Original Article

Carbamylated erythropoietin ameliorates cardiomyopathy via ERK (44/42) activation in rats with diabetic cardiomyopathy

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Abstract: This study aimed to investigate the potential mechanisms underlying the protective effects of carbamylated erythropoietin (CEPO) on diabetic-related myocardial fibrosis. Wistar rats were fed a high-fat/high-carbohydrate diet and intraperitoneally injected with streptozotocin to induce diabetic cardiomyopathy (DCM). The rats were then treated with recombinant human erythropoietin (rhEPO) or CEPO. The lipid and glucose levels of the rats were recorded 4 and 8 weeks after the treatment. Myocardial samples were analyzed through Massons trichrome staining and transmission electron microscopy. The expression levels of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) were also detected. Moreover, myocardial apoptosis and extracellular signal-regulated kinase (ERK) signaling were investigated. Induction of the diabetic model significantly promoted myocardial cell apoptosis and upregulated the protein expression levels of TGF-β1 and CTGF. Treatment with CEPO or rhEPO decreased the number of apoptotic cells, ameliorated the dissolution of myocardial myofilaments and damage of mitochondria, and downregulated TGF-β1 and CTGF expression levels in the myocardium of diabetic rats. Furthermore, CEPO increased ERK (44/42) protein expression levels in the hearts of diabetic rats. Overall, it was found that myocardial fibrosis may contribute to myocardial injury in diabetes mellitus and that CEPO might protect the myocardium of diabetic rats against fibrosis through the ERK (44/42) signaling pathway.

Keywords: Carbamylated erythropoietin, fibrosis, ERK, diabetic cardiomyopathy

Introduction

The major pathological changes associated with diabetic-related heart disease include oxidative stress, inflammation, cardiac fibrosis, and myocardial apoptosis. These changes induce myocardial relaxation and systolic dysfunction in diabetic cardiomyopathy [1]. Myocardial fibrosis in diabetes mellitus is affected by several factors. Transforming growth factor-β1 (TGF-β1) can promote myocardial fibrosis and cardiac hypertrophy [2, 3] and is activated by high levels of blood glucose and angiotensin II [4]. TGF-β1 exists in five isoforms in vertebrates; of these isoforms, TGF-β1-3 are mainly found in mammals. TGF-β1 is widely present in normal and transforming cells in the human body and plays an important role in cell growth, differentiation, apoptosis, inflammation and gene expression [5, 6].

Erythropoietin (EPO) is a glycoprotein growth factor produced and secreted by renal interstitial cells. Recombinant human erythropoietin (rhEPO) possesses similar molecular structure and biological activity to natural EPO, which is mainly used for the treatment of anemia. rhEPO can inhibit the expression of TGF-β1 and protect myocardial tissues against fibrosis [7, 8]. However, long-term application of high doses of rhEPO may cause hypertension, thromboembolism, and other side effects because this hormone affects hematocrit [9]. Carbamylated EPO (CEPO), a derivative of EPO, completely lacks bioactivity in hematopoiesis but retains the cardioprotective effect of EPO on myocardial cells [10-13].

CEPO reduces renal tubulointerstitial fibrosis by inhibiting TGF-β1 expression [14]. However, the effect of CEPO on myocardial fibrosis remains...
unclear. This study aimed to investigate whether CEPO can reduce myocardial fibrosis and to determine the underlying mechanism in diabetic rats.

Materials and methods

Main reagents

rhEPO (Category [Cat.] no. E10053) was obtained from Kai Mao Biomedical Co., Ltd. (Shanghai, China). Streptozotocin (STZ; Cat. no. S0130), diethyl pyrocarbonate, sodium borate, and potassium cyanate were purchased from Sigma-Aldrich (St. Louis, MO, USA). A p-ERK (44/42)MAPK (Cat. no. 9101 s) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). TGF-β1 (Cat. no. bs-4538R) and connective tissue growth factor (CTGF; Cat. no. bs-0743R) antibodies were obtained from Boaosen Biological Manufacture Co., Ltd. (Beijing, China). All reagents were of analytical pure grade.

Carbamylation of EPO

CEPO was synthesized using a method described by Leist et al. [10]. Potassium cyanate was added to a mixture (1 mol/l) of 500 μL of rhEPO (1 mg/ml) and 500 μl of 1 M sodium borate. The mixture was incubated at 37°C for 24 h to form the reaction solution. Excess potassium cyanate was removed from the reaction solution by dialysis, and the solution was concentrated by ultrafiltration (membrane cut-off, 10 kDa). Protein concentration was measured by Coomassie brilliant blue staining, and absorbance was read at 335 nm.

Animal groups

A total of 120 healthy male Wistar rats aged 7 weeks (220±20 g, License: SCXK-(Ji) 2007-0003) were obtained from the Experimental Animal Center of Jilin University (Changchun, China). This study was approved by the ethics committee of the First Bethune Hospital of Jilin University. The high-fat/high-carbohydrate diet fed to the rats was composed of 66% basic rat chow, 20% sucrose, 10% lard, 3% egg yolk, and 0.4% cholesterol. Twenty rats were randomly designated as controls, and the remaining 100 rats were fed the high-fat/high-carbohydrate diet for 4 weeks and then injected with STZ (50 mg/kg, intraperitoneally) to establish a diabetic model. After 1 week, a second injection of the same dose of STZ was administered. Seventy-five diabetic rats were obtained based on the criterion of a fasting blood glucose concentration > 18 mmol/L.

The experiment was divided into two parts. The first part analyzed dose-effects. Rats were divided into the following groups: group A (control, healthy rats, n = 10), group B (DCM, n = 9), group C (500 IU/kg CEPO, n = 9), group D (1,000 IU/kg CEPO, n = 9), group E (2,000 IU/kg CEPO, n = 9), and group F (1,000 IU/kg rhEPO, n = 10). Groups B-F comprised rats with DCM. CEPO or rhEPO was subcutaneously injected twice a week for 4 weeks. Subsequently, the final numbers of surviving rats in groups A-F were 10, 6, 7, 7, 8, and 7. The second part of the study focused on the investigation of time-effects. This part included groups A, B, D, and F as well as a long-term (8-week) intervention panel, which consisted of the following groups: group A’ (control, healthy rats, n = 10), group B’ (DCM, n = 9), group D’ (1,000 IU/kg CEPO, n = 10), and group F’ (1,000 IU/kg rhEPO, n = 10). In the long-term intervention panel, the final numbers of the surviving rats in groups A’, B’, D’, and F’ were 10, 6, 8, and 7, respectively.

Routine blood examination

After 4 and 8 weeks, rats were fasted for 8 h and anesthetized with 10% chloral hydrate (0.30 g/kg). Serum was collected from the right ventricles of diabetic rats to detect glucose, triglyceride, and total cholesterol levels. Blood samples were sent for routine hematological examination at the clinical laboratory of the First Bethune Hospital of Jilin University (Changchun, China).

Masson’s trichrome staining of myocardial tissues

Hearts were obtained, fixed in 10% neutral buffered formalin, and embedded in paraffin. Paraffin sections (4 μm) were acquired and subjected to Masson’s trichrome staining after routine deparaffinization and hydration.

Transmission electron microscopy (TEM) of myocardial tissues

Myocardial tissue was fixed in 4% glutaraldehyde at 4°C, then processed successively with 1% osmic acid fixations for 3 hours, dehy-
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TUNEL investigation of myocardial cell apoptosis

Eight weeks after the experiment, the rats’ hearts were perfused and fixed with 10% neutral buffered formalin and then paraffinembedded. Sections measuring 4 μm were used for the detection of myocardial cell apoptosis with a TUNEL kit (In Situ Cell Death Detection kit, POD). Apoptosis was quantified using Image Pro Plus 6.0 image analysis software (Media Cybernetics, USA).

Immunohistochemistry

Myocardial tissue sections were prepared as described above and immunohistochemically stained through the streptavidin-peroxidase method to detect the protein expression of TGF-β1 and CTGF. Cells were defined as positive if the cytoplasm was stained brown. The integrated optical density of positive staining was measured using Image-Pro Plus 6.0 software (Media Cybernetics, USA).

Western blot analysis of phosphorylated ERK protein expression

A small quantity of myocardial tissue was cut into fragments and homogenized with radioimmunoprecipitation assay buffer (1 mM phenylmethylsulfonyl fluoride) for protein extraction. After centrifugation of the lysate (13,750×g for 15 min at 4°C), the supernatant was quantified using a bicinchoninic acid protein assay. The protein concentration in the loading samples was adjusted to 80 μg/20 μl, and the samples were separated through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples were then transferred to a polyvinylidene fluoride (PVDF) solid-phase membrane. The membrane was incubated with p-ERK(44/42)MAPK (1:1,000) and β-actin (1:1,000) antibodies for 90 min and then conjugated with secondary anti-immunoglobulin IgG antibody (1:2,000) for 90 min. The immunoblotted proteins were detected using enhanced chemiluminescence.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software (IBM-SPSS, Inc., Armonk, New York, USA). All data are expressed as the mean ± standard deviation. Differences between the means of the two groups were compared using Student’s t test. Multiple means were compared using one-way ANOVA, which was employed for intergroup comparisons. Two-sided p values <0.05 were considered statistically significant.

Results

Changes in serum levels of GLU, TG, and TC

As shown in Table 1, the serum levels of GLU were higher in the rats with DCM compared with those in the control group (p<0.05). EPO and CEPO had no significant effect on the serum levels of GLU. The serum levels of TC were higher in the rats with DCM compared with those in the control group (p<0.05). CEPO significantly decreased the serum TC levels in the rats with DCM (p<0.05), but there was no significant difference between different doses of treatment. Eight weeks of treatment with CEPO resulted in a lower TC level than 4 weeks

Table 1. GLU, TG and TC in different groups

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>A'</th>
<th>B'</th>
<th>D'</th>
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</tr>
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<tbody>
<tr>
<td>GLU</td>
<td>5.18±0.49</td>
<td>32.62±6.77</td>
<td>28.50±2.06</td>
<td>29.19±5.70</td>
<td>27.12±6.56</td>
<td>35.96±7.72</td>
<td>5.03±0.17</td>
<td>36.53±2.26</td>
<td>30.05±3.87</td>
<td>28.91±5.94</td>
</tr>
<tr>
<td>TG</td>
<td>0.91±0.14</td>
<td>2.44±0.29</td>
<td>0.92±0.28</td>
<td>0.54±0.26</td>
<td>0.47±0.14</td>
<td>1.62±0.44</td>
<td>0.97±0.15</td>
<td>2.61±0.31</td>
<td>0.66±0.19</td>
<td>0.81±0.14</td>
</tr>
<tr>
<td>TC</td>
<td>1.30±0.25</td>
<td>2.84±0.37</td>
<td>1.81±0.43</td>
<td>1.78±0.03</td>
<td>1.92±0.21</td>
<td>2.63±0.24</td>
<td>1.41±0.26</td>
<td>2.93±0.28</td>
<td>1.52±0.14</td>
<td>1.96±0.49</td>
</tr>
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</table>

CEPO, carbamylated erythropoietin; DCM, diabetic cardiomyopathy; No., number of rats; GLU, glucose; TG, triglyceride; TC, total cholesterol. Rats were assigned to the following groups for a fourweek treatment intervention: A, control group; B, DCM group; C, CEPO (500 IU/kg) group; D, CEPO (1000 IU/kg) group; E, CEPO (2000 IU/kg) group; and F, rhEPO (1000 IU/kg) group. For the eightweek treatment intervention, the rats were assigned to the following groups: A’, control group; B’, DCM group; D’, CEPO (1000 IU/kg) group; and F’, rhEPO (1000 IU/kg) group. *, P<0.05 vs. control group; †, P<0.05 vs. DCM group; ‡, P<0.05 vs. CEPO (500 IU/kg) group; §, P<0.05 vs. CEPO (1000 IU/kg) group; II, P<0.05 vs. CEPO (2000 IU/kg) group; and ‟, P<0.05 vs. the corresponding four-week intervention group.
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The effect of CEPO was more significant than that of the same dose of EPO (p<0.05). The serum levels of TG were higher in the rats with DCM compared with those in the control group (p<0.05). CEPO significantly decreased the serum TG levels in the rats with DCM (p<0.05), but the difference was not significant between different times and doses of treatment. The effect of CEPO was more significant than that of the same dose of EPO (p<0.05).

CEPO decreases the number of apoptotic cells and ameliorates the ultrastructure of cells

TUNEL assays were performed to examine whether diabetes mellitus induced myocardial cell apoptosis and whether CEPO exerted an antiapoptotic effect. As shown in Figures 1, 2, apoptotic myocardial cells were occasionally observed in normal control rats. By contrast, the number of apoptotic myocardial cells significantly increased in the DCM group. The extent of apoptosis was significantly lower (p<0.05) in the EPO (4 week intervention: 14.48±2.80 mm² and 8 week intervention: 13.42±3.90 mm²) and CEPO (4 week intervention: 13.08±2.37 mm² and 8 week intervention: 11.93±1.64 mm²) groups compared with the untreated DCM group (4 week intervention: 20.53±3.20 mm² and 8 week intervention: 17.26±2.68 mm²).
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The Masson's trichrome staining results are shown in Figure 3. In the normal control rats, myocardial cells were stained red, collagen was stained blue and green, and red blood cells were stained orange. Less myocardial collagen was observed, with only a small amount surrounding the vessels. The distribution of collagen fibers in the myocardium was uniform and fine. In the rats with DCM, there was a significant increase in the amount of collagen tissue. Thick collagen fibers were connected to each other in a network and were unevenly distributed, with a large amount surrounding myocardial cells and small blood vessels. CEPO and rhEPO decreased the amount of myocardial collagen, particularly after prolonged treatment.

As shown in Figure 3, TEM analysis of the myocardial cells demonstrated that in the DCM group, there was a reduction in the myocardial myofilaments in the cytosol, and a number of
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Myocardial myofilaments dissolved. Cytoplasmic matrices were cavitated. The intercalated discs were moderately separated. The number of mitochondria increased. Several mitochondria were pyknotic in appearance. The number of myocardial myofilaments slightly decreased after treatment with CEPO. Dissolution only occurred in small sections of the sarcomeres. A small number of mitochondria were small and circular in shape. The difference was not significant between EPO and CEPO treatment.

CEPO inhibits the protein expression of TGF-β1 and CTGF

As shown in Figures 4-7, the protein expression levels of TGF-β1 and CTGF in the myocardial cells of the rats with DCM significantly increased compared with those of the control rats (p<0.05). In comparison with the DCM rats, CEPO treatment dose-dependently decreased TGF-β1 and CTGF protein expression levels in myocardial cells (p<0.05). TGF-β1 and CTGF protein expression levels were not significantly different between the same-dose CEPO and rhEPO groups (p>0.05), but they significantly differed between the 4 and 8-week treatment regimens (p<0.05).

CEPO promotes activation of the p-ERK(44/42)MAPK protein

The protein expression levels of p-ERK(44/42) MAPK in the myocardial cells of the rats with DCM significantly increased compared with those of the control rats (p<0.05). Rats given middle and high doses of CEPO showed increased protein expression levels of p-ERK(44/42) MAPK in myocardial cells compared with the rats with DCM (p<0.05). p-ERK(44/42)MAPK protein expression levels significantly differed between the same-dose CEPO and rhEPO groups (p<0.05), but they were not significantly different between the 4 and 8-week treatment regimens (Figures 8, 9, Supplementary Figure 1).
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Discussion

In this study, we revealed that CEPO, a carbamyl derivative of EPO, decreased serum levels of TG and TC and partly attenuated myocardial pathological damage through the activation of the p-ERK(44/42)MAPK signaling pathway. This pathway is known to regulate myocardial cell apoptosis in diabetic rats.

Previous studies have revealed that CEPO does not bind to the classical EPO receptor (EPOR) and does not show any hematopoietic activity in human cell signaling assays or after chronic dosing in different animal species [10]. CEPO exerts cytoprotective effects in vitro and confers neuroprotection against stroke, spinal cord compression, diabetic neuropathy, and experimental autoimmune encephalomyelitis [10]. However, whether CEPO confers protection against DCM remains unknown.

CEPO and rhEPO exert varied effects on erythropoiesis. rhEPO binds to the classic receptor (EPOR) 2 and stimulates the proliferation and differentiation of red blood cells [10]. By contrast, CEPO does not bind to (EPOR) 2 and does not increase the risk of blood clot formation caused by excessive red blood cell production.
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EPOR can physically interact with the common β receptor (βcR), also known as CD-131, to provide increased ligand-binding affinity to the receptor complex. EPOR is also a signal-transducing subunit shared by the granulocyte-macrophage colony stimulating factor and IL-3 and IL-5 receptors [16, 17]. CEPO exerts anti-apoptotic effects by binding to EPOR-CD131 [18].

In this study, CEPO did not affect GLU levels but significantly decreased serum TG and TC levels in rats with DCM. EPO reduced the levels of serum TG and TC but did not affect GLU levels. The effect of CEPO was more significant than that of the same dose of EPO. These results suggest that EPOR and rhEPO decreased the amount of myocardial collagen. These results suggest that CEPO may ameliorate cardiac fibrosis in the development of DCM.

TGF-β, a cytokine important in the pathogenesis of myocardial fibrosis [19], functions as a profibrotic cytokine and as a growth factor in several pathophysiological processes [20]. TGF-β1 activity, an important factor of myocardial fibrosis and failure, is enhanced as a result of high levels of blood glucose and induction of angiotensin II [5, 21, 22]. TGF-β1 affects ventricular remodeling, myocardial hypertrophy, and fibrosis; the plasma level and activity of TGF-β1 in myocardial tissues provides laboratory standards for the early diagnosis and prevention of heart diseases associated with diabetes [23]. Recent studies have found that reducing the expression of TGF-β1 protein can inhibit myocardial fibrosis in high-fat and STZ-induced diabetic rats [24-26].

CTGF, a downstream mediator of the TGF-β pathway, plays a major role in adverse remodeling through promotion of myocardial fibrosis and ECM production in connective tissues [26-28].

In this study, the protein expression levels of TGF-β1 and CTGF increased in diabetic rats; after administration of CEPO, however, these levels decreased. CEPO treatment exhibited a dose- and time-dependent relationship to TGF-β1 and CTGF expression. Thus, we conclude that CEPO dose-dependently inhibits myocardial fibrosis in diabetic rats.

Mitogen-activated protein kinases (MAPKs) are multifunctional regulators that play indispensable roles in a number of biological processes in the heart [29, 30]. These processes include cell proliferation, survival, apoptosis, actin reorganization and cytokine production [29]. The MAPK subfamilies include extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal kinases, p38 kinase, and large MAPKs (BMK or ERK5) [30-32].

Some studies have suggested that activation of ERK signaling is one of the major components of the reperfusion injury salvage kinase pathway [33, 34]. In the present study, our findings indicated that CEPO could significantly activate...
the ERK (44/42) signaling pathway in the myocardium of diabetic rats. However, CEPO treatment did not exhibit a dose- or time-dependent relationship to the protein expression of ERK (44/42). Meanwhile, p-ERK (44/42) protein expression levels significantly differed between the same-dose CEPO and rhEPO groups. These results indicate that another pathway may be involved in ERK (44/42) activation.

In conclusion, CEPO partially protects the myocardium from fibrosis through the activation of the ERK (44/42) pathway.

Disclosure of conflict of interest

None.

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References


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**Supplementary Figure 1.** ERK(44/42)MAPK signal pathway protein expression in different groups. Western blot (from left to right: A, B, C, D, E, F, A’, B’, D’, F’).