Delayed erythropoietin therapy reduces post-MI cardiac remodeling only at a dose that mobilizes endothelial progenitor cells

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Prunier F, Pfister O, Hadri L, Liang L, del Monte F, Liao R, Hajjar RJ. Delayed erythropoietin therapy reduces post-MI cardiac remodeling only at a dose that mobilizes endothelial progenitor cells. Am J Physiol Heart Circ Physiol 292:H522–H529, 2007. First published September 22, 2006; doi:10.1152/ajpheart.00357.2006.—We examined the cardiac effects of chronic erythropoietin (EPO) therapy initiated 7 days after myocardial infarction (MI) in rats. A single high dose of EPO has been shown to reduce infarct size by preventing apoptosis when injected immediately after myocardial ischemia. The proangiogenic potential of EPO has also been reported, but the effects of chronic treatment with standard doses after MI are unknown. In this study, rats underwent coronary occlusion followed by reperfusion or a sham procedure. Infarcted rats were assigned to one of three treatment groups: 1) 0.75 µg/kg darbepoetin (MI+darb 0.75, n = 12); 2) 1.5 µg/kg darbepoetin (MI+darb 1.5, n = 12); 3) vehicle (MI+PBS, n = 16), once a week from day 7 postsurgery. Sham rats received the vehicle alone (n = 10). After 8 wk of treatment, the animals underwent echocardiography, left ventricular pressure-volume measurements, and peripheral blood endothelial progenitor cell (EPC) counting. MI size and capillary density in the border zone and the area at risk (AAR) were measured postmortem. The AAR was similar in the three MI groups. Compared with MI+PBS, the MI+darb 1.5 group showed a reduction in the MI-to-AAR ratio (20.8% vs. 38.7%; P < 0.05), as well as significantly reduced left ventricle dilatation and improved cardiac function. This reduction in post-MI remodeling was accompanied by increased capillary density (P < 0.05) and by a higher number of EPC (P < 0.05). Both darbepoetin doses increased the hematocrit, whereas MI+darb 0.75 did not increase EPC numbers or capillary density and had no functional effect. We found that chronic EPO treatment reduces MI size and improves cardiac function only at a dose that induces EPC mobilization in blood and that increases capillary density in the infarct border zone.

EPO has also been shown to have proangiogenic potential in a number of animal models. EPO increases the number of endothelial cells in vitro (1, 2) and increases the mobilization of circulating endothelial progenitor cells (EPC) both in experimental animals (15) and in human peripheral blood (5). EPO triggers a concentration-dependent increase in neonatal cardiomyocyte proliferation in culture (36) and stimulates neovascularization in chick embryos (28). It is particularly worthy to note that EPO has similar angiogenic potential to VEGF in vitro (16).

Cardioprotective effects have been shown with high doses of EPO injected during the first hour after ischemic injury, but the effect of chronic treatment beginning a few days after coronary artery occlusion, before the reperfused myocardium has healed, is unknown. Therefore, the aim of this study was to determine whether chronic treatment with clinically relevant doses of EPO beginning 1 wk after I/R reduces postinfarction cardiac remodeling. We postulated that EPO would be beneficial by mobilizing EPC and by increasing capillary density.

METHODS

The experiments complied with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, and published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996). The experimental protocol was approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital.

Myocardial infarction model. Male Sprague-Dawley rats weighing 200–250 g were anesthetized with 60 mg/kg ip pentobarbital sodium and ventilated with an endotracheal tube (SAR-830; CWE, Ardmore, PA). Through a central thoracotomy, myocardial infarction (MI) was induced by ligating the left anterior descending coronary artery (LAD) with 7-0 monofilament suture slip knot ~5 mm from its origin. Ischemia was confirmed by myocardial blanching. Rats without a clear myocardial blanching were rejected. At 5 min into ischemia, 300 µl of fluorescent 10-µm Fluosphere microspheres (Molecular Probes) were injected into the left ventricle (LV) while the pulmonary artery and aorta were briefly clamped. After 40 min, the LAD ligation was released and reperfusion was confirmed visually. Sham-operated rats had the same surgery, including microsphere injection, but without LAD ligation.

Erythropoietin (EPO) was first characterized as a hematopoietic growth factor and has been used clinically during the past decade for the treatment of anemia (12). Recent observations that EPO receptors are expressed by neurons (19), endothelial cells (2), and cardiomyocytes (37) expand the biological role of EPO beyond hematopoiesis. Animal studies show that EPO protects the brain (30, 32) and the heart against acute ischemia (8, 17, 20, 21, 25, 26, 35). A single injection of a high dose of EPO (1,000–5,000 U/kg), 12–24 h before ischemia-reperfusion (I/R), has been shown to confer preconditioning protection on the heart, preventing cardiomyocyte apoptosis and cardiac dysfunction (7, 25). Furthermore, when injected at the onset of ischemia (18, 33) or immediately after reperfusion, a similar dose of EPO markedly reduces the infarct size, attenuates cardiomyocyte apoptosis, and improves left ventricle functional recovery (8, 18, 25, 26, 34).

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Seven days after ligation, rats were randomized to one of the following treatment groups: darbepoetin alfa, 0.75 μg·kg\(^{-1}\)·wk\(^{-1}\) (MI+darb 0.75); darbepoetin alfa, 1.5 μg·kg\(^{-1}\)·wk\(^{-1}\) (MI+darb 1.5); and vehicle alone (MI+PBS). Sham-operated rats received vehicle alone (Sham). Darbepoetin alfa and vehicle were injected intraperitoneally once per week from day 7 postsurgery. Darbepoetin alfa (Aranesp; Amgen, Thousand Oaks, CA) was diluted in PBS immediately before injection. Darbepoetin alfa doses of 0.75 and 1.5 μg/kg are approximately equivalent to 150 and 300 U/kg recombinant EPO (Amgen).

After 8 wk of this treatment, the animals underwent echocardiography and hemodynamic evaluations. After death, blood samples and heart tissues were collected for hematocrit assay, peripheral blood EPC counting, MI size assessment, area at risk (AAR), and capillary density measurements.

**Echocardiography.** Echocardiographic evaluation was performed with a commercial system (VIVID7; GE Medical System, Milwaukee, WI) equipped with a 13-MHz probe. Rats were anesthetized with ketamine 80 mg/kg ip, and M-mode tracings were recorded at the level of the papillary muscles with two-dimensional image guidance. LV wall and cavity diameters were measured as recommended by the American Society of Echocardiography (31).

**Hemodynamic measurements.** Six consecutive randomly selected rats from each group were anesthetized with ketamine and xylazine, 85 and 13 mg/kg ip, respectively. A conductance catheter (Mikro-Tip 2-Fr pressure-volume catheter, SPR-838; Millar Instruments, Houston, TX) was introduced through the right internal carotid artery into the aorta to record arterial pressure and was then pushed into the LV. The catheter was connected to a pressure-conductance unit (MPV5-400; Millar Instruments). The continuous digital pressure and conductance signals were recorded with ChartV5 software (Powerlab; AD Instruments) and analyzed with Millar PVAN3.3 software (Millar Instruments). Volume was calculated with the relative volume units/ductance signals were recorded with ChartV5 software (Powerlab; AD Instruments). Volume was corrected for parallel conductance, by using the saline injection method through the cuvette method. The volume was corrected for parallel conductance, which was calculated by using the saline injection method through the internal jugular vein (4). After baseline measurements, inferior vena cava occlusions were performed to reduce preload, by applying direct pressure through an abdominal incision. The left ventricular end-systolic pressure-volume relationship was obtained for each rat at the varying preload status produced by vein occlusion. The maximum elastance and preload-recruitable stroke work of the LV were obtained from a series of pressure-volume relationship regression curves at various preloads.

**Infarct size.** MI size and AAR were determined as previously described in five consecutive randomly selected rats from each MI group (9). Hearts were sectioned from apex to base into six 1-mm sections by using a coronal heart slicer matrix (Braintree Scientific). Sections were incubated in 1% triphenyltetrazolium chloride (TTC; Sigma) in PBS at 32°C for 5 min and then fixed in 10% formalin. For each section, the AAR and MI area were quantified by planimetry using ImageJ software (NIH, Bethesda, MD). The AAR was defined as the myocardial area delineated by the absence of microspheres. The percentage of MI was calculated as the total infarcted area, unstained by TTC, divided by the total AAR of the same heart.

**Capillary density.** To detect capillary endothelial cells, midpapillary slices from six consecutive randomly selected rats per group were embedded in tissue-freezing medium (Triangle Biomedical Sciences) and frozen. Eight-micrometer-thick sections were stained with rabbit anti-human von Willebrand factor (Chemicon International) and with Alexa fluor 555 goat anti-rabbit (Molecular Probes) and then mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories). The number of capillary vessels was counted in the peri-infarct area in 5–10 random high-power fields (×400 magnification) per heart and then averaged. Only vessels <10 μm in diameter were taken into account, to exclude venules and small arterioles. These analyses were performed by two examiners who were blinded to the treatments.

**Hematocrit and fluorescence-activated cell sorter analysis.** Arterial blood was collected in EDTA tubes from the aorta at the time of death from all infarcted rats and from six sham rats. Five rats were excluded from the hematocrit analysis because a clot formed in the syringe at the time of blood collection. The microhematocrit was measured after 5 min of centrifugation at 12,000 g. Mononuclear cells were isolated from 0.5 ml of peripheral blood by density-gradient centrifugation with Histopaque (Sigma) (13) in five rats from each MI group and from four sham rats. Approximately 10⁶ cells/ml from each animal were suspended in HBSS (Invitrogen) containing 2% FCS and 10 mMol/L HEPES in the presence of phycoerythrin-labeled monoclonal mouse anti-rat CD31 antibodies (Pharmingen) for 30 min on ice. An irrelevant antibody of the same isotype (Pharmingen) was used as a

### Table 1. Echocardiographic and hemodynamic parameters

<table>
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<tr>
<th></th>
<th>Sham</th>
<th>MI + PBS</th>
<th>MI + darb 0.75</th>
<th>MI + darb 1.5</th>
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<td>3.668±346*</td>
<td>4.918±302*‡</td>
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Values are means ± SE; n = no. of rats. MI, myocardial infarction; darb 0.75, 0.75 μg/kg darbepoetin; darb 1.5, 1.5 μg/kg darbepoetin; BW, body weight; HR, heart rate; AW, anterior wall thickness in diastole (d) and systole (s); LVEDD, left ventricle (LV) end-diastolic diameter; LVESD, LV end-systolic diameter; SF, shortening fraction; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; PRSW, preload-recruitable stroke work; dP/dt, first derivative of LV pressure. *P < 0.05 vs. Sham; †P < 0.05 vs. MI + PBS; ‡P < 0.05 vs. MI + darb 0.75.
For MI/H11001 PBS, MI darb 1.5) were included 7 days after surgery. There was no significant difference in the mortality rate among the MI groups during the 2 mo of treatment (18.8, 16.7, and 16.7% in MI+PBS, MI+darb 0.75, and MI+darb 1.5, respectively; P > 0.05). One sham-operated rat (10.0%) died during this period.

Echocardiography. Forty minutes of myocardial ischemia in the LAD area reduced anterior wall thickness 2 mo after surgery but did not induce a thin akinetic wall. However, myocardial damage resulted in LV dilatation and decreased systolic function in untreated MI rats compared with Sham (Table 1). No significant difference was observed between untreated MI rats and infarcted rats receiving the lower dose of EPO. In contrast, the higher dose of EPO prevented anterior wall thinning and LV dilatation and preserved LV systolic function (Table 1).

Hemodynamics. We failed to introduce the conductance catheter into the LV chamber of one untreated MI rat. Invasive hemodynamic data are shown in Table 1. LV contraction (preload-recruitable stroke work and +dP/dt) and LV relaxation (−dP/dt) were both clearly impaired in untreated MI rats. LV end-diastolic pressure was also significantly elevated in this group, indicating higher filling pressures. The lower dose of EPO had no significant effect on these parameters. The beneficial effect of the higher dose of EPO was confirmed by

<table>
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<tr>
<td>HW/BW, mg/g</td>
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<tr>
<td>LVW/BW, mg/g</td>
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<td>RVW/BW, mg/g</td>
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Values are means ± SE; n = no. of rats. HW, heart weight; LVW, left ventricular weight; RVW, right ventricular weight.
a 30% increase in \(dP/dt\) compared with untreated infarcted rats and to rats receiving the lower dose of EPO (Table 1 and Fig. 1). Maximum elastance, a load-independent index of systolic contractility, obtained from the pressure-volume relationship, was improved in MI rats treated with the higher dose of darbepoetin but not with the lower dose (Fig. 1).

**MI size and anatomic parameters.** Anatomic parameters are shown in Table 2. Heart weight and LV weight indexed to body weight were not significantly different among the groups. Despite a thinner anterior wall in MI+PBS and MI+darb 0.75 compared with Sham (based on echographic data), heart weight and LV weight were similar in these groups, possibly owing to hypertrophy of the remote myocardium. The ischemic area induced by LAD ligation (%AAR) did not differ among the three MI groups, as shown in Fig. 2. TTC staining showed a similar percentage of MI (MI-to-AAR ratio) between untreated MI rats and MI+darb 0.75 rats. Rats treated with MI+darb 1.5 exhibited a significant reduction in MI size (20.8% vs. 38.7%; \(P < 0.05\)) (Fig. 3).

**Capillary density.** Two rats (1 MI+darb 0.75 and 1 Sham) were excluded from the capillary density analysis because of inadequate staining for von Willebrand factor. Quantitative analysis showed that capillary density in the MI border zone was significantly higher in MI+darb 1.5 than in MI+PBS rats (698.4 ± 42.8 vs. 449.5 ± 59.5 mm², \(P < 0.005\); Fig. 4). There was no significant difference among MI+darb 0.75, MI+PBS, and sham animals (Fig. 4).

**Dose-dependent mobilization of EPC by darbepoetin.** Previous studies have demonstrated that EPO can mobilize EPC from bone marrow (19). To determine the effect of the darbepoetin doses used here on EPC mobilization, peripheral blood mononuclear cells were analyzed for CD31 expression, a candidate marker of EPC and endothelial cells (22). Rats treated with the lower darbepoetin dose (0.75 \(\mu g/kg\)) had numbers of CD31-expressing peripheral blood mononuclear cells similar to those of PBS-treated animals (8.6 ± 1.8% and 8.8 ± 1.5%, respectively). Interestingly, however, rats receiving the higher dose of darbepoetin (1.5 \(\mu g/kg\)) showed a significant increase in circulating CD31-positive mononuclear cells (15.3 ± 0.9%, \(P \leq 0.05\)) compared with animals treated with the lower dose or with PBS (Fig. 5). Relative to PBS, both darbepoetin doses significantly increased the hematocrit level (Table 3).

**Apoptosis assessment 7 days after I/R.** Seven days after I/R, 27% of myocytes were apoptotic in the border zone. Figure 6
illustrates TUNEL staining of peri-infarct myocardium 7 days after MI.

**DISCUSSION**

These results show that chronic EPO treatment beginning 7 days after MI reperfusion in rats attenuates cardiac remodeling and improves cardiac function. These effects occurred only with an EPO dose that induced EPC mobilization in blood and increased capillary density in the MI border zone. The improvement in cardiac contractility was clearly related to EPC mobilization. To our knowledge, this is the first study suggesting beneficial effects of chronic EPO therapy at standard doses after I/R.

**Neovascularization.** Seven days after reperfusion, chronic EPO therapy initiated, at a dose commonly used to treat anemia (1.5 μg/kg), significantly increased capillary density in the MI border zone. The improvement in cardiac contractility was clearly related to EPC mobilization. To our knowledge, this is the first study suggesting beneficial effects of chronic EPO therapy at standard doses after I/R.

Fig. 4. Representative tissue sections immunostained for von Willebrand Factor (red) from negative control (A), MI+darb 0.75 (B), and MI+darb 1.5 (C) rats. 4',6-Diamidino-2-phenylindole-stained nuclei are blue. D: tabulated data for capillary density. For MI+PBS, n = 6; for MI+darb 0.75, n = 5; for MI+darb 1.5, n = 6; for Sham, n = 5. *P < 0.05 vs. MI+PBS.

Fig. 5. Fluorescence-activated cell sorter analysis of peripheral blood mononuclear cells after anti-CD31 labeling. Representative histograms of MI+PBS rats (A), MI+darb 1.5 rats (B), and negative isotype control rats (C). D: Tabulated data. For MI+PBS, n = 5; for MI+darb 0.75, n = 5; for MI+darb 1.5, n = 5; for Sham, n = 4. FSC, forward scatter. R16 defines the gate depicting CD31-positive cells of total mononucleated cells. *P < 0.05 vs. Sham.
border zone. It was recently reported that chronic treatment with a very high dose of EPO (40 μg/kg ip darbepoetin alfa once every 3 wk), initiated 3 wk after permanent coronary ligation, increased myocardial capillary density (34). This latter study also showed beneficial effects on cardiac remodeling and LV function. Our data suggest that EPC mobilization is involved in these positive effects. Although the lower dose of EPO used here (0.75 μg/kg ip darbepoetin once per week) significantly increased the hematocrit, it had no significant effect on EPC mobilization, capillary density, or LV remodeling relative to rats treated with the vehicle alone. Our results provide further evidence that EPO is a potent regulator of EPC proliferation and differentiation (5). EPO has been shown to increase the number of circulating EPC in patients with renal failure and to induce the formation of tubulike structures in vitro (5). A prospective study recently showed that serum EPO levels are markedly elevated after reperfused MI in humans (23). The significant associations observed between the serum EPO level, the circulating EPC count (peak 7 days after reperfusion), and the LV ejection fraction evaluated 6 mo later suggest that EPO stimulates EPC-mediated vasculogenesis and prevents post-MI cardiac remodeling.

Hematopoietic effect. When injected at the onset of ischemia or at the time of reperfusion at supratherapeutic doses (1,000–5,000 U/kg), EPO has anti-apoptotic properties. Because a high hematocrit may increase the risk of vascular thrombosis, hypertension, and death (6), such doses cannot be used for chronic therapy. In the only other study of chronic EPO therapy on post-MI remodeling, van der Meer et al. (34) injected 40 μg/kg darbepoetin (equivalent to 8,000 U/kg recombinant human EPO) once every 3 wk, starting 3 wk after coronary artery ligation in rats. Cardiac function improved, and capillary density increased; however, the hematocrit reached 62% 3 wk after the first injection, and blood pressure also increased significantly. Because low EPO doses used to treat renal anemia have been shown to mobilize active EPCs in humans (5), we chose to test doses suitable for chronic treatment (10, 11). Darbepoetin doses of 0.75 and 1.5 μg·kg⁻¹·wk⁻¹ both significantly increased the hematocrit, which nevertheless remained at a clinically acceptable level in animals receiving the higher dose. No impact on systemic blood pressure was observed with either dose.

Antiapoptotic effect. The rat I/R model closely mimics human acute MI, with early reperfusion creating regions of cellular necrosis and AAR in the MI border zone, where cells are in a state of reversible injury. Cell recovery after infarction is severely limited in this portion of the myocardium, and a large percentage of myocytes die by apoptosis, thereby increasing the infarct size (24). Architectural rearrangement of the LV cavity by side-to-side slippage of cells within the myocardium has been shown to be a major contributor to post-MI remodeling. It seems strongly related to the extent of apoptosis, especially in the border zone, in response to increased wall stress (3). Importantly, a significant number of apoptotic myocytes are found up to 10 days after MI in humans (24) and up to 1 mo after MI in rats (29). A single high dose of EPO, when injected at the time of coronary ligation or at the reperfusion time, inhibits apoptosis and reduces MI size (7, 8, 18, 20, 21, 25–27, 34, 35, 37). Very little is known about the effects of chronic EPO therapy started after the acute phase of MI. In the rat I/R model, repeated administration of recombinant human EPO (5,000 U/kg daily, beginning at the time of reperfusion) reduces cardiomyocyte loss by 50% and normalizes hemodynamic function within 1 wk (8). In contrast, the same treatment

![Fig. 6. Detection of apoptotic cells by fluorescence microscopy.](http://ajpheart.physiology.org/)

**Table 3. Hematocrit**

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<th>Sham</th>
<th>MI + PBS</th>
<th>MI + darb 0.75</th>
<th>MI + darb 1.5</th>
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<td>Hematocrit, %</td>
<td>41.6±1.2</td>
<td>41.2±0.6</td>
<td>46.4±0.6†</td>
<td>51.3±1.5*‡</td>
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Values are means ± SE; n = no. of rats. *P < 0.05 vs. Sham; †P < 0.05 vs. MI + PBS; ‡P < 0.05 vs. MI + darb 0.75.
applied to the permanently ligated coronary artery rat model fails to reduce MI size (14). EPO seems to restrict cell death to myocytes that are completely cut off from the blood supply and to prevent apoptosis in the border zone where cells typically undergo a delayed form of death. We chose to begin EPO treatment 7 days after reperfusion to avoid acute effects of EPO on the very early phases of apoptosis. EPO reduced the size of the infarct zone and prevented thinning of the myocardial wall submitted to I/R, suggesting that 7 days after reperfusion represents a "window of opportunity" for EPO therapy in this model. We did not assess apoptosis in rats treated with EPO, but it is likely that the beneficial effect of EPO administered chronically from 7 days after reperfusion is due to a combination of antiapoptotic and proangiogenic actions. When we quantified apoptosis 7 days after I/R in another group of rats, 27% of myocytes in the border zone were found to be undergoing apoptosis. Recently, genetically engineered cardiac fibroblasts producing recombinant human EPO were injected into scar tissue 7 days after MI in rats: angiogenesis was strongly enhanced, and cardiomyocyte apoptosis evaluated 1 mo after transplantation was drastically reduced (29).

Limits. We used only CD31 staining to identify EPCs, whereas detailed phenotypic descriptions of circulating EPCs are based on coexpression of several other endothelial and hematopoietic antigens such as CD34, VE-cadherin, VEGF receptor 2, and Tei2. However, EPC mobilization by EPO has been clearly shown in species other than rats, including humans. Bahlmann et al. (5) demonstrated that chronic administration of low-dose EPO (50 U/kg per wk) for 16 wk after transplantation was drastically reduced (29).

References


