for GATA-4 and Bcl-2,[18, 34] we evaluated which MAP kinases were responsible for EPO mediated cardioprotection against I/R injury. In HG-sham, EPO enhanced ERK phosphorylation in myocardium compared with control (data not shown). In HG rats, phosphorylated and acetylated GATA-4 protein levels were down-regulated by I/R injury, which were attenuated with EPO pretreatment (Figure 5).

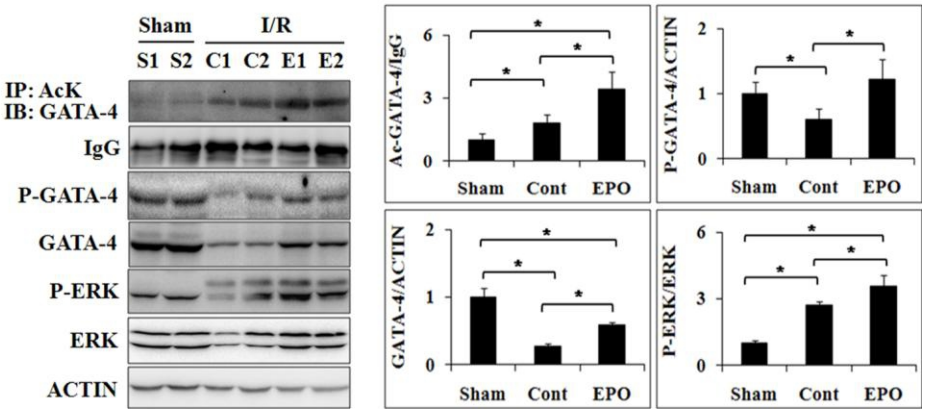

Figure 5. EPO increased GATA-4 phosphorylation and acetylation in hyperglycemic rat myocardium. The hyperglycemic (HG) rats were subjected to ischemia (30 min) and reperfusion (4 h) and EPO was pretreated before ischemia. The hearts were collected at the end of reperfusion. Immunoprecipitation (IP) and IB analysis were then performed. Ack, anti-acetylated lysine antibody. Right panels were quantified signal by scanning densitometry. Values are means ± standard deviation from 5 independent experiments. Bars with different superscript letters are significantly differentiation from each other (*P < 0.05). S=Sham, C=control, E=EPO group.

7. EPO increased GATA-4 phosphorylation and acetylation via ERK in HG in H9c2 cells

Additionally, *in vitro*, we confirmed that EPO-mediated PTMs of GATA-4 occurred via ERK signaling pathway in HG. To further explore the specific role of EPO mediated cardioprotection regarding ERK signaling pathway under HG, U0126, an inhibitor of MEK1/2, or the constitutively active MEK1 (*ca*MEK1) plasmid were administrated in H9c2 cells. As shown Figure 6A, in HG medium, EPO stimulated GATA-4 phosphorylation and acetylation compared to the control, whereas U1026 treatment attenuated these responses. Since endogenous GATA-4 is expressed at a low level in cells,[35] the experiments employed exogenous GATA-4 overexpression. Furthermore, to clarify the association between EPO induced GATA-4 stability and ERK phosphorylation in HG, experiments were carried out with transiently transfected H9c2 cells with pcDNA or *ca*MEK1 expression vectors for GATA-4 overexpression following administration of U0126. *ca*MEK1 overexpression alone enhanced GATA-4 phosphorylation similar to EPO pretreatment and synergistically increased GATA-4 acetylation and protein levels in H9c2 cells in HG medium (Figure 6B).

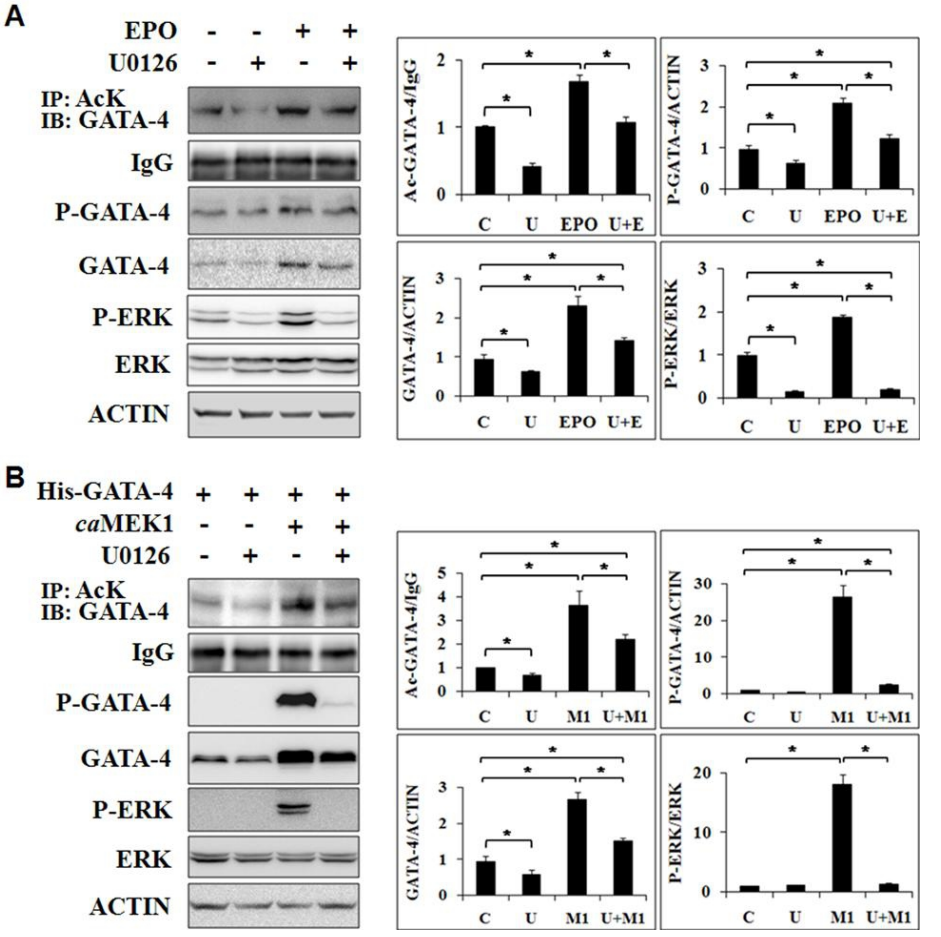

Figure 6. EPO increased GATA-4 phosphorylation and acetylation in H9c2 cells in hyperglycemic (HG) medium.

(A) H9c2 cells were pretreated with vehicle (DMSO) or U0126 for 1 h and treated with EPO for 1 h and further exposed to hypoxia for 2 h followed by reoxygenation for 4 h in HG medium and then immunoprecipitation (IP) and immunoblot (IB) analysis were performed. (B) H9c2 cells were transiently transfected with pcDNA, His-GATA-4, and constitutively active MEK1

(*ca*MEK1) expression vectors and incubated for 16 h. These cells were pretreated with vehicle (DMSO) or U0126 for 1 h and further exposed to hypoxia for 2 h followed by reoxygenation for 4 h in high glucose (HG) medium and then IP and IB analysis were performed. Ack, anti-acetylated lysine antibody. Right panels were quantified signal by scanning densitometry. Values are means ± standard deviation from 4 independent experiments. Bars with different superscript letters are significantly differentiation from each other (*P < 0.05). C=control, U=U0126, E=EPO, M1= *ca*MEK1.

8. EPO diminished H/R-induced GATA-4 ubiquitination via ERK in HG in H9c2 cells

Since GATA-4 stability is related with ubiquitination,[26] we analyzed whether EPO activated ERK phosphorylation affected endogenous GATA-4 ubiquitination. In the presence of MG132 (a proteasomal inhibitor), H/R-induced GATA-4 ubiquitination was protected by EPO treatment while U0126 pretreatment diminished this protective effect in H9c2 cells in HG (Figure 7A). In H9c2 cells in NG medium, similar results were observed (data not shown). Overexpression of *ca*MEK1 attenuated hypoxia/reoxygenation (H/R)-induced GATA-4 ubiquitination in HG medium in H9c2 cells (Figure 7B).

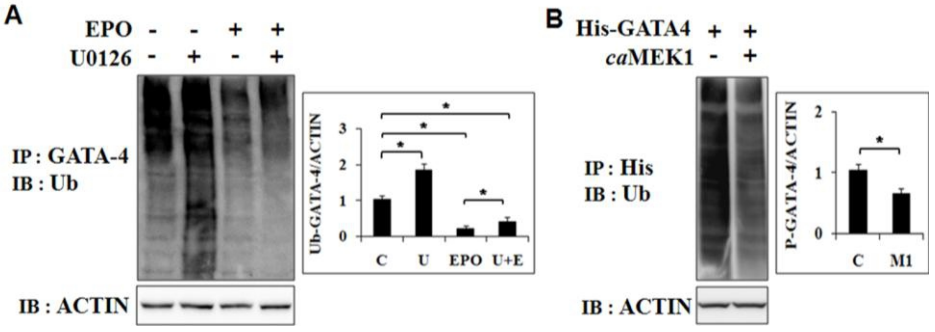

Figure 7. EPO diminished H/R-induced GATA-4 ubiquitination via ERK in H9c2 cells in high glucose (HG) medium. (A) H9c2 cells were treated with MG132 (20 μM) and vehicle (DMSO) or U0126 for 1 h and further incubated in the presence or absence of EPO for an additional 6 h during hypoxia/reoxygenation (H/R) in HG medium. IP and IB analysis were then performed. (B) H9c2 cells were transiently transfected with His-GATA-4, pcDNA, or *ca*MEK1 expression vectors and incubated for 16 h under normoxia. Cells were incubated in the presence of MG132 (20 μM) for 6 h during H/R in HG medium. IP and IB analyses were then performed. Ub, anti-ubiquitin antibody. Right panels were quantified signal by scanning densitometry. Values are means ± standard deviation from 4 independent experiments. Bars with different superscript letters are significantly differentiation from each other (*P < 0.05). C=control, U=U0126, E=EPO, M1= *ca*MEK1.

IV. DISCUSSION

HG is commonly encountered in acute disease states and perioperative period, which often complicates the patients' prognosis by aggravating inflammatory response and ischemic organ damage.[36, 37] Acute and chronic HG were reported to be associated with increased apoptosis, mitigation of the activities targeted to organ protective pathways, impaired nitric oxide (NO) availability,[38] and excessive ROS production during I/R.[2] In conjunction, recent studies have depicted that even transient HG episodes during I/R exerted significant influence on patients' outcome.[3] Thus, accounting for the frequent occurrence of HG in clinical theatre and its' potential to aggravate myocardial I/R injury, studies addressing efficient protective strategies seem to be mandatory. Of interest, previous studies have shown that aggravated myocardial I/R injury in HG rats could be attenuated by increasing the intensities of protective measures if the degree of HG was moderate.[1, 2, 4, 9] This implies that powerful protective measures strong enough to overcome HG-induced adverse influence on cell survival would still provide cardioprotection even under HG.

EPO is an innate hormone mainly produced from the kidney. Apart from its hematopoietic effect, numerous literatures support the organ protective role of EPO against I/R injury through its anti-oxidative, anti-inflammatory, and anti-apoptotic effects.[39, 40] In addition to its pluripotent action, EPO was reported to up-regulate the activities of GATA-4, which is also involved various

survival signaling pathways of cardiac myocytes including regulation of anti-apoptotic protein Bcl-xl.[15, 18, 41] Thus, the cardioprotective potency of EPO was considered to be able to overcome the aggravation of myocardial I/R injury by HG and we investigated the underlying molecular mechanisms in HG condition.

In the present study, EPO reduced both infarct size and degree of cardiomyocyte apoptosis following I/R injury in HG rats as well as NG rats (Figure 1, 2). The cardiomyocytes apoptosis after I/R injury was greater in the HG rats than in the NG rats (Figure 2), which are in accordance with the results of previous studies that HG exaggerated myocardial infarct size and cardiomyocyte injury.[1, 42]

One of the cysteine proteinases, Caspase-3 has been shown to be specifically involved in the initiation of apoptosis signaling pathway and is also known to cleave bcl-2.[30, 43, 44] As previously reported,[45] I/R induced capspase-3-dependent apoptosis, and we hereby confirmed that EPO was cardioprotective effect via attenuation of caspase-3 activity in HG (Figure 3). Bcl-2, an anti-apoptotic protein, is cleaved by caspase-3 during apoptosis, and is important for preventing myocardial injury.[18, 29, 30] The caspase-3 inhibition by EPO ameliorated I/R-induced Bcl-2 down-regulation in HG (Figure 4A).

Additionally, GATA-4 partly mediated regulation of Bcl-2 protein expression in anti-apoptotic signaling pathway plays an important role in cardioprotection.[33] Phosphorylated GATA-4 binds to Bcl-2 gene promoter and

subsequently, promotes Bcl-2 gene expression and activates cell survival signaling pathway.[31, 33] In NG, GATA-4 induced cardioprotection against myocardial I/R injury was reported to be associated with Bcl-2 regulation.[18] We confirmed the Bcl-2 mRNA expression was partly abolished by knock-down GATA-4 in H9c2 cells in HG medium (Figure 4B). These down-regulations of Bcl-2 and GATA-4 were significantly diminished with pretreatment of EPO in HG condition *in vivo* and *in vitro*, which were accompanied with EPO-activated ERK phosphorylation (Figure 4, 5). Role of increased ERK phosphorylation for EPO induced cardioprotection has been discussed well in previous studies.[18] Furthermore, EPO was also associated with increased GATA-4 acetylation, which was decreased following I/R injury in the HG-IRC group (Figure 5). In H9c2 cells in HG medium, pretreatment with U0126, an inhibitor of EPO induced ERK phosphorylation, abolished GATA-4 phosphorylation and acetylation (Figure 6A).

Besides of GATA-4 phosphorylation and acetylation, EPO diminished H/R-induced GATA-4 ubiquitination in H9c2 cells in HG medium (Figure 7A). The decrease of GATA-4 ubiquitination by EPO pretreatment enhanced GATA-4 stability against I/R injury and that is partly involved in up-regulation of Bcl-2 expression in HG. The same results were also observed in GATA-4 overexpressed H9c2 cells that underwent H/R in HG medium. *ca*MEK1 stimulated GATA-4 phosphorylation via ERK phosphorylation leading to increased GATA-4 acetylation and diminished H/R-induced GATA-4

ubiquitination in H9c2 cells in HG medium (Figure 6B, 7B). The results suggest the sustained cardioprotective effects of EPO against H/R or I/R injury in HG condition involved regulations of various GATA-4 PTMs, which were similar in NG condition. In particular, decreased GATA-4 ubiquitination in HG, a distinct feature of EPO signaling transduction in GATA-4 regulation, is a novel finding of the current study, which further aids elucidating the associated mechanisms of EPO induced cardioprotection.

In contrast to the results of the current study, DM abrogated EPO induced cardioprotetive effect against I/R injury in isolated heart model.[12] EPO (1000 IU/kg) administered immediately after I/R injury failed to increase the reperfusion injury salvage kinase pathway including Akt and ERK1/2 in type I diabetes models. Differences in animal model as well as timing and dose of EPO should affect the diverse results compared to the current study. Above all, duration of HG should also exert influences in that regard. Generally, longer duration of DM or hyperglycemia more significantly repressed organ protective pathways and mitigate protective effects of pharmacologic conditioning in vivo and in vitro experiment.[46, 47] Thus, it remains to be proven whether EPO could provide cardioprotection against I/R injury in DM as well. Yet, considering the frequent occurrence of transient HG in acute disease states and perioperative period, and the raised concerns regarding its adverse influence on the patients' outcome regardless of the presence of DM,[1, 2] the results of this study might have significant clinical relevance. Furthermore, the use of a single dose of EPO

for organ protection has already been validated in clinical field in terms of safety and convenience. It merits further studies to translate the results of the current study into the clinical field.

V. CONCLUSION

In transient moderate HG condition, EPO significantly diminished cardiomyocyte apoptosis and myocardial infarct size. It is attributable to EPO induced Bcl-2 expression through the inhibition of caspase-3 and EPO related ERK activation leading to sustained stability of GATA-4. These findings suggest the regulation of GATA-4 post-transcriptional modification may be one of the protective mechanisms by EPO and the feasibility of the use of an EPO treatment as a therapeutic strategy to provide myocardial protection against I/R injury under acute hyperglycemia.

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ABSTRACT (IN KOREAN)

Caspase-3 억제와 GATA-4 조절을 통해 bcl-2을 증가시킴으로서 고혈당증에 의해 악화된 심근의 허혈-재관류 손상을 감소시키는 erythropoietin의 효과

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전나형

Erythropoietin (EPO)은 erythroid precursor cells의 생존과 증식을 촉진시킴으로써 erythropoiesis를 조절하는 물질로, 허혈에 의한 심근의 손상을 줄여준다. 고혈당증은 급성심근 경색이나 심장 수술 중에 흔히 발생하며 장기 손상을 증가시키고 심근보호를 위한 치료의 효과를 감소시킨다. 본 연구는 EPO가 정상혈당 쥐에서 뿐 만 아니라 고혈당쥐에서도 심근 허혈 재관류 손상을 줄여주는지 알아보고자 하였다.

마취유도 후 정상혈당 쥐와 고혈당증을 유발한 쥐들을 대상으로 하여 각각 sham군과 허혈-재관류군 및 EPO 치료군의 모두 여섯 군으로 나누어 실험을 진행하였다. 고혈당군 쥐들에게는 킬로그램당 덱스트로즈 1.2그램을 주입하였고 정상혈당 군 쥐에게는 동량의

생리식염수를 투여하였다. 허혈 재관류 손상은 30분의 허혈과 4시간의 재관류를 동해 이루어졌고 EPO 투여 군의 경우 허혈 재관류 손상 1시간 전에 킬로그램당 4000IU의 EPO를 주입하였다

정상혈당 군 뿐만 아니라 고혈당 군에서도 EPO를 전처리 하였을 때 그렇지 않은 군에 비해 심근 경색 정도와 세포사멸 수, 그리고 caspase-3 activity가 감소하였다. 고혈당 조건에서 EPO 전처리시 심근의 허혈 재관류 손상에 의한 bcl-2의 감소 정도가 확연히 줄어들었다. EPO 주입시 GATA-4의 분해가 감소하였고 이는 허혈 재관류 손상 후 ERK에 의한 GATA-4의 post-translational modification을 통해 GATA-4의 안정성이 증가되었기 때문이다.

이와 같은 결과는 고혈당증에서의 심근의 허혈 재관류 손상 전에 EPO 투여에 의한 심근보호 효과는 caspase-3 activity의 감소와 ERK 활성화에 의한 GATA-4 안정성의 증가를 통해 bcl-2가 증가되었기 때문임을 나타낸다.

핵심되는 말: 허혈-재관류 손상, erythropoietin, 고혈당증, 세포사멸, 세포 신호전달 체계, GATA-4, bcl-2