Phosphatidylinositol-3-Kinase Signaling Is Required for Erythropoietin-Mediated Acute Protection Against Myocardial Ischemia/Reperfusion Injury

Zheqing Cai, PhD; Gregg L. Semenza, MD, PhD

Background—Parenteral administration of recombinant human erythropoietin (rhEPO) to rats induces protection against myocardial ischemia/reperfusion injury 24 hours later. However, the mechanisms by which rhEPO mediates protection have not been determined.

Methods and Results—rhEPO was perfused into isolated rat hearts over 15 minutes immediately before 30 minutes of no-flow ischemia and 45 minutes of reperfusion. Compared with saline-perfused control hearts, recovery of left ventricular developed pressure was increased in rhEPO-perfused hearts. rhEPO also increased AKT activity and decreased apoptosis. All of these effects were blocked when the phosphatidylinositol-3-kinase inhibitor wortmannin was infused with rhEPO.

Conclusions—rhEPO provides immediate protection against ischemia/reperfusion injury in the isolated perfused rat heart that is mediated by the phosphatidylinositol-3-kinase pathway. (Circulation. 2004;109:2050-2053.)

Key Words: erythropoietin • ischemia • reperfusion • myocardial infarction

Novel therapies are needed to reduce cardiac cell dysfunction and death in patients presenting with acute myocardial ischemia.1,2 Administration of recombinant human erythropoietin (rhEPO) to rats has a dramatic protective effect against cardiac injury when administered 24 hours before global ischemia/reperfusion, as measured by improved recovery of left ventricular developed pressure (LVDP), reduced numbers of apoptotic cells, and reduced activation of caspase 3 in the hearts of treated animals.3 Treatment with rhEPO also reduces apoptosis and increases functional recovery after in vivo coronary artery occlusion/reperfusion.4–6 These initial studies did not define the molecular mechanisms underlying the observed protective effects of rhEPO in the heart. The present study was designed to determine whether rhEPO has an immediate protective effect on the isolated perfused heart and whether protection mediated by rhEPO is dependent on signaling via phosphatidylinositol-3-kinase.

Methods

Rat Heart Perfusion

Hearts isolated from male Sprague-Dawley rats (Harlan, Indianapolis, Ind) were perfused in Langendorff mode with Krebs-Henseleit buffer at 37°C at a constant pressure of 100 cm H2O.3 A latex balloon was inserted into the left ventricle (LV) and inflated to an initial LV end-diastolic pressure (LVEDP) of 4 to 8 mm Hg. Saline or rhEPO was infused through an aortic cannula by syringe pump at a rate of 2 mL/h for 15 minutes. Hearts were subjected to 30 minutes of no-flow ischemia followed by 45 minutes of reperfusion in buffer alone. LVEDP, LVDP (LV systolic pressure–LVEDP), and coronary flow rate (CFR) were monitored continuously (PowerLab, AD Instruments). A third group of hearts was infused with rhEPO and 1 μmol/L wortmannin (Sigma Chemical Co) before ischemia, and wortmannin was included in the buffer during reperfusion. Statistical analyses were performed by ANOVA.

Immunoblot Assays

Hearts were lysed in 50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, and 1 mmol/L EGTA, and aliquots were subjected to immunoblot assays with the use of antibodies that recognize the following: phosphorylated or total AKT (Biosource International); phosphorylated or total p70s6k (Santa Cruz Biotechnology); cleaved or noncleaved caspase 3 (Cell Signaling Technology); EPO receptor (R&D Systems); phosphatidylinositol-3-kinase (Upstate Biotechnology); and tubulin (Santa Cruz). Signals from scanned immunoblots were quantified with the use of Image J1.30 software (National Institutes of Health). Immunoprecipitation was performed with the use of anti–phosphatidylinositol-3-kinase antibody.

Laddering Assay

DNA was isolated from heart homogenates by phenol/chloroform/isoamyl alcohol extraction followed by ammonium acetate/isopropanol precipitation. DNA aliquots (2 μg) were analyzed by 1.5% agarose gel electrophoresis followed by ethidium bromide staining.

Results

Saline or rhEPO was administered directly to isolated, Langendorff-perfused rat hearts over 15 minutes (t=0 to 15 minutes; Figure 1) at a dose of 100 U/kg donor body weight. For a 300-g donor rat and a CFR of 12.5 mL/min, the concentration of rhEPO in the perfusate was 0.16...
LVDP of saline-treated hearts at 90 minutes was before ischemia, a 65% recovery of function. The mean LVDP of rhEPO-treated hearts (Figure 1A). At the 90-minute time point (after 45 minutes of reperfusion), the mean LVDP of rhEPO-treated hearts was associated with a significant reduction in LVEDP compared with controls (40.3±3.9 versus 71.1±4.5 mm Hg at 90 minutes; Figure 1B). rhEPO treatment was also associated with a modest but significant increase in CFR immediately after the period of ischemia (Figure 1C). rhEPO had no effect on heart rate.

AKT (protein kinase B), a serine-threonine kinase that is activated by phosphatidylinositol-3-kinase, plays a critical role in promoting cell survival by inhibiting the activation of caspases, which function as effectors of the apoptotic program.8 Expression of an activated form of AKT protects against cardiac ischemia in vivo.9 EPO treatment activates AKT and promotes the survival of cultured endothelial cells10 and cardiomyocytes5,6 subjected to O2 deprivation. Phosphatidylinositol-3-kinase signaling, which is stimulated by binding of EPO to its receptor (EPOR),11 leads to the activation of AKT by phosphorylation on threonine-308 and serine-473.8 Analysis of an activated caspase 3.9 Analysis of activated (phosphorylated) AKT compared with control hearts (Figure 2B). To demonstrate that phosphatidylinositol-3-kinase activity is required for the activation of AKT in response to rhEPO treatment, wortmannin, an inhibitor of phosphatidylinositol-3-kinase, was added to the perfusate. Wortmannin blocked the phosphorylation of AKT but had no effect on total AKT protein levels or on the association of EPOR and p85.

Analysis of lysates prepared from hearts that were subjected to ischemia/reperfusion revealed that rhEPO increased activation of AKT, which was completely blocked by addition of wortmannin to the perfusate (Figure 2C). Phosphorylation of p70 S6 kinase (p70S6K), a downstream target of AKT, was induced by rhEPO in a phosphatidylinositol-3-kinase–dependent manner (Figure 2D). rhEPO significantly decreased the cleavage of caspase 3 to its activated form, whereas addition of wortmannin increased the levels of activated caspase 3 (Figure 2E). Ischemia/reperfusion induced apoptotic DNA laddering and caspase 3 activation, effects that were blocked by rhEPO in the absence, but not in the presence, of wortmannin (Figure 2F). Finally, the increased recovery of LVDP and CFR and reduction in LVEDP in rhEPO-treated hearts after ischemia/reperfusion were completely blocked by coadministration of wortmannin (Figure 1).

Discussion
The results of this study support 2 major conclusions. First, infusion of low doses of rhEPO induces a direct and immediate protective effect in the isolated heart subjected to ischemia/reperfusion. Second, rhEPO-mediated protection is dependent on phosphatidylinositol-3-kinase activity. Although induction of AKT phosphorylation in response to in vivo administration of rhEPO was recently reported,5 no evidence was presented that the phosphatidylinositol-3-kinase pathway was required for the protective response mediated by rhEPO. Our results show that the phosphatidylinositol-3-kinase inhibitor wortmannin completely blocks all of the beneficial effects mediated by rhEPO in isolated rat hearts after ischemia/reperfusion and demonstrate the essential role of phosphatidylinositol-3-kinase signaling in protection against myocardial injury.
Treatment with rhEPO induced an increase in CFR at the onset of reperfusion that may contribute to the reduced LVEDP and increased LVDP observed in rhEPO-treated hearts. This effect of rhEPO on CFR may be due to activation of endothelial NO synthase via AKT-mediated phosphorylation, leading to NO-mediated vascular dilatation.\(^1^2\),\(^1^3\) rhEPO may also promote endothelial cell survival.\(^1^0\)

In addition to potential effects of rhEPO on endothelium, our data demonstrate that the rhEPO-mediated reduction in myocardial apoptosis and caspase 3 activation after ischemia/reperfusion is dependent on phosphatidylinositol-3-kinase signaling. The antiapoptotic effect of AKT is well established via its direct phosphorylation and inactivation of multiple proapoptotic proteins, including caspase 9, an upstream activator of caspase 3.\(^3\),\(^8\) Phosphatidylinositol-3-kinase/AKT signaling has been implicated in cardiac protection induced by ischemic preconditioning, insulin-like growth factor 1, and insulin.\(^1^4\)–\(^1^6\) Thus, therapeutic strategies designed to induce activation of the phosphatidylinositol-3-kinase/AKT signal-transduction pathway may protect patients against ischemia/reperfusion injury. In addition to reducing infarct size, EPO may have additional therapeutic effects in vivo, such as recruitment of vascular progenitor cells,\(^1^7\) that may promote tissue repair.

Acknowledgments
This work was supported by National Institutes of Health grant P01-HL65608. We thank Dr Jaime Caro (Thomas Jefferson University, Philadelphia, Pa) for providing rhEPO.

References


Phosphatidylinositol-3-Kinase Signaling Is Required for Erythropoietin-Mediated Acute Protection Against Myocardial Ischemia/Reperfusion Injury

Zheqing Cai and Gregg L. Semenza

Circulation. 2004;109:2050-2053; originally published online April 26, 2004;
doi: 10.1161/01.CIR.0000127954.98131.23

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/17/2050

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/