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Expression of calcitonin gene-related peptide, adenosine A2a receptor and adenosine A1 receptor in experiment rat migraine models

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Abstract. A migraine is a disabling neurovascular disorder characterized by a unilateral throbbing headache that lasts from 4 to 72 h. The headache is often accompanied by nausea, vomiting, phonophobia and photophobia, and may be worsened by physical exercise. The trigeminovascular system (TVS) is speculated to have an important role in migraines, although the pathophysiology of this disorder remains to be elucidated. Trigeminal ganglion (TG) and spinal trigeminal nucleus caudalis (TNC) are important components of the TVS. Several clinical cases have provided evidence for the involvement of the brainstem in migraine initiation. Electrical stimulation of the trigeminal ganglion (ESTG) in rats can activate TVS during a migraine attack. Calcitonin gene-related peptide (CGRP) is an important vasoactive compound produced following TVS activation. Numerous studies have revealed that adenosine and its receptors have an important role in pain transmission and regulation process. However, only a few studies have examined whether adenosine A2a receptor (A2aR) and adenosine A1 receptor (A1R) are involved in migraine and nociceptive pathways. In the present study, CGRP, A2aR and A1R expression levels were detected in the TG and TNC of ESTG models through reverse transcription-quantitative polymerase chain reaction and western blot analysis. Tianshu capsule (TSC), a type of Chinese medicine, was also used in the ESTG rat models to examine its influence on the three proteins. Results demonstrated that CGRP, A2aR and A1R mediated pain transmission and the regulation process during migraine and the expression of the three proteins was regulated by TSC.

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Introduction

A migraine is more than just a headache; it is a complex neurological disorder that involves altered sensory perception and processing, affecting ~15% of the population, and the mechanisms underlying migraine have not been completely elucidated (1). The trigeminovascular pathway and neuropeptide calcitonin gene-related peptide (CGRP) have important roles in migraines (2-5). Trigeminovascular doctrine combines nerves, blood vessels and neurotransmitters to explain certain animal experiments results and clinical manifestations of migraines. Electrical stimulation of the trigeminal ganglion (ESTG) in humans leads to increased extracerebral blood flow and local release of CGRP and substance P (6). This phenomenon causes extravasation of plasma proteins and neurogenic inflammation, which can stimulate spreading of the trigeminal nerve vascular fiber and nerve impulses in the brainstem, hypothalamus and cortex, eventually leading to migraine.

The pathogenesis of a migraine remains to be elucidated, and a variety of theories offer different targets for migraine treatment. Serotonin receptor agonists and nonsteroidal anti-inflammatory drugs have a clear therapeutic effect on migraines. Other targets, such as glutamate receptors, nitric oxide synthase and adenosine Al receptor (A1R), have been recently found to treat migraine, but they have not been clinically used (7,8). The physiological effects of adenosine, an important neurotransmitter, are mediated by adenosine receptor. Four adenosine receptor subtypes have been discovered, which are A1R, adenosine A2a receptor (A2aR), A2bR and A3R (9). A1R and A2aR are high-affinity receptors, which have an important role in pain information transmission and regulation. In addition to the aforementioned drugs, several Chinese medicines, including Tianshu capsule (TSC), also have an important role in migraine treatment. In China, a number of neurologists apply TSC as an acute and prophylactic drug treatment for migraines (10).

The present study aimed to determine whether CGRP, A2aR and A1R are involved in migraine pain information transmission in the ESTG migraine rat model. The possible mechanisms of TSC for migraine treatment were also explored.

Materials and methods

Animals. A total of 40 male Sprague-Dawley rats weighing 280-320 g (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were used. All the rats were maintained under standard laboratory housing conditions with a 12 h-light-dark cycle and had free access to food and water. All the experimental protocols were approved by the Ethics Committee for the Use of Experimental Animals at Binzhou Medical University (Binzhou, China). All the procedures were performed with utmost caution to minimize animal suffering. All the rats were randomly divided into four groups: Blank (n=10), ESTG (n=10), sham-operated (SO; n=10) and TSC groups (n=10).

Experimental protocols

ESTG. The rats in the ESTG model group were anesthetized with 10% chloral hydrate [4 ml/kg, intraperitoneal injection (i.p.)] and placed in a stereotaxic frame (ZH-B; Zhenghua Biological Instrument Co., Ltd., Huaibei, China). The calvarium was exposed through a midline incision. A hole was made with a cranial drill 3.2-3.4 mm posteriorly to and 2.8-3.2 mm laterally from the bregma. A disposable concentric needle electrode (DCN37; Alpine Biomed Corp., Fountain Valley, CA, USA) was lowered into the right TG (at a depth of ~9.2 mm from the dura mater). TG was electrically stimulated for 30 min with square pulses at 10 Hz and 0.5 mA, with a pulse duration of 5 msec. Correct electrode placement of the electrode needle was confirmed through ipsilateral contraction of the masseter muscle during stimulation.

The rats in the SO group underwent a surgical procedure similar to that performed in the rats of the ESTG group. However, the concentric bipolar electrode was only lowered into the right TG and was maintained for only 30 min. The TG was not electrically stimulated.

Drug administration. The rats in the TSC group received intragastric administration of TSC (Jiangsu Kangyuan Pharmaceutical, Jiangsu, China), which was dissolved in saline at a dose of 3.5 mg/kg/day for 7 days. Subsequently, the rats were anesthetized with 10% chloral hydrate (4 ml/kg; i.p.) and subjected to ESTG 30 min after the last drug administration.

All the rats were sacrificed after 30 min of stimulation. The trigeminal nucleus caudalis (TNC) and ipsilateral TG were immediately removed and stored at -80˚C for western blot analysis or reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR. The TG and TNC of mice were rapidly isolated for total RNA preparation as described in the previous section. Total RNA (1 μg) was reverse transcribed in a 25-μl reaction volume using Premix Ex Tap II (Takara Bio, Shiga, Japan). PCR amplifications and fluorescence detections were performed using the CFX96™ RT-PCR detection system C1000 according to the manufacturer's protocol. The PCR conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. A relative quantity of each sample was calculated using the $2^{-\Delta\Delta Ct}$ method. Nucleotide sequences of the specific primers for the selected genes were as follows: β-actin (forward, AGAGCTATGAGC TGCCTGACG and reverse, CTTCTGCATCCTGTCAGC GAATGC); CGRP (forward, TCCTGGTTGTCAGCATCTTG and reverse, CTCAGCCTCCTGTTCCTCCT); A1R (forward, GGCCACAGACCTACTTCCAC and reverse, ACCGGAGAG GGATCTTGACT); and A2aR (forward, GTCCTCACGCAG AGTTCCAT and reverse, CACCTGTCACCAAGCCATT).

Western blot analysis. The tissues were lyzed in lysis buffer (Beyotime Biotechnology, Shanghai, China) that contained freshly added protease inhibitor. Equivalent amounts of protein lysates and loading buffer were loaded on 7.5/12.5% polyacrylamide gels, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequently electrophoretically transferred onto PVDF membranes. These membranes were blocked with 5% non-fat milk in Tris-buffered saline + 0.1% Tween-20 buffer at room temperature for 1 h and incubated overnight at 4˚C with the following primary antibodies: Rabbit polyclonal anti-CGRP (1:500; cat. no. ab47027), rabbit polyclonal anti-adenosine receptor A2a (1:1,000; cat. no. ab3461), rabbit polyclonal anti-adenosine receptor A1 (1:500; cat. no. ab82477) (all from Abcam, Shanghai, China) and β-actin (1:1,000; cat. no. AA128; Beyotime Biotechnology). The membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h, and the proteins were visualized using an electrochemiluminescence kit (1:5,000; cat. no. A0208; both from Beyotime Biotechnology).

Statistical analysis. All the values are presented as the mean ± standard deviation. Independent Student's t-test was used to compare the data from two groups. One-way analysis of variance followed by Tukey's post-hoc test was applied when more than two groups of data were compared. $P<0.05$ was considered to indicate a statistically significant difference. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Effect of electrical stimulation on CGRP, A1R and A2aR expression in the TG and TNC. According to the western blot analysis and RT-qPCR results, electrical stimulation significantly increased CGRP and A2aR expression in the TNC and ipsilateral TG compared with the blank groups and SO groups ($P<0.05$) (Figs. 1 and 2). Electrical stimulation also significantly decreased A1R expression in the TNC and ipsilateral TG compared with the blank groups and SO groups ($P<0.05$) (Figs. 1 and 2), in which no differences were detected.

Effect of pretreatment with TSC on CGRP, A1R and A2aR expression in the TG and TNC. According to the western blot results, TSC pretreatment with TSC significantly decreased CGRP and A2aR expression in the TNC and ipsilateral TG compared with the ESTG group ($P<0.05$) (Fig. 2). TSC significantly increased A1R expression compared with the ESTG group ($P<0.05$) (Fig. 2). No differences were detected between the blank and TSC groups.

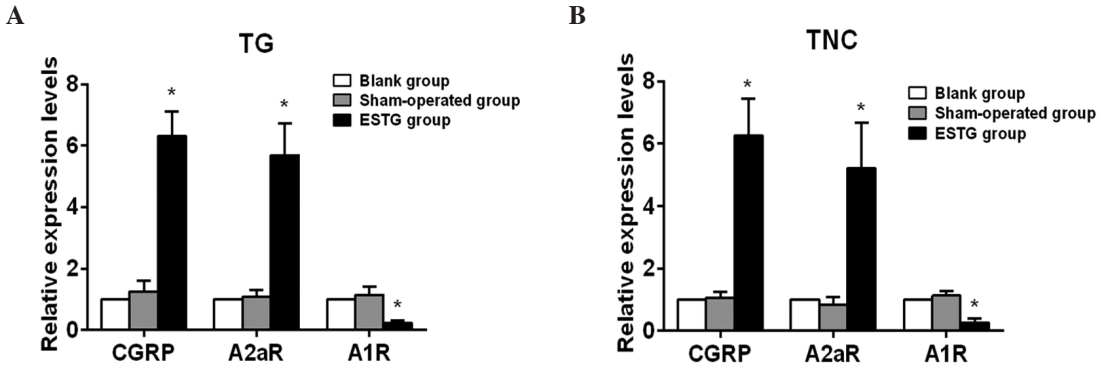

Figure 1. Results of RT-qPCR analysis. A relative quantity of each sample was calculated using the $2^{-\Delta\Delta Ct}$ method. (A) RT-qPCR analysis of CGRP, A2aR and A1R mRNA expression levels in ipsilateral TG. No significant differences in the three genes expression were observed between the blank and SO groups, while electrical stimulation significantly increased CGRP and A2aR mRNA expression in ipsilateral TG compared with the blank and SO groups. Electrical stimulation also significantly decreased A1R mRNA expression in ipsilateral TG compared with the blank and SO groups. (B) RT-qPCR analysis of CGRP, A2aR and A1R mRNA expression in the TNC. No significant differences in the three gene expressions were observed between the blank and SO groups, while electrical stimulation significantly increased CGRP and A2aR mRNA expression in the TNC compared with the blank and SO groups. Electrical stimulation also significantly decreased A1R mRNA expression in the TNC compared with the blank and SO groups. *P<0.05 compared with the blank and SO group. RT-qPCT, reverse transcription-quantitative polymerase chain reaction; CGRP, calcitonin gene-related peptide; A2aR, adenosine A2a receptor; A1R, adenosine A1 receptor; TG, trigeminal ganglion; TNC, trigeminal nucleus caudalis; SO, sham-operated.

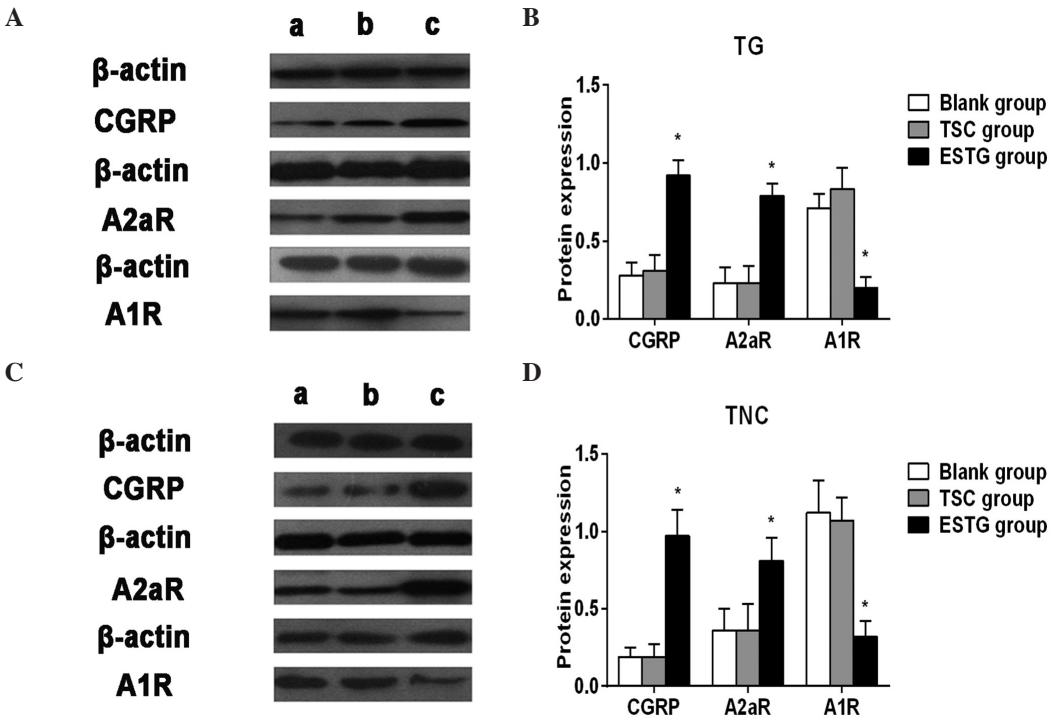

Figure 2. (A) Representative images of CGRP, A2aR and A1R expression in ipsilateral TG. (B) Western blot analysis of the expression of CGRP, A2aR and A1R in ipsilateral TG. No significant differences in the the three proteins expression were observed between the (a) blank groups and the (b) TSC groups, while pretreatment with TSC significantly decreased CGRP and A2aR expression in ipsilateral TG compared with the (c) ESTG groups. TSC significantly increased A1R expression compared with the ESTG groups. (C) Representative images of CGRP, A2aR and A1R expression in the TNC. (D) Western blot analysis of the expression of CGRP, A2aR and A1R in the TNC. No significant differences in the three proteins expression levels were observed between the blank and the TSC groups, while pretreatment with TSC significantly decreased CGRP and A2aR expression in the TNC compared with the ESTG groups. TSC significantly increased A1R expression compared with the ESTG groups. *P<0.05 compared with the blank and TSC groups. CGRP, calcitonin gene-related peptide; A2aR, adenosine A2a receptor; A1R, adenosine A1 receptor; TG, trigeminal ganglion; TNC, trigeminal nucleus caudalis; TSC, Tianshu capsule.

Discussion

Trigeminovascular theory is currently dominating the pathogenesis of migraine. This theory suggests that the activation of the trigeminovascular system (TVS) results in increased CGRP release that causes neurogenic inflammation, which is characterized by meningeal vascular expansion, plasma protein leakage and mast cell degranulation (11). Neurogenic inflammation is an important part of the pathogenesis of migraine. CGRP is essential vasoactive compound that cause neurogenic inflammation. Studies have identified that the CGRP expression levels in the external jugular vein, cubital vein and cerebrospinal fluid were significantly higher in patients with migraines compared to those in the healthy

controls, and CGRP content is greater in the external jugular vein than that in the cubital vein (12). These results suggest that trigeminal nerve endings, which dominate the cerebravl blood wall, can release CGRP. Further research has found that CGRP nerve fibers dominating cerebrovascular are mainly from the TG (13). The present study showed that the expression levels of CGRP protein and CGRP mRNA in TG were higher in the migraine model rats compared to the in normal rats, further suggesting that the elevated CGRP levels during migraine attacks may be the result of CGRP release from the TG neuron. The results also showed that the ESTG model rats can stimulate TVS activation during a migraine attack.

TNC includes secondary neurons that are responsible for integrating the nociceptive information from the TG neurons. TNC and TG activities reflect the activation of trigeminal nerve nociceptive pathway in the central nervous system. TNC is a key component of pain transmission process. CGRP may be involved in the neuron activity adjustment (14). The present study identified that the CGRP protein and mRNA levels in TNC were higher in the ESTG model rats than those in normal rats. This finding suggests that CGRP is important in migraine transmission.

Adenosine, a strong vasoactive compound, and its receptors have an important role in pain transmission and information regulation. Adenosine injection in the vein, spine and intraventricular area has an analgesic effect (15-17). Numerous animal experiments (18-20) showed that A1 receptor activation can have a role in pathological neuralgia, inflammation and pain in animal models. Other studies showed that selective A1R agonists can inhibit neurogenic vasodilation and CGRP release, thereby inhibiting migraine generation and transfer (21). Combined with the experimental results, the A1R expression in TNC and TG in migraine model rats was lower than that in normal rats. This result suggests that decreased expression of A1R has an important role in the pathogenesis of migraine, whereas activation or increased expression of adenosine A1R may suppress the occurrence of migraines.

A2aR is widely distributed in the central nervous system and mediates the pathophysiology of numerous diseases, such as neuronal protection, stroke, pain and neurodegenerative disease, through interaction with other important receptors (22-24). Adenosine can directly stimulate the nociceptive nerve through A2aR and A2aR-knockout mice have a high pain threshold (25). The present study found that the A2aR expression in TG and TNC of the ESTG group was higher than that of the blank control group, which was similar with CGRP expression. This result suggests that CGRP and A2aR may cause migraine and pain transmission when combined. In addition, a previous study showed that CGRP cannot affect synaptic transmission in the brain neurons but can enhance excitatory synapse potential depending on A2aR activation and A1R inhibition (26). Another study revealed that A2aR activation can inhibit A1R, suggesting that transmission of migraine information requires multiple neurotransmitter interactions (27).

TSC is a modern Chinese medicine that consists of Chuanxiong and Tianma. TSC can promote blood circulation to remove blood stasis and activate meridians to alleviate pain. This Chinese medicine is mainly used for migraine treatment and has a beneficial effect; however, the underlying mechanism remains to be elucidated. The experiments showed that the CGRP, A1R and A2aR expression in the TSC intervention group did not significantly differ with that in the blank group, whereas no significant differences were found between the ESTG and TSC groups, suggesting that TSC can improve the levels of the three proteins involved in the pathogenesis of migraine for relief. Other studies showed that TSC can adjust the expression of vasoactive compounds, such as nitric oxide, CGRP and neurotransmitters, including serotonin, during migraine attacks, thereby relieving migraine symptoms (28,29). In conclusion, TSC can adjust to a variety of vasoactive compounds and neurotransmitters involved in the pathogenesis of migraines, thereby affecting the onset and progression of migraine.

The CGRP, A2aR and A1R expression in TG and TNC significantly changed the migraine rats more than the normal rats. CGRP not only had an important role in neurogenic inflammation, but may also have mediated migraine pain information with adenosine. TSC can adjust the expression of these three proteins, thereby providing a new experimental basis and targets for the pathogenesis and treatment of migraines. However, the interaction mechanisms of CGRP, A1R and A2aR in the pathogenesis of migraines require further study.

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