

# **$\beta$ -Endorphin attenuates collagen-induced arthritis partially by inhibiting peripheral pro-inflammatory mediators**

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**Abstract.** The classical analgesic pathway of opioids by binding their receptors in the nervous system is well known. However, little is known regarding opioid analgesia through the anti-inflammatory pathway. The present study aimed to investigate the analgesic and anti-inflammatory effect of  $\beta$ -endorphin on inflammatory pain. A rat model of collagen-induced arthritis (CIA) was generated by intradermal injection of bovine type II collagen. Rats were divided into the CIA + saline group and the CIA +  $\beta$ -endorphin group, in which rats were intraperitoneally injected with  $\beta$ -endorphin once every other day from day 18 following the injection of CII until day 28. Thermal hyperalgesia as determined by tail flick latency (TFL), as well as paw arthritis index and swelling. Tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6 mRNA expression in synovial tissue and their protein levels in paw inflammatory tissue were measured. The rat CIA model was successfully induced as indicated by the significantly decreased TFL, increased paw arthritis index and percentage of swelling on day 18.  $\beta$ -endorphin treatment significantly increased the TFL, while decreasing the paw arthritis index and swelling in CIA rats. It also significantly downregulated TNF- $\alpha$  and IL-1 $\beta$  mRNA expression in synovial tissue and their protein levels in inflammatory tissue of the paws of CIA rats, while it had no significant effect on the levels of IL-6. These results indicated that  $\beta$ -endorphin suppresses peripheral pro-inflammatory mediators in collagen-induced arthritis, which may contribute to its analgesic effect.

## **Introduction**

The analgesic effect of opioids has been well demonstrated in animal behavioral studies as well as human clinical trials (1,2). Opioids are potent analgesics, which exert their pharmacological effects by binding and activating specific receptors in the nervous system (3,4). Accumulating evidence has demonstrated that opioids also exert an immunomodulatory function (5). Under inflammatory conditions, local inflammatory cells, including monocytes, granulocytes, lymphocytes and macrophages, synthesize and release opioids (6). Opioids also bind to receptors on immune cells to regulate inflammatory cytokines, which may constitute another mechanism of opioid-induced analgesia (7).

Rheumatoid arthritis (RA) is a chronic, systemic type of autoimmune inflammatory arthritis, which may lead to severe joint pain, as well as destruction of cartilage and bone (8). A large number of cytokines, including interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$  are active in the joints of patients with RA. These cytokines have a fundamental role in RA pathology, causing inflammation, pain and articular destruction (9). Previous studies have indicated that injection of opioids into knee joints after surgery caused prolonged post-operative analgesia in patients (10-12), which may be associated with their immunomodulatory function. Based on these results, the present study hypothesized that opioids also produce analgesic effects on RA via regulating peripheral inflammation.

In the present study, a rat model of collagen-induced arthritis (CIA) was generated. The effects of  $\beta$ -endorphin on pain and inflammation in CIA rats were assessed. The mRNA levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in synovial tissue and their protein levels in inflamed tissue of the paws were also measured in order to determine whether the analgesic effect of  $\beta$ -endorphin on RA is associated with its anti-inflammatory activity.

## **Materials and methods**

**Animals.** A total of 30 male Wistar rats (weight, 100-120 g; age, 4 weeks), were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Rats were housed in temperature-controlled animal

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cages at  $25\pm 1^{\circ}\text{C}$  with a relative humidity of  $55\pm 5\%$ , under a standard 12-h light/dark cycle (8:00 a.m.-8:00 p.m.), with free access to food and water. All experiments were performed between 8:00 a.m. and 4:00 p.m. All animals were treated in accordance with the regulations of the State Science and Technology Commission of China for the care and use of laboratory animals (State Science and Technology Commission order no. 2, 1988), and the protocols were approved by the Ethics Committee of Zhejiang Chinese Medical University (Hangzhou, China).

**Induction of arthritis with collagen.** Bovine type II collagen (CII; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved overnight in 0.1 mol/l acetic acid (2 mg/ml) at  $4^{\circ}\text{C}$  and emulsified with incomplete Freund's adjuvant (IFA; Sigma-Aldrich; Merck KGaA) to a final concentration of 1 mg/ml. Rats were anesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneal) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and placed on a heated small animal operating table (Harvard Apparatus, Cambridge, USA) in a prone position. No rats exhibited signs of peritonitis. Bovine CII (2 mg/kg) was intradermally injected into six sites at the base of the tail and back of the rats.

**$\beta$ -Endorphin treatment.** In the experiment, a CIA model was successfully established in total of 22 rats. These 22 CIA rats were randomly divided into the CIA + saline group and CIA +  $\beta$ -endorphin group ( $n=11$  per group).  $\beta$ -Endorphin (Bachem, Bubendorf, Switzerland) was dissolved in saline at a concentration of 5 nmol/ml. Rats were intraperitoneally injected with 1 ml  $\beta$ -endorphin once every other day from day 18 after the injection of CII until day 28. The rats in the CIA + saline group were intraperitoneally injected with 1 ml saline.

**Tail flick latency (TFL) test.** The TFL was assessed to determine thermal hyperalgesia using a tail-flick apparatus (Ugo Basile, Comerio, Italy). Rats were immobilized except for free tail movement. Heat from an infrared source was administered to the tail with a radiation intensity of  $30\text{ mW/cm}^2$ . The TFL was defined as the time of heat exposure until withdrawal of the tail, which was recorded by a single blinded observer. In order to avoid tissue damage, a cut-off time of 10 sec was implemented, with this duration being defined as representing the maximum analgesic effect. The average TFL was obtained from three consecutive trials with an interval of 3 min performed on 1/3 of the tail at the distal end.

**Arthritis evaluation.** Paw arthritic signs characterized by edema and erythema were inspected daily following the CII/IFA injection. To evaluate the incidence and severity of arthritis, lesions on all four paws of each rat were graded by using an arthritic scoring system (13). Lesions were graded using a scale from 0 to 4 according to the extent of edema and erythema of the periarticular tissues; 16 was the potential maximum of the combined arthritic scores per animal. The severity scores were defined as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the mid-foot (tarsals) or ankle joint; 2, erythema and mild swelling extending from the ankle to the foot; 3, erythema and

moderate swelling extending from the ankle to the metatarsal joints; and 4, erythema and severe swelling encompassing the ankle, foot and digits.

**Paw volume measurement.** The paw volume was measured by a water displacement plethysmometer (Ugo Basile). The hind paws were respectively immersed in an electrolyte solution up to the boundary between the hairy and non-hairy skin, and the volume displacement in the chamber was determined electronically. The percentage of the paw swelling was calculated by the following formula: % Swelling =  $(V_t - V_0)/V_0 \times 100$ , where  $V_t$  represents the paw volume after saline/ $\beta$ -endorphin injection and  $V_0$  represents the basal paw volume.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in synovial tissue.** Rats were anesthetized with 10% (w/v) chloral hydrate at a dose of 350 mg/kg (intraperitoneal) on day 28 after the injection of CII, the synovial tissues of rat knees were isolated and removed and subsequently frozen in liquid nitrogen and immediately stored at  $-80^{\circ}\text{C}$ . No rats exhibited signs of peritonitis. After homogenization of synovial tissue, total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and purified with the Prime Script<sup>®</sup> RT reagent Kit with gDNA Eraser (cat. no. PR0474A; Takara Bio Inc., Tokyo, Japan). Total RNA (1  $\mu\text{g}$ ) was then reverse-transcribed into complementary DNA using the abovementioned kit. Gene expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 was analyzed by real-time qPCR using the CFX96<sup>™</sup> real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH served as an internal control for detecting the expression of target genes. The primers for TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and GAPDH were designed with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) (primer sequences are listed in Table I) and were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The PCR mixture of 20  $\mu\text{l}$  contained 10  $\mu\text{l}$  SsoFast EvaGreen supermix (Bio-Rad Laboratories, Inc.), 0.3  $\mu\text{l}$  sense and anti-sense primers (400 nM), 1  $\mu\text{l}$  template complementary DNA and 8.4  $\mu\text{l}$  RNase/DNase-free water. The thermocycling conditions were as follows: Initial denaturation at  $95^{\circ}\text{C}$  for 30 min, followed by 40 cycles of 10 sec at  $95^{\circ}\text{C}$  and 30 sec at  $61.9^{\circ}\text{C}$ . Each sample was measured in triplicate. The generation of specific PCR products was confirmed by melting curve and gel analysis. The expression ratio was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method (14).

**ELISA for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in paw inflammatory tissue.** Samples were pulverized in liquid nitrogen, resolved in cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing protease inhibitor cocktail (10% v/v; Bio Basic Inc., Markham, ON, Canada), sonicated on ice (5x5 sec), stored for 1 h at  $4^{\circ}\text{C}$ , and then centrifuged at  $13,201 \times g$  for 30 min at  $4^{\circ}\text{C}$  to obtain the protein extract. TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were determined using a Rat TNF- $\alpha$  ELISA Kit (cat. no. ELR-TNFalpha-001), Rat IL-1 $\beta$  ELISA Kit (cat. no. ELR-IL1beta-001) and Rat IL-6 ELISA Kit (cat. no. ELR-IL6-001; all Raybiotech Corp., Atlanta, GA, USA), respectively.

**Statistical analysis.** All values are expressed as mean  $\pm$  standard error of the mean, and differences between

Table I. Primer sets used for polymerase chain reaction.

Gene	Sense primer	Anti-sense primer
IL-1 $\beta$	5'-GACCTGCTAGTGTGTGATGTTTC-3'	5'-GCTCATGGAGAATACCACTTG-3'
IL-6	5'-TTCCAATGCTCTCCTAATGG-3'	5'-CTTAGGCATAGCACACTAG-3'
TNF- $\alpha$	5'-TCACACTCAGATCATCTTC-3'	5'-CAGCCTCCTCTCTGCCATCAAG-3'
GAPDH	5'-TGCTGAGTATGTCGTGGAG-3'	5'-GTCTTCTGAGTGGCAGTGAT-3'

TNF, tumor necrosis factor; IL, interleukin.

groups were analyzed using the independent-samples t-test.  $P < 0.05$  was considered to indicate a statistically significant difference. All statistical analyses were conducted using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

## Results

**Effect of  $\beta$ -endorphin on TFL.** The mean TFL in the CIA + saline group and CIA +  $\beta$ -endorphin group is presented in Fig. 1. No significant difference in the TFL between the two groups was identified prior to  $\beta$ -endorphin injection. The TFL was significantly increased in the CIA +  $\beta$ -endorphin group when compared with that in the CIA + saline group on days 22 and 28 ( $P < 0.01$ ).

**Effect of  $\beta$ -endorphin on arthritis index.** As presented in Fig. 2, no significant difference in the arthritis index was present between the CIA + saline group and the CIA +  $\beta$ -endorphin group on day 18 after the injection of CII prior to  $\beta$ -endorphin treatment. The arthritis index was significantly reduced in the CIA +  $\beta$ -endorphin group when compared with that in the CIA + saline group on days 22 and 28 ( $P < 0.05$  and  $P < 0.01$ , respectively).

**Effect of  $\beta$ -endorphin on paw swelling.** The results on the percentage of paw swelling are displayed in Fig. 3. Prior to  $\beta$ -endorphin treatment (day 18), no significant difference in paw swelling was noted between the CIA + saline group and the CIA +  $\beta$ -endorphin group. Of note, on days 22 and 28, paw swelling in the CIA +  $\beta$ -endorphin group was significantly suppressed when compared with that in the CIA + saline group ( $P < 0.05$ ).

**Effect of  $\beta$ -endorphin on TNF- $\alpha$ , IL-1 $\beta$  and IL-6 mRNA expression in synovial tissue.** As presented in Fig. 4, the mRNA levels of TNF- $\alpha$  and IL-1 $\beta$  in synovial tissue of the CIA +  $\beta$ -endorphin group were significantly downregulated when compared with those in the CIA + saline group ( $P < 0.05$ ) on day 28. However,  $\beta$ -endorphin treatment had no significant effect on the IL-6 mRNA level of CIA rats.

**Effect of  $\beta$ -endorphin on the protein levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in paw inflammatory tissue.** As presented in Fig. 5, the protein levels of TNF- $\alpha$  and IL-1 $\beta$  in inflammatory paw tissue of the CIA +  $\beta$ -endorphin group were downregulated when compared with those in the CIA + saline group ( $P < 0.01$ ). No significant difference in the protein levels of

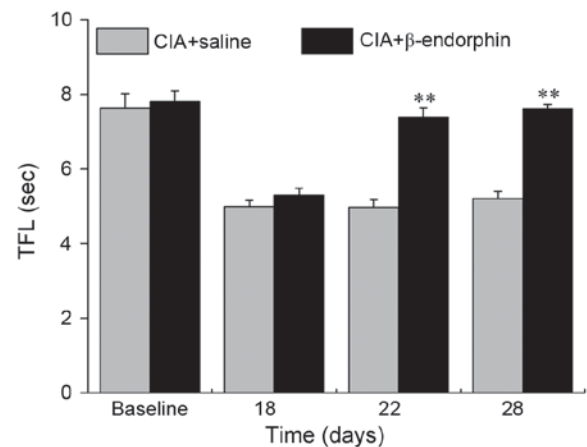


Figure 1. Effect of  $\beta$ -endorphin on TFL at the indicated time-points. Values are expressed as the mean  $\pm$  standard error of the mean ( $n=11$ ). \*\* $P < 0.01$ , vs. CIA + saline group. TFL, tail flick latency; CIA, collagen-induced arthritis.

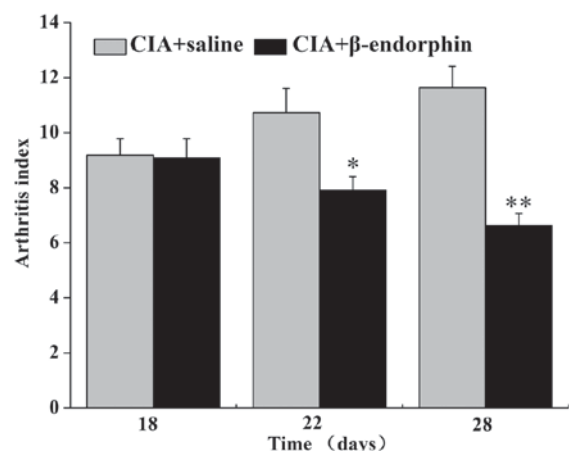


Figure 2. Effect of  $\beta$ -endorphin on arthritis index. Values are expressed as the mean  $\pm$  standard error of the mean ( $n=11$ ). \* $P < 0.05$  and \*\* $P < 0.01$ , vs. CIA + saline group. CIA, collagen-induced arthritis.

IL-6 was identified between the CIA + saline group and the CIA +  $\beta$ -endorphin group.

## Discussion

It is well known that opioids have a potent analgesic effect by activating their receptors in peripheral sensory nerve fibers and their terminals or the central nervous system. It has been

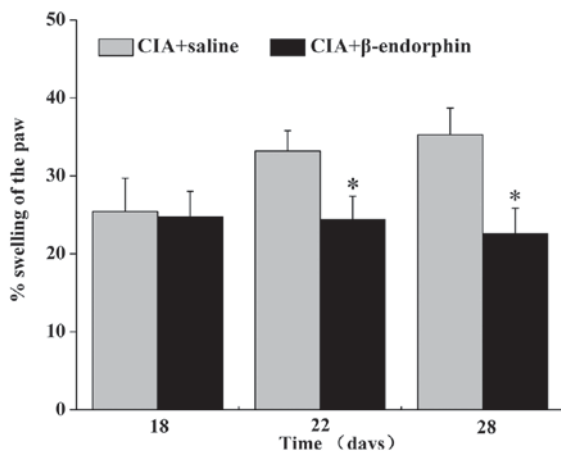


Figure 3. Effect of  $\beta$ -endorphin on percentage swelling of the paw. Values are expressed as the mean  $\pm$  standard error of the mean (n=11). \*P<0.05 vs. CIA + saline group. CIA, collagen-induced arthritis.

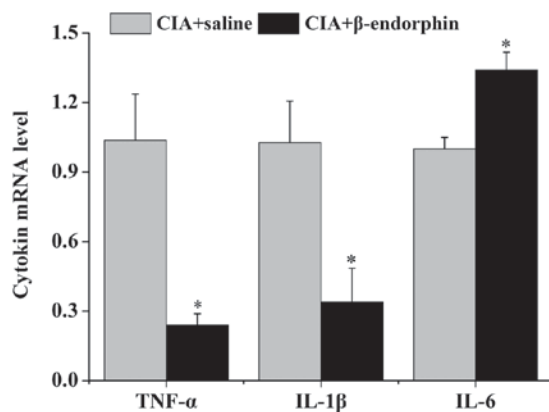


Figure 4. Effect of  $\beta$ -endorphin on the expressions of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 mRNA in synovial tissue. Values are expressed as the mean  $\pm$  standard error of the mean (n=11). \*P<0.05 vs. CIA + saline group. CIA, collagen-induced arthritis; TNF, tumor necrosis factor; IL, interleukin.

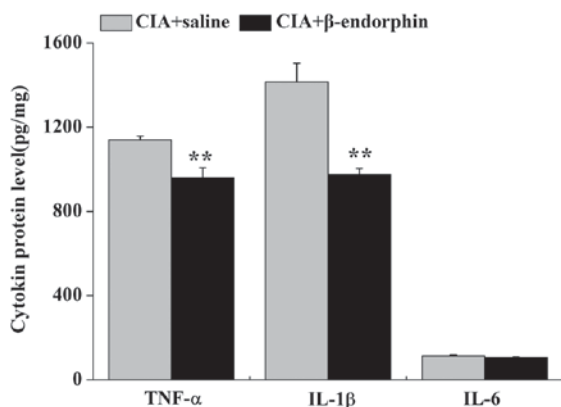


Figure 5. Effect of  $\beta$ -endorphin on TNF- $\alpha$ , IL-1 $\beta$  and IL-6 protein levels in paw inflammatory tissue. Values are expressed as the mean  $\pm$  standard error of the mean (n=11). \*\*P<0.01, vs. CIA + saline group. CIA, collagen-induced arthritis; TNF, tumor necrosis factor; IL, interleukin.

demonstrated that opioid receptors are also distributed on immune cells (15). In the present study, it was demonstrated that administration of exogenous  $\beta$ -endorphin not only produces

an analgesic effect, but also exerts an anti-inflammatory effect in rats with CIA. It selectively suppresses IL-1 $\beta$  and TNF- $\alpha$  in synovial tissue and paw inflammatory tissue.

The animal model of CIA is a commonly adopted experimental arthritis model, since its manifestations are similar to the clinical presentation of patients with RA (16). In the present study, CIA rats exhibited hyperalgesia, and their arthritis score and paw swelling were increased, indicating that the collagen-induced inflammatory pain was successfully established. The results indicated that intraperitoneal injection of exogenous  $\beta$ -endorphin effectively attenuated hyperalgesia in CIA rats. Furthermore, it also suppressed inflammation, as indicated by lowered arthritis score and paw swelling. Thus, it was suggested that the analgesic effect of  $\beta$ -endorphin on CIA rats was not only exerted through activating its receptors on nerve terminals, but that it may also be associated with the intervention with the inflammatory process.

In the present study, the levels of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in synovium and paw inflammatory tissue were then determined. Numerous studies have indicated that the physiological mechanisms of RA involve the release of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (17-20). TNF- $\alpha$  has a key role in the pathogenesis of RA (17,21), which comprises its ability to induce the production of other pro-inflammatory cytokines, including IL-1 $\beta$  and IL-6. Together, these cytokines induce the production and release of chemokines that attract leukocytes from the blood into the inflamed tissue (22). The present RT-qPCR and ELISA results indicated that intraperitoneal injection of  $\beta$ -endorphin decreased TNF- $\alpha$  and IL-1 $\beta$  levels in the paws and synovium, while it had no significant effect on IL-6 levels. Apart from the nervous system, opioid receptors have been reported to be distributed on immune cells (15). The downregulation of TNF- $\alpha$  and IL-1 $\beta$  levels by  $\beta$ -endorphin observed in the present study may result from the activation of its receptor on immune cells that produced an inhibitory effect on the inflammatory process. The reason why  $\beta$ -endorphin selectively suppressed TNF- $\alpha$  and IL-1 $\beta$  but not IL-6 requires further investigation.

In summary, the present study indicated that  $\beta$ -endorphin suppressed inflammation and partially downregulated peripheral pro-inflammatory mediators in inflammatory pain. The anti-inflammatory effect of  $\beta$ -endorphin may have a role in its analgesic effect.  $\beta$ -Endorphin may be of great value in the control of inflammatory pain.

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## Availability of data and materials

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

## Authors' contributions

YJ and JF designed and performed the experimental protocols. XH, XY, YS and YW performed the animal experiments. LH performed ELISA, SQ performed RT-qPCR, XH wrote the initial draft of the manuscript and performed statistical analysis and image acquisition. YJ and JF supervised the data analysis, manuscript design and revisions.

## Ethical approval and consent to participate

The study protocols were approved by the Ethics Committee of Zhejiang Chinese Medical University (Hangzhou, China).

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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