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Research Paper

Nonhematopoietic erythropoietin derivative protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis☆

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Abstract

Objective: Carbamylated EPO(CEPO) is a derivative of erythropoietin(EPO) by subjecting it to carbamylation. It does not stimulate erythropoiesis, but effectively protects tissue from injury. The present study was to investigate the effect of CEPO treatment using *in vitro* models of hypoxia/reoxygenation(H/R). **Methods:**Cardiomyocytes were exposed to hypoxia(95% N_2 and 5% CO_2) for 1 hour followed by 4 hours of reoxygenation(95% O_2 and 5% CO_2). CEPO was administered after hypoxia, just before reoxygenation. The apoptotic cardiomyocytes were determined by flow cytometry. The level of protein was assessed by western blot analysis. **Results:** CEPO treatment significantly decreased the apoptotic cardiomyocytes by 54.20% compared with H/R group. Western blot analysis showed that CEPO administration increased the level of Bcl-2(an antiapoptotic protein) by 62.22% compared with H/R group. **Conclusion:** Acute administration of CEPO protected cardiomyocytes from H/R-induced apoptosis. CEPO protected cardiomyocytes with a concomitant upregulation of Bcl-2 after H/R injury.

Key words: carbamylated erythropoietin; hypoxia/reoxygenation; cardiomyocytes; apoptosis

INTRODUCTION

Current studies suggest that erythropoietin(EPO) protects the brain and the spinal cord from ischemic and traumatic injury[1,2], the peripheral nerve from diabetic damage[3], the kidney[4,5] and the heart from ischemia[6-8]. The observed protective effects of EPO depend on an antiapoptotic effect of this cytokine[6,9]. It has been reported that the cytoprotective effect of EPO requires higher doses than those used to treat anemia[10,11]. However, many clinical trials have suggested that higher doses of rhEPO are likely to be associated with undesired side effects such as thrombosis[11,12]. Furthermore, the fact that EPO is cytoprotective for cardiomyocytes *in vitro*[6,13] indicates that it has some direct cardioprotective effects independent of changes in erythrocyte concentration.

Recently, it has been proved that tissue protection of EPO does not depend on homodimeric EPOR, but utilizes EPOR and βcR heteroreceptor[14]. Carbamylated EPO (CEPO), a derivative of EPO by subjecting it to carbamylation, is reported to signal not through the homodimeric EPOR but the EPOR and βcR heteroreceptor[12], so that it can effectively protects tissue from injury in cardiomyocytes[11,14] and spinal cord neurons[12,15] without stimulating erythropoiesis. Recent studies showed that long-time CEPO administration can reduce cardiomyocyte loss, limit the extent of the myocardial scar, and improve cardiac function[11,16] so that attenuate myocardial ischemic injury. Furthermore, CEPO significantly attenuated staurosporine-induced apoptosis of adult rat or mouse cardiomyocytes *in vitro*, the effect of which is comparable with the effect of EPO[11]. However, whether acute administration of CEPO will protect cardiomyocytes from hypoxia/reoxygenation (H/R)-induced apoptosis and the dependent anti-apoptotic protein which is involved in CEPO-induce cytoprotection have not been elucidated entirely.

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It is well known that Bcl-2 is an antiapoptotic protein which belongs to Bcl-2 family[17], overexpression of Bcl-2 attenuates apoptosis and against myocardial I/R injury in transgenic mice[18]. Therefore, increases in the expression of Bcl-2 would be antiapoptotic[17,19]. Based on the data, we hypothesized that Bcl-2 was involved in the protection of CEPO.

In the current study, we determined whether acute administration of CEPO will protect cardiomyocytes from hypoxia/reoxygenation(H/R)-induced apoptosis. An additional aim was to evaluate whether CEPO-induced cytoprotection involves modulation of the expression of the Bcl-2.

MATERIALS AND METHODS

Preparation of CEPO

CEPO was prepared as described in reference[12], by using rhEPO(Shenyang Sunshine pharmaceutical Co., Ltd). Briefly, one volume of EPO(1 mg/ml) was mixed with one volume of 1 mol/L Na-borate, (pH8.8) and recrystallized KOCN was added to a final concentration of 1 mol/L. The mixture was incubated at 37℃ for 24 h. Samples were immediately dialysed against milli-Q water, and subsequently against 20 m mol/L sodium citrate in 0.1 mol/L NaCl, pH6.0. After dialysis, the samples were concentrated to 2 mg/ml by Centricon (Millipore).

Analysis of CEPO

The molecular weight of CEPO was detected by SDS-PAGE and the protein content determined by the BCA method of Pierce. The purity was verified according to the protocols in reference[12]. Briefly, samples(20-200 μg/ml) were dialysed against 0.1 mol/L sodium bicarbonate pH8.5. To 500 μl of sample, 250 μl 0.01% TNBS solution was added and the mixture was incubated at 37℃. After 2 h, 250 μl of 10% SDS and 125 μl M HCL were added, and the absorbance of the solution was measured at 335 nm.

Hematopoietic activity of CEPO was determined by using BALB/c mice(6-7 weeks of age). Animals were randomly divided into three groups: mice treated with saline(control); mice treated with CEPO(CEPO); mice treated with EPO(EPO). CEPO or EPO was administered intraperitoneally to mice twice weekly for 5 weeks at doses of 100 μl/kg-body weight. Serum erythrocyte concentrations were determined by Traditional Chinese Medical Hospital of Jiangsu Province.

Cell culture and experimental design

Primary cardiac myocytes were isolated from neonatal rats and cultured as described previously[20]. Briefly, the hearts were collected and atria were removed. The ventricles were cut into pieces and subjected to 10 rounds of enzymatic digestion with 0.06% trypsin. Cardiomyocytes were plated on 6-well cell culture dishes and cultured for 36 hours in Dulbecco's modified Eagle's medium(DMEM) supplemented with 12% FBS, penicillin/streptomycin(100 units/ml), and L-glutamine (2 mmol/L). When the cardiomyocytes were in 75%-80% confluence in 6-well culture dishes, cardiomyocytes were exposed to hypoxia(95% N_2 and 5% CO_2) for 1 hour followed by 4 hours of reoxygenation (95% O_2 and 5% CO_2) as described previously[21]. CEPO(100 ng/mL) was administered after hypoxia, just before reoxygenation. The experimental design consisted of three groups of cardiomyocytes: normal(normal); H/R, not treated(H/R);H/R,treated with CEPO(H/R+CEPO). Cardiomyocytes were harvested with lysis buffer.

Activity assay of caspase-3 like in apoptosis of cardiomyocytes by flow cytometry(FCM)

Caspase-3 activity was determined by CaspGLOWTM fluorescein caspase-3 staining kit(Biovision). Cardiomyocytes were collected after 4 hours of reoxygenation and incubated with CaspGLOWTM in situ marker, FITC-DEVD-MK, for 60 minutes at 37℃ incubator with 5% CO_2 according to the supplie's protocols. Subsequently, resuspended cardiomyocytes were analyzed by FCM. UL and UR in FCM were indicative of the percentage of normal cells and apoptotic cells respectively.

Western blot analysis

After harvested cardiomyocytes, total proteins extracts were isolated. To detect Bcl-2 in myocardial tissues after CEPO administration, western blot was performed with a commercially available kit(Bcl-2 antibody kit, Biovision). Protein extracts were electrophoresed in a 12% SDS-polyacrylamide gel. Then the gel was transferred onto PVDF membranes, and the membrane was incubated with the Bcl-2 antibody(1:800), and β-actin (1:2000, sigma) and subsequently probed with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG(1:2000) according to the supplier's protocols. Proteins were detected using enhanced chemiluminescence(ECL) western blotting detection kit. The membranes were exposed to photographic film, and scanned by using a document scanner. Densitometry was performed by using NIH Image 1.63.

Statistical analysis

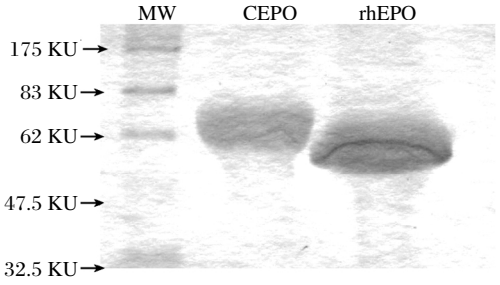
Data were presented as mean±SD. Group differences were analyzed with Scheffe or post-hoc test according to Bartlett's test for equal variances. $P < 0.05$ was considered as significant.

RESULTS

CEPO lacked hematopoietic activity

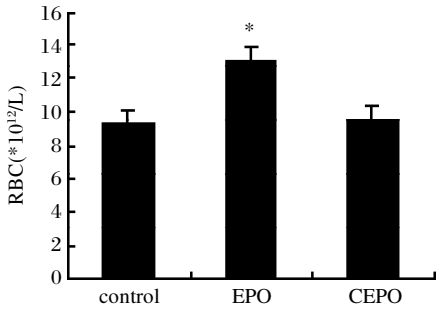
As shown in ***Fig 1***, Molecular weight of CEPO was

about 6 KD higher than that of EPO, consistent with the data in reference[12]. The purity of CEPO was about 97.65%. Hematopoietic activity of CEPO was then determined. EPO was also given to one group of mice which served as positive control. As shown in ***Fig 2***, there was no significant difference between CEPO-treated group and normal group while EPO treatment increased erythrocyte concentrations by 41.01% (13.1 ± 0.40 *vs* 9.29 ± 0.17, $n = 8$, $^{*}P < 0.05$) compared with normal group.



The molecular weight of CEPO was about 65 KD while EPO was about 59KD

Fig 1 The molecular weight of CEPO was detected by SDS-PAGE



CEPO treatment did not stimulate erythropoiesis. Compared with control group, $^{*}P < 0.05 (n = 6)$.

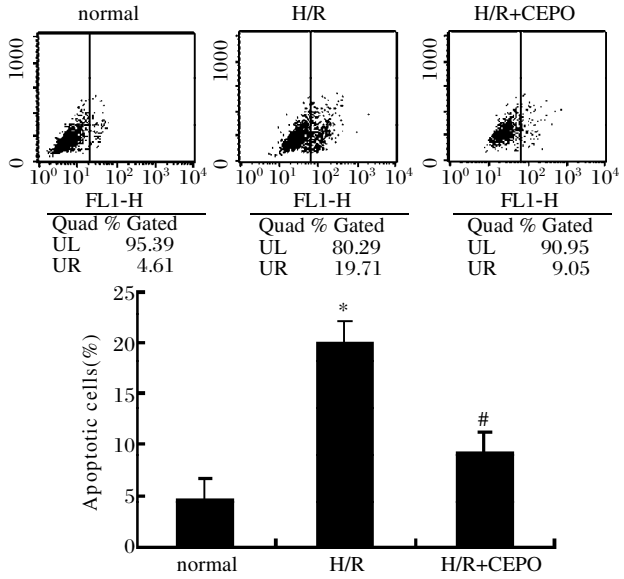
Fig 2 The result of serum erythrocyte concentrations

CEPO administration attenuated H/R-induced apoptosis

To detect the effect of CEPO on H/R-induced cardiomyocytes apoptosis, we performed in vitro experiments using a model of H/R. As shown in ***Fig 3***, H/R significantly increased the level of apoptotic cardiomyocytes by 300.60%(19.98 ± 0.55 *vs* 4.64 ± 0.32, $n = 3$, $^{*}P < 0.05$) compared with normal group. CEPO significantly decreased the apoptotic cardiomyocytes by 54.20%($9.15\% \pm 0.14$ *vs* $19.98\% \pm 0.55$, $n = 3$, $^{\#}P < 0.05$) compared with H/R.

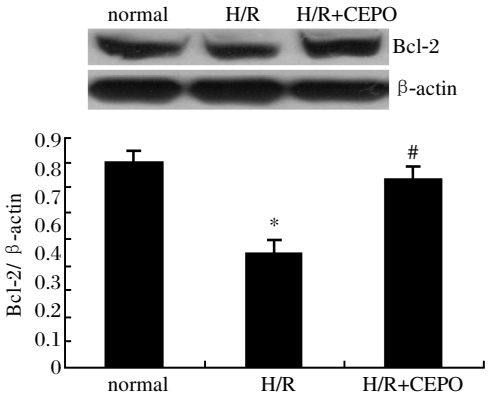
CEPO administration increased the level of Bcl-2

As shown in ***Fig 4***, H/R significantly decreased the level of Bcl-2 by 43.75%(0.45 ± 0.04 *vs* 0.80 ± 0.12, $n = 3$, $^{*}P < 0.05$) compared with normal group. CEPO administration increased the level of Bcl-2 by 62.22% (0.73 ± 0.09 *vs* 0.45 ± 0.04, $n = 3$, $^{\#}P < 0.05$) compared with H/R group.



CEPO treatment decreased H/R-induced apoptosis. Compared with normal group, $^{*}P < 0.05$; compared with H/R group, $^{\#}P < 0.05 (n = 3)$.

Fig 3 The apoptotic cardiomyocytes were determined by flow cytometry



CEPO administration increased the level of Bcl-2 in cardiomyocytes. Compared with normal group, $^{*}P < 0.05$; compared with H/R group, $^{\#}P < 0.05 (n = 4)$.

Fig 4 The protein extracts were assessed by Western blot analysis

DISCUSSION

Carbamylated EPO(CEPO), a derivative of EPO by subjecting it to carbamylation, completely lacks hematopoietic activity, but effectively protects tissue from injury in cardiomyocytes[11,12,14]. Recent studies showed that CEPO treatment can reduce cardiomyocyte loss and improve cardiac function after experimental myocardial infarction of rats. CEPO can also significantly attenuate staurosporine-induced apoptosis of adult rat or mouse cardiomyocytes *in vitro*[11]. In this paper, we examined whether CEPO administration will protect cardiomyocytes from H/R-induced apoptosis. Our results suggested that untreated cardiomyocytes after H/R

ex-hibited severe apoptosis while administration of CEPO significantly decreased apoptotic cells following H/R. These data indicated that acute administration of CEPO protected cardiomyocytes from H/R-induced apoptosis As well, CEPO was found to significantly increase the level of Bcl-2, an antiapoptotic protein which belongs to Bcl-2 family. It has been proved that Bcl-2 mediates in ischemia/reperfusion-induced apoptosis[18,19]. The level of Bcl-2 has been shown to be down-regulated or loss under H/R. It has been demonstrated to be in part responsible for the apoptosis observed with H/R injury, an effect we confirmed in the present study. Our new finding is that CEPO restored Bcl-2 expression after H/R injury. These data indicate that CEPO-treatment reduced the cardiomyocytes apoptosis by repressing apoptosis possibly through up-regulation of Bcl-2, an antiapoptotic protein of Bcl-2 family.

In summary, CEPO can protect cardiomyocytes from H/R-induced apoptosis.CEPO protected cardiomyocytes with a concomitant upregulation of Bcl-2 after H/R injury. The precise mechanism for CEPO-induced alleviation of H/R injury remains to be further investigeted.

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