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Erythropoietin protects cardiac myocytes against anthracycline-induced apoptosis

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Abstract

The cardiotoxic adverse effects of anthracycline antibiotics limit their therapeutic utility as essential components of chemotherapy regimens for hematologic and solid malignancies. Here we show that the hematopoietic cytokine erythropoietin attenuates doxorubicin-induced apoptosis of primary neonatal rat ventricular cardiomyocytes in a dose-dependent manner. Erythropoietin treatment induced rapid, time-dependent phosphorylation of MAP kinases (MAPK) Erk1/2 and the phosphatidylinositol 3-kinase substrate Akt. Treatment of cardiomyocytes with inhibitors of phosphatidylinositol 3-kinase (LY294002) or Akt (Akti-1/2) abolished the protective effect of erythropoietin, whereas treatment with MAPK kinase (MEK1) inhibitor U0126 did not. Erythropoietin also induced the phosphorylation of GSK-3 β , a downstream target of PI3K-Akt. Because phosphorylation is known to inactivate GSK-3 β , we investigated whether GSK-3 β inhibition is cardioprotective. We found that GSK-3 β inhibitors SB216763 or lithium chloride blocked doxorubicin-induced cardiomyocyte apoptosis in a manner similar to erythropoietin, suggesting that GSK-3 β inhibition is involved in erythropoietin-mediated cardioprotection. Erythropoietin may serve as a novel cardioprotective agent against anthracycline-induced cardiotoxicity.

Keywords

Erythropoietin; cardiac myocyte; apoptosis; anthracycline; kinase inhibitors

INTRODUCTION

The anthracycline chemotherapeutic agents are widely used as important and effective components of the treatment regimens for several different types of hematologic and solid malignancies, such as leukemias, lymphomas and breast cancer. The major clinical toxicity of anthracyclines such as doxorubicin (DOX) involves cardiac toxicity which may occur as an acute syndrome or cumulative and dose-dependent toxicity leading to cardiac dysfunction, thereby limiting the therapeutic usefulness of the drug. Among the mechanisms responsible for anthracycline-mediated cardiac toxicity are formation of free reactive oxygen radicals, direct DNA damage and immunologic reactions [1]. In addition, previous studies have provided evidence that acute toxicity of anthracyclines is associated with the induction of cardiomyocyte apoptosis [2-4]. Identification of mechanisms of anthracycline-induced cardiotoxicity has been important for the development of strategies to prevent the cardiotoxic effects of anthracyclines without interfering with its anti-tumor effects. For instance, the antioxidant, chelating agent

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dexrazoxane is used clinically as a cardioprotective drug in cancer patients receiving anthracyclines.

Erythropoietin (Epo) was originally characterized as a hematopoietic cytokine that is required for the regulation of mammalian erythropoiesis. The physiologic effects of Epo in hematopoietic cells are mediated through its interaction with its specific cellular receptor EPOR, a member of the type I superfamily of transmembrane cytokine receptors. A series of recent studies have provided evidence for Epo as a pleiotropic cytokine with diverse non-hematopoietic functions. For instance, Epo-EPOR signaling has been implicated in the regulation of physiologic angiogenesis that occurs during embryogenesis [5,6], wound healing [7], and in the female reproductive tract [8]. Furthermore, the recombinant form of human Epo, when administered exogenously, has been shown to exhibit tissue-protective effects in various experimental models of ischemic injury involving the brain and kidney [9-11]. Our laboratory and others previously described EPOR expression in the rat heart and in isolated cardiac myocytes [12-14]. Recent preclinical studies have shown that Epo exhibits cardioprotective effects in various experimental models of ischemic injury, reducing infarct size and improving recovery of left ventricular function [14-18]. The remarkable cardioprotective effects of Epo during ischemic cardiac injury has led us to hypothesize that Epo may protect cardiac myocytes against anthracycline-induced apoptosis.

MATERIALS AND METHODS

Reagents

Recombinant Epo (Procrit) was purchased from the Duke University Medical Center pharmacy. Phospho-specific and total Erk1/2, Akt, GSK-3 β antibodies and PI3K inhibitor LY294002 were from Cell Signaling Technologies (Beverly, MA). Doxorubicin, bovine serum albumin (BSA), monoclonal sarcomeric α -actinin antibody, GSK3 β inhibitors (lithium chloride and SB-216763) were from Sigma-Aldrich (St. Louis, MO). MEK1 inhibitor U0126 was from EMD Biosciences (San Diego, CA), Akt inhibitor (Akti-1/2) was from Calbiochem (San Diego, CA).

Cultures of neonatal rat ventricular cardiomyocytes and treatments

Primary cardiomyocyte cultures isolated from newborn rat heart ventricles (NRCM) were established using a kit from Cellutron (Highland Park, NJ). Animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication No. 85-13, revised 1996) under a protocol approved by the Duke Institutional Animal Care and Use Committee. Hearts were harvested from 2-3 day old Sprague-Dawley rats. NRCMs were cultured at 37°C for 48 hours (density of 7×10^4 cells/cm² for experiments involving Epo stimulation for protein analyses or 4×10^4 cells/cm² for other procedures) in DMEM supplemented with 10% horse serum (Sigma-Aldrich), 5% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 0.1 mmol/L bromodeoxyuridine (Sigma-Aldrich). The cell preparations were observed to beat spontaneously. The purity of cardiomyocyte cultures were >97% as assessed by immunocytochemical staining using cardiac muscle sarcomeric α -actinin antibody, and fluorescence microscopy. NRCMs were cultured in serum-free-medium for either 10 or 18 hours prior to addition of 0.5 μ M DOX in fresh serum-free-medium for 14 hours. Epo was added at indicated concentrations 45 minutes prior to DOX treatment. In experiments involving kinase inhibitors, cardiac myocytes were cultured in serum-free-medium for 10 hours following which the inhibitors LY294002 (50 μ M), Akti-1/2 (5 μ M), lithium chloride (3mM), SB-216763 (3 μ M) or U0126 (10 μ M) were added one hour prior to Epo and/or DOX treatments. All inhibitors were freshly constituted in DMSO and protected from light except for lithium chloride which was constituted in DMEM.

Protein Analysis

NRCMs were incubated in serum-free-medium (DMEM with 0.5% BSA) for 18 hours before Epo stimulation. Kinase inhibitors were added 1 hour prior to Epo. Following treatments, cells were washed with ice-cold PBS and lysed in a buffer containing 1% Triton-X100, 10% glycerol, 150mM NaCl, 20mM Tris-HCl (pH 7.4), 5mM EDTA, supplemented freshly with leupeptin 10 μ g/ml, aprotinin 10 μ g/ml, pepstatin 1 μ g/ml, 1mM PMSF, 1mM Na₃VO₄, 10mM NaF. Proteins were separated by SDS-PAGE using 4-12% Tris-glycine gels (Invitrogen, Carlsbad, CA), transferred to Immobilon PVDF membranes (Millipore, Billerica, MA), blocked with 5% non-fat milk in TBST (20mM Tris-HCl, 137mM NaCl, 0.1% Tween-20, pH 7.5) for 1 hour, incubated with primary antibodies at 4°C overnight, followed by HRP-conjugated secondary anti-rabbit antibody (Pierce Biotechnology, Rockford, IL). Proteins were detected using enhanced chemiluminescence and bands were visualized by autoradiography. Membranes were stripped and reprobed with antibodies against total Erk1/2, Akt or GSK-3 β to normalize the phosphorylated protein levels. Densitometry was performed using NIH ImageJ software.

Apoptosis Assays

Apoptosis was assessed using three different assays. Monolayers of NRCMs were labeled using TUNEL assay (In-Situ Cell Death Detection Kit (TMR red), Roche Diagnostics Corporation, Indianapolis, IN). The nuclei were counter-stained using DAPI (4,6-diamidino-2-phenylindole, Molecular Probes, Eugene, OR). The apoptotic and non-apoptotic nuclei were visualized using an Olympus IX70 fluorescence microscope and 10X/0.30 numeric aperture objective (Olympus Optical Co., Ltd., Tokyo, Japan). Images were acquired using Optronics digital camera system (model S60800, Goleta, CA) equipped with Optronic Magnafire Camera Imaging and Control (Version 1.0) software, and the digital images were colored and processed with Photoshop 5.5 (Adobe Systems, San Jose, CA). At least 1,500 DAPI-stained cells in multiple random fields were counted in each treatment group to assess the fraction of apoptotic cells determined by dividing the number of TUNEL-positive cells (red) by the total number of DAPI-positive cardiac myocyte nuclei (blue). A second assay consisted of a quantitative nucleosome ELISA (Cell Death Detection ELISA kit, Roche) to detect histone-associated DNA fragments (mono- and oligo-nucleosomes) generated in apoptotic cardiac myocytes. NRCMs were cultured in 24-well plates (7.5 $\times$ 10⁴ cells/well) for 2 days prior to the treatments and both adherent and detached cells were analyzed. Absorbance was measured at 405nm using a microplate reader. To measure caspase-3 activity, NRCMs were cultured in 6-well plates (2 $\times$ 10⁶ cells/well) for 10 hours in serum-free medium followed by treatment with DOX (0.5 μ M) for 14 hours in the presence or absence of Epo as indicated. Control cells were left untreated. Caspase-3 activity was determined with the ApoAlert R Caspase-3 Colorimetric Assay kit (Clontech, Mountain View, CA) according to the manufacturer's instructions.

Statistical analyses

The data are presented as mean $\pm$ s.e.m. Statistical analyses were performed using GraphPad InStat version 3.0 software (San Diego, CA). Comparisons between multiple groups were performed using analysis of variance (ANOVA) followed by Bonferroni multiple comparisons post-hoc test. All *P* values were two-sided and a value of *p*<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Epo Protects Cardiomyocytes against DOX-induced Apoptosis in a Dose-dependent Manner

Cells were treated with DOX in the presence or absence of increasing concentrations of Epo (Fig. 1A and 1B). Following DOX treatment, there was a 7.6-fold increase in the percentage

of apoptotic cells from $8.6 \pm 0.6\%$ in the controls to $65.8 \pm 5.5\%$ in the DOX-treated group ($p < 0.001$). Epo treatment resulted in a significant, dose-dependent reduction in cardiomyocyte apoptosis (Fig. 1B, left panel). When cells were incubated in serum free medium for 10 hours instead of 18 hours, DOX-induced apoptosis was nearly completely inhibited by Epo (Fig. 1B, right panel). To further investigate the anti-apoptotic effect of Epo, we used an ELISA-based assay to quantify histone-associated DNA fragmentation generated in apoptotic NRCMs (Fig. 1C). DOX-treatment of NRCMs resulted in a significant 2.7 ± 0.1 -fold increase in DNA fragmentation compared to control, untreated cells (Fig. 1C, $p < 0.001$). Treatment of cells with a lower Epo concentration (1 unit/ml) did not have a significant effect, whereas Epo at 10 units/ml and 100 units/ml significantly reduced DNA fragmentation by 24% and 33%, respectively. Caspase-3 activity in DOX-treated cardiomyocytes significantly increased by 1.6-fold as compared with control cells (Fig. 1D). Epo at a concentration of 100 units/ml significantly inhibited DOX-induced caspase-3 activation to 1.2-fold of control. These results demonstrate that Epo protects cardiomyocytes against DOX-induced apoptosis in a dose-dependent manner using TUNEL, DNA fragmentation and caspase-3 activity assays.

Epo induces the phosphorylation of Erk1/2 and Akt in primary cardiomyocytes

We investigated Epo-dependent activation of intracellular signal transduction in primary cardiomyocytes. In hematopoietic cells, Epo-EPOR signaling is associated with activation of the PI3K-Akt and p44/42 MAP kinases, pathways that have been previously implicated in cardiomyocyte apoptosis associated with DOX [19-21]. NRCMs were stimulated with Epo and whole cell lysates were analyzed by immunoblotting with antibodies against phospho-p44/42 MAPK (Fig. 2A) or phospho-Akt (Fig. 2B, 2C). Epo treatment induced a significant increase in the phosphorylation of p44/42 MAP kinases Erk1 and Erk2 at 5 minutes (4.4 ± 0.2 -fold increase compared to control), then decreased gradually approaching the basal phosphorylation level by 20 minutes (Fig. 2A). Epo treatment also resulted in a significant and transient increase in the phosphorylation of Akt (Ser473 and Thr308) that peaked at 5 minutes after Epo stimulation (Fig. 2B and 2C, respectively). Treatment with MEK1 inhibitor U0126 blocked the phosphorylation of Erk1/2 (Fig. 2D), and PI3K inhibitor LY294002 or Akt inhibitor Akti-1/2 blocked Epo-induced phosphorylation of Akt (Fig. 2E, 2F).

Protective Effect of Epo during DOX-induced Apoptosis is Blocked by Inhibitors of PI3K and Akt, but not by MEK1 Inhibitor

We investigated the mechanisms involved in Epo-mediated protection of cardiomyocytes during DOX-induced apoptosis. Previous studies have implicated p44/42 MAPK as a protective pathway during anthracycline-induced cardiac injury where expression of Erk2 was reported to protect cardiomyocytes from daunomycin-induced apoptosis [19]. Other studies have implicated PI3K-Akt as a protective pathway during DOX-induced cardiomyocyte apoptosis. In one study, expression of a constitutively active PI3K inhibited DOX-induced cardiomyocyte apoptosis [20]. In another study, sustained activation of the PI3K-Akt pathway by hepatocyte growth factor was required to inhibit DOX-induced cardiomyocyte apoptosis, whereas transient activation of Akt by epidermal growth factor was not associated with inhibition of apoptosis [21]. We found that in the presence of LY294002 or Akti-1/2 kinase inhibitors, Epo no longer protected against cardiomyocyte apoptosis induced by DOX (Fig. 3). In contrast, when cells were treated with MEK1 inhibitor U0126, there was no significant effect on the protection conferred by Epo. Similar results were obtained using DNA fragmentation assays (data not shown). These data demonstrate that Epo-mediated protection against DOX-induced apoptosis involves the activation of the PI3K-Akt but not MEK1-Erk1/2 pathway.

Epo Phosphorylates GSK-3 β through a PI3K-Akt-dependent Pathway and GSK-3 β Inhibition is Cardioprotective

We investigated the role of GSK-3 β , a downstream target of PI3K-Akt, in Epo-mediated protection during DOX-induced apoptosis. GSK-3 β has been implicated in diverse biological processes including Wnt/wingless pathway-associated developmental patterning, β -catenin driven tumorigenesis as well as cell survival and protective signaling in cardiomyocytes [22, 23]. Epo treatment of cardiomyocytes stimulated a significant increase in the phosphorylation of GSK-3 β (Fig.4A), an effect that was blocked by LY294002 or Akti-1/2 (Fig.4B). Unlike most protein kinases, phosphorylation leads to the inactivation of GSK-3 β [22]. We therefore postulated that GSK-3 β inhibition through its phosphorylation by Epo may represent a downstream mechanism by which Epo protects against DOX-induced apoptosis. To further investigate the role for GSK-3 β in DOX-induced apoptosis, we employed specific GSK-3 β inhibitors including SB-216763 [23-25] and lithium chloride [26-29] to determine whether GSK-3 β inhibition is protective. Treatment with GSK-3 β inhibitors lithium chloride and SB216763 resulted in significant inhibition of DOX-induced apoptosis, mimicking the protective effect of Epo (Fig.4C). Similar results were observed using DNA fragmentation assays (data not shown). These data suggest that Epo is cardioprotective through a PI3K-Akt-dependent pathway involving inhibition of GSK-3 β that leads to decreased DOX-induced apoptosis.

The ability of Epo treatment to stimulate red blood cell production in the bone marrow has resulted in the clinical use of Epo to prevent or treat anemia associated with several chronic disorders such as renal failure, inflammatory diseases and cancer. The discovery of the significant protective properties of Epo in various non-hematopoietic tissues, including the heart, has resulted in the exploration of new biologic functions and clinical applications. For instance, the neuroprotective effects of Epo characterized in pre-clinical studies have paved the way to a clinical trial of Epo in acute ischemic stroke patients which demonstrated significant improvements in clinical recovery scores and outcomes as a result of Epo administration[30]. A recent study in an in vivo model of DOX-induced cardiomyopathy reported that Epo may attenuate cardiac dysfunction [31]. Further pre-clinical studies are warranted to determine the role of Epo as a protective agent against anthracycline-induced cardiotoxicity.

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Fig. 1.
EPO inhibits DOX-induced apoptosis of primary cardiomyocytes in a dose-dependent manner. **A.**-Apoptotic cells were detected by TUNEL assays for labeling apoptotic nuclei (red), and DAPI counter-staining for all nuclei (blue). NRCMs were incubated in serum-free-medium and then treated with DOX in the presence or absence of Epo (10 or 100 units/ml). Control cells cultured in serum-free medium were left untreated. Photomicrographs of representative images are illustrated. **B.**-Quantification of apoptotic NRCMs following DOX and Epo treatments. NRCMs were incubated in serum-free-medium for either 18 hours (left panel) or 10 hours (right panel) prior to DOX treatment in the presence of indicated concentration of Epo. The fraction of apoptotic cells was determined in 6 or 7 random

microscopic fields totaling at least 1,500 cells/group and expressed as fold increase over control. Results are representative of four independent experiments. (* $p < 0.001$, ** $p < 0.05$). **C.**-Histone-associated DNA fragmentation generated in apoptotic NRCMs was quantified by ELISA assays. NRCMs were either left untreated (control) or treated with DOX in the absence or presence of increasing concentrations of Epo as indicated (1, 10 or 100 units/ml). The optical density (405nm) of the untreated control samples was arbitrarily designated 1. (n=11 replicates in each group). * $p < 0.05$, ** $p < 0.01$ compared to DOX group. Data is representative of six independent experiments. **D.**-Caspase-3 activity was determined in NRCMs following DOX treatment in the presence or absence of Epo (10 or 100 units/ml) using ELISA assays. The optical density of the untreated control samples was arbitrarily designated 1. * $p < 0.01$ compared to control and ** $p < 0.05$ compared to DOX group (n=6 in each group).

Fig. 2.

Epo-induced phosphorylation of Erk1/2 and Akt in primary cardiomyocytes. Isolated NRCMs were either left untreated (Control) or treated with Epo (10 units/ml) for the indicated durations. Whole cell lysates were analyzed by Western blotting using phospho-specific antibodies for **A.**-Erk1/2 (Thr202/Tyr204), **B.**-Akt (Ser473), **C.**-Akt (Thr308). The phosphorylated proteins are indicated by the arrows (upper panels). Comparable loading in each lane and protein integrity were confirmed by stripping the blots and hybridizing to respective antibodies to detect total Erk1/2 or Akt protein (lower panels). The relative positions of the molecular weight markers are indicated in each blot. Quantitative representation of phosphorylated proteins using densitometry are shown (mean±sem values from 3 independent

experiments, * $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$, NS: not significant compared to control group). **D-F.**-Isolated NRCMs were either left untreated (–) or treated (+) with Epo (10 units/ml) for 5 minutes in the presence or absence of **D.**-MEK1 inhibitor U0126, **E.**-PI3K inhibitor LY294002, or **F.**-Akt inhibitor Akti-1/2. Western blotting was performed using phospho-specific antibodies as indicated (upper panels). The blots were stripped and hybridized to antibodies against total Erk1/2 or Akt (lower panels).

Fig. 3.
Protective effect of Epo on DOX-induced apoptosis is blocked by PI3K and Akt inhibitors but not MEK1 inhibitor. NRCMs were incubated in serum-free medium for 10 hours followed by DOX treatment (0.5 μ M) for 14 hours with or without kinase inhibitors LY294002, Akti-1/2, or U0126, and in the presence or absence of Epo (100 units/ml). Quantitative analysis of apoptotic cardiomyocytes was expressed as fold increase of TUNEL-positive apoptotic NRCMs compared to untreated control. * $p < 0.001$, ** $p < 0.01$, NS: not significant.

Fig. 4.**Epo stimulates the phosphorylation of GSK-3β and treatment with GSK-3β inhibitors**

lithium chloride and SB-21673 are protective. **A.**-NRCMs were either left untreated (Control) or treated with Epo (10 units/ml). Whole cell lysates were analyzed by Western blotting using phospho-specific (Ser9) antibody for GSK-3β (upper panel) followed by stripping and incubation with total GSK-3β antibody (lower panel). Quantification of phosphorylated GSK-3β is shown (mean±sem values from 3 independent experiments, *p<0.05, NS: not significant compared to control). **B.**-PI3K and Akt inhibitors block Epo-induced stimulation of GSK-3β phosphorylation. Cells were either left untreated (–) or treated (+) with Epo in the presence or absence of LY294002 (left panel) or Akti-1/2 (right panel).

Western blots for phosphorylated and total GSK-3 β protein were performed. C.-Isolated NRCMs were incubated with Epo, or GSK-3 β inhibitors lithium chloride (Li) or SB216763 (SB) during DOX treatment. Quantitative analysis of apoptotic cardiomyocytes was expressed as fold increase of TUNEL-positive apoptotic NRCMs compared to untreated controls.*p<0.001 compared to DOX-treated group.