Int J Clin Exp Pathol 2017;10(5):5875-5881
www.ijcep.com /ISSN:1936-2625/IJCEP0047603

Original Article

Central distribution sites and protein expression of human HK-1 in mice

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Received December 28, 2016; Accepted March 20, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: Human hemokinin-1 (human HK-1) is a member of the mammalian tachykinin family encoded by Pptc gene, whose biological activities are needed further investigated. In our previous studies, human HK-1 could induce moderate analgesic effect when injection in mice via intracerebroventricular (i.c.v.) route with less potent than the analgesic effect of rat/mouse HK-1, via the opioid-responsive neurons. In present study, we aimed to investigate the central distribution sites and the expression level changes of interested proteins when human HK-1 was injected into mice via i.c.v. route, compared with the corresponding results of recently published work about rat/mouse HK-1. In our present research, we found that the distribution sites of human HK-1 were similar with the distribution sites of rat/mouse HK-1 at 10 min when injection via i.c.v. route. In addition, the expression level for POMC protein was increased significantly while the expression level of NK1R was unchanged after injection of human HK-1 via i.c.v. route, which was consistent with the results of rat/mouse HK-1. At 5, 10 and 20 min after injection of human HK-1, the protein expression levels of POMC were 1.06±0.14, 1.40±0.13 and 1.75±0.24, respectively. However, at 5, 10 and 20 min after injection of human HK-1, the protein expression levels of MOR were decreased significantly with the potency of 0.90±0.04, 0.75±0.07 and 0.62±0.16, respectively, which was a totally opposite result compared with the result of rat/mouse HK-1. Our present results, as well as further investigations about the possibility of HK-1 peptides on opioid analgesia and tolerance, may give new insights into the investigation and design of new strategies of pain.

Keywords: Human HK-1, fluorescent labeled peptides, intracerebroventricular injection, protein expression

Introduction

Hemokinin-1 (HK-1) is a member of mammalian tachykinins identified in 2000 from mouse [1] and in 2002 from rat and human [2]. HK-1 was encoded by *Pptc* gene sharing the conserved C-terminal motif FXGLM-NH_2 of mammalian tachykinins [1, 2]. The human Pptc polypeptide could generate full length human HK-1 and a truncated version (human HK-1 (4-11)) after cleavage at the end of amino-terminal of human Pptc polypeptide via two monobasic cleavage sites [2]. The amino acids sequence of human HK-1 (TGKSQFFGLM-NH_2) [2] is different from that of murine HK-1 (RSRTRQFYGLM-NK_2) [1], just sharing only five of eleven amino acids. In mammalian tachykinin family, there are some classical peptides, such as substance P, neurokinin A and neurokinin B, which are encoded by *Ppta* and *Pptb* gene, respectively [3].

There are three types of neurokinin receptors (NKR) denoted as NK1R, NK2R and NK3R, which mediate the biological actions of mammalian tachykinins, and the NKRs are subsumed in the class of G protein-coupled receptors [3]. HK-1 has the properties of high selectivity and specificity for NK1R as compared to NK2R and NK3R [4-6]. Several biological activities of murine HK-1 peptide are similar to the classical tachykinin, substance P [1]. However, murine HK-1, but not substance P, promoted the survival of B lineage cells or pre-B cells [1]. Our previous results showed that murine and human HK-1, as well as human HK-1 (4-11), could induce marked analgesic effect in varying degrees in mice after injection via intraventricular (i.c.v.) route [7, 8]. The distribution sites, as well as the pharmacological mechanisms involved in the analgesic effect of murine HK-1 and human HK-1 (4-11), however,

are different [7-10]. The main objective in this research was to study the fluo-human HK-1 distribution sites and the effect of human HK-1 on the expression of NK1R, MOR and POMC after injection in mice via i.c.v. route. At present, our data showed the similar distribution sites between human HK-1 and murine HK-1, but the mechanism in molecular level involved in analgesic effect among human and murine HK-1, as well as human HK-1 (4-11) is different.

Materials and methods

Ethics and animals

This research was approved by ethic committee of animal research and followed guidelines of animal research in Hangzhou Normal University. As our previous reports [9, 10], ICR mice were obtained from the animal center of Hangzhou Normal University (male or female with the bodyweight of 20±1 g). Mice were raised with a 12-hour phase shift in the light/dark cycle and were enabled to adapt to our environment for at least 3 days before the experiments (five per cage at 23-25°C).

Peptides

The fluo-human HK-1 and human HK-1 with a purity of more than 98% were purchased from Chinese Peptide Company Hangzhou, China, which were synthesized by solid-phase peptide method. 5-carboxyfluorescein was coupled to the amino group of human HK-1 to create the fluo-human HK-1 peptide using the standard coupling method [11]. Human HK-1 peptide in the dosage of 1.5 mM was prepared in stroke-physiological saline solution. Fluo-human HK-1 in the dosage of 15 mM was dissolved with dimethyl sulfoxide (DMSO, 50%) and conserved at -80°C. When usage, the stock was diluted in stroke-physiological saline solution stroke-physiological saline solution to a final dosage of 1.5 mM.

The processing of mice and tissue preparation

The injection methods via i.c.v. route were as previous reports [9, 10, 12] with the concentration of 6 nmol/mouse (4 μl) of fluo-human HK-1 or human HK-1 according to our former report [8].

Our former study showed marked analgesic effect at the concentration of 6 nmol/mouse of human HK-1, and it was maintained within 60 min, in which the peak analgesic effect occurred around 10 min [8]. According to our previous study, the three time points (5, 10 and 20 min) were selected in our present study to sacrifice mice via decapitation. The mice brains injected with fluo-human HK-1, human HK-1 or stroke-physiological saline solution were stored as our previous reports [9, 10]. Briefly, for fluorescence detection of fluo-human HK-1, the brains were removed quickly, fixed for 24 hours in 4% paraformaldehyde (Sigma). Then the brains were cryoprotected with 30% sucrose before embedding and freezing using OCT regent (LEICA, Germany). For Western blotting analysis after injection of human HK-1 or stroke-physiological saline solution, the brains were stripped and stored at -80°C for subsequent experiments quickly.

Observation of coronal sections with light microscopy

The methods used for coronal sections of each brain and for observation with light microscopy were the same with the procedures in our reports [9, 10]. In brief, each brain embedded by OCT was frozen at -20°C for at least 30 min before section into 10 μm slices and placed immediately on microscopy slide after section for observation with the inverted fluorescence microscope (NIKON TE2000-U), and the fluorescent sites of human HK-1 were investigate according to the book of adult mouse brain atlas [13, 14].

Western blotting

The methods for isolation of protein samples from each mouse brain were the same with our previous works [9, 10]. In brief, each brain tissue was lysed in RIPA lysis buffer and the protein concentrations were determined using BCA Protein Assay Kit (Tiangen) [15, 16]. Equal amounts of protein from each brain were separated in 12% SDS-PAGE (Bio-Rad) [17]. The proteins were subsequently electrotransferred onto PVDF membranes. Protein expression was determined by Western blotting with specific antibodies of GAPDH (1:20000 dilution, Bioworld), POMC (1:1000 dilution, Bioword), Muopipid receptor (MOR) (1:1000 dilution, Bioworld) or NK1R (1:1000 dilution, Bioworld) at 4°C overnight. Subsequently, HRP-second antibodies (1:20000 dilution, Bioworld) were used to incubate each membrane and protein expression signals were visualized by Chemiluminescence using ECL detection reagent. The densitometric quantification of bands obtained

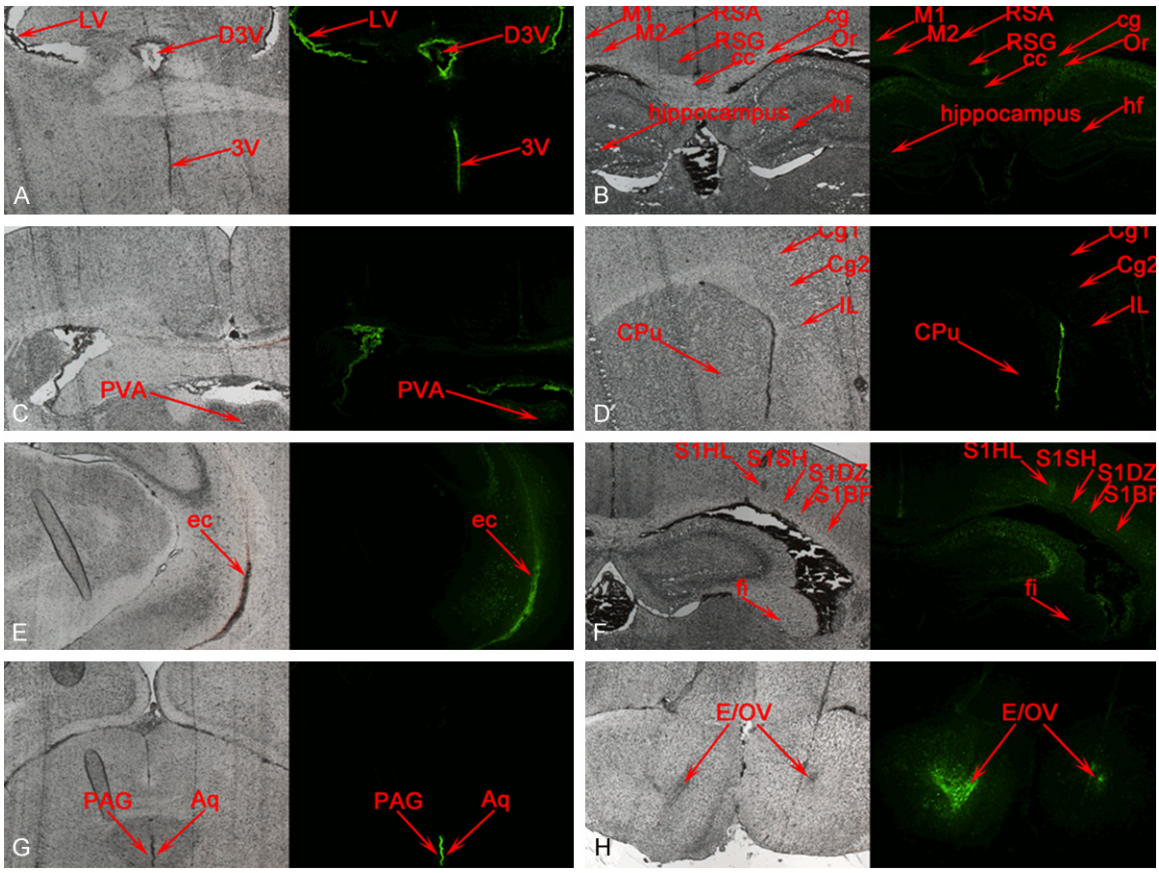

Figure 1. The central distribution sites of human HK-1 in mice administration via i.c.v. route. Representative photographs were filmed by using the phase contrast microscope (A-H left), and all data are from an independent experiments (n≥3). The same coronal sections observed with inverted fluorescence microscope (A-H right) corresponding to each phase-contrast photograph. Scale bar = 50 μm. The fluorescence of FAM-human HK-1 was green. Abbreviations: Aqueduct (Aq); Corpus callosum (cc); Periaqueductal central gray (PAG); Paraventricular thalamic nucleus, anterior part (PVA); Cingulate cortex, area 1 (Cg1); Cingulate cortex, area 2 (Cg2); Caudate putamen (striatum) (CPu); External capsule (ec); Cingulum (cg); Ependymal and subendymal layer/olfactory ventricle (E/OV); Lateral ventricle (LV); 3rd ventricle (3V); Dorsal 3rd ventricle (D3V); Oriens layer of the hippocampus (Or); Fimbria of the hippocampus (fi); Primary motor cortex (M1); Secondary motor cortex (M2); Retrosplenial agranular cortex (RSA); Retrosplenial granular (RSG); S1 cx, barrel field (S1BF); Infralimbic cortex (IL); Cingulate cortex (cg); Primary somatosensory cortex, shoulder region (S1SH); Primary somatosensory cortex, dysgranular region (S1DZ); Primary somatosensory cortex, hindlimb region (S1HL).

in Western Blotting results were scanned using Quantity One Software (Bio-Rad), which were normalized with that of a control band and further normalized to control samples.

Data analysis and statistics

All data were presented as mean ± SEM of an independent experiments (n≥3) and tested for significance by the Dunnett's test and Fisher's exact tests with the help of the statistical software package SPSS. In all experiments, differences between the experimental groups were analyzed using one way of variance taking a *P* value <0.05 as significant.

Results

Central distribution sites of human HK-1 (injection via i.c.v. route) in conscious mice

Our former results showed that human HK-1 could induce marked analgesia in a dose- and time-dependent manner at the concentrations of 0.3 nmol to 6 nmol per mouse after injection via i.c.v. route, in which the peak analgesic effect was around 10 min after injection of human HK-1 [8]. So the concentration of 6 nmol per mouse and the time points of 5 min, 10 min and 20 min after i.c.v. injection were selected in our present study. Also, the analgesic effect of

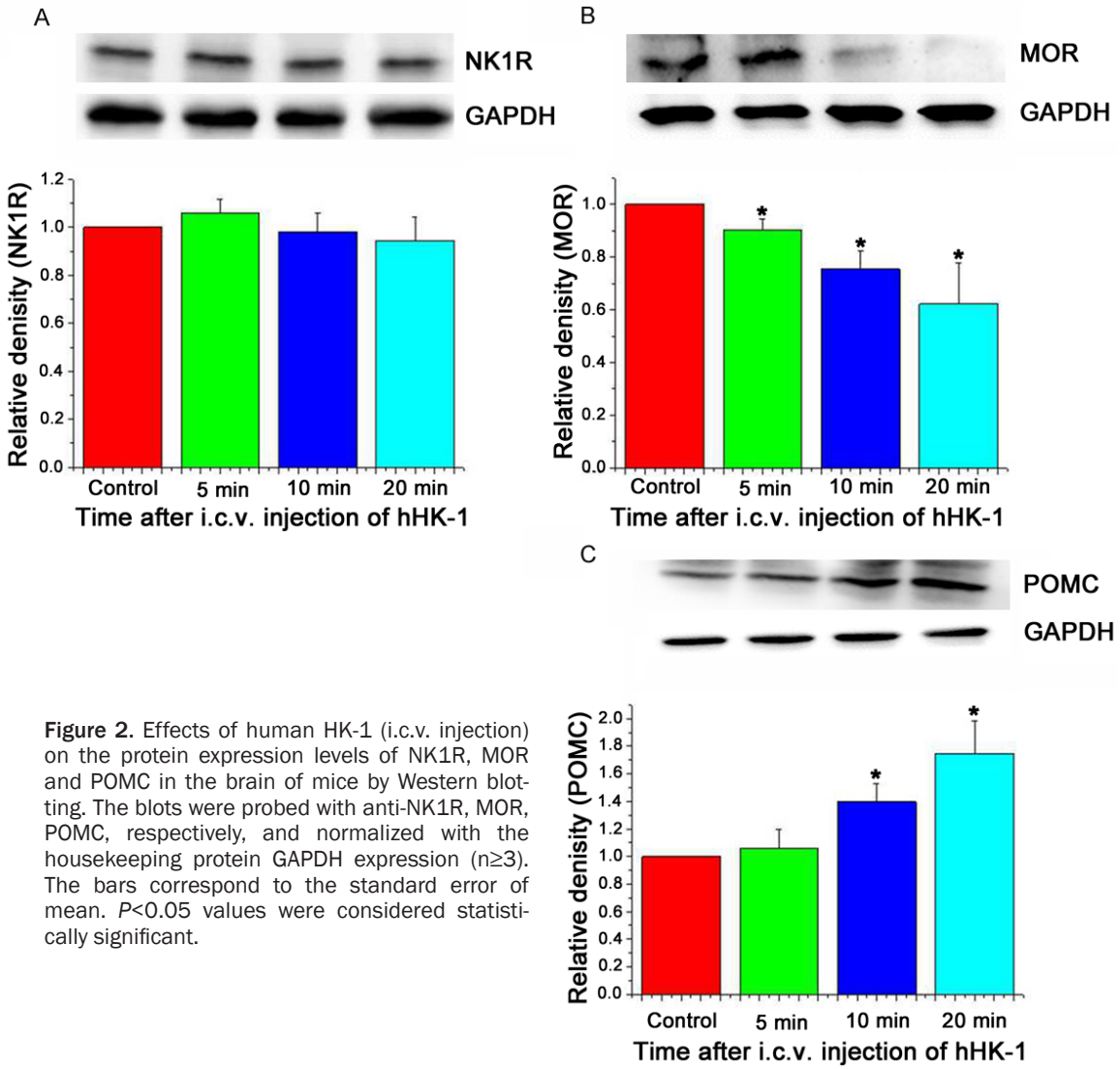

Figure 2. Effects of human HK-1 (i.c.v. injection) on the protein expression levels of NK1R, MOR and POMC in the brain of mice by Western blotting. The blots were probed with anti-NK1R, MOR, POMC, respectively, and normalized with the housekeeping protein GAPDH expression (n≥3). The bars correspond to the standard error of mean. *P*<0.05 values were considered statistically significant.

human HK-1 was verified in our present study using the method of tail-flick test, which was consistent with our previous conclusion (data not shown).

The fluo-human HK-1 peptides were excited the fluorescence in green in our present study observed with fluorescence microscope, and the green fluorescence distribution sites were mainly located among the cerebroventricular walls, and some juxta-ventricular structures (**Figure 1**). In detail, the bright green fluorescence sites were located in ventricles (**Figure 1A**), corpus callosum (**Figure 1B**), external capsule (**Figure 1E**), periaqueductal central gray (**Figure 1G**), ependymal and subendymal layer/olfactory ventricle (**Figure 1H**), which is highly similar with the data of fluo-rat/mouse HK-1 in our recent results [10].

Protein expression of POMC and MOR were changed significantly after injection of human HK-1 via i.c.v. route in mice

In recent published results, we demonstrated that the protein expression levels of endogenous opioid receptors, as well as opioid peptides were changed markedly, but not NK1R, after treatment with rat/mouse HK-1 or human HK-1 (4-11) in mice via i.c.v. route [9, 10]. Accordingly, we detected the protein concentrations of NK1R, MOR and POMC in our present study. Our results showed that POMC (pro-opiomelanocortin) protein expression level was increased significantly after treatment with human HK-1 with the levels of 1.06±0.14, 1.40±0.13 and 1.75±0.24 at 5, 10 and 20 min, respectively (**Figure 2C**), while there was no sig-

nificant change in NK1R protein expression (**Figure 2A**). These results are consistent with our published work of rat/mouse HK-1 [10]. However, in the human HK-1-treated group, a significant decrease in MOR (mu opioid receptor) protein expression was detected with the expression levels of 0.90±0.04, 0.75±0.07 and 0.62±0.16 at 5 min, 10 min and 20 min, respectively, (**Figure 2B**), which is an entirely opposing result compared with the expression levels change of MOR at each time point treatment with rat/mouse HK-1 via i.c.v. route [10].

Discussion

Previous studies showed that human HK-1 is a uterine stimulant in the human with the characteristics of partial agonist for NK1R and NK2R [18]. The expression pattern of human HK-1 mRNA was determined in human lymphocytes, monocytes, neutrophils and eosinophils by Klassert et al., indicating that human HK-1 peptide has an important role in the regulation of inflammatory pathophysiology process [19]. In the immune system, IL-17A and IFN-γ production, but not IL-4 and IL-10 production, were upregulated by human memory $CD4^+$T cells induced by substance P and hHK-1 treatment through NK1R, indicating that substance P and human HK-1 are novel Th17 cell-inducing factors and that SP and human HK-1 may effect on memory T cells locally to enhance inflammatory process [20]. The binding assay indicated that the affinity of human HK-1 and human HK-1 (4-11) with NK1R was about 14 and 70 times lower than the affinity of substance P and rat/mouse HK-1 with NK1R [2]. Our previous study showed that the tachykinin peptides rat/mouse HK-1, human HK-1 and human HK-1 (4-11) had obvious analgesic effect in mice injected via i.c.v. route and the potency of their analgesic activities was rat/mouse HK-1> human HK-1> human HK-1 (4-11) [8], which was consistent with the binding activities. In our present study, we reported the fluorescent sites of human HK-1 in central system via i.c.v. route in mice firstly, and the characteristics of human HK-1 and rat/mouse HK-1, but not human HK-1 (4-11), had the similar fluorescent distribution sites [9, 10].

Our previous study also showed that the analgesic activity of human HK-1 and rat/mouse HK-1 could be antagonized by the classical opioid receptor antagonist naloxone, as well as by the β-FNA (an irreversible mu opioid receptor) and naloxonazine (an irreversible mu_1 opioid receptor), indicating that human HK-1 and rat/mouse HK-1 have a potential effect to induce the produce of endogenous opioid peptides and to induce analgesic activity by the activation of mu opioid receptor [8]. In the molecular level, our Western blotting data showed that the bands intensity for MOR and POMC were elevated significantly after treatment with rat/mouse HK-1 [10]. However, only the expression level of POMC after treatment with human HK-1 in mice was increased markedly in this study. In contrast, bands intensity of MOR was decreased significantly, which was different from that of rat/mouse HK-1. As the endogenous ligands for NK1R, the central distribution sites of rat/mouse HK-1 and human HK-1 after i.c.v. operation in mice were similar while the molecular events were somewhat different, which may be due to the differences in amino acid sequence between both peptides (just sharing only five of eleven amino acids) [1, 2]. In addition, our recently study showed that hHK-1 (4-11), just absence of the three amino acids at the N-terminal of human HK-1, had no potency to affect the expression levels of MOR and POMC proteins after i.c.v. injection, while had obvious potency to enhance the expression level of DOR (delta opioid receptor) [9], further indicating that the amino acids in the N-terminal of HK-1 peptide are very important for the molecular events induced by these peptides. Moreover, our former results also indicated that rat/mouse HK-1 had the ability to enhance the analgesic effect of morphine [21] and pethidine [22] via a pharmacological convergence effect of opioid-responsive neurons, respectively. Previous study suggests that neuropathic pain was insensitivity to opioid analgesics, which could be due to the phenomenon of mu opioid receptors were reduced significantly in the axotomized sensory neurons and in interneurons in the dorsal horn of the spinal cord [23], as well as that the downregulation of mu opioid receptor may contribute to opioid tolerance in vivo [24]. In our present study, the downregulation of mu opioid receptor after treatment with human HK-1 may partially be related to the lower potency of analgesic activity of human HK-1 than the analgesia of rat/mouse HK-1 [8]. Considering the fact that human HK-1 could induce a downregulation of mu opioid receptor,

more further studies will be designed to investigate the effect of human HK-1 on the analgesia of morphine and pethidine (compared with the corresponding effect of rat/mouse HK-1), as well as the possibility of human HK-1 on the insensitivity to opioid analgesics and to opioid tolerance, which may provide some new insights into the opioid analgesics and tolerance.

In brief, our present data indicated firstly that, as the endogenous ligands for NK1R, the distribution sites of human HK-1 after i.c.v. injection are quite similar with the distribution sites of rat/mouse HK-1 while the molecular events induced by these peptides were somewhat different. Our present results, as well as further investigations about the possibility of HK-1 peptides on opioid analgesia and tolerance, may pave the way for the design of new strategies to control of pain.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (No. 31470071, 31000496), the New Century 151 Talent Project of Zhejiang Province, the 521 Talent Foundation of Zhejiang Sci-Tech University, the Open Foundation from Key Laboratory of Tumor Molecular Diagnosis and Individualized Medicine of Zhejiang Province (No. ZJZLSYS004).

Disclosure of conflict of interest

None.

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