Reduction of inflammatory cytokine expression and oxidative damage by erythropoietin in chronic heart failure

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Abstract

Objectives: Late treatment with erythropoietin (EPO), as well as the administration before the onset of or during the acute stage of myocardial infarction (MI), has recently been shown to mitigate post-MI heart failure. We investigated the mechanisms, including the downstream signaling pathways, for the beneficial effect of late treatment with EPO on chronic post-MI heart failure.

Methods and results: EPO (1500 U/kg, twice a week) was administered to mice beginning 6 weeks after induction of large MI. The EPO treatment for 4 weeks diminished left ventricular dilatation and improved function. It significantly reduced inflammatory cell infiltration and fibrosis, and increased vascular density in noninfarcted areas. The elevated levels of the inflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor-α and transforming growth factor-β1 seen in the failing hearts were returned nearly to control levels by EPO treatment. Oxidative damage in surviving cardiomyocytes was also significantly attenuated by EPO. Expression of EPO receptor was upregulated in failing hearts, and EPO treatment led to myocardial activation of signal transducer and activator of transcription-3 (Stat3), Stat5, and Akt. These in vivo effects of EPO were confirmed in vitro in experiments that showed the anti-inflammatory and anti-oxidant effects of EPO to be mediated via Stat and Akt activation. Finally, the beneficial effects of EPO were found to persist for 4 weeks after discontinuing treatment.

Conclusions: It thus appears that Stat-mediated reduction of inflammation and cytokine production and Akt-mediated attenuation of oxidative stress accompany the beneficial effects of late treatment with EPO on chronic post-MI heart failure.

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This article is referred to in the Editorial by Qingping Feng (pages 615–617) in this issue.

1. Introduction

Erythropoietin (EPO) is a hypoxia-induced hormone that is essential for normal erythropoiesis and has been broadly used in patients with anemias having a variety of etiologies. In addition, early studies in heart failure patients with anemia suggest that EPO therapy is a safe and effective method of reducing left ventricular hypertrophy, enhancing exercise performance and increasing ejection fraction [1,2]. Notably, however, expression of the EPO receptor (EPOR) within the cardiovascular system, including on cardiomyocytes and endothelial cells, suggests EPO exerts cardiovascular effects beyond hematopoiesis [3–5]. For instance, based on the observations that recombinant human EPO exerts cardioprotective effects in the infarcted heart and following ischemia–reperfusion injury of the myocardium—i.e., EPO administration either prior to or during ischemia significantly enhances functional recovery after reperfusion—it was suggested
that one cellular mechanism contributing to this protective effect was inhibition of cardiomyocyte apoptosis, resulting in decreased infarct size within the area at risk [6,7].

Given that large myocardial infarctions (MIs) are a major cause of heart failure, it is noteworthy that van der Meer et al. [8] recently showed that EPO treatment started 3 weeks after the onset of MI (late treatment) improved cardiac function in a rat model of post-MI heart failure. This beneficial effect was accompanied by increases in the capillary density and the capillary-to-myocyte ratio and a shift in the expression of the myosin heavy chain phenotype. In addition, evidence from both animal and human studies suggests that increased inflammatory cytokines and oxidative stress are associated with a poor prognosis following MI and may play an important role in the pathogenesis and progression of heart failure: they influence heart contractility by inducing hypertrophy and promoting apoptosis or fibrosis, thereby contributing to the continuous myocardial remodeling process [9–13]. In neurons, on the other hand, EPO exerts anti-inflammatory [14,15] and anti-oxidant [16,17] effects by suppressing production of inflammatory cytokines and reactive oxygen species. A recent study has shown that EPO decreases inflammation during myocardial ischemia and reperfusion [18]. Similar effects of EPO have yet to be documented in chronic heart failure [5], however, and the molecular signaling pathways related to the effects of EPO in failing hearts remain largely unknown.

Our aims in the present study, therefore, were first to confirm the beneficial effect of late treatment with EPO on an established case of heart failure; second, to investigate whether the expression of inflammatory cytokines and oxidative stress is involved in its effect; and third, to begin to characterize the molecular signaling upstream of the outcome.

2. Methods

2.1. Experimental heart failure

This study was approved by our Institutional Animal Research Committee. MI was induced in 70 male 12-week-old C57BL/6 mice (Chubu Kagaku Shizai) by ligating the left coronary artery as previously described [19]. Six weeks after the operation, there were 40 surviving mice (survival rate, 57%). The surviving animals underwent echocardiographic examination and were entered into the in vivo studies. For sham-operated mice (n=18), the suture was passed around the artery but was not tied; all mice survived for 6 weeks after surgery.

2.2. In vivo treatment with EPO

2.2.1. Protocol-1

MI-bearing mice were randomly assigned to two groups, resulting in similar levels of echocardiography-based cardiac function in both. In one group (n=10) recombinant human EPO (Chugai Pharmaceutical Co.) was subcutaneously administered twice a week at a dosage of 1500 U/kg in saline, beginning 6 weeks post-MI. The dose of EPO was selected because it was within the range of known dosages providing organ protection [5,6]. The injections were continued for 4 weeks, until 10 weeks post-MI. The other group (control; n=10) received the same volume of saline alone over the same period. Sham-operated mice were treated with saline or EPO (n=9 each) in the same manner. At the end of the protocol, cardiac geometry and function were examined physiologically (see below), and necropsies were performed for histological and biochemical analyses.

2.2.2. Protocol-2

Continuous treatment with EPO such as described in Protocol-1 may be impossible in actual clinical situations because of various adverse effects (e.g., severe polycythemia). In another protocol, therefore, we also examined how long the effects of EPO persist after discontinuing treatment. Beginning 6 weeks post-MI, EPO or saline (n=10 each) was given for 4 weeks, as in Protocol-1, then after an additional 4 weeks (14 weeks post-MI) cardiac function was examined physiologically (see below).

2.3. Blood sampling

Blood used for hemocounts was drawn from the tail vein before and during treatment and from the interior vena cava at sacrifice.

2.4. Physiology studies

Physiological studies were carried out as previously described [19]. Animals were anesthetized with halothane (induction, 2%; maintenance, 0.5%) in a mixture of N2O and O2 (0.5 l/min each) via a nasal mask. Echocardiograms were recorded using an echocardiographic system (Aloka) equipped with a 7.5-MHz imaging transducer 6 weeks post-MI (before treatment) and at sacrifice. Following echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 671, Millar Instrument) and advanced into the aorta and then into the left ventricle (LV) for recording pressure and maximal and minimal dp/dt.

2.5. Histological analysis

After making the physiological measurements, the mice were killed and the hearts removed. The hearts were then cut into two transverse slices through the middle of the infarct area, and the apical specimens were quickly frozen in liquid nitrogen or used to prepare cryosections. The basal specimens were fixed with 10% buffered formalin and embedded in paraffin. Four-micrometer-thick deparaffinized sections were stained with hematoxylin-eosin, Masson’s trichrome or Sirius red. Quantitative assessments of MI size, cell population, cardiomyocyte size and fibrotic area were made using a LUZEX F multipurpose color image processor (Nireco, Tokyo, Japan). The size of the MI and the fibrotic
area in the noninfarcted region was measured by searching the entire ventricle. Cardiomyocyte size (expressed as the transverse diameter of the myocyte cut at the level of the nucleus) and cell populations were assessed in 20 randomly chosen high-power fields in each section.

2.6. Immunohistochemistry

The 4-μm-thick deparaffinized sections and 8-μm-thick cryosections were incubated with a primary antibody against Flk-1 (Santa Cruz), F4/80 (Biomedicals AG), CD45 (Pharmingen) or 8-hydroxy-2′-deoxyguanosine (8-OHdG; Japan Institute of The Control of Aging, Shizuoka, Japan), which is the DNA base-modified product most commonly used for the evaluation of oxidative DNA damage [20]. An ABC kit (DAKO) with which DAB served as the chromogen was then used to immunostain the sections, after which they were observed under a light microscope. For all of the sections, the interpretations of the staining by two observers blinded to the specimens’ group were in accord. The percentage of immunopositive cells present was calculated in 20 randomly chosen high-power fields in each section.

In addition, cultured cardiomyocytes were fixed with 4% paraformaldehyde, triple-stained with anti-8OHdG antibody followed by Alexa Fluor 488-conjugated secondary antibody (Molecular Probes), rhodamine-phalloidin (Molecular Probes) and Hoechst 33342. They were observed under a confocal microscope (LSM510, Zeiss).

2.7. In vitro treatment with EPO

Cardiomyocytes and cardiac fibroblasts were isolated from neonatal C57BL/6 mice and cultured separately in DMEM supplemented with 10% FBS as previously described [21]. The cells were subjected to oxidative stress by incubating them in 100 μmol/l H₂O₂ for 1 h or 6 h to induce inflammatory cytokine production and oxidative damage. To assess the capacity of EPO to attenuate cytokine production and oxidative damage, some cells were pretreated with EPO (0 to 10,000 U/ml) for 1 h prior to H₂O₂ exposure. In addition, to investigate the molecular signaling mediating the effects of EPO on cardiac cells exposed to H₂O₂, in some cases parthenolide (1 μg/ml; Sigma), an inhibitor of signal transducer and activator of transcription (Stat) signaling [22], or wortmannin (16 ng/ml; Sigma), a widely used inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt signaling, was added to the cells along with EPO. Cell death was evaluated by the 3-[4,5-2-yl]2,5-diphenyltetrazolium bromide (MTT) method.

2.8. Western analysis

Protein extracted from hearts or cultured cells was subjected to 10% polyacrylamide gel electrophoresis and then transferred to PVDF membranes, after which the membranes were probed using primary antibodies against EPO receptor (EPOR, M-20, Santa Cruz) and transforming growth factor (TGF)-β1 (Promega). The activation of Stat3 and Stat5, Akt, and extracellular signal-regulated protein kinase (ERK) was assessed using antibodies against their phosphorylated forms (p-Stat3, p-Stat5, p-Akt and p-ERK; all from Cell Signaling). Three to five hearts from each group were subjected to Western analysis. The blots were visualized using an ECL kit (Amersham), and the signals were quantified by densitometry. α-Tubulin (analyzed using an antibody from Sigma) served as the loading control.

2.9. ELISA

The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β in myocardial tissue and cultured cells were quantified using ELISA kits (Quantikine M®, R&D Systems) according to the supplier’s instructions.

2.10. Statistical analysis

Values are shown as means±SD. The significance of differences between means was evaluated using t tests followed by Newman–Keul’s multiple comparison test. Values of p<0.05 were considered significant.

Fig. 1. Comparison of hematocrit levels and echocardiography- and cardiac catheterization-based cardiac geometry and function in untreated and EPO-treated mice 10 weeks after surgery (sham operation or induction of MI) in Protocol-1. *p<0.05 vs. sham operated, #p<0.05 vs. the untreated MI group.
3. Results

3.1. Effect of late treatment with EPO on LV remodeling and function in failing hearts (In vivo experiment: Protocol-1)

Immediately prior to starting treatment with either vehicle (saline) or EPO (6 weeks post-MI), all of the mice bearing MIs showed significant LV dilatation and dysfunction, as compared to the sham-operated mice. Four weeks later (10 weeks post-MI), all of the mice in each group were still alive. The hematocrit level was significantly higher in the EPO-treated groups (Fig. 1A) and marked LV enlargement and the signs of diminished cardiac function – i.e., reduced LV ejection fraction (EF), LV developed pressure (LVEDP), and maximal and

![Fig. 2. Histological and immunohistochemical preparations of hearts from untreated and EPO-treated mice 10 weeks post-MI (Protocol-1). (A) Ventricular transverse slices stained with hematoxylin-eosin. (B) Sirius red stain showing fibrosis in noninfarcted and infarcted areas. (C) CD45 immunostaining showing leukocytes. (D) F4/80 immunostaining showing macrophages. (E) Flk-1 immunostaining showing endothelial cells. (F) 8-OHdG immunostaining showing oxidative DNA damage in surviving cardiomyocytes. Scale bars, 1 mm in panel A; 50 μm in panel B; and 20 μm in panels C–F.](image-url)
minimal \( dp/dt \) and increased LV end-diastolic pressure (LVEDP) – persisted in the vehicle-treated mice. By contrast, all of these parameters were significantly improved by the EPO treatment (Fig. 1B–C). Systolic blood pressure was elevated by the EPO treatment in the sham-operated groups (EPO (−), 87±4 vs. EPO (+), 93±7 mm Hg, \( p<0.05 \)), but it was similar in the groups with MI (EPO (−), 80±8 vs. EPO (+), 83±9 mm Hg, \( p=ns \)). The heart rate was similar among the groups. EPO had no effect on cardiac function except for systolic blood pressure in sham-operated mice.

3.2. Effects of EPO on the structure of failing hearts

At necropsy, LV dilatation was less marked in animals treated with EPO than untreated animals (Fig. 2A), and the heart-to-body (Fig. 3A) and lungs-to-body weight ratios (not shown) were significantly smaller. On the other hand, there was no difference in the size of the MI area relative to the total LV area in the untreated and EPO-treated groups (Fig. 3B). The size of the surviving cardiomyocytes was increased in MI-bearing mice, probably due to compensatory hypertrophy, and somewhat unexpectedly it did not differ between the two groups (Fig. 3C). Although the region of the 10-week-old infarct showed substantial fibrosis in both groups, the intensity of Sirius red staining appeared weaker in the EPO-treated group (Fig. 2B). Moreover, there was a significantly greater amount of interstitial fibrosis in the noninfarcted areas of the untreated hearts than in those of the EPO-treated hearts (Figs. 2B and 3D). The population of Flk-1-positive vessels did not differ in the infarcted areas of the untreated and EPO-treated hearts, but it was significantly greater in the noninfarcted areas of the EPO-treated hearts (Figs. 2E and 3F). CD45-positive leukocytes and F4/80-positive macrophages were found to have infiltrated both the infarcted and noninfarcted areas of hearts with 10-week-old MIs, but the populations were significantly smaller in the EPO-treated group (Figs. 2C, D and 3G, H).

![Fig. 3. Morphometric analysis of hearts collected from untreated and EPO-treated mice 10 weeks after sham operation or induction of MI (Protocol-1). (A) Heart-to-body weight ratios. (B) Infarct area expressed as a percentage of total LV area. (C) Cardiomyocyte size (transverse diameter through the nucleus). (D) Percentage fibrosis in the noninfarcted area. (E) Incidence of 8-OHdG positivity among cardiomyocytes. (F–H) Numbers of Flk-1 (F) CD45 (G) and F4/80-positive (H) cells in the noninfarcted and infarcted areas. \( N=9 \) each from sham operation groups and \( n=10 \) each from MI groups. In panels A–E, *\( p<0.05 \) vs. the untreated sham group, †\( p<0.05 \) vs. the untreated MI group; in panels F–H, *\( p<0.05 \) vs. the MI area, †\( p<0.05 \) vs. the untreated MI group.]

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3.3. Effect of EPO on inflammatory cytokine expression and oxidative damage in failing hearts

ELISAs revealed that the myocardial levels of IL-1β, IL-6 and TNF-α were significantly higher in untreated mice 10-weeks post-MI than in sham-operated mice, and that levels were partially or nearly entirely restored to control by EPO treatment (Fig. 4A). In addition, Western analysis showed that myocardial expression of TGF-β1 was significantly upregulated in the untreated mice 10-weeks post-MI, but that expression was significantly attenuated in EPO-treated mice (Fig. 4B).

Cardiomyocytes positive for 8-OHdG (indicative of oxidative damage to DNA) were frequently found in the non-infarcted areas of hearts with 10-week-old MIs (12±0.7%), but significantly fewer were found in the hearts treated with EPO (8.2±1.2%, p<0.05), indicating that EPO mitigated the oxidative damage to the DNA (Figs. 2F and 3E).

3.4. EPOR expression and its downstream signaling in failing hearts

Western analysis revealed that myocardial expression of EPOR was significantly higher in hearts with 10-week-old MIs than in sham-operated hearts, and that EPO treatment further increased EPOR expression in MI-bearing hearts (Fig. 5). Receptor-associated Janus family tyrosine kinase (Jak)/Stat, PI3K/Akt, and mitogen-activated protein kinase (MAPK)/ERK are all known to be downstream mediators of EPOR signaling in cardiac cells both in vitro and in vivo [6]. It is therefore consistent with the observed increase in EPOR

Fig. 4. ELISAs and Western analysis of the myocardium from untreated and EPO-treated mice carried out 10 weeks after sham operation or induction of MI (Protocol-1). (A) ELISAs for IL-1β, IL-6 and TNF-α. (B) Western blot of TGF-β1 and its densitometry. N=6 each from the groups. *p<0.05 vs. the untreated sham group, #p<0.05 vs. the untreated MI group.

Fig. 5. Western analysis of EPOR, p-Stat3, p-Stat5, p-Akt and p-ERK expression in the myocardium of untreated and EPO-treated mice 10 weeks after sham operation or the induction of MI (Protocol-1). N=6 each from the groups. *p<0.05 vs. the untreated sham group, #p<0.05 vs. the untreated MI group.
Fig. 6. (A) Dose-dependent effect of EPO on H$_2$O$_2$-induced expression of the indicated inflammatory cytokine in cultured cardiac fibroblasts. (B) Parthenolide (P) but not wortmannin (W) inhibits the effects of EPO on H$_2$O$_2$-induced expression of inflammatory cytokines. N=6 each from the groups. *p<0.05 vs. untreated, #p<0.05 vs. EPO alone.
that we also found that Stat3, Stat5 and Akt, but not ERK, were all significantly activated in hearts with 10-week-old MIs, and that the level of activation was further increased by EPO (Fig. 5).

3.5. Direct effects of EPO on cardiac cells and their molecular mechanisms (in vitro experiments)

The direct effects of EPO on cardiac cells were investigated using cultured neonatal cardiomyocytes and cardiac fibroblasts. Oxidative stress has been reported to play a critical role for progression of heart failure [23], and we here treated cardiac cells with H₂O₂. In response to 100 μmol/l H₂O₂, cardiac fibroblasts produced substantial amounts of inflammatory cytokines (IL-1β, IL-6, TNF-α, and TGF-β1), an effect that was significantly attenuated by pretreatment with EPO (Fig. 6A). In cardiomyocytes exposed to H₂O₂, intense 8-OHdG immunostaining was observed mainly in the perinuclear regions of cells, suggesting oxidative damage was primary to the mitochondrial DNA (Fig. 7A, upper panel). Approximately 60% of the cells were affected, but the incidence of 8-OHdG-positivity was dose-dependently suppressed by pretreatment with EPO (Fig. 7A, lower panel). According to the MTT assay, no significant cell death was noted at 1 and 6 h after H₂O₂ addition in either cardiomyocytes or fibroblasts (data not shown).

To investigate the molecular signaling involved in the anti-inflammatory and anti-oxidative effects of EPO on cardiac cells exposed to H₂O₂, parthenolide, a specific inhibitor of Stat signaling, or wortmannin, an inhibitor of PI3K/Akt signaling, was added simultaneously with EPO to the cardiac cells prior to treatment with H₂O₂. The concentrations of parthenolide and wortmannin used here were found to inhibit activation of Stat and Akt in cardiac fibroblasts and cardiomyocytes, respectively (Figs. 6B, upper panel and

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Fig. 7. (A) Dose-dependent effect of EPO on H₂O₂-induced 8-OHdG production (oxidative damage) in cultured cardiomyocytes; top panels, confocal microphotographs showing actin (red), 8-OHdG (green) and nuclei (blue). Scale bars, 10 μm. (B) Wortmannin (W) but not parthenolide (P) inhibits the effects of EPO on H₂O₂-induced 8-OHdG production (oxidative damage). N=6 each from the groups. *p<0.05 vs. untreated, †p<0.05 vs. EPO alone.
Table 1
Functionality, fibrosis, cytokine expression and oxidative damage in hearts with 14-week-old MIs (Protocol-2)

<table>
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<tr>
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<th>No treatment (n=10)</th>
<th>EPO treatment (n=10)</th>
<th>p value</th>
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<tr>
<td><strong>LV geometry and function</strong></td>
<td></td>
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<tr>
<td>LVDD (mm)</td>
<td>5.7±0.16</td>
<td>5.1±0.10</td>
<td>0.0101</td>
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<td>LVEF (%)</td>
<td>38±1.4</td>
<td>49±1.2</td>
<td>&lt;0.0001</td>
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<tr>
<td>SBP (mm Hg)</td>
<td>77.4±2.8</td>
<td>83.6±3.2</td>
<td>0.2290</td>
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<tr>
<td>Heart Rate (beats/min)</td>
<td>553.8±6.4</td>
<td>551.3±17.9</td>
<td>0.9323</td>
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<tr>
<td>LVEDP (mm Hg)</td>
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<td>+dp/dt (mm Hg/s)</td>
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<td>5416±318</td>
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<td>−dp/dt (mm Hg/s)</td>
<td>−3926±372</td>
<td>−4874±243</td>
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<td><strong>Fibrosis in noninfarcted area</strong></td>
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</tr>
<tr>
<td>Sirius red-positive area (%)</td>
<td>6.3±0.38</td>
<td>2.8±0.35</td>
<td>0.0001</td>
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<tr>
<td><strong>Inflammatory cells in noninfarcted area</strong></td>
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<tr>
<td>CD45+ cells (/HPF)</td>
<td>5.4±0.82</td>
<td>2.6±0.57</td>
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<td>F4/80+ cells (HPF)</td>
<td>2.1±0.50</td>
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<td><strong>Myocardial cytokine levels</strong></td>
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<tr>
<td>IL-1β (pg/mg)</td>
<td>25.1±3.94</td>
<td>13.3±1.45</td>
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<tr>
<td>IL-6 (pg/mg)</td>
<td>13.4±2.82</td>
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<td>TNF-α (pg/mg)</td>
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<td>TGF-β1 (relative value)</td>
<td>100±0</td>
<td>69.5±2.60</td>
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<tr>
<td><strong>Oxidative damage in cardiomyocytes</strong></td>
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<tr>
<td>8-OHdG* cells (%)</td>
<td>6.4±0.39</td>
<td>3.1±0.15</td>
<td>0.0002</td>
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</table>

SBP: systolic blood pressure. See text for other abbreviations.

7B, left panel). Parthenolide also completely suppressed the inhibitory effect of EPO on inflammatory cytokine expression in cardiac fibroblasts, while wortmannin diminished the protective effect of EPO against oxidative damage in cardiomyocytes (Figs. 6B, lower panels and 7B, right panel). Collectively then, these findings suggest that the anti-inflammatory and anti-oxidant effects of EPO are respectively mediated by the Jak/Stat and PI3K/Akt pathways.

3.6. The beneficial effect of EPO persists even after discontinuing treatment in failing hearts (In vivo experiment: Protocol-2)

Mice with 6-week-old MIs were treated with EPO for 4 weeks in the same manner as Protocol-1, after which treatment was stopped for 4 weeks prior to examination. Notably, even after 4 weeks without treatment (14 weeks post-MI), we found significantly better cardiac function, lower inflammatory cytokine levels and less oxidative damage in EPO-treated hearts than in untreated ones (Table 1).

4. Discussion

Van der Meer et al. recently described the ability of EPO treatment to improve cardiac function in post-MI heart failure in rats, even when the treatment was started during the chronic stage of MI (3 weeks post-MI) [8]. We have confirmed this finding using a murine model (6 weeks post-MI) in the present study, and also showed that the beneficial effect persists long after (4 weeks) discontinuing the treatment. Although rapid recanalization of the occluded coronary artery is presently the best clinical approach to the treatment of acute MI, unfortunately most patients miss the chance for that therapy because, to be effective, it must be performed within a few hours after the onset of MI [24]. Patients who escape death during the acute stage of a large MI are at high risk of developing heart failure during the chronic stage [25], so that patients with post-MI heart failure account for nearly half of the candidates for cardiac transplantation [26]. Thus, development of effective therapies for post-MI heart failure that are efficacious even when begun during the chronic stage of MI would be highly desirable, which highlights the clinical importance of the present study as well as the study by van der Meer et al. [8].

It is now clear that EPO possesses direct beneficial cardiovascular effects [5]; however, the mechanisms underlying the effects on chronic heart failure are not yet fully understood. In their study, van der Meer et al. found that EPO increased angiogenesis and reversed the modulation of the myosin heavy chain phenotype in failing rat hearts [8]. In the present study, we not only confirmed that EPO increased capillary density, but also found that EPO reduces inflammation, cytokine production, fibrosis and oxidative damage in the failing myocardium. Several studies have shown that in both animals and human with failing hearts, levels of inflammatory cytokines (e.g., IL-1β, IL-6 and TNF-α) are increased in plasma [9,10] and circulating leukocytes [27], as well as in the myocardium itself [11,28,29]. Our observation that levels of IL-1β, IL-6 and TNF-α were elevated in the failing myocardium of mice 10 and 14 weeks post-MI is consistent with those earlier findings. Importantly, expression of these inflammatory cytokines is reportedly directly related to the degree of heart failure and inversely related to survival [11,30]. Moreover, the results of animal studies and some clinical pilot trials have suggested that suppression of inflammatory cytokines may improve cardiac performance [31,32]. Though speculative, it is therefore conceivable that reduction of inflammatory cytokines may be one of the mechanisms involved in the beneficial effect of EPO on failing hearts.

The present study also revealed the anti-oxidant effect of EPO on failing myocardium; EPO significantly reduced the numbers of cardiomyocytes positive for 8-OHdG, a commonly used marker of oxidative DNA damage [20]. Along with inflammatory cytokines, the overproduction of reactive oxygen species is also thought to be involved in myocardial remodeling [12]. Apparently, inflammatory cytokines can induce oxidative stress – e.g., TNF-α can directly induce mitochondrial reactive oxygen species production in cardiomyocytes, causing damage to mitochondrial DNA [33] – while oxidative stress can increase levels of inflammatory cytokines, leading to the development of a vicious cycle in failing hearts [34,35]. It is therefore noteworthy that our in vitro study confirmed that oxidative stress caused by H2O2...
induced both oxidative damage in cardiomyocytes and cytokine production in cardiac fibroblasts.

We also observed that EPO treatment reduced the elevated TGF-β1 expression otherwise seen in the failing myocardium. Induction of TGF-β1 expression, as well as supplementation with exogenous TGF-β1, can protect cardiomyocytes against ischemia/reperfusion injury [36], but this beneficial effect appears only during the early phase of the cytokine’s expression. Sustained expression, by contrast, leads to LV remodeling and failure following MI [37]. In addition, TGF-β1 is a major stimulator of tissue fibrosis, influencing fibroblast proliferation and extracellular matrix production, collagen and fibronectin in particular, while reducing degradation of these components [38]. The EPO-induced attenuation of the myocardial levels of TGF-β1 seen in the present study may be responsible for the observed reduction in fibrosis.

We also found that expression of EPOR is upregulated in failing hearts, possibly as a compensatory mechanism. Among downstream targets of EPOR signaling are Stat3, Stat5 and Akt, all of which showed significant activation in the hearts of EPO-treated mice. Moreover, our in vitro experiments showed that inhibiting Jak/Stat signaling with parthenolide completely blocked the EPO-induced reduction of inflammatory cytokine production in cardiac fibroblasts, while inhibiting PI3K/Akt signaling with wortmannin blunted EPO-induced attenuation of oxidative damage in cardiomyocytes. These findings suggest that the anti-inflammatory effect of EPO is elicited via the Jak/Stat pathway, while its anti-oxidative effect is via the PI3K/Akt pathway. Consistent with that idea, downregulation of TNF-α, IL-1 and IL-6 by IL-10 is known to be mediated by activation of Stat1 and Stat3 [39]. In addition, mice with cardiomyocyte-restricted deletion of Stat3 are susceptible to inflammation-induced heart damage and show a dramatic increase in cardiac fibrosis [40]. On the other hand, cardiothrophin-1 is known to protect cardiomyocytes from oxidative stress-induced cell death via gp130 and the PI3K/Akt and ERK intracellular cascades [41]. It is also noteworthy that in heart failure, excessive inflammatory cytokine production can reduce EPO secretion from the kidney, interfere with EPO activity in bone marrow, and reduce the iron supply to the bone marrow [42].

Finally, we recently showed that EPO improves cardiac function in a murine model of doxorubicin (DOX)-induced non-ischemic cardiomyopathy [43]. In that model, EPO-induced molecular signaling downstream of EPOR differed substantially from that seen in the present MI model. ERK activation was suppressed in DOX-induced cardiomyopathy and was restored to a significant degree by EPO, which had no effect on Stat or Akt activation. Thus, EPO’s downstream signaling via EPOR apparently varies in different models of heart failure.

In summary, EPO treatment during post-MI heart failure improves cardiac function by reducing expression of inflammatory cytokines and oxidative damage, at least in part through activation of the Jak/Stat and PI3K/Akt pathways.

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References


