Original Paper

NPY and CGRP Inhibitor Influence on ERK Pathway and Macrophage Aggregation during Fracture Healing

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Key Words

Fracture Healing • NPY • CGRP • ERK • Macrophage

Abstract

Aim: The aims of this study are to investigate the effects of neurotransmitters NPY and CGRP on ERK signaling in fracture healing, and to identify the correlation between macrophage aggregation and fracture healing. **Methods:** Male Sprague-Dawley rats were used to build a fracture model. The neurotransmitter receptor inhibitors were injected intraperitoneally into the rats. Immunofluorescence staining and ELISA were employed to determine the expression of NPY and CGRP in fracture area and the peripheral blood, respectively. Micro-CT together with histological staining were utilized to assess the fracture healing conditions. Relative protein expression was determined using western blot. Immunofluorescence staining was used to detect the aggregation of macrophages in the injury area. **Results:** During fracture healing, the serum NPY and CGRP significantly increased. The levels of NPY and CGRP reached a peak in the 8th week and reduced significantly thereafter. NPY and CGRP inhibitors could inhibit fracture healing and down-regulate the phosphorylated ERK. Macrophages (NPY+ and CGRP+) aggregated in the injury area. **Conclusion:** NPY and CGRP participated in fracture healing, in which they were also shown to influence phosphorylated ERK expression. In addition, macrophages are involved in the fracture healing process.

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Introduction

Bones can repair or regenerate in response to a significant injury. The repair process restores the injury tissues while the regeneration process forms new tissues and increases the skeletal tissue volume [1]. Fracture healing is a complex process involving the interplay of various types of cells, cytokines, growth factors and extracellular environment [2-4]. The inflammatory response after injury also plays a significant role in bone healing by regulating endochondral ossification and bone remodeling [5]. Several investigations identifying the

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diverse regulatory molecules during fracture recovery have been conducted, among which the neurotransmitters were of our particular interest and have been considered as critical regulators in fracture healing.

Neuropeptide Y (NPY), a 36-amino-acid peptide widely extant in the autonomic nervous system, is believed to play a vital role in osteogenesis and bone remodeling [6, 7]. NPY functioning is mediated by the activating of 5 distinct G-protein coupled receptors (Y1, Y2, Y4, Y5 and Y6) and participates in various physiological processes [4, 8]. Among the receptors, Y1 and Y2 have been identified for their specific influence on bone formation in mice [6]. Baldock et al. have investigated the role of Y2 receptor in bone formation, and they found that mice with hypothalamic Y2 receptors deletion demonstrated intensified osteoblast activities and accelerated bone formation [9]. In 2005, they again showed that both Y2 receptor and serum leptin influenced osteoblast activity, which is responsible for bone development [10]. Y2 receptor is thus regarded as an important agent in bone healing therapy. Later in 2007, the same team discovered that Y1 receptor signaling regulated skeletal homeostasis, which potentially inhibited bone formation via a single pathway with Y2 receptors [11]. Consistent with previous studies, Lee et al. have recently illustrated that NPY system inhibited osteoblasts' activities via Y1 receptor signaling by suppressing the development and mineral-producing of osteoblastic cells [6]. All existing evidence suggests that the NPY system can inhibit bone formation.

The effects of the nervous system on bone metabolism have been the focus of many studies [12]. Calcitonin gene-related peptide (CGRP), a neuropeptide consisting of 37 amino acids, has been identified as a potential transmitter that may influence bone homeostasis [12]. Li et al. found that CGRP had a positive effect on mineralization and remodeling of bone by regulating NOS [13]. Zhang et al. claimed that CGRP participated in bone metabolism and promoted swifter fracture healing [14]. Moreover, in a recent study, Tian et al. confirmed that CGRP promoted osteogenic differentiation as well as proliferation [15]. Despite various proposed mechanisms, it is certain that CGRP is a pro-osteogenic neurotransmitter. Still, more needs to be done to fully explore the mechanism by which CGRP influences osteogenesis.

Another notable factor during fracture repair is the role of macrophages, a type of white blood cell and a member of the adaptive immune system [16]. It has been reported that macrophages can convert to a pro-healing phenotype and promote limb generation [17]. Macrophages could also help maintain bone homeostasis and promote fracture healing by enhancing the differentiation process of mesenchymal progenitors [2]. Herein we inferred that macrophage aggregation would likely present in the femoral fracture area.

Considering the sophisticated role of neuropeptides during fracture healing, we designed the present study to explore the effects of NPY and CGRP on bone healing. We endeavoured to explore the mechanism by which NPY and CGRP influence fracture healing and investigate the correlation between macrophages and bone healing. We intended to provide stronger theoretical basis for further study of fracture healing.

Materials and Methods

Animals

Thirty male Sprague-Dawley rats (aged 6-8 months, weight 336 ± 10 g) (purchased from the Laboratory Animal Center of the Fourth Military Medical University, Xi'an, China) were used in the experiments. The rats were housed under controlled conditions, while food and water were given *ad libitum*. All experiments were approved by the Animal Ethics Committee of Xijing Hospital, the Fourth Military Medical University. The study complied with relevant guidelines and regulations.

Surgical techniques

The fracture surgery method was based on one described in a previous study [18]. Before the surgery started, intramedullary fixation was performed on bilateral femurs of rats. Firstly, anesthesia was induced using 1% pentobarbital sodium (3mg/kg, Sigma-Aldrich, St. Louis, MO, USA) via intraperitoneal injection.

Small transverse incisions were made on the lateral side of bilateral knee joints to expose knees and patella. Fossa intercondylar femora were then exposed and a Kirschner rod (1.5 mm-diameters) was used to drill a hole on the femurs. A Kirschner rod with appropriate size was inserted into the medullary canal of the bilateral femurs of the rats. We then sewed the wound (muscle, fascia and skin) using 4-0 nylon suture (Ethicon Inc., Somerville, NJ, USA). Finally, under general anesthesia, a femoral fracture was created by hitting the middle of femurs with an impactor. The femoral fracture was confirmed by radiography using an X-ray machine (MinXray, Inc., Northbrook, IL, USA). All surgical procedures were done under sterile conditions by an experienced veterinarian.

Drugs and grouping

All drugs were dissolved in 10 μ L saline and injected intraperitoneally into anesthetized rats. NPY Y1 receptor antagonists (BIBP3226, 20 μ g, MedChem Express, Monmouth Junction, NJ, USA) or CGRP receptor antagonists (BIBN4096BS, 20 μ g, MedChem Express, Monmouth Junction, NJ, USA) were injected every two days, for a duration of two weeks.

The rats were randomly divided into five groups (6 rats per group): sham surgery group (blank control), model group (fracture rat model), negative control group (intraperitoneal injection of saline into fracture rat model), NPY inhibitors group (intraperitoneal injection of BIBP3226 into fracture rat model), and CGRP inhibitors group (intraperitoneal injection of BIBP3226 into fracture rat model).

Bone micro-CT analysis

High-resolution micro-CT analyses were performed on the femurs using a SkyScan 1174 compact micro-CT scanner (SkyScan, Aartselaar, Belgium). The femurs were imaged using an X-ray tube voltage of 49Kv and a tube current of 795 μ A. SkyScan was employed to calculate the callus tissue volume, callus bone volume and callus BV/TV after scans.

Histological analysis

The fractured femurs were fixed in 4% formaldehyde, decalcified in 10% EDTA, and embedded in paraffin. The embedded samples were sliced into sections (5 μ m in thickness). The sections were subjected to Safranin-O staining or immunohistochemical staining.

For immunohistochemical (IHC) staining, the sectioned tissues were incubated with Phosphate Buffer Saline (PBS) containing 10% goat serum for 1h, followed by incubation with primary antibodies (For the single staining, rabbit anti-rat NPY1R (5 μ g/ml) or rabbit anti-rat CGRP (5 μ g/ml) primary antibodies were incubated with the sections overnight. For double staining, rabbit anti-rat NPY1R or CGRP together with rabbit anti-rat F4/80 (1:100) primary antibodies were incubated with sections overnight. On the following day, the sections were incubated with secondary antibodies (single staining, goat anti-rabbit FITC 1:1000) secondary antibodies were incubated with the sections. For double staining, goat anti-rabbit TRITC (1:1000) secondary antibodies were incubated with sections). Images were captured using confocal microscopy (Olympus Fluo View FV-1000, JAPAN). All antibodies were purchased from ABCAM (Cambridge, MA, USA).

Scanning electron microscopy (SEM) analysis

For all rat groups, samples were collected after infusion and immobilization with 2.5% glutaraldehyde. Gradient dehydration was then performed. Scanning Electron Microscopy (HITACHI S-3400, JAPAN) was used to observe the repair and regeneration of fracture sites.

Enzyme-linked immunosorbent assays (ELISA)

To determine the protein levels of NPY and CGRP, ELISA was performed on the cardiac blood sample supernatants using Immuno Assay System kits (AMEKO, USA). A microplate reader (Tecan Infinite M200, Austria) was used to measure the optical density of the supernatants at 450 nm.

Western blot

Samples were collected and lysed in RIPA lysis buffer. The insoluble material was sedimented by centrifugation (12,000g, 10min, 4°C), while the supernatants were collected and stored at -80°C. Bradford Protein Assay Kit (Pierce, USA) was used to determine the protein concentration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the proteins. The proteins were

then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked and incubated with the primary antibodies (goat anti-human, 1:800) overnight at 4°C, following by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit anti-goat, 1:2000) for 1 hour at 37°C. The protein bands were observed with ECL Plus chemiluminescence reagent and the Lab Works 4.5 software. GAPDH was used as the internal loading control.

Statistical analysis

Statistical analyses were carried out using the Statistical Program for Social Sciences (SPSS). Numerical data were expressed as the mean ± standard deviation (SD). Student's *t*-test and one-way ANOVA were performed between and among groups, respectively. $P < 0.05$ was considered statistically significant.

Results

Expression of NPY and CGRP in the fracture repair process

The NYP and CGRP levels in the fracture area 4, 8 and 12 weeks after the fracture surgery were determined using IHC. The expression levels of NYP and CGRP were significantly elevated and reached a peak in the 8th week ($P < 0.05$) in the model group compared with the sham surgery group. In the 12th week after surgery, the expression level of NYP and CGRP were lower than that in the 8th week after surgery but still higher than that in the 4th week after surgery ($P < 0.05$) (Fig. 1).

The NYP and CGRP protein levels in the peripheral blood were measured using ELISA. Compared with the sham surgery group, the expression levels of NYP and CGRP in the model group rats rose constantly as time went by, and significant differences were observed at each time point ($P < 0.05$). The expression levels of NYP and CGRP in the 12th week after surgery

Fig. 1. Immunohistochemistry staining was used to detect NPY (A) and CGRP (B) expression in the fracture area. Data were presented as mean ± SD (repeated 5 times). *Compared with Sham group, $P < 0.05$. #Compared with 4w group, $P < 0.05$. °Compared with 8w group, $P < 0.05$. Scale bars: 150 μm. Giemsa staining images on the top indicates where we were looking at the callus (scale bars: 500 μm).

Fig. 2. ELISA was used to detect levels of NPY (A) and CGRP (B) in peripheral blood during the fracture repair process. Data were presented as mean $\pm$ SD (repeated 5 times). *Compared with Sham group, $P < 0.05$. #Compared with 4w group, $P < 0.05$. °Compared with 8w group, $P < 0.05$.

Fig. 3. Micro-CT was used to analyze the callus tissue volume, callus bone volume and callus BV/TV. Callus tissue volume (A), callus bone volume (B) and callus BV/TV (C) in the fracture area during the repair process. *Compared with 4w group, $P < 0.05$. #Compared with 8w group, $P < 0.05$. The callus tissue volume (C), callus bone volume (D) and callus BV/TV (E) of different groups 4 weeks after fracture surgery. Data were presented as means $\pm$ SD (repeated 5 times). *Compared with model group, $P < 0.05$. #Compared with NC group, $P < 0.05$.

were higher than that in the 8th week post-surgery ($P < 0.05$). Similarly, the expression levels of NYP and CGRP in the 8th week after the surgery were substantially higher than that in the 4th week post-surgery ($P < 0.05$) (Fig. 2).

NPY and CGRP affect the development of callus in the fracture repair process

Micro-CT was used to detect the callus development in the fracture area during the 4th, 8th and 12th week after surgery.

In the model group on the 4th week callus tissue and callus bone volumes were easily visible in the fracture area. The callus tissue volume and callus bone volume reduced in the 8th and 12th weeks, compared with those in the 4th week after surgery ($P < 0.05$). Callus BV/TV in the 8th and 12th week was significant lower than that in the 4th week as well ($P < 0.05$). A significant difference of callus BV/TV was also observed between the 8th and 12th week ($P > 0.05$) (Fig. 3A-C).

Fig. 4. Histological analysis was adopted to analyze the development of callus area 4 weeks after surgery. (A) Staining results of the four experimental groups (bone tissues were stained in green and cartilage tissues were stained in red). (B-C) Statistical analysis of callus tissue areas (B) and cartilage tissue areas (C). Data were presented as mean $\pm$ SD (repeated 5 times). *Compared with model group, $P < 0.05$. #Compared with NC group, $P < 0.05$. Scale bars: 500 μ m.

No significant differences in callus tissue volume, callus bone volume or callus BV/TV were observed between the model group and the control group ($P > 0.05$). Compared with the model group and the control group, rats in the NPY inhibitors group and CGRP inhibitors group showed significant decrease in callus tissue volume, callus bone volume and callus BV/TV ($P < 0.05$) (Fig. 3D-F).

Aside from this, histological analysis showed that 4 weeks after the surgery, the total callus area of rats in NPY inhibitors group and CGRP inhibitors group were both significantly lower than the model group and the control group, whereas the cartilage areas were significant higher ($P < 0.05$, Fig. 4).

NPY and CGRP affect the activation of ERK pathway in the fracture repair process

Western blot was used to detect the levels of ERK1/2 and p-ERK1/2 in the fracture area. The expression of ERK1/2 in experimental groups (model, NC, NPY inhibitors, CGRP inhibitors groups) showed no significant difference compared with the sham surgery group ($P > 0.05$, Fig. 5).

In terms of the p-ERK1/2 expressions, there was no significant difference between the model and the NC groups, however, rats in the model and NC groups showed substantially higher p-ERK1/2 levels than those in the sham surgery group ($P < 0.05$). The level of p-ERK1/2 in rats in the NPY inhibitors group and CGRP inhibitors group were significantly lower compared with the model group ($P < 0.05$).

Macrophages aggregate in fracture area

SEM results showed that 8 weeks after the surgery, spherical cells of various sizes were proliferating and aggregating in the fracture area (Fig. 6).

Fig. 5. Western blot was used to detect expression of ERK1/2 and p-ERK1/2 in fracture area 4 weeks after the surgery. Data were presented as mean $\pm$ SD (repeated 5 times). *Compared with sham group, $P < 0.05$. #Compared with model group, $P < 0.05$. °Compared with NC group, $P < 0.05$.

Fig. 6. Scanning electron microscope (SEM) was used to detect the presence of macrophages in fracture areas. (A) One field of fracture area with different magnifications. (B) Another field of fracture area with different magnifications. (C) Different fields of fracture area with the same magnification. The scale bars are shown on the images. The regularly global grey cells are macrophages.

The single and double immunohistochemistry staining results showed that a huge amount of F4/80-expression positive cells were present in the fracture area (Fig. 7), indicating the presence of macrophages in the fracture area. The counterstaining of NPY/

Fig. 7. Immunohistochemistry staining was used to detect macrophages in the 4th, 8th, and 12th week after surgery in the fracture area (marker protein F4/80 was stained in red). The HE staining image (scale bar: 500 μ m) on the very top of Fig. 7 identified where in the callus we were observing. NPY and CGRP were stained in green at the same time. Scale bars: 150 μ m.

CGRP and F4/80 showed that macrophages were NPY- and CGRP-expression positive, and the number of NPY/CGRP-expression positive macrophages reached a peak in the 8th week and had reduced in the 12th week.

Discussion

To discover the effects of CGRP and NPY during fracture healing, we firstly studied their expression during bone repair and regeneration process in adult SD rats, finding high concentrations of CGRP and NPY in the fracture regions. Previous studies have supported this conclusion regarding the effects of both CGRP and NPY on bone healing. For instance, Li et al. found a positive effect of CGRP on bone mineralization and remodeling, while Lau et al. found that high density of CGRP-positive fibers were widely distributed in bone formation areas during fracture healing [13, 19]. Similarly, Long et al. demonstrated that NPY increased during the early and late phases of fracture healing and Ladock et al. suggested NPY as an important factor in bone regulation and energy homeostasis [7, 11]. Notably, Song et al. discovered that the fracture-healing process was accelerated in traumatic brain injury (TBI) patients and based on the evidence he declared that the overexpression of CGRP originated from TBI. According to them, CGRP was secreted from the injured brain and was transported to the fracture region where it accelerated bone healing. [20]. This finding shed some light upon the process by which CGRP influences fracture recovery. Consistently, another

research study had a similar result when a bone fracture combined with cerebral cortex injury demonstrated a faster healing process [14]. Although in our study we did not deeply investigate the relationship between brain injury and fracture healing, we discerned the high levels of NPY and CGRP during healing process in the injured areas, indicating a potential relationship between these two types of neurotransmitters and fracture healing. Not surprisingly, the two kinds of transmitters were also found increased in the rats' sera during the healing process, which again confirmed that NPY and CGRP were systematically involved in fracture healing. Interestingly, the level of the two peptides in the fracture region came to a peak in the eighth week after the surgery and slightly decreased thereafter, while they kept increasing until the twelfth week in sera. This discrepancy might be due to a delayed effect, i.e. while in fracture area the neurotransmitters dispensed rapidly 4 weeks after the fracturing, those in the peripheral blood dispensed very slowly. Nonetheless, both the IHC staining and the ELISA analyses demonstrated that NPY and CGRP were overexpressed in the model rats group.

The micro-CT scanning results demonstrated that the callus tissue volume, callus bone volume and the BV/TV ratio significantly increased immediately after the fracture and then gradually decreased over the next few weeks. Fracture healing is a complex process and generally divided into four stages: inflammation, soft callus (fibrocartilage) formation, hard callus formation and bone remodeling [21]. The inflammation process accounts for the broken tissue initially repairing while the fracture is bridged by both soft and hard calluses. The hard callus eventually remodels the broken bone tissue to re-establish both its structure and function [3]. Rats treated with NPY and CGRP inhibitors showed a remarkable reduction in callus bone and tissue volumes as well as BV/TV compared with the model and negative control groups in week 4, indicating a delay of bone healing. Sousa et al. found that the lack of the entire coding region of NPY in rats hindered the rate of bone healing, as seen in a decrease of bone callus volume and strength [4]. The result from their research showed a significant decrease in callus bone as well as callus tissue in NPY inhibition group compared with the wild type group in weeks 3 and 6. Likewise, the histological analysis of our study suggested a decrease of total callus area in peptides-inhibited group as well as an increase of cartilage area. These results illustrated that the deletion of NPY hindered the early stage of fracture healing, during which the cartilage callus removal was delayed and the formation of hard callus was suppressed [4].

Ras/extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway regulates the differentiation as well as cytoskeletal function of several cell lines including cells that are pivotal to bone formation and remodeling [22, 23]. We herein investigated the effects of NPY and CGRP on ERK signaling, in an effort to elaborate upon the possible molecular mechanism through which the neurotransmitters regulate fracture repair. p-ERK1/2 was substantially overexpressed in the model and NC groups compared with the sham surgery group. We also found an under-expression of p-ERK1/2 in the neurotransmitter inhibitors groups compared with the model group, indicating a possible pro-healing role of ERK signaling during fracture healing and an inhibitory role of CGRP and NPY on ERK pathway. Sharma et al. previously showed that the activation of ERK could enhance the osteoblast progenitor differentiation [23]. We thus believe that GCRP and NPY can activate ERK signaling, which then promotes fracture healing.

In addition, macrophages were detected in fracture regions with positive NPY and CGRP expression. Macrophages have been reported to play a functional role during bone development and regeneration previously [2]. In our study, the macrophage white blood cells were found aggregated in the fracture area from the 4th week to the 8th week but dispersed after this period. Given the validated effects of NPY and CGRP in bone healing, we suspect that there may be a relationship between the neurotransmitters and macrophage aggregation during the fracture repair process and that together they participate in this healing.

We herein propose that the neurotransmitters NPY and CGRP are correlated with ERK signaling during fracture healing, and that macrophages aggregate in fracture areas which may also play a role in the healing process.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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