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Pharmacological characterization of rat VD-hemopressin(α), an α -hemoglobin-derived peptide exhibiting cannabinoid agonist-like effects in mice

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ABSTRACT

Hemopressin and related peptides have shown to function as the endogenous ligands or the regulator of cannabinoid receptors. Moreover, hemopressin and its truncated peptides were also reported to produce a slight modulatory effect on opioid system. In the present work, based on the amino acid sequence analyses of hemoglobin subunit α , rat VD-hemopressin(α) [(r)VD-Hp α] was predicted as a cannabinoid peptide derived from rat α -hemoglobin. Furthermore, (r)VD-Hp α was synthesized and characterized in a series of in vitro and in vivo assays. Our results demonstrated that (r)VD-Hp α induced neurite outgrowth in Neuro 2A cells via CB₁ receptor. In the tail-flick assay, (r)VD-Hp α dose-dependently exerted central antinociception through CB₁ receptor, but not CB2 and opioid receptors. In mice, supraspinal administration of (r)VD-Hpa produced dose-dependent hypothermia, which was partially reduced by the CB₁ receptor antagonist AM251, but not by the antagonists of CB₂ and opioid receptors. In addition, (r)VD-Hp α caused hypoactivity after intracerebroventricular injection, and this effect was insensitive to the antagonists of cannabinoid and opioid receptors. Further assessment of the side-effects demonstrated that (r)VD-Hp α evoked the limited effects on gastrointestinal transit at antinociceptive doses, but repeated i.c.v. injection of (r)VD-Hpa induced development of antinociceptive tolerance. Taken together, these data suggest that the predicted peptide (r)VD-Hp α produces antinociception, hypothermia and hypoactivity via different pharmacological mechanisms, at least partially, which may offer an attractive strategy for separating cannabinoid analgesia from hypoactivity. Moreover, it implies that (r)VD-Hp α has therapeutic potential in pain management with limited side-effects.

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1. Introduction

Cannabinoids have shown potent therapeutic roles in clinical trials and in animal models of various pain (Bushlin et al., 2010; Krustev et al., 2016; Pacher et al., 2006; Richardson, 2000). Cannabinoid receptors including CB₁ and CB₂ receptors are class A members of the G protein coupled receptor superfamily (Pertwee, 1997). The previous data

http://dx.doi.org/10.1016/j.npep.2016.12.006 0143-4179/© 2016 Elsevier Ltd. All rights reserved. demonstrated that CB₁ receptor was widely distributed in the central nervous system and peripheral tissues, while CB₂ receptor was mainly expressed in immune cells (Pacher et al., 2006; Pertwee, 1997, 2001). However, both CB₁ and CB₂ receptors were recently proved to play a critical role in pain modulation (Agarwal et al., 2007; Ibrahim et al., 2006; Pertwee, 2001).

Hemopressin and related peptides were identified as the novel ligands of cannabinoid receptors (Bomar and Galande, 2013; Gomes et al., 2009; Heimann et al., 2007). Hemopressin behaved as an inverse agonist or antagonist of the CB₁ receptor in vitro and in vivo (Dodd et al., 2010; Heimann et al., 2007). Firstly, hemopressin was reported as a novel bioactive peptide derived from hemoglobin, which could inhibit peripheral hyperalgesic responses in the inflammatory pain model, independently of opioid receptors (Dale et al., 2005a, 2005b). In addition, central or systemic administration of hemopressin caused antinociceptive actions, anorectic activities and anxiogenic-like effects (Bomar and Galande, 2013; Dodd et al., 2010; Fogaca et al., 2015; Heimann et al., 2007; Toniolo et al., 2014). However, hemopressin recently has shown to specifically activate CB₁ receptor in the

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Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; CB₁, cannabinoid receptor type 1; CB₂, cannabinoid receptor type 2; CP55,940, (2)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; dNPA, *D*.NP(*N*-Me)AFLFQPQRFamide; Hu-210, Δ^8 -tetrahydrocannabinol dimethyl heptyl; i.c.v., intracerebroventricular; MPE, maximum percentage effect; PBS, Phosphate Buffer Solution; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; (m)VD-Hp α , mouse VD-hemopressin(α); WIN55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1- napthalenvlmethanone.

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 $[^{35}S]$ GTPγS assays (Szlavicz et al., 2015). Furthermore, three hemopressin related peptides were identified as the endogenous agonists of cannabinoid receptors in vitro (Gomes et al., 2009). In addition, our recent data demonstrated that mouse VD-hemopressin(α) [(m)VD-Hp α , VDPVNFKLLSH-OH] was involved in pain modulation, cardiovascular action, food intake, locomotor activity, gastrointestinal and hypothermic modulation (Han et al., 2014; Li et al., 2014; Li et al., 2016; Pan et al., 2014). Central injection of (m)VD-Hp α produced antinociceptive effects in a CB₁ receptor-dependent manner (Han et al., 2014). Similar to the classical cannabinoid WIN55,212-2, (m)VD-Hp α induced antinociceptive tolerance after i.c.v. administration (Han et al., 2014; Pan et al., 2014). At the supraspinal level, (m)VD-Hp α also evoked hypothermia and hypoactivity in mice (Han et al., 2014). However, intrathecal injection of (m)VD-Hp α decreased arterial pressure via a non-CB₁ and non-CB₂ mechanism (Li et al., 2014).

A vast body of evidence suggests an important role of hemopressin and related peptides in the development of peptide-based pharmacological tools for the study of the endocannabinoid system (Bauer et al., 2012; Bomar and Galande, 2013; Dale et al., 2005b; Gomes et al., 2009; Heimann et al., 2007; Toniolo et al., 2014). The amino acid sequence of hemoglobin subunit α was reported to have apparent homology in different mammalian (Fig. 1). Based on these sequence analyses, we predicted that rat VD-hemopressin(α) [(r)VD-Hp α , VDPVNFK**F**LSH-OH] would be produced from α -chain of rat hemoglobin. It is worthy to note that hemopressin related peptides including (m)VD-Hp α were isolated from mouse brain. Thus, the C-terminal amino acid sequence of (r)VD-Hp α quite resembled that of hemopressin (PVNFKFLSH-OH).

To date, the reported data have shown that hemopressin and related peptides induced complex biological activities via different mechanisms, and the structure-activities relationship of these cannabinoid peptides remains unclear (Bauer et al., 2012; Dvoracsko et al., 2016; Gomes et al., 2009; Heimann et al., 2007; Szlavicz et al., 2015). In order to understand the structural and pharmacological properties of hemopressin and related peptides, different peptidic ligands of

	10	20	30	40	
Human	MULSPADETNUE	DODVE ABAGE	NO FATARME	LSEPTT	4
Bat	MULSADDETNIKNO		VGPEALORME	AAFPTT	4
Mouse	MVLSGEDKSNIK	A GE TE GEGAE	NG EAL PRME	ASEPTT	4
Bovine	MULSABDEGNUE	O GEVECEAAE	YGREALPRME	LSEPTT	4
Horse	MVLSAADKINVKAA	2 SKVCGBAGE	FGAEALERME	LCFPTT	4
Chimpanzee	MVLSPACKTNVKAR	NGKVGAHAGE	YGAEALERME	LSEPTT	4
African elephant	MVLSDNDKINVK	WSKVGDHASI	YVAEALERME	FSEPTT	4
	50	60	70	80	
Human	KTYFEHEDISEGS	CARCHERKAN	DATINEVARY	DEMENA	8
Rat	KTYESHIDVSEGS	CVKAHGRKVA	DALARADAD	DIEGA	8
Mouse	KTYFEHEDVSEGS	CVKCHGRKVA	DALANPAGHI	DLEGA	8
Bovine	KTYFEHEDLSHGS	CVKGHGAKVA	AALTRAVEHI	DDLEGA	8
Horse	KTYFEHEDLSHGS	CVKAHGEKVC	DAITLAVGHI	DDLEGA	8
Chimpanzee	KTYFEHEDLSEGS	CVKCHGSKVA	DALTNEVARY	DMENA	8
African elephant	KTYFEHEDLGHGS	QVKAHGSKVC	DALICAVGHI	DIFSA	8
	90	100	110	120	
Human	90 ISALSDIHAHKIRV	100 DEVNER	110	120 EADETE	12
Human Rat	90 LSALSDLHAHKLRV LSTLSDLHAHKLRV	100 DEVNEKTISH	110 CLINTLAADI CLINTLACDE	120 FAEFTP FGDFTP	12
Human Rat Mouse	90 LSALSDLHAHKLRV LSTLSDLHAHKLRV LSALSDLHAHKLRV	100 /DPVNFKILSH /DPVNFKILSH /DPVNFKILSH	110 CLUVIUAAHI CLUVIUACHI CLUVIUASHI	120 FAEFTP FGDFTP FADFTP	12 12 12
Human Rat Mouse Bovine	90 ISALSDLHAHKLRV LSTLSDLHAHKLRV LSALSDLHAHKLRV LS <mark>B</mark> LSDLHAHKLRV	100 DEVNERTLSE DEVNERTLSE DEVNERTLSE DEVNERTLSE	110 CLIVTDAAHI CLIVTDACHH CLIVTDASHH SLIVTDASHI	120 PAEFTP EGDFTP FADFTP ESDFTP	12 12 12
Human Rat Mouse Bovine Horse	90 LSALSDLHAHKLRV LSTLSDLHAHKLRV LSALSDLHAHKLRV LSELSDLHAHKLRV LSNLSDLHAHKLRV	100 VDEVNER LSH VDEVNER LSH VDEVNER LSH VDEVNER LSH VDEVNER LSH	110 CLIVIDACHI CLIVIDACHI CLIVIDACHI SLIVIDASHI CLISIDAVHI	120 FAEFTP FGDFTP FADFTP FSDFTP FNDFTP	12 12 12 12 12
Human Rat Mouse Bovine Horse Chimpanzee	90 LSALSDIHAHKIRV LSTISDIHAHKIRV LSALSDIHAHKIRV LSALSDIHAHKIRV LSALSDIHAHKIRV	100 VDEVNEK LSH VDEVNEK LSH VDEVNEK LSH VDEVNEK LSH VDEVNEK LSH VDEVNEK LSH	110 CLIVIDAAHI CLIVIDACHI CLIVIDASHI SLIVIDASHI CLISIDAVHI CLIVIDAAHI	120 FAEFTP FGDFTP FADFTP FNDFTP FNDFTP FAEFTP	12 12 12 12 12
Human Rat Mouse Bovine Horse Chimpanzee African elephant	90 LSALSDLHAHKLRV LSTLSDLHAHKLRV LSDLSDLHAHKLRV LSDLSDLHAHKLRV LSALSDLHAHKLRV LSALSDLHAHKLRV LSALSDLHAHKLRV	100 VDEVNEK VDEVNEK VDEVNEK VDEVNEK VDEVNEK VDEVNEK VDEVNEK VDEVNEK	110 CHIVTHAFI CHIVTHACE CHIVTHASE SHIVTHASE CHIVTHASE CHIVTHASE CHIVTHASE CHIVTHSE CHIVTHSE	120 FADFTP FGDFTP FADFTP FSDFTP FADFTP FADFTP FTDFTP	12 12 12 12 12 12 12
Human Rat Mouse Bovine Horse Chimpanzee African elephant	90 ISALSDIHAHKIRV ISTISDIHAHKIRV ISPISDIHAHKIRV ISPISDIHAHKIRV ISPISDIHAHKIRV ISPISDIHAHKIRV ISPISDIHAHKIRV	100 VDPVNFK LSH VDPVNFK LSH VDPVNFK LSH VDPVNFK LSH VDPVNFK LSH VDPVNFK LSH VD-Hpa	110 CLIVIDAE CLIVIDACE SLIVIDASE SLIVIDASE CLIVIDASE CLIVIDASE CLIVIDASE CLIVIDASE	120 EAD FTP EAD FTP ESD FTP END FTP EAD FTP EAD FTP	12 12 12 12 12 12 12
Human Rat Mouse Bovine Horse Chimpanzee African elephant Human	90 ISALSDIHAHKIRV ISTISDIHAHKIRV ISPISDIHAHKIRV ISPISDIHAHKIRV ISALSDIHAHKIRV ISALSDIHAHKIRV 130 MHASIDKFIASVS	100 VDEVNEKT LSE VDEVNEKT LSE VDEVNEKT LSE VDEVNEKT LSE VD-HPA STVLTSKYR	110 CHATRAFI CLIVILACE CLIVILASEI CLIVILASEI CLIVILASEI CLIVILASEI CLIVILSEC	120 FADFTP FGDFTP FADFTP FSDFTP FNDFTP FADFTP FDFTP	12 12 12 12 12 12 12 12
Human Rat Mouse Bovine Horse Chimpanzee African elephant Human Kat	90 LSALSDIHAHKIRV LSTLSDIHAHKIRV LSALSDIHAHKIRV LSALSDIHAHKIRV LSALSDIHAHKIRV LSALSDIHAHKIRV 130 AVHASLDKFIASVS MHASLDKFIASVS MHASLDKFIASVS	100 VEPVNEKISH VEPVNEKISH VEPVNEKISH VEPVNEKISH VEPVNEKISH VEPVNEKISH VEPVNEKISH VEPNEKISH VEPNEKISH VEPKEKISH VEFKEKISH	110 CHATTRAGI CLUTIAGI CLUTIAGI CLUTIAGI CLUTIAGI CLUTIAGI CLUTIGSI CLUTISSI CLUTISSI	120 FADETP FGDETP FADETP FSDETP FNDETP FADETP FADETP	120 120 120 120 120 120 120 120 120
Human Rat Mouse Bovine Horse Chimpanzee African elephant Human Rat Mouse	90 ISALSDIHAHKIRV ISTISDIHAHKIRV ISDISDIHAHKIRV ISDISDIHAHKIRV ISALSDIHAHKIRV ISALSDIHAHKIRV 130 AVHASIDKFIASV AVHASIDKFIASV AVHASIDKFIASV AVHASIDKFIASV	100 DEVNEKISH DEVNEKISH DEVNEKISH DEVNEKISH DEVNEKISH DEVNEKISH VD-HPa TVJISKYR SIVJISKYR	110 CLIVITACE CLIVITACE SCLIVITASE SCLIVITASE CLISITASE CLIVITASE CLIVITASE CLIVITASE	120 FADETP FGDETP FADETP FSDETP FADETP FADETP FTDETP	12 12 12 12 12 12 12 12 12 12 12 12 12 1
Human Rat Mouse Bovine Horse Chimpanzee African elephant Human Kat Mouse Bovine	90 ISALSDIHAHKIRV ISTISDIHAHKIRV ISPISDIHAHKIRV ISPISDIHAHKIRV ISPISDIHAHKIRV ISALSDIHAHKIRV 130 AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS	100 VDFVNFKILSF VDFVNFKILSF VDFVNFKILSF VDFVNFKILSF VDFVNFKILSF VDFVNFKILSF VD-HPA STVLTSKYR STVLTSKYR STVLTSKYR	110 CHATRAFI CHATRASI CHATRASI SHATRASI SHATRASI CHATRASI CHATRASI CHATRASI CHATRASI CHATRASI	120 FAETP FGDFTP FADTTP FSDFTP FNDFTP FAETP FIDFTP	12 12 12 12 12 12 12 12 12 12 12 12 12 1
Human Rat Mouse Bovine Horse Chimpanzee African elephant Human Kat Mouse Bovine Horse	90 LSALSDIHAHKIRV LSTLSDIHAHKIRV LSALSDIHAHKIRV LSALSDIHAHKIRV LSALSDIHAHKIRV LSALSDIHAHKIRV 130 AVHASLDKFIASV AVHASLDKFIASV AVHASLDKFIASV AVHASLDKFIASV AVHASLDKFIASV AVHASLDKFIASV	100 VDFVNFRILSF VDFVNFRILSF VDFVNFRILSF VDFVNFRILSF VDFVNFRILSF VDFVNFRILSF VDFVNFRILSF VDFVNFRILSF VDFVNFRILSF STVLTSKYR STVLTSKYR STVLTSKYR	110 CLIVITAAEI CLIVITASEI SLIVITASEI SLIVITASEI CLIVITASEI CLIVITASEI CLIVITASE	120 FASTP FGDFTP FASTP FSDFTP FNDFTP FASTP FTSTP	121 121 121 121 121 121 121 121 121 121
Human Rat Mouse Bovine Horse Chimpanzee African elephant Human Rat Mouse Bovine Horse Chimpanzee	90 ISALSDIHAHKIRV ISTISDIHAHKIRV ISDISDIHAHKIRV ISDISDIHAHKIRV ISALSDIHAHKIRV ISALSDIHAHKIRV 130 AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS AVHASIDKFIASVS	100 DEVNER LSF DEVNER LSF DEVNER LSF DEVNER LSF DEVNER LSF VDEVNER LSF VDEVNER LSF VDEVNER LSF VD-Hpa STVLTSKYR STVLTSKYR STVLTSKYR STVLTSKYR STVLTSKYR	110 CHATRAGI CHATRAGI CHATRAGI SLIVIIASI CHATRAGI CHATRAGI CHATRAGI CHATRAGI	120 FAETP FGITTP FAITP FSIETP FNIETP FAETP FAETP	12 12 12 12 12 12 12 12 12 12 12 12 12 1

Fig. 1. Alignment of the peptide sequences of the hemoglobin subunit α of human, rat, mouse, bovine, horse, chimpanzee and African elephant. VD-hemopressin(α) (VD-Hp α) is underlined. The predicted peptide sequence of (r)VD-Hp α is VDPVNFKFLSH-OH. The NCBI Protein accession numbers are as follows: human hemoglobin subunit α , NP_000508; rat hemoglobin subunit α , NP_037228; mouse hemoglobin subunit α , NP_032244; bovine hemoglobin subunit α , NP_001070890; chimpanzee hemoglobin subunit α , NP_001036091; African elephant hemoglobin subunit α , NP_001267813.

cannabinoid receptors will be further investigated. Recently, hemopressin and its truncated peptides were reported to produce a slight modulatory effect on opioid system (Szlavicz et al., 2015). In competitive receptor binding assays, both hemopressin and hemopressin(1–7) displayed a slight mu-opioid affinity by measuring in whole rat brain membrane homogenate and the CHO cells overexpressing mu-opioid receptors (Szlavicz et al., 2015). Moreover, [³⁵S]GTP γ S binding assays also revealed the G-protein-stimulating effect of hemopressin in the cells expressing mu-opioid receptors (Szlavicz et al., 2015). Therefore, the present work was designed to study the total pharmacological profiles of the predicted peptide (r)VD-Hp α in a series of classical functional models. In addition, the role of cannabinoid and opioid receptors in (r)VD-Hp α -induced activities was also investigated by using the selective antagonists of CB₁, CB₂ and opioid receptors.

2. Materials and methods

2.1. Drugs

The peptides including (r)VD-Hp α (VDPVNFKFLSH-OH) and (m)VD-Hp α (VDPVNFKLLSH-OH) were synthesized by manual solid-phase Fmoc (N-fluorenylmethoxycarbonyl) chemistry, as described previously (Han et al., 2014). The crude peptide was purified by preparative reversed-phase high-performance liquid chromatography using a C18 column as the solid. The identity of these peptides was confirmed using an electrospray ionization mass spectrometer (ESI-Q-TOF maXis-4G, Bruker Daltonics). The purity of the peptides was determined using analytical reversed-phase high-performance liquid chromatography.

In this study, WIN55,212-2, and naloxone were obtained from Sigma-Aldrich. The selective cannabinoid receptors antagonists AM251 and AM630 were purchased from Tocris. SR141716 were purchased from Cayman Chemical. Furthermore, in vitro, WIN55,212-2 and SR141716 were dissolved in 2% dimethyl sulfoxide/PBS. In in vivo studies, WIN55,212-2, AM251, AM630 and naloxone were dissolved in the vehicle (a 1:1:18 ratio of cremophor:dimethyl sulfoxide:saline solution). All other drugs were dissolved in sterilized saline, and the solutions were divided into aliquots and stored in 1.5 ml tubes at -20 °C. The aliquots were thawed and used on the day of the experiment.

2.2. In vitro neurite outgrowth assay

Neuro 2A cells were plated in 60 mm dishes and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1‰ penicillin-streptomycin. In the neurite outgrowth assay, Neuro 2A cells were plated in 12-well plates and treated for 16 h in medium containing drugs as described previously (Gomes et al., 2009; Heimann et al., 2007; Jordan et al., 2005). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂. For each culture condition, randomly chosen regions of the plate containing 300 cells were scored under the microscope (Nikon TE2000-S). The cell having cellular projection of length at least two times longer than its diameter was considered neurite outgrowth of Neuro 2A cells.

2.3. Animals

Male Kunming (KM) mice (18-22 g) were provided by Animal Center of Lanzhou University and were housed in a climate-controlled room $(22 \pm 1 \text{ °C})$, with food and water available ad libitum. All animals were cared for and experiments were carried out in accordance with the European Community guidelines for the use of experimental animals (2010/63/EU). In addition, all the protocols in the present study were approved by the Ethics Committee of Lanzhou University, China. In the present behavior tests, each animal was used only once.

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