0022-3565/08/3241-160–169$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 324:160–169, 2008

Vol. 324, No. 1
125773/3287076
Printed in U.S.A.

Erythropoietin Protects against Doxorubicin-Induced Cardiomyopathy via a Phosphatidylinositol 3-Kinase-Dependent Pathway

Kyoung-Han Kim, Gavin Y. Oudit, and Peter H. Backx

Departments of Physiology and Medicine, Heart and Stroke/Richard Lewar Centre of Excellence and Division of Cardiology, University Health Network, University of Toronto, Toronto, Ontario, Canada

Received May 14, 2007; accepted October 9, 2007

ABSTRACT

Doxorubicin (DOX) is an effective antineoplastic agent whose use has been limited by its cardiotoxic side effects. Recent studies have established that erythropoietin (EPO), a cytokine essential for red blood cell production, protects against ischemic injury in the heart and other organs. The purpose of this study was to assess whether EPO protects the heart against cardiotoxicity induced by DOX. We found that DOX-induced apoptosis and impaired heart function in mice were largely prevented by EPO administration. To investigate the mechanism of protection by EPO, cultured neonatal mouse ventricular myocytes were treated with EPO at therapeutic levels (i.e., 1 U/ml), before application of DOX (0.1–1.0 μM). EPO protected against DOX-induced cardiomyocyte death (by $\approx$50%) and apoptosis assessed by annexin-V labeling, DNA fragmentation, and caspase-3 activity. DOX-mediated increases in reactive oxygen species, which trigger cardiotoxicity, were also reversed by preconditioning with EPO. These functional effects of EPO correlated with increased Akt/protein kinase B ($\sim$2-fold) and glycogen synthase kinase 3 (GSK-3; $\sim$1.3-fold) phosphorylations, suggesting protection by EPO was mediated by phosphatidylinositol 3-kinase activation. Indeed, preventing Akt and GSK-3β phosphorylations by phosphatidylinositol 3-kinase (PI3K) inhibition abolished protection by EPO against cardiomyocyte loss, apoptosis, and oxidative stress. Thus, pretreatment with therapeutic levels of EPO can protect the myocardium against DOX-induced impaired heart function and cardiomyocyte apoptosis by activating PI3K-Akt cell survival pathways.

Although the anthracycline antibiotic doxorubicin (DOX) has been used effectively to treat a broad range of human malignancies for decades, its clinical usage and efficacy are limited by side effects, especially cardiomyopathy and heart failure (Gewirtz, 1999). The mechanism for the cardiac toxicity associated with DOX treatment remains unclear. DOX-induced cardiomyopathy has been linked to apoptosis and DNA damage, free radical formation, and alterations of calcium metabolism (Takemura and Fujiwara, 2007). Recent studies showed that the programmed cardiomyocyte death induced by DOX was related to elevated reactive oxygen species, mitochondrial impairment (Takemura and Fujiwara, 2007), *Fas*-mediated pathway activation (Nitobe et al., 2003), and ceramide generation (Delpy et al., 1999).

Clearly, preventing cardiomyopathy could increase the use and enhance the efficacy of DOX. In this regard, the cytokine erythropoietin (EPO), which stimulates both the production and maturation of red blood cells, has been shown to be cytoprotective against ischemic injury in the heart and other organs (Chong et al., 2002b; Parsa et al., 2003). The effects of EPO have been linked to its ability to reduce apoptosis in neurons (Chong et al., 2002b), vascular smooth muscle cells (Akimoto et al., 2000), vascular endothelial cells (Chong et al., 2002a), and cardiomyocytes (Parsa et al., 2003). Due to its effects on erythropoiesis, EPO is currently used to treat ane-

This study was supported by a grant from the Canadian Institutes for Health Research (to P.H.B). P.H.B is a Career Investigator of the Heart and Stroke Foundation of Ontario. K.H.K is the recipient of a studentship from the Heart and Stroke Richard/Lewar Centre of Excellence at the University of Toronto. Some of the results were presented at the *American Society of Clinical Oncology 41st Annual Meeting* in May 2005, Orlando, FL. American Society of Clinical Oncology, Alexandria, VA.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.107.125773.

ABBREVIATIONS: DOX, doxorubicin; EPO, erythropoietin; EPOR, erythropoietin receptor; PI3K, phosphatidylinositol 3-kinase; NMVM, neonatal mouse ventricular myocyte; SFM, serum-free media; LDH, lactate dehydrogenase; pNA, *p*-nitroaniline; AMVM, adult mouse ventricular myocyte; PKB, protein kinase B; GSK-3, glycogen synthase kinase-3; ROS, reactive oxygen species; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; DCF, 2′,7′-dichlorofluorescein; TUNEL, terminal deoxynucleotidyl transferase nick-end labeling; bpm, beats per minute; CTRL, control; HR, heart rate; P-, phosphorylated-; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; circ/s, circumferences/second.

mia and to improve the quality of life in patients with chronic renal failure, human immunodeficiency virus, and congestive heart failure as well as cancer patients treated with DOX (Venturini et al., 1996; Patton et al., 2004). It is conceivable that the use of EPO might be extended to prevent or reduce the cardiomyopathy associated with DOX treatment.

Because EPO exerts its effects via erythropoietin receptors (EPOR) that activate several signaling cascades, leading to the production of several antiapoptotic proteins included in the Bcl-2 gene family, Janus tyrosine kinase 2, signal transducer and activator of transcription, PI3K, and mitogen-activated protein kinases (Constantinescu et al., 1999), we examined whether EPO could protect the myocardium from DOX-induced toxicity. We found that EPO administration decreased myocardial damage after DOX injection and that it improved cardiac function. This protection was associated with PI3K-dependent Akt activation and reduced apoptosis in cardiomyocytes.

Materials and Methods

Animal Models of DOX-Mediated Cardiomyopathy. Male CD1 8-week-old mice (Charles River Laboratories, Inc., Wilmington, MA) were housed in temperature- and humidity-controlled rooms with 12-h light/dark cycles. All experimental protocols conformed to the standards of the Canadian Council on Animal Care. Mice were randomly divided into four groups (placebo + vehicle, placebo + EPO, DOX + vehicle, and DOX + EPO), and all the drugs were administered by i.p. injection. Mice received either three equal doses of 5 mg/kg DOX (cumulative DOX dose of 15 mg/kg) or equal volumes of saline (i.e., vehicle) every 7 days (Fig. 1A). Mice also received either 1000 U/kg EPO (Epoetin Alfa, EPREX; Ortho Biotech, Toronto, ON, Canada) or an equal volume of saline every third day for 3 weeks. This level of EPO (1000 U/kg) is equivalent to the doses used in human patients (Patton et al., 2004), whereas the DOX concentration (15 mg/kg) corresponds to the recommended maximum cumulative dose of DOX (550 mg/m^2) (Sawyer et al., 1999; Kang et al., 2000; Takemura and Fujiwara, 2007). The mice in all groups were assessed echocardiographically 3 weeks following their final injection.

We also examined the effects of DOX and EPO on a more acute time scale (Fig. 1B). For these studies, mice were injected i.p. either with 2000 U/kg EPO at time 0 followed by additional injection of 1000 U/kg 24 h later. Control animals were injected with equal volumes of saline i.p. at the same time points. Fifteen minutes after the last injection of EPO or saline, the mice received a single dose of 20 mg/kg DOX or an equal volume of saline.

Neonatal Mouse Ventricular Cardiomyocyte Culture. Neonatal mouse ventricular cardiomyocytes (NMVMs) were isolated using a method adapted from rats (Zobel et al., 2002). In brief, 1-day-old CD1 mouse hearts were digested with 50 U/ml collagenase type II (Worthington Biochemicals, Lakewood, NJ) and 0.5 mg/ml trypsin (Invitrogen Canada Inc., Burlington, ON, Canada) in calcium- and bicarbonate-free Hanks' buffer with HEPES. Dissociated cells were collected every 3 to 5 min. Fibroblast and endothelial cell numbers were minimized by differential plating. Cardiomyocytes were counted, plated at a density of 2×10^5 cells/ml, and cultured at 37°C in growth media containing Dulbecco's modified Eagle's medium/Ham's F-12 [1:1 (v/v); Sigma-Aldrich, St. Louis, MO], 10% fetal bovine serum, and 100 units/ml penicillin/streptomycin (Invitrogen Canada Inc.) supplemented with 0.1 mM bromodeoxyuridine (Sigma-Aldrich) and 20 μM arabinosylcytosine (Sigma-Aldrich) to inhibit proliferation of nonmyocytes such as fibroblasts and endothelial cells. After 24 h in culture, the medium was replaced by serum-free medium (SFM) supplemented with 1% insulin-transferrin-selenium supplements-X (Invitrogen Canada Inc.) in Dulbecco's modified Eagle's medium/Ham's F-12 [1:1 (v/v)]. After 24 h in SFM (i.e., after 48 h in culture), the cardiomyocytes were treated with 1 U/ml EPO or vehicle (control). This was followed 24 h later (i.e., after 3 days in culture) with the addition of fresh EPO (or vehicle) along with various concentrations of DOX or vehicle 30 min later. Measurements were made at the end of day 4 in culture.

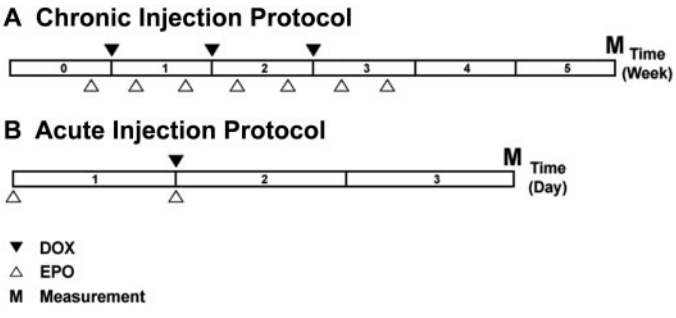

Fig. 1. Experimental time protocols of in vivo studies. A, chronic injection model was designed to examine the protective effect of EPO by mimicking clinical side effect induced by DOX. B, acute injection model was planned to investigate DOX-induced apoptosis and antiapoptotic mechanism of EPO in the heart.

Measurements of Myocyte Viability and Apoptosis in NMVM Cultures. Cell viability was determined at 24 h after treatment with DOX, EPO, or both by counting cardiomyocytes identified immunohistochemically using cardiac α-actinin antibodies. This was achieved by fixing and permeabilizing cultures with 4% paraformaldehyde phosphate buffer containing 0.2% Triton X-100. After incubation with mouse monoclonal anti-α-actinin antibodies (A7811; Sigma-Aldrich), a fluorescent anti-mouse IgG antibody was applied (Alexa Fluor 488; Invitrogen, Carlsbad, CA). Nuclei were also stained with 4,6-diamidino-2-phenylindole (VECTASHIELD; Vector Laboratories, Burlingame, CA). Fluorescent images were captured from 12 to 14 random fields per slide at 100× magnification (Leica Microsystems, Bannockburn, IL). Cardiomyocyte numbers were determined by counting the number of nuclei present in cell showing α-actinin staining. The counting was performed by a blinded investigator with the assistance of Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Cardiomyocyte viability was also assessed by measuring the lactate dehydrogenase (LDH) activity in the culturing media using the TOX-7 (Sigma-Aldrich). LDH concentration was standardized by LDH (catalog no. L2625; Sigma-Aldrich).

Apoptosis was quantified in three ways. First, caspase-3 activity was measured in cardiomyocytes using the ApoAlert assay kit (BD Biosciences, San Jose, CA) 8 h after treatment with DOX, EPO, or both. This method uses the specific substrate Asp-Glu-Val-Asp-*p*-nitroaniline, which is metabolized to the chromophore *p*-nitroaniline by caspase-3. The caspase-3 activities were standardized by *p*-nitroaniline (pNA) and calibrated by the amount of proteins measured by bicinchoninic acid method. Second, apoptosis was assessed by flow cytometry of cardiomyocytes labeled with annexin-V. For this method, after 12 h after DOX, EPO, or combined treatments, cardiomyocytes were suspended by trypsinization and incubated with Annexin-V FLUOS (Roche Diagnostics, Laval, QC, Canada) in the dark for 15 min at room temperature. Then, 10,000 cells were subjected to FACScan (excitation, 488 nm; emission, 515 nm; BD Biosciences). Third, apoptosis was determined using DNA laddering. For these studies, DNA was isolated from NMVMs after 16 h from DOX and EPO administrations. The collected cardiomyocytes were lysed with a solution containing 7.5 mM NaCl, 0.5% SDS, 10 mM Tris, 10 mM EDTA, pH 8.0, and 0.15 mg/ml proteinase K (Sigma-Aldrich). Lysates were incubated at 50°C for 3 h and incubated with 200 μg/ml RNase A (10 μg/μl; Sigma-Aldrich) at 37°C for 1 h. DNA was extracted by phenol-chloroform and precipitated with 2 volumes of ethanol. After centrifugation, DNA was washed with 70% ethanol, resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and resolved on 2% agarose gel with 0.5 mg/ml ethidium bromide.

Western Blot Analysis. Western blots analyses were carried out on 2-day cultured NMVMs, 2-day-cultured neonatal nonmyocyte cells, freshly isolated adult mouse ventricular myocytes (AMVMs), neonatal mouse hearts, and whole adult mouse heart at 8 weeks of age. Samples were lysed in buffer containing 50 mM Tris, pH 7.5, 20 mM EDTA, 100 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Roche Diagnostics), 1 mM sodium orthovanadate, and phosphatase inhibitor cocktail (Sigma-Aldrich). Proteins were resolved on SDS-polyacrylamide gel electrophoresis, transferred to membrane, and incubated with the primary antibodies at 4°C overnight followed by blocking with 5% milk or bovine serum albumin. After 1-h incubation with secondary antibodies at room temperature, the signals were visualized by enhanced chemiluminescence (GE Healthcare, Arlington Heights, IL) and quantified by a densitometry program, GeneGenome (Syngene, Cambridge, UK). EPOR (sc-697) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-Akt/PKB (9271), Akt/PKB (9272), phospho-GSK-3β (9336), and GSK-3β (9332) were purchased from Cell Signaling Technology Inc. (Danvers, MA).

Detection of Reactive Oxygen Species. ROS was measured using the cell-permeant indicator 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Invitrogen Canada Inc.) 5 h after DOX, EPO, or combined treatments in NMVMs. After washing with phosphate-buffered saline, cells were incubated with 10 μM CM-H_2DCFDA in SFM for 30 min, recovered for 5 min in full growth media, and suspended in phosphate-buffered saline by trypsinization. The fluorescent intensity of the oxidized product, 2′,7′-dichlorofluorescein (DCF) was determined using FACSCalibur (excitation, 488 nm; emission, 515–545 nm; BD Biosciences).

Terminal Deoxynucleotidyl Transferase Nick-End Labeling Assay in Mouse Hearts. TUNEL staining was performed in mouse hearts isolated at 2 days after acute injection of EPO and DOX. Hearts were fixed with 10% formaldehyde and embedded with paraffin. Sectioned hearts were stained with biotinylated nucleotides, followed by peroxidase-conjugated avidin. Images were captured from 10 random fields of TUNEL-stained slides using a CoolSNAP digital camera (Roper Scientific, Atlanta, GA). TUNEL-positive cells and total cells were counted using Image Pro Plus (Media Cybernetics).

Echocardiography. Transthoracic 2D, M-mode, and Doppler echocardiographic examinations were performed with a Sequoia C256 system (Acuson Corporation, Mountain View, CA) equipped with a 15-MHz linear transducer (15L8) in mice anesthetized with isoflurane/oxygen (0.75%/100%).

Statistics. All results are expressed as mean ± S.E. Statistical significance of differences among the multiple groups were determined by analysis of variance with post hoc analysis Student-Newman-Keuls. Differences at $P < 0.05$ were considered statistically significant. Calculations and statistical test were performed using SPSS (SPSS Inc., Chicago, IL).

Results

The effects of DOX on heart function and morphology were examined (Fig. 1A) by injecting DOX (three injections of 5 mg/kg every 7 days), as described previously (van Acker et al., 1996; Taniyama and Walsh, 2002). Compared with saline-injected controls, DOX-treated mice showed reductions ($P < 0.05$; $n = 4$) in heart rate (DOX, 417 ± 10 bpm versus CTRL, 507 ± 23 bpm), fractional shortening (DOX, 33.0 ± 0.2% versus CTRL, 48.7 ± 1.5%), and velocities of circumferential shortening (DOX, 6.84 ± 0.0 circ/s versus CTRL, 9.47 ± 0.46 circ/s), without evidence of altered chamber size ($P = 0.142$) or anterior left ventricular wall thickness ($P = 0.068$). The administration of EPO (1000 U/kg every 3 days) protected ($P < 0.05$; $n = 4$) hearts from DOX-induced changes in heart rate (500 ± 20 bpm), fractional shortening (50.9 ± 5.0%), and velocity of circumferential shortening (9.94 ± 0.52 circ/s). EPO treatment alone had no effect on cardiac function.

The protective effects of EPO in DOX-treated mouse hearts might seem somewhat surprising, since previous studies concluded that the expression of the EPOR does not occur in the adult heart and that its expression in the developing heart is limited to the mesothelial epicardium with no expression in myocardium (Wu et al., 1999; Stuckmann et al., 2003). However, as shown in Fig. 2A, EPOR expression increases progressively during development in the mouse heart. This EPOR expression seems to originate from cardiomyocytes since robust EPOR expression was also observed (Fig. 2B) in Western blots of fresh enzymatically isolated AMVMs. Conversely, EPOR expression was not detected in nonmyocyte cells isolated from neonatal mouse hearts.

To assess the mechanism of protection by EPO against DOX-induced cytotoxicity, we used cultured NMVMs. Under our experimental conditions, more than 87% of the cultured cells were spontaneously beating, which coincided with positive immunostaining for cardiac α-actinin in 93.3 ± 1.6% of the cells. Although the results in Fig. 2A demonstrate that EPOR expression is very low in neonatal mouse hearts, as reported previously (Wu et al., 1999), Fig. 2B demonstrates that, after serum-withdrawal for 24 h, EPOR expression in cultured NMVMs is robust, consistent with serum starvation inducing an adult phenotype (Dubus et al., 1993). Figure 3, A and B, shows that DOX treatment for 24 h decreased ($P < 0.05$; $n = 6–8$) the number of surviving cultured cardiomyocytes in a dose-dependent manner (0.1 μM DOX, 75.2 ± 4.9%; 0.5 μM DOX, 62.2 ± 4.7%; and 1.0 μM DOX, 47.8 ± 5.5%), compared with non-DOX-treated cardiomyocytes. Consistent with the benefits of EPO on heart function (Table 1) in DOX-treated mice, 1 U/ml EPO increased ($P < 0.05$; $n = 6–8$) the number of surviving cardiomyocytes at all doses of DOX (0.1 μM EPO + DOX, 89.5 ± 3.0%; 0.5 μM EPO + DOX, 80.8 ± 2.2%; and 1.0 μM EPO + DOX, 68.1 ± 5.1%), whereas EPO treatment alone had no effect on cardiomyocyte survival (EPO, 97.8 ± 2.8%). These actions of DOX and EPO were mirrored in LDH activity measurements (Fig. 3C). Specifically, 1.0 μM DOX treatment increased ($P < 0.01$; $n = 7$) LDH activity in the culture media by more than 2-fold compared with control group, and these increases were attenuated ($P < 0.01$; $n = 9$) by EPO.

The cause of cardiomyocyte loss by DOX is unclear. However, routine microscopic inspection of cultured NMVMs, il-

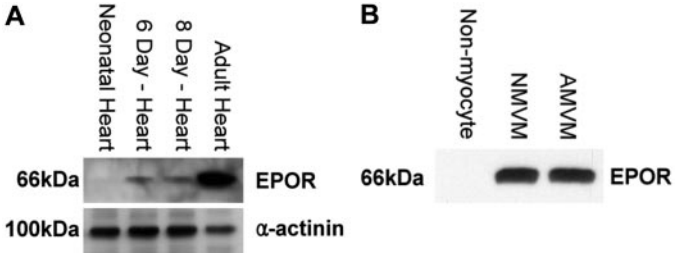

Fig. 2. A, EPOR expression in the heart. Hearts were collected from 1-day-, 6-day-, 8-day-, and 8-week-old mice. EPOR expression in the mouse heart increases progressively during development. B, EPOR expression in cultured nonmyocyte cells, cultured NMVMs, and freshly isolated AMVMs. EPOR is expressed in NMVMs and AMVMs, whereas no EPOR was detected in nonmyocyte cells. The figures are representative of two separate experiments.

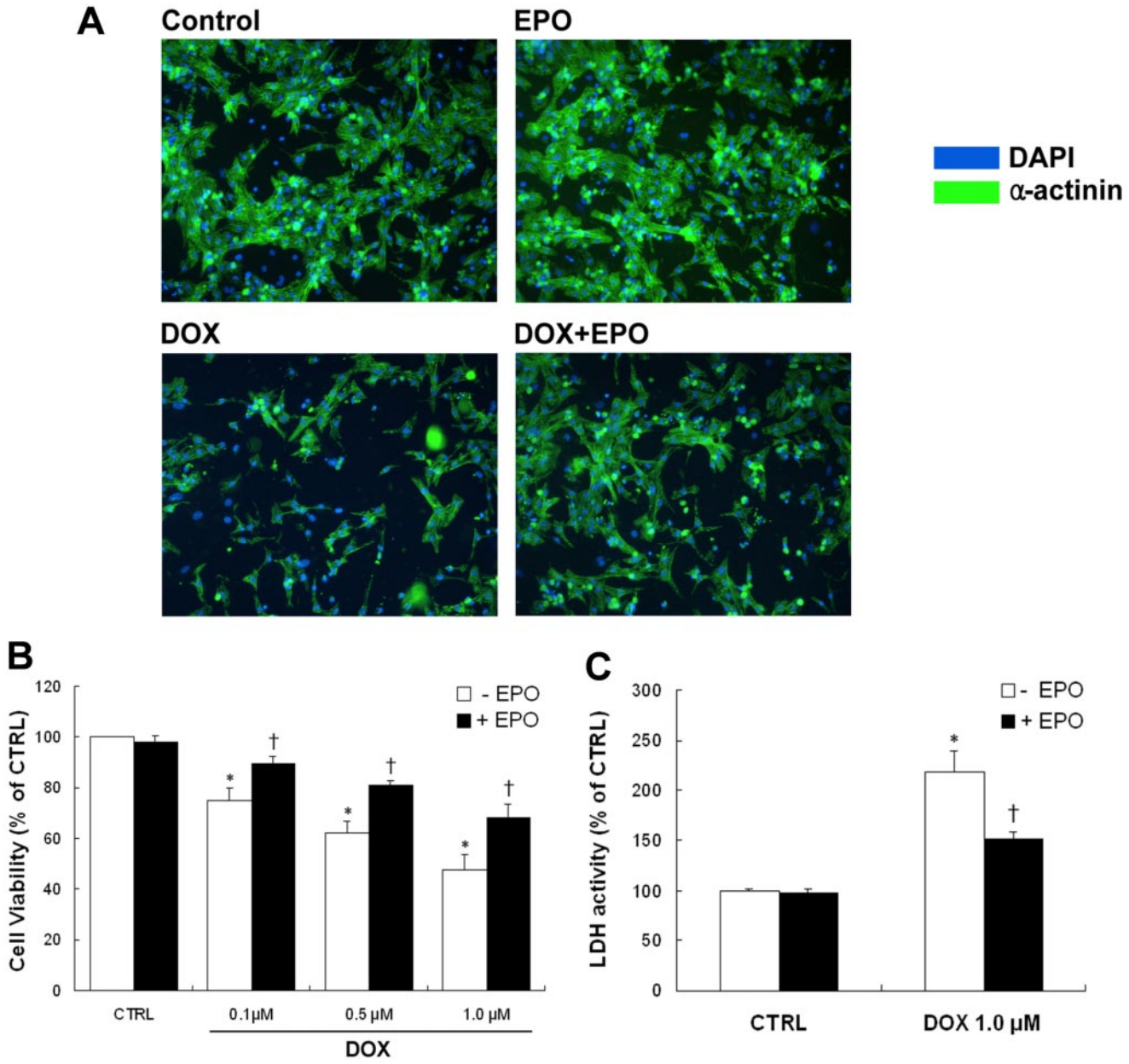

Fig. 3. Protective effect of EPO on NMVMs against DOX-induced cytotoxicity. NMVMs were treated twice with 1 U/ml EPO or physiological saline at 24 h and 30 min before DOX treatment. The cells were then incubated with the three different concentrations of DOX (0.1, 0.5, or 1.0 μM) for 24 h. A, representative immunofluorescent figure of NMVMs, which were treated with 1 U/ml EPO, 1.0 μM DOX, or both describes protective effect of EPO against DOX-induced cytotoxicity. 4,6-Diamidino-2-phenylindole-labeled nuclei and monoclonal antibody-sarcomeric α-actinin labeling are shown in blue and green, respectively. B, cell viability was quantified by the number of live NMVMs. *, $P < 0.05$ versus DOX. †, $P < 0.05$ versus DOX + EPO. C, cytotoxicity was also quantified by LDH activity measurements after DOX, EPO, or combined administrations. *, $P < 0.05$ versus CTRL. †, $P < 0.05$ versus DOX.

lustrated in Fig. 4A, revealed that DOX treatment reduced cell volume in conjunction with changes in cardiomyocyte morphology and cellular fragmentation, suggestive of cardiomyocyte apoptosis (Arola et al., 2000). Consistent with DOX-induced apoptosis, Fig. 4B shows that 1.0 μM DOX induced a 3.5-fold increase ($P < 0.05$; $n = 8$) in caspase-3 activity compared with control (DOX, 45.3 ± 2.2 versus CTRL, 12.8 ± 1.7 nmol pNA/mg), and these increases in caspase-3 activity were attenuated ($P < 0.01$; $n = 7$) by treatment with 1 U/ml EPO (DOX + EPO, 33.1 ± 2.2 nmol pNA/mg). Figure 4, C to E, also shows that EPO treatment reduced DNA laddering and reversed the increased ($P < 0.01$; $n = 4$) annexin-V staining induced by DOX.

Previous studies have established that PI3K-dependent activations of Akt and GSK-3 are powerful regulators of cell survival, replication, and apoptosis as well as oxidative stress (Hanada et al., 2004; Juhaszova et al., 2004; Salinas et al., 2004). To examine whether EPO activates Akt and GSK-3, we measured the levels of phosphorylation on Akt at Ser473 (P-Akt) and GSK-3β at Ser9 (P-GSK-3β). Figure 5, A and B, shows that EPO treatment increased P-Akt (1.90 ± 0.39-fold; $n = 7$) and P-GSK-3β (1.26 ± 0.05-fold; $n = 4$), relative to control. Treatment with the PI3K inhibitor LY294002 at 10 μM blocked the activation of Akt and GSK-3β by EPO, reducing ($P < 0.05$) P-Akt (0.16 ± 0.07-fold; $n = 5$) and P-GSK-3β (0.54 ± 0.08-fold;

TABLE 1
Echocardiographic parameters in mice chronically injected with DOX and EPO

	Placebo + Vehicle	Placebo + EPO	DOX + Vehicle	DOX + EPO
HR (bpm)	507 ± 23	506 ± 17	417 ± 10†	500 ± 20**
Anterior wall thickness (mm)	0.77 ± 0.02	0.74 ± 0.02	0.69 ± 0.04	0.74 ± 0.02
Posterior wall thickness (mm)	0.77 ± 0.04	0.74 ± 0.01	0.69 ± 0.04	0.78 ± 0.03
Left ventricular end-diastolic dimension (mm)	4.23 ± 0.12	4.14 ± 0.10	4.51 ± 0.11	3.89 ± 0.49
Left ventricular end-systolic dimension (mm)	2.17 ± 0.09	2.16 ± 0.24	3.02 ± 0.08†	1.92 ± 0.42*
Fractional shortening (%)	48.7 ± 1.5	48.0 ± 4.5	33.0 ± 0.2†	50.9 ± 5.0**
Ejection time corrected for HR (s)	51.5 ± 2.1	50.8 ± 2.5	48.3 ± 0.4	51.2 ± 3.2
Peak aortic velocity corrected for HR (cm/s)	95.4 ± 4.6	99.8 ± 2.1	91.7 ± 8.5	99.5 ± 14.2
Velocity of circumferential shortening corrected for HR (circ/s)	9.47 ± 0.46	9.45 ± 0.44	6.84 ± 0.00†	9.94 ± 0.52*

* $P < 0.01$ compared with DOX + vehicle.
** $P < 0.05$ compared with DOX + vehicle.
† $P < 0.05$ compared with all other groups.

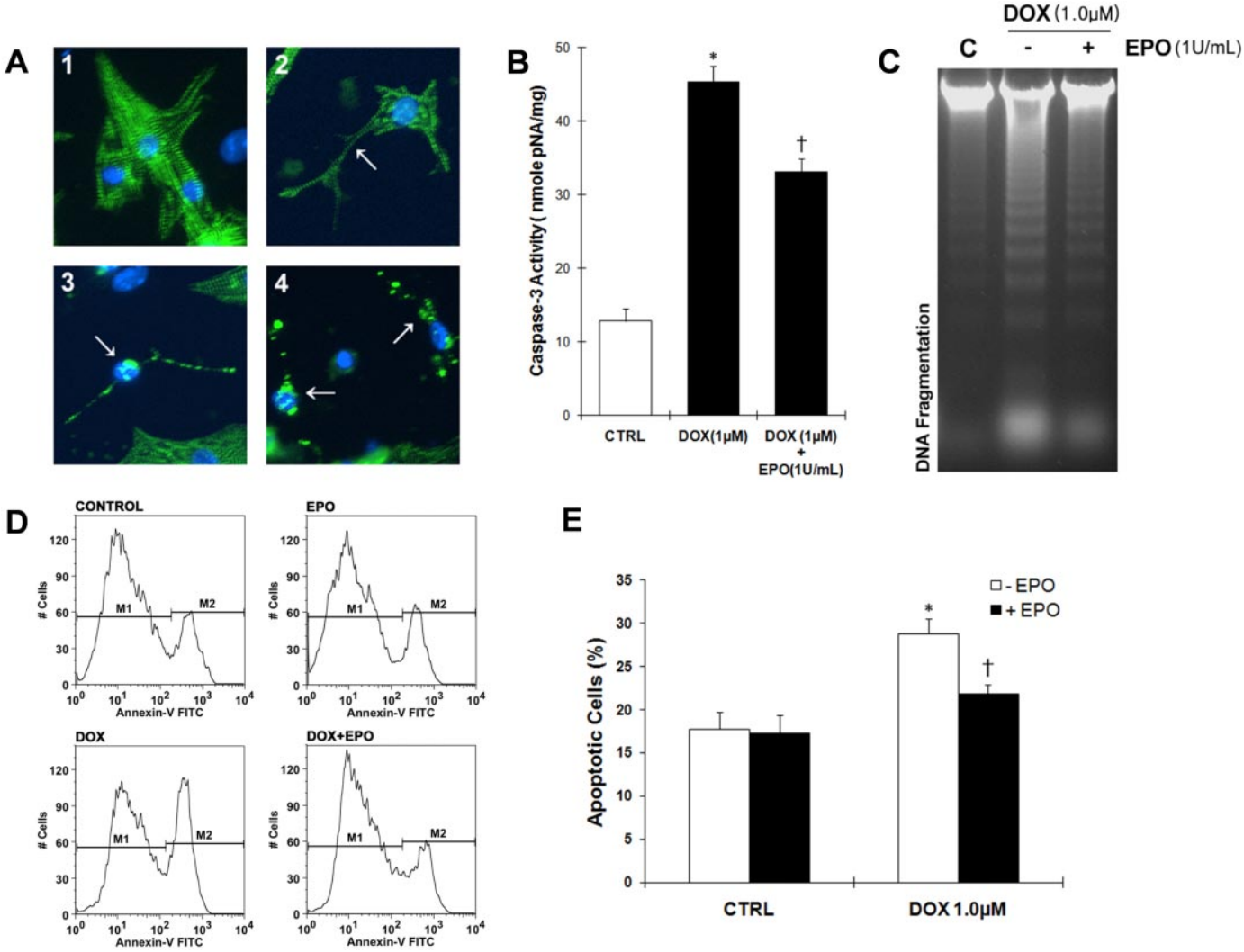

Fig. 4. Antiapoptotic effect of EPO in NMVMs against DOX. A, DOX treatment induced apoptotic morphological alteration in NMVMs such as thin cytoplasmic extensions (2), cellular volume losses (3), and fragmentation into membrane-bound bodies (4), compared with control (1). B, NMVMs pretreated with EPO or saline were harvested after 8-h incubation of DOX. Caspase-3 activity was then measured by the spectrophotometric detection of the chromophore pNA. *, $P < 0.05$ versus CTRL. †, $P < 0.05$ versus DOX. C, DNA fragmentation was illustrated by electrophoresis of genomic DNA extract from NMVMs that were collected after 16 h of DOX administration. D, representative histograms of flow cytometric analysis of annexin-V positivity after 12-h incubation with DOX in the presence or absence of EPO. E, percentage of annexin-V-positive cardiomyocytes demonstrated EPO-mediated protective effect against DOX-induced apoptosis. *, $P < 0.05$ versus CTRL. †, $P < 0.05$ versus DOX.

$n = 4$) to levels below those observed in control. Consistent with the involvement of PI3K-mediated activation of Akt and GSK-3β in cellular protection induced by EPO, PI3K inhibition with 10 μM LY294002 diminished ($P < 0.05$; $n = 7$) cardiomyocyte survival to 45.9 ± 2.4% compared with the DOX + EPO group, without affecting ($P = 0.82$) cell survival in the DOX or control groups (Fig. 6, A and C). In addition, LY294002 treatment increased ($P < 0.05$) LDH

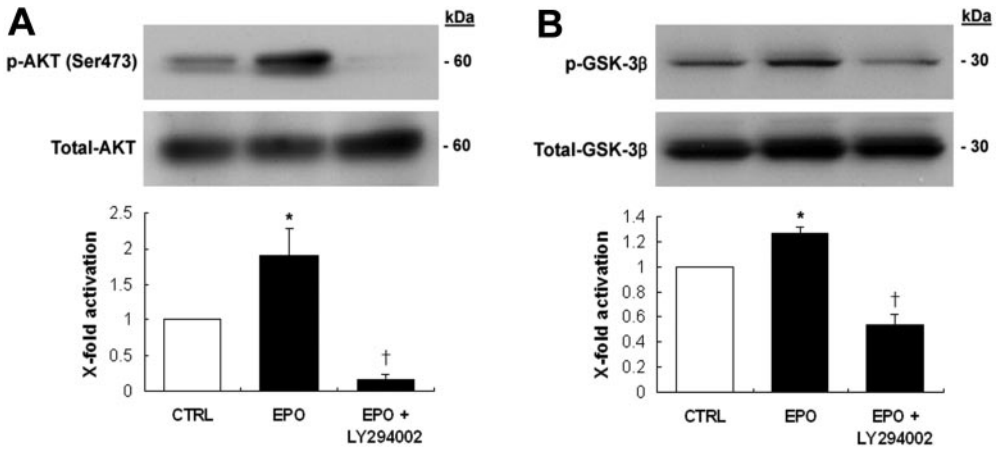

Fig. 5. EPO-mediated Akt/PKB and GSK-3 phosphorylations (top, representative immunoblots; bottom, densitometry data). The figures are representatives of multiple experiments. NMVMs were incubated with EPO for 15 min in the presence or absence of PI3K inhibitor LY294002. Equal amounts of protein lysate from cardiomyocytes were electrophoresed on 8 to 12% SDS-polyacrylamide gel electrophoresis gels and immunoblotted with phospho-specific antibodies for Akt/PKB (A) and GSK-3β (B). *, $P < 0.05$ versus CTRL. †, $P < 0.05$ versus EPO.

activity (202.1 ± 4.0%; $n = 6$), annexin-V staining (30.8 ± 4.4%; $n = 4$) and caspase-3 activities (45.5 ± 3.6 nmol pNA/mg; $n = 6$) in cardiomyocytes treated with DOX + EPO back to the levels observed with DOX treatment alone (Fig. 6, B, D, E, and F). However, LY294002 also increased ($P < 0.05$; $n = 3$) caspase-3 (20.0 ± 3.2 nmol pNA/mg) activity in control cultures without affecting cardiomyocyte survival (Fig. 6, C and F).

Our results in cultured cardiomyocytes suggest that the protective actions of EPO against the proapoptotic effects of DOX are mediated by the activations of Akt and GSK-3β. To explore whether the protective effects of EPO on DOX-in-

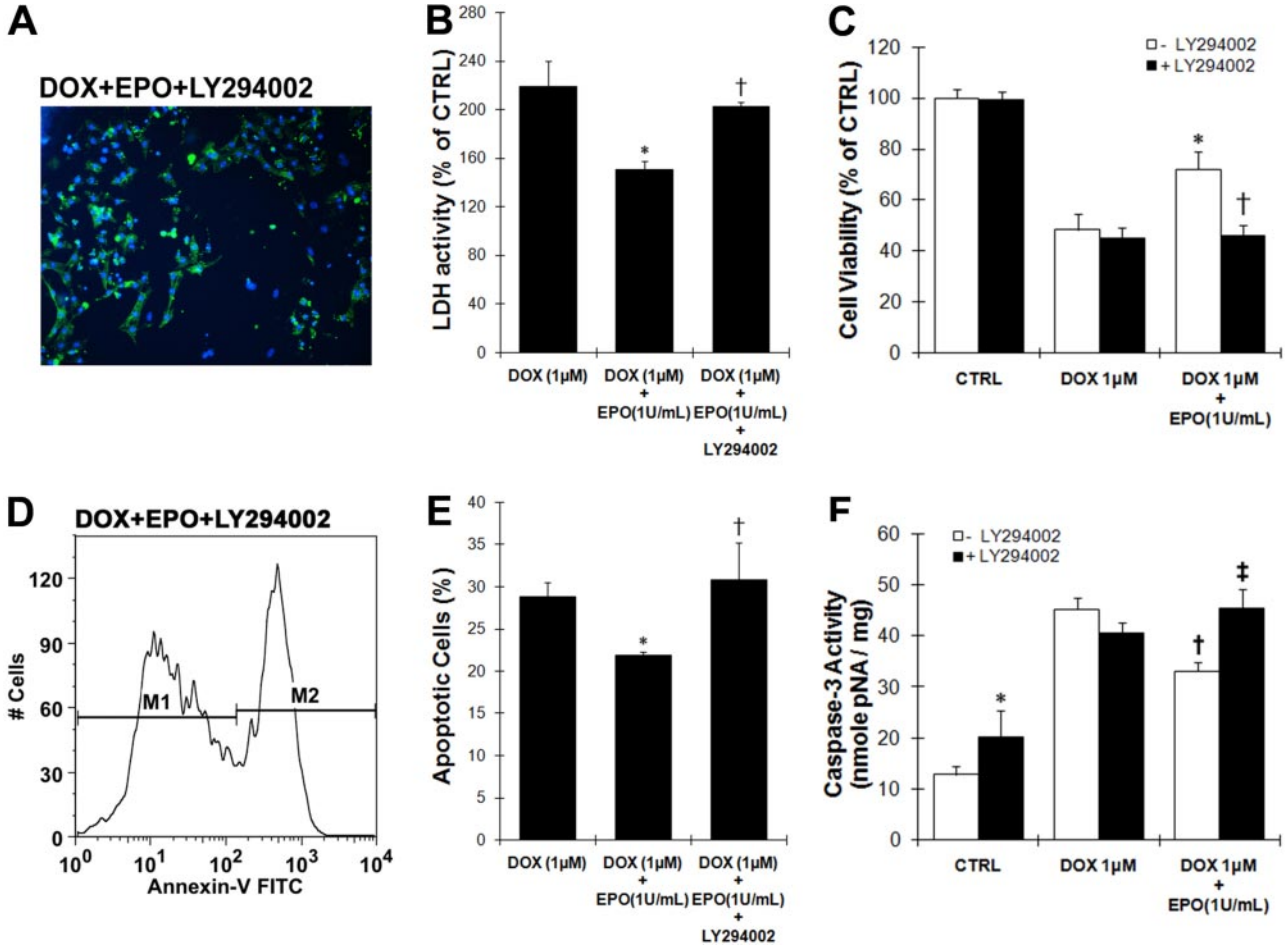

Fig. 6. Protective effect of EPO is abolished by a PI3K inhibitor LY294002. A, representative immunofluorescent figure of NMVMs after DOX + EPO + LY294002 treatments. LY294002 cotreatment (10 μM) eliminated the protective effect of EPO against DOX-induced cytotoxicity, assessed by LDH activity measurement (B) and quantified cell viability (C). *, $P < 0.05$ versus DOX. †, $P < 0.05$ versus DOX + EPO. D, representative histogram of flow cytometric analysis of annexin-V positivity after LY294002 treatment. Consistent with cell survival results, EPO-mediated reductions in both the percentage of apoptotic cardiomyocytes (E) and caspase-3 activity (F) increased by DOX were abrogated by cotreatment of LY294002. *, $P < 0.05$ versus CTRL. †, $P < 0.05$ versus DOX. ‡, $P < 0.05$ versus DOX + EPO.

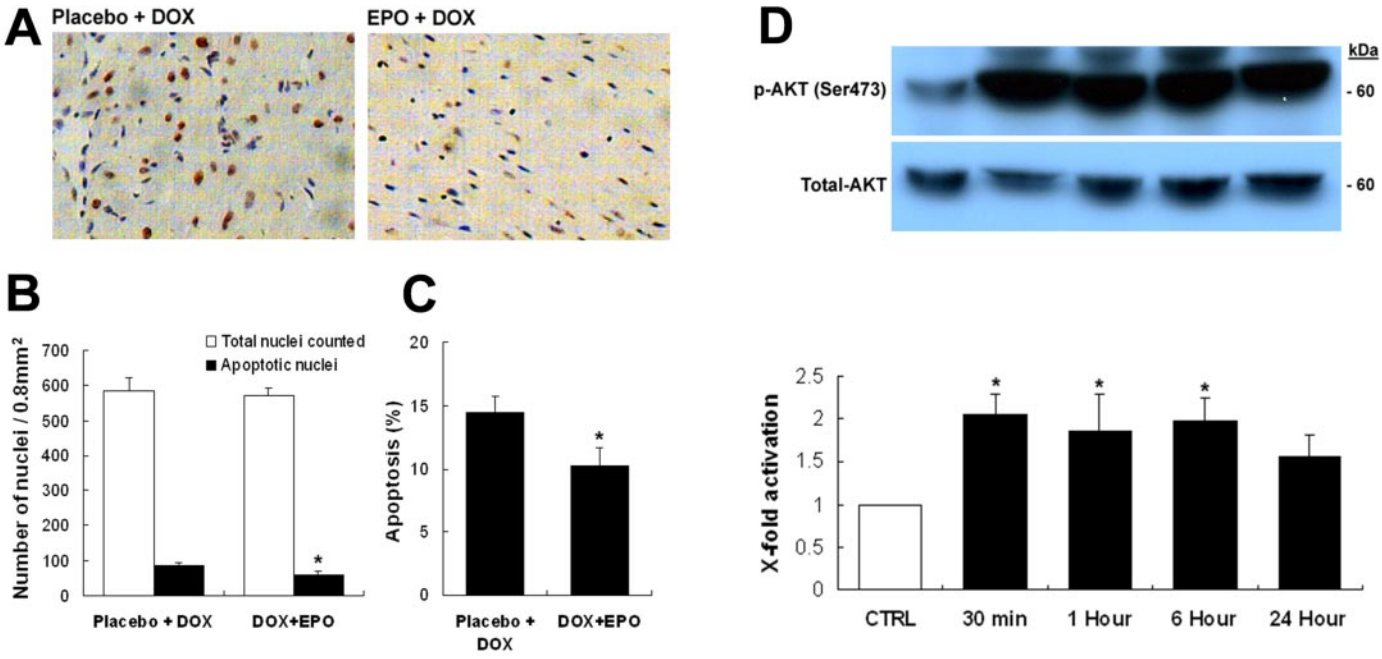

Fig. 7. In vivo antiapoptotic effect of EPO administration (1000 and 2000 U/ml i.p.) against single acute injection of 20 mg/kg i.p. DOX. Mouse hearts were isolated at 2 day from the DOX injection and labeled with TUNEL assay methods. A, representative pictures of TUNEL assay. B, quantitative analysis of apoptosis is carried out with counting the number of total nuclei and TUNEL-positive nuclei from 10 random fields (=0.8 mm^2) per heart. *, $P < 0.05$ versus DOX. C, percentage of apoptosis under DOX administration in the presence or absence of EPO injection. *, $P < 0.05$ versus DOX. D, in vivo Akt/PKB phosphorylations were assessed after EPO injection (1000 U/ml). Hearts were isolated at different time points after EPO administration. *, $P < 0.05$ versus CTRL.

duced impairment of cardiac function are associated with reduced myocardial apoptosis in intact mouse heart, we examined apoptosis in DOX-treated mice. Despite our NMVM results and other previous studies showing DOX-mediated apoptosis (Sawyer et al., 1999), we were unable to detect apoptosis in hearts 3 weeks following the final injection of DOX, consistent with previous reports (Arola et al., 2000). Thus, following a previously published protocol (Kang et al., 2000), we examined the effects of EPO treatment in mouse hearts treated more acutely with a single injection of 20 mg/kg DOX (Fig. 1B). Figure 7, A and B, demonstrates that mouse hearts treated with DOX + EPO showed fewer ($P < 0.05$; $n = 4$) TUNEL-positive nuclei (59.9 ± 2.7/0.8 mm^2) compared with mouse hearts injected with DOX alone (85.5 ± 11.2/0.8 mm^2). Accordingly, the percentage of apoptotic cells to nonapoptotic cells in DOX + EPO group (10.2 ± 0.5%) was lower ($P < 0.05$) compared with DOX + placebo group (14.4 ± 1.4%), as shown in Fig. 7C. To determine whether the antiapoptotic actions of EPO were associated with Akt activation in adult mouse heart, Akt phosphorylation was measured at 0.5, 1, 6, and 24 h after 1000 U/kg EPO i.p. injection. As shown in Fig. 7D, EPO increased ($P < 0.05$) the levels of P-Akt relative to control at several time points after EPO injection (i.e., after 30 min, 1 h, and 6 h, P-Akt was elevated by 2.06 ± 0.23-, 1.87 ± 0.42-, and 1.98 ± 0.260-fold, respectively; $n = 3$–4).

A proposed mechanism for the cardiotoxic effects of DOX involves the induction of ROS, which can induce apoptosis in cardiomyocytes (Sawyer et al., 1999; Takemura and Fujiwara, 2007). This mechanism seems particularly attractive because EPO can reduce oxidative stress in the heart (Li et al., 2006b). Consistent ROS activation by DOX, Fig. 8 shows that, compared with control, DOX treatment increased ($P < 0.02$; $n = 6$) DCF fluorescence (DOX, 124.2 ± 6.1%), an indicator of intracellular ROS. It is noteworthy that EPO reduced DCF fluorescence in both the DOX + EPO (100.8 ± 7.2%; $P < 0.01$; $n = 6$) and control (73.6 ± 5.5%; $P < 0.02$; $n = 3$), confirming the antioxidant role of EPO. More importantly, a PI3K inhibitor LY294002 blocked ($P < 0.001$; $n = 5$) antioxidant effect of EPO in the presence of DOX (DOX + EPO + LY294002, 139.9 ± 6.3%).

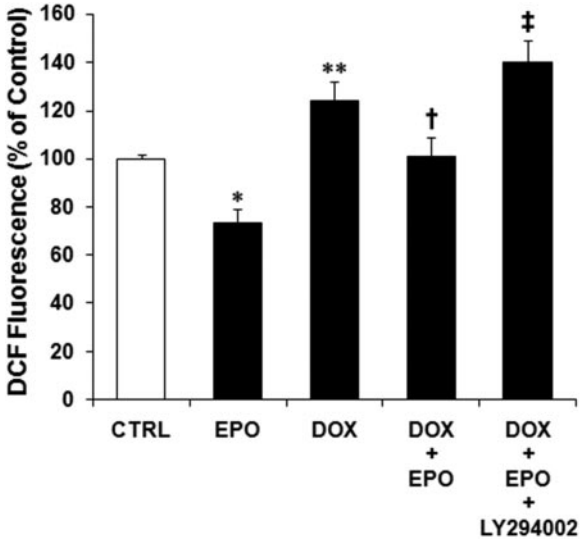

Fig. 8. Intracellular ROS was determined by measuring the fluorescent intensity of DCF oxidized from CM-H_2DCFDA using flow cytometer. *, $P < 0.05$ versus CTRL. **, $P < 0.05$ versus CTRL. †, $P < 0.05$ versus DOX. ‡, $P < 0.05$ versus DOX + EPO.

Discussion

To study the potential protective actions of EPO against the cardiotoxic effects of DOX, we developed a mouse model, as described previously (Taniyama and Walsh, 2002). In this model, DOX injection over a 2-week period reduced heart rate and myocardial contractility measured 3 weeks after DOX injection. The cumulative dose of DOX administered (15 mg/kg) was comparable with doses typically given to cancer patients, which causes bradycardia (van Acker et al., 1996) and reduces cardiac contractility (Taniyama and Walsh, 2002) in many cancer patients. The underlying mechanism of DOX-induced cardiomyopathy is unclear, although previous studies have suggested that DOX causes cardiomyocyte apoptosis (Delpy et al., 1999; Sawyer et al., 1999). However, no apoptosis was observed in our DOX-treated mice 3 weeks following DOX injection, consistent with previous reports (Arola et al., 2000; Li et al., 2006a). The absence of apoptosis at the 3-week time point is not unexpected, because cardiomyocytes are rapidly phagocytized after the initiation of apoptosis (Arola et al., 2000). Consequently, following the work of others (Kang et al., 2000), mice were acutely treated with DOX at doses comparable with our chronic model. Two days after acute DOX treatment, increased TUNEL-positive nuclei were observed in the heart, consistent with previous animal studies (Arola et al., 2000; Kang et al., 2000). These results suggest that DOX causes cardiomyocyte loss via apoptosis shortly after administration, leading, or contributing, to cardiac dysfunction, cardiomyocyte atrophy, fibrosis, inflammatory cell infiltration and altered gene expression observed weeks after DOX injection (Li et al., 2006a). It is noteworthy that EPO injection at doses similar to those used in patients (Patton et al., 2004), and in animals models of ischemia-related (Parsa et al., 2003; Cai and Semenza, 2004) and DOX-induced injuries (Li et al., 2006a) provided protection against both cardiac apoptosis and dysfunction induced by DOX. Importantly, the protection against DOX-related injury by EPO in our studies might be related to EPO exposure before DOX administration. Indeed, a previous study found that EPO was ineffective if applied after DOX treatment (Li et al., 2006a).

The protective actions of EPO against DOX toxicity in the heart could involve several mechanisms originating from actions on cardiomyocytes, noncardiomyocytes, or both. Previous studies concluded that EPOR expression in mouse and chick hearts is limited to nonmyocardial epicardium with EPOR expression disappearing in late gestation (Wu et al., 1999; Stuckmann et al., 2003). Consistent with these findings, EPORs were virtually undetectable in neonatal mouse hearts, but they were highly expressed in adult hearts and AMVMs. In addition, EPORs were not expressed in cultured myofibroblasts isolated from neonatal hearts. These findings support the conclusion that the cardioprotective actions of EPO are mediated via direct activation of EPORs in cardiomyocytes.

To more directly explore whether the protective effects of EPO against DOX-induced cardiotoxicity were related to actions of EPO on cardiomyocytes, we used high-density NMVM cultures (1×10^6 cells/35-mm plate) that were starved after 24 h to induce an adult cardiac phenotype (Dubus et al., 1993). Indeed, unlike freshly isolated NMVMs, serum-starved NMVMs robustly express EPORs, which were functionally active (see below). In our NMVM cultures, DOX treatment for 24 h induced dose-dependent alterations in cardiomyocyte morphology and cardiomyocyte loss that were associated with elevated caspase-3 activity, annexin-V staining, and DNA fragmentation, supporting cardiomyocyte apoptosis as seen in our in vivo studies. The mechanism for DOX-induced apoptosis is unclear, but DOX also strongly increased ROS, a known trigger of apoptosis. These effects of DOX were largely reversed by EPO treatment at doses (1 U/ml) similar to the peak plasma concentration of EPO (1–2 U/ml) achieved in patients (1200 U/kg parenteral injections) (Ramakrishnan et al., 2004) when EPO was added before DOX. Conversely, EPO had no effect in the absence of DOX, and it did not induce proliferation as assessed by [^{3}H]thymidine uptake measurements (data not shown), contrary to previous findings in cultured neonatal rat cardiomyocytes (Wald et al., 1996). The lack of EPO effect on NMVM proliferation versus rat cultures may reflect species differences, or it could result from differences in the timing of EPO treatment (Soonpaa and Field, 1998). Taken together, our results in cultured NMVMs support our findings in acutely treated in vivo mouse hearts, and they establish that physiologically relevant EPO doses can provide EPOR-mediated protection against DOX-induced cardiotoxicity (see below). Moreover, the protective effects of EPO do not result from the absence of serum in our cultures.

Although EPOR stimulation activates multiple signaling pathways, including Janus tyrosine kinase-signal transducer and activator of transcription, ras-mitogen-activated protein kinase, and PI3K (Chong et al., 2002b), we focused on Akt because of its profound antiapoptotic actions via regulating many key proteins involved in cell survival (Hanada et al., 2004). We found that, at therapeutically relevant EPO doses, Akt and GSK-3β phosphorylation was increased in both NMVM and whole hearts. Although a recent study suggested that extracellular signal-regulated kinase 1/2 activation might mediate the protective actions of EPO (Li et al., 2006a), we found that blocking PI3K activation with LY294002 abolished EPO-mediated protection, whereas it had no effect in the absence of EPO. Therefore, our results support the conclusion that EPO protects against DOX-induced cardiomyopathy by activating the PI3K-Akt-GSK-3 signaling cascade, leading to reduced cardiomyocyte apoptosis. In support of this conclusion, Akt-overexpression mice are protected against cardiac dysfunction induced by DOX (Taniyama and Walsh, 2002), whereas neuregulin-1 activates Akt and also suppresses DOX-induced cardiomyocyte apoptosis (Fukazawa et al., 2003). Moreover, EPO reduces cardiomyocyte apoptosis induced by ischemia via PI3K-Akt activation (Parsa et al., 2003; Cai and Semenza, 2004). A more recent report further showed that EPO prevented DOX-induced apoptosis in cultured neonatal rat ventricular myocytes by activating Akt but only at EPO doses that were 10- to 100-fold higher than in our studies where therapeutically relevant doses (i.e., 1 U/ml) were used (Fu and Arcasoy, 2007). This discrepancy in the doses of EPO required for protection could reflect species differences or originate from differences in the timing of EPO application. Indeed, unlike the previous study where EPO was added simultaneously with DOX, we started EPO treatment 24 h before DOX application, thereby potentially producing a "preconditioning" effect. The importance of applying EPO before DOX administration is consistent with a previous study (Li et al., 2006a) and suggests that low therapeutic EPO doses, if judiciously administered in advance of DOX treatment, could be protective in patients receiving DOX.

It is noteworthy that our results in NMVMs revealed that EPO at 1 U/ml only provided modest protection against DOX, similar to other studies (Li et al., 2006a; Fu and Arcasoy, 2007). It is conceivable that higher EPO doses would have provided greater protection by more fully activating cell survival pathways. Conversely, full cardioprotection may require the simultaneous activation of several signaling pathways not directly modulated by EPO. Alternatively, some of the toxic effects of DOX may simply not be preventable by activation of protective pathway. By contrast, our in vivo studies, like those of others (Li et al., 2006a), showed very potent protection of the DOX-induced impairment of cardiac function by EPO. This might suggest that actions of EPO involve other cell types in the heart. For example, EPO could provide additional protection to cardiomyocytes via crosstalking or by paracrine release of other cardioprotective factors. Indeed, EPO was reported to induce endothelial cells to release endothelin-1 (Liefeldt et al., 1998), which is known to inhibit apoptosis in various types of cells, including cardiomyocytes. Clearly, more studies will be required to determine the optimal timing and dosing levels required to maximize the benefit of EPO in treating DOX-induced cardiac injury and dysfunction and to assess other potential cardioprotective mechanisms of EPO against DOX.

Despite our findings and those of several other studies, the clinical relevance of the contribution of apoptosis in DOX-induced cardiomyopathy is still controversial (Takemura and Fujiwara, 2007). Thus, it is conceivable that protection by EPO is related to alternate mechanisms that may, or may not, require PI3K-Akt activation. Consistent with this possibility, EPO reduced oxidative damage in heart disease (Li et al., 2006b). In addition, our findings and those of others (Sawyer et al., 1999; Takemura and Fujiwara, 2007) showed that DOX increased ROS levels, which we found are reduced in NMVMs by EPO. The mechanism whereby EPO reduces ROS and provides cardioprotection will require further investigation, although it is interesting to recognize that increased ROS by DOX is linked to mitochondrial intrinsic apoptotic pathway (Takemura and Fujiwara, 2007) as well as Fas-mediated extrinsic apoptotic pathway (Nitobe et al., 2003). Alternatively, EPO-mediated protection against DOX injury could be related to heme oxygenase-1 (Katavetin et al., 2007), which degrades heme and releases reduced iron (Fe^{2+}), leading to reduced oxidative stress and cardioprotection (Das et al., 2006). Moreover, heme oxygenase-1 is regulated by PI3K-Akt signaling (Salinas et al., 2004), which our results show is required for the protective actions of EPO against DOX injury. Further investigation is clearly required to test this hypothesis.

In summary, our findings suggest that the judicious use of EPO might be useful in reducing cardiomyopathy in patients treated with DOX. This could be particularly relevant because EPO is already commonly used to treat anemia in breast cancer patients undergoing DOX chemotherapy (Larsson et al., 2004). Conceivably, EPO-mediated cardioprotection could enable higher dosages of DOX to be used, thereby improving the efficacy of antineoplastic therapy (Venturini et al., 1996). However, in addition to reducing apoptosis in the heart, EPO might also diminish apoptosis in cancer cells, particularly those expressing EPORs. Indeed, EPOR expression in human breast cancer cells (Arcasoy et al., 2002) is linked to the adverse outcomes in patients receiving combined EPO and chemotherapy (Leyland-Jones et al., 2005), whereas EPOR expression correlates with reduced survival in cancer patients receiving EPO (Henke et al., 2006). However, other studies showed that EPO does not interfere with antineoplastic drugs on carcinoma cell lines expressing EPOR (Gewirtz et al., 2006; LaMontagne et al., 2006). Because cardiomyocyte apoptosis induced by DOX seems to be a relatively acute event, it will be of some interest to determine whether a brief course of EPO treatment before the DOX administration will maximize cardiac benefit while minimizing interference with antineoplastic actions of DOX. This strategy may prove particularly effective if combined with screens for the presence of EPOR in tumor cells.

Acknowledgments

We thank Dr. Thomas Parker and Dr. Robert Tsushima for expert advice and Rachel D. Vanderlaan, Ivona Kozieradzki, and Kiwon Ban for technical assistance.

References

Akimoto T, Kusano E, Inaba T, Iimura O, Takahashi H, Ikeda H, Ito C, Ando Y, Ozawa K, and Asano Y (2000) Erythropoietin regulates vascular smooth muscle cell apoptosis by a phosphatidylinositol 3 kinase-dependent pathway. *Kidney Int* **58:**269–282.

Arcasoy MO, Amin K, Karayal AF, Chou SC, Raleigh JA, Varia MA, and Haroon ZA (2002) Functional significance of erythropoietin receptor expression in breast cancer. *Lab Invest* **82:**911–918.

Arola OJ, Saraste A, Pulkki K, Kallajoki M, Parvinen M, and Voipio-Pulkki LM (2000) Acute doxorubicin cardiotoxicity involves cardiomyocyte apoptosis. *Cancer Res* **60:**1789–1792.

Cai Z and Semenza GL (2004) Phosphatidylinositol-3-kinase signaling is required for erythropoietin-mediated acute protection against myocardial ischemia/reperfusion injury. *Circulation* **109:**2050–2053.

Chong ZZ, Kang JQ, and Maiese K (2002a) Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. *Circulation* **106:**2973–2979.

Chong ZZ, Kang JQ, and Maiese K (2002b) Hematopoietic factor erythropoietin fosters neuroprotection through novel signal transduction cascades. *J Cereb Blood Flow Metab* **22:**503–514.

Constantinescu SN, Ghaffari S, and Lodish HF (1999) The erythropoietin receptor: structure, activation and intracellular signal transduction. *Trends Endocrinol Metab* **10:**18–23.

Das S, Fraga CG, and Das DK (2006) Cardioprotective effect of resveratrol via HO-1 expression involves p38 map kinase and PI-3-kinase signaling, but does not involve NFkappaB. *Free Radic Res* **40:**1066–1075.

Delpy E, Hatem SN, Andrieu N, de Vaumas C, Henaff M, Rucker-Martin C, Jaffrezou JP, Laurent G, Levade T, and Mercadier JJ (1999) Doxorubicin induces slow ceramide accumulation and late apoptosis in cultured adult rat ventricular myocytes. *Cardiovasc Res* **43:**398–407.

Dubus I, Rappaport L, Barrieux A, Lompre AM, Schwartz K, and Samuel JL (1993) Contractile protein gene expression in serum-free cultured adult rat cardiac myocytes. *Pflugers Arch* **423:**455–461.

Fu P and Arcasoy MO (2007) Erythropoietin protects cardiac myocytes against anthracycline-induced apoptosis. *Biochem Biophys Res Commun* **354:**372–378.

Fukazawa R, Miller TA, Kuramochi Y, Frantz S, Kim YD, Marchionni MA, Kelly RA, and Sawyer DB (2003) Neuregulin-1 protects ventricular myocytes from anthracycline-induced apoptosis via erbB4-dependent activation of PI3-kinase/Akt. *J Mol Cell Cardiol* **35:**1473–1479.

Gewirtz DA (1999) A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol* **57:**727–741.

Gewirtz DA, Di X, Walker TD, and Sawyer ST (2006) Erythropoietin fails to interfere with the antiproliferative and cytotoxic effects of antitumor drugs. *Clin Cancer Res* **12:**2232–2238.

Hanada M, Feng J, and Hemmings BA (2004) Structure, regulation and function of PKB/AKT—a major therapeutic target. *Biochim Biophys Acta* **1697:**3–16.

Henke M, Mattern D, Pepe M, Bezay C, Weissenberger C, Werner M, and Pajonk F (2006) Do erythropoietin receptors on cancer cells explain unexpected clinical findings? *J Clin Oncol* **24:**4708–4713.

Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, et al. (2004) Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* **113:**1535–1549.

Kang YJ, Zhou ZX, Wang GW, Buridi A, and Klein JB (2000) Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases. *J Biol Chem* **275:**13690–13698.

Katavetin P, Inagi R, Miyata T, Shao J, Sassa R, Adler S, Eto N, Kato H, Fujita T, and Nangaku M (2007) Erythropoietin induces heme oxygenase-1 expression and attenuates oxidative stress. *Biochem Biophys Res Commun* **359:**928–934.

LaMontagne KR, Butler J, Marshall DJ, Tullai J, Gechtman Z, Hall C, Meshaw A, and Farrell FX (2006) Recombinant epoetins do not stimulate tumor growth in

erythropoietin receptor-positive breast carcinoma models. *Mol Cancer Ther* **5:**347–355.
Larsson AM, Landberg G, Pahlman S, and Albertsson M (2004) Erythropoietin enhances response to treatment in patients with advanced breast cancer. *Acta Oncol* **43:**594–597.
Leyland-Jones B, Semiglazov V, Pawlicki M, Pienkowski T, Tjulandin S, Manikhas G, Makhson A, Roth A, Dodwell D, Baselga J, et al. (2005) Maintaining normal hemoglobin levels with epoetin alfa in mainly nonanemic patients with metastatic breast cancer receiving first-line chemotherapy: a survival study. *J Clin Oncol* **23:**5960–5972.
Li L, Takemura G, Li Y, Miyata S, Esaki M, Okada H, Kanamori H, Khai NC, Maruyama R, Ogino A, et al. (2006a) Preventive effect of erythropoietin on cardiac dysfunction in doxorubicin-induced cardiomyopathy. *Circulation* **113:**535–543.
Li Y, Takemura G, Okada H, Miyata S, Maruyama R, Li L, Higuchi M, Minatoguchi S, Fujiwara T, and Fujiwara H (2006b) Reduction of inflammatory cytokine expression and oxidative damage by erythropoietin in chronic heart failure. *Cardiovasc Res* **71:**684–694.
Liefeldt L, Schmidt-Ott KM, Orzechowski HD, Distler A, and Paul M (1998) Transcriptional regulation of endothelin-1 by erythropoietin in endothelial cells. *J Cardiovasc Pharmacol* **31 (Suppl 1):**S464–S466.
Nitobe J, Yamaguchi S, Okuyama M, Nozaki N, Sata M, Miyamoto T, Takeishi Y, Kubota I, and Tomoike H (2003) Reactive oxygen species regulate FLICE inhibitory protein (FLIP) and susceptibility to Fas-mediated apoptosis in cardiac myocytes. *Cardiovasc Res* **57:**119–128.
Parsa CJ, Matsumoto A, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Annex BH, Stamler JS, et al. (2003) A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* **112:**999–1007.
Patton J, Kuzur M, Liggett W, Miranda F, Varsos H, and Porter L (2004) Epoetin alfa 60,000 U once weekly followed by 120,000 U every 3 weeks increases and maintains hemoglobin levels in anemic cancer patients undergoing chemotherapy. *Oncologist* **9:**90–96.
Ramakrishnan R, Cheung WK, Wacholtz MC, Minton N, and Jusko WJ (2004) Pharmacokinetic and pharmacodynamic modeling of recombinant human erythropoietin after single and multiple doses in healthy volunteers. *J Clin Pharmacol* **44:**991–1002.
Salinas M, Wang J, Rosa de Sagarra M, Martin D, Rojo AI, Martin-Perez J, Ortiz de Montellano PR, and Cuadrado A (2004) Protein kinase Akt/PKB phosphorylates heme oxygenase-1 in vitro and in vivo. *FEBS Lett* **578:**90–94.
Sawyer DB, Fukazawa R, Arstall MA, and Kelly RA (1999) Daunorubicin-induced apoptosis in rat cardiac myocytes is inhibited by dexrazoxane. *Circ Res* **84:**257–265.
Soonpaa MH and Field LJ (1998) Survey of studies examining mammalian cardiomyocyte DNA synthesis. *Circ Res* **83:**15–26.
Stuckmann I, Evans S, and Lassar AB (2003) Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation. *Dev Biol* **255:**334–349.
Takemura G and Fujiwara H (2007) Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. *Prog Cardiovasc Dis* **49:**330–352.
Taniyama Y and Walsh K (2002) Elevated myocardial Akt signaling ameliorates doxorubicin-induced congestive heart failure and promotes heart growth. *J Mol Cell Cardiol* **34:**1241–1247.
van Acker SA, Kramer K, Voest EE, Grimbergen JA, Zhang J, van der Vijgh WJ, and Bast A (1996) Doxorubicin-induced cardiotoxicity monitored by ECG in freely moving mice. A new model to test potential protectors. *Cancer Chemother Pharmacol* **38:**95–101.
Venturini M, Del Mastro L, Testore F, Danova M, Garrone O, Lanfranco C, Latini F, Sertoli MR, Lionetto R, Queirolo P, et al. (1996) Erythropoietin and granulocyte-macrophage colony-stimulating factor allow acceleration and dose escalation of cyclophosphamide/epidoxorubicin/5-fluorouracil chemotherapy: a dose-finding study in patients with advanced breast cancer. *Cancer Chemother Pharmacol* **38:**487–494.
Wald MR, Borda ES, and Sterin-Borda L (1996) Mitogenic effect of erythropoietin on neonatal rat cardiomyocytes: signal transduction pathways. *J Cell Physiol* **167:**461–468.
Wu H, Lee SH, Gao J, Liu X, and Iruela-Arispe ML (1999) Inactivation of erythropoietin leads to defects in cardiac morphogenesis. *Development* **126:**3597–3605.
Zobel C, Kassiri Z, Nguyen TT, Meng Y, and Backx PH (2002) Prevention of hypertrophy by overexpression of Kv4.2 in cultured neonatal cardiomyocytes. *Circulation* **106:**2385–2391.

Address correspondence to: Dr. Peter H. Backx, Departments of Physiology and Medicine, Heart and Stroke/Richard Lewar Centre of Excellence, Room 71, FitzGerald Bldg. 150 College St., Toronto, ON M5S 3E2, Canada. E-mail: p.backx@utoronto.ca