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Study on the distribution sites and the molecular mechanism of analgesia after intracerebroventricular injection of rat/mouse hemokinin-1 in mice

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ABSTRACT

Hemokinin-1 is a peptide encoded by *Pptc*, which belongs to the family of mammalian tachykinins. Our previous results showed that rat/mouse hemokinin-1 (r/m HK-1) produced striking analgesia after intracerebroventricular (i.c.v.) injection in mice, and the analgesia could be blocked by the NK_1 receptor antagonist and the opioid receptor antagonist, respectively. However, the precise distribution sites and the molecular mechanism involved in the analgesic effect after i.c.v. administration of r/m HK-1 are needed to be further investigated deeply. Using the fluorescence labeling method, our present results directly showed that r/m HK-1 peptides were mainly distributed at the ventricular walls and several juxta-ventricular structures for the first time. Our results showed that the mRNA expressions of NK_1 receptor, PPT-A, PPT-C, KOR, PDYN, DOR and PENK were not changed markedly, as well as the protein expression of NK_1 receptor was hardly changed. However, both the transcripts and proteins of MOR and POMC were up-regulated significantly, indicating that the analgesic effect induced by i.c.v. administration of r/m HK-1 is related to the activation of NK_1 receptor first, then it is related to the release of endogenous proopiomelanocortin, as well as the increased expression level of μ opioid receptor. These results should facilitate further the analysis of the analgesia of r/m HK-1 in the central nerval system in acute pain and may open novel pharmacological interventions.

1. Introduction

The mammalian tachykinins are a family of closely related peptides sharing a common C-terminal sequence (FXGLM-NH_2), whose best known members are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) [36]. Hemokinin-1 was identified firstly in 2000 from mouse as a hematopoietic-specific tachykinin that regulates B lymphopoiesis [44], while in 2002, hemokinin-1 was also identified from rat [22], which has the identical sequence with that from mouse (RSRTRQFYGLM-NH_2) [22,44], so they are both named as rat/mouse hemokinin-1 (r/m HK-1). The mammalian tachykinins are derived from three distinct genes: *Ppta* encodes for SP, NKA and two additionally extended forms of NKA (NPK and NPγ) [31], *Pptb* encodes for NKB [21], and *Pptc* encodes for HK-1 [22,44].

The biological actions of the mammalian tachykinins are mediated by at least three different transmembrane G-protein coupled receptors, namely NK_1 receptor (substance P-preferring), NK_2 receptor (neurokinin A-preferring) and NK_3 receptor (neurokinin B-preferring) [26]. Recent pharmacological and functional studies with r/m HK-1 revealed that it acted as a full agonist at the NK_1, NK_2 and NK_3 receptor with highest affinity for NK_1 [1,2,6,35]. The mammalian tachykinins have been implicated to have a wide variety of biological actions including smooth muscle contraction, vasodilatation, pain transmission, emotional behavior, neurogenic inflammation, and the activation of the immune system [19,38,39]. In modulation of pain transmission, Endo et al. reported that intrathecal administration of r/m HK-1 as well as SP at 10^{-3} M caused pain-related behavior such as scratching [7]. Additionally, our previous studies showed that r/m HK-1 has dual roles in pain modulation after intracerebroventricular (i.c.v.) administration in mice, and the analgesic effect of r/m HK-1 could be blocked by the NK_1 antagonist (SR140333) and the opioid receptor antagonist (naloxone), respectively [11]. Furthermore, our further studies showed that r/m HK-1 (i.c.v.) could significantly potentiate the antinociceptive effects of morphine which was injected at the peripheral and supraspinal level [13]. However, r/m HK-1 (i.c.v.) remarkably enhanced the antinociceptive extent of pethidine administered at the peripheral level, but not at the supraspinal level [12].

Obviously, our previous results just provide a phenomenon of r/m HK-1 in the modulation of pain after i.c.v. administration in mice. However, the precise distribution sites after i.c.v.

Abbreviations: r/m HK-1, rat/mouse hemokinin-1; FAM, carboxyfluorescein; PPT, preprotachykinin; MOR, μ-opioid receptor; POMC, proopiomelanocortin; KOR, κ-opioid receptor; PDYN, prodynorphin; DOR, δ-opioid receptor; PENK, proenkephalin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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administration of r/m HK-1 in mice are needed to be further investigated. Moreover, the molecular mechanisms involved in analgesic effect induced by i.c.v. administration of r/m HK-1 are also needed to be investigated deeply. So in this study, the technologies of fluorescence labeling, quantitative RT-PCR and Western blotting were used to explore the distribution sites and regulatory molecules of analgesia after i.c.v. administration of r/m HK-1 in mice.

2. Material and methods

2.1. Animals

Male or female ICR mice (20 ± 1.0 g) were supplied randomly by the animal center of Hangzhou Normal University. Mice were housed five per cage in a temperature (22 ± 1 °C) and humidity-controlled (55 ± 10%) room with a 12:12 h light/dark cycle (light between 08:00 and 20:00 h). The animals were allowed to adapt to this environment for a period of three days before the experiments. Food and water were available ad libitum.

All experimental procedures and animal husbandry were conducted according to standard ethical guidelines from the China Council on Animal Care and approved by the guidelines of the Ethics Committee of Animal Experiments at Zhejiang Sci-Tech University. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments.

2.2. Peptides

Fluo-r/m HK-1 and r/m HK-1 peptides were synthesized in Chinese Peptide Company by the solid-phase peptide method and purified by high-performance liquid (HPLC) with a purity of more than 98%. FAM was carboxyfluorescein and the standard coupling method was used to couple 5-carboxyfluorescein to the amino group of the r/m HK-1 peptide [15]. The peptide of r/m HK-1 was dissolved in normal saline at a working concentration of 0.75 nmol/μl. Fluorescein-labeled (FAM) r/m HK-1 was solubilized in 50% DMSO (dimethyl sulfoxide) at the concentration of 7.5 nmol/μl for stock solution. Then it was diluted in normal saline at the working concentration of 0.75 nmol/μl with 5% DMSO before the experiments.

2.3. Intracerebroventricular injection and tissue preparation

The method of intracerebroventricular (i.c.v.) injections was performed following the method described by Haley and McCormick in conscious mice [18] and the injection site was the same as our previous reports [11,13] (1.5 mm from the middle, 1 mm from the bregma and 3 mm from the surface of the skull). Drugs were administered in a volume of 4 μl at a constant rate of 10 μl/min using a 25 μl Hamilton microsyringe. The final concentration of r/m HK-1 or fluo-r/m HK-1 was 3 nmol/mouse.

Our previous studies showed that the maximum analgesia time of r/m HK-1 was around 10 min after i.c.v. injections [11], so the time points of 5, 10 and 20 min were selected for this research. After injection of FAM-r/m HK-1 or r/m HK-1 (3 nmol/mouse according to our previous report [11]), the mice were sacrificed by decapitation at each time point, and the proper injection site was verified by microscopic measurement. As the control, 4 μl of normal saline were injected into the sites of i.c.v. in mice. Then the brains administrated of r/m HK-1 or normal saline were quickly removed and stored at −80 °C for further studies (including RT-PCR and Western blotting). The brains administrated of FAM-r/m HK-1 were quickly removed and fixed in 4% paraformaldehyde (Amresco) in 0.1 M sodium phosphate buffer at pH 7.4 for 24 h, and cryoprotected with 30% sucrose before OCT (JUNG Tissue freezing medium, LEICA, Germany) embedding and freezing.

2.4. Coronal sections and light microscopy

Each brain was embedded in OCT (JUNG Tissue freezing medium, LEICA, Germany, Order number: 0201 08926) placed in a rubber ring (width 1 mm) of 1.5 cm height and of the same diameter. The embedded brains were frozen at −20 °C in a LEICA CM1900 cryostat and the frozen blocks containing brains were kept at −20 °C before sectioning in the same device. Each of the blocks containing brains was sectioned coronally into 10 μm slices by using a cryostat (LEICA CM1900), and the freshly cut sections were placed individually on pre-cooled (−20 °C) microscopy slides using cool tweezers. Sections were stored at 4 °C, but never longer than 12 h before observation. Samples were observed and photographed in an inverted phase contrast and fluorescence microscope (NIKON TE2000-U). Anatomical structures were identified according to an adult mouse brain atlas [9].

2.5. mRNA levels of target genes

2.5.1. Total RNA extraction

The frozen brain tissue sample was ground under liquid nitrogen and RNA was isolated from tissue using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Briefly, approximately 100 mg frozen brain was homogenized in 1 ml TRIzol Reagent using a mortar and pestle until they became red-violet. The amount of the purified RNA (A_{260}/A_{280} ratio was ≥1.9) was determined by spectrophotometry and their quality assessed by electrophoresis on a 1% agarose gel.

2.5.2. Reverse transcription and real-time polymerase chain reaction (PCR)

In all experiments, 5 μg of total RNA was reverse transcribed with M-MLV reverse transcriptase (TIANGEN) according to product instructions, using oligo dT (12–18 mer, Gibco BRL) into cDNA in a final volume of 50 μl. Negative controls were performed in which all of the components were included except reverse transcriptase.

The expression of NK_1 receptor, PPT-C, PPT-A, MOR, POMC, KOR, PDYN, DOR and PENK mRNA was assessed using relative quantitative real-time PCR. In order to control variations in the amount of the mRNA between samples, GAPDH was used as an endogenous internal control gene. The PCR primers and products sizes of each gene were summarized in Table 1, in which the primers of PENK gene were designed according to that in rat [43]. Negative controls were performed by replacing cDNA with distilled water in the PCR. An ABI 7300 real-time PCR detection system apparatus was used to perform the quantitative expression study. Each sample was analyzed in triplicate in a total reaction volume of 25 μl consisting of 2 μl of each cDNA, 1.2 μl of each forward/reverse primer (5 mM of each primer), 12.5 μl of 2 × SYBR Green qPCR mix (DongSheng Biotech, China) which contained 100 mM KCl, 4 mM $MgCl_2$, 400 μM dNTP mix, 0.2 U/μl Taq DNA polymerase and dd H_2O, and 8.1 μl of dd H_2O. The cycling conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s. For each experiment, a non-template reaction was included as negative control. The specificity of the PCR reactions was confirmed by melting curves analysis of the products as well as by size verification of the amplicon in a conventional 2% (wt/vol) agarose gels containing 0.05 mg/100 ml ethidium bromide. The threshold cycle (Ct) was used for quantification of the input target numbers. The normalized expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method [24], where