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Research Article

C-Jun N-Terminal Kinase Mediates Calcitonin Gene-Related Peptide Expression in Rat Trigeminal Ganglion in Vitro

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Summary

Objective: Calcitonin gene-related peptide (CGRP) plays a prominent role in migraine pathophysiology. However, the underlying molecular mechanisms that are responsible for regulating CGRP expression are not fully understood. Using an in-vitro model of organ culture of rat trigeminal ganglion (TG), the present study aims to investigate the role of c-Jun N-terminal kinase (JNK) in regulation of CGRP.
Methods: The TG was isolated from Sprague-Dawley (SD) rats and then organ cultured in presence of inflammatory cytokines tumor necrosis factor-alpha (TNF-α) (50μg/L) or interleukin-1β (IL-1β) (25μg/L) with and without a specific JNK inhibitor SP600125 (10^{-5M}) or an anti-migraine drug sumatriptan (0.5g/L) for 24h. Real-time polymerase chain reaction (RT-PCR) and Western blotting were used to evaluate the CGRP-mRNA expression and phosphorylated JNK (pJNK) protein in rat TG after the organ culture.
Results and Conclusions: The JNK inhibitor significantly reduced the organ culture and cytokines-induced up-regulation of CGRP-mRNA expression compared with control ($P<0.05$), indicating that a transcriptional mechanism was involved. Interestingly, the anti-migraine drug sumatriptan had similar effect. Western blotting showed that the cytokines significantly increased pJNK protein level, while this increase could decrease by sumatriptan and SP600125 ($P<0.05$), demonstrating that JNK mediated the up-regulation of CGRP expression induced by the cytokines. This may suggest a novel pharmacotherapeutical target for migraine treatment.

Key words: CGRP; migraine; sumatriptan; JNK; cytokines

C-Jun N-Terminal Kinaz Sıçan Trigeminal Ganglionunda in Vitro Olarak Kalsitonin Gene-bağlı Peptid Yapımını Düzenler

Özet

Amaç: Kalsitonin gene-bağlı peptid (CGRP) migren patofizyolojisinde önemli bir rol oynar. Bununla beraber CGRP oluşumunun düzenlenmesinde altta yatan moleküler mekanizma tam olarak anlaşılmış değildir. Bu çalışmada sıçan trigeminal ganglion (TG) organ kültürü in vitro modeli kullanılarak c-Jun N-terminal kinazın CGRP regülasyonundaki rolü araştırılmaktadır.
Yöntem: Sprague-Dawley (SD) sıçanlarının TG'ları izole edildi ve ortamda yangısal sitokinler tümör nekroz faktörü-alfa (TNF- α) (50μg/L) ya da interlökin-1β (IL-1β) (25μg/L)

ile bir spesifik JNK inhibitörü SP600125 (10^{-5M}) birlikte veya olmaksızın ya da bir migren ilacı olan sumatriptan (0.5g/L) bir gün boyu uygulandı. Organ kültürü sonrası sıçan TG'nda fosforilize olan JNK (pJNK) proteini ve CGRP-mRNA miktarı değerlendirilmesi için gerçek zamanlı polimeraz zincir reaksiyonu (RT-PCR) ve Western blotting kullanıldı.
Sonuç ve Yorum: JNK inhibitörü, transkripsiyonel bir mekanizmayı işaret edercesine, organ kültürü ve sitokinin oluşturduğu CGRP-mRNA miktarının regülasyonunu kontrollere göre önemli ölçüde azalttı ($P<0.05$). İlginç olarak bir migren ilacı olan sumatriptan da benzer etki gösterdi. Western blotting sitokinlerin pJNK protein seviyelerini önemli ölçüde yükselttiğini ve bu yükselişi sumatriptanın ve SP600125'in azaltabildiğini göstererek JNKnin sitokinlerin oluşturduğu CGRP'yi düzenlediğini ortaya koydu. Bu durum migren tedavisinde yeni bir farmakolojik tedavi hedefini gösterdi.

Anahtar Kelimeler: CGRP; migren; sumatriptan; JNK; sitokinler

INTRODUCTION

Migraine is a genetically related recurrent headache syndrome appearing with neuro-psychological and gastrointestinal symptoms, implicating both external triggers and internal pathophysiology. According to the World Health Organization (WHO) statistic, migraine is the 19th among all diseases with regard to years lived with disability[13]. It affects approximately 18% of females and 6% of males, and has more profound negative effect on quality of migraineurs' life[1]. The common comorbidities of migraine include stroke, hypertension, coronary heart disease, sub-clinical vascular brain lesions, epilepsy and other disorders[28], and results in serious and huge socio-economic burden to society as well as to the individual. However, attention has present been paid to the burden of migraine is far from enough in scale and scope.

Based on migraine pathophysiological change, activation of trigeminovascular system mediates the release of calcitonin gene-related peptide (CGRP), as a major neuropeptide but not substance P (SP) or neurokinin A. More and more experimental and clinical studies support that CGRP plays a crucial role in the generation and maintenance of pain during acute migraine attacks by facilitating cellular events which contribute to peripheral sensitization of TG neurons and central sensitization of second order neurons involved in nociceptive transmission[9,23]. However, information describing the underlying molecular mechanisms of CGRP expression in TG neurons remains obscure presently.

Mitogen-activated protein kinases (MAPK) is a family of Ser/Thr kinases which transmit various extracellular signals to the nucleus, and then induce gene expression, cell proliferation, differentiation, apoptosis[12,21]. MAPKs are essential for intracellular signal transduction and play critical roles in regulating neural plasticity and inflammatory responses[4,11,12,21]. Three distinct MAPK cascades have been elucidated: extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK. The JNK pathway is activated by pro-inflammatory cytokines and a large number of environmental stressors[20,29]. Accumulating evidence shows that JNK pathway contribute to pain sensitization after tissue and nerve injury via particular cellular and molecular mechanism[2,10,18], but much less is known about the involvement of JNK pathway in regulatory mechanism of CGRP expression in rat TG after organ culture. Here we examine if CGRP up-regulation induced by organ culture in TG is related with JNK signal transduction pathway.

In the present study, we used rat TG in vitro organ culture in improved serum-free DMEM treated with inflammatory

cytokines TNF-α or IL-1β, sumatriptan and the specific inhibitor for JNK to identify if JNK signaling pathway was involved in regulating CGRP expression. Furthermore, we also evaluated whether blocking the critical kinases of JNK signal transduction pathways would be a logical therapeutic target for migraine acute attacks.

MATERIAL AND METHODS

Animals and reagents

Male SD rats (250～300g) were provided by the Experimental Animal Center of Xi'an Jiaotong University College of Medicine (China). All animals were kept under standard conditions of controlled temperature (20～24℃) and humidity (40%～70%), with a light-dark cycle of 12 hours, and were given free access to tap water and rat chow. All procedures were approved by the Animal Ethics Committee of Xi'an Jiaotong University.

Inflammatory cytokines TNF-α and IL-1β (Sigma, USA) were dissolved in phosphate buffer saline (PBS) to make a stock solution of 50mg/L. Sumatriptan (Sigma, USA) was dissolved in sterile double distilled water to 20mg/L. The JNK specific inhibitor SP600125 (Sigma, USA) was dissolved in dimethylsulfoxide (DMSO) and further diluted in saline solution to 10^{-5M} used in the experiments. The primary antibody used for Western blotting analyses was anti-P-JNK (9258, Cell Signaling).

Rat TG after organ culture in vitro model

Adult male SD rats were asphyxiated under inhaling CO2 sedation, and then TGs were isolated in ice-cold PBS (PH7.4) and subsequently transferred to improved serum-free DMEM supplemented with penicillin (100U/mL), streptomycin (100μl/mL) and amphotericin B (25μg/mL). The TGs were incubated for up to 24h at 37°C in humidified incubator containing 5% CO_2, the medium was replaced per 24h. Different intervening reagent has been separately added into DMEM medium co-cultured with TG acting as experimental group. Simply TG culturing in DMEM medium without intervention reagent acted as control group, while fresh group TG without incubation.

Real time polymerase chain reaction (RT-PCR)

Total RNA was extracted from rat TG using FastPrepA kit according to the supplier's guidelines (QBIOgene, CA, USA). The quantity of extracted RNA was determined spectrophotometrically. cDNA was reverse transcribed by Oligo (dT) primed reverse transcription using the Gene Amp RT kit (PE Applied Biosystems) in a Perkin-Elmer 2400 GeneAmp PCR system (Perkin Elmer, MA, USA). Real time PCR was performed using GeneAmp SYBR® Green PCR kit (PE Applied Biosystems) in a GeneAmp 5700 Sequence Detection System. Table 1 lists the used primer sequences and cycling conditions. All primers were designed using the Primer Express 2.0 software (Perkin Elmer, Applied Biosystems) and synthesized by Invitrogen Corporation. Dissociation curves were run after the real-time PCR to identify the specific PCR products.

Data were analyzed with the comparative cycle threshold (CT) method. To evaluate the amount of CGRP mRNA in a sample, β-actin mRNA was simultaneously assessed in the same sample. The C_T values of β-actin mRNA were used as a reference to quantify the relative amount of CGRP mRNA. The relative amount of mRNA was calculated with the C_T values of CGRP mRNA in relation to the CT values of β-actin mRNA in the sample.

Western blotting

Total proteins of TG were extracted in RIPA buffer (Cell Signaling Technology, USA), following a centrifugation at 12000×g for 20 minutes at 4℃, protein concentration was determined using the BCA protein assay with bovine serum

albumin as a standard. Equal amounts of protein were subjected to SDS-PAGE and transferred by electroblotting onto nitrocellulose membranes. The membranes were blocked in 5% non-fat milk for 1h at room temperature and followed by overnight incubation at 4℃ with primary antibodies for pJNK, or β-actin (Cell Signaling Technology, USA; dilution 1:1000), and then washed in TBST and incubated with biotinylated secondary antibodies for 2h at room temperature. Proteins were visualized using an enhanced chemiluminescence (ECL) plus kit. Immunoblots were scanned by an electronically cooled CCD camera system (Fujifilm LAS-1000, Japan). The intensity of each band was quantified by Image Gauge version 4.0 (Fuji Photo Film Co., Ltd., Japan). The expression level of pJNK was corrected by comparison with β-actin.

Statistical analysis

Data are presented as mean ± standard error (SEM). Statistical analyses among groups are performed using one-way analysis of variance (ANOVA), between-group comparisons are done with Dunnett's post test using the software SPSS 17.0 (SPSS software, Chicago, IL, USA). GraphPad 5.0 software (GraphPad, La Jolla, CA, USA) is used for data analysis and charting. For these tests, a value of $P<0.05$ is regarded as statistically significant.

Table 1: Sequences and cycling conditions of all primers for RT-PCR

Gene	Primer sequences (5'-3')	Den.	Ann.	Elo.
CGRP	forward GAGGCAGCTACAAGGTTCAGG	95℃	60℃	72℃
	reverse AGGTGTTGGTGCTGGACACA	15s	30s	30s
β-actin	forward CTATCGGCAATGAGCGGTTCC	95℃	58℃	72℃
	reverse TGTGTTGGCATAGAGGTCTTTACG	15s	30s	30s

RESULTS

Effect of JNK inhibitor on organ culture-induced up-regulation of CGRP-mRNA

The relative expression levels of CGRP-mRNA in TG were investigated following 24h of culture in the presence of JNK inhibitor SP600125, control solvent, and in non-cultured fresh TG. RT-PCR analysis revealed a strongly significant up-regulation of CGRP-mRNA in TG after cultured in serum-free DMEM medium for 24h compared with fresh rat TG ($p<0.01$), but levels of CGRP-mRNA decreased remarkably in rat TG after co-cultured with JNK specific inhibitor 10^{-5M} SP600125 compared with control group (Figure 1, $P<0.05$).

Effect of JNK inhibitor on TNF-α, IL-1β-induced up-regulation and sumatriptan induced down-regulation of CGRP-mRNA

To study whether JNK pathway involves in process of inflammatory cytokines up-regulate CGRP, the effect of rat TG co-incubated with 10^{-5M} JNK inhibitor SP600125, 50μg/L TNF-α and 25μg/L IL-1β in the serum-free DMEM medium for

24h was investigated. Generally, there was a further up-regulation of CGRP in 50μg/L TNF-α and 25μg/L IL-1β group compared with control group (Figure 2A& B, P<0.05). We previously experiment indicated that optimal concentration sumatriptan can significantly down-regulated CGRP-mRNA expression in TG[17]. Therefore, 10^{-5} M SP600125 was added into DMEM medium 30min prior to 0.5g/L sumatriptan co-cultured with TG for 24hrs in order to determine whether JNK pathway involved in sumatriptan induced CGRP-mRNA down-regulation (Figure 2C). As determined through RT-PCR, levels of CGRP-mRNA lowered strikingly in rat TG pretreated with SP600125 in comparison with rat TG co-cultured with optimal concentration of TNF-α, IL-1β and sumatriptan for 24h respectively (Figure 2, P<0.05). These results are in accordance with previous research[22,30,31].

Effect of cytokine TNF-α, IL-1β and sumatriptan on pJNK protein expression after organ culture

Additionally, we assessed the influence of JNK pathway on cytokine of TNF-α, IL-1β induced up-regulation and sumatriptan induced down-regulation of CGRP-mRNA expression by means of Western blotting. TG was treated as simply cultured, cultured in the presence of 50μg/L TNF-α, 25μg/L IL-1β, 0.5g/L sumatriptan and 10^{-5M} JNK inhibitor SP600125, respectively. Following organ cultured for 24 hrs, the phosphorylated JNK protein was quantified by Western blotting. Results demonstrated that pJNK protein levels of rat TG strikingly increased co-culturing with cytokine TNF-α and IL-1β, whereas pJNK reduced markedly treating with 0.5g/L sumatriptan comparing to control group (Figure 3, P<0.05).

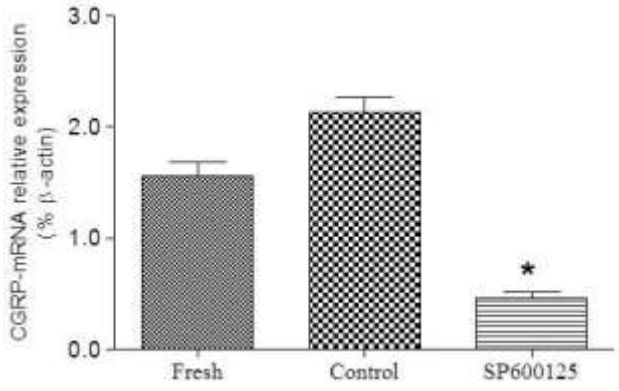

Figure 1: *RT-PCR analysis of CGRP-mRNA expression level in rat TG following 24 hrs organ culture with control DMEM medium or JNK inhibitor 10^{-5M} SP600125. *P<0.05 vs. control group.*

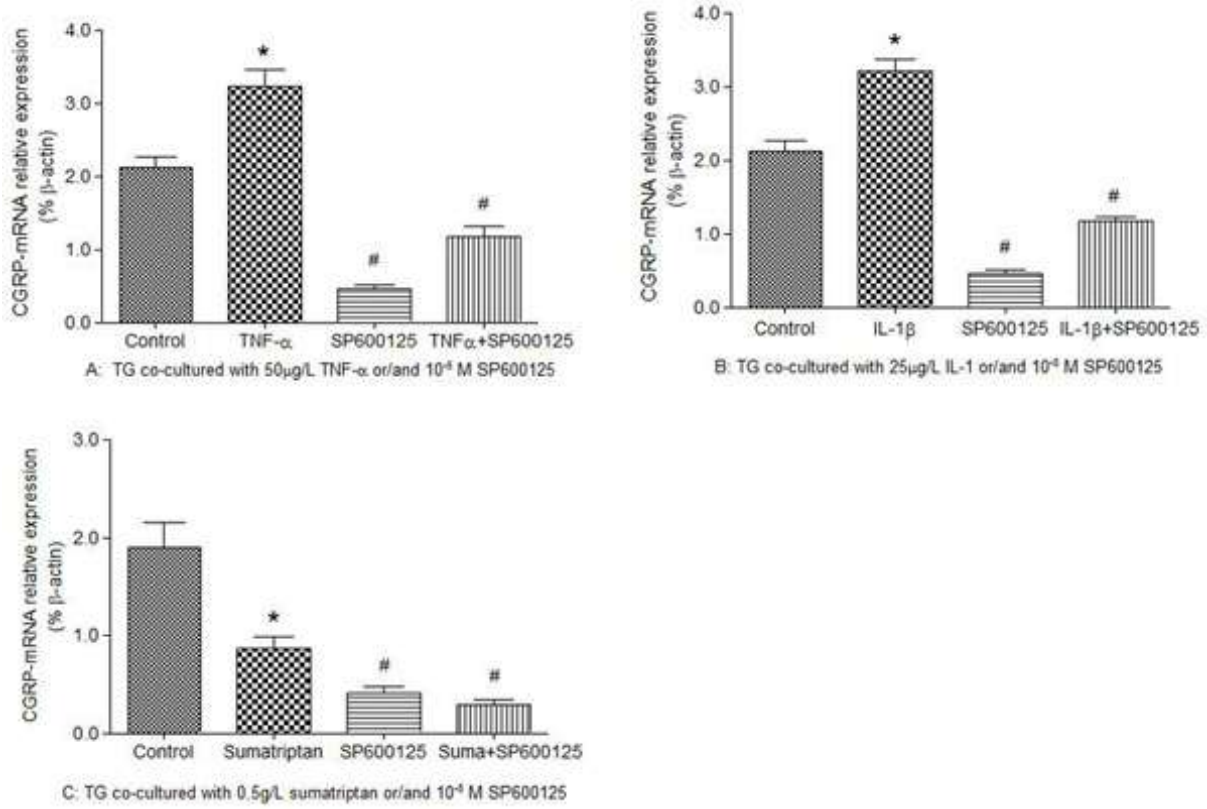

Figure 2: *CGRP-mRNA expression in TG after respectively co-incubated with 50μg/L TNF-α, 25μg/L IL-1β, 0.5g/L sumatriptan pretreated with 10^{-5M} SP600125. *P<0.05 vs. control group, #P<0.05 vs. TNF-α, IL-1β or sumatriptan, respectively.*

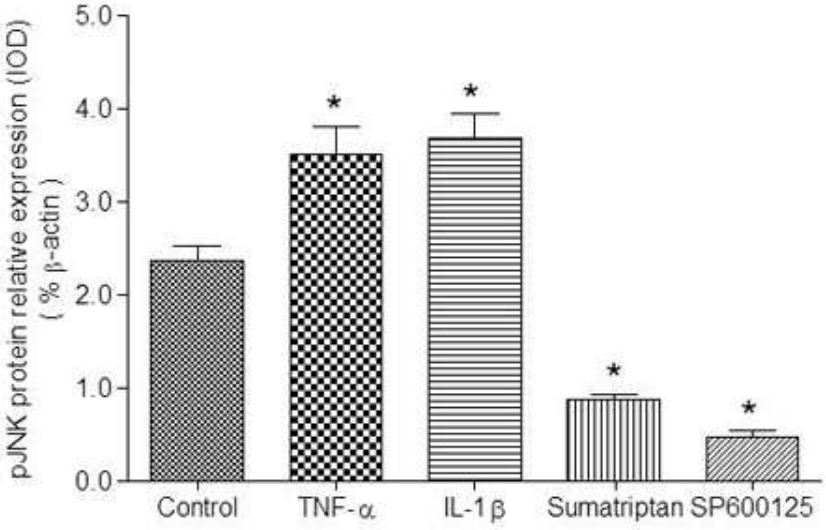

Figure 3: *Western blotting analysis of pJNK protein levels in TG co-cultured with severally 50μg/L TNF-α, 25μg/L IL-1β, 0.5g/L sumatriptan and 10^{-5M} SP600125 for 24 hrs. *P<0.05 vs. control group.*

DISCUSSION

Mounting evidence suggests that the JNK pathway plays vital roles in the pathogenesis of neurogenic inflammation. It is believed that JNK pathway is a critical signaling transduction pathway in triggering neuropathic pain via particular mechanism in the dorsal root ganglia (DRGs) and spinal cord[10,18]. Current tools for analgesia are often merely partially successful, thus investigations of new targets for migraine therapy stimulate great interest. However, the relative contribution of JNK pathway in regulation of CGRP expression during migraine attacks remains unclear. In this study, we used rat TG after organ culture in vitro model to explore the relationship between JNK pathway activity and CGRP expression, and to relate the role of JNK involve in regulation mechanism of CGRP at molecular and cellular levels.

Increasing studies show that organ culture increases the level of CGRP protein and mRNA expression and the upregulation could be a non-specific intracellular inflammation process[7,14,24]. However, the specific intracellular signaling transduction mechanism behind CGRP upregulation remains unclear currently. Inspired by some studies of the arteries from rat in organ culture[15,16] the mechanism of enhanced CGRP expression was studied by adding the JNK inhibitor SP600125 into serum-free DMEM medium co-incubated with TG for 24hrs. Our results revealed that JNK signaling transduction pathway is responsible for the increased expression of CGRP, this is similar as it is seen in the arteries following organ culture. Rat TG after organ culture in vitro model is based on the organ level and retains integrity of TG. Rat TG incubated in serum-free DMEM medium lack of nutrients, oxygen and blood, and then causes the secretion of inflammatory cytokines and nerve growth factor etc., which induces stress to the system accompanied with local neurogenic inflammation , and in turn activates JNK pathway, which subsequently results in increased release of CGRP. Therefore, we deduce that CGRP synthesis and expression may occur after activated pJNK transfer to nucleus and initiate gene expression. Blocking JNK signaling transduction pathway reduces the enhanced CGRP expression may provide novel options for the treatment of migraine.

This study showed that inflammatory cytokines TNF-α, IL-1β induce over-expression of CGRP in rat TG and that upregulation is transcriptionally regulated via the JNK signal transduction pathway. A growing number of studies suggested that a relationship exists between migraine and neurogenic inflammation. CGRP is believed to be involved in several pathophysiological processes, such as dilation of cerebral and dural blood vessels, release of inflammatory mediators from mast cells and transmission of nociceptive information from intracranial blood vessels to the nervous system[8,17,23,25]. Recent findings have now shed light on that glial cells, astrocytes, microglia, monocytes/macrophages or lymphocytes release a series of pro-inflammatory cytokines like TNF-α, IL-1β, IL-2, IL-6 etc., which play a pivotal role in the mediation and maintenance of inflammation and neuropathic pain during migraine attacks[17,22,30,31]. Stimulation with TNF-α increased the synthesis and secretion of CGRP in TG neurons suggesting a link between cytokines and CGRP release[3]. In addition, CGRP is shown to differentially regulate cytokines secretion from cultured TG glia[5]. CGRP demonstrates modest pro-inflammatory effects per se on trigeminal satellite cells, while it significantly enhanced inflammatory cytokines TNF-α, IL-1β actions[3,5,17].

To investigate the underlying molecular mechanism of TNF-α and IL-1β increased CGRP expression, 10^{-5M} SP600125 was

severally added into medium 30 min prior to co-cultured with inflammatory cytokines 50μg/L TNF-α and 25μg/L IL-1β for 24h. The results reveal that SP600125 could strikingly decrease CGRP-mRNA over-expression induced by TNF-α and IL-1β respectively, which is in line with the findings of others in rat TG[3,5,17,22,30,31]. TG is comprised of neuronal cells and two types of glial cells, satellite cells and Schwann cells. During the period of TG cultured in serum-free DMEM, stress stimulus such as ischemia, hypoxia, the lack of nutrients etc. initiate JNK signaling pathways in small-to mid-size neuronal cells and glial cells to increase synthesis, storage and secretion of CGRP. Enhanced CGRP activates the receptors of CGRP on surface of small glial cells and astrocyte cells, in turn initiates JNK pathway followed by sequently activation of adenylate cyclase, protein kinase A, phospholipase C, inositol triphosphate, protein kinase C etc., which finally induces CGRP gene expression and neuropeptide release from sensory neurons[2,10,18,26]. Furthermore, TG glial cells produce more inflammatory cytokines TNF-α and IL-1β by means of autocrine or paracrine self-feedback loop to further increase the synthesis of CGRP[5]. Activated protein kinase Ras, Raf get into the nucleus so as to activate transcription factors nuclear factor-kappa B and activator protein-1, which up-regulate the levels of TNF-α and IL-1β expression in astrocyte cells. Stimulation with IL-1β increases cycloxygenase 2 expression and then in a dose and time dependent manner induces prostaglandin E_2 synthesis and prolong CGRP release in TG neurons[17], this further strengthen inflammation and excessive dilation of cerebral and dural blood vessels. The positive feedback loop of inflammatory cytokines-CGRP-vasomotor dysfunction has been formed in the trigeminovascular system, this may be the mechanism of continuous migraine attacks, chronification of recurrent and central pain sensitization.

Sumatriptan is the first artificial selective serotonin 5-hydroxytryptamine ($5\text{-HT}_{1B/1D}$) agonists thought to treat migraine attacks by several mechanisms, including specific intracranial vasoconstriction, inhibition of activation of 5-HT receptor on presynaptic membrane, reduction of neurotransmitters and inflammatory mediators release from activated trigeminal neurons[6,19,27]. It has been observed that triptans reduce the release of CGRP during primary headaches attacks, putatively that occurs via presynaptic/ganglionic receptors, this is in concert with the work of Durham and colleagues who revealed that triptans can reduce the activity of the CGRP promoter via a calcium dependent mechanism and hence modify the expression of CGRP-immunoreactivity in culture. In our previous study, we found that 0.5g/L sumatriptan could significantly decrease CGRP expression levels in TG after organ culture. An important finding from our present study was that the inhibitory effect of sumatriptan on CGRP expression is mediated by JNK signaling pathway. Results from our study provide a plausible explanation of the reported benefits of sumatriptan to treat migraine because it represses signaling protein in TG. We found that blocking the critical kinases of JNK signal transduction pathways would be a logical therapeutic target for migraine acute attacks.

In conclusion, the result of our study indicates that JNK pathway participates in the regulation mechanism of CGRP expression in rat TG following organ culture. Our findings imply that JNK protein play a key role in migraine pathogenesis, and provide insight into the underlying molecular mechanism and experimental evidence for pharmacotherapeutic targets of migraine.

Disclosure statement

The authors declare that there are no conflicts of interest.

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