European Journal of Pharmacology 745 (2014) 1–9

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

Erythropoietin protects myocardium against ischemia–reperfusion injury under moderate hyperglycemia

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ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form
23 September 2014
Accepted 23 September 2014
Available online 16 October 2014

Keywords:
Erythropoietin
Hyperglycemia
GATA binding protein-4
Bcl-2
Caspase-3
Cardioprotection

ABSTRACT

Erythropoietin (EPO), an essential hormone for erythropoiesis, provides protection against myocardial ischemia/reperfusion (I/R) injury. Hyperglycemia during acute myocardial infarction aggravates organ damage and attenuates the efficacies of various protective measures. This study aimed to investigate the protective role of EPO against myocardial I/R injury under a clinically relevant moderate hyperglycemic condition and its associated mechanisms. Eighty-two Sprague-Dawley rats were randomly assigned to six groups: normoglycemia-Sham, normoglycemia-I/R-control-saline (IRC), normoglycemia-I/R-EPO (IRE), hyperglycemia-Sham, hyperglycemia-IRC, and hyperglycemia-IRE. The rats received 1.2 g/kg dextrose or 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 number of apoptotic cells and the infarct size compared with those of the control groups. EPO increased GATA-4 phosphorylation and acetylation against I/R in hyperglycemic myocardium. It also enhanced ERK induced GATA-4 post-translational modifications such as increased GATA-4 phosphorylation and acetylation, and decreased GATA-4 ubiquitination following hypoxia-reoxygenation in H9c2 cells in hyperglycemic medium. Increased GATA-4 stability by EPO diminished I/R-related down-regulation of Bcl-2 and reduction of caspase-3 activities in hyperglycemic myocardium. In conclusion, EPO pretreatment before I/R injury conveyed significant myocardial protection under moderate hyperglycemic condition through mechanisms involved in reduction of caspase-3 activity and up-regulation of Bcl-2 in association with enhanced ERK-induced GATA-4 stability.

1. Introduction

Hyperglycemia (HG) is known to worsen cardiovascular outcome regardless of the presence of diabetes mellitus (DM) (Capes et al., 2000; Ceriello, 2005). Even a transient HG-episode has been shown to aggravate myocardial ischemia/reperfusion (I/R) injury incurring poor prognosis (Su et al., 2007). Furthermore, HG is known to attenuate the cardioprotective effects of various pharmacologic measures against I/R injury in both experimental and clinical settings (Duncan et al., 2010; Kersten et al., 1998; Sowers et al., 1993). Plausible mechanisms for this loss of cardioprotective effect under HG include increased 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 (Di Filippo et al., 2005; Schultz and Gross, 2001; Su et al., 2007; Tanaka et al., 2004).

Of note, the severity of HG was reported to critically influence the extent of myocardial injury following I/R injury in HG (Kersten et al., 2001; Xu et al., 2004). Negative influence of HG could be ameliorated by increasing the dose of the applied protective agents in moderate HG (blood glucose level <300 mg/dl) but not in profound HG (blood glucose level 300–600 mg/dl) (Ghaboura et al., 2011; Kersten et al., 2001). Thus, under transient moderate HG, which is commonly encountered in the perioperative period or in acute disease states, a powerful pluripotent pharmacologic measure might reduce myocardial injury following I/R.

Apart from its primary function on erythropoiesis, erythropoietin (EPO) has been demonstrated to reduce organ damage by I/R injury through multiple mechanisms (Ehrenreich et al., 2004; Maiese et al., 2005; Riksen et al., 2008). Its beneficial influence is manifested by phosphatidylinositol 3 kinase (PI3K)/AKT and

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http://dx.doi.org/10.1016/j.ejphar.2014.09.038
0014-2999/

extracellular signal-regulated kinase (ERK) 1/2, and increased GATA 4 activities via ERK activation (Asaumi et al., 2007; Smith et al., 2003). EPO is also involved in anti-apoptotic pathways such as Bcl-2 expression (Chen et al., 2007; Shan et al., 2009). However, most of the evidence stem from studies performed in normoglycemic (NG) condition, and the effects and molecular mechanisms of EPO against myocardial I/R injury in HG have barely been elucidated heretofore. Considering the multidisciplinary mechanisms of EPO, we hypothesized that EPO may provide myocardial protection against I/R injury in moderate HG as well.

The aim of this study was to investigate the protective effect of EPO against myocardial I/R injury in moderate HG and its related mechanisms regarding Bcl-2 and caspase-3 regulation in association with GATA-4 activities.

2. Material and methods

2.1. 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 (National Research Council, 1996). 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 d. Depth of anesthesia was considered adequate when the heart rate (HR) was regular in the absence of pedal and palpebral reflexs.

The rats were intubated with a 16-gauge (G) catheter and artificially ventilated (Harvard Apparatus 683, Holliston, MA) at 50–55 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 a power lab system (ML845 PowerLab with ML132; AD Instruments, Colorado Springs, CO) for monitoring the mean arterial pressure (MAP) and HR. The body temperature was maintained around 37 °C using a heating pad and continuously monitored throughout the experiment.

2.2. Experimental models and study groups

We employed a rodent model of myocardial I/R injury as described previously (Kim et al., 2010). Rats underwent 30 min of left anterior descending coronary artery occlusion followed by 4 h of reperfusion. I/R were confirmed by the appearance of regional cyanosis and akinesia or bulging, and marked hyperemic response on the corresponding distal myocardium, respectively. Lead II electrocardiography was also continuously monitored via skin needle electrodes, which were placed after anesthesia, and ST segment changes were also taken into consideration. MAP and HR were continuously monitored during the procedures and serially recorded (baseline, during ischemia, after reperfusion).

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

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 point. The dose of EPO was chosen based on our preliminary results in which 4000 IU/kg exerted maximum influence on GATA-4 (Fig. 3A). The rats in the HG groups received dextrose (1.2 g/kg) 1 h before ischemia to induce moderate HG, while the NG groups received equivalent volume of normal saline via the IV catheter at the same time point. A blood glucose concentration >11.1 mmol/L was considered as HG (Hirose et al., 2008). Blood glucose concentrations were serially monitored. Hearts were collected at the end of reperfusion and store at −80 °C, and used for protein extraction for immunoblot analysis.

2.3. Cell culture and transient transfection

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 g/ml streptomycin. Culture media and supplements were purchased from WelGENE (Seoul, Korea). High-glucose (30 mM) DMEM was 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. H9c2 cells were treated with 0.1% (v/v) DMSO as a vehicle control. 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 for 2 h following reoxygenation for 4 h.

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

Details regarding the His-GATA-4 expression plasmid were previously reported (Jun et al., 2013a). 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).

2.5. 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 were performed as previously described (Jun et al., 2011). 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′ and reverse 5′-GTTTCATGGTC-CATCCTTG-3′ for Bcl-2; forward 5′-CTGGAGAAACCTGCCAAGTA-3′ and reverse 5′-AGACAACCTGGTCCTCAGTG-3′ for GAPDH.

2.6. Ubiquitination, immunoprecipitation and immunoblot analyses

After appropriate treatments, cells from each group underwent ubiquitination or immunoprecipitation assay as previously described (Jun et al., 2013a; Jun et al., 2013b). For ubiquitination assay under each condition, cells were pre-treated with 20 μM MG132 (a proteasome inhibitor, Sigma) and subjected to 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