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The N-terminal domain of human hemokinin-1 influences functional selectivity property for tachykinin receptor neurokinin-1

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ABSTRACT

Human hemokinin-1 (hHK-1) is a substance P-like tachykinin peptide preferentially expressed in non-neuronal tissues. It is involved in multiple physiological functions such as inflammation, hematopoietic cells development and vasodilatation via the interaction with tachykinin receptor neurokinin-1 (NK1). To further understand the intracellular signal transduction mechanism under such functional multiplicity, current study was focused on the differential activation of Gs and Gq pathways by hHK-1 and its C-terminal fragments, which is termed as functional selectivity. We demonstrated these hHK-1 and related peptide fragments can independently activate Gs and Gq pathways, showing a relative bias toward Gq over Gs pathway. The T1, K3 and Q6 of hHK-1 might play roles in the activation of adenylate cyclase mediated by Gs, while having negligible effect on Gq mediated intracellular calcium release. The stepwise truncation of N-terminal amino acid of hHK-1 caused gradual decrease in ERK1/2 phosphorylation level and NF-κB activity. However, it had little influence on the induction of NK1 receptor desensitization and internalization. Taken together these data support that hHK-1 and its C-terminal fragments are human NK1 receptor agonists with different functional selectivity properties and that such functional selectivity leads to differential activation of downstream signaling and receptor trafficking.

1. Introduction

The mammalian tachykinins are some small bioactive peptides classically described as neurotransmitters. They share the common C-terminal region of -FXGLM-NH_2 where X is hydrophobic amino acid. They have included three members, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), until 2000, hemokinin-1 (HK-1) was identified as the fourth tachykinin [1]. Ensuing studies investigated the pharmacology of hemokinin-1 at each of the three tachykinin receptors, neurokinin-1 (NK1), neurokinin-2 (NK2) and neurokinin-3 (NK3). Human hemokinin-1 was a full agonist at tachykinin receptor NK1, NK2 and NK3, showing remarkable selectivity for NK1 receptor [2,4,46]. Unlike that other known mammalian tachykinins are identical through all species, human hemokinin-1 (hHK-1) does not show complete homology with rat or mouse hemokinin-1. More especially, HK-1 is demonstrated to be primarily expressed in hematopoietic cells rather than the predominant neuronal expression of the other known mammalian tachykinins [2]. These unique features imply that this new-found member of tachykinin family may have some characteristic biological and pharmacological properties of its own.

Up to now, the investigations on the biological roles of hHK-1 have been widely involved in the immune cells [1,23,29], central and peripheral neural system [40,41,47], cardiovascular system [4,38], reproductive system [30,39,48], playing multiple roles in inflammation, hematopoietic cells development, vasodilatation, nociception, etc. The preferential expression in immune cells makes HK-1 a more likely regulator of immune system compared to SP and other tachykinins. In human glial cell U-251 MG, hHK-1 can increase the expression of several cytokines such as IL-1β, IL-6, LIF and GM-CSF [23]. In hematopoietic system, HK-1 promoted the proliferation and survival of lymphoid precursors *in vitro* and blocking its action with the NK1 antagonist impaired lymphoid development *in vivo* [1,3]. Recently hHK-1 was demonstrated to rescue bone marrow-derived dendritic cells from apoptosis and increased the dendritic cells longevity in *vivo* through NK1 receptor signaling, leading to enhanced and prolonged effector cellular immunity [5]. Wang et al. showed that hHK-1 also acted as

Abbreviations: cAMP, cyclic adenosine monophosphate; NF-κB, nuclear factor kappa B; ERK1/2, extracellular regulated protein kinases1/2.

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a co-stimulatory factor for B cell activation possibly through synergistic activation of the MAPK pathway and induction of transcription factors critical for plasmacytic differentiation [6].

NK1 belongs to the class A G protein-coupled receptor (GPCR) and couples to two distinct signaling pathways: a Gs pathway that activates adenylate cylcase inducing intracellular cAMP accumulation; a Gq pathway that activates phospholipase Cβ (PLCβ) initiating inositol phosphate formation and intracellular calcium release [7]. During the past years, such multi-G-protein coupling situation has been found in several other GPCRs like cannabinoid receptor, dopamine receptor, μ and δ opioid receptor. GPCR can independently activate a variety of signaling effectors; distinct receptor ligands can do so with different potencies and efficacies (intrinsic efficacy). This selective activation of independent pathways by ligands has been termed functional selectivity [8,9]. Although a new concept of pharmacology, functional selectivity of GPCR has become widely accepted. The conceptual basis for this is that GPCRs can adopt multiple conformation states and that different ligands can stabilize distinct active conformations with different intrinsic efficacies, thus leading to differential sets of cellular signaling and behavior [10,11]. Taking functional selectivity into account, it has become more and more evident that the properties of a ligand should be evaluated not only about its receptor subtype-selectivity, but also signal-pathway and ligand trafficking selectivity [32–34]. However, as an important endogenous ligand of NK1 receptor, hHK-1 is mainly characterized by its relative binding affinity to the different receptor subtypes. The evaluation of the functional pharmacology is often limited to its ability to induce intracellular calcium mobilization [2,3,23]. The structural requirements for hHK-1 to activate NK1 receptor are not clear. NK1-coupled Gs activation and downstream signaling have not been established, not to say the discrimination of the role of each pathway in physiological and pathological conditions. Considering the recent renaissance of interest in HK-1 biofunctions [5,6], the knowledge about its detailed mechanism of activation on NK1 receptor is warranted especially based on functional selectivity.

In this study, therefore, human HK-1 was investigated for its intrinsic efficacies on two distinct signaling pathways, receptor desensitization and endocytosis in a CHO cell system stably expressing human NK1 receptor. Since the diverse N-terminal domains of tachykinins are considered to determine the intrinsic property of ligand-receptor interaction [35,36], the role of hHK-1 N-terminal domain in human NK1 activation and downstream signaling was also discussed in current study. In this CHO cell system, hHK-1 was demonstrated to act in a fashion of functional selectivity upon activating human NK1. The N-terminal domain, especially the one, three and six amino acid position, influenced its functional selectivity properties.

2. Materials and methods

2.1. Reagents

The plasmid pcDNA3.1-3 × HA-NK1 was purchased from Missouri S&T cDNA Resource Center (www.cdna.org). cAMP-Glo assay kit, luciferase assay system and TMB One solution substrate were from Promega Corporation (Madison, WI, USA). The enhanced chemoluminescence (ECL) detection system and 16% formaldehyde were from Thermo (Rockford, IL, USA). G418, probenecid, NK1 antagonist L-732,138, calcium chelator BAPTA-AM, phosphatase inhibitors 3-isobutyl-1-methylxanthine (IBMX) and Ro 20-1724 were obtained from Sigma–Aldrich (St. Louis, Missouri, USA). RPMI 1640 medium, Opti-MEM medium, fetal bovine serum (FBS), Fluo-4 AM, Pluronic F-127 and transfection reagent Lipofectamine2000 were the products of Invitrogen (Carlsbad, CA, USA). PKA inhibitor H89, anti-HA antibodies, anti-phospho-ERK1/2(Thr202/Tyr204) antibodies, anti-ERK1/2 antibodies and HRP-conjugated secondary antibody were purchased from Cell Signaling Technology Inc. (Danver, MA, USA).

2.2. Peptides synthesis

hHK-1 (TGKASQFFGLM-NH_2) and its C-terminal fragment peptides were synthesized using the Fmoc method on a solid-phase peptide synthesis system, as described previously [37]. The identity of all peptides was confirmed using ESI-TOF mass spectrometry. All peptides were determined to be >95% pure by reversed-phase high-performance liquid chromatography using a C_{18} column as the solid phase and a H_2O: acetonitrile gradient as the solution phase.

2.3. Establishment of CHO cells stably expressing NK1 receptor

CHO cells were cultured in RPMI 1640 medium supplemented with 10% FBS. The eukaryotic vector containing HA-tagged human NK1R, pcDNA3.1-3 × HA-NK1, was introduced into CHO cells by Lipofectamine2000 according to the manufacture's instruction. The day after transfection, G418 (200 μg/ml) was added to the medium for two weeks. Then the antibiotic-resistant clones derived from single cell were selected and further characterized by RT-PCR and Western-blotting to ensure the expression of human NK1 receptor. On the base of the strength of calcium mobilization induced by hHK-1, the cell clones that positively expressed functional human NK1 receptor were chosen and referred as CHO-hNK1. The cellular functions of NK1 receptor were further confirmed by the NK1 antagonist L-732,138. Briefly, CHO-hNK1 cells were pre-incubated with or without 1 μM L-732,138 in RPMI1640 medium for 30 min. Then the cells were stimulated with 1 μM hHK-1 to measure the calcium mobilization or the cAMP accumulation level.

2.4. cAMP accumulation assay

The intracellular cAMP level was measured using the commercial available cAMP-Glo assay kit. Briefly, 5000 NK1-expressing CHO cells were seeded in 96-well plate with RPMI 1640 medium containing 10% FBS and incubated in 37 °C for 24 h. Remove the medium, then 20 μl treatment buffer (PBS containing 0.5 mM IBMX and 0.1 mM Ro 20-1724, pH 7.4) with or without hHK-1 or its C-terminal fragments was added to the cells and incubated at 37 °C for 15 min. 20 μl/well of the cAMP-Glo lysis buffer was added to the cells, shaking for 15 min at room temperature before being developed with the detection buffer and substrate supplied by the cAMP-Glo assay kit. Finally luminescent signal was measured by a plate-reading luminometer (Infinite M200, Tecan, Switzerland).

2.5. Calcium mobilization assay

CHO-hNK1 cells were seeded in a 96-well-plate at a density of 20,000/well and cultured for 24 h. The cells were rinsed three times with assay buffer (130 mM NaCl, 5 mM KCl, 10 mM HEPES, 8 mM D-glucose, 1.2 mM $MgCl_2$ and 1.5 mM $CaCl_2$, pH 7.4). The cells were then incubated with this buffer supplemented with the organic anion transport inhibitor probenecid (2.5 mM), 1 μM Fluo 4-AM and 0.1% Pluronic F-127 for 60 min at 37 °C. Before the measurement, cells were rinsed three times with assay buffer then placed in a FlexStation II plate reader (Molecular Devices Corp., Palo Alto, CA, USA) at 37 °C. The fluorescence emission at 525 nm following excitation at 480 nm was measured as hHK-1 or its C-terminal fragments were added. The peak fluorescent value was used as an index of intracellular calcium mobilization.

2.6. ERK1/2 phosphorylation and receptor desensitization

CHO-hNK1 cells were seeded in Corning 12-well plate at a density of 200,000/well, grown in the medium of RPMI 1640 with 2%FBS overnight and then starved in RPMI 1640 medium for at least 4 h at 37 °C. For rapid ERK1/2 phosphorylation assay, the cells were treated with hHK-1 or its C-terminal fragments at different concentrations, incubated at 37 °C for 10 min, then lysed in RIPA lysis buffer containing 10 μM PMSF. To assess the effects of intracellular cAMP accumulation and calcium mobilization on ERK1/2 phosphorylation, the CHO-hNK1 cells were pre-incubated with 20 μM PKA inhibitor H89 or 20 μM cell-membrane permeating calcium chelator BAPTA-AM for 30 min, then treated with 1 μM hHK-1 for 10 min. For receptor desensitization assay, the cells were pretreated with 1 μM hHK-1 or its C-terminal fragments for 4 h at 37 °C. Then the cells were rinsed with PBS and exposed to 1 μM hHK-1 for 10 min at 37 °C. The incubation was terminated by removing the medium and adding RIPA lysis buffer containing 10 μM PMSF. The untreated cells were used as control in all experiments. Total amount of 20 μg protein from each sample was loaded and separated on a 10% SDS-PAGE gel. After electrophoresis, the samples were transferred onto a PVDF membrane. The membranes were probed with primary antibody against phospho-ERK1/2 or ERK1/2 followed by incubation with HRP-conjugated secondary antibody. The signal was detected by an enhanced chemoluminescence detection system.

2.7. Luciferase reporter assay for NF-κB release activity

2,000,000 CHO-hNK1 cells were seeded in a 60 mm-dish and cultivated at 37 °C overnight. 8 μg reporter plasmid pNF-κB-luc plasmid of high purity was transfected into cells with Lipofectamine2000 following the instructions of manufactures. 24 h later the transfected cells were trypsinized and seeded in 24-well plate at a density of 50,000, grown for another 24 h with RPMI 1640 containing 10% FBS. Then the cells were exposed to 1 μM hHK or its C-terminal fragments for 6 h at 37 °C. Untreated cells were used as control. Then the cells were lysed and NF-κB-driven luciferase activity was measured with the luciferase assay system and the plate-reading luminometer mentioned in Section 2.4. The untreated control was defined as 1.0. The luciferase activity was expressed as fold induction relative to untreated control.

2.8. Quantitative internalization assay

Quantitative internalization assay was performed as described before [24,31]. Briefly, cells were seeded in Corning 24-well plate at a density of 200,000/well and cultured at 37 °C for 24 h. After washed with PBS, cells were pre-incubated with mouse anti-HA antibody (1:500) in Opti-MEM for 2 h at 4 °C. For induction of receptor internalization, cells were treated with 1 μM hHK-1 or its C-terminal fragments for 60 min at 37 °C and then chilled on ice to terminate the receptor internalization. The untreated cells were used as control. After the fixation with 4% formaldehyde in PBS, cells were incubated with HRP-conjugated goat-anti-mouse antibody (1:1000) for 2 h at room temperature. Washed with PBS 3 times, plate was developed with 300 μl TMB substrate. After 5 min, the reaction was stopped by adding 300 μl 1 M HCl. 200 μl Substrate solution were transferred to a 96-well-plate and read at 450 nm using a microplate reader (Model 680, BioRad). Receptor internalization was quantified as the percent loss of cell surface receptor in agonist-treated cells.

2.9. Data analysis

Curve-fitting and statistical analysis were conducted by use of GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, CA). Statistical significance of the differences between more than two groups was calculated by one-way ANOVA, followed by Turkey's post-test.

3. Results

3.1. Effect of sequential N-terminal truncation of hHK-1 on intracellular cAMP accumulation

The intracellular cAMP accumulation was measured to monitor Gs activation induced by peptide-NK1 interaction. hHK-1 induced cAMP accumulation can be abolished by the NK1 selective antagonist L-732,138 (Fig. 1C). Sequential truncation of N-terminal amino acid residues of hHK-1 had significant effect on the Gs-mediated adenylate cyclase (AC) activity. When applied to the NK1-expressing CHO cells, the hHK-1 peptide fragments with length between hHK-1 and hHK-1(7-11) resulted in a dose-dependent cAMP accumulation. There was no statistical difference in the maximal cAMP accumulation level elicited by the peptides though the maximal response to hHK-1(5-11) and hHK-1(6-11) occurred at 10 μM, while the potency (EC_{50}) was dramatically changed (Fig. 1A). It is interesting that hHK-1(2-11) had much lower EC_{50} value for cAMP accumulation than either hHK-1 or hHK-1(3-11). The third amino acid Lys should be crucial in determining the potency of the peptides to stimulate AC since the EC_{50} value of hHK-1(4-11) was almost 50 folds higher than that of hHK-1(3-11). Another important amino acid residue is the sixth position Gln because hHK-1(7-11) and hHK-1(8-11) lose their ability to stimulate the cAMP production even at a high concentration of 1 μM. These results indicated that peptide with the last 6 amino acids, which represented hHK-1(6-11), was the least length needed to activate the AC pathway.

3.2. Effect of sequential N-terminal truncation of hHK-1 on intracellular calcium mobilization

Intracellular calcium mobilization was detected to monitor Gq activation. Fig. 1C indicated that hHK-1 promoted intracellular calcium mobilization was abolished by the NK1 selective antagonist L-732,138. Fig. 1B showed that the potency of hHK-1 promoted intracellular calcium mobilization was marginally influenced by the N-terminal amino acid truncation until the hHK-1(7-11) truncation. The hHK-1 peptide fragments with lengths between hHK-1 and hHK-1(7-11) had similar EC_{50} and maximal response for intracellular calcium mobilization. The conserved C-terminal structure of tachykinins, hHK-1(7-11), was enough to be a full NK1 receptor agonist for Gq activation. The EC_{50}s of tested peptides for cAMP accumulation and calcium mobilization are compared in Table 1.

3.3. ERK1/2 phosphorylation induced by NK1 receptor activation

ERK1/2 phosphorylation could result from both Gs and Gq activation. Next ERK1/2 phosphorylation was evaluated in CHO cells expressing NK1 receptor. Fig. 2A demonstrated that hHK-1 and its C-terminal fragments strongly stimulated ERK1/2 phosphorylation in 10 min in a concentration-dependent way. Compared with hHK-1, all of the tested hHK-1 C-terminal fragments were able to activate ERK1/2 at a concentration of 1 μM, but the magnitude of the phosphorylation was gradually reduced (Fig. 4A). Though much lower than hHK-1, hHK-1(7-11) and hHK-1(8-11) did induce considerable extent of ERK1/2 phosphorylation. Both PKA inhibitor H89 and calcium chelator BAPTA-AM could significantly reduce the hHK-1 induced rapid ERK1/2 phosphorylation (Fig. 2B).

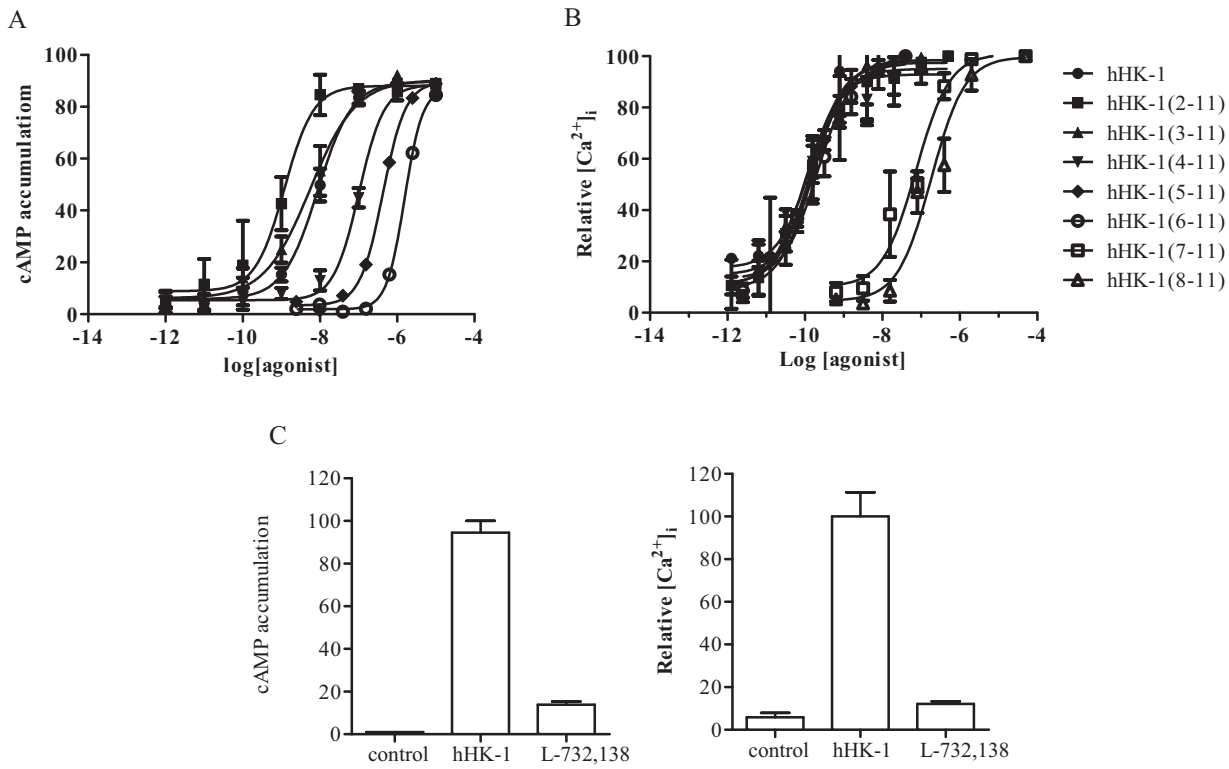

Fig. 1. Concentration-dependent curves of hHK-1 and its C-terminal fragments for (A) cAMP accumulation and (B) intracellular calcium mobilization. CHO cells expressing hNK1 receptor were stimulated with increasing concentrations of the indicated peptide agonists as described in Section 2. (C) NK1 selective agonist L-732,138 was used to identify that NK1 receptor was responsible for the intracellular cAMP accumulation and calcium mobilization. The CHO-hNK1 cells were pre-treated with or without L-732,138, and then stimulated with 1 μM hHK-1. Data shown were the means ± S.E.M. of three independent experiments ($N = 9$).

3.4. NF-κB activity enhanced by NK1 receptor activation

NK1 agonist led to NF-κB activation in different cell types. In the cells co-expressing NF-κB-luc and human NK1, using untreated cells as control, NF-κB-driven luciferase gene expression was significantly enhanced by hHK-1 and its C-terminal peptides ($p < 0.001$, Fig. 3). The level of luciferase activity was also demonstrated to gradually decrease with the progressive truncation of N-terminal amino acids of hHK-1 in a way similar to ERK1/2 phosphorylation. Meanwhile, compared with hHK-1, hHK-1(7-11) induced luciferase level was moderately reduced ($p < 0.05$) whereas hHK-1(8-11) induced luciferase level was significantly decreased ($p < 0.001$).

Table 1
Characterization of potencies for cAMP accumulation and Ca^{2+} mobilization induced by hHK-1 and its C-terminal fragments in CHO-hNK1 cells. Values represented means ± S.E.M. of three independent experiments, each performed in triplicate.

Peptide	EC_{50} (nM)	
	cAMP	Ca^{2+} release
hHK-1	8.79 ± 3.5	0.15 ± 0.05
hHK-1(2-11)	1.20 ± 0.8	0.14 ± 0.04
hHK-1(3-11)	5.59 ± 3.7	0.18 ± 0.07
hHK-1(4-11)	327.6 ± 25.6[a]	0.11 ± 0.03
hHK-1(5-11)	426.4 ± 36.5[a]	0.16 ± 0.06
hHK-1(6-11)	1378 ± 160.6[a]	0.22 ± 0.05
hHK-1(7-11)	NA[b]	74.5 ± 19.9[a]
hHK-1(8-11)	NA	183 ± 48[a]

[a] EC_{50} value was significantly different compared with hHK-1, $p < 0.001$ (one-way ANOVA followed by Turkey's post-teat).
[b] NA = not applied.

3.5. Receptor desensitization and endocytosis

GPCR signaling was terminated through receptor desensitization and endocytosis. The ability of the peptides for the NK1 receptor desensitization was determined by ERK1/2 phosphorylation assay due to the multi-pathway-convergent nature of ERK1/2 signaling, shown in Fig. 4. hHK-1 was able to induce almost 100% desensitization in the cells. The stepwise truncation of N-terminal amino acid of hHK-1 did not display a stepwise decrease of receptor desensitization. At a concentration of 1 μM, all the fragments except hHK-1(7-11) and hHK-1(8-11) are equal to hHK-1 in inducing receptor desensitization; while hHK-1(7-11) and hHK-1(8-11) are still capable to induce 80.4% and 57% receptor desensitization respectively.

The receptor internalization provoked by the indicated peptides was examined using a quantitative ELISA method. As depicted in Fig. 5, the rank order of the magnitude of NK1 receptor endocytosis was hHK-1(5-11) ≥ hHK-1(6-11) ≥ hHK-1(4-11) > hHK-1, while the rest of the tested peptides had similar abilities for NK1 receptor endocytosis to hHK-1.

3.6. Assessment of agonist functional selectivity

We compared the potencies of hHK-1 and its N-terminal truncated fragments to induce Gs and Gq responses. The amino acid sequences of peptides and binding affinity reported previously were listed in Table 2. All the tested peptides were compared for the potencies of intracellular cAMP accumulation and calcium mobilization (Table 1). The relative levels of cAMP accumulation, calcium mobilization, ERK1/2 phosphorylation, NF-κB activation, receptor desensitization and endocytosis were compared in Table 3, using hHK-1 as a reference "full agonist" set at 100%. All the

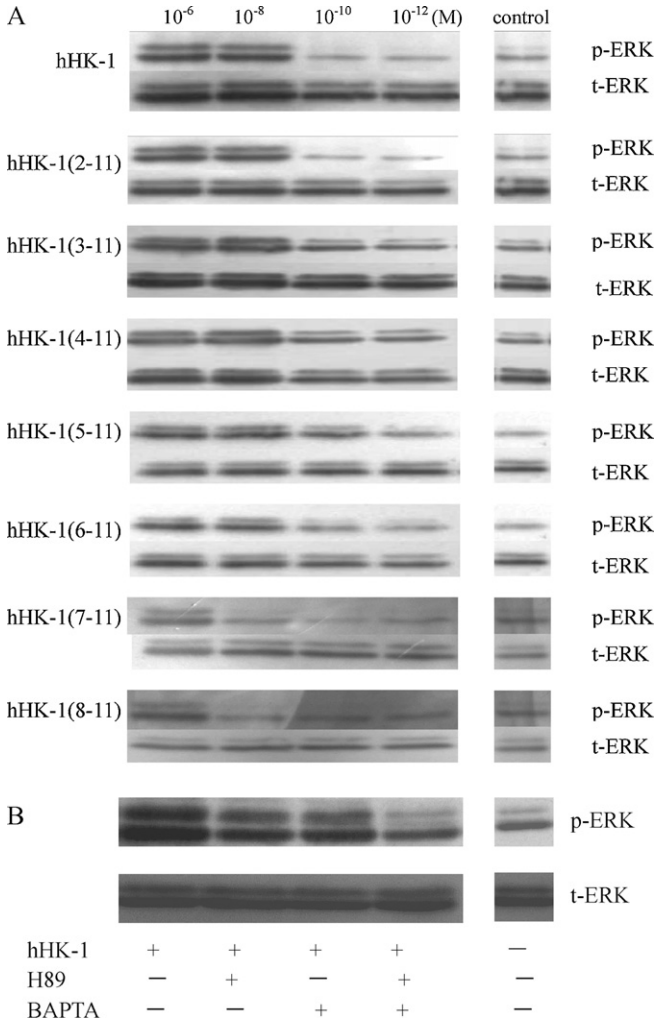

Fig. 2. (A) Concentration-dependent ERK1/2 phosphorylation stimulated by hHK-1 and its C-terminal fragments in human NK1 receptor expressed CHO cells. CHO-hNK1 cells were treated with increasing concentrations of hHK-1 or its C-terminal fragments (1 pM – 1 μM). (B) CHO-hNK1 cells were pre-incubated with 20 μM H89 (PKA inhibitor) or 20 μM BAPTA-AM (calcium chelator) and then stimulated with 1 μM hHK-1. Cells were lysed, and phosphorylated ERK1/2 was detected using Western-blotting method. The results were representative of at least three independent experiments.

peptides showed greater potency for calcium mobilization than for cAMP accumulation. The rank order of potencies for cAMP accumulation was hHK-1(2-11) > hHK-1(3-11) > hHK-1 ≫ hHK-1(4-11) > hHK-1(5-11) ≫ hHK-1(6-11). On the contrary, the potencies for calcium release were similar: hHK-1 ≈ hHK-1(2-11) ≈ hHK-1(3-11) ≈ hHK-1(4-11) ≈ hHK-1(5-11) ≈ hHK-1(6-11) ≫ hHK-1(7-11) ≫ hHK-1(8-11). However, it was noteworthy that the stepwise truncation of N-terminal amino acids caused gradual decrease in ERK1/2 phosphorylation level. Both PKA inhibitor H89 and cell-membrane permeating calcium chelator BAPTA-AM could reduce agonist-promoted ERK1/2 phosphorylation in this CHO cell system. ERK1/2 could be viewed as an indicator of the convergence of Gs and Gq pathway. The receptor desensitization was similar among the peptides except hHK-1(7-11) and hHK-1(8-11), showing that the N-terminal truncations having trivial effect on the induction of receptor desensitization. hHK-1(6-11) should be considered as the active core peptide fragment retaining both activities to stimulate cAMP production, calcium mobilization and the downstream ERK1/2 phosphorylation meanwhile inducing total receptor desensitization.

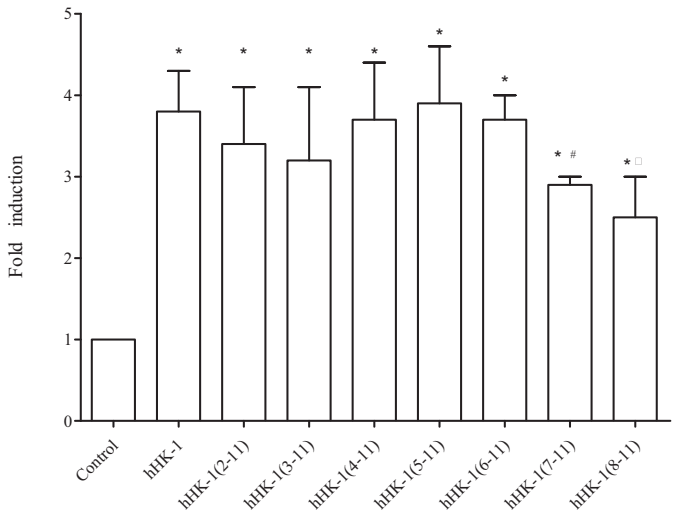

Fig. 3. Effects of hHK-1 and its C-terminal fragments on stimulation of NF-κB in CHO cells co-expressing human NK1 and NF-κB-luc. CHO-hNK1 cells transiently transfected with NF-κB-luc reporter gene were incubated with 1 μM hHK-1 or its C-terminal fragments. Untreated cells were used as controls. The results were presented as relative fold of untreated controls, which was defined as 1.0. The data were representative of three independent experiments with triplicates. *Value was significantly different from untreated control ($p < 0.001$, one-way ANOVA followed by Turkey's post-teat). #Value was significantly different from hHK-1 treated cells ($p < 0.05$, one-way ANOVA followed by Turkey's post-teat).

4. Discussion

In present work we demonstrated that NK1 receptor could be activated by hHK-1 and its C-terminal fragments in a way of functional selectivity, leading to differential receptor signaling, desensitization and endocytosis. To investigate the ligand-directed receptor activation of NK1 receptor, a series of N-terminal truncated fragments of hHK-1 were synthesized and tested by two different functional assays. The sequential truncation of N-terminal amino acid of hHK-1 significantly decreased its potency for cAMP accumulation, while having almost no effect on the potency of intracellular calcium mobilization. All peptides showed relative bias toward Gq over Gs activation. According to their potencies for cAMP accumulation and calcium mobilization, they could be divided into three groups: hHK-1, hHK-1(2-11) and hHK-1(3-11), which show nanomolar EC_{50}s for cAMP accumulation and subnanomolar EC_{50}s for calcium mobilization; hHK-1(4-11), hHK-1(5-11) and hHK-1(6-11), which show micromolar EC_{50} for cAMP accumulation and subnanomolar EC_{50} for calcium mobilization; hHK-1(7-11) and hHK-1(8-11), which show relative higher EC_{50} for calcium mobilization and no ability for cAMP accumulation. These results exhibited differential functional selectivity of NK1 receptor by hHK-1 and its C-terminal fragments.

We observed that the removal of T1 improved the potency of cAMP accumulation, while the truncation of the first three amino acids (TGK) caused the EC_{50} value of cAMP accumulation increasing dramatically from 6.9 nM to 327.6 nM. hHK-1(7-11) and hHK-1(8-11), even at high concentration (1 μM), were not able to stimulate cAMP accumulation. hHK-1 mostly resembled SP among the mammalian tachykinins. They were both undecapeptide ligands for NK1 and identical with the last six amino acids (Table 2). Previous receptor modeling studies had predicted that the N terminal residues R1 and K3 of SP might interact with the N-terminus of rat NK1. The Q6 might have interaction with the core of the receptor [14,15]. It has been described that NK1 had two high-affinity binding states of which the higher one corresponds to Gs signaling and the lower one corresponds to Gq signaling [16]. Our results were consistent with these conclusions. First, the abilities

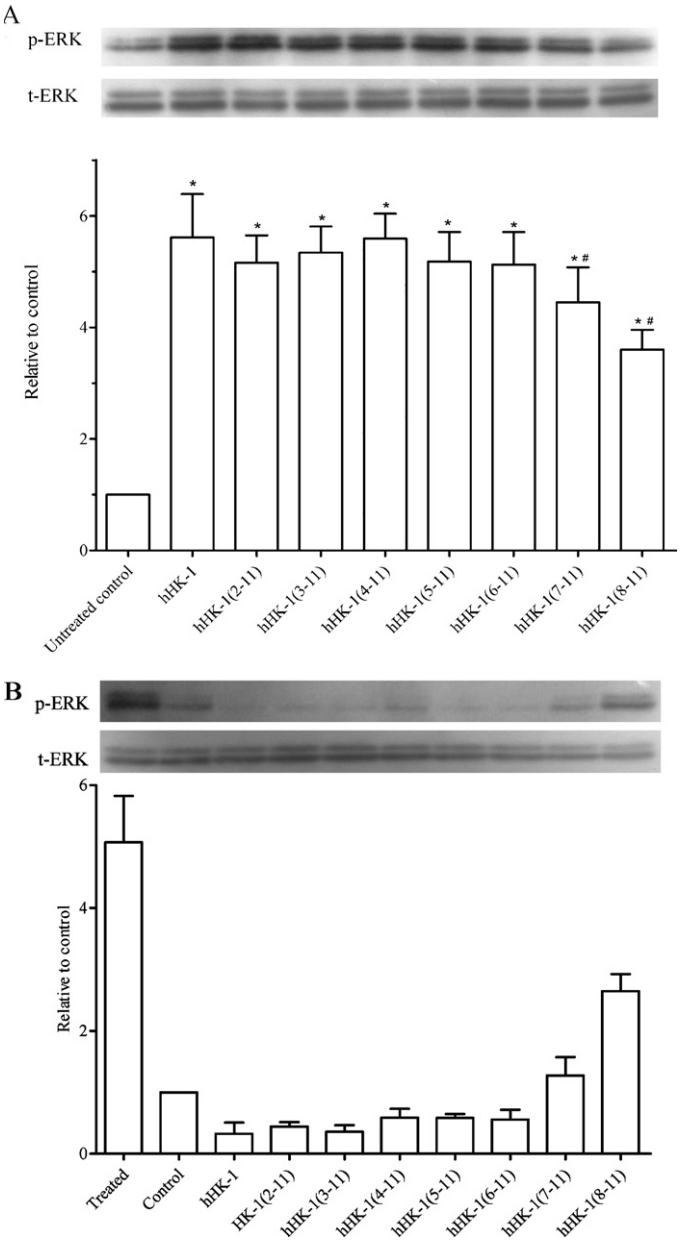

Fig. 4. Human NK1 receptor desensitization induced by hHK-1 and its C-terminal fragments measured as ERK1/2 phosphorylation level in CHO cells expressing human NK1 receptor. (A) Rapid ERK1/2 phosphorylation induced by 1 μM hHK-1 or its C-terminal fragments in CHO-hNK1R cells was gradually decreased. (B) Human NK1 receptor desensitization by hHK-1 and its C-terminal fragments was equal except hHK-1(7-11) and hHK-1(8-11). The results were representative of at least three independent experiments. CHO-hNK1 cells were grown in RPMI 1640 medium containing 2% FBS for 24 h and serum-starved for 4 h, then treated as described in Section 2. Cells were lysed and total amount of 20 μg protein were loaded to detect the total and phosphorylation ERK1/2 respectively. Data were normalized by total ERK1/2 and presented as relative fold to untreated control which was defined as 1.0. For each sample, data were quantified by densitometric analysis shown as means ± S.E.M. for three independent experiments. *Value was significantly different from untreated control ($p < 0.001$, one-way ANOVA followed by Turkey's post-teat). #Value was significantly different from hHK-1 treated cells ($p < 0.001$, one-way ANOVA followed by Turkey's post-teat).

for cAMP accumulation of hHK-1 and its C-terminal fragments were correspondent with their binding affinities of NK1 receptor (Tables 1 and 2). Second, the loss of the amino acids at 1, 3 and 6 position would impair the Gs-mediated AC activation. Of course, more work was still needed to ensure the role of these three amino acids in the hHK-1-NK1 interaction.

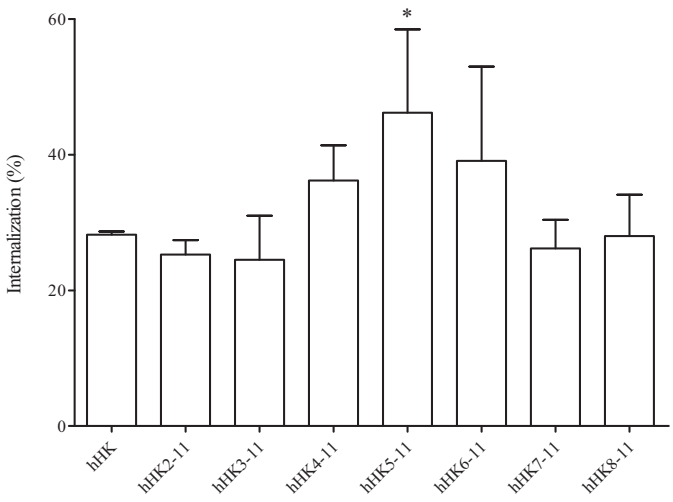

Fig. 5. Human hemokinin and its C-terminal fragments (1 μM) induced human NK1 internalization in CHO cells. Internalization of HA-tagged human NK1 was determined by ELISA method as described in Section 2. Using untreated cells as control, receptor internalization was quantified as the percent loss of cell surface receptor in agonist-treated cells. Data were represented as the means ± S.E.M. for 3–6 independent experiments performed in triplicate. *Value was significant different from hHK-1 treated cells ($p < 0.001$, one-way ANOVA followed by Turkey's post-teat).

ERK1/2 was one of the important members of mitogen-activated protein kinase (MAPK) family. It could be activated by various G proteins and even non G-Proteins such as β-arrestins [17,18]. Significant rapid ERK1/2 phosphorylation was observed when the CHO-hNK1 cell were treated with hHK-1. Using PKA inhibitor H89 and calcium chelator BAPTA-AM, it could be seen that both Gs and Gq activation had effect role in the ERK1/2 phosphorylation (Fig. 2B). Rapid ERK1/2 phosphorylation was compared among all the tested peptides. The progressive truncation of N-terminal amino acid of hHK-1 caused a gradual loss of rapid ERK1/2 phosphorylation in this CHO cell system, which was not totally parallel with either cAMP accumulation or calcium mobilization. This phenomenon was reported here for the first time. At the concentration of 1 μM, hHK-1(5-11) and hHK-1(6-11) showed significant decrease of cAMP accumulation but maintained full efficacy for calcium mobilization, having moderate declination of ERK1/2 phosphorylation (~20%); while hHK-1(7-11) and hHK-1(8-11) showed no activity for cAMP accumulation and relative lower efficacies for calcium mobilization, having significant declination of ERK1/2 phosphorylation (~40%). This result reinforced that both of the second messengers contributed to the downstream rapid ERK1/2 phosphorylation. Further investigation was needed to understand how and at what extent these two messengers induced the rapid ERK1/2 phosphorylation. Generally ERK1/2 were mainly related to anabolic processes, such as cell division, growth, and differentiation. For tachykinin agonists of NK1, it has been evidenced that SP-induced ERK1/2 phosphorylation controlled NF-κB-dependent chemokine expression by regulating the transactivation activity of p65 subunit [19,20]. NF-κB was a ubiquitous transcription factor that played a critical role in the expression of pro-inflammation genes in different cell systems [21,22]. Here we examined the influence of N-terminal truncation on the NF-κB activity stimulated by hHK-1. Our result showed that the magnitude of NF-κB activity induced by each peptide was similar to the correspondent ERK1/2 phosphorylation level (Table 3).

Because both of receptor desensitization and endocytosis regulate the magnitude of receptor signaling, they were examined to evaluate the effect of hHK-1 N-terminal truncation on NK1 receptor desensitization and endocytosis. Due to the multi-

Table 2
Sequences of amino acid and binding affinities of SP, hHK-1 and hHK-1 C-terminal fragments for binding to NK1.

	Position											Binding affinity for NK1R			
	1	2	3	4	5	6	7	8	9	10	11	IC_{50} (nM)	Radiolabled ligand	Receptor resource	Reference
SP	R	P	K	P	Q	Q	F	F	E	L	M-NH_2	0.1	[I125]SP	Recombinant human NK1R	Kurtz et al. [2]
												2.1	[H3][Pro9]SP	Rat urinary bladder	Torrens et al. [13]
hHK-1	T	G	K	A	S	Q	F	F	E	L	M-NH_2	1.8	[I125]SP	Recombinant human NK1R	Kurtz et al. [2]
hHK-1(2-11) (EKA/B)		G	K	A	S	Q	F	F	E	L	M-NH_2	0.8	[I125]SP	Recombinant human NK1R	Kurtz et al. [2]
hHK-1(3-11)			K	A	S	Q	F	F	E	L	M-NH_2	NA			
hHK-1(4-11)				A	S	Q	F	F	E	L	M-NH_2	9.2	[I125]SP	Recombinant human NK1R	Kurtz et al. [2]
hHK-1(5-11)					S	Q	F	F	E	L	M-NH_2	NA			
hHK-1(6-11)						Q	F	F	E	L	M-NH_2	NA	[I125]SP	Recombinant human NK1R	Torrens et al. [13]
hHK-1(7-11)							F	F	E	L	M-NH_2	NA			Liu et al. [12]
hHK-1(8-11)								F	E	L	M-NH_2	NA			

Table 3
Comparison of efficacies of hHK-1 and its C-terminal fragments to activate distinct functional pathways. Cells were treated with 1 μM indicated peptides and intracellular cAMP accumulation, calcium mobilization, ERK1/2 phosphorylation, NF-κB activity, receptor desensitization and endocytosis were measured as described in Section 2. Using hHK-1 as a reference "full agonist" set at 100%, the relative efficiencies were expressed as that of hHK-1 at 1 μM.

Peptide	Relative efficacy to hHK-1 at 1 μM (%)					
	cAMP	Ca^{2+} release	ERK1/2 phosphorylation	NF-κB activity	Desensitization	Internalization
hHK-1	100.0	100.0	100.0	100.0	100.0	100.0
hHK-1(2-11)	97.8	100.6	94.6	94.6	98.1	89.5
hHK-1(3-11)	103.7	102.7	94.1	96.3	99.1	86.8
hHK-1(4-11)	100.1	105.0	94.2	88.0	96.3	128.5
hHK-1(5-11)	65.8	104.9	86.6	93.7	96.3	164.0
hHK-1(6-11)	17.2	103.6	80.6	91.8	97.0	138.5
hHK-1(7-11)	<1	84.7	68.9	77.3	83.4	92.9
hHK-1(8-11)	<1	48.0	60.7	60.3	59.2	99.1

pathway-convergent nature of ERK1/2 phosphorylation, it was determined by stimulation with individual peptide and re-stimulation with hHK-1 to evaluate the receptor desensitization. In Fig. 4, it can be seen that unlike ERK1/2 phosphorylation, the sequential truncation of N-terminal amino acid of hHK-1 did not induce the gradual loss of the ability for receptor desensitization. For receptor internalization, our results demonstrated for the first time that the systematical removal of N-terminal amino acid of hHK-1 does not decrease the magnitude of NK1 endocytosis. Taken together, these data indicated that the abilities of a tachykinin NK1 receptor agonist to produce the signal activation, to induce receptor desensitization and to provoke receptor internalization could be disassociated in a ligand-dependent way.

The potential influence of functional selectivity has been realized by many laboratories investigating different GPCRs. This phenomenon not only provided an intriguing picture of mechanisms but also a new perspective of drug development. One example was the agonist-directed regulation of μ opioid receptor. Both morphine and DAMGO were potent agonists of μ receptor for the inhibition of cAMP production and activation of G protein-coupled inward rectifying potassium channel [24,42,45]. However, using fluorescence microscopy, it was visualized that only DAMGO could induce receptor endocytosis with high efficacy while morphine did not [43,44]. The deficiency of morphine for the induction of μ receptor endocytosis was considered to be highly correlated with morphine-induced opioid-tolerance in clinical therapy [25,42]. As to NK1, its functional selectivity by different tachykinin agonists has been reported by several groups. Vigna demonstrated that the C-terminal fragments with different chain lengths from SP initiated the same maximal level of inositol phosphate formation but were only able to partially induce receptor desensitization [26]. SP and its analogues such as rantakinin C were shown to preferentially produce signal activation or desensitization of NK1 [27]. Correspondent with the complexity of receptor signaling and trafficking, NK1 played a plethora of biological roles including pain transmission, immune regulation and vasodilatation. Among the tested peptides, hHK-1, hHK-1(2-11) (which is the same peptide sequence as EKA/B) and hHK-1(4-11) were the predicted productions of *tac*-4 gene [2,4,28]. It was a reasonable speculation that *in vivo* functional selectivity might result from differential pre-tachykinin processing or larger tachykinin peptides degradation. Even more complicated was that the procedure of pre-tachykinin processing and tachykinin peptides degradation could be tissue-type dependent. These meant that our findings might have some interesting implications for both the biofunction exploration and drug development of NK1 receptor. First, in organisms containing several ligands for NK1, functional selective mechanisms could be provided for distinct ligands to transfer specific information through this single receptor. It would be helpful to explain the diversity of the biofunctions mediated by the single receptor. Secondly, once the detailed relationships between NK1 signaling pathways and its biofunctions were illustrated, especially the function of specific signaling pathway set in different physiological and pathological conditions, it was possible to design ligands with particular intrinsic activity for NK1 signaling to improve the therapeutic efficiency or decrease the side effects.

In a summary, though more work was needed to examine our results in physiological or pathological condition, which may be different from our model system owing to the availability of the respective signaling molecules, however, our work would facilitate both the further exploration of hHK-1 and related peptides mediated biological roles through NK1 activation and the future development of the synthetic ligands.

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