Erythropoietin Decreases the Occurrence of Myocardial Fibrosis by Inhibiting the NADPH-ERK-NF- κ B Pathway

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Key Words

Myocardial fibrosis · Erythropoietin · Cardiac fibroblasts · Proliferation

Abstract

Objectives: The aim of this study was to investigate the protective role of erythropoietin (EPO) against myocardial fibrosis (MF). **Methods:** Pressure-overloaded rats were established by abdominal aortic constriction, the rats were randomly divided in a double-blind manner into 3 groups (n = 12 for each group): sham-operated rats (sham), operated rats receiving physiological saline (vehicle) and operated rats receiving 4,000 U/kg rhEPO (EPO group). The vehicle and drugs were administered to rats by intraperitoneal injection. In addition, cultured adult rat cardiac fibroblasts (CFs) were utilized to investigate the role of EPO in CF proliferation and collagen secretion. **Results:** After 4 weeks, besides an increase in blood pressure, myocardial hypertrophy, collagen deposition in the myocardium and decreased cardiac function were observed in the pressure-overloaded rats. The expression of NADPH oxidase (Nox2 and Nox4) and inflammatory cytokines (CD45, F4/80 and MCP-1) was also significantly increased. All these alterations were prevented by EPO. TGF- β promoted CF proliferation, collagen secretion, ROS production and Nox2/Nox4 expression, which was inhibited

by EPO. In addition, the TGF- β -induced increase of ERK1/2 phosphorylation and NF- κ B expression were attenuated by EPO. **Conclusion:** EPO inhibited rat MF induced by pressure overload and improved myocardial function by decreasing CF proliferation and differentiation via inhibition of the NADPH-ERK-NF- κ B pathway.

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Introduction

Myocardial fibrosis (MF), characterized by the excessive proliferation of cardiac fibroblasts (CFs) and the abundant accumulation of matrix proteins within the extracellular space, is a prominent pathological process accompanying hypertension and myocardial infarction, which ultimately participates in the progression towards heart failure. CFs are the main effector cells of MF, and they play a vital role in the maintenance of myocardial structure and function. With physical and chemical stimulation (e.g. pressure overload, Ang II and TGF- β), CFs will undergo a phenotypic transformation (differentiation of fibroblasts into myofibroblasts) and begin to heavily proliferate and deposit collagen; these processes represent the main changes that occur during MF [1]. Therefore, inhibiting these structural and functional changes in

CFs represents a potential target for antifibrotic therapies.

Mounting evidence indicates that the cytokine hormone erythropoietin (EPO) may be a powerful cardioprotective agent and a potential component for antifibrotic therapies. Naturally, EPO originates primarily in the kidneys and promotes erythroid progenitor cell proliferation, differentiation and the suppression of apoptosis. However, recently, research has revealed that in addition to its hematopoietic functions, EPO plays an important cell-protective role within the heart [2, 3], brain [4, 5], kidney and liver [6]. In the heart, endogenous EPO may contribute to tissue maintenance and repair. After myocardial ischemia-reperfusion, EpoR-null mice show an increase in infarct size compared to wild-type mice [7]. EPO pretreatment before ischemia-reperfusion injury conveys significant myocardial protection under moderate hyperglycemic condition through mechanisms involved in the reduction of caspase-3 activity and the up-regulation of Bcl-2 in association with enhanced ERK-induced GATA-4 stability [8]. EPO can ameliorate the myocardial inflammatory response in ischemia-reperfusion models, and this beneficial effect is mediated by eNOS-derived NO via PI3-kinase-dependent activation of AP-1 [9]. During heart failure, following the permanent ligation of the coronary artery, EPO administration can reduce the left ventricle (LV) infarct size, improve LV ejection fraction (LVEF) and enhance capillary density. Recent studies provide evidence that EPO exerts a protective effect on the myocardium by also suppressing cardiomyocyte apoptosis [7, 10, 11]. It is currently unknown whether EPO affects the behaviors of myocardial fibroblasts. It may have deleterious effects that can outweigh its cardioprotective effects. Therefore, the molecular mechanisms of EPO treatment require further elucidation to optimize future therapy regimens [12, 13].

This study was designed to investigate the regulatory role of EPO in rat CFs and to determine whether it participates in the inhibition of MF using molecular biology approaches. Our results demonstrate that EPO inhibits CF proliferation and differentiation by interfering with NADPH-ERK-NF- κ B pathway signaling to serve a protective role in MF.

Methods

Reagents

Recombinant EPO (rhEPO) was purchased from Promega (Madison, Wis., USA). Antibodies against Nox2 and Nox4 were obtained from Sigma-Aldrich (St. Louis, Mo., USA). Antibodies

against CD45, F4/80, and MCP-1 were obtained from Millipore Corporation (Billerica, Mass., USA). Antibodies against collagen I, collagen III, matrix metalloproteinase (MMP)-2 and MMP-9 were obtained from Abcam (USA), while anti- α -SMA, used to assess the conversion from CFs to myofibroblasts, was purchased from Beijing Biosynthesis Biotechnology (Beijing, China). MTT and the DCFH-DA kit and other chemicals used in this study were obtained from Sigma-Aldrich.

Preparation of Pressure-Overload Hypertension Rat Model

Male Sprague-Dawley rats (230–250 g each) obtained from the Laboratory Animal Centre of North China University of Science and Technology were housed in a standard environment on a 12-hour light/dark cycle at a constant temperature ($22 \pm 2^\circ\text{C}$) and 45–50% humidity. The animals were allowed ad libitum access to water and standard rat chow. Pressure overload was produced by constriction of the abdominal aorta according to a previously published method [14]. Briefly, under intraperitoneal (i.p.) sodium pentobarbital anesthesia (50 mg/kg), the abdominal aorta was constricted at a point proximal to the left renal artery with a 7-0 silk suture and a blunted 10-gauge needle, which was removed later. Sham-operated rats underwent similar surgical procedures minus the abdominal aorta binding. The rats were then randomly divided into the following 3 groups ($n = 12$ in each group): sham-operated rats (sham), operated rats receiving 0.1 ml/day of physiological saline (vehicle) and operated rats receiving 4,000 U/kg rhEPO dissolved in 0.1 ml physiological saline. The vehicle and drugs were administered to rats by i.p. injection, beginning 1 h after operation and then twice a week for 4 weeks.

Four weeks after aortic banding, the rats were weighed and their blood pressure was measured with a tail-cuff system (BP-98A; Softron, Tokyo, Japan). Under anesthesia with sodium pentobarbital (40 mg/kg i.p.), cardiac function was measured by ultrasonic cardiogram and hemodynamics measurement, then blood was collected from the abdominal aorta and the hearts were isolated and weighed in order to calculate the LV weight/body weight (LV/BW) ratio.

Preparation of Adult Rat CFs

Adult rat CFs were isolated and cultured using a modified procedure, as described previously [15]. Briefly, male Sprague-Dawley rats (8–12 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg i.p.). The hearts were quickly excised and placed in cold phosphate-buffered saline (PBS). The atria and valves were removed. The ventricles were then minced into small pieces in sterile PBS and digested with 0.04% collagenase and 0.08% trypsin for 10 min at 37°C . The digestion was repeated 4–5 times until the tissue was completely digested. Cell suspensions were centrifuged (1,000 g for 10 min), and the cells were collected and suspended in Dulbecco's modified Eagle's medium (DMEM) with 5 mM glucose (Gibco), 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 U/ml) and streptomycin (100 mg/ml). After a 90-min incubation in flasks at 37°C in a humidified atmosphere with 5% CO_2 , the unattached cells were discarded, and the attached cells were continuously cultured, changing the medium twice a week. The passage culture was made at 80% confluence.

Experiments were performed with the cells obtained from the initial passage. Before the cells were treated with different chemical interventions, the FBS in the medium was reduced to 0.5% for 24 h to induce growth arrest.

Masson's Trichrome Staining and Immunohistochemistry

Myocardial tissue was fixed in formalin and then embedded in paraffin. Deparaffinized sections (5 μ m in thickness) were stained with hematoxylin and eosin (HE) and Masson's trichrome reagent. Images were digitized (6 fields/heart) under a microscope ($\times 400$; BX-51; Olympus, Tokyo, Japan). The collagen volume fraction in the interstitial region was calculated as the percentage of the collagen-stained area (blue color) to the total LV area, using Image-Pro Plus 6.0 analysis software [16]. All the measurements were performed in a double-blind manner.

The expression of α -smooth-muscle actin (α -SMA), CD45, F4/80 and MCP-1 in the myocardium was immunolocalized with anti- α -SMA (1:500), anti-CD45 (1:200), anti-F4/80 (1:200) and anti-MCP-1 (1:200) antibodies accordingly, and visualized with diaminobenzidine. A negative control was prepared by incubating the tissue with an anti-IgG antibody without primary antibody.

Cell Proliferation Assays

Cell proliferation was determined by the MTT assay. Briefly, rat CFs were plated in 96-well plates at a density of 1×10^4 cells/well in 200 μ l DMEM containing 10% FBS. After incubation with DMEM containing 0.5% FBS for 24 h, CFs were incubated with TGF- β in the presence or absence of EPO. EPO was used to pre-treat the cells for 30 min prior to the addition of TGF- β . After 24 h of culture, 20 μ l of PBS-buffered MTT (5 mg/ml) was added to each well, and the cells were maintained at 37°C for an additional 4 h. The medium was then removed and 100 μ l of DMSO was added to each well to dissolve the purple formazan crystals and, finally, the absorbance at 490 nm was measured. Results were standardized to control group values.

Western Blot Analysis

The expression of collagen, inflammation-related markers and NADPH oxidase in the myocardium was determined by Western blot analysis as described previously [14, 17]. The myocardium or the CFs were dissected, and the total protein was extracted using ice-cold modified RIPA buffer [60 mM Tris-HCl, 0.25% sodium dodecyl sulfate (SDS), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 10 μ g/ml aprotinin and leupeptin]. The lysates were then centrifuged at 13,000 g for 15 min at 4°C. After transferring the supernatant to a fresh, ice-cold tube, the protein concentration was determined with a BCA protein assay. Equal concentrations of proteins were mixed with SDS sample buffer and denatured at 100°C for 10 min. The samples were separated on a 10% polyacrylamide gel and then transferred to a PVDF membrane at 90 V for 2 h in transfer buffer containing 20 mM Tris, 150 mM glycine and 20% methanol. The membranes were blocked with 5% skimmed-milk powder in TBST (0.1% Tween-20) for 1 h. After blocking, the blots were incubated in primary anti- α -SMA (1:1,000), anti-CD45 (1:300), anti-F4/80 (1:300), anti-MCP-1 (1:150), anti-Nox2 (1:200), anti-Nox4 (1:200), anti-collagen I (1:300), anti-collagen III (1:300), anti-ERK (1:150), anti-phosphorylated (p)ERK (1:150) and anti-NF- κ B (1:100) antibodies at 4°C overnight and then incubated with HRP-conjugated secondary antibodies (1:3,000) for 1 h at room temperature. The bound antibodies were detected with an enhanced chemiluminescence detection system (ECL, Beyotime, China) and quantified by densitometry using a ChemiGenius 2 bioimaging system (Syngene, Cambridge, UK). To ensure equal sample loading, the ratio of experimental band intensity to GAPDH was determined to quantify the relative protein expression level.

Hydroxyproline Assay

To observe collagen production, heart tissue samples were harvested after the animals were sacrificed and then digested at 95°C for 20 min. The hydroxyproline concentration in the supernatants was measured following the instructions of the hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Determination of ROS Generation

Generation of ROS was determined by microscopic fluorescence imaging using DCFH-DA. Cells (1×10^6 cells/well) were seeded in 60-mm dishes. After treatment with TGF- β in the presence or absence of EPO for 24 h, cells were incubated in 500 μ l of 10 μ mol/l DCFH-DA at 37°C for 30 min. Cells were collected and placed in a 96-well plate to assess cell fluorescence intensity using a fluorescence microplate reader (Varioskan Flash, Thermo Scientific, USA) with an excitation wavelength of 488 nm and an emission wavelength of 605 nm. The final values were expressed as the percentage of fluorescence intensity relative to control wells.

Statistical Analysis

Results are presented as mean $\pm$ SEM. Paired and/or unpaired Student's *t* tests were used to evaluate the statistical significance of differences between the means of 2 groups, while analysis of variance (ANOVA) was performed to determine significance across multiple groups. A difference of $p < 0.05$ was considered significant. All graphs were prepared using Sigma Plot 10.0 software.

Results

The Effect of EPO on Blood Pressure and LV/BW Induced by Pressure Overload

Figure 1 illustrates the alterations in blood pressure and the LV/BW of rats after abdominal aortic ligation for 4 weeks on different treatments. Compared with the sham-operated group, the blood pressure and LV/BW were significantly increased in the vehicle group ($p < 0.01$ vs. sham), which were prevented by treatment with 4,000 U/kg/day rhEPO ($p < 0.01$ vs. vehicle). These results suggest that EPO treatment can reduce the blood pressure and myocardial hypertrophy of rats with abdominal aortic ligations.

The Role of EPO in Regulating Cardiac Function

Tables 1 and 2 show the changes in cardiac function and the homodynamic index via echocardiography and hemodynamic methods. Results indicate that the LVEF, short axial shortening (LVFS), LV contraction rate of pressure (LVESP) and LV pressure at the end of the maximum rising and falling rate ($+dP/dt_{max}$) increased significantly after EPO treatment ($p < 0.01$ vs. vehicle); relatively, the LV contraction at the end of the inner diameter (LVESD), the LV end-diastolic diameter (LVEDD) and

Fig. 1. Cardiovascular parameters of pressure-overloaded rats. Rats received sham operation (sham) or abdominal aortic constriction for 4 weeks with different pharmaceutical interventions, physiological saline (vehicle) or 4,000 U/kg rhEPO (EPO). The treatment began 1 h after the operation, and then twice a week for 4 weeks. **a** Blood pressure. **b** LV/BW ratio (n = 12; ** p < 0.01 vs. sham; ## p < 0.01 vs. vehicle).

Table 1. The result of ultrasonic cardiogram

Treatment	n	LVESD, mm	LVEDD, mm	LVEF, %	LVFS, %
Sham	12	4.09±0.97	5.69±0.34	90.27±9.01	59.24±3.29
Vehicle	12	11.23±0.24 ^{##}	10.17±0.99 ^{**}	49.91±5.42 ^{**}	29.33±5.33 [*]
EPO	12	5.01±0.77 ^{**}	6.33±0.35 [#]	87.63±7.21 [#]	43.21±4.71 [#]

Values express means ± SD. ** p < 0.01, * p < 0.05 versus sham; ## p < 0.01, # p < 0.05 versus vehicle.

Table 2. The result of hemodynamics measurement

Treatment	n	LVESP, mm Hg	LVEDP, mm Hg	+dp/dtmax, mm Hg/s	-dp/dtmax, mm Hg/s
Sham	12	119.24±10.97	5.34±0.67	5,281.72±199.77	4,097.22±123.29
Vehicle	12	78.29±9.64 [#]	11.89±1.32 ^{**}	3,521.92±167.72 [*]	2,531.64±165.73 ^{**}
EPO	12	101.11±7.71 [*]	6.22±1.52 ^{##}	4,981.64±271.23 [#]	3,679.51±194.81 [#]

Values express means ± SD. ** p < 0.01, * p < 0.05 versus sham; ## p < 0.01, # p < 0.05 versus vehicle.

the LV end-diastolic pressure (LVEDP) were decreased (p < 0.01 vs. vehicle) after EPO treatment (tables 1, 2). These results suggest that EPO improves the cardiac function of rats with pressure overload.

The Role of EPO in Collagen Deposition in Myocardial Tissue

Masson's trichrome staining was performed to evaluate the degree of cardiac fibrosis. As shown in figure 2, abdominal aorta constriction caused significant collagen deposition in the myocardial interstitial space. Collagen

deposition was significantly reduced by treatment with 4,000 U/kg/day rhEPO (n = 12; p < 0.01 vs. vehicle). These results suggest that EPO attenuates the increased collagen deposition in the hearts subjected to aortic constriction for 4 weeks.

The Effect of EPO on CF Proliferation and Differentiation

To investigate whether EPO is involved in regulating cell proliferation in rat CFs induced by TGF-β, an MTT assay was performed to measure cell proliferation. Fig-

Fig. 2. Collagen contents in the myocardium of rats after 4 weeks of abdominal aortic constriction with different interventions. **a** Representative LV myocardium of rats with Masson's trichrome staining after abdominal aortic constriction. Rats received i.p. in-

jections of vehicle or rhEPO (4,000 U/kg, twice a week) for 4 weeks. Myocardium was stained red and collagens were blue (colors refer to the online version only). $\times 400$. **b** Quantitative analysis of collagen contents ($n = 12$; ** $p < 0.01$ vs. sham; ## $p < 0.01$ vs. vehicle).

ure 3a, b displays cell proliferation profiles in cells treated with TGF- β (1–10 ng/ml) and TGF- β (5 ng/ml) plus different doses of rhEPO (1–8 U/ml) for 24 h; 5 and 10 ng/ml TGF- β significantly increased cell proliferation ($n = 10$; $p < 0.01$ vs. control) and the effect was antagonized by coapplication of 5 ng/ml TGF- β and rhEPO (5 and 8 U/ml; $n = 10$; $p < 0.01$ vs. TGF- β alone).

Figure 3c–e illustrates the expression of α -SMA, a chief marker of myofibroblast differentiation. Immunohistochemical and Western blot results suggest that the expression of α -SMA in CFs treated with 5 ng/ml TGF- β for 24 h significantly increased ($p < 0.01$ vs. control), while this effect was greatly reduced in cells pretreated with EPO (5 U/ml) for 30 m ($n = 10$; $p < 0.01$ vs. TGF- β alone). These results suggest that EPO is involved in decreasing cell proliferation and transformation in cultured rat CFs induced by TGF- β .

The Role of EPO in Regulating Collagen Secretion of Cultured Adult Rat CFs

To further study the role of EPO in the TGF- β -mediated proliferation of cultured adult rat CFs, collagen secretion was determined in cells treated with TGF- β

(5 ng/ml) and TGF- β plus 5 U/ml EPO. Western blot results showed that EPO reduced the increased expression of collagen I and collagen III in cultured adult rat CFs induced by TGF- β ($p < 0.01$; fig. 4a, b). Hydroxyproline detection yielded similar results (fig. 4c). These results suggest that EPO reduces collagen secretion in TGF- β -stimulated cultured adult rat CFs.

We also detected the expression of MMPs in cultured adult rat CFs; these are key enzymes involved in collagen metabolism. Figure 4d, e shows that TGF- β increased the secretion of MMP-2 and MMP-9 ($n = 8$; $p < 0.01$). Interestingly, in the EPO group, MMP-2 and MMP-9 secretion were higher than in rats treated with TGF- β . Combined with the above results, it would appear that EPO can promote collagen degradation in the myocardium.

The Effect of EPO on NADPH Protein Expression in the Myocardium

Studies have demonstrated that increased expression of ROS induced by the activation of NADPH is an important process in MF. We therefore determined the effect of EPO on the expression of NADPH in the LV myocardium. Figure 5 shows that the expression of the Nox2 and

Fig. 3. Effects of TGF- β and EPO on cultured adult rat CF proliferation and differentiation. **a** Concentration-dependent effects of TGF- β on cell proliferation in adult rat CFs. CFs were cultured in the absence or presence of TGF- β (1, 3, 5 and 10 ng/ml) for 24 h. Cell proliferation was determined by MTT and shown as the relative viability to cells treated with control alone ($n = 12$; # $p < 0.05$, ## $p < 0.01$ vs. control). **b** Concentration-dependent effects of EPO on cell proliferation induced by TGF- β (5 ng/ml) in adult rat CFs. CFs were precultured with EPO (1, 3, 5 and 8 U/ml) for

30 min, then added to TGF- β (5 ng/ml) and cultured for 24 h. Cell proliferation was determined by MTT and shown as the relative viability to cells treated with control alone ($n = 12$; ## $p < 0.01$ vs. control). **c** Immunohistochemical staining of α -SMA in CFs treated with TGF- β (5 ng/ml) and TGF- β plus EPO (5 U/ml) for 24 h. $\times 200$. **d** Western blot images of α -SMA protein in CFs in **c**. **e** Quantitative analysis of α -SMA in CFs ($n = 10$; ** $p < 0.01$ vs. control; # $p < 0.05$ vs. TGF- β alone).

Nox4 proteins were significantly increased by pressured overload ($p < 0.05$ or $p < 0.01$), and that treatment with EPO reduced these changes ($p < 0.05$ or $p < 0.01$).

To study the cellular source of myocardial NADPH, we also detected ROS production and NADPH expression in CFs. Figure 6a, b displays the ROS fluorescence intensity in CFs, as determined by DCFH-DA. Treatment with 5 ng/ml TGF- β for 24 h significantly increased ROS production in CFs by $29 \pm 3.8\%$ ($n = 8$; $p < 0.01$ vs. control). EPO fully antagonized ROS production in TGF- β -treated cells ($n = 8$; $p < 0.01$ vs. TGF- β). As the main source of ROS, Nox2 and Nox4 protein expression also

increased by TGF- β ($n = 8$; $p < 0.01$ vs. control), and EPO inhibited this effect ($n = 8$; $p < 0.01$ vs. TGF- β ; fig. 6c, d). These results demonstrate that EPO reduces the oxidative stress levels induced by TGF- β .

The Effect of EPO on the Expression of Inflammation-Related Factors

Immunohistochemistry and Western blot were employed to observe the changes in the expression of inflammation-related factors in the myocardium. As shown in figure 7, the expression of inflammation-related factors, including CD45, CD3 (leukocyte differentiation anti-

Fig. 4. Effects of TGF-β and EPO on collagen production and MMP secretion of cultured adult rat CFs. **a** Western blot images of collagen I and collagen III protein expression in cultured adult rat CFs treated with TGF-β (5 ng/ml) and TGF-β plus EPO (5 U/ml) for 24 h. **b** Quantitative analysis of collagen I and collagen III protein in myocardium (n = 12; ** p < 0.01 vs. control, ## p < 0.01; # p < 0.05 vs. TGF-β). **c** Mean values of myocardial hydroxyproline

content in cells treated as in **a** (n = 12; ** p < 0.01 vs. control; ## p < 0.01; # p < 0.05 vs. TGF-β). **d** Western blot images of MMP-2 and MMP-9 protein in cultured adult rat CFs treated with TGF-β (5 ng/ml), TGF-β plus EPO (5 U/ml) for 24 h. **e** Quantitative analysis of MMP-2 and MMP-9 protein expression (n = 8; ** p < 0.01 vs. control; ## p < 0.01; # p < 0.05 vs. TGF-β).

Fig. 5. Expression of NADPH oxidase (Nox2 and Nox4) in the myocardium of rats after 4 weeks of abdominal aortic constriction with different interventions. Western blots (**a**) and relative protein levels (to GAPDH; **b**) of Nox2 and Nox4 in LV myocardium of rats treated with i.p. injections of vehicle, rhEPO (4,000 U/kg, twice a week) for 4 weeks (n = 10; ** p < 0.01 vs. sham; ## p < 0.01 vs. vehicle alone).

Fig. 6. Effect of EPO on ROS generation and in NADPH expression in CFs induced by TGF- β . **a** ROS generation in CFs treated with TGF- β (5 ng/ml) and TGF- β plus EPO (5 U/ml) for 24 h with a DCFH-DA kit. **b** Fluorescence intensity of ROS in CFs relative to control (n = 8; ** p < 0.01 vs. control; ## p < 0.01 vs. TGF- β group).

c Western blot images of Nox2 and Nox4 protein in CFs treated with TGF- β (5 ng/ml) and TGF- β plus EPO (5 U/ml) for 24 h. **d** Quantitative analysis of Nox2 and Nox4 protein expression (n = 8; ** p < 0.01 vs. control; ## p < 0.01 vs. TGF- β group).

gens), F4/80 (a macrophage surface marker) and MCP-1 were enhanced in the myocardium of rats subjected to abdominal aortic constriction (n = 12; p < 0.01 vs. sham). Treatment with rhEPO (5 U/l) reduced the expression of these inflammation-related factors (n = 12; p < 0.05 or p < 0.01 vs. vehicle). These results suggest that EPO decreases the inflammatory reaction induced by TGF- β .

The Effect of EPO on the Phosphorylation of ERK1/2 and the Activation of NF- κ B in CFs

To further study whether the effect of EPO on CF proliferation is related to the phosphorylation of ERK1/2, we examined the total and phosphorylated levels of ERK1/2 in cells treated with TGF- β (5 ng/ml) and TGF- β plus 5 U/ml EPO. Figure 8(a, b) shows Western blots and the mean values of relative levels of p-ERK1/2; these levels were significantly increased in cells treated with TGF- β for 24 h (n = 10; p < 0.01 vs. control). The TGF- β -induced increase in p-ERK1/2 was antagonized by pretreatment of rhEPO (n = 10; p < 0.01 vs. TGF- β alone).

Studies have shown that ERKs translocate to the nucleus to activate NF- κ B [17]. We therefore determined whether activation of NF- κ B was involved in EPO-mediated

inhibition of CF proliferation. The potential role of NF- κ B in regulating CFs was investigated by Western blot (fig. 8c, d). We found that the expression of NF- κ B was increased in cells treated with 5 ng/ml of TGF- β . 5 U/ml rhEPO effectively attenuated TGF- β -induced NF- κ B expression (n = 10; p < 0.01). These results suggest that the effect of EPO on MF is likely related to downregulation of the ERK-NF- κ B pathway.

Discussion

In this study, we demonstrated that (1) EPO significantly prevents cardiac fibrosis by alleviating an increase in oxidative stress levels in the myocardium while reducing the expression of myocardial inflammation-related factors in rats following abdominal aorta constriction, ultimately improving cardiac function, and (2) EPO inhibits rat CF proliferation, transformation and collagen deposition by targeting the NADPH-ERK-NF- κ B pathway.

EPO is mainly synthesized and secreted by renal tubular juxtaglomerular cells at the junction of the renal cortex and medulla oblongata [18]. It is a member of the type I

Fig. 7. Expression of inflammatory markers in the myocardium of rats after 4 weeks of abdominal aortic constriction with different interventions. **a** Immunohistochemical staining of CD45, F4/80 and MCP-1 in myocardium. $\times 200$. Rats received i.p. injections of vehicle, rhEPO (4,000 U/kg, twice a week) for 4 weeks. **b** Western

blot images of inflammation factors in myocardium as described in **a**. **c** Quantitative analysis of inflammation factors in myocardium ($n = 12$; ** $p < 0.01$, * $p < 0.05$ vs. sham; ## $p < 0.01$; # $p < 0.05$ vs. vehicle).

cytokine family and stimulates bone marrow hematopoiesis. Recently, a large number of studies have shown that EPO exhibits a wide range of nonhematopoietic functions. It protects endothelial cells and inhibits the apoptosis of cardiomyocytes, conferring a protective effect on cells in animal and human models of ischemic injury [12, 19–22]. It reduces the production of inflammatory cytokines in the ischemic heart [23], inhibiting the apoptosis of myocardial cells by reducing the myocardial oxidative stress reaction [24]. It can promote endothelial progeni-

tor cell migration, increase vascular endothelial growth factor levels and promote angiogenesis around infarction sites to reduce the infarction area [25–27]. However, it is unknown whether the action of EPO is linked to MF. In this study, we investigated the role of EPO in the formation of MF. Using multiple approaches of molecular biology and electrophysiology, we demonstrated that EPO plays an important protective role in this context.

Chronic pressure overload is known to induce the development of concentric hypertrophy and cardiac fibro-

Fig. 8. Effect of EPO on phosphorylation of ERK1/2 and activation of NF-κB in CFs induced by TGF-β. **a** Western blot images of total ERK1/2 and p-ERK1/2 in rat CFs treated with TGF-β (5 ng/ml) and TGF-β plus 5 U/ml EPO for 24 h. **b** Quantitative analysis of ERK1/2 and p-ERK1/2 protein expression (n = 10; ** p < 0.01 vs.

control; ## p < 0.01 vs. TGF-β group). **c** Western blot images of NF-κB expression in rat CFs treated as in **a**. **d** Quantitative analysis of NF-κB protein expression (n = 10; ** p < 0.01 vs. control; ## p < 0.01 vs. TGF-β group).

sis, which is characterized by an increase in collagen in the interstitium and/or perivascular regions of the myocardium [14]. In this study, we concentrated our attention on the events following pressure overload in adult rats, which was induced by abdominal aortic constriction. We observed the typical changes of myocardial hypertrophy, collagen deposition and decreasing cardiac function after 4 weeks of pressure overload. In addition, mean blood pressures were increased in pressure-overloaded rats (fig. 1). However, treatment with EPO inhibited these changes. These results demonstrate that EPO assisted in the maintenance and recovery of cardiac function in rats with pressure overload.

The excessive proliferation of CFs and the abundant accumulation of matrix proteins in the extracellular space is an important pathological process that accompanies MF [15, 17]. In our study, we found that TGF-β induced rat CF proliferation, the conversion to myofibroblasts and the synthesis/deposition of collagen. However, these changes were decreased by EPO treatment. Another study has suggested that EPO also plays a protective role in treatment with antitumor drugs, reducing cardiotoxicity by regulating the balance of MMP-2/TIMP-2 [28]. Therefore, we also examined MMP expression. Our re-

sults showed that EPO increased MMP-2 and MMP-9 expression compared to treatment with TGF-β alone; this may be more beneficial for collagen degradation. These results demonstrate that EPO exhibits resistance to MF.

It is likely that ROS induces myocardial cell apoptosis and the myocardial hypertrophic responses that contribute to cardiovascular disease [29, 30]. NADPH oxidase is the main source of myocardial ROS. Studies show that the accumulation of fibroblasts and deposition of collagen were reduced in myocardial-infarction mice and that the mice had lower blood pressures after Nox2 knockout, while the excessive proliferation of blood vessels induced by Ang II was prevented [31]. Nox4 plays an important role in myocardial hypertrophy, fibrosis and myocardial apoptosis in pressure-overloaded mice and is important in the process of ageing of the heart [32–34]. It has been demonstrated that endothelial dysfunction, macrophage infiltration and NADPH oxidase-dependent superoxide production are attenuated by EPO in the streptozotocin-induced diabetic rat aorta [35]. Combined with enalapril, EPO caused additive reduction of cardiac fibrosis and microvessel disease in 5/6 nephrectomized rats, presumably by decreasing myocardial oxidative stress [36]. In our study, we found that pressure overload resulted in

an increased expression of Nox2 and Nox4 in the myocardium, while the effect was decreased in rats treated with rhEPO. In cultured CFs, TGF- β induced increased expression of Nox2 and Nox4, while EPO attenuated this change. We also found that EPO inhibited inflammatory cytokine protein expression (CD45, F4/80 and MCP-1) induced by pressure overload. These results suggest that EPO can inhibit myocardial oxidative stress, and then decrease the myocardial inflammatory response.

TGF- β -induced proliferation of CFs has been shown to be related to the activation of the ERK1/2 cascade [15, 36, 37]. To demonstrate whether the role of EPO in the modulation of myocardial oxidative stress is mediated by activating ERK1/2, the effects on total and p-ERK1/2 levels were determined in cultured rat CFs. TGF- β treatment increased the level of p-ERK1/2, and the effect was countered in the cells pretreated with EPO. Studies suggest that EPO reduces inflammatory cell infiltration by decreasing neutrophil infiltration and by weakening NF- κ B and protein 1 (AP-1) activity [25]. We found that TGF- β treatment increased NF- κ B expression in CFs, while EPO attenuated this increase. This result suggests that the in-

hibition of ERK1/2 phosphorylation and the subsequent inactivation of NF- κ B are involved in the EPO-mediated effects on the proliferation of cultured rat CFs induced by TGF- β .

Conclusion

We demonstrate that EPO alleviates the cardiac fibrosis induced by pressure overload, and this is related to its inhibitory action on the NADPH-ERK-NF- κ B pathway. Our findings indicate that the inhibition of the NADPH-ERK-NF- κ B signaling pathway may represent a novel application of EPO in preventing the progression of cardiac fibrosis.

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