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Research article

Effects of rizatriptan on the expression of calcitonin gene-related peptide and cholecystokinin in the periaqueductal gray of a rat migraine model

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HIGHLIGHTS

- Investigate the expression of CGRP and CCK in the PAG during migraine attacks.
- Assess the influence of rizatriptan on midbrain CGRP and CCK expression.
- Provide useful insights into how rizatriptan regulates the endogenous pain modulatory system.

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ABSTRACT

Triptans are serotonin 5-hydroxytryptamine receptor 1B/D agonists that are highly effective in the treatment of migraine. We previously found that rizatriptan can reduce the expression of proenkephalin and P substance in the rat midbrain, suggesting that rizatriptan may exert its analgesic effects by influencing the endogenous pain modulatory system. Calcitonin gene-related peptide (CGRP) and cholecystokinin (CCK) are mainly responsible for antagonizing the analgesic effects of opioid peptides in the endogenous pain modulatory system. In this study, we investigated the effects of rizatriptan on the expression of CGRP and CCK in the periaqueductal gray (PAG), a key structure of the endogenous pain modulatory system, in a rat migraine model induced by nitroglycerin. We found that the mRNA and protein levels of CGRP and CCK in the PAG of migraine rats were significantly increased compared to those in control rats, and these levels were significantly reduced upon treatment with rizatriptan in migraine rats ($P < 0.05$). Our results suggest that the expression of CGRP and CCK in the endogenous pain modulatory system may be increased during migraine attacks, which further antagonizes the analgesic effects of endogenous opioid peptides and induces sustained migraine. Rizatriptan, however, significantly reduces the levels of CGRP and CCK to enhance the inhibition of pain signals via the endogenous pain modulatory system, resulting in effective treatment of migraine.

1. Introduction

The periaqueductal gray (PAG) is an important structure that plays a central role in the endogenous pain modulatory system because most of the analgesic effects caused by the activation of higher centers have been proven to be achieved only via the PAG [15,22]. Pain-related neuropeptides such as endogenous opioid peptides, substance P, calcitonin gene-related peptide (CGRP), and cholecystokinin (CCK) are important mediators of the endogenous pain modulatory system and participate in regulating its functions [4,17]. Endogenous opioid peptides and opioid receptor agonists play a critical role in the central analgesic effect by inhibiting neuronal discharges of pain and activating the descending inhibitory pain modulation system in the brain [17]. Substance P has been found to exert its analgesic effect by inducing the release of encephalin from the PAG [29]. CGRP and CCK in the endogenous

Abbreviations: 5-HT, 5-hydroxytryptamine; $5HT_{1B/D}$, 5-hydroxytryptamine receptor 1B/D; CGRP, calcitonin gene-related peptide; CCK, cholecystokinin; PAG, periaqueductal gray.

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pain modulatory system are thought to antagonize the analgesic effect of endogenous opioid peptides [24].

Triptans are serotonin 5-hydroxytryptamine receptor 1B/D ($5HT_{1B/D}$) agonists that are highly effective in the treatment of migraine. The mechanism of action for triptans is attributed to the constriction of dilated cerebrovascular and brain meningeal blood vessels via their agonist effects on serotonin $5HT_{1B/D}$ receptors [9,16]. We previously found that rizatriptan can reduce the expression of proenkephalin and P substance in the rat midbrain, suggesting that rizatriptan may exert its analgesic effects by influencing the endogenous pain modulatory system [27]. Rizatriptan has been found to affect the CGRP level in the trigeminal vascular system [5,19]; however, its effect on the CGRP level in the endogenous pain modulatory system remains unclear. Also, CCK is present in the nervous system, especially in the cerebral regions related to pain modulation [14]. Unfortunately, there has been no studying reporting the effect of rizatriptan on CCK level in the endogenous pain modulatory system. In this study, we established a rat migraine model via induction with nitroglycerin [18] and investigated the effects of rizatriptan on the mRNA and protein levels of CGRP and CCK in the PAG of migraine model rats. Our results provide useful insights into the mechanism by which rizatriptan regulates the endogenous pain modulatory system in the treatment of migraine.

2. Materials and methods

2.1. Animals and treatments

Wistar adult rats (24 males and 24 females, body weight of 200–220 g) were obtained from the Laboratory Animal Center at the Jilin University (Changchun, China) [Certificate No.: SCXK(JI):2003-001]. Rats were maintained at a constant temperature (21 ± 1 °C) and humidity (40–70%) with a 12-h light/dark cycle. The animals were given ad libitum access to food and water. All animal-related procedures conformed to the guidance of the care and use of laboratory animals issued by the Ministry of Science and Technology of the People's Republic of China [20]. These 48 rats were randomly divided into the following four groups (n = 12/group): control (A), migraine (B), rizatriptan (C), and migraine + rizatriptan (D). In Groups B and D, 10 mg/kg body weight of nitroglycerin was subcutaneously administered into rat buttocks to establish an experimental migraine model. Three to five minutes later, the rats exhibited characteristics of head discomfort such as binaural redness, frequent forelimb scratching of head, increased attempts to climb the cage, tail biting, and reciprocating motion [26]. Thus, the rat migraine model was usually established 5 min after the administration of nitroglycerin, which is consistent with previous reports of the incubation period of migraine [30,31]. Five minutes after the administration of nitroglycerin, the rats were in the acute period of migraine, which is the therapeutic time of rizatriptan [30]. Then the rats were immediately subjected to intragastrical administration of 2 ml/kg body weight of saline (Group B) or 1 mg/kg (i.e., 2 ml/kg) body weight of rizatriptan (Group D). In Groups A and C, 2 ml/kg body weight of saline was subcutaneously administered into rat buttocks, and then rats were subjected to intragastrical administration of 2 ml/kg body weight of saline (Group A) or 1 mg/kg body weight of rizatriptan (Group C). The dosage of rizatriptan was calculated according to the single oral dose used clinically in adults [7].

2.2. Real-time RT–PCR

Two hours after treatments, rats (n = 6/group) were anesthetized with 10% chloral hydrate (3 ml/kg body weight) and decapitated. The isolated midbrain was immediately immersed in liquid nitrogen and stored at −70 °C until use for real-time RT–PCR.

Total RNA was extracted with RNAiso Reagent (TaKaRa, China) according to the manufacturer's instructions. The cDNA was reversed from 0.25 μg of total RNA using the Reverse Transcription Kit (TaKaRa, China) and was stored at −70 °C until use.

To prepare the standards for CGRP and CCK, we amplified the above cDNA by PCR with specific CGRP and CCK primers synthesized by TaKaRa. The primers used in the study were: CGRP forward 5′-AAGTTCTCCCCTTTCCTGGT-3′, reverse 5′-GGTGGGCACAAAGTTGTCCT-3′; and CCK forward 5′-CCCGATACATCCAGCAGGTC-3′, reverse 5′-AAATCCATCCAGCCCATGTAGTC-3′. The PCR parameters were: 94 °C for 2 min, and then 35 cycles of 94 °C for 30 s, 53 (CGRP) or 57 °C (CCK) for 30 s, and 72 °C for 30 s. The PCR products were subjected to 2% agarose gel electrophoresis, and target bands were excised and recovered using an AxyPrep DNA Gel Extraction Kit and an AxyPrep Plasmid Miniprep Kit obtained from Axygen Biosciences (Union City, CA, USA). The recovered and purified target gene (CGRP or CCK) was cloned into the pEASY-T1 vector and transformed in *Escherichia coli* DH5α competent cells. The plasmid was extracted after ampicillin selection, digested by *BamH* I and *EcoR* V, and subjected to sequencing. The sequence of the cloned gene had 100% homology with the sequence of the target gene (CGRP or CCK). The optical density at 260 nm (OD_{260}) of the extracted plasmid was measured, and its copy number was calculated accordingly. The extracted plasmids were stored at −20 °C as standards of CGRP and CCK.

Amplification was undertaken on the ABI PRISM 7500 real time PCR system (Applied Biosystems, USA) with the following steps: 94 °C for 2 min, and then 40 cycles of 94 °C for 30 s, 53 (CGRP) or 57 °C (CCK) for 30 s, and 72° C for 30 s. Fluorescent signals were detected at the end of the annealing stage of each cycle. The detection threshold was set at the threshold cycle (Ct) where the fluorescent signal entered the inflection point of an exponential increase from the background during the amplification of PCR. Melting curves were prepared from conditions of 95 °C for 15 s, 60 °C for 20 s, and 95 °C for 15 s.

2.3. Immunohistochemistry

Two hours after treatments, rats (n = 6/group) were anesthetized with 10% chloral hydrate (3 ml/kg body weight). Isotonic saline was perfused through the left ventricle for about 3 min followed by a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS) for about 5 min, and then the brainstem was quickly dissected. The isolated midbrain was immediately fixed in 10% neutral-buffered formalin for 48 h. The midbrain was dehydrated by an 80–100% alcohol gradient, vitrified by dimethylbenzene, embedded in paraffin, and cut into 4-μm-thick sections.

Immunohistochemistry was performed as previously described [28]. Briefly, paraffin-embedded brain sections were first subjected to dewaxing and rehydration, and then antigen retrieval was performed at 92–95 °C for 20 min. Endogenous peroxidase activity was quenched with 0.3% H_2O_2, and unspecific binding sites were blocked with 2% normal goat serum in PBS containing 0.3% Triton X-100 for 30 min at room temperature. The sections were rinsed in PBS and incubated with normal rabbit IgG (negative control) or primary polyclonal rabbit anti-CGRP or anti-CCK-8 (1:1000; Beijing Biosynthesis Biotechnology, China) overnight at 4 °C. Sections were then rinsed in PBS and incubated with biotinylated goat-anti-rabbit antibody (1:2000; Beijing Biosynthesis Biotechnology) for 10 min. Following three rinses in PBS, sections were incubated in avidin-biotin peroxidase complex for 10 min. Following another three rinses in PBS, sections were incubated for 5–10 min in 3,3′-diaminobenzidine (DAB) substrate solution, which produced a