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Experimental acute myocardial infarction in rats: HIF-1α, caspase-3, erythropoietin and erythropoietin receptor expression and the cardioprotective effects of two different erythropoietin doses

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ABSTRACT

The cardioprotective effects of two different doses of erythropoietin administration were analyzed in rats with experimental myocardial infarction. None, saline, standard-dose (5000 U kg^{-1}) and high-dose (10,000 U kg^{-1}) of human recombinant erythropoietin alpha were administered intraperitoneally in Wistar rats with myocardial infarction induced by coronary artery ligation. Infarct sizes measured after triphenyltetrazolium chloride staining, levels of biochemical markers, histopathology examined by light and electron microscopy, and immunohistochemical expressions of erythropoietin, erythropoietin receptor, hypoxia inducible factor-1α and caspase-3, were analyzed. Lower scores of infarction and hemorrhage, lower number of macrophages and higher score of vascularization surrounding the infarct area were observed in the erythropoietin administered groups ($p < 0.05$). Erythropoietin administration after myocardial infarction reduced the area of infarction and hemorrhage. There were hypoxia inducible factor-1α and caspase-3 expressions in the marginal area, and erythropoietin and erythropoietin receptor expression in both marginal and normal areas ($p < 0.001$). Vascularization, erythropoietin expression in the normal area and vascular erythropoietin expression were positively correlated with human erythropoietin levels. The cardioprotective effects of erythropoietin treatment were independent of endogenous erythropoietin/erythropoietin receptor activity. Moreover exogenous erythropoietin treatment did not suppress endogenous erythropoietin. Erythropoietin administration after myocardial infarction reduced caspase 3 expression (apoptotic activity) and induced neovascularization around the infarct area. Higher erythropoietin administration did not provide an additional benefit over the standard-dose in myocardial protection.

Introduction

Erythropoietin (Epo) is a glycoprotein hormone essential for normal erythrocyte production in bone marrow. It is released from renal peritubular cells and various extrarenal tissues including: liver, spleen, brain, lungs, bone marrow, and reproductive organs. Epo induces erythropoiesis under hypoxic conditions (Sasaki et al., 2000; Chong et al., 2002; Jelkmann, 2004; Marzo et al., 2008; Paschos et al., 2008). Interaction of Epo with its receptor decreases programmed death of erythroid progenitor cells and promotes their differentiation in bone marrow (Fisher et al., 1996; Sasaki et al., 2000).

The protective effects of Epo against tissue ischemia are mediated by erythropoietin receptor (EpoR) (Brines et al., 2004). In addition to erythroid progenitor cells, a varied group of cells including neurons, endothelial cells, vascular smooth muscle cells, and cardiac myocytes, express EpoR (Anagnostou et al., 1994; Digicaylioglu et al., 1995; Akimoto et al., 2000; Ogilvie et al., 2000; Ammarguellat et al., 2001; Digicaylioglu and Lipton, 2001; Tramontano et al., 2003; Jelkmann and Wagner, 2004; Marti, 2004). The cardioprotective effects of Epo have become a topical issue after detection of EpoR expression on cardiomyocytes

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(Marzo et al., 2008). The interaction of Epo with EpoR on these cells exerts protective effects against tissue ischemia (Junk et al., 2002; Cai et al., 2003; Calvillo et al., 2003; Moon et al., 2003; Parsa et al., 2003; Cai and Semenza, 2004; Lipsic et al., 2004; Sharples et al., 2004; Solaroglu et al., 2004; Wu et al., 2006; Guneli et al., 2007). Epo inhibits apoptosis and limits infarct size as seen using triphenyltetrazolium chloride (TTC) staining during ischemia and reperfusion through activation of various intracellular signaling pathways, especially the PI3K-Akt pathway (Parsa et al., 2003). Epo also has anti-inflammatory, anti-oxidative, and angiogenic potential (Paschos et al., 2008). The cardioprotective effects of Epo are independent of its hematopoietic effects (Parsa et al., 2003). Similar antiapoptotic and cardioprotective effects of Epo, independent of its hematopoietic effects, have been shown with carbamylated Epo, a non-erythropoietic derivative of Epo, and with helix B-surface peptide, a peptide mimicking the 3D structure of Epo (Fiordaliso et al., 2005; Ueba et al., 2010; Ahmet et al., 2011).

Several studies regarding the cardioprotective effects of Epo have been tested using a similar standard dose of Epo treatment including 3000 U kg^{-1} or 5000 U kg^{-1} (Calvillo et al., 2003; Moon et al., 2003; Parsa et al., 2003; Tramontano et al., 2003; Rui et al., 2005). It remains to be clarified whether a higher-dose of the Epo treatment may improve the cardioprotective effects over the standard-dose treatment. In this study, cardioprotective effects of two different doses of Epo treatment were analyzed using biochemical, morphological, and immunohistochemical methods in rats with myocardial infarction induced by coronary artery ligation.

Materials and methods

The study was approved by Gazi University Animal Experiments Local Ethics Committee (project number G.Ü.ET-08.059) and conformed to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. All procedures on laboratory animals were performed in Gazi University Laboratory Animals Care and Experimental Research Center, Ankara, Turkey.

Male Wistar rats weighing 250–300 g were divided into five groups: Group 1: Rats without treatment and sacrificed 1 h after coronary ligation ($n = 8$); Group 2: Rats receiving a single intraperitoneal (i.p) injection of saline immediately after coronary ligation and sacrificed 6 h after surgery ($n = 7$); Group 3: Rats receiving a single i.p. injection of standard-dose (5000 U kg^{-1}) Epo (human recombinant erythropoietin alpha) (Eprex 4000 IU/0.4 mL pre-filled syringe, Janssen-Cilag AG, Schaffhausen, Switzerland) immediately after coronary ligation and sacrificed 6 h after surgery ($n = 9$); Group 4: Rats receiving a single i.p. injection of high-dose (10,000 U kg^{-1}) Epo immediately after coronary ligation and sacrificed 6 h after surgery ($n = 9$); Group 5: Sham-operated control rats and sacrificed 6 h after surgery ($n = 3$).

Myocardial ischemia model

Rats were anesthetized with 45 mg kg^{-1} ketamine (Alfamine 10%, Alfasan International BV, Woerden, The Netherlands) and 5 mg kg^{-1} xylazine (Alfazyne 2%, Alfasan International BV, Woerden, The Netherlands) administered intraperitoneally. Basal electrocardiograms of all rats were taken using a data acquisition system (MP 150 Data Acquisition System, BIOPAC Systems, Goleta, CA, USA). Rats were intubated through tracheotomy and ventilated with room air using a volume controlled rodent ventilator (Inspira ASV, Harvard Apparatus, Holliston, MA, USA). Thoracotomy through the fourth intercostal space was performed to expose the heart. Left anterior descending coronary artery (LAD) ligation was obtained by placing a 7–0 polypropylene suture around the space between the pulmonary artery and the left auricle. Cessation of normal contractions of the myocardium in the LAD perfusion area was accepted as a marker of adequate LAD ligation. Following LAD ligation, saline, standard-dose erythropoietin and high-dose erythropoietin were administered intraperitoneally in Groups 2, 3, and 4, respectively. The thoracotomy was closed without residual pneumothorax, and the tracheotomy was repaired after weaning from the ventilator. Control electrocardiograms of all rats were taken. ECG recordings taken after left anterior descending coronary artery (LAD) ligation showed ST segment changes indicating myocardial infarction. Rats were allowed to recover in a warm and oxygen rich compartment until they were fully active, and then they were transferred to their cages. No additional analgesia was required over the initial anesthesia. None of the rats died before euthanasia.

Rats were fully anesthetized again with the same dose of ketamine and xylazine before euthanasia, and were re-intubated via the previous tracheotomy. The heart was exposed through a midline sternotomy and excised quickly after a blood sample was withdrawn from the right atrium. The occurrence of acute myocardial infarction in each rat was verified qualitatively using a commercially available troponin T test kit (Trop T, Roche Diagnostics, West Sussex, UK).

Biochemistry

Rat Epo, rat high sensitivity C-reactive protein (hsCRP), and rat cardiac troponin T (cTnT) levels in rat serum were measured using commercially available enzyme-linked immunosorbent assay (ELISA) rat sensitive kits (Cusabio Biotech, Newark, DE, USA, Catalog no: CSB-E07323r, CSB-E08618r, and CSB-E11305r, respectively) on a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The detection limits of Epo, hsCRP, and cTnT in rat serum were 0.2 mIU mL^{-1}, 0.16 ng mL^{-1}, and 15.6 pg mL^{-1}, respectively. Human Epo levels in rat serum were measured using a DRG EPO ELISA kit (DRG International, East Mountainside, NJ, USA, Catalog no: EIA-3646).

Light microscopy

The hearts were fixed in 10% buffered formalin, and processed for embedding in paraffin wax by routine protocols and cut into 5-μm-thick sections by a microtome. The sections were stained using hematoxylin and eosin (H&E) and examined using a photomicroscope (Axioskop 40 Microscope and AxioCam ICc3 Microscope Camera, Carl Zeiss, Göttingen, Germany). Five sections were randomly chosen from the mid-ventricular level of the heart in each animal. From each section, five areas were randomly selected for the histopathological examination. The presence of vascularization around the infarct area, the score of infarction area, hemorrhage, inflammatory cell infiltration (increase in neutrophils), and the number of macrophages were assessed. Macrophages were counted (40× objective) in five different areas surrounding the infarct area.

Semi-quantitative analysis

Semi-quantitative analysis of the infarct size in the left ventricle, hemorrhage, and leukocyte increase were scored as: none (0), weak (1), moderate (2), strong (3) and very strong (4). The semi-quantitative lesion scoring system was adapted from Azevedo Filho et al. (2004). Each section received a score according to the percentage of the infarct size in the left ventricle (score 0, 1, 2, 3 and 4). Score 0 corresponded to the absence of infarct; score 1 (weak) corresponded to infarct size of 1–25% of the area of the segment; score 2 (moderate) corresponded to infarct size of 26–50%; score 3 (strong) corresponded to infarct size of 51–75%; and score 4 (very