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Rizatriptan benzoate influences the endogenous pain modulatory system in a rat model of migraine[☆]

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Abstract

The present study utilized a nitroglycerin-induced rat model of migraine to detect the effects of rizatriptan benzoate on proenkephalin and substance P gene expression in the midbrain using real-time quantitative polymerase chain reaction and investigate whether rizatriptan benzoate can regulate the endogenous pain modulatory system. The results showed that rizatriptan benzoate significantly reduced expression of the mRNAs for proenkephalin and substance P. Rizatriptan benzoate may inhibit the analgesic effect of the endogenous pain modulatory system.

Key Words: proenkephalin; substance P; migraine; rizatriptan benzoate; midbrain; real-time quantitative polymerase chain reaction; pain

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INTRODUCTION

The endogenous pain modulatory system has been the focus of a number of studies of the cause, pathogenesis and medication of migraine^[1-4]. Substance P (SP) and endogenous opioid peptides are important mediators in the pain modulatory system, and they participate in central analgesia^[5-6]. Endogenous opioid peptides include enkephalin, endorphin and dynorphin, in addition to orphanin and endomorphin. As 5-hydroxytryptamine (5-HT_{1B/1D}) receptor agonists, triptans represent a specific medicine for migraine treatment, acting *via* several pathways^[7]: stimulating 5-HT_{1B} receptors to induce contraction of dilated cerebral vessels and meningeal vessels; stimulating presynaptic 5-HT_{1D} receptors in the trigeminal nerve to inhibit duramatral neurogenic inflammation and blood plasma exosmosis; and stimulating brain stem 5-HT_{1B} and 5-HT_{1D} receptors to inhibit excitation of the nuclei of the trigeminal nerve. However, the effects of triptans on the endogenous pain modulatory system remain poorly understood.

The present study utilized a nitroglycerin-induced rat model of migraine^[8] to assess the influence of rizatriptan benzoate on midbrain proenkephalin (PENK) and SP mRNA expression to investigate the possible mechanism by which rizatriptan

benzoate treats migraine.

RESULTS

Quantitative analysis of experimental animals

A total of 24 adult Wistar rats were equally and randomly assigned to four groups. Rizatriptan benzoate treatment and model groups were subcutaneously injected with nitroglycerin to establish the migraine model (successful model establishment was confirmed by the presence of ear flushing, scratching the head frequently using forelimbs, increased activities to climb the cage, biting tails, and a reciprocating motion^[9]), while normal control and rizatriptan benzoate control groups were injected with normal saline. The rizatriptan benzoate treatment and rizatriptan benzoate control groups were intragastrically perfused with rizatriptan benzoate for 7 days prior to modeling. Twenty-four rats were included in the final analysis.

PCR products

The absorbance (A_{260nm}/A_{280nm}) ratio of extracted RNA from brain samples in each group ranged between 1.9 and 2.0, indicating a high purity of extracted RNA. Amplification products of the PENK and SP genes were obtained using PCR, purified, retrieved, and conjugated to pMD-18T vector to form a recombinant plasmid. Correct recombinant plasmids were confirmed by double-enzyme digestion in

accordance with expectation. TaKaRa sequencing results showed 100% homology between cloned sequences and target gene sequences.

Real-time quantitative PCR standard curves for PENK and SP gene standards

The PENK and SP plasmid standards were used to plot PCR standard curves. The linear scale for PENK spanned seven orders of magnitude, ranging from 1.2×10^9 copies/ μL to 1.2×10^3 copies/ μL (Figure 1A). The regression equation was defined as $Ct = -3.367\log(x) + 42.28$, $r = 0.991$ (supplementary Figure 1 online). The linear scale for SP spanned six orders of magnitude, ranging from 1.6×10^9 copies/ μL to 1.6×10^4 copies/ μL (Figure 1B). The regression equation was defined as $Ct = -3.428\log(x) + 43.18$, $r = 0.995$ (supplementary Figure 1 online).

Specificity of PCR amplification products

Melting curve analysis showed that the peak melting

curve value for the PENK gene PCR product was maintained at 85°C, and that of the SP gene standard PCR product was maintained at 82°C. Both products displayed a uniform melting temperature and a sharp peak shape under varied range of dilutions (supplementary Figure 2 online), indicating that they were specific products.

PENK and SP mRNA expression in rats with migraine

PENK mRNA levels were similar between normal control and model groups ($P > 0.05$), but the SP mRNA levels were significantly lower in the model group compared with the normal control group ($P < 0.05$). PENK and SP mRNA levels in the rat midbrain were similar between the two rizatriptan benzoate groups ($P > 0.05$). Compared with the normal control and model groups, PENK and SP mRNA levels in the rat midbrain were significantly reduced in both rizatriptan benzoate groups ($P < 0.05$; Table 1).

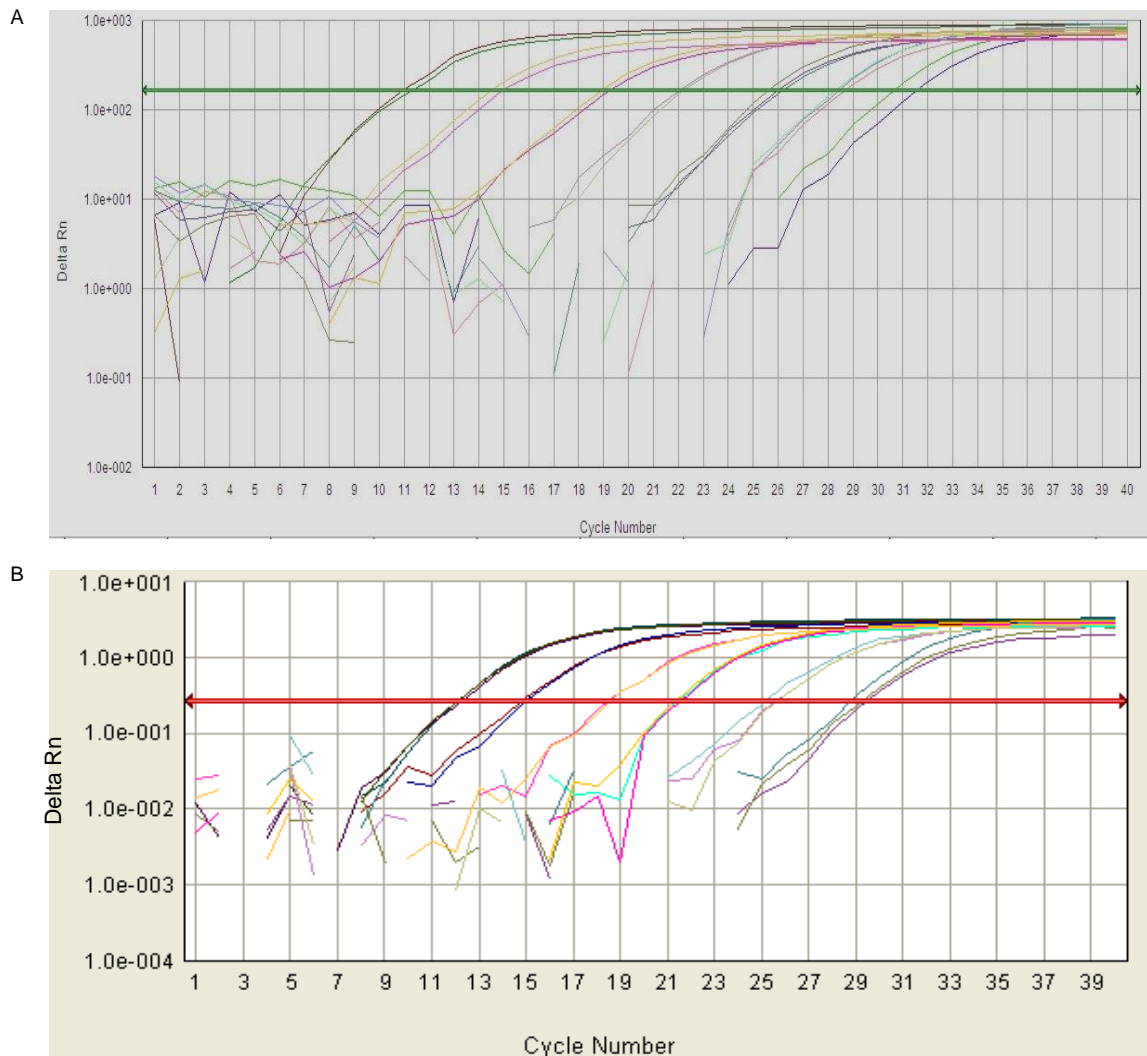


Figure 1 Amplification curves for proenkephalin (PENK) and substance P (SP) from serial dilutions of RNA. The copy numbers are in the range 1.2×10^9 copies/ μL to 1.2×10^3 copies/ μL for PENK (A) and in the range 1.6×10^9 copies/ μL to 1.6×10^4 copies/ μL for SP (B) from left to right in the amplification plot.

Table 1 Proenkephalin and substance P mRNA levels (target gene mRNA copies per 250 ng total RNA, $\times 10^4$) in the rat midbrain of each group

Group	Proenkephalin	Substance P
Normal control	2.54 \pm 1.07	8.38 \pm 3.05
Model	2.00 \pm 0.45	6.00 \pm 1.23 ^a
Rizatriptan benzoate control	0.39 \pm 0.15 ^{ab}	3.09 \pm 0.71 ^{ab}
Rizatriptan benzoate treatment	0.38 \pm 0.19 ^{ab}	3.45 \pm 0.39 ^{ab}

Results are expressed as mean \pm SD of six rats in each group.

^a $P < 0.05$, vs. normal control group; ^b $P < 0.05$, vs. model group (F test).

DISCUSSION

The present study investigated the effects of the triptan rizatriptan benzoate on migraine. The endogenous pain modulatory system, with the midbrain periaqueductal gray at its center and including the ventral medial medulla oblongata, regulates primary afferent activities in the spinal posterior horn *via* descending inhibition^[1]. Endogenous opioid peptides and SP are important mediators in the endogenous pain modulatory system. SP transmits nociceptive information to the center and plays an analgesic role in the central nervous system. SP can influence midbrain function by regulating neuronal currents in the midbrain periaqueductal gray matter^[10]. The results of the present study showed that SP mRNA levels in rat midbrain are significantly lower in the model group compared with the normal control. When the level of midbrain SP is reduced, the analgesic effects are weakened^[11]. However, the mechanism underlying midbrain SP reduction following nitroglycerin-induced migraine have required further investigation. Rizatriptan benzoate significantly reduced SP mRNA levels in the midbrains of normal and model group rats, indicating that rizatriptan benzoate can downregulate SP gene expression in the rat midbrain. Rosen *et al*^[12] proposed that increasing SP release in the periaqueductal gray matter can enhance the analgesic effects of opioids. In the present study, rizatriptan benzoate reduced SP mRNA expression in the rat midbrain, possibly attenuating the analgesic effects of endogenous opioids. Opioid peptides and opioid receptor agonists exert strong analgesic effects by inhibiting neuronal pain-evoked discharges and activating the pain modulatory descending inhibitory system^[5]. Enkephalin is classified into two forms according to its structure: met-enkephalin and leu-enkephalin. They are derived from a single precursor, namely, PENK^[13]. The results of the present study revealed no significant difference in midbrain PENK expression levels between model and normal control groups, indicating that migraine does not directly influence midbrain PENK expression. However, the effects of migraine on opioid peptide expression require further study. Rizatriptan benzoate significantly reduced midbrain PENK mRNA expression, decreasing

the levels of midbrain met-enkephalin and leu-enkephalin, and thereby weakening the analgesic effects of the endogenous pain modulatory system.

In addition, SP has been shown to stimulate enkephalin release from the periaqueductal gray^[14]. In the present study, rizatriptan benzoate reduced SP and PENK mRNA expression in the midbrain. However, whether there is a correlation between these two reductions remains to be fully investigated.

In conclusion, rizatriptan benzoate decreased expression of the mRNAs for SP and PENK in the midbrain, possibly inhibiting the analgesic effects of the endogenous pain modulatory system.

MATERIALS AND METHODS

Design

A randomized, controlled, animal neuropharmacology experiment.

Time and setting

This study was performed at the Laboratory Animal Center and the Central Laboratory at the Second Hospital of Jilin University from 2010 to 2011.

Materials

A total of 24 healthy, adult, Wistar rats, of clean grade and weighing 200–220 g, irrespective of gender, were provided by the Animal Center, School of Basic Medicine, Jilin University, China (license No. SCXK (Ji) 2003-001). The rats were housed at 20–26°C and 40–70% humidity, with typical day-night illumination, and allowed free access to water and food. Experimental protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[15].

Methods

Migraine model establishment and interventions

Rizatriptan benzoate control and treatment groups were intragastrically perfused with rizatriptan benzoate (Hubei Huayuan Shiji Pharmaceutical Co., Ltd., Hubei, China), 1 mg/kg per day (according to the adult daily dose), and normal control and model groups were perfused with normal saline 2 mL per day. After 7 days, nitroglycerin (10 mg/kg; Shanxi Kangbao Biological, Shanxi, China) was subcutaneously injected into the buttocks of the rizatriptan benzoate treatment and model groups to induce migraine^[11]. Normal saline (2 mL/kg) was injected into the normal control and rizatriptan benzoate control groups.

Midbrain tissue sample preparation

Two hours after nitroglycerin injection, rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g) and then sacrificed. The midbrains were isolated, immediately placed in liquid nitrogen, and were stored at -70°C for real-time PCR detection^[8].

Total RNA extraction

Midbrain tissues (50–100 mg) were mixed with 1 mL RNAiso reagent, and RNA was extracted according to the manufacturer's instruction (TaKaRa, Dalian, China).

Following gel electrophoresis, total RNA quality and concentration were determined by ultraviolet spectrophotometry (Shimadzu, Kyoto, Japan). RNA concentration was calculated according to the following formula: RNA concentration ($\mu\text{g}/\mu\text{L}$) = $A_{260\text{ nm}}$ (absorbance at 260 nm) \times dilution multiple \times 40/1 000^[16].

cDNA synthesis

Reactions comprised 250 ng of RNA, 4 μL of MgCl_2 , 2 μL of 10 \times RT buffer, 2 μL of dNTP mixture (10 mM), 0.5 μL of RNase inhibitor, 1 μL of AMV reverse transcriptase, 1 μL of random 9-mers, and RNase-free dH_2O to a total volume of 20 μL . Reverse transcriptase was performed under the following conditions: 30°C for 10 minutes, 42°C for 30 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. Synthesized cDNA was stored at -70°C.

Quantitative standard sample preparation

Using PENK-specific^[17] upstream 5'-TCC CGG CGA CAT CAA CTT C-3' and downstream 5'-AAC TCG GGC TTG GAC ACC TG-3' primers, the amplification fragment length was 111 bp. The PCR reaction parameters were as follows: 94°C pre-denaturation for 2 minutes, 94°C denaturation for 30 seconds, 62°C annealing for 30 seconds, and 72°C extension for 30 seconds, for a total of 35 cycles. Using SP-specific^[18] upstream 5'-TGG CGG TCT TTT TTC TCG TT-3' and downstream 5'-GCA TTG CCT CCT TGA TTT GG-3' primers, the amplification fragment length was 114 bp. The PCR reaction parameters were as follows: 94°C pre-denaturation for 2 minutes, 94°C denaturation for 40 seconds, 54°C annealing for 50 seconds, and 72°C extension for 60 seconds, for a total of 35 cycles. The PCR products were electrophoresed in a 2% agarose gel, the target band was excised, and the cDNA was recovered using the AxyPrep DNA Gel Extraction Kit (TaKaRa). The purified target gene was conjugated with the pMD-18T vector (TaKaRa) and transformed into *E. coli* DH5 α competent cells (TaKaRa). Subsequent to ampicillin screening, plasmid extraction was digested with endonuclease (*Sal*I and *Eco*RI for PENK; *Hind*III and *Bam*HI for SP), followed by sequencing, and identification. The absorbance value of the extracted plasmid at 260 nm was measured, and the copy number was calculated. Following 10-fold serial dilution in sterile water and subpackaging, the standard samples were stored at -20°C.

SYBR green real-time quantitative PCR

Twenty-microliter reactions comprised 10 μL of SYBR Premix Ex TaqTM (TaKaRa), 0.4 μL of upstream and downstream primers (10 μM), 0.4 μL of ROX Reference Dye (TaKaRa), 2.0 μL of cDNA, and 6.8 μL of dH_2O . Different concentrations of plasmid standard samples (1.2×10^3 – 1.2×10^9) copies/ μL were processed by quantitative PCR. Each sample was run in triplicate. Reaction conditions were as follows: 94°C pre-denaturation for 2 minutes, 94°C denaturation for 30 seconds, 62°C annealing for 30 seconds, 72°C extension for 30 seconds, for a total of 40 cycles.

Fluorescence signals were measured at the end of annealing in each cycle with the critical point for measurement defined during PCR amplification, *i.e.* the value of the threshold cycle corresponding to the inflection point of fluorescence signals entering the exponential growth phase above background level. A melting curve analysis was performed in a pattern of 95°C for 15 seconds, 60°C for 20 seconds, and 95°C for 15 seconds.

Statistical analysis

Data were statistically analyzed using SAS 6.12 software (SAS Software Institute, Kerry, North Carolina, USA). Results are expressed as mean \pm SD, and *F* tests were used to make comparisons among groups. A level of *P* < 0.05 was considered statistically significant.

Author contributions: Gang Yao participated in the study concept and design, experimental implementation, data integrity and analysis, and wrote the manuscript. Yuhong Man and Xiangdan Luo and Lin Ji conducted the experiments. Tingmin Yu participated in the study concept and design and revised the manuscript.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Jilin University, China.

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Supplementary information: Supplementary data associated with this article can be found in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 2, 2012, after selecting the "NRR Current Issue" button on the page.

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