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Full Length Research Paper

Tetrandrine attenuates NF-κB activation in trigeminal ganglia by blocking calcium channel in a rat model of migraine

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Glial cells are now recognized as an active participant in the initiation and maintenance of migraine. NF-κB, a nuclear transcription factor, is the center of inflammatory response. Nitroglycerin (NTG)-induced NF-κB activation has been shown to play a critical role in the pathogenesis of migraine. Nitroglycerin (NTG) can activate NF-κB transcription, leading to the translocation of p65 subunit of NF-κB into the nucleus. Tetrandrine (Tet), a traditional Chinese medicine as a calcium channel blocker and an inflammation antagonist, has been applied in the treatment of various neurologic diseases. Evidence has demonstrated tetrandrine as a potent inhibitor of NF-κB activation in cells. The present study aimed to investigate whether NF-κB activation in the satellite cells can lead to the release of inflammatory cytokines involving in the migraine and to explore the relationship between NF-κB and Tet in the pathology of migraine. Male Sprague Dawley rats received injection with nitroglycerin to introduce migraine. Immunohisto chemistry, Western blot, and RT-PCR were used to determine the NF-κB levels and study the changes under basal conditions in response to tetrandrine injection. A significant increase in the nuclear p65, an indicator of NF- κB activation, was detected in the trigeminal ganglia of rats following injection with nitroglycerin. However, the nitroglycerin-induced NF-κB activation in the trigeminal ganglia was attenuated by pretreatment with Tet in a dose-dependent fashion. NF-κB activation in the trigeminal ganglia is implicated in the pathology of migraine, and Tet may be a novel and promising candidate for future treatment or prevention of migraine via inhibiting NF-κB activation in the trigeminal ganglia.

Key words: Tetrandrine, migraine, nuclear factor kappa B and satellite cells.

INTRODUCTION

Migraine is one of the most prevalent disabling nervous system diseases affecting the patients' quality of life (Afridi et al., 2005a, Afridi et al., 2005b). It is a complex disorder with characteristic episodic activation of the trigeminal system (Longoni and Ferrarese, 2006). Although, the exact mechanisms are not known, growing evidence indicates that the neurogenic inflammation is closely associated with the pathogenesis of migraine. Glial cells are now recognized as an active participant in the initiation and maintenance of migraine (Tassorelli et al., 2007). At the same time, receptors and mediators secreted by glial cells have important roles in the regulation of neuronal function, and can activate the glial cells to produce and release pro-inflammatory cytokines involving in the migraine. It has been confirmed (Daugaard et al., 2010; Kim et al., 2008) that trigeminal neurons are surrounded by the specialized glial cells known as satellite cells and neuron-glial cells interact via the gap junctions in the trigeminal ganglion in the migraine patients (Greco et al., 2005; Tian et al., 2003). NF-κB, a nuclear transcription factor involving in the inflammatory response and being regarded as the center of inflammatory response, is able to regulate the

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expression of various inflammatory cytokines. Nitroglycerin (NTG) can specifically activate the NF-κB transcription, resulting in the translocation of p65 subunit of NF-κB into the nucleus (Joseph et al., 1996). The p65 subunit then binds to the specific recognition elements in the promoter regions of inflammation and stress related genes (Daugaard et al., 2010; Ramachandran et al., 2010). Previous studies indicated that NTG could activate trigeminovascular system (TVS) (Reuter et al., 2002) and induce NF-κB activation in the trigeminal nucleus caudalis (TNC) at the neuronal level (Greco et al., 2005). Taken together, since NF-κB is activated in satellite cells, to block its activation may have therapeutic potentials in treatment and prevention of migraine (Bakkar and Guttridge, 2010). The focus on migraine's treatment has been a topic of great interest for a long time. However, a specific has not yet found. Most of the current drugs aim to alleviate the damage to the neurons and disregard the function of glial cells, especially the glial cell induced inflammatory response. This may be one of causes of refractory characteristic of migraine. Therefore, to find a drug that can attenuate the inflammatory response of glial cells and associates with NF-κB may offer a new strategy in treatment and research of migraine.

A variety of traditional Chinese medicines have been applied in the treatment of nervous system diseases and proved to be effective in both prevention and treatment of these diseases. In particular, the traditional Chinese medicines not only have the unique superiority of reduced side effects but also possess the effectiveness on the neurogenic inflammation. Tetrandrine (Tet) (6, 6′, 7, 12 – tetranmethoxy - 2, 2′ - dimethyberbaman, $C_{38}H_{42}O_6N_2$, 622.7), as a traditional Chinese medicine, is a bisbenzylisoquinoline alkaloid which is derived from the Chinese herb of Radix Stephania tetrandra (Chinese name Hanfangji). It has been applied in the treatment of various diseases in China (Shen et al., 2001) due to its multiple biological activities, including anti- inflammation (Shen et al., 2001; Wang and Lemos, 1995). In addition, it also acts as a natural non-specific calcium channel blocker (CCBs) (Wu and Ng, 2007). Numerous studies have confirmed that Tet can inhibit the NF-κB activation and the expressions of pro-inflammatory cytokines (Lin et al., 2008; Yin et al., 2009). Therefore, we hypothesized that NF-κB activation in the satellite cells would trigger the release of inflammatory cytokines involving in the migraine, and the NF-κB activation in the trigeminal ganglia might be attenuated by Tet treatment in a NTG-induced migraine model. Our results may provide a novel and promising candidate for the future treatment or prevention of migraine.

MATERIALS AND METHODS

Animals

A total of 64 Male Sprague-Dawley rats weighting 200 to 230 g were purchased rom the Experimental Animal Center of Chongqiong Medical University Animal care and procedures were in accordance with the guidelines of Institutional and National Institutes of Health. Animals were housed in cages in a 12-h light/dark cycle and given ad libitum access to food and water. Rats were randomly divided into four groups:1) CONT group (n = 16): normal animals received an intraperitoneal injection of saline; 2) NTG group (n = 16): normal animals received subcutaneous injection of NTG (10 mg/kg) in the back of the neck ; 3) Tet Group (1 mg/ml) + NTG (n = 16): normal animals received an intraperitoneal injection of Tet (10 mg/kg) at 30 min before receiving an intraperitoneal injection of NTG (10 mg/kg); 4) Tet Group (5 mg/ml) + NTG (n = 16): normal animals received an intraperitoneal injection of Tet (50 mg/kg) at 30 min before receiving an intraperitoneal injection of NTG (10 mg/kg). During the study, the changes in the behaviors of these animals were recorded. Four hours after NTG injection, the rats were sacrificed for the following experiments. This time point was chosen because preliminary study revealed the expression of nuclear NF-κB protein was the highest (Jim, 2009).

RNA extraction and quantitative fluorescent-PCR

Trigeminal ganglia were collected from the animals in each group. Total RNA was extracted from the homogenized trigeminal ganglia using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Aliquots (1 μg) of total RNA were reverse transcribed into cDNA using ReverTra Ace -α- cDNA synthesis kit (TOYOBO, Japan). Quantitative fluorescent real-time polymerase chain reaction (QF RT-PCR) was performed to detect the expressions of NF-κB and IL-1βusing 7500 Real-Time PCR instrument (ABI Laboratories, USA). Amplification was performed using the SYBR Green PCR master mix (Bio-Rad Laboratories). The primer sequences used for amplification were as follows: NF-κB sense 866: 5'-GTGGAGTTTGGGAAGGATTTG-3', antisense 1061: 5'-TTGTCTTTGATTTCGGGGTAGT-3'; IL-1β sense 270: 5'-GGGATGATGACGACCTGC-3', antisense 402: 5'-GGAGAATACCACTTGTTGGCTTA-3'; GAPDH sense 231: 5'-GCAAGTTCAACGGCACAG-3', antisense 370: 5'-GCCAGTAGACTCCACGACAT-3'. PCR conditions included pre-denaturation at 95°C for 30 s, and 40 cycles of denaturation at 95°C for 10 s, 60°C for 10 s and 72°C for 40 s. Then, mRNA expressions of NF-κB and IL-1β were normalized to that of GAPDH. In order to evaluate the real-time PCR efficiency, 10-fold serial dilutions of cDNA were used for each amplicon. The slope values from protocol were substituted in the following equation: Efficiency = [10(1/slope)]. All primer sets had the efficiency of (100% (+/-) 10%). Quantization was performed by the comparative-threshold cycle (CT) method against the expression level of GAPDH. Data are compared to those from biopsy specimens cultured in the medium alone after normalization to GAPDH expression. Each experiment was performed in triplicate.

Fluorescent Immunohistochemistry

For immunohistochemistry, trigeminal ganglia were collected and cryosections (10 μm) were obtained on a cryostat (CM1900, Leica, Germany) followed by double-immunofluorescent staining. In brief, the sections were fixed in 4% paraformaldehyde phosphate buffer for 15 min and then rinsed with 0.01 M PBS for 5 min thrice. Free-floating sections were subsequently incubated for 10 min with 0.1% Triton X-100 in 0.01 M PBS and washed in 0.01 M PBS followed by incubation in 3% peroxide for 10 min and washing in 0.01 M PBS. After pre-incubation for 2 h in 10% normal goat serum (Zhongshan Golden Bridge, China), the sections were incubated with the primary antibody (rabbit anti-NF-κB p65) (1:100, Bios, China) for 1 h and then overnight at 4°C. Sections were washed extensively with

Table 1. Number of head scratching in different groups ($\overline{X}$ ±S, n=6). * *P<0.05* vs. control group; # *P<0.05* vs. NTG group. Comparisons were done with one-way ANOVA followed by post hoc Scheffe test.

Group	n	Number of head scratching			
		0-1 h	1-2 h	2-3 h	3-4 h
Control	6	8.00±2.83	9.50±3.07	7.00±2.61	2.33±1.21
NTG	6	69.83±9.45*	35.00±5.29*	20.83±4.49*	9.00±1.79*
Tet (1 mg/ml) + NTG	6	33.00±5.40#	18.83±4.54#	11.67±3.78#	8.00±1.67
Tet (5 mg/ml) + NTG	6	15.33±3.93#	15.17±3.31#	9.50±2.43#	6.00±1.41

0.01 M PBS and subsequently incubated with the secondary Taxas Red-conjugated Affinipure Goat Anti-Rabbit IgG (Cali-Bio, USA) for 1 h at room temperature and then overnight at 4℃. After extensively washing with 0.01 M PBS, the sections were incubated with GFAP mouse mAb (Cell Signaling, USA) overnight at 4°C. Images were captured by confocal laser-scanning microscopy (Leica LCS-SP2, Germany). Each image was converted to grayscale picture using Olympus MicroSuite Five image processing software and the intensity was detected.

Western blot assay

To investigate the changes in the expressions of migraine related proteins, trigeminal ganglia were collected, cut into pieces and used for the preparation of nuclear extraction. Proteins in the nuclear and cytoplasmic compartments were extracted via a commercial kit (Active Motif by Alexis). Protein concentration was measured with BCA method. Proteins (20 to 80 μg/lane) were subjected to sodium dodecyl sulfonate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. The membrane was blocked in 5% non-fat milk in TBS for 2 h at room temperature and then incubated with the primary antibody (rabbit anti-NF-κB p65) (1:200, Bios, China) overnight at 4°C. Proteins of equal amount were loaded to detect the expression of β-actin, an internal reference. Finally, an enhanced chemiluminescence system (Bio-Rad, USA) was used for visualization. For quantitative analysis, optical density was detected and analyzed using Image-Pro Plus software (Version 6.0 for Windows, USA).

Intracellular calcium concentration

Trigeminal ganglias were collected from untreated animals and animals injected with NTG or NTG and Tet, and immediately washed with D-Hanks-buffered saline solution (0.4 g/L KCl, 0.06 g/L KH_2PO_4, 8 g/L NaCl, 0.35 g/L $NaHCO_3$, 0.09 g/L $Na_2HPO_4.7H_2O$). According to the description of Fluo-3 (AM; dissolved in DMSO; Biotium, USA), ganglia was cut into pieces and ground sufficiently. Tissue suspensions were filtered by sieve mesh and then centrifuged at 1500 rpm for 5 min. The supernatant was removed and cells were re-suspended in D-Hanks followed by detection of cell viability. Then, these cells were seeded in 96-well plates (1×10^5cells/well). The cells were incubated in DMEM-F12 (Hyclone, USA) without fetal bovine serum (FBS) and 5 μM Fluo-3 AM at 37°C for 60 min, then washed in D-Hanks thrice and centrifuged. Finally, the suspensions were incubated with DMEM-F12 without FBS at 37°C for 30 min. The fluorescence intensity of calcium ion was determined by SpectraMax M2e Multifunctional ELIASA (Molecular Devices Corporation, USA). The experiments were repeated three times in duplicate.

Statistical analysis

Data were expressed as means ± standard error of the mean (S.E.M.). Statistical analysis was done with SPSS version 10.0 statistic software package. Comparisons were carried out with one way analysis of variance (ANOVA), followed by post hoc Scheffe test. A value of P <0.05 was considered statistically significant.

RESULTS

Head scratching

The behaviors of rats can reflect the influence of treatment. In the present study, the changes in the behaviors of treated rats were recorded, especially the head scratching. In general, the head scratching in rats treated with NTG alone (NTG group) increased significantly when compared with that in the control group (P<0.05*)*. But the rats pretreated with Tet (Group Tet) had significantly decreased head scratching when compared with animals in the NTG group (P<0.05). In addition, head scratching frequency varied from different time ranges. In 60 min, head scratching, occurred occasionally in the control group. The head scratching became frequent in the NTG group *(P<0.05*), and the frequency reached a maximal level in the NTG group. From 1 to 2 h, the head scratching frequency was still at the peak level in the NTG group. However, significant difference in the frequency of head scratching was observed between Tet group and NTG group (P<0.05). From 2 h to 3 h, the head scratching frequency decreased, but there was still significant difference between control group and NTG group *(P<0.05*), and between NTG group and Tet group (P<0.05). From 3 h to 4 h, the frequency of head scratching in the NTG group decreased gradually and head scratching was absent before being sacrificed. However, the number remained at its peak compared with the control group *(P<0.05*) (Table 1).

Inhibitory effects of Tet on mRNA expressions of NF-κB and IL-1β

Previous studies showed that both the cytoplasm and

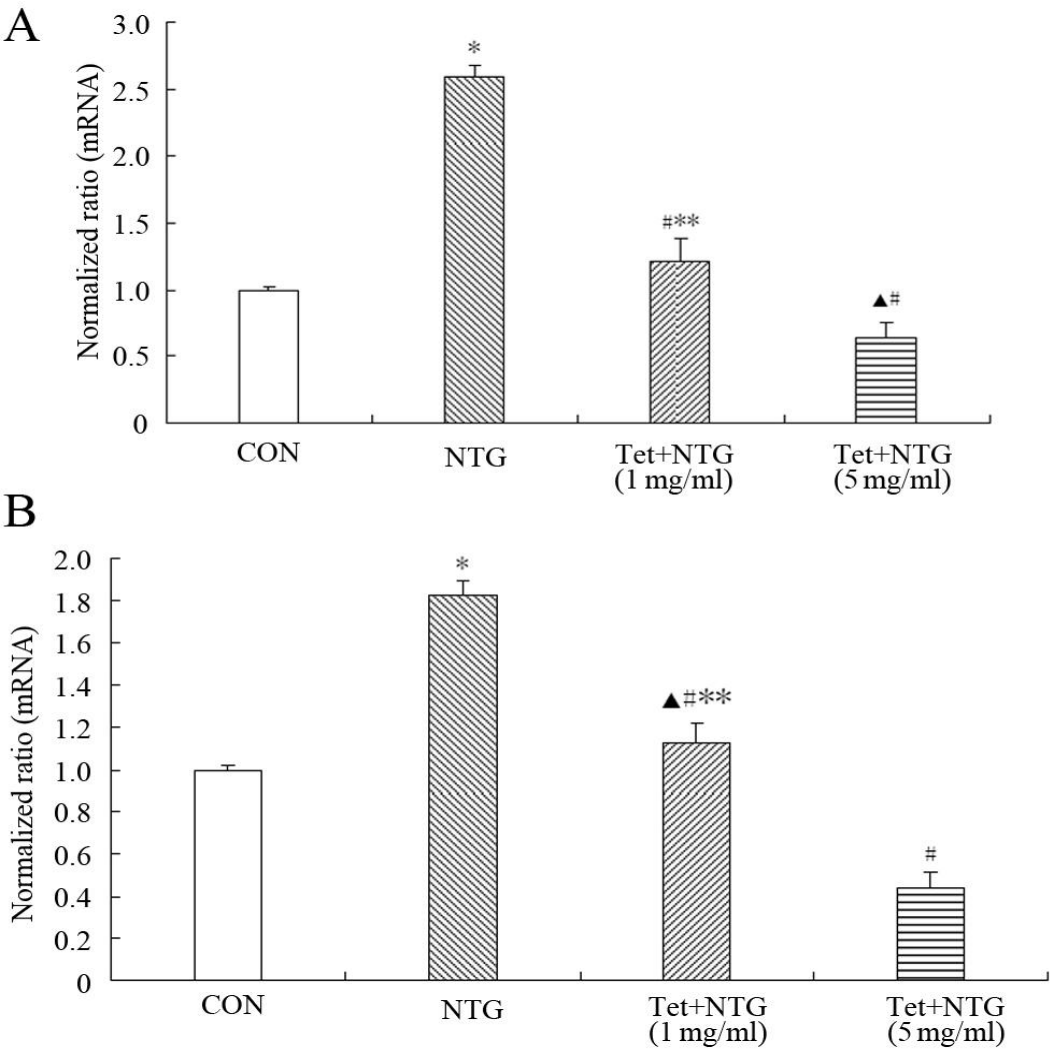

Figure 1. mRNA expressions of NF-κB and IL-1β in rat trigeminal ganglia were decreased by pretreatment of Tet. The mRNA levels of NF-κB (A) and IL-1β (B) in the trigeminal ganglia were significantly higher in the NTG group than those in the CON group and the increase of NF-κB and IL-1β induced by NTG was significantly reduced by Tet pretreatment (*P < 0.01 vs. CON group, ▲P < 0.05 vs. CON group, #P < 0.01 vs. NTG group, **P < 0.05 vs. Tet group (50 mg/kg) + NTG (one-way ANOVA with post hoc Scheffe test).

nucleus of neurons in the trigeminal nucleus caudalis have expression of NF-κB p65, even under activation condition. As a result, we chose three aspects to locate the expression of NF-κB. QF RT-PCR was performed to detect the mRNA expression of NF-κB in the trigeminal ganglia. Figure 1A shows the NF-κB and GAPDH expressions in our groups. A significant increase of NF-κB expression was observed in the NTG group when compared with the control group (P<0.01). However, marked reduction of NF-κB expression was noted in Tet pretreated animals even NTG was administered subsequently, when compared with NTG group (P<0.01). Furthermore, the NF-κB level was lower in high dose Tet group (5 mg/ml) than in low dose Tet group (1 mg/ml) (P<0.05). Moreover, when ompared with the control group, the NF-κB expression in the high dose Tet group (5 mg/ml) was markedly decreased (P<0.05), but there was no significant difference between low dose Tet group (1 mg/ml) and control group (P>0.05). In order to verify the release of inflammatory cytokines in the trigeminal ganglia in migraine animals, the mRNA expression of IL-1β was detected. Results showed similar trend to the expression of NF-κB (Figure 1B).

Inhibitory effects of Tet on protein expression of NF-κB

Similar findings were acquired in the protein expression of NF-κB p65 in the trigeminal ganglia by Western blot

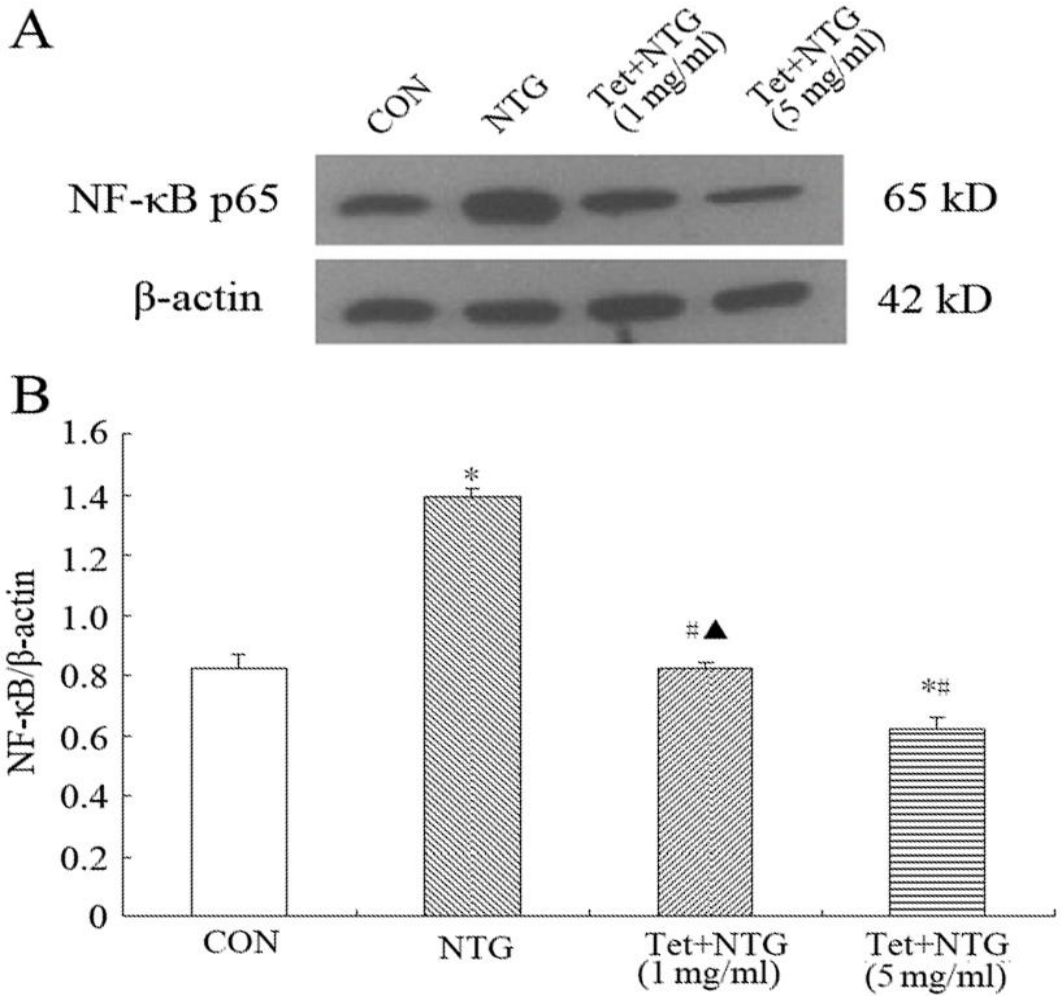

Figure 2. Protein expression of NF-κB in rat trigeminal ganglia was attenuated by Tet pretreatment. The expression of nuclear p65 in the trigeminal ganglia was measured by western blot assay (A). The level of nuclear p65 in the trigeminal ganglia was significantly higher in NTG group than that in CON group, and the increase of nuclear p65 expression induced by NTG was significantly reduced by Tet pretreatment (B, *P<0.01 vs. CON group, #P<0.01 vs. NTG group, ▲P<0.01 vs. Tet group (50 mg/kg) + NTG) (one-way ANOVA with post hoc Scheffe test).

assay. As illustrated in Figure 2, the protein expression of NF-κB p65 shared similar trend to the mRNA expression of NF-κB p65. In the NTG group, protein expression of p65 was increased significantly when compared with the control group (P<0.01). But p65 expression in the Tet group was markedly decreased as compared to NTG group (P<0.01). In addition, there was a significant difference between low dose Tet group (1 mg/ml) and high dose Tet group (5 mg/ml) (P<0.01).

Locating the NF-κB

To locate the NF-κB in the glial cells, immunohistochemistry was performed to detect the NF-κB expression. In the trigeminal ganglia, the glial distribution of NF-κB p65 was determined by double immunostaining with one antibody against GFAP and the other against NF-κB p65. As a marker for satellite cells, the GFAP-(+) immunofluorescent staining was shown in the trigeminal ganglia (Figure 3 green, arrows). As shown in Figure 3, the NF-κB p65 was observed in both nucleus and cytoplasm indicating the activation of NF-κB (Figure 3. red, arrows). Co-expression of NF-κB p65 and GFAP indicated the activation of NF-κB p65 in the satellite cells of the trigeminal ganglia (Figure 3. yellow, arrows).

Inhibitory effects of Tet on intracellular calcium concentration

Calcium is an important factor involving in the pathogenesis of migraine. However, no definite evidence has demonstrated the influence of calcium on NF-κB

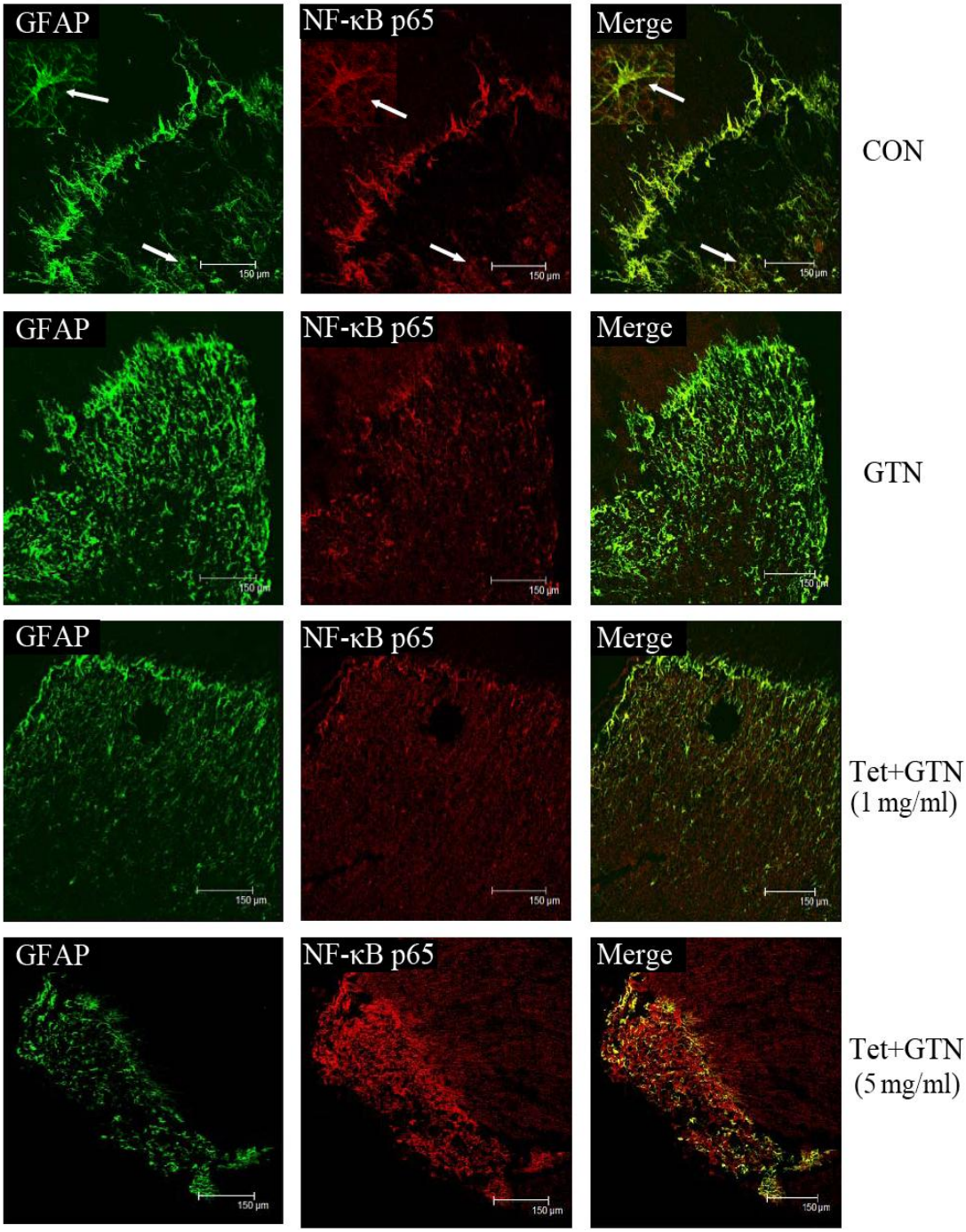

Figure 3. Activation of NF-κB in the satellite cells of rat trigeminal ganglia was decreased by Tet pretreatment. Satellite cells: green; NF-κB p65: red and merge: yellow.

activation in the trigeminal ganglia. To investigate the possible association between the increase of $[Ca^{2+}]i$ and NF-κB activation, the intracellular calcium concentration was detected in the trigeminal ganglia cells. Results are shown in Figure 4. In the NTG group, the intracellular $[Ca^{2+}]$ was significantly higher than that in the control group ($P<0.01$). In the Tet + NTG groups, the $[Ca^{2+}]i$ was markedly reduced when compared with the control group ($P<0.05$), but no remarkable difference was noted between low dose Tet group (1 mg/ml) and high dose Tet Group (5 mg/ml) ($P>0.05$). Moreover, there was a pronounced decrease of $[Ca^{2+}]i$ in the Tet + NTG group when compared with the NTG group (*P<0.05*). These findings were generally consistent with the effect of Tet

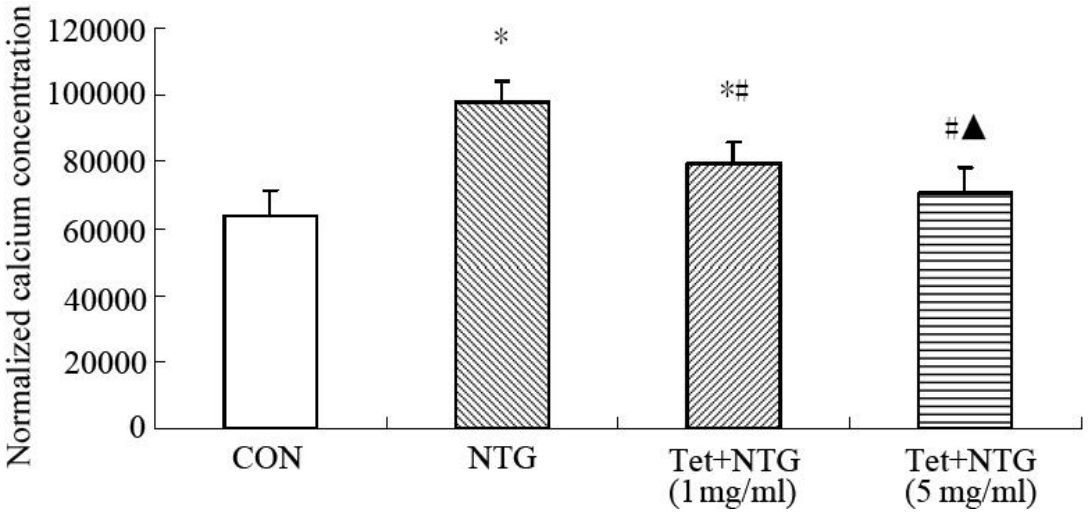

Figure 4. Calcium concentration in rat trigeminal ganglia was decreased by Tet pretreatment. The calcium concentration in the trigeminal ganglia was significantly higher in NTG group than that in CON group and the NTG induced increase of $[Ca^{2+}]i$ was significantly reduced by Tet pretreatment (*P<0.01 vs. CON group, ▲P< 0.05 vs. CON group, #P<0.01 vs. NTG group), (one-way ANOVA with post hoc Scheffe test).

on NF-κB activation.

DISCUSSION

A variety of traditional Chinese medicines have been applied in the treatment of nervous system diseases and proved to be effective in both prevention and treatment of these diseases. Tet, a traditional Chinese herb with multiple biological activities, can reduce the NF-κB activation. It is now believed to be a potent inhibitor of NF-κB activation and can effectively inhibit the expressions of pro-inflammatory cytokines. In the field of migraine, it has been shown that glia cells can interact with peripheral and central neurons and directly modulate the functions and state of excitability of these neurons. The cell bodies of neurons are surrounded by satellite cells in the trigeminal ganglion. As suggested in a recent study, satellite cells in the trigeminal ganglion not only are activated but also involved in the initiation and maintenance of peripheral sensitization together with the neurons. A variety of neuroexcitatory substances including reactive oxygen species, nitric oxide and inflammatory cytokines are produced and released when the microglia and astrocytes are activated (Watkins et al., 2003). It has been confirmed that astrocytes can synthesize a lot of cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), which promote the development of neurodegeneration by amplifying the brain inflammation and neuronal injury (Moynagh, 2005). Herein, we demonstrated that the production of inflammatory cytokine such as IL-1β was increased in the trigeminal ganglion, which could be reduced by Tet pre-treatment in a NTG-induced migraine model. Consequently, we focused on the potential contribution of satellite cell activation to the neural hypersensitivity and pain.

There is a general agreement that NF-κB activation plays an important role in the pathophysiology of migraine. Increasing evidence indicates that NF-κB can be activated in the neurons of superficial laminae I and II of the trigeminal nucleus caudalis (TNC) (Greco et al., 2005; Yin et al., 2009) and dura mater (Reuter et al., 2002) of rats by NTG, as well as in the monocytes of internal jugular venous blood from migraine patients without aura (Sarchielli et al., 2006). NF-κB is a transcription factor involving in the inflammatory responses (Reuter et al., 2002), which suggests NF-κB is a mediator of pro-inflammatory cytokines and other neurochemical signals leading to migraine attacks in the presence of NTG induced increase of NF-κB transcriptional activity (Jim, 2009). In the present study, we confirmed that NF-κB was released from the satellite cells in the NTG-induced migraine. This finding explains the release of inflammatory cytokine from satellite cells participating in the pathophysiology of migraine. In addition, the protein and mRNA expressions of NF-κB in the trigeminal ganglion were also increased significantly suggesting that NF-κB is a critical factor involving in the migraine. Moreover, our results demonstrated that the NTG-induced NF-κB activation could be inhibited by Tet pre-treatment. Further studies have indicated that Tet possesses the inhibitory effects on NF-κB activation. This may be attributed to its ability to prevent the degradation of IκBα, a cytoplasmic inhibitor of NF-κB by inhibiting nuclear translocation of p65 which depends on blocking the IκBα kinases α and β activities (Lin et al., 2008). However, it is unknown whether there is earlier event

which precedes the NTG-induced NF-κB activation and influences the inhibitory effect of Tet on NF-κB activation.

Previous studies have shown that NF-κB activation is dependent on the increase of cytosolic Ca^{2+} concentration. Calcium channel is a determinant of calcium influx in the cells, and can lead to nuclear translocation in the T lymphocytes and mast cells (Wu and Ng, 2007; Ho et al., 2004). In addition, Ca^{2+} is a critical factor involved in the pathogenesis of migraine (Amery et al., 1985). It is reasonable to postulate that NF-κB activation is related to the Ca^{2+} influx in the trigeminal ganglion. Therefore, we detected the intracellular calcium concentration of the trigeminal ganglion and results showed the increased NF-κB activation occurred simultaneously with the increase of cytosolic calcium concentration in the NTG-induced migraine. Moreover, Tet pre-treatment could decrease the cytosolic calcium concentration. The finding that NF-κB activation has a positive correlation with calcium elevation is in agreement with recent studies showing that the increase of intracellular calcium was an upstream event of NF-κB activation. Tet, as a natural non-specific CCB, has been applied in the treatment of numerous diseases (Wang and Lemos, 1995). We speculate that Tet can regulate the NF-κB activation by blocking the calcium channel and hence preventing Ca^{2+} influx. But more studies are required to confirm our findings.

In summary, our findings provided evidence that NF-κB activation was elevated in a NTG-induced migraine model and could induce the production of inflammatory cytokine. Furthermore, our results also highlighted the NF-κB activation in the satellite cells of trigeminal ganglia, and confirmed the role of glial cells in the pathogenesis of migraine. Finally, we also demonstrated that Tet could alleviate the head scratching symptoms of rats with migraine, which may be attributed to the suppression of NF-κB activation by Tet blocking the cytosolic calcium channel in the glial cells of rat trigeminal ganglia. Therefore, Tet may be used as a novel and promising candidate for the future treatment or prevention of migraine.

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