Neuroscience Letters 632 (2016) 157–162

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Local intramuscular injection of a plasmid encoding human proenkepahlin attenuates incision pain in rats

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HIGHLIGHTS

- Surgical incision resulted in mechanical allodynia and thermal hyperalgesia in rats.
- Local intramuscular injection of pVAX1-PENK attenuated nocifensive behaviors.
- Analgesic effect of pVAX1-PENK was blocked by methylnaltrexone.

ARTICLE INFO

Article history:
Received 26 February 2016
Received in revised form 25 August 2016
Accepted 30 August 2016
Available online 31 August 2016

Keywords:
Incision
Postoperative pain
Plasmid
Proenkephalin
Gene therapy

ABSTRACT

We investigated the antinociceptive effect of local intramuscular injection of a plasmid encoding human proenkephalin (pVAX1-hPPE) on postoperative pain in rats. Male Sprague-Dawley rats with incision-induced pain were intramuscularly injected into injured plantaris muscle with empty vector (pVAX1) or pVAX1-hPPE, respectively. Paw mechanical threshold and thermal latency in the 200 μg pVAX1-hPPE treated rats were significantly higher at 6 h and on 1 day, and lasted until day 7 after intramuscular administration, respectively. The analgesic effects were reversed by methylnaltrexone, suggesting that the antinociceptive effect of pVAX1-hPPE was mediated through peripheral opioid receptor pathway. In contrast, incisional or pVAX1-treated rats did not significantly affect pain thresholds. These results demonstrated that single intramuscular injection of pVAX1-hPPE attenuated incision-induced pain in rats, and it is worthy of further study as a potential gene therapy for postoperative pain.

1. Introduction

Postoperative pain is a major clinical problem with few effective therapeutic interventions. At present, treatment with drugs such as opioids, nonsteroidal anti-inflammatory drugs (NSAIDs), and topical anesthesia are commonly used to prevent postoperative pain. However, the analgesia agents widely used in clinical treatment usually have been limited by significant side effects, including gastrointestinal complications, respiratory depression, tolerance and addiction, and effectiveness [1,2]. Therefore, novel and effective analgesic strategies are required for the management of postoperative pain.

Endogenous opioid peptides, such as enkephalin, endorphin and dynorphin, play a substantial role in the pain relief and proved to be safe in clinic, but they are limited for clinical applications due to their fast metabolism [3,4]. Gene therapy is a novel and suitable strategy to enhance continuously expression of endogenous opioid peptides in target tissues and provides improved efficacy while minimizing potential systemic side effects. Proenkephalin (PPE), the precursor to the opioid peptides Met- and Leu-enkephalin (EK), has the widest tissue distribution and play an important role in the control of pain perception [5]. Several studies reported that overexpression of exogenous PPE in the spinal cord, dorsal root ganglia (DRG) or neurons resulted in an analgesic effect on CFA, formalin and capsaicin-induced inflammatory pain, SNL, CCI-induced neuropathic pain [6–11]. However, few studies have focused on whether local gene transfer of PPE into injured tissue produce antiallodynic effects on postoperative pain. If PPE can produce a stable and prolonged analgesic effect, it will provide a new treatment strategy for postoperative pain.

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http://dx.doi.org/10.1016/j.neulet.2016.08.058
0304-3940/

Therefore, in this study, we evaluated the antinociceptive effect of local intramuscular injection of a plasmid encoding human PPE (pVAX1-hPPE) on incisional induced postoperative pain in rats.

2. Materials and methods

2.1. Plasmid construction and sequencing

The hPPE gene fragments were amplified by PCR from plasmid pEGFP-N1-PENK-GFP (purchased from GeneChem co. Ltd. Shanghai, China) as a template using primers (*Kpn* I-tailed forward: 5′-GCGGGTACCACCATGGCGCGGTTCCTGAC-3′ and *Xba* I-tailed reverse: 5′-GCTCTAGATTAAAATCTCATAAATCCTC-3′). The hPPE gene PCR fragments were double digested with *Kpn* I and *Xba* I, respectively. Then, the target fragments were isolated and ligated using T4 DNA ligase and subsequently constructed a recombinant plasmid named as pVAX1-hPPE. The plasmid was further sequenced to confirm the sequences by Invitrogen Life Technologies Company.

2.2. Plasmid production

Plasmid production process was carried out as described previously [12]. Briefly, the fed-batch fermentation was performed to produce *E.coli* DH5α bearing interesting plasmid. The purification process included continuous alkaline lysis, separation of DNA from RNA on a Sepharose 6 Fast Flow column, selective supercoiled plasmid DNA purification with PlasmidSelect Xtra and final refining with a Source 15Q column. The plasmid solutions at 2 mg/ml and 4 mg/ml concentrations were obtained for further *in vivo* research.

2.3. Animals

Adult male Sprague-Dawley rats (180–220 g) obtained from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. were adapted to the laboratory for one week. Animals were housed in plastic cages in a room with constant temperature (22 ± 2 °C) and relative humidity between 45% and 60% with a 12:12 h light-dark cycle in group of three rats per cage. Animal study was approved by the Institutional Animal Care and Use Committee at Beijing Institute of Radiation Medicine and all experiments adhered to the ethical guidelines of the International Association for the Study of Pain.

2.4. Incision surgery

The rat hind paw plantar incision model was performed as previously described by Brennan [13]. Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg w.t.), and a 1 cm longitudinal incision was made through skin and fascia of the plantar aspect of the left hind paw, starting 0.5 cm from the proximal edge of the heel and extending toward the digits. The underlying flexor muscle is elevated with forceps and incised longitudinally. The muscle origin and insertion are kept intact. After hemostasis with gentle pressure, the skin was closed with 2 mattress sutures of 5–0 nylon on FS-2 needle and administrated gentamicin by intramuscular injection for anti-infection.

2.5. Experimental animal groups

After assessment of baseline behavior, rats were randomly divided into six groups (8 rats for each group): (1) Naïve group: rats did not receive any operation; (2) incision group: rats received only incision operation; (3) incision + pVAX1 group: incisional rats were injected intramuscularly with 200 μg empty vector (pVAX1, 2 mg/ml, produced as same as pVAX1-hPPE); (4) incision + 100 μg pVAX1-hPPE group: incisional rats were injected intramuscularly with 100 μg pVAX1-hPPE (1 mg/ml); (5) incision + 200 μg pVAX1-hPPE group: incisional rats were injected intramuscularly with 200 μg pVAX1-hPPE (2 mg/ml). (6) incision + 400 μg pVAX1-hPPE group: incisional rats were injected intramuscularly with 400 μg pVAX1-hPPE (4 mg/ml). All injections were administered into the plantaris muscle of incisional paw immediately after incision surgery. In order to further confirm whether opioid receptors are involved in the anti-hyperalgesic effects of hPPE in the peripheral nerve system, the mechanical allodynia and thermal hyperalgesia were measured within 15 min after intraperitoneal injection of methylnaltrexone (MNTX), which was given 3 days after intramuscular with pVAX1 or pVAX1-hPPE.

2.6. Detection of enkephalin in the muscle

Enkephalin in the target muscle was assayed using immunohistochemistry (ZSGB-BIO, Beijing, China) and Elisa kit (USCNK, Wuhan, China), respectively. For immunohistochemistry, flexor muscles were taken from naïve, pVAX1 and pVAX1-hPPE group rats at 7 day after gene transfer, and fixed in 4% formalin for 48–72 h. Thereafter, all samples were embedded in paraffin, cut into 5 μm thick sections. To remove endogenous peroxidase activity, the sections were incubated for 15 min with 3% H_2O_2 in PBS. Tissue sections were washed in PBS and incubated overnight at 4 °C with a 1:100 dilution of primary antibody Met-EK (Abcam, product code: ab22620). The sections were washed three times in PBS and incubated with a 1:500 solution of biotinylated goat anti-rabbit in PBS for 1 h at 37 °C, followed by incubating with avidin and biotinylated HRP complex for 30 min and visualized with 3,3′-diaminobenzidine. The immunostained sections were mounted with permount after dehydrated through ethanol. Subsequently, sections were examined using a microscope (OLYMPUS, model: CX41RF) at a magnification of 10, and images of each slice were captured with a digital camera (OPTPro DV500, Chongqing Optec Instrument Co. Ltd.). The muscle of naïve, pVAX1 and pVAX1-hPPE group rats were analyzed for optical density using Image Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD, USA).

For Elisa assay, the muscle of naïve, pVAX1, and pVAX1-hPPE group rats were taken at 1, 3, 5, 7 days after gene transfer. Briefly, muscle tissues (50 mg) were homogenized in 500 μl ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, China) with protease inhibitor cocktails. After incubation on ice for 30 min, homogenates were centrifuged at 10,000*g* for 20 min at 4 °C. The supernatants were used to determine the levels of human Leu-EK according to ELISA kit manufacturer's recommendation.

2.7. Behavioral testing

Mechanical allodynia was detected by von Frey hairs (Stoelting, wood Dale, IL). The 50% withdrawal threshold was determined using the up-down method as described previously by Dixon and Chaplan[14,15]. Briefly, rats were allowed to adapt a metalgrid for 30 min on the morning of the test day. Starting with a von Frey filament of 2.0 g weight force, each filament was applied for 5 s. with sufficient force to cause slight bending against the paw. A positive response was defined as rapid withdrawal and/or licking of the paw immediately upon application of the stimulus, which was then followed by application of the next finer von Frey filament. When a negative response was appeared, the next higher von Frey filament was applied. Rats did not respond to a pressure of 26 g that was assigned to cutoff value.

Thermal hyperalgesia was measured as the time of withdrawal from a radiant thermal stimulus directly under the hindpaw using a Hargreaves apparatus (plantar test apparatus; IITC, woodland hills, CA) [16]. In brief, rats were placed in individual enclosure on a glass