Effects of rhBNP on myocardial fibrosis after myocardial infarction in rats

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Received February 13, 2015; Accepted April 12, 2015; Epub June 1, 2015; Published June 15, 2015

Abstract: Purpose: We aimed to observe the effects and mechanism of rhBNP treatment on myocardial fibrosis (MF) after myocardial infarction (MI). Methods: SPF rats were separated into 3 groups: normal, MI (ligation of left coronary artery), and MI + rhBNP (recombinant human brain natriuretic peptide). Rats in MI + rhBNP group were given 30 μg/kg for 2 days before modeling and for 4 weeks after modeling. mRNA levels and the expression levels of TGF-β1 (transforming growth factor) and CTGF (connective tissue growth factor) in 3 groups were analyzed using the RT-qPCR and western blotting analysis, respectively. Furthermore, myocardial volume fraction (CVF) was analyzed using the Sirius Red F3B (SR) while the percentage of type I and III collagen in 3 groups were analyzed using the immunohistochemical staining. Results: Compared with the normal group, the levels of TGF-β1, CTGF, CVF, type I and III collagen were higher in MI group. However, mRNA levels of TGF-β1 and CTGF were significantly decreased in MI + rhBNP compared to MI groups. Expression of TGF-β1 was lower while that of CTGF was higher in MI + rhBNP group than that in MI group. Besides, CVF, and type I and III collagen were lower in MI + rhBNP group compared with MI group. Conclusion: rhBNP could significantly decrease the TGF-β1 and CTGF levels in post-MI so as to inhibit the type I and III collagen deposition in MF of post-MI. rhBNP will be benefit for the improvement of MF.

Keywords: Recombinant human brain natriuretic peptide (rhBNP), TGF-β1, connective tissue growth factor (CTGF), myocardial infarction, myocardial fibrosis

Introduction

Myocardial infarction (MI) is one of the main diseases that leading to death or disability worldwide [1]. The main cause for MI is the sustained and serious myocardial ischemia resulted by myocardial necrosis due to the imbalance of myocardial blood supply and demand [2]. Myocardial fibrosis (MF), a subsequent development of MI, is characterized by the abnormal level and excess deposition of collagen in myocardial matrix that leading to the dysfunction of ventricular and heart failure [3]. Although many studies have devoted to the exploration of MI control and treatment methods, the mechanisms of MI still remain largely unknown. Therefore, it will be of great significance to investigate the mechanism of MI and dig several biomarkers or useful drugs for MI treatment.

Previous studies referred that inflammatory factors participated in MF progression of post-MI. For instance, tumor necrosis factor (TNF-α) contributes to the MF in post-MI [4] and interleukin-1β (IL-1β) levels is strongly associated with the myocardial remodeling in post-MI [5]. Also, IL-17A promotes the ventricular remodeling in post-MI [6]. On the other hand, the newly reported connective tissue growth factor (CTGF) is another factor that functions in myocardial remodeling and MF. The activated CTGF is related to the left ventricular remodeling after MI [7]. In addition, the TGF-β-stimulated cardiac fibroblast collagen synthesis and CTGF expression are decreased by the soluble expoxide hydrolase in post-MI [8].

Recombinant human brain natriuretic peptide (rhBNP) is a heart failure treatment drug that approved by America Food and Drug Administration (FDA) in 2001 [9]. The role of rhBNP on MF in post-MI has been discussed in previous studies. For example, BNP is an important prognostic drug for heart failure [10] and rhBNP improves the symptoms in patients with acute decompensated heart failure [11]. Also, the
combined rhBNP therapy and bone mesenchymal stem cell transplantation is benefit for the heart failure treatment [12]. In spite of many explorations of rhBNP in improving MF. However, few studies demonstrated the mechanisms of rhBNP in the TGF-β1/CTGF-stimulated MF of post-MI.

In this study, we constructed a rat model of MI and treated the MI rat with rhBNP. Comprehensive experimental methods were used to detect the mRNA level and protein levels of TGF-β1 and CTGF in MI model and MI + rhBNP models compared to the normal groups. Further experiments were conducted to detect the CVF, type I and III collagen of myocardial cells. We aimed to explore the intervention effect and possible mechanism of rhBNP on MF in post-MI. This study might provide basis for the future research of rhBNP on MF in post-MI.

Materials and methods

Modeling of rat MI

All experimental procedures in this study were approved by the Institute of Health Services Research at Harbin Medical University on the protection of animal used for scientific purpose. The manuscript was prepared according to the guidelines of the declaration of Helsinki for biomedical research. The SPF C57BL/6J mice (Shanghai laboratory animal center, Chinese Academy of Sciences) weighting 18-20 g were individually housed in a rodents feeding room with the adjustable temperature, humidity, light, and pressure. The environment was maintained under a 12/12 hour light/dark cycle (lights on at 150-200 LX), with the relative humidity at 50-70%, the temperature set at 22 ± 1°C, and noise < 50 dB. A total of 12 mice were separated into 3 treatment groups (each group containing 4 repeats): sham-operated, MI, and MI + rhBNP. Rats in MI + rhBNP group were intravenous injected with 30 μg/kg rhBNP for 2 days before modeling.

Anesthesia was conducted using the anesthesia apparatus and maintained with 2% isoflurane. Endotracheal intubation was completed via oral, and then rats were connected with HSE-HA MiniVent (USA). Chest at the fourth rib was open to exposure the heart, the left coronary artery was ligated (with the 8.0 nylon suture) about 1 mm from their origin. The immediate color change of the heart surface (pale appearance about 1 min) and the slower movement of ventricular wall were the signs of successful coronary ligation (Figure 1). The diaphragm was sutured with an absorbable 5.0 suture starting at the muscular segment towards its tendinous part, and final suture was tightened after the air was expelled from thoracic cavity. Rats in MI + rhBNP group were given 30 μg/kg rhBNP for 4 weeks after modeling, while rats in the normal and MI groups were given the same dose of water.

Quantitative RT-PCR

The fresh heart tissues of rats collected at week 4 were grinded in the liquid nitrogen and then washed with the PBS buffer (PH 7.4). The
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Table 1. Primers used for the targets amplification in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>sense</td>
<td>5’-GGCGGTGCTCGCTTTGTA-3’</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>anti-sense</td>
<td>5’-GTTGCGGTCCACCATTAGC-3’</td>
<td>19</td>
</tr>
<tr>
<td>CTGF</td>
<td>sense</td>
<td>5’-TCTTCGGTGCGTCTCGTA-3’</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>anti-sense</td>
<td>5’-GGCAGCTTGACCTCTCG-3’</td>
<td>19</td>
</tr>
<tr>
<td>β-actin</td>
<td>sense</td>
<td>5’-CCACACGTGAGGGAAATC-3’</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>anti-sense</td>
<td>5’-AAGGAAGGCTGAAAAAGAC-3’</td>
<td>20</td>
</tr>
</tbody>
</table>

Total RNA was extracted from heart tissues using the Trizol reagent [13] (Invitrogen life technologies, Carlsbad, CA) and treated with RNase-free Dnase I (Promega Biotech). The concentration and purity of extracted RNA was detected using SMA4000 UV-VIS (Merinton, Shanghai, China). The purified RNA that adjusted to 0.5 μg/μL with nuclease-free water was used for cDNA synthesis using the PrimerScript 1st Strand cDNA Synthesis Kit (Takara, China). Primers for the targets amplification were shown in Table 1. Then RT-PCR was carried out in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). The reaction system of 20 μL volume containing 1 μL cDNA from the above PCR, 10 μL SYBR Premix EX Taq, 1 μL each of the primers (10 μM), and 7 μL dd H₂O. The PCR program was as follows: denaturation at 95°C for 2 min; followed by 45 cycles of 95°C for 10 s, 59°C for 20 s, and 72°C for 30 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected.

Western blotting analysis

Tissues harvested at 4 weeks post-MI were lapped in RIPA (radioimmunoprecipitation assay, Sangon Biotech) lyase containing PMSF (phenylmethanesulfonyl fluoride) and then were centrifuged at 12,000 rpm/min for 10 min at 4°C. The supernatant was collected to determine the concentration of protein using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rochford, IL).

A total of 30 μg protein per cell lysates was subjected to a 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and then transferred onto a Polyvinylidene delfluoride (PVDF) membrane (Mippore). The membrane was blocked in TBST (Tris Buffered Saline Tween) with 5% non-fat milk for 1 h, and subsequently incubated with rabbit anti-human TGF-β1 or CTGF monoclonal antibodies (1:100 dilution) overnight at 4°C, followed by incubation with horseradish peroxidase labeled goat anti-rat secondary antibody (1:100 dilution) at room temperature for 1 h. The PVDF was washed using the 1 x TBST buffer for 10 min with 3 times. Detection was performed using the development of X-ray after chromogenic substrate with an enhanced CEL (chemiluminescence) method. In addition, β-actin (Sigma-Aldrich, USA) served as the internal control.

Sirius Red F3B (SR) for myocardial volume fraction (CVF)

One third part of the fresh heart tissues harvested at 4 weeks post-MI was removed with a scalpel along the heart transverse section, and were washed with the precooling PBS, then fixed in the 10% formalin (100 mL formalin + 900 mL PBS) for 24 h. Briefly, paraffin was sliced and then dewaxed to water. Tissues were put into the Sirius Red F3B liquid for 1 h, and then washed with distilled water for 5 min. Then the nuclear was treated with hematoxylin (Mayer) for 5 min, followed by washed with distilled water for 10 min. Xylene was used to transparent the staining tissues and neutral gum was used to seal the tissues. Sections were observed in at least five areas at × 200. The percentage of type I/III collagen (the ratio of red area and yellow area in a field vision) was analyzed using the Image-Pro plus 6.0 software.

Immunohistochemical staining

Immunohistochemical staining was conducted to detect the expressions of type I and III collagen in MI heart tissues. The fresh rat heart tissues harvested at 4 weeks were fixed in 4% formaldehyde, and then dehydrated with an automatic dehydration device (70% alcohol for 30 min, 80% alcohol for 2 h, 95% alcohol for 2 h with 2 cycles, 100% alcohol for 2 h with 2 cycles). The dehydrated tissues were transparent in xylene for 30 min and embedded in paraffin (melting point 52°C) for 1 h with 3
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Cycles, and then 6 μm sections were cut series. Sections were treated with xylene 3 times for 15 min and then treated with 99% ethanol 3 times for 10 min. The sections were immersed in 3% H₂O₂ solution and incubated at room temperature for 10 min to deactivate internal peroxidase and then washed 3 times for 5 min using the PBS buffer (PH 7.4).

Non-specific binding was blocked by incubation in 1.5% normal goat serum for 1 h at room temperature. The sections were incubated overnight at 4°C with the following primary antibodies: rabbit anti-human type I collagen monoclonal antibody (BOSTER, China, 1:100 dilution); Rabbit anti-human type III collagen (BOSTER, China, 1:1000). Sections were incubated at 37°C for 1 h with the horseradish peroxidase (HRP) labeled goat anti-rabbit (Jackson, 1:100 dilution). At lase, 3'-diaminobenzidine (DAB) was used for color development and hematoxylin for counterstaining. The sections were mounted, examined and photographed. In negative control slides, phosphate buffer solution (PBS) was used instead of primary antibody.

After treated with the above steps, pictures from sections were observed in at least five areas at × 200 using the Olympus BX53 digital microscope (Olympus BX53, Tokyo, Japan).

**Statistical analysis**

The data were expressed as mean ± standard error of mean (SEM). The independent sample t-test method was used to calculate the difference among groups (normal vs. MI, MI vs. MI + rhBNP) with the Prism 5.0 software (GraphPad Prism, San Diego, CA). The P < 0.05 was defined as statistically significant.

**Results and discussion**

**mRNA expression levels of TGF-β1 and CTGF**

We used RT-qPCR to analyze the mRNA levels of TGF-β1 and CTGF in MI rats (Figure 2). The RT-qPCR products were analyzed using the agarose gel electrophoresis (Figure 2A). The results showed us that mRNA levels of TGF-β1 and CTGF significantly increased in MI group compared with the normal group (P < 0.05). Besides, the mRNA levels of TGF-β1 and CTGF were lower in MI + rhBNP groups than that in MI groups (Figure 2B and 2C).

**Western blotting for the expressions of TGF-β1 and CTGF protein**

To identify the association of rhBNP on TGF-β1 and CTGF in heart tissues of post-MI, we con-
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**Figure 3.** Western blotting analysis of TGF-β1 and CTGF protein expression levels. A: Expression levels of TGF-β1 and CTGF protein in 3 groups, B: The relative fold of TGF-β1 in 3 groups, C: The relative fold of CTGF in 3 groups. β-actin served as the internal control.

Conducted the western blotting analysis to further detect their expression (Figure 3A). The relative expression levels of TGF-β1 and CTGF in MI group were lower than that in normal group (Figure 3B and 3C). However, TGF-β1 level in MI group was higher than that in MI + rhBNP group (Figure 3B), while the CTGF level in MI group was lower than that in MI + rhBNP group (Figure 3C).

**Sirius Red F3B (SR) for myocardial volume fraction (CVF)**

The CVF of heart in each kind group was analyzed using the Sirius Red F3B (SR) method (Figure 4A). The results displayed that myocardial collagen fibers were stained with red, while myocardial cells were stained with yellow. Also, the percentage of CVF was calculated using the Image-Pro plus 6.0 software (Figure 4B).

**Figure 4.** Sirius Red F3B (SR) for CVF. A: The Sirius red F3R for CVF in 3 groups, B: The area percentage CVF in 3 groups. *: the significance between Normal and Model group (P < 0.05), #: the significance between Model group and Model + rhBNP group (P < 0.05).
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Compared to the normal group, CVF was significantly increased in MI group (\(P < 0.05\)), while CVF significantly decreased in MI + rhBNP group compared with the MI group (\(P < 0.05\)).

**Immunohistochemical staining of type I and III collagen in post-MI tissues**

Immunohistochemical analysis on paraffin sections displayed that post-MI cells expressed strong reactivity for type I and III collagen in post-MI tissues (Figure 5A and 5B). Compared with the normal groups, expressions of type I and type III collagen were significantly higher in MI group (\(P < 0.05\)) (Figure 5C and 5D). Besides, both the type I and III collagen levels in MI groups were higher than that in MI + rhBNP groups. No statistical significance was observed of type I and III collagen between MI group and MI + rhBNP group (\(P > 0.05\)).

MF in post-MI is a disease that associated with the heart dysfunction and characterized by the excess deposition or abnormal higher level of collagen in normal heart tissues [1, 3]. Studies have devoted to the mechanisms of some useful drugs on cytokines and other factors in the MF of post-MI, such as aminomethylbenzoic acid and angelica sinensis [14, 15]. In this study, we built a rat MI model and treated the MI rat with rhBNP to explore the mechanism of rhBNP on MF in post-MI. The mRNA level and protein levels of TGF-\(\beta\)1 and CTGF in MI were significantly higher than that in normal groups. rhBNP could significantly decrease the TGF-\(\beta\)1 and CTGF levels in MI. Moreover, the deposition of CVF, type I and III collagen were inhibited by rhBNP in MI + rhBNP group compared with that in MI group.

The complement in immune system was activated in post-MI, characterized by the leukocytes gathered together to the damage heart. TGF-\(\beta\)1 is a member of the TGF-\(\beta\) family proteins that involved in embryogenesis, cell differentiation, and inflammatory reaction and damage repairers in the early stage of MI [16]. Baxter et al. proved that TGF-\(\beta\)1 alleviated the areas of myocardial apoptosis and infraction via activating the p42/p44 MAPK in MI [17]. Also, TGF-\(\beta\)1 prevented the apoptosis of cardiac fibroblast through the Smad3 signaling pathways [18]. Besides, TGF-\(\beta\)1 level could be decreased with the long-term treatment of rhBNP in heart failure [19] and rhBNP prevent-
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ed the serum TGF-β1 level and induced its fibrosis in heart failure [20]. Hence, rhBNP might play roles in lowering the TGF-β1 level in MI. On the other hand, CTGF (also named CCN2) is a mitogen which is secreted by vascular endothelial cells and plays a role in the osteogenesis, tissue repair, and fibrosis [21]. CTGF was correlated with the cardiac diastolic dysfunction in heart failure [22]. A high activity of CTGF in post-MI was related to attenuate the remodeling of left ventricular via inhibiting the cell apoptosis and some inflammatory responses [23]. Therefore, CTGF may be a contributor for MI. It has been reported that CTGF might cooperate with TGF-β1 during the myocardial fibrosis of heart via mapping their mRNA levels in the infarct zone [24]. Also, Dean et al. said that TGF-β1 was involved in the initial at the acute stage of inflammation and repair after MI, while CTGF was involved in the ongoing fibrosis of heart [25]. Thus, TGF-β1 might induce the CTGF expression in the cardiac fibrosis of post-MI. Ritchie et al. said that rhBNP had the potential role to protect the ischemic of myocardial [26]. Also, the continuous application of exogenous rhBNP reduced the infarction areas of heart and alleviated the ventricular remodeling in post-MI [27]. Our data showed that both the mRNA levels and the protein levels of TGF-β1 and CTGF in MI group were high while these levels were low in MI + rhBNP group, indicating that rhBNP may be an inhibitor of MI in early stage via suppressing the expression of TGF-β1 and CTGF. Thus, rhBNP may be the suppressor drug for MI treatment in early stage.

Meanwhile, CVF and the deposition of collagen were the most important factors that affecting the MF formation. Deposition of type I collagen might be associated with the MF in myocardium [28]. The level of collagen type III was high in rats of cardiac fibroblast [29]. Myocardial collagen metabolism was significantly enhanced in patients underwent surgical ventricular restoration [30]. rhBNP is a potential marker for the prognosis of patients with acute CAD [31] and clinical diagnosis of heart failure can be detected by the rhBNP [32]. Kapoun et al. proved that rhBNP could prevent cardiac remodeling in pathological conditions of heart failure by inhibiting TGF-β-induced effects on cardiac fibroblasts [33]. Hypertension et al. proved that TGF-β1 activated the synthesis of myofibroblasts and increased the collagen production in rats cardiac fibroblasts [34] and the synthesis of collagen induced by TGF-β was mediated via the down-regulate cAMP [35]. Thus, type I and III collagen deposition might be induced by TGF-β1 and CTGF. The association between rhBNP and type I and III collagen in MI has not been fully discussed. However, rhBNP could suppress the serum TGF-β1 and CTGF levels in MI. Also, Magga et al. said that rhBNP was the antifibrotic factor in heart and degradation of type I collagen was regulated by rhBNP [36]. Based on our results, type I and III collagen level were high in MI rats while that was low in MI + rhBNP group, we speculated that rhBNP might prevent the MI progression by decreasing the expression of TGF-β1 and CTGF to inhibit the expression of type I and III collagen. Therefore, rhBNP may be a therapeutic drug for the improvement of MF in MI.

In conclusion, our findings suggest that the levels of TGF-β1, CTGF, CVF, and type I and III collagen are high in the MI rats. Treatment with rhBNP can better suppress the expression levels of TGF-β1, CTGF, as well as can improve the deposition of CVF, and type I and III collagen in MI, suggesting that rhBNP may be associated with the inhibition of MF in post-MI, which may be one of the mechanisms of rhBNP inhibition role in the MI therapeutics.

Disclosure of conflict of interest

None.

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References

[3] Soriano FG, Guido MC, Barbeiro HV, Caldini EG, Lorigados CB and Nogueira AC. Endotox-
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[34] Petrov VV, Fagard RH and Lijnen PJ. Stimulation of collagen production by transforming growth factor-β1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 2002; 39: 258-263.
