Effects of Parathyroid Hormone-Related Peptide Fragments on Neovascularization after Myocardial Infarction

Hao Zhang (✉ zhanghao1991cz@163.com)

The Affiliated Hospital of Yangzhou University

Guangwei Xia

The Affiliated Hospital of Yangzhou University

Yahong Qin

The Affiliated Hospital of Yangzhou University

Dan Wu

The Affiliated Hospital of Yangzhou University

Pei Zhao

The Affiliated Hospital of Yangzhou University

Yang Gao

The Affiliated Hospital of Yangzhou University

Research Article

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Abstract

Purpose Restoration of blood supply to infarction area plays an extremely important role in the prognosis of myocardial infarction (MI). Consequently, the effects of various fragments of parathyroid hormone-related peptide (PTHrP) on the peri-infarction neovascularization and cardiac function after MI were studied.

Methods Wild-type mice were used to construct a MI model. Together with the sham operation group (sham group), the mice were divided into four groups with 11 animals in each group. Four groups of mice were injected with saline (sham group and MI group), PTHrP (1-84) and PTHrP (87-139) subcutaneously into the abdomen at a dose of 80ug/kg, once a day for 28 days. After 4 weeks, cardiac color Doppler ultrasound examination was performed to evaluate the cardiac function, and the heart tissues of mice in each group were taken for immunohistochemical experiments.

Results Compared with the sham group, the heart function of the MI group mice was significantly decreased. After treatment with PTHrP (87-139), the heart function was significantly improved, while PTHrP (1-84) further reduced the heart function. Masson staining results showed that PTHrP (87-139) significantly reduced the peri-infarction myocardial fibrosis after MI, while PTHrP (1-84) aggravated the degree of myocardial fibrosis. In addition, PTHrP (87-139) significantly increased the peri-infarction microvessel density (MVD) after MI, and the expressions of various relevant vascular factors, while PTHrP (1-84) exerted the opposite effect.

Conclusion PTHrP (87-139) promoted angiogenesis in the peripheral area of MI and improved cardiac function, while PTHrP (1-84) exerted the opposite effect.

1. Introduction

As for ischemic diseases, effective restoration of blood supply exerts an extremely important part in the progression and prognosis of diseases¹. Interventional surgery and thrombolytic therapy are the two strategies commonly used to restore blood supply of myocardial infarction in clinical practice, while not suitable for all patients. At the same time, studies have shown that promotion of peri-infarction neovascularization, especially the establishment of good collateral circulation, also exerts an effective role in controlling the progression of such diseases². As a humoral factor, PTHrP has been proved to be closely related to the migration, proliferation and apoptosis of vascular smooth muscle cells (VSMCs), which are the premise and basis of neovascularization; meanwhile, PTHrP also dilates arteries and increases regional myocardial blood flow³. PTHrP is similar with the well-known parathyroid hormone (PTH) in spite of many differences. The similarity lies in the fact that the N-terminal amino acid sequences of PTHrP and PTH are homologous in spatial conformation, and both play a physiological role through the same PTH/PTHrP receptor. The difference lies in the fact that PTHrP is expressed in almost all system tissues involving the cardiovascular system, while the concentration of which in the circulation is very low, and PTHrP mainly exerts its physiological effects through autocrine and paracrine,

while PTH is mainly secreted and expressed by parathyroid glands and plays a role in the form of endocrine. Secondly, the physiological functions of PTHrP and PTH are not the same⁴. The PTHrP precursor forms four fragments after transcription, and each of which performs a unique function⁵. N-terminal (1-36) mainly plays the role of calcineurin. The middle section regulates the transportation of placental calcium. The nuclear localization sequence (NLS, 87-107) can be transported into the nucleus and has the function of promoting cell proliferation and inhibiting cell apoptosis. The C-terminal (108-139) regulates bone resorption, which is indispensable for the function of NLS⁶.

Previous studies have shown that PTHrP binds to the PTH/PTHrP1 type receptors and leads to the increase of intracellular cAMP, which in turn arrests cell development in the G1 phase, and thus significantly reducing the DNA synthesis and inhibiting the proliferation of VSMCs⁷. However, other experiments show that PTHrP promotes the proliferation of VSMCs and accelerates neovascularization due to the increase of the relative percentage of VSMCs in S phase and G2/M phase⁸. Obviously, the above experimental results are contradictory. However, it can be determined that PTHrP has a significant effect on the proliferation of VSMCs, while the specific mechanism is still unclear.

NLS exerts a pivotal role in the physiological effects of PTHrP. Consequently, two proteins including PTHrP (87-139) containing NLS and C-terminus, and PTHrP (1-84) without NLS or C-terminus, were prepared to study the influence on peri-infarction angiogenesis after myocardial infarction, which is possibly promising in the prevention and treatment of myocardial infarction in the future.

2. Materials And Methods

2.1 Animals and grouping

In current study, eight-week-old C57BL/6 wild-type male mice weighted about 25 g were selected to construct the myocardial infarction model by ligating the anterior descending branch of the heart. All mice were obtained from the SPF Experimental Animal Center of the Bone and Stem Cell Experimental Center of Nanjing Medical University Rearing. The policy was approved by the animal experiment ethics committee. Gene recombination method was used to prepare two kinds of protein including PTHrP (87-139) with nuclear localization sequence (NLS) and C-terminal, and PTHrP (1-84) without NLS or C-terminal.

The mice that finally survived were randomly divided into 4 groups, with 11 mice in each group. After opening the chest, the mice in the sham group were undergone needling of the anterior descending branch of the heart instead of ligation and on the next day injected with normal saline subcutaneously into the abdomen. The anterior descending branch of the mice in MI group was ligated to construct a myocardial infarction model, and normal saline was injected subcutaneously into the abdomen one day later. The mice in MI+ PTHrP (1-84) group were subjected to ligation of the anterior descending branch of the heart to construct a myocardial infarction model and subcutaneous injection of MI+PTHrP (1-84) into the abdomen 1 day later. The mice in the MI+PTHrP (87-139) group were subjected to ligation of the

anterior descending branch of the heart to construct a myocardial infarction model and subcutaneous injection of MI+PTHrP (87-139) into the abdomen 1 day later. All mice were injected with a dose of 80 ug/kg⁹ once a day for 4 weeks for the following experiments.

2.2 Experimentation of PTHrP (1-84) and PTHrP (87-139) on HUVEC

The HUVEC (Chinese Academy of Sciences, Beijing, China) were stored in Dulbecco's modified Eagle's medium (DMEM) , which is supplemented using 100 U/mL penicillin , 100 ug/mL streptomycin and 10% fetal bovine serum (FBS) in a cell incubator with 5% CO₂ and 95% O₂ at 37°C. In order to keep the medium fresh, it should be replaced at least once every two days. To study the effects of PTHrP (1-84) and PTHrP (87-139) on the survival of cells, HUVECs were cultured with the above two proteins (1×10^{-7} mmol/L) or bovine serum albumin (BSA) as control respectively for 24 hours and 48 hours, and then washed using phosphate-buffered saline (PBS) three times before following the subsequent assay.

Cell viability was detected by mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as described previously¹⁰. In general, 10-15uL of MTT solution was supplied to the cell supernatant before incubation (4 hours at 37°C). The medium was then discarded, and lysing cells with 2-propanol and solubilizing formazan. The absorbance of formazan was detected by a microplate reader at 570 nm (Bio-Rad Laboratories, Inc., Hercules, CA).

Under normal conditions, the absorbance of formazan produced by untreated cells was taken as 100%.

2.3 Determination of heart function

High-frequency ultrasound imaging system (Vevo 2100, Visualsonics, Canada) was used to measure the left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular ejection fraction (LVEF) and left ventricular short axis shortening rate (LVFS). Each parameter was measured for 3 times and the average value was recorded.

2.4 Histology and immunohistochemistry

Four weeks later, animals of each groups were anesthetized and then inoculated using 25 mL of PBS and 20 mL of phosphate-buffered formalin (PBF, 4%). After taken out, each hearts were first fixed in PBF for two days at 4°C; All samples were then embedded in paraffin. The heart tissues were cut into 5um sections. Immunostaining and Masson's trichrome staining (MTS) were performed. MTS was performed using a D026 Masson detection kit (Nanjing Bioengineering Institute, China) following the instructions provided by the manufacture. CD31 immunostaining (Abcam, Cambridge, England) in the granulation tissue at the border zone of MI was carried out, which is used to assess blood vessel density. The Vector ABC Vectastain Elite Kit (Vector Laboratories, Burlingame, CA) was used to visualize the brown colored reaction product, and the 3,3'-diaminobenzidine (DAB) substrate will be used. Stained vessels which are CD31 positive spot were detected in ten different fields per section under a light microscopy at x400 magnification (IPP 6.0, Media Cybernetics, Inc., Bethesda, MD).

2.5 Western-blot

Different kinds of proteins are extracted by different methods. The homogenized heart tissues and a standard method were used for total protein; Mem-PER™ Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo, MA) was used for membrane protein following the manufacturer's instructions. The bicinchoninic acid assay (BCA) Protein Assay Kit was used to detect protein concentration. The proteins were transferred electrophoretically to nitrocellulose membranes (Millipore, Billerica, MA) after loading onto an sodium dodecyl sulfate polyacrylamidegel electrophoresis (SDS-PAGE) gel. The blocking method was done using 5% nonfat milk in PBS/Tween 20 for two hours at 37°C. All membranes were incubated overnight with the following primary antibody of the recommended dilution at 4°C: GAPDH (Abcam, Cambridge, England), VEGF (Abcam, Cambridge, England), VEGFR2 (Abcam, Cambridge, England). The membranes were washed subsequently, and further incubated with an horseradish peroxidase (HRP)-conjugated secondary antibody at 37°C for 1 hour. chemiluminescence system (Tanon, Beijing, China) was used to visualize the immunoreactive bands and the quantification was used by densitometry.

2.6 Statistical analysis

SPSS 21.0 software was used for statistical analysis. The measurement resources were expressed as mean±standard deviation. Single-factor analysis of variance and t test were used for comparison between groups, and $P<0.05$ indicated that the difference was statistically significant.

3. Results

3.1 The effects of PTHrP fragments on mouse cardiac function after myocardial infarction

After 4 weeks, cardiac color Doppler ultrasound examination was conducted on the mice of all groups. As shown in table 1, compared with the Sham group, the cardiac function of the mice in the MI group was significantly decreased, and the heart volume was significantly increased. After treating the mice in the MI+ PTHrP(1-84) group with PTHrP(1-84), the cardiac function was further decreased and the heart volume was further increased compared with mice in the MI group. However, after treating the mice in the MI+PTHrP (87-139) group with PTHrP (87-139), the heart function was significantly improved and the cardiac volume was also decreased significantly compared with the mice in the MI group.

3.2 Detecting the effects of PTHrP (1-84) and PTHrP (87-139) on the proliferation of HUVEC with MTT experiment

The effects of PTHrP(1-84) and PTHrP(87-139) on cell survival, especially on cell proliferation was explored by incubating HUVECs with above two protein or BSA as control for two time nodes (24 hours and 48 hours). The MTT experiment confirmed that the PTHrP(87-139) treatment was beneficial to the survival and proliferation of HUVEC and this positive effect displayed a time-dependent manner, and PTHrP(1-84) has an opposite effect on HUVEC proliferation (Fig 1).

3.3 The effect of PTHrP (1-84) and PTHrP (87-139) on peri-infarction angiogenesis of wild-type mice

The role of PTHrP(1-84) and PTHrP(87-139) treatment on the levels of angiogenesis after MI was detected by counting capillary density per field through anti-CD31 immunohistochemistry staining 4 weeks after operation. The capillary density between MI group and sham group was not observed Significant difference. Compared with mice in the MI group which were treated with normal saline, the MI mice treated with PTHrP (1-84) exhibited significantly decreased peri-infarction blood vessel density, while that of the MI mice treated with PTHrP (87-139) were increased significantly (Fig 2).

3.4 The effect of PTHrP(1-84) and PTHrP(87-139) on relevant angiogenic factors in the peri-infarction area of wild-type mice

Four weeks after the surgery, the proteins in the peripheral regions of myocardial infarction of mice in the four groups were extracted for Western-blot experiment. The results showed that the expression levels of VEGF and VEGFR-2 in the MI+saline group were significantly lower than those in the sham group. After treatment with PTHrP(1-84), the expression levels of the two angiogenic factors were further decreased compared with the MI+saline group. After treatment with PTHrP (87-139) protein, the expression levels of the two angiogenic factors were significantly higher than those in the MI+saline group (Fig 3).

3.5 The effect of PTHrP (1-84) and PTHrP (87-139) on peri-infarction myocardial fibrosis of wild-type mice

Four weeks after the surgery, the degree of myocardial fibrosis in the peripheral area of myocardial infarction was detected by MTS and expressed as the percentage of fibrosis area. The results showed that the degree of peri-infarction myocardial fibrosis of the MI mice was significantly higher than that of the Sham mice (MI vs Sham: $15.80 \pm 1.35\%$ vs $1.78 \pm 0.58\%$) (Fig 4). After treatment with PTHrP(1-84), the degree of peri-infarction fibrosis was further aggravated (MI+PTHrP(1-84) vs MI: $27.45 \pm 2.16\%$ vs $15.80 \pm 1.35\%$), and after the treatment with PTHrP(87-139), the degree of peri-infarction myocardial fibrosis was significantly decreased (MI+PTHrP(87-139) vs MI: $6.46 \pm 0.76\%$ vs $15.80 \pm 1.35\%$) (Fig 4).

4. Discussions

This study was mainly conducted to explore the effects of different fragments of PTHrP, especially NLS, on mouse cardiac function after myocardial infarction. It was shown that: (1) PTHrP (87-139) containing NLS significantly improved mouse cardiac function after myocardial infarction, while PTHrP (1-84) without NLS exhibited the opposite effect; (2) PTHrP (87-139) promoted peri-infarction angiogenesis and reduced myocardial fibrosis, while PTHrP(1-84) inhibited peri-infarction angiogenesis and aggravated myocardial fibrosis.

The importance of NLS to PTHrP's physiological role involving cell proliferation, apoptosis, differentiation and transformation has been increasingly accepted.

Henderson et al. initially conducted the researches on NLS in 1995¹¹. They transfected chondrocyte CFK2 cells which normally didn't express PTHrP with two kinds of plasmids (one contained the whole-length

sequence of PTHrP, and the other contained PTHrP sequence without NLS). In the absence of serum culture, the CFK2 cells without NLS largely died, while those expressed NLS obviously tolerated the condition, indicating that NLS exerted an important role in anti-apoptosis of cells. Similar conclusions were also confirmed by Bhatia et al. in the experiment of colon cancer cell line LoVo in 2009¹². The effect of PTHrP promoting the proliferation of VSMCs was found to be relevant to the translocation of PTHrP into nucleus, which was dominated by NLS¹³. However, the mechanism by which NLS regulates the proliferation of VSMCs is still unclear.

Vascular endothelial growth factor (VEGF) is one of the most widely used vascular growth factors, which is closely related to angiogenic activity¹⁴. VEGF stimulates the formation of immature and disordered vascular networks, which indicates that VEGF is able to gradually regulate the formation of stable and functional microvascular networks to restore the blood supply of different tissues, especially ischemic tissues¹⁵. The results showed that PTHrP (87-139) not only promoted the expression of VEGF in the peripheral area of myocardial infarction, but also promoted the expression of its receptor VEGFR-2. The immunohistochemical results also showed that the expressions of CD31 and other representative indicators of blood vessel density were significantly increased. MTT experiments also confirmed that PTHrP (87-139) promoted the proliferation of HUVECs, suggesting that PTHrP (87-139) promoted the formation of new blood vessels around myocardial infarction, which is very important for the recovery of effective blood supply to the myocardium. However, PTHrP(1-84) exerted the opposite effect. Our work also explained the contradictions in previous studies on the effect of PTHrP on cell proliferation to a certain extent, which was mainly determined by the existence of NLS.

It has also been confirmed by in vivo experiments that PTHrP lack of NLS and C-terminal inhibits the cell cycle process of VSMCs and the formation of vascular intima¹⁶. At the same time, studies have also shown that mice with NLS and C-terminal being knocked out exhibits poor vascular development, and the blood vessel walls of which are thin and easy to rupture¹⁷. All these work have similarities with our results. In addition, our research also showed that PTHrP (87-139) significantly reduced the degree of myocardial fibrosis in the peripheral area after myocardial infarction, and improved the cardiac function of mice, while PTHrP (1-84) exerted a significantly aggravating effect on cardiac function after myocardial infarction, which was possibly due to whether the blood supply was effectively improved.

However, the mechanism of PTHrP regulating cell proliferation and angiogenesis remained still unclear, which was suspected to be related to the P27 gene. P27 gene has been confirmed as an inhibitor of various cells including VSMCs^{18,19}, and multiple studies have shown that PTHrP exerts its physiological effects by regulating the P27 gene¹⁸⁻²⁰. In the future, we will also explore the mechanism of P27 gene by construction of P27 knockout mice.

In spite of vast attempts made previously, treating ischemic diseases by promoting angiogenesis has not been successfully applied to the clinic²¹. The concept of angiogenesis was first proposed by Folkman in 1971, involving complex biological processes, including endothelial cell activation, migration, basement

membrane degradation, and vascular lumen formation, which also requires the activation and participation of various cells. The current work is based on this, and will be verified through relevant experiments, which will provide a new reference for the treatment of ischemic diseases in the future.

5. Conclusions

This study is essential to the understanding of physiological functions of PTHrP, especially NLS. Meanwhile, the effects of PTHrP fragments on related ischemic diseases, especially myocardial infarction are revealed, which possibly provide another choice for patients with myocardial infarction, especially for those who are inappropriate for interventional treatment.

6. Declarations

Not applicable

7. Funding

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8. Conflicts of interest/Competing interests

All authors declare no conflicts of interest.

9. Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

10. Code availability

Not applicable

11. Authors' contributions

HZ and YG designed the study. HZ, GX and YG drafted the manuscript; HZ, GX, DW, YQ, and PZ conduct experiments and collect data; HZ and YG are responsible for statistics and data analysis of data. The final manuscript was approved by all authors.

12. Ethics approval

All the research was approved by the institutional review board of Ethical ommittee of Yangzhou University.

13. Consent to participate

The administrative permissions were acquired by our team to access the data we used in our research.

14. Consent for publication

All participants in this study had no objection to the publication of the article.

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Table

Table 1: The effects of PTHrP fragments on mouse cardiac function after myocardial infarction

The values are means $\pm$ SD. LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVIDd, left ventricular internal diastolic diameter; LVIDs, left ventricular internal systolic diameter; MI, myocardial infarction; PTHrP, parathyroid hormone-related peptide. * $P < 0.05$ vs sham group. # $p < 0.05$ vs MI group & $P < 0.05$ vs MI+PTHrP(1-84) group. n = 11 per group.

	Sham	MI	MI+PTHrP(1-84)	MI+PTHrP(87-139)
LVEF, %	63.66 ±1.79	38.01 ±1.27 [*]	29.02±2.28 [#]	53.33± 4.33 ^{#&}
LVFS, %	32.98 ± 1.11	17.55 ± 1.27 [*]	11.25±1.98 [#]	26.53 ± 2.68 ^{#&}
LVIDd,mm	3.80 ±0.13	4.32 ± 0.49 [*]	5.26±0.45 [#]	3.76 ± 0.09 ^{#&}
LVIDs, mm	2.63 ± 0.09	3.58 ± 0.58 [*]	4.29±0.35 [#]	2.84 ± 0.16 ^{#&}

Figures

Figure 1

Detecting the effects of PTHrP (1-84) and PTHrP (87-139) on the proliferation of HUVEC with MTT experiment Cells were treated with PTHrP (1-84) and PTHrP (87-139) for 24 hours and 48 hours then analyzed for viability by the MTT assay. Data are shown as mean ± SD. The results are representative of three independent experiments. Compared with control group, *P < 0.05 and **P < 0.01. HUVECs, human umbilical vein endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PTHrP, parathyroid hormone-related peptide.

Figure 2

The effect of PTHrP (1-84) and PTHrP (87-139) on peri-infarction angiogenesis of wild-type mice A, Representative digital micrographs revealing capillary density/CD31 immunostaining 28 days after surgical intervention in four groups, (A) Sham group; (B) MI group; (C) MI+PTHrP(1-84) group; (D) PTHrP(87-139) group. Magnification: $\times 400$. B, Quantitative analysis of capillary density in counts per field. The values are means $\pm$ SD. * $p < 0.05$ vs Sham group, # $p < 0.05$ vs MI group, & $p < 0.05$ vs PTHrP(1-84) group. $n = 6$ per group. MI, myocardial infarction; PTHrP, parathyroid hormone-related peptide.

Figure 3

The effect of PTHrP(1-84) and PTHrP(87-139) on relevant angiogenic factors in the peri-infarction area of wild-type mice A, Western blot assay of VEGF and VEGFR-2 in the border zone of the three groups of

mice. B, Quantitative data of the expression of VEGF in the three groups of mice. C, Quantitative data of the expression of VEGFR-2 in the three groups of mice. The values are means $\pm$ SD. * p < 0.05 vs Sham group, # p < 0.05 vs MI group, & p < 0.05 vs PTHrP(1-84) group. n = 6 per group.

Figure 4

The effect of PTHrP (1-84) and PTHrP (87-139) on peri-infarction myocardial fibrosis of wild-type mice A, Representative images of Masson's trichrome-stained heart sections in four groups of mice 28 days after operation; (A)Sham group; (B) MI group; (C)MI+PTHrP(1-84) group; (D)PTHrP(87-139) group. B, Quantitative analysis of myocardial interstitial fibrosis.The values are means $\pm$ SD. * p < 0.05 vs Sham group, # p < 0.05 vs MI group, & p < 0.05 vs PTHrP(1-84) group. n = 6 per group.