Biochemical Pharmacology 81 (2011) 661–668

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

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

Lingyun Mou [a,b], Yanhong Xing [a,b], Ziqing Kong [a,b], Ying Zhou [a,b], Zongyao Chen [a,b], Rui Wang [a,b,c,*]

[a] Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Medicine, Lanzhou University, 222 Tian Shui South Road, Lanzhou 730000, PR China
[b] Institute of Biochemistry and Molecular Biology, School of Life Sciences, Lanzhou University, 222 Tian Shui South Road, Lanzhou 730000, PR China
[c] State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong, PR China

ARTICLE INFO

Article history:
Received 20 September 2010
Accepted 8 December 2010
Available online 17 December 2010

Keywords:
Hemokinin-1
Functional selectivity
Neurokinin-1 receptor
ERK1/2
NF-κB

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.

* Corresponding author at: Institute of Biochemistry and Molecular Biology, School of Life Sciences, Lanzhou University, 222 Tian Shui South Road, Lanzhou 730000, PR China. Tel.: +86 931 8912567; fax: +86 931 8912567.
E-mail addresses: wangrui@lzu.edu.cn, bcrwang@polyu.edu.hk, muly2007@lzu.cn (R. Wang).

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.12.007

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.