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Study on the molecular mechanism of antinociception induced by ghrelin in acute pain in mice

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ABSTRACT

Ghrelin has been identified as the endogenous ligand for the GHS-R1α (growth hormone secretagogue receptor 1 alpha). Our previous experiments have indicated that ghrelin (i.c.v.) induces antinociceptive effects in acute pain in mice, and the effects were mediated through the central opioid receptors and GHS-R1α. However, which opioid receptor (OR) mediates the antinociceptive effects and the molecular mechanisms are also needed to be further explored. In the present study, the antinociceptive effects of ghrelin (i.c.v.) could be fully antagonized by δ-opioid receptor antagonist NTI. Furthermore, the mRNA and protein levels of δ-opioid peptide PENK and δ-opioid receptor OPRD were increased after i.c.v injection of ghrelin. Thus, it showed that the antinociception of ghrelin was correlated with the GHS-R1α and δ-opioid receptors. To explore which receptor was firstly activated by ghrelin, GHS-R1α antagonist [D-Lys^3]-GHRP-6 was co-injection (i.c.v.) with deltorphin II (selective δ-opioid receptor agonist). Finally, the antinociception induced by deltorphin II wasn't blocked by the co-injection (i.c.v.) of [D-Lys^3]-GHRP-6, indicating that the GHS-R1α isn't on the backward position of δ-opioid receptor. The results suggested that i.c.v. injection of ghrelin initially activated the GHS-R1α, which in turn increased the release of endogenous PENK to activation of OPRD to produce antinociception.

1. Introduction

Ghrelin, an acylated 28-amino-acid peptide, has been identified as the endogenous peptide ligand which binds to GH secretagogue receptor (GHS-R) [11]. Ghrelin has gained increasing attention as a brain-gut hormone since it was discovered from endocrine cells of the stomach in rats in 1999 [12,10]. GHS-R includes two forms: GHS-R1α and GHS-R1β [19]. Almost all the documented activities of ghrelin have been discovered to bind to GHS-R1α whose selective antagonist is [D-Lys^3]-GHRP-6 [10,3,17]. Ghrelin and its receptor GHS-R1α express in many other organs, such as ovaries and testis [2,18], liver, lung [7], kidney, etc. [26]. Ghrelin is characterized with the presence of an *n*-octanoylation on the position of Ser^3 [3]. The special structure is important for ghrelin's physiological activities and help ghrelin to cross the blood-brain barrier (BBB) [21,1].

Ghrelin displays several physiological functions through GHS-R1α, such as promoting food intake [28] and GH, prolactin (PRL), ACTH secretion [20,21], regulating of cardiovascular roles, etc. [14]. Furthermore, The mRNAs or proteins of GHS-R1α and ghrelin have been shown to express in central nervous system such as the midbrain, the medulla oblongata, the sensorimotor area of the cortex and the hypothalamus, where is the regions relating to pain transmission [5,25,31].

Therefore, researchers have been interested in the role and mechanisms of ghrelin in the modulation of pain perception. Ghrelin has been reported to suppress the inflammatory pain through the central opioid receptors and other receptors [22,23]. Other studies have showed that ghrelin prevents mechanical hyperalgesia and cachexia induced by cisplatin [6], attenuates chronic neuropathic pain [8,13,30], and reduces diabetic neuropathy, etc. [24].

In our previous experiments, i.c.v. injection of ghrelin (1 nmol) can evoked antinociceptive effects, which are mediated through

Abbreviations: GHS-R1α, growth hormone secretagogue receptor 1 alpha; β-FNA, β-funaltrexamine; NTI, naltrindole; nor-BNI, nor-binaltorphimine; POMC, proopiomelanocortin; OPRM, μ-opioid receptor; PENK, proenkephalin; OPRD, δ-opioid receptor; PDYN, prodynorphin; OPRK, κ-opioid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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the central opioid system and GHS-R1α in acute pain in mice [27]. In addition, our further study has revealed that ghrelin (i.c.v.) can attenuate the antionciception of morphine which is administrated at the peripheral leval [29]. However, less attention has been given to investigate which opioid receptor (OR) mediates the antinociceptive effects of ghrelin. It is still unclear which receptor activated firstly and which receptor activated secondly by ghrelin. Moreover, the molecular mechanisms of antinociception induced by ghrelin (i.c.v.) are not known. So, in the present study, various opioid receptor antagonists will be used to explore which opioid receptors are involved in the antinociceptive effects of ghrelin (i.c.v.). It will be investigated which receptor was activated by ghrelin firstly in this test. Moreover, the methods of quantitative Real-Time PCR (qRT-PCR) and Western blot will be used to investigate the molecule mechanisms of antinociceptive effects after i.c.v. injection of ghrelin in mice. The present study examined effects of the opioid receptor antagonists, naloxone (general), beta-funaltrexamine (mu), naltrindole (delta) and nor-binaltorphamine (kappa) upon the antinociceptive effects of ghrelin.

2. Materials and methods

2.1. Animals

Male Kunming mice, weighing 18–22 g was provided by the Laboratory Animal Center of Medical College of Nanchang University. The experiments were all approved by the Ethics Committee of Animal Experiments at Nanchang University. Mice were housed under standard laboratory conditions in a 12/12 h light-dark cycles with standard water and food ad libitum. The mice must adapt to this surroundings for at least 3 days before the study. All tests were undertaken during the light (09:00-16:00). The mice were used only once. All efforts were taken to minimize the suffering and number of mice which were used in the following experiments.

2.2. Peptides and compounds

Ghrelin, [D-Lys^3]-GHRP-6 and deltorphin II were purchased from Phoenis Pharmaceuticals, Inc. Naloxone hydrochloride dihydrate was purchased from Fluka, β-funaltrexamine hydrochloride (β-FNA), nor-binaltorphimine dihydrochloride (nor-BNI) and naltrindole hydrochloride (NTI) were purchased from Sigma. β-FNA, nor-BNI and NTI are the antagonists of opioid receptors. All drugs were freshly dissolved in physiological saline solution.

2.3. Drugs injection procedure

The approach of intracerebroventricular (i.c.v.) administration was operated as described by Haley and McCormick [9]. Mice received 3 μl i.c.v. injection of drugs using the 25 μl microsyringe. The constant rate of injection was 10 μl/min. After behavioral testing, the right injection site was confirmed by injection of methylene blue dye.

2.4. Tail withdrawal test

According to our previous similar experiments [27], the antinociceptive effects of the drugs were measured using the tail withdrawal test. The behavioral tester was uninformed of the specific drug conditions at testing. The tail of mouse was immerged in water set at 48.5 ± 0.5 °C. The time before the mice withdraw the tail from the water was set as the tail withdrawal latency (TWL). The latency of mouse was measured through putting its tail into the water and measuring the response time. Those mice were selected whose baseline latency was 3–5 s for following study. A cut-off latency time was set at 15 s to minimize tail damage. The measurements of post-drug latency were executed at 5, 10, 20, 30, 40, 50 and 60 min. After testing, the proper injection site was confirmed by injection of methylene blue dye. Only the results with right i.c.v. injection sites in mice were used in the study. Data are expressed as the maximum percentage effect (MPE) calculated as: MPE (%) = 100 × [(post-drug response−baseline response)/(cut-off response−baseline response)]. The original data were calculated to area under the curve (AUC) over the period 0–60 min. The data were showed as mean value ± SEM of every group of 8–12 mice.

Table 1
Sequences and the sizes of forward (F) and reverse (R) primers of target genes for quantitative real-time PCR.

Gene	Sequences	Size (bp)
Ghrelin(F)	GAATCCAAGAAGCCACCAGC	
Ghrelin(R)	ACAGCTTGATGCCAACATCG	142
GHSR(F)	GAGATCGCGCAGATCAGTCA	
GHSR(R)	GAAGTTTGAACACGGCCACC	127
POMC(F)	AGATTCAAGAGGGAGCTGGA	
POMC(R)	CTTCTCGGAGGTCATGAAGC	159
Oprm(F)	ATCCTCTCTTCTGCCATTGGT	
Oprm(R)	TGAAGGCGAAGATGAAGACA	148
PENK(F)	AACAGGATGAGAGCCACTTGC	
PENK(R)	CTTCATCGGAGGGCAGAGACT	474
Oprd(F)	AACCTCTCGGACGCCTTTC	
Oprd(R)	CGATGCCAAACATGACGAGC	172
PDYN(F)	CGGAACTCCTCTTGGGGTAT	
PDYN(R)	TTTGGCAACGGAAAAGAATC	154
Oprk1(F)	CCGATACACGAAGATGAAGAC	
Oprk1(R)	GTGCCTCCAAGGACTATCGC	342
GAPDH(F)	AGGAGCGAGACCCCACTAACAT	
GAPDH(R)	GTGATGGCATGGACTGTGGT	313

2.5. Tissue preparation

In our study, the best antinociceptive effect of ghrelin (1nmol) emerged around 10 min after i.c.v. injection. The antinociception of ghrelin (1nmol) after i.c.v. injection was within 20 min. So, the points-in-time of 5, 10 and 20 minafter i.c.v. administration of drugs were selected for next research. As a control, 3 μl physiological saline was conducted into the intracerebroventricular sites in mice. After administration of drugs, the mice were decapitated at the exact point-in-time. The right site of injection was confirmed by microstructure measurement. Then the brain samples which were injected ghrelin or physiological saline were rapidly transferred and stored at −80 °C for further researches.

2.6. Total RNA isolation and quantitative real-time PCR (qRT-PCR)

The total RNA was extracted from the brain samples with Trizol Reagent (TaKaRa, China), and the RNA sample were reverse-transcribed in a 20 μl reaction mixture (TaKaRa, China). The primers used are listed in Table 1. The mRNA levels of these genes were evaluated by qRT-PCR with an internal control of GAPDH. QRT-PCR was performed in a 20 μl reaction volume using the 7500 Fast Real-Time PCR System. Negative controls consisted of samples with distilled water which replaced cDNA template. The thermalcycling criteriafor qRT-PCR were: 95 °C for 30s, followed closely by 40cycles at 95 °Cfor 5s, 60 °C for 34 s and 1cycles at 95 °C for 15 s, 60 °Cfor 1 min, end up with 95 °C for 15 s. The purity of PCR products were confirmed by the analysis of melting curve and agarose gel electrophoresis results. The raw Ct (threshold cycle) was selected to quantitative analysis using the $2^{-\Delta\Delta Ct}$method [15]. Each reaction was conducted in triplicate and reduplicated at least three times, respectively.