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Ethyl pyruvate retains its protective effect against myocardial ischemia-reperfusion injury under hyperglycemic condition

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Directed by Professor Jae-Kwang Shim

The Doctoral Dissertation
submitted to the Department of Medicine,
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Sarah Soh

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Sarah Soh

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ABSTRACT

Ethyl pyruvate retains its protective effect against myocardial ischemia-reperfusion injury under hyperglycemic condition

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(Directed by Professor Jae-Kwang Shim)

High mobility group box 1 (HMGB1) is one of the key inflammatory mediators known to exert its influence via receptors of advanced glycation end products (RAGE)/toll-like receptors (TLR)–NF-κB signaling pathway in myocardial ischemia/reperfusion (I/R) injury. Also, HMGB1–TLR axis has been shown to play a pathognomonic role in triggering cardiomyocyte apoptosis in myocardial I/R injury. Hyperglycemia, which is common in acute disease state such as acute myocardial infarction or cardiac surgery, is known to aggravate myocardial I/R injury. To complicate matters, hyperglycemia attenuates the protective efficacies of known preventive strategies. Proposed mechanisms include amplification of the HMGB1–RAGE/TLR–NF-κB pathway activation by hyperglycemia. Therefore, under hyperglycemic condition, blockade of the foremost event of this signaling pathway, HMGB1, may ameliorate the

myocardial I/R injury. In that context, ethyl pyruvate (EP) could be an efficient protective measure because of its antagonistic effect on HMGB1 release and downstream pathway. This study aimed to investigate the protective role of EP against myocardial I/R injury under a clinically relevant moderate hyperglycemic condition and its associated mechanisms.

Sprague-Dawley rats (n = 104) were randomly assigned to eight groups: normoglycemia-Sham (NG-sham, n = 10), normoglycemia-I/R-control-saline (NG-IRC, n = 14), normoglycemia-EP treatment before ischemia (NG-pre-EP, n =14), normoglycemia-EP treatment upon reperfusion (NG-post-EP, n = 14), hyperglycemia-Sham (HG-sham, n = 10), hyperglycemia-I/R-control-saline (HG-IRC, n = 14), hyperglycemia-EP treatment before ischemia (HG-pre-EP, n = 14), and hyperglycemia-EP treatment upon reperfusion (HG-post-EP, n = 14). The rats received 1.2 g/kg dextrose or same volume of normal saline depending on the group before procedure. I/R was induced by a 30 minute-period of left anterior descending coronary artery ligation followed by reperfusion for 4 hours. At one hour before ischemia (pre-EP) or upon reperfusion (post-EP), intravenous 50 mg/kg of EP was administered.

Hyperglycemia resulted in exacerbation of myocardial infarct size with amplification of HMGB1–RAGE/TLR–NF-κB pathway activation compared to normoglycemia following I/R. As compared with IRC and pre-EP, post-EP significantly reduced myocardial infarct size and myocardial apoptosis after myocardial I/R under normoglycemia and this protective effect of EP was preserved under hyperglycemic condition. EP treatment upon reperfusion decreased HMGB1 protein level and the expression of TLR-2 and TLR-4 in the

area of induced ischemia. The decreased NF-κB phosphorylation by EP treatment upon reperfusion was associated with diminished tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 expression under hyperglycemia. EP treatment upon reperfusion also attenuated I/R-related down-regulation of Bcl-2 and up-regulation of Bax, and decreased I/R-induced cardiomyocytes apoptosis.

In conclusion, EP treatment upon reperfusion conveyed significant myocardial protection against the myocardial I/R injury under both normoglycemic and hyperglycemic conditions. Associated mechanisms involved attenuated increase in HMGB1 level and suppression of its down-stream pathways, which were common to both glycemic conditions.

Key words: ethyl pyruvate, high-mobility-group box-1, hyperglycemia, ischemia-reperfusion, receptors of advanced glycation end products

I. INTRODUCTION

Hyperglycemia is often present in acute disease state. Accordingly, patients with acute myocardial infarction (MI) frequently exhibit hyperglycemia, and even in non-diabetic patients, transient hyperglycemia is present in up to 40% of ischemic patients.[1] In extension, hyperglycemia is also commonly observed during cardiac surgery with cardiopulmonary bypass (CPB) because of systemic inflammation and insulin resistance.[2,3]

While previously regarded as a natural response to the imposed acute illness, cumulating evidence indicated adverse influence of transient hyperglycemia on outcome. Indeed, hyperglycemia could increase myocardial infarct size and myocyte apoptosis, exaggerate heart failure, and decrease survival after myocardial ischemia and reperfusion (I/R) injury by increasing inflammation and oxidative stress, which even resulted in abolished protective effect of ischemic preconditioning.[4-7] Moreover, it has been shown that many of the experimentally proven protective measures' efficacies against I/R injury are mitigated under hyperglycemia.[6,8,9] Therefore, an efficient therapeutic regimen still requires to be identified, which remains elusive.

Recently, high-mobility-group box-1 (HMGB1) was reported as a key activator that plays a central role in mediating injury and inflammation in diabetes via signaling through receptors of advanced glycation end products (RAGE) and toll-like receptors (TLR) that trigger NF-κB activation.[10] Even in non-diabetic models, hyperglycemia *per se* induced by glucose infusion, was associated with increased serum HMGB1 levels,[11] which played a major role in inducing myocardial I/R injury.[12] Also, HMGB1 and its receptor TLR-4 contributed to

cardiomyocyte apoptosis after I/R injury,[13] and inhibition of HMGB1 was shown to protect cardiomyocytes against apoptosis under hyperglycemia.[14] Therefore, we hypothesized that the aggravated myocardial I/R injury and apoptosis under hyperglycemia was associated with increased HMGB1–RAGE/TLR–NF-κB pathway activation, and blockade of the foremost event of this signaling pathway, HMGB1, could ameliorate the myocardial I/R injury and decrease cardiomyocyte apoptosis even under hyperglycemia.

In that context, ethyl pyruvate (EP) is a noteworthy pharmacologic intervention for myocardial protection against I/R injury under a clinically relevant moderate hyperglycemic condition. EP is an ester formed from the naturally occurring anti-oxidant pyruvate combined with ethanol for its chemical stability in aqueous solution.[15] It is an inhibitor of HMGB1[16] and the protective effect of EP on I/R injury has been reported in various organs including the kidney, liver and heart under normoglycemic condition.[17-19] Yet, albeit its solid theoretical background, it has never been tested whether EP retains its protective effect against myocardial I/R injury even in hyperglycemic condition. Of note, EP's proven clinical safety would enable its prompt clinical application, once its efficacy is proven in animal models mimicking the clinical scenario. Therefore, this study aimed to investigate the protective role of EP against myocardial I/R injury under hyperglycemia and seek the relevant mechanistic insights of EP in terms of the regulation of HMGB1–RAGE/TLR–NF-κB pathway.

II. MATERIALS AND METHODS

1. Animal preparation

All animal procedures 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. After being anesthetized with Rompun (Vial Korea, 10 mg/kg) plus Zoletil 50 (Virbac Korea, 30 mg/kg), 10-12 weeks old Sprague-Dawley rats (250-300 g) were intubated with a 16-gauge catheter and artificially ventilated (Harvard Apparatus 683, Holliston, MA) at 30-35 cycles/min. Rats were placed on an electric heating pad to maintain the body temperature at 37°C. Heparin (200 IU/ kg) was given before ischemia intravenously. The right femoral artery was cannulated to monitor mean arterial pressure (MAP) and collect blood. Heart rate (HR) was monitored by subcutaneous stainless steel electrodes connected to the power lab system (ML845 PowerLab with ML132; AD Instruments, Colorado Springs, CO).

2. Study groups and experimental models.

The animals were randomly assigned to eight groups: normoglycemia-Sham (NG-sham, n = 10), normoglycemia-I/R-control-saline (NG-IRC, n = 14), normoglycemia-EP treatment before ischemia (NG-pre-EP, n =14), normoglycemia-EP treatment upon reperfusion (NG-post-EP, n = 14), hyperglycemia-Sham (HG-sham, n = 10), hyperglycemia-I/R-control-saline (HG-IRC, n = 14), hyperglycemia-EP treatment before ischemia (HG-pre-EP, n

= 14), and hyperglycemia-EP treatment upon reperfusion (HG-post-EP, n = 14).

For hyperglycemia or normoglycemia, the rats received 1.2 g/kg dextrose or same volume of normal saline, respectively. A blood glucose concentration >11.1 mmol/L was considered as hyperglycemia.[20] The blood glucose concentration was determined at baseline, during ischemia, and after reperfusion. Pre-EP treatment group rats received intravenous 50 mg/kg of EP (Sigma, St. Louis, MO) at one hour before ischemia and post-EP treatment group rats received it upon reperfusion (Fig. 1).

For myocardial I/R injury *in vivo*, a thoracotomy through a left parasternal incision was performed to expose the anterior wall of the left ventricle. A 4-0 silk suture on a small curved needle was passed through the myocardium beneath the middle segment of the left anterior descending (LAD) coronary artery. A small vinyl flake was passed into both ends of the suture, which was then fixed by clamping the tube with a mosquito hemostat. A successful myocardial I/R model was confirmed by ST segment elevation on the electrocardiogram and regional cyanosis of the myocardial surface. The rats underwent a 30 minutes occlusion of the LAD, followed by a 4 h reperfusion. In the sham-operated animals, the same procedure was performed with the exception of the LAD ligation. The MAP and HR were continuously monitored during the procedures and recorded (baseline, during ischemia, and after reperfusion). The rats were euthanized and the hearts were harvested 4 h after reperfusion.

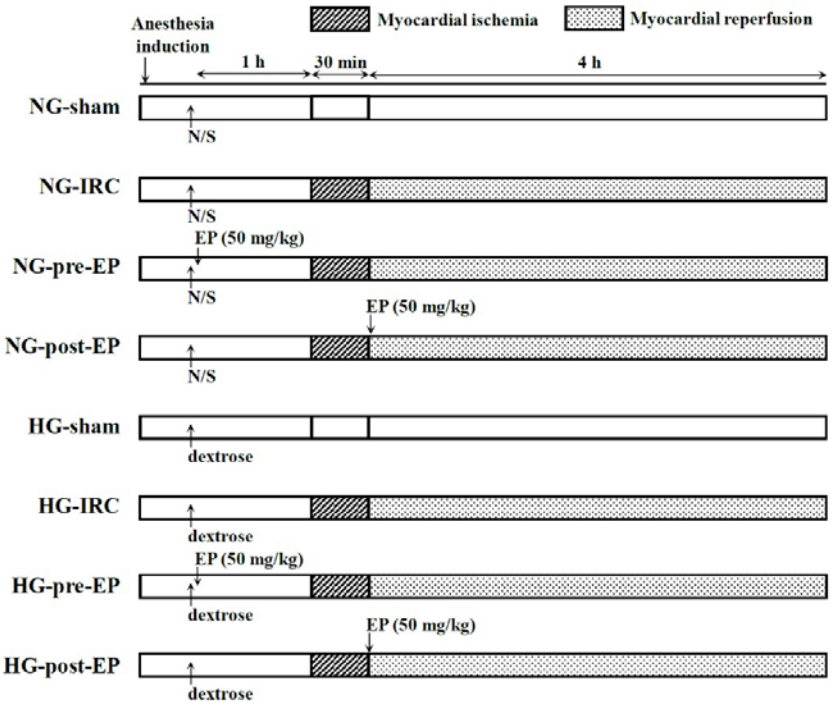

Figure 1. Study groups and experimental models. The animals were randomly assigned to eight groups: normoglycemia-Sham (NG-sham), normoglycemia-I/R-control-saline (NG-IRC), normoglycemia-EP treatment before ischemia (NG-pre-EP), normoglycemia-EP treatment upon reperfusion (NG-post-EP), hyperglycemia-Sham (HG-sham), hyperglycemia-I/R-control-saline (HG-IRC), hyperglycemia-EP treatment before ischemia (HG-pre-EP), and hyperglycemia-EP treatment upon reperfusion (HG-post-EP). For hyperglycemia or normoglycemia, the rats received 1.2 g/kg dextrose or same volume of normal saline, respectively. Pre-EP group rats received intravenous 50 mg/kg of EP at one hour before ischemia and post-EP group rats received it upon reperfusion.

NG; normoglycemia, IRC; ischemia/reperfusion-control-saline, EP; ethyl pyruvate, HG; hyperglycemia.

3. Histopathology examination

At the end of 4 h reperfusion, LAD was re-ligated at the original site and 1 ml of 2% Evans blue dye (Sigma-Aldrich) was injected intravenously. The left ventricle was sliced transversely into four to five slices (~2 mm). The sections were then incubated in phosphate-buffered saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) staining at 37°C for 20 minutes. The area at risk (AAR) was calculated as a percentage of left ventricle exhibiting absence of staining with Evans blue. The slices were incubated in 1% TTC in phosphate buffer (pH 7.4) at 37°C for 10 minutes and photographed with a digital camera. The borders of the infarct in each heart slice (TTC-staining area vs. TTC-staining negative area) were outlined and the area quantified using Image J software. Based on these measurements, Infarct size was calculated as a percentage of the AAR.

4. TUNEL assay

Detection of apoptosis on paraffin sections from each group was examined using the terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick end labeling (TUNEL) as described previously.[21] Five visual fields from each sample block were randomly selected and analyzed by a blind observer using an Olympus microscope (× 400). The apoptotic index was analyzed (apoptotic cells/total cells × 100%) from a total of 20 fields per sample.

5. Immunofluorescence

Immunofluorescence staining was examined for TLR-2 and TLR-4 on paraffin-embedded sections of myocardial tissue. Tissue sections were stained

with rabbit anti-TLR-2 and anti-TLR-4, followed by staining with FITC-conjugated secondary antibody (all Santa Cruz, CA). Slides were viewed with an Olympus U-LH100HG fluorescence microscope (Olympus America, NY) equipped with an Olympus DP71 digital camera (× 20)

6. Immunoblot analysis

After appropriate treatments, equal amount of protein from each group underwent immunoblot assay as described previously.[22] Proteins were separated on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with anti-HMGB1 (Abcam, Cambridge), anti-RAGE, anti-TLR-2, anti-TLR-4 (SantaCruz, CA), anti-Bcl-2, anti-Bax, anti-phospho-NF-κB, anti-NF-κB, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (all Cell Signaling Technology, Beverly, MA). Each experiment was performed at least three times.

7. Real-time polymerase chain reaction

Levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α messenger ribonucleic acid (mRNA) expression were examined by real-time polymerase chain reaction (PCR). Total RNA was isolated using RNeasy-mini kits (Qiagen, Valencia, CA, USA). Complementary deoxyribonucleic acid (cDNA) was synthesized from 1 μg of total RNA by using SuperScript™ II RNase H-Reverse Transcriptase kits (Thermo Fisher Scientific, USA). Real-time PCR analysis was performed using SensiFAST™ SYBR Hi-ROX kit (BIOLINE, USA) and AB7500 Fast Real-Time PCR System (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. Each sample was

analyzed in quadruplicate and target genes were normalized to the reference housekeeping gene, GAPDH. Fold differences were then calculated for each group using normalized C_T values for the sham groups. The primer sequences for real-time PCR as follows: forward 5'- GGTTTGCCATTTCATACCAG-3' and reverse 5'-GGTACATGGGCTCATACCAG-3' for TNF-α, forward 5'-ACAAGGAGAGACAAGCAACG-3' and reverse 5'-TTGTTTGGGATCCACACTCT-3' for IL-1β, forward 5'-CCCTTCAGGAACAGCTATGA-3' and reverse 5'-AGTCTCCTCTCCGGACTTGT-3' for IL-6, forward 5'-CTGGAGAAACCTGCCAAGTA-3' and reverse 5'-AGACAACCTGGTCCTCAGTG-3' for GAPDH.

8. Statistical Analysis

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

III. RESULTS

1. Hemodynamic and glycemic parameters

Hemodynamic data including MAP and HR, and level of serum glucose are given in Table 1. The trends of MAP and HR over time were similar among the groups ($p = 0.902$ and $p = 0.859$). MAPs measured during ischemia were lower compared to their corresponding baseline values in all groups. We confirmed that baseline glucose level was similar between the hyperglycemia and normoglycemia groups ($p = 0.461$), and after the administration of dextrose, blood glucose concentrations during ischemia and after reperfusion were significantly higher in the hyperglycemia groups compared to the normoglycemia groups (Table 1).

Table 1. Changes in hemodynamic parameters and serum glucose level

Parameter	Group							
MAP (mmHg)	**NG-sham**	**NG-IRC**	**NG-pre-EP**	**NG-post-EP**	**HG-sham**	**HG-IRC**	**HG-pre-EP**	**HG-post-EP**
Baseline	100 ± 5	83 ± 24	86 ± 23	87 ± 20	92 ± 7	94 ± 7	94 ± 8	93 ± 16
During ischemia		57 ± 22*	60 ± 13	60 ± 20		71 ± 16*	72 ± 8*	70 ± 6
After reperfusion		75 ± 1	73 ± 25	75 ± 6		78 ± 13	79 ± 7	76 ± 29
HR (beat/min)	**NG-sham**	**NG-IRC**	**NG-pre-EP**	**NG-post-EP**	**HG-sham**	**HG-IRC**	**HG-pre-EP**	**HG-post-EP**
Baseline	352 ± 17	346 ± 29	351 ± 25	351 ± 47	360 ± 19	328 ± 17	329 ± 37	339 ± 35
During ischemia		363 ± 17	352 ± 26	325 ± 39		359 ± 30	336 ± 9	350 ± 32
After reperfusion		334 ± 10	335 ± 10	356 ± 7		360 ± 32	351 ± 33	333 ± 40
Glucose (mmol/L)	**NG-sham**	**NG-IRC**	**NG-pre-EP**	**NG-post-EP**	**HG-sham**	**HG-IRC**	**HG-pre-EP**	**HG-post-EP**
Baseline	5.6 ± 0.3	5.6 ± 0.2	5.7 ± 0.2	5.6 ± 0.2	5.6 ± 0.2	5.7 ± 0.1	5.6 ± 0.2	5.7 ± 0.1
During ischemia		5.5 ± 0.2	5.6 ± 0.2	5.6 ± 0.1		17.6 ± 0.2 *,#	17.4 ± 0.4 *,#	17.5 ± 0.4 *,#
After reperfusion		5.5 ± 0.2	5.6 ± 0.2	5.6 ± 0.1		12.9 ± 0.6 *,#	12.6 ± 0.4 *,#	12.9 ± 0.4 *,#

Data are presented as mean ± standard deviation. Baseline, 15 minutes after starting the anesthetics; during ischemia, 15 minutes after myocardial ischemia; after reperfusion, 15 min after starting reperfusion.

MAP; mean arterial blood pressure, HR; heart rate, NG; normoglycemia, EP; Ethyl pyruvate, IRC; ischemia/reperfusion-control, pre-EP; ethyl pyruvate treatment before ischemia, post-EP; ethyl pyruvate treatment upon reperfusion, HG; hyperglycemia. *$P < 0.05$ compared with the baseline; #$P < 0.05$ compared with the normoglycemia groups.

2. EP treatment decreased infarct size and apoptotic cardiomyocytes death following I/R injury

The infarct size was significantly increased in the hyperglycemia groups than in the normoglycemia groups, while there were no significant differences in AAR/left ventricle (%) among all groups (Fig. 2). Both pre- and post-EP treatment significantly reduced the infarct size under normoglycemic, as well as hyperglycemic condition (Fig. 2). To determine the quantitative degree of I/R induced apoptosis, we used TUNEL assay and the proportion of apoptotic cardiomyocytes to total cardiomyocytes was assessed (Fig. 3). Both pre- and post-EP treatment decreased I/R-induced apoptotic cell deaths after I/R injury under normoglycemic condition, while under hyperglycemic condition, only post-EP treatment significantly reduced I/R-induced apoptotic cell deaths (Fig. 3). Considering the practical applicability of a treatment strategy upon reperfusion rather than before ischemia, and the result that post-EP was more efficient in terms of preventing apoptosis, further studies were only done with post-EP models.

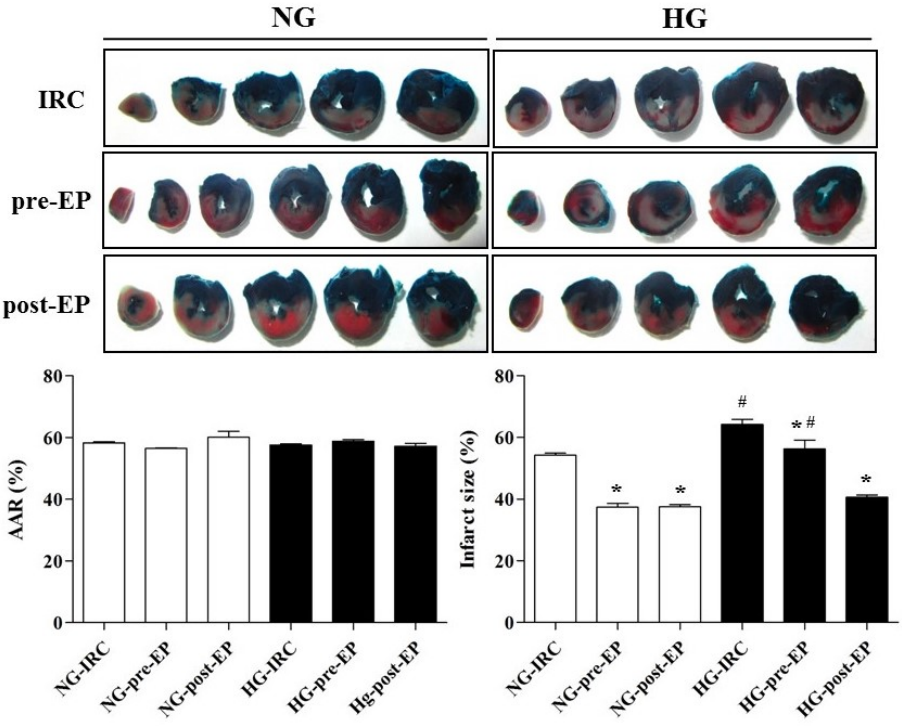

Figure 2. Effect of ethyl pyruvate treatment on infarct size following ischemia and reperfusion injury. There were no significant differences in area at risk /left ventricle (%) among all groups and the infarct size was significantly increased in the hyperglycemia groups than in the normoglycemia groups. Both pre- and post-treatment of ethyl pyruvate significantly reduced the infarct size under normoglycemic, as well as hyperglycemic condition.

NG; normoglycemia, HG; hyperglycemia, EP; ethyl pyruvate, IRC; ischemia/reperfusion-control-saline, pre-EP; EP treatment before ischemia, post-EP; EP treatment upon reperfusion, AAR; area at risk. $^{*}P < 0.05$ compared with the IRC; $^{\#}P < 0.05$ compared with the normoglycemia groups.

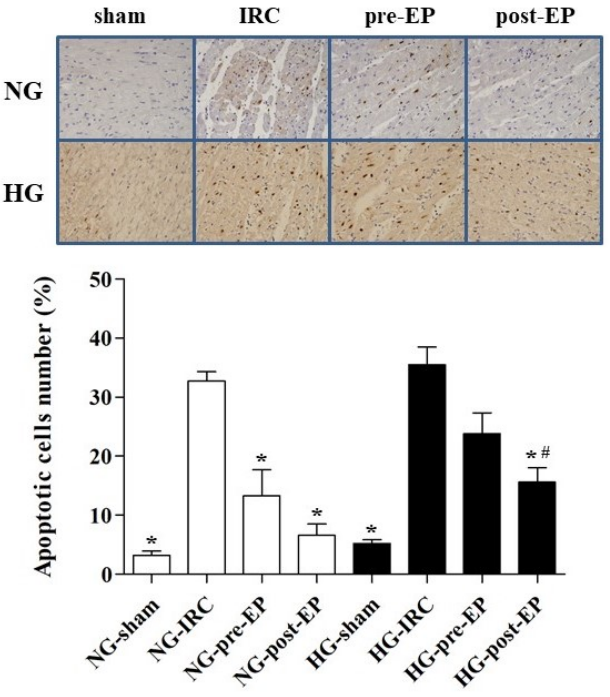

Figure 3. Effect of ethyl pyruvate treatment on the number of apoptotic cardiomyocytes after ischemia and reperfusion injury. Ethyl pyruvate treatment before ischemia or upon reperfusion decreased the number of apoptotic cardiomyocytes after ischemia and reperfusion injury under normoglycemic condition. However, under hyperglycemic condition, only ethyl pyruvate treatment upon reperfusion significantly reduced ischemia and reperfusion-induced apoptotic cell deaths. (TUNEL assay, ×400)

NG; normoglycemia, HG; hyperglycemia, EP; ethyl pyruvate, IRC; ischemia/reperfusion-control-saline, pre-EP; EP treatment before ischemia, post-EP; EP treatment upon reperfusion. $^{*}P < 0.05$ compared with the IRC; $^{\#}P < 0.05$ compared with the normoglycemia groups.

3. Post-EP treatment reduced HMGB1, RAGE, TLR-2 and TLR-4, and NF-κB phosphorylation following I/R injury

To investigate the association between aggravated myocardial I/R injury under hyperglycemic condition and HMGB1–RAGE/TLR axis, the levels of HMGB1, RAGE, TLR-2 and TLR-4 in rat myocardium were measured using immunoblot analysis. The degree of HMGB1, RAGE, TLR-2 and TLR-4 expression induced by I/R under hyperglycemic condition were significantly higher compared to normoglycemic condition (Fig. 4A). Post-EP treatment suppressed the expression of HMGB1 and its receptors in both normoglycemic and hyperglycemic conditions (Fig. 4A). The increase in TLR-2 and TLR-4 expressions following I/R and the attenuation of those increases by post-EP treatment under normoglycemia and hyperglycemia were also verified using immunofluorescence analysis (Fig. 4B).

The common signaling pathway involves activation of NF-κB, resulting in production of pro-inflammatory cytokines, adhesion molecules, and the overexpression of receptors for HMGB1.[10] Therefore, we investigated the triggered activation of NF-κB and the effect of post-EP treatment upon it. The ratio of phosphorylated NF-κB to NF-κB expression following I/R under hyperglycemic condition was significantly higher compared to normoglycemic condition (Fig. 5). Post-EP treatment was associated with a reduction in I/R-induced increase in NF-κB phosphorylation in both normoglycemic and hyperglycemic conditions (Fig. 5).

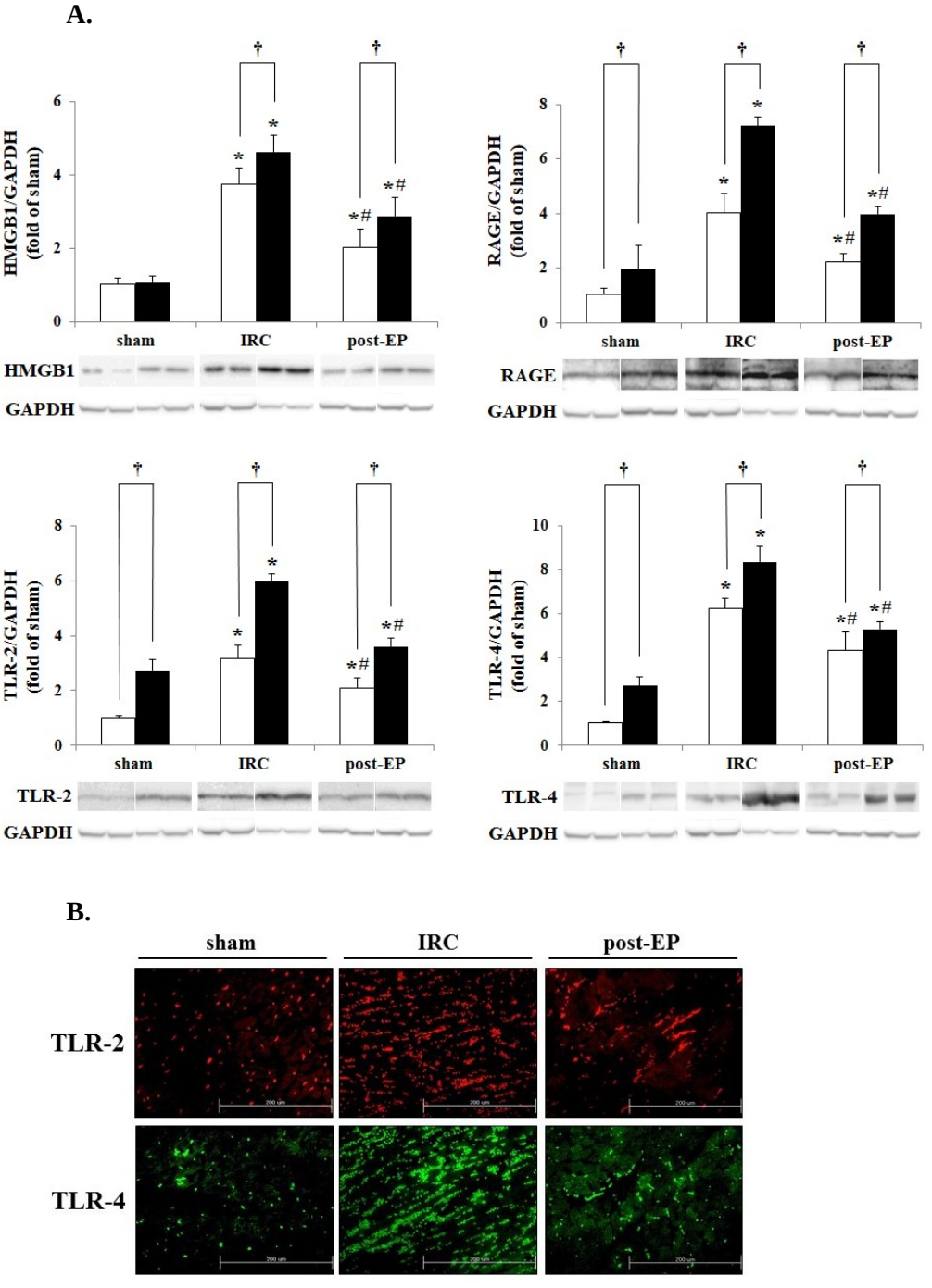

Figure 4. Effect of ethyl pyruvate treatment upon reperfusion on high-

mobility-group box-1 and its receptors expression following ischemia and reperfusion injury. The white boxes indicate the normoglycemia groups and the black boxes indicate the hyperglycemia groups. The degree of high-mobility-group box-1 (HMGB1), receptors of advanced glycation end products (RAGE), toll-like receptors (TLR)-2 and TLR-4 expressions induced by ischemia and reperfusion under hyperglycemic condition were significantly higher compared to normoglycemic condition (A). Also, ethyl pyruvate treatment upon reperfusion suppressed the expression of HMGB1 and its receptors under normoglycemia, and this effect of ethyl pyruvate treatment on HMGB1 and its receptors was preserved under hyperglycemic condition (A). The increase in TLR-2 and TLR-4 expressions following ischemia and reperfusion and the attenuation of those increases by ethyl pyruvate treatment upon reperfusion under hyperglycemia were also measured using immunofluorescence analysis (B).

IRC; ischemia/reperfusion-control-saline, EP; ethyl pyruvate, post-EP; EP treatment upon reperfusion, HMGB1; high-mobility-group box-1, RAGE; receptors of advanced glycation end products, TLR; toll-like receptors, GAPDH; glyceraldehyde 3-phosphate dehydrogenase. $^{*}P < 0.05$ compared with the sham; $^{\#}P < 0.05$ compared with the IRC; $^{\dagger}P < 0.05$ compared with the normoglycemia groups.

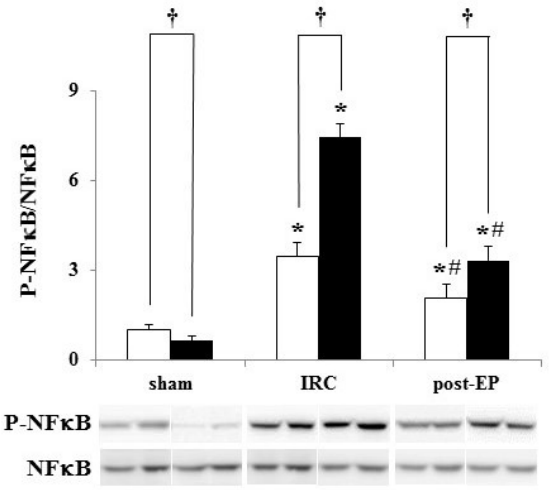

Figure 5. Effect of ethyl pyruvate treatment upon reperfusion on NF-κB phosphorylation following ischemia and reperfusion injury in rat myocardium. We investigated the triggered activation of NF-κB and the effect of ethyl pyruvate treatment upon it. The white boxes indicate the normoglycemic groups and the black boxes indicate the hyperglycemia groups. The degree of NF-κB activation induced by ischemia and reperfusion under hyperglycemic condition was significantly higher compared to normoglycemic condition. Ethyl pyruvate treatment upon reperfusion exhibited a reduction of ischemia and reperfusion-induced NF-κB phosphorylation under both normoglycemia and hyperglycemia.

IRC; ischemia/reperfusion-control-saline, EP; ethyl pyruvate, post-EP; ethyl pyruvate treatment upon reperfusion, p-NF-κB; phosphorylated NF-κB. $^{*}P < 0.05$ compared with the sham; $^{\#}P < 0.05$ compared with the IRC; $^{\dagger}P < 0.05$ compared with the normoglycemia groups.

4. Post-EP treatment diminished I/R-induced Bcl-2 reduction and Bax increase

Regulation of mitochondrial dysfunction and membrane permeabilization by proteins of the Bcl-2 family including anti-apoptotic Bcl-2 and pro-apoptotic Bax plays a key role in myocardial I/R-induced injury via apoptosis.[23] HMGB1 has been shown to exert critical influence on cardiomyocytes apoptosis induced by myocardial I/R via Bax up-regulation following I/R injury.[24] Also, reduced Bax/Bcl-2 ratio by inhibition of HMGB1 under hyperglycemic condition was reported.[14] To identify whether post-EP treatment could favorably affect the modulation of Bcl-2 family under hyperglycemic condition, the degree of anti-apoptotic Bcl-2 and pro-apoptotic Bax expression was detected using immunoblotting analysis. After I/R injury, Bcl-2 was down-regulated and Bax was up-regulated resulting in a significant increase in the Bax/Bcl-2 ratio. Post-EP treatment significantly attenuated the increase in Bax/Bcl-2 ratio compared with IRC under both normoglycemic and hyperglycemic conditions (Fig. 6).

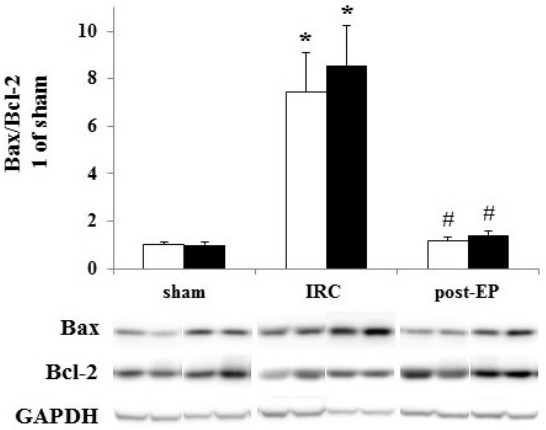

Figure 6. Effect of ethyl pyruvate treatment upon reperfusion on ischemia and reperfusion-induced Bcl-2 reduction and Bax increase in rat myocardium. The white boxes indicate the normoglycemia groups and the black boxes indicate the hyperglycemia groups. The immunoblotting analysis showed that Bcl-2 was down-regulated and Bax was up-regulated following ischemia and reperfusion under both normoglycemic and hyperglycemic conditions. Ethyl pyruvate treatment upon reperfusion significantly attenuated the increase in Bax/Bcl-2 ratio compared with ischemia/reperfusion-control under both normoglycemic and hyperglycemic conditions.

IRC; ischemia/reperfusion-control-saline, EP; ethyl pyruvate, post-EP; ethyl pyruvate treatment upon reperfusion, GAPDH; glyceraldehyde 3-phosphate dehydrogenase. $^{*}P < 0.05$ compared with the sham; $^{\#}P < 0.05$ compared with the IRC.

5. Post-EP treatment decreased TNF-α, IL-1β, and IL-6 mRNA expressions under hyperglycemia following I/R injury

NF-κB activation by HMGB1 induces the transcription of pro-inflammatory genes, such as IL-1β, IL-6, TNF-α,[25] and those cytokines were described as key mediators in the pathophysiology of myocardial I/R injury.[26,27] For the quantitation of TNF-α, IL-1β, and IL-6 mRNA expressions under hyperglycemia, RT-PCR was used. Following I/R, TNF-α, IL-1β, and IL-6 mRNA expressions were significantly increased and, post-EP treatment mitigated the I/R-induced increases in TNF-α, IL-1β and IL-6 under hyperglycemia (Fig. 7).

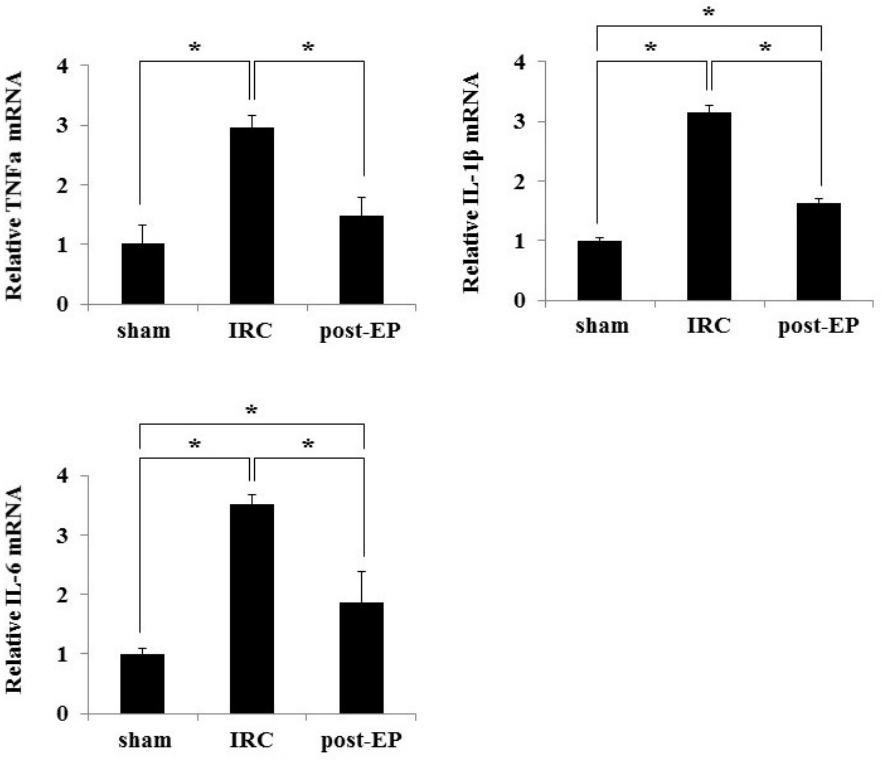

Figure 7. Effect of ethyl pyruvate treatment upon reperfusion on tumor necrosis factor-α, interleukin-1β and interleukin-6 messenger ribonucleic acid expressions under hyperglycemia following ischemia and reperfusion injury. Following ischemia and reperfusion, tumor necrosis factor-α, interleukin-1β and interleukin-6 expressions were significantly increased, and ethyl pyruvate treatment upon reperfusion mitigated the ischemia and reperfusion-induced increases in those cytokines under hyperglycemia.

TNF- α; tumor necrosis factor-α, mRNA; messenger ribonucleic acid, IRC; ischemia/reperfusion-control-saline, EP; ethyl pyruvate, post-EP; ethyl pyruvate treatment upon reperfusion, IL-1β; interleukin-1β, IL-6; interleukin-6. $^{*}P < 0.05$

IV. DISCUSSION

In the current study, we could confirm that hyperglycemia exacerbated myocardial I/R injury via increased HMGB1 release and activation of its downstream pathways mediated by RAGE/TLR–NF-κB compared with normoglycemia. EP treatment upon reperfusion conveyed beneficial influence in terms of attenuating the amplified activation of HMGB1–RAGE/TLR–NF-κB axis by hyperglycemia. In extension, it was associated with favorable modulation of pro-apoptotic Bax/anti-apoptotic Bcl-2 ratio, and decreased expressions of TNF-α, IL-1β, and IL-6. Ultimately, we could confirm that EP treatment upon reperfusion could retain its myocardial protective efficacy against I/R injury under hyperglycemic condition as in normoglycemia in terms of decreased infarct size and apoptosis.

An association between hyperglycemia and an increased risk of poor prognosis and mortality after acute MI was well noted in patients with or without diabetes.[28,29] Additional studies reported that acute hyperglycemia in acute MI patients without diabetes was independently associated with greater enzymatic infarction and higher long-term mortality rates.[30] In terms of cardiac surgery with CPB, hyperglycemia has also been consistently shown to be associated with detrimental outcome.[31]

Experimentally, hyperglycemia was shown to be related to decreased ischemic tolerance and increased severity of the myocardial I/R injury through excessive oxidative stress and aggravated inflammatory response.[4-7] In cultured

ventricular myocytes under hyperglycemic condition, free radical generation, pro-inflammatory cytokine concentrations and the number of apoptotic myocytes were markedly increased.[32] Previous studies also showed that hyperglycemia-induced oxidative stress increased the expression of RAGE and RAGE ligands.[33] Separately, hyperglycemia has been shown to be associated with increased serum HMGB1 levels.[11]

HMGB1, a constitutional DNA chaperon, was demonstrated to act as a critical mediator of organ damage,[34] and cardiomyocytes apoptosis,[13] when it is either passively released from the nuclei of necrotic cells or actively secreted by inflammatory cells following an I/R injury. Subsequently, inhibition of HMGB1 was proven to exert protective effect against I/R injury and I/R-induced apoptosis.[12,14] Furthermore, the activation of NF-κB induced by HMGB1–RAGE/TLR axis leads to increases in pro-inflammatory cytokines and overexpression of receptors for HMGB1.[10] These findings implicate that activation of the HMGB1–RAGE/TLR–NF-κB axis could be associated with aggravated myocardial I/R injury and I/R-induced apoptosis under hyperglycemic condition. Thus, we hypothesized that the hyperglycemia-induced exacerbation of myocardial I/R injury may be attenuated by inhibiting the release of HMGB1, the foremost critical mediator linking the initial ischemic insult related to oxygen deprivation with the ensuing inflammation that contributes to further organ damage.

EP is an ester formed from pyruvic acid, a naturally occurring metabolic intermediate having a scavenging activity of reactive oxygen species, combined with ethanol in order to sustain its stability in aqueous solution.[15] Since Ulloa et

al. reported substantial anti-inflammatory effect of EP via inhibition of HMGB1 release,[16] many subsequent studies demonstrated protective effect of EP on inflammatory disorder,[35] and myocardial I/R injury.[19] In the current literature, evidence is lacking in terms of an effective protective measure for myocardial I/R injury under hyperglycemia along with relevant molecular mechanisms, which would be of high priority considering its clinical prevalence and influence on outcome. Based on the available evidence, EP may be able to ameliorate myocardial I/R injury under hyperglycemia by inhibiting the release of HMGB1. Previously, Lin et al. demonstrated myocardial protective effect of EP in I/R injury under normoglycemic condition only.[19] As yet, the hypothesis that EP could retain its efficacy to ameliorate myocardial I/R injury under hyperglycemia has not been investigated. In addition, the approved clinical safety of EP would enable its immediate clinical translation,[36] once its protective efficacy has been validated in a clinically relevant animal model.

As our results indicate, we could verify significant increase in myocardial injury under hyperglycemia compared to normoglycemia. We could also observe accentuated increase in HMGB1 level with amplified activation of the RAGE/TLR-NF-κB-mediated downstream inflammatory and apoptotic pathways under hyperglycemia compared with normoglycemia, which are in agreement with the results of previous studies.[11,33] Thus, our results clearly implicate that even in the absence of diabetes, an acute hyperglycemic condition *per se* can exacerbate the myocardial I/R injury, which correlates with the clinical findings observed in patients with acute MI or in those undergoing cardiac surgeries showing hyperglycemia as a portent of adverse outcome.[30,31]

In terms of validating an efficacious therapy in that regard, we could observe a significant decrease in infarct size and apoptotic cell deaths with EP administration upon reperfusion, both in normo- and hyperglycemic conditions. We also demonstrated that EP ameliorated myocardial I/R injury in association with attenuated activation of the HMGB1–RAGE/TLR–NF-κB axis and concomitant suppressions in the mRNA expressions of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6). These results are in line with those of previous studies that verified NF-κB activation by HMGB1 resulted in enhanced transcriptions of pro-inflammatory genes linked to expressions of IL-1β, IL-6, and TNF-α,[25] which were elucidated as key mediators of myocardial I/R injury.[26,27] Moreover, favorable modulation of the ratio of pro-apoptotic Bax/anti-apoptotic Bcl-2, was also observed in the current study that ultimately lead to decreased apoptotic myocardial cell deaths. Regulations of mitochondrial dysfunction and membrane permeabilization by proteins of the Bcl-2 family (Bcl-2 and Bax) are critical for mediating apoptotic cell deaths related to myocardial I/R injury.[23] In agreement with our result, HMGB1 was demonstrated to play a key role in cardiomyocytes apoptosis induced by myocardial I/R injury via up-regulation of the pro-apoptotic Bax.[24] In conjunction, reduced ratio of the pro-apoptotic Bax/anti-apoptotic Bcl-2 by inhibition of HMGB1 under hyperglycemic condition was reported.[14]

Noticeably, the protective efficacy of EP seemed to be well preserved under hyperglycemic condition, while many of the known protective measures against I/R injury have been reported to be ineffective under hyperglycemia.[1,6,37] The retained ability of EP to convey its protective effect under hyperglycemia may be explained by its action mechanism targeting the key pathogenic factor, increased

HMGB1 release, in both hyperglycemia- and I/R-induced injury. Thus, the current study provides primary evidence that EP administration upon reperfusion is able to protect the myocardium against the I/R injury regardless of the glycemic condition.

The chosen dose of EP (50 mg/kg) in the current study was from a previous study dealing with EP and I/R injury,[19] and our preliminary study in which we tested the efficacy of various EP dosages from 30 mg/kg in 10 mg/kg increments and found that 50 mg/kg yielded maximum myocardial protective effects in terms of infarct size while no further benefit could be provided by a higher dosage.

This study is subject to following limitations. First, although we clearly demonstrated a significant increase in HMGB1 after I/R under hyperglycemia compared to normoglycemia, hyperglycemia alone without the I/R injury (sham) was associated with significant increases in TLRs and RAGE without an increase in HMGB1. This may be attributable to the induction of other damage-associated molecular pattern molecules by hyperglycemia that interact with TLR or RAGE,[10] which were beyond the scope of this study and thus, not addressed properly. Nonetheless, these findings further support the adverse influence of acute hyperglycemia, even in the absence of diabetes or an overt I/R injury. Second, phosphorylated NF-κB was actually lower in the hyperglycemia sham group compared with the normoglycemia sham group, which did not conform to our hypothesis. Yet, the absolute levels of the phosphorylated NF-κB were very low in both sham groups, while they showed marked increases after I/R injury. Moreover, despite the intergroup differences in the sham group, increase in phosphorylated NF-κB was more prominent in the hyperglycemia group

compared to the normoglycemia group after I/R injury supporting its contribution to aggravated myocardial injury under hyperglycemia.

V. CONCLUSION

In conclusion, exacerbation of the myocardial I/R injury under hyperglycemic condition was associated with enhanced activation of the HMGB1–RAGE/TLR–NF-κB axis, and increased expressions of pro-inflammatory cytokines and apoptosis. EP treatment upon reperfusion could protect the myocardium from I/R injury through retained suppressive efficacy of the above-mentioned pathways related to HMBG1. It was also associated with favorable modulations of related down-stream pathways involving the expressions of TNF-α, IL-1β, IL-6, and Bax/Bcl-2, even under hyperglycemia.

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ABSTRACT (IN KOREAN)

고혈당 상태에서 심장 허혈-재관류 손상에 대한
ethyl pyruvate의 심근 보호 효과

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High mobility group box 1 (HMGB1)은 심근 허혈/재관류 (ischemia/reperfusion, I/R) 손상 동안 receptors of advanced glycation end products (RAGE)/toll-like receptors (TLR)–NF-κB 신호 전달 경로를 통해 그 영향을 발휘하는 것으로 알려진 염증 매개체 중 하나이다. 또한 HMGB1–TLR 경로는 심근 I/R 손상 중 심근세포 사멸 촉진에 중요한 병태학적 역할을 한다. 고혈당은 심근경색이나 심장 수술과 같은 급성 질환 상태에서 흔히 나타나며, 심근 I/R 손상과 심근세포 사멸을 악화시킨다고 알려져 있다. 더욱이 고혈당 상태에서는 이미 알려진 심근 보호 수단의 효능이 감소한다는 점도 보고되었다. 고혈당으로 인한 심근 I/R 손상 악화의 기전 중 하나로 HMGB1–RAGE/TLR–NF-κB 신호 전달 경로의 활성화의 증폭을 가정해볼 수 있다. 그러므로 이 신호 전달 경로의 가장 첫 단계인 HMGB1 활성을 차단한다면, 고혈당 상태에서 심근 I/R 후 심근의 손상 및 심근세포 사멸을 감소시킬 가능성이 있다. Ethyl pyruvate (EP)의 경우 HMGB1 분비 및 그 아래

단계의 신호 전달 경로에 길항 작용을 하므로 고혈당 상태에서도 심근 보호 효과를 가질 가능성이 있다. 이와 같은 이론적 배경을 바탕으로, 본 연구는 임상적인 연관성이 있는 중등도 고혈당 상태에서 I/R 손상에 대한 EP의 심근 보호 효과와 그에 관련된 메커니즘을 조사하기 위해 계획되었다.

Sprague-Dawley 쥐 (n = 104)는 다음과 같은 8개의 그룹으로 나누어 배정되었다; 정상혈당-sham (NG-sham), 정상혈당-I/R-대조-식염수군 (NG-IRC), 정상혈당-pre-EP군 (NG-pre-EP), 정상혈당-post-EP군 (NG-post-EP), 고혈당-sham (HG-sham), 고혈당-I/R-대조-식염수군 (HG-IRC), 고혈당-pre-EP군 (HG-pre-EP), 및 고혈당-post-EP군 (HG-post-EP). 쥐들은 정상혈당/고혈당 그룹에 따라 1.2 g/kg 덱스트로오스 또는 같은 양의 생리 식염수를 투여 받았다. I/R은 좌전 하행 관상 동맥 허혈 30분 후 4시간 재관류에 의해 유도되었다. Pre-나 post-EP군에 따라 허혈 1시간 전 (pre-EP)이나 혹은 재관류 직후 (post-EP) 50 mg/kg의 EP를 정맥 내 투여 받았다.

고혈당은 정상혈당 상태에 비교하여 심근 I/R 후, 심근 경색 크기를 증가시켰고, HMGB1–RAGE/TLR–NF-κB 신호 전달 경로 활성화를 증폭시켰다. 정상혈당 상태에서 IRC군이나 pre-EP군에 비해, post-EP군에서 심근 I/R 후 심근 경색 크기와 심근 세포 사멸을 유의하게 감소되었고, 이와 같은 EP의 심근 보호 효과는 고혈당 상태에서도 유지되었다. 재관류 직후 EP 투여는 심근 허혈 부위의 HMGB1 단백질 수치

뿐 아니라, 심근 TLR-2과 TLR-4의 발현도 감소시켰다. 또한, 재관류 직후 EP 투여는 NF-κB 인산화의 감소시키고, 이는 고혈당 상태에서 tumor necrosis factor-α, interleukin (IL)-1β, 및 IL-6 발현의 감소로 이어졌다. 마지막으로, 재관류 직후 EP 투여는 I/R로 인해 촉진된 심근세포 사멸을 감소시켰고 I/R 관련 Bcl-2의 하향-조절과 Bax 상향-조절을 약화시켰다.

결론적으로 심장의 I/R 손상 시, 재관류 직후 EP 투여는 HMBG1 및 아래 신호 전달 경로의 활동 억제를 통해 중등도 고혈당 상태에서도 심근 보호 효과를 나타냈다.

핵심되는 말: ethyl pyruvate, high-mobility-group box-1, 고혈당, 허혈-재관류, receptors of advanced glycation end products