

# Erythropoietin, Modified to Not Stimulate Red Blood Cell Production, Retains Its Cardioprotective Properties

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## ABSTRACT

Erythropoietin (EPO), a hematopoietic cytokine, possesses strong antiapoptotic, tissue-protective properties. For clinical applications, it is desirable to separate the hematopoietic and tissue-protective properties. Recently introduced carbamylated erythropoietin (CEPO) does not stimulate the erythropoiesis but retains the antiapoptotic and neuroprotective effects. We tested the ability of CEPO to protect cardiac tissue from toxin-induced and oxidative stress in vitro and ischemic damage in vivo and compared these effects with the effects of EPO. CEPO reduced by 50% the extent of staurosporine-induced apoptosis in isolated rats' cardiomyocytes and increased by 25% the reactive oxygen species threshold for induction of the mitochondrial permeability transition. In an experimental model of myocardial infarction induced by permanent ligation of a coronary artery in rats, similarly to EPO, a single bolus injection of 30

$\mu\text{g}/\text{kg}$  b.wt. of CEPO immediately after coronary ligation reduced apoptosis in the myocardial area at risk, examined 24 h later, by 50%. Left ventricular remodeling (ventricular dilation) and functional decline (fall in ejection fraction) assessed by repeated echocardiography were significantly and similarly attenuated in CEPO- and EPO-treated rats. Four weeks after coronary ligation, the myocardial infarction (MI) size in CEPO- and EPO-treated rats was half of that in untreated coronary-ligated animals. Unlike EPO, CEPO had no effect on hematocrit. The antiapoptotic cardioprotective effects of CEPO, shown by its ability to limit both post-MI left ventricular remodeling and the extent of the myocardial scar in the model of permanent coronary artery ligation in rats, demonstrate comparable potency to that of native (nonmodified) EPO.

Erythropoietin (EPO) is a well known hematopoietic cytokine produced by the kidney in response to hypoxia (Yousoufian et al., 1993). Recombinant human EPO (rhEPO) is widely used to treat the anemia related to surgery, cancer, and kidney failure (Jelkmann, 1994). However, EPO possesses much broader salutary effects than merely stimulation of red blood cell production. EPO receptors, originally thought to be confined only to hematopoietic tissue in adults, were also found in other tissues, for example, neural tissue (for review, see Masuda et al., 1999). Many recent studies have demonstrated the neuroprotective effects of rhEPO in different animal models (Sadamoto et al., 1998; Bernaudin et

al., 1999; Brines et al., 2000) and in a phase II clinical trial in cerebral ischemia (Ehrenreich et al., 2002).

In several recent studies, the effects of systemic administration of rhEPO have been extended to include cardioprotection from ischemia. The antiapoptotic effects of rhEPO on cardiomyocytes have been reported in tissue culture and in vivo animal models of ischemia-reperfusion injury (for review, see Smith et al., 2003; Bogoyevitch, 2004). The recent discovery of EPO receptors in cardiomyocytes of adult rat solidified these findings (Wright et al., 2004).

In a rat model of myocardial ischemia using permanent ligation of a coronary artery, we have shown that in comparison with untreated animals, a single systemic injection of 30  $\mu\text{g}/\text{kg}$  b.wt. of rhEPO immediately after coronary artery ligation reduced apoptosis in the myocardial area at risk 24 h later by 50%. Left ventricular remodeling was suppressed in rhEPO-treated rats, and 8 weeks after coronary ligation, the

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**ABBREVIATIONS:** EPO, erythropoietin; rhEPO, recombinant human erythropoietin; MI, myocardial infarction; STAT, signal transducer and activator of transcription; PI3K, phosphatidylinositol 3 kinase; MPT, mitochondrial permeability transition; CEPO, carbamylated erythropoietin; LV, left ventricle; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; SH, sham (S); EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; ANOVA, analysis of variance.

myocardial infarct (MI) scar was 4-fold smaller than in untreated coronary artery-ligated animals (Moon et al., 2003).

A number of signaling pathways reportedly have been involved in the mechanism of EPO-induced cardioprotection. Jak-2/STAT signaling was implicated as well as PI3K signaling (Parsa et al., 2003), protein kinase C, p38, and p42/44 mitogen-activated protein kinase activation, affecting sarcolemmal and mitochondrial potassium channels,  $K_{ATP}$  (Shi et al., 2004), and Akt signaling (Calvillo et al., 2003; Parsa et al., 2003). We have recently reported that the end effector of cardioprotection by rhEPO is the permeability transition pore complex: rhEPO limits the induction of mitochondrial permeability transition (MPT) in cardiomyocytes and, thus, promotes their survival during adverse conditions (Juhászova et al., 2004).

Thus, a number of convincing preclinical experiments suggest that systemic administration of rhEPO presents a new therapeutic approach to limit myocardial damage and subsequent heart remodeling after ischemia (Maiese et al., 2005). However, the classic property of EPO to activate production of red blood cells and thrombocytes is the weakness of such therapy, limiting it to a single application and, even as such, might be contraindicated in some patients. The attendant elevation of hematocrit associated with repeated rhEPO treatment may have an adverse effect on the outcome of MI (Spiess, 1999).

A modification of EPO by subjecting it to carbamylation has recently been introduced for tissue protection (Leist et al., 2004). This carbamylated EPO (CEPO) completely lacks bioactivity in hematopoiesis bioassays and in vivo animal testing with repeated high-dose injection but effectively protects isolated neural cells from induced apoptosis. Moreover, in in vivo experiments, CEPO does not bind to EPO receptors (Leist et al., 2004). Further experiments established that CEPO's pharmacodynamic parameters are similar to that of rhEPO, and it mimics rhEPO efficacy in experimental models of brain ischemia, spinal cord and nerve damage, and autoimmune encephalomyelitis (Leist et al., 2004). In a temporary coronary ligation (ischemia-reperfusion model) in the rat (Fiordaliso et al., 2005), CEPO was shown to be cardioprotective in preventing increases in LV end-diastolic pressure, LV wall stress in systole and diastole, and improving the LV response to dobutamine. Protection against staurosporine-induced cardiomyocyte apoptosis in vitro was also observed.

The objective of this study is to establish the relationship between the efficacy of CEPO as a cardioprotective compound in vitro and in vivo and the mechanism of protection operating through induction of the MPT independently of its effects on hematopoiesis. We hypothesized that CEPO would demonstrate antiapoptotic properties in isolated cardiomyocytes undergoing hypoxia/reoxygenation stress and enhance their survival by limiting induction of the MPT. We also hypothesized that similar to EPO, systemic administration of a single dose of CEPO immediately after coronary ligation in rats would 1) reduce apoptosis in the area of myocardium at risk [area at risk (AAR)] 24 h later, 2) would attenuate the ensuing left ventricular remodeling and functional decline in the following weeks, and 3) would result in a smaller MI size at the end of 4 weeks of observation.

## Materials and Methods

### Materials

CEPO was produced by Warren Pharmaceuticals, Inc. (Ossining, NY) by subjecting rhEPO (Dragon Pharmaceutical, Vancouver, BC, Canada) to carbamylation—the process by which all lysines were transformed to homocitrulline (Leist et al., 2004). The dosages of CEPO used in the in vivo experiments were equivalent to EPO in terms of weight; i.e., 3000 IU/kg b.wt. EPO and 30  $\mu$ g/kg b.wt. CEPO.

### I. In Vitro Protocols

**Left Ventricular Myocytes Isolation for Experiments on Cell Culture.** Left ventricular cardiomyocytes were isolated from adult Sprague-Dawley rats (250–300 g; Taconic Farms, Germantown, NY) in a perfusion chamber using Adumyts (Cellutron, Highland Park, NJ) proprietary buffers. Twenty minutes before sacrifice, animals were given 5000 U/kg heparin (Sigma-Aldrich, St. Louis, MO). Hearts were isolated rapidly, perfused through the aorta, and gassed with 85%  $O_2$  and 15%  $N_2$  at 37°C. Myocytes were then isolated by mechanical dissociation, separated by differential centrifugation, and plated on laminin (Sigma-Aldrich)-coated dishes (Calvillo et al., 2003). After 1 h, the medium was changed, and CEPO or EPO (100 ng/ml) or control buffer was added to the myocytes 30 min before induction of apoptosis by staurosporine (0.1  $\mu$ M; Sigma-Aldrich). After 16-h incubation, myocytes were washed with ice-cold Hanks' solution (Invitrogen, Carlsbad, CA), fixed for 20 min in 10% MeOH-free formaldehyde (Polysciences, Warrington, PA) at 4°C, washed in ice-cold Hanks' solution, stored in  $-20^\circ\text{C}$  70% EtOH overnight, and processed for in situ terminal deoxynucleotidyl transferase assay (Roche, Minneapolis, MN) for detection of apoptosis.

**Left Ventricular Myocytes Isolation for Mitochondrial Permeability Transition Experiments.** Single ventricular myocytes were isolated via a previously described technique with minor modifications (Capogrossi et al., 1986). Briefly, 2- to 4-month-old Sprague-Dawley rats were anesthetized with sodium pentobarbital, and hearts were rapidly excised and perfused with 40 ml of nominally  $Ca^{2+}$ -free bicarbonate buffer gassed with 95%  $O_2$  to 5%  $CO_2$  at 37°C. The composition of buffer was the following: 116.4 mM NaCl, 5.4 mM KCl, 1.2 mM  $MgSO_4$ , 1.2 mM  $NaH_2PO_4$ , 5.6 mM glucose, and 26.2 mM  $NaHCO_3$ , pH 7.4. Hearts were continuously perfused with bicarbonate buffer containing 0.1% collagenase type B, 0.04 mg/ml protease XVI, and 0.1% bovine serum albumin type V for 4 min, and 50  $\mu$ M  $Ca^{2+}$  was added. After 10-min perfusion, the left ventricle was minced and incubated in bicarbonate buffer containing 100  $\mu$ M  $Ca^{2+}$  for 10 min at 37°C. Myocytes were then resuspended in HEPES buffer with gradually increasing  $Ca^{2+}$  concentration up to 1 mM and kept at room temperature until use. The composition of the HEPES buffer was the following: 137 mM NaCl, 4.9 mM KCl, 1.2 mM  $MgSO_4$ , 1.2 mM  $NaH_2PO_4$ , 15 mM glucose, 20 mM HEPES, and 1.0 mM  $CaCl_2$  (adjusted pH to 7.4). Cardiac myocytes viability was typically 70 to 80%.

**Confocal Microscopy and Determination of MPT-ROS Threshold.** Experiments were conducted as described previously (Juhászova et al., 2004), using a method to quantify the ROS susceptibility for the induction of MPT in individual mitochondria within cardiac myocytes (Zorov et al., 2000). Briefly, isolated cardiac myocytes were exposed in vitro to conditions that mimic oxidative stress by repetitive laser scanning of a row of mitochondria in a myocyte loaded with tetramethylrhodamine methyl ester (TMRM; see Fig. 2). This results in incremental, additive exposure of only the laser-exposed area to the photodynamic production of ROS and consequent MPT induction. The occurrence of MPT is clearly identified by the immediate dissipation of  $\Delta\Psi$ . Myocytes were loaded with 125 nM TMRM for at least 1 h at room temperature and imaged with an LSM-510 inverted confocal microscope (Carl Zeiss Inc., Jena, Germany) (Fig. 2A). Line scan images at 2 Hz were recorded from mitochondria arrayed along individual myofibrils with excitation at

568 nm and collecting emission at  $>560$  nm, using a Zeiss Plan-Apochromat 63 $\times$ /1.4 numerical aperture oil immersion objective, and the confocal pinhole was set to obtain spatial resolutions of 0.4  $\mu\text{m}$  in the horizontal plane and 1  $\mu\text{m}$  in the axial dimension. Images were processed by MetaMorph software (Universal Imaging, Downingtown, PA). The ROS threshold for MPT induction ( $t_{\text{MPT}}$ ) was measured as the average time necessary to induce MPT in a row consisting of  $\sim 25$  mitochondria (Fig. 2B). Experiments were carried out at 23°C. The cardioprotective action of insulin, which normally results in an enhancement of the MPT-ROS threshold by  $\sim 35$  to 40% (Juhászova et al., 2004), was used as a positive control in the present experiments. In parallel experiments, cells were exposed to CEPO (10, 100, or 250 ng/ml for 20 min prior to  $t_{\text{MPT}}$  measurements). Wortmannin (50  $\mu\text{M}$ ) was also applied in certain protocols.

## II. In Vivo Experiments

**Animals and Experimental Design.** Eighty male Sprague-Dawley rats, 2 months of age, were housed and studied in conformance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, Manual 3040-2 (1999), with institutional Animal Care and Use Committee approval. After baseline echocardiography, animals were randomly divided into coronary artery-ligated (MI;  $n = 54$ ) or sham (SH;  $n = 16$ ) groups and, under inhalation anesthesia by isoflurane, subjected to ligation of the left anterior descending coronary artery to induce myocardial infarction (MI) or to a sham operation, as previously described (Moon et al., 2003). Animals in the MI group were either treated with a single systemic injection of CEPO ( $n = 18$ ) or EPO ( $n = 18$ ) or remained untreated ( $n = 18$ ). Both CEPO and EPO, 30  $\mu\text{g}/\text{kg}$  b.wt., were given i.v. in 0.3 ml of saline immediately ( $<5$  min) after surgery. Untreated animals received a single i.v. injection of 0.3 ml of saline at the same time. SH animals were either injected with CEPO ( $n = 8$ ), or with saline ( $n = 8$ ) in a dose and manner similar to MI animals. Therefore, the experimental design consisted of five groups of rats: sham, not treated (SH-SALINE); sham, treated with CEPO (SH-CEPO); MI, not treated (MI-SALINE); and MI, treated with CEPO (MI-CEPO), and MI, treated with EPO (MI-EPO). Six animals from each of the MI-CEPO, MI-EPO, and the MI-SALINE groups were killed 24 h after surgery, and their hearts were harvested for appropriate immunohistochemical staining to assess the early effect of CEPO treatment on the extent of post-MI apoptosis. In the remainder of the operated animals, LV function was assessed by echocardiography 1 and 4 weeks after surgery, at which time all animals were killed using a bolus injection of 4 ml of 0.5 M KCl under general anesthesia with sodium pentobarbital (50 mg/kg b.wt., i.p.), and their hearts were harvested for histological analyses.

**Echocardiography.** Cardiac function was assessed by echocardiography (HP Sonos 5500 equipped with a 12-MHz phase array linear transducer, S12, allowing a 150-mm/s maximal sweep rate; Hewlett Packard, Palo Alto, CA) under general anesthesia with pentobarbital sodium (30 mg/kg b.wt., i.p.) as described previously (Moon et al., 2003). Briefly, parasternal long-axis views were obtained and recorded, ensuring that the mitral and aortic valves and the apex were visualized. Endocardial area tracings using the leading-edge method were performed in the two-dimensional mode (short- and long-axis views) from digital images captured on cineloop. LV end-diastolic volume (EDV) and LV end-systolic volume (ESV) were calculated by a modified Simpson's method from the long-axis view. LV ejection fraction (EF %) was derived as  $\text{EF} = (\text{EDV} - \text{ESV})/\text{EDV} \times 100$ . All measurements were made by one observer who was blinded with respect to the identity of the tracings. All measurements were averaged over three to five consecutive cardiac cycles. The reproducibility of measurements was assessed at baselines by two sets of measurements in 10 randomly selected rats. The repeated measure variability did not exceed  $\pm 5\%$ .

**Infarct Size Measurement.** Hearts were excised and placed in 10% phosphate-buffered formalin. The fixed tissue was then embedded in paraffin and serially cut from the apex to the level just below

the coronary artery ligation site; transverse 6- $\mu\text{m}$ -thick sections were cut at 600- $\mu\text{m}$  distances such that 10 to 12 sections were obtained from each heart. Sections were stained with hematoxylin/eosin and azan, and morphological analysis was performed by computerized video imaging using an Axioplan microscope (Zeiss) and NIH IMAGE software (Bethesda, MD). The myocardial infarct size of each section was calculated as the ratio of infarction area to the area of total LV section (area method) and as the average of ratios of the outer infarction length to the outer LV circumference and the inner infarction length to the inner LV circumference (perimeter method). The infarct size of all sections for both area and perimeter methods was averaged and expressed as the percentage of LV for each heart.

**LV Posterior Wall Thickness Measurement.** The thickness of LV posterior wall was measured and averaged in each LV section where the myocardial infarct size was measured.

**Assessment of Apoptosis in Hearts.** Twenty-four hours after coronary artery ligation or sham operation, under general anesthesia with pentobarbital sodium (50 mg/kg b.wt., i.p.), 2 ml of 5% Evans blue was injected into the right ventricular chamber via the right jugular vein. The rats were killed immediately by a bolus injection of 4 ml of 0.5 M KCl, and the hearts were removed, rapidly rinsed in phosphate-buffered saline, and snap-frozen in liquid nitrogen. Serial, 6- $\mu\text{m}$ -thick cryostat sections were prepared. Processing of subsequent sections alternated between tetrazolium chloride and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining. The parts unstained by Evans blue containing a combination of dead tissue and underperfused but viable myocardium (AAR) were incubated for 20 min in tetrazolium chloride and then transferred into 4% paraformaldehyde. In all resulting sections, the AAR of myocardial tissue was stained in red, whereas dead tissue remained white (Bialik et al., 1997). The AAR on every other section was further subjected to TUNEL staining for detection of apoptotic cells by the nick-end labeling method using a commercially available kit (Roche) as directed by the manufacturer. Slides were examined by light microscopy. In each section, the number of cardiomyocytes and the number of TUNEL-positive cardiomyocyte nuclei were counted and totaled in 10 randomly selected fields of the AAR at  $\times 400$  amplification. Only nuclei that were clearly located in cardiomyocytes were counted.

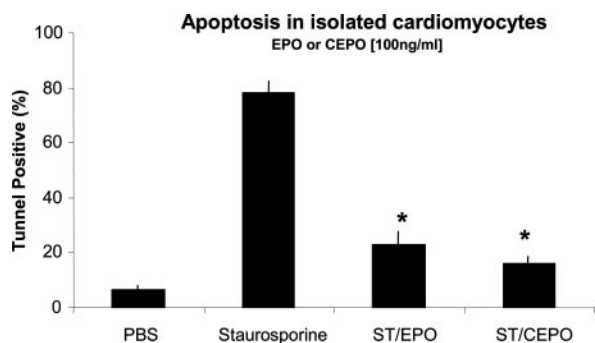
**Statistical Analyses.** Sonographic indices of morphometric and functional assessment at each time point were expressed as a percentage of change from the baseline (measurements taken before surgery). All values were corrected for body mass. Statistical significance of differences among groups with regard to changes of these indices over time was determined using ANOVA for repeated measurements, specifically noting group  $\times$  time interactions. A post hoc pair comparison between MI-SALINE, MI-CEPO, and MI-EPO groups was conducted for the 4<sup>th</sup>-week data. Statistical significance of differences between groups with regard to infarct size and apoptosis was determined using a one-way ANOVA following by a post hoc pair comparison. The same approach was used in vitro experiments. Statistical significance was assumed at  $p < 0.05$ .

## Results

### In Vitro Experiments

**Apoptosis in Isolated Cardiomyocytes.** After 16 h of staurosporine exposure, 78% of untreated myocytes were apoptotic. In the presence of 100 ng/ml rhEPO or CEPO, the number of apoptotic myocytes was reduced by 77 and 87%, respectively (Fig. 1).

**Assessment of MPT-ROS Threshold.** Figure 2C presents the cardioprotective effects of CEPO in isolated cardiac myocytes as indexed by the ROS threshold for MPT induction ( $t_{\text{MPT}}$ ). CEPO exposure at 10, 100, and 250 ng/ml resulted in increased MPT-ROS threshold by 20 to 25% above the un-



**Fig. 1.** The antiapoptotic effect of rhEPO and CEPO on isolated rat cardiomyocytes. After a 30-min *in vitro* exposure of isolated rat ventricular cardiomyocytes to 100 ng/ml CEPO or EPO, apoptosis was induced by staurosporine. After 16-h incubation, myocytes were processed for *in situ* terminal deoxynucleotidyl transferase assay for detection of apoptosis. PBS, phosphate-buffered saline. \*,  $p < 0.05$  versus staurosporine (post hoc comparison).

treated control. This protective effect was completely blocked by 50 nM wortmannin. For comparison, insulin, used as a positive control, increased  $t_{MPT}$  by 35%.

### In Vivo Experiments

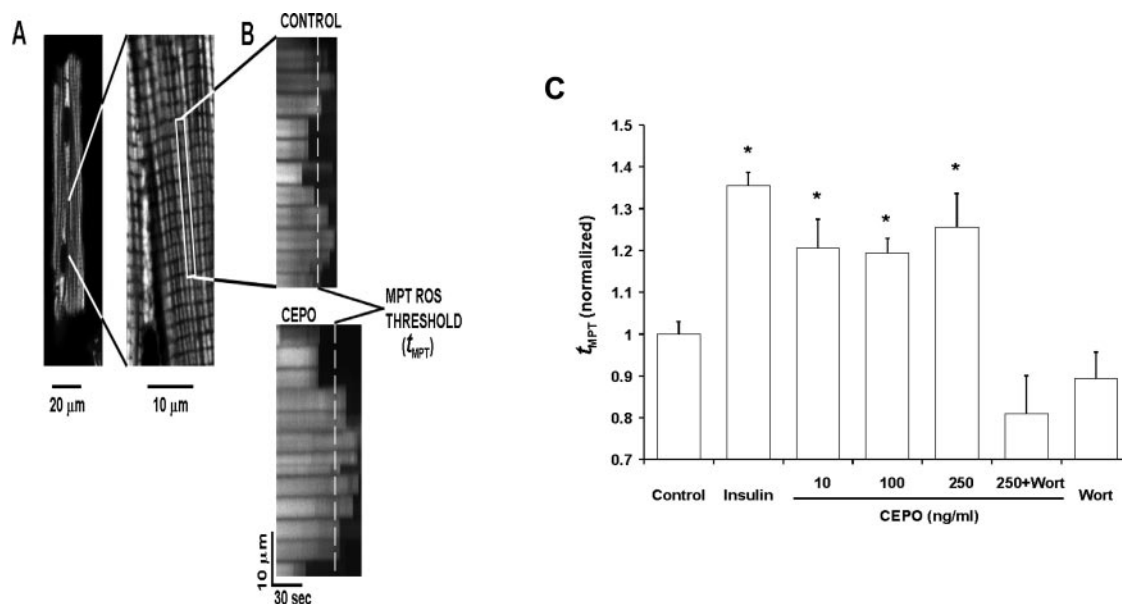
**Mortality and Final Number of Animals.** Three animals died among coronary artery-ligated CEPO-treated animals, four among coronary artery-ligated EPO-treated animals, and two among untreated rats. One animal died in the sham-operated group. No mortality was registered after the first 24 h. Thus, the final number of animals per group in 24-h study was: MI-SALINE, six; MI-CEPO, six; and MI-EPO, six. The final number of animals per group in 4-week study was SH-SALINE, seven; SH-CEPO, eight; MI-SALINE, 10; MI-CEPO, nine; and MI-EPO, eight.

**The Effect of CEPO and EPO on Hematocrit.** One week after MI induction, the hematocrit values increased on

average by 5.1% in EPO-treated rats ( $p < 0.05$ ) but did not change in CEPO-treated animals ( $-0.7%$ ,  $p > 0.05$ ).

**Echocardiography.** At baseline, before coronary artery ligation or sham operation, echocardiographic indices of LV volumes and EF are presented in Table 1. There were no statistical differences at baseline among coronary artery-ligated or sham-operated animals untreated or treated with CEPO or EPO in EDV, ESV, or EF. Average changes of these parameters from baseline during 4 weeks of observation are illustrated in Fig. 3. Treatment of sham-operated animals with CEPO (S-CEPO) did not affect the direction or magnitude of changes during the 4 weeks after surgery relative to untreated animals (S-SALINE). In nontreated ligated animals (MI-SALINE), there was a gradual enlargement of LV over time; by week 4, this averaged a 26% and approximately 140% increase of baseline LV volumes at end-diastole and end-systole, respectively. The EF in MI-SALINE animals fell by more than 50% by week 4. The magnitude and pattern of changes of all indices in MI-SALINE group were significantly different from those of both sham-operated groups (the ANOVA-derived group  $\times$  time interaction,  $p < 0.05$ ).

The pattern and magnitude of changes reflecting the extent of LV remodeling and functional decline were less pronounced in both the MI-CEPO and MI-EPO groups than in MI-SALINE group. In fact, contrary to the MI-SALINE group, the evaluation of LV remodeling (ANOVA-derived group  $\times$  time interaction) showed that there were no statistical differences between MI-CEPO or MI-EPO and sham groups for all three presented indices. Moreover, EF and ESV were significantly different between either MI-CEPO or MI-EPO and MI-SALINE. Post hoc paired comparisons also revealed that both MI-CEPO and MI-EPO groups statistically differed at week 4 from MI-SALINE group with respect to EF and ESV: ESV at week 4 was significantly smaller and EF was significantly higher in both MI-CEPO and MI-EPO

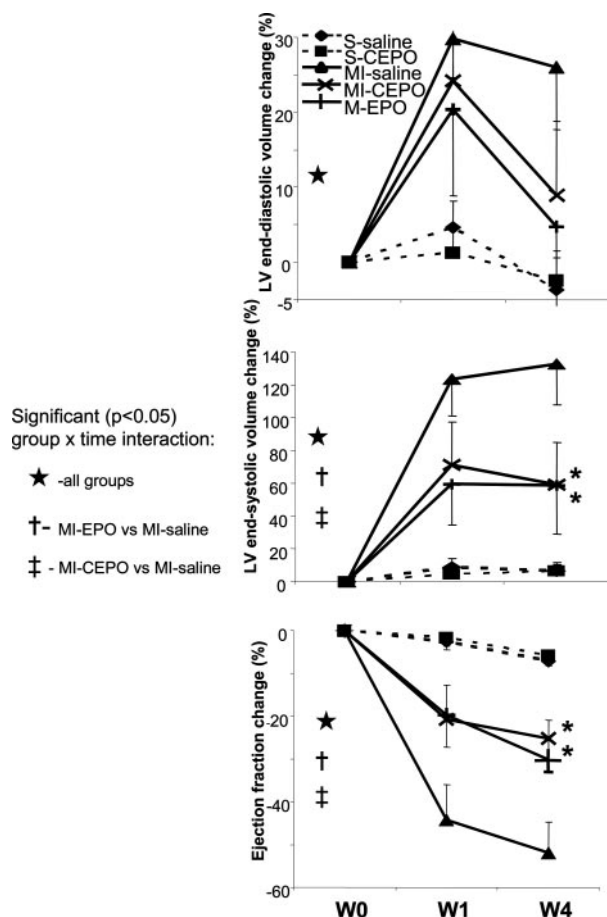


**Fig. 2.** Cellular mechanism of cardioprotection. A and B, methodology used to determine the ROS threshold of MPT induction, the index of cardioprotection. Mitochondria in isolated rat cardiac myocytes stained with TMRM (A) were laser line-scanned until MPT induction (B). The average time required for the standardized photoproduction of ROS to cause MPT induction ( $t_{MPT}$ ) is taken as the index of the ROS threshold in that cell (see text and references, Zorov et al., 2000; Juhaszova et al., 2004). C, CEPO reduces the MPT susceptibility to ROS ( $t_{MPT}$ ) via PI3K-dependent signaling in cardiac myocytes. Isolated cells were exposed to 30 nM insulin (as the positive control) or to 10, 100, or 250 ng/ml CEPO for 20 min prior to  $t_{MPT}$  measurement (see text). Wortmannin (50 nM) was used to inhibit PI3K. \*,  $p < 0.01$  versus control.

TABLE 1

Baseline (week 0) echocardiographic indices of LV volumes and EF (mean  $\pm$  S.E.)

	SH-SALINE ( <i>n</i> = 7)	SH-CEPO ( <i>n</i> = 8)	MI-SALINE ( <i>n</i> = 10)	MI-CEPO ( <i>n</i> = 9)	MI-EPO ( <i>n</i> = 8)
EDV (ml)	0.35 $\pm$ 0.02	0.34 $\pm$ 0.01	0.35 $\pm$ 0.01	0.33 $\pm$ 0.01	0.33 $\pm$ 0.01
ESV (ml)	0.14 $\pm$ 0.004	0.14 $\pm$ 0.008	0.14 $\pm$ 0.005	0.13 $\pm$ 0.004	0.13 $\pm$ 0.005
EF (%)	59.5 $\pm$ 0.9	58.5 $\pm$ 1.2	60.5 $\pm$ 0.8	61.4 $\pm$ 0.4	60.0 $\pm$ 1.3

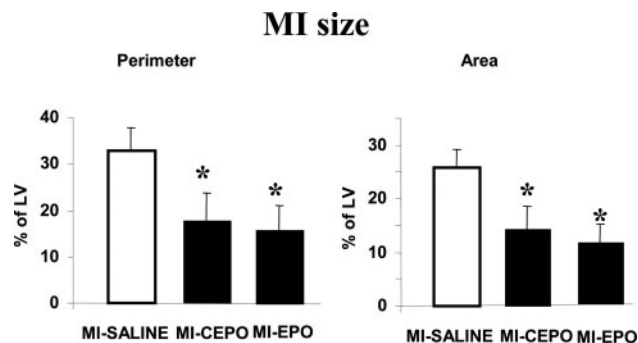


**Fig. 3.** Changes in echocardiographic indices of LV volume and function (ejection fraction) during 4 weeks after coronary artery ligation (MI) or sham (S) operation in CEPO-, EPO-, and saline-treated (SALINE) rats. All indices are derived from images obtained from the long-axis view in two-dimensional mode echo, adjusted for body mass, and expressed as the percentage of change from the baseline values (see Table 1). Statistically significant ( $p < 0.05$ ) group  $\times$  time interactions (ANOVA for repeated measurements) are indicated by the following: ★, among all groups; ‡, MI-SALINE versus MI-CEPO; and †, MI-SALINE versus MI-EPO. \*, significantly different ( $p < 0.05$ ) in post hoc comparison between MI-SALINE and MI-CEPO (or MI-EPO) groups at week 4.

groups in comparison with MI-SALINE. In any of presented indices, the MI-CEPO and MI-EPO groups did not differ from each other either in the pattern of changes over time or at any specific time point.

**Infarct Size.** The average infarct size, expressed as a percentage of LV, in MI-SALINE, MI-CEPO, and MI-EPO group is presented in Fig. 4. Regardless of the technique used to estimate the MI size, perimeter, or area calculation, the average MI size in either MI-CEPO or MI-EPO groups was half of that in MI-SALINE group ( $p < 0.05$ ).

**Posterior Wall Thickness.** The thickness of LV posterior wall measured histologically at the same sections the MI size was measured was similar in MI-EPO and MI-CEPO groups



**Fig. 4.** MI size 4 weeks after ligation of a coronary artery in untreated rats and rats treated with CEPO or EPO. \*,  $p < 0.05$  post hoc comparison.

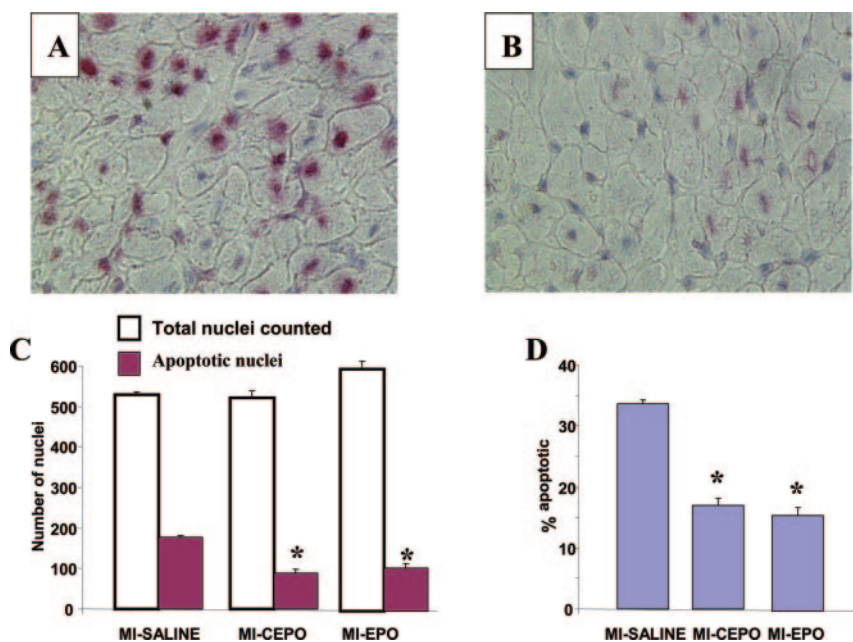
( $0.89 \pm 0.03$  mm) and not different from that in MI-SALINE group ( $0.84 \pm 0.02$  mm,  $p > 0.05$ ).

**Determination of Extent of Apoptosis within the Area at Risk.** Figure 5, A and B, illustrates the TUNEL staining at 24 h after coronary artery ligation in representative histological slides of comparable AARs in hearts from MI-SALINE and MI-CEPO groups, respectively. More apoptotic nuclei are clearly observed in the untreated heart (Fig. 5A). Figure 5C shows the average number of apoptotic nuclei in comparison with a total number of counted nuclei in the AAR of untreated hearts and hearts treated with EPO or CEPO. Only  $17 \pm 1.2\%$  of nuclei were TUNEL-positive in the MI-CEPO group and  $15.3 \pm 1.2\%$  in the MI-EPO group, compared with  $33.6 \pm 0.8\%$  in MI-SALINE group ( $p < 0.01$ ) (Fig. 5D).

## Discussion

CEPO is a recently introduced, engineered cytokine that was designed to retain the tissue-protective (antiapoptotic) characteristics of EPO but not trigger erythropoiesis (Leist et al., 2004). CEPO's pharmacokinetic parameters are very similar to those of EPO, but even injected daily for 4 weeks in doses as high as  $200 \mu\text{g}/\text{kg}$  b.wt. it fails to increase hematocrit in mice (Leist et al., 2004). However, CEPO's pharmacodynamics is remarkably different from that of EPO, because CEPO does not signal the classic EPO receptor (Leist et al., 2004). Nevertheless, extensive testing demonstrated strong neuroprotective properties of CEPO that are comparable with that of EPO. Antiapoptotic effects of CEPO have been shown in vitro on isolated neural cells and in vivo in cerebral infarct and spinal injury models in rats (Leist et al., 2004). Similar tissue protective properties of EPO and CEPO and the lack of hemopoietic properties of CEPO led to the recent suggestion that the tissue protection by EPO is mediated through a heteroreceptor complex comprising both the EPO receptor and a common  $\beta$  receptor subunit, also known as CD131 (Brines et al., 2004).

We (Moon et al., 2003) and others (see review in Bogoyevitch, 2004) have reported the cardioprotective properties of EPO in different experimental models of myocardial



**Fig. 5.** Representative examples of TUNEL staining in the AAR of myocardium 24 h after coronary artery ligation in untreated rats (A) and in rats treated with CEPO (B) (magnification  $\times 400$ ). C, the average number of counted and TUNEL-positive nuclei in the area at risk in untreated MI rats and rats treated with CEPO or EPO. D, the percentage of TUNEL-positive nuclei in the AAR of the hearts from coronary-ligated rats. \*,  $p < 0.05$  versus MI-SALINE.

ischemia. Our experiments have shown that a single 3000 IU/kg systemic injection of EPO after permanent ligation of a coronary artery in rat resulted 24 h later in a 50% reduction of apoptosis in the myocardial area at risk. LV remodeling at 8 weeks was significantly attenuated in treated animals, and the MI size was only 15 to 25% of that in untreated animals (Moon et al., 2003). Recently, using a temporary ligation model of myocardial ischemia, cardioprotection using CEPO was also demonstrated (Fiordaliso et al., 2005). In the present study, we showed in a permanent ligation model that, as in an experiment with EPO (Moon et al., 2003), a single dose of CEPO (30  $\mu\text{g}/\text{kg}$  b.wt. i.v.) immediately after permanent coronary artery ligation in rat reduced the apoptosis in the AAR 24 h later by 50%. During 4 weeks of post-MI observation, the LV remodeling and functional decline were similarly and significantly attenuated in both CEPO- and EPO-treated animals. The MI scar at the end of 4 weeks was significantly smaller in EPO- or CEPO-treated rats than in untreated animals. The LV posterior wall thickness was not different from that of untreated animals. However, with such a significant LV dilation and obvious posterior wall thinning in untreated animals, and with such a remarkable reduction in LV and MI size in treated animals, one would expect the posterior wall would be significantly thicker in treated animals. The lack of such thickness either suggests the possibility that both EPO and CEPO therapy suppress the myocardial hypertrophy or that 4-week observation is not sufficient to reveal the difference in posterior wall thickness.

The results of in vitro experiments on the culture of isolated cardiomyocytes also were similar for CEPO and EPO: CEPO added to culture protected the myocytes from apoptosis induced by staurosporine, and the effect was comparable with the effect of EPO. This experiment confirms a direct effect of CEPO on cardiomyocytes rather than an indirect effect, which could not be ruled out in the in vivo experiments. The remarkable similarities of outcomes of experiments in which permanent coronary ligation in rats followed by a single injection of EPO or CEPO suggest that both

compounds probably engage the same mechanism of cardioprotection, which is, at least in part, antiapoptotic. This conclusion is supported by experiments with TUNEL staining in cardiomyocyte cultures or in myocardial tissue 24 h after coronary ligation as well as by experiments measuring the MRT-ROS threshold of single cardiomyocytes—a final common pathway for antiapoptotic signaling. The particular signaling pathway involved in EPO-CEPO-induced cardioprotection remains less certain. In different studies of EPO effects, many possible antiapoptotic signaling pathways have been reported: Jak-2/STAT, PI3K, protein kinase C, p38, p42/44 mitogen-activated protein kinase activation,  $K_{\text{ATP}}$ , and Akt (Brines et al., 2000; Calvillo et al., 2003; Parsa et al., 2003; Ghezzi and Brines, 2004; Shi et al., 2004). Introduction of CEPO allowed us to narrow the possibilities. Because CEPO does not bind to classic EPO receptors (Leist et al., 2004) its effects would not necessarily involve transcription factors STAT-5 or Jak2, a downstream kinase directly activated upon ligand binding to EPO receptors. On the other hand, the finding that the PI3-kinase inhibitor wortmannin completely blocked the beneficial effect of CEPO on MPT-ROS threshold suggests that the PI3-kinase signaling pathway is definitely involved in CEPO-mediated protection against ischemia, similar to that observed with rhEPO (Juhászova et al., 2004). The very effect of CEPO on MPT-ROS threshold gives additional weight to the idea that both EPO and CEPO exert their tissue-protective properties not through affinity to classic homodimeric EPO receptors but rather to heteromeric receptor complexes containing at least one EPO receptor subunit (Brines et al., 2004; Leist et al., 2004).

In summary, the demonstration of strong antiapoptotic effects of CEPO on ischemic myocardium, comparable with that of EPO, in conjunction with CEPO's lack of hematopoietic activity suggests the possibility of its use in treatment of myocardial infarction or myocardial ischemia in situations when repeated dosing is clinically desirable or the use of EPO is prohibitive due to its procoagulant and prothrombotic effects (Stohlawetz et al., 2000). Moreover, since death of car-

diac myocytes due to apoptosis is now considered a major causative factor in evolution of chronic heart failure to end-stage dilated cardiomyopathy (Wencker et al., 2003), CEPO might be suitable for a long-term treatment of the late LV remodeling.

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