

# The Role of CREB and NF- $\kappa$ B in the p38MAPK-induced Activation of Spinal Cord Astrocytes

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**ABSTRACT Objective:** To investigate the role of CREB and NF- $\kappa$  B in the p38MAPK-induced activation of spinal cord astrocytes, p38MAPK signal transduction pathway in the activation of spinal cord astrocytes cell. **Methods:** Astrocytes, cultured from spinal cord of SPF rat, were grouped into the normal group, the SP stimulus group (SP group), the SP stimulus + SB203580 interrupt group (SP+SB group), the SP stimulus + PD98059 interrupt group (SP+PD group) and the SP stimulus + SN50 interrupt group (SP+SN group) in which SP (10-7 mol/L), SB203580 (10  $\mu$  mol/L), PD98059 (10  $\mu$  mol/L) and SN50 (10  $\mu$  mol/L) were added to the supernatant for 12h. The WB method, immunofluorescence method and ELISA method were used to determine the changes of p-p38, p-CREB, NF- $\kappa$  B p65, GFAP, TNF- $\alpha$ , IL-1 $\beta$  of astrocytes or supernatant at 12h and 24h. **Results:** p-p38, p-CREB, NF- $\kappa$  B p65 level in the SP group increased significantly. In the same time, GFAP, TNF- $\alpha$  and IL-1 $\beta$  level increased significantly too. When p38MAPK pathway was inhibited by SB203580 in the SP+SB group, p-p38, p-CREB, NF- $\kappa$  B p65 level and GFAP, TNF- $\alpha$  and IL-1 $\beta$  was significantly reduced compared with those in the SP group. In the SP+PD group, p-CREB level was significantly reduced compared with those in the SP group. In the SP+SN group, NF- $\kappa$  B p65 level was significantly reduced compared with those in the SP group. **Conclusion:** Astrocytes from spinal cord were significant activated after stimulated by SP in vitro, Inflammatory factors levels from glial cells were significantly increased through CREB and NF- $\kappa$  B signaling pathways after p38MAPK activation.

**Key words:** astrocytes; activation; p38MAPK; CREB; NF- $\kappa$  B

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## Introduction

In the model of rat chronic prostatitis pain, the excitatory neurotransmitter expression of substance P (substance P, SP), located in L<sub>5</sub>-S<sub>2</sub> of the spinal cord, is observed increased significantly. In the same position, the astrocytes of the spinal cord were activated and inflammatory changes could be observed. Furthermore, the SP could induce the astrocyte activation in vitro directly, which leads to inflammatory factors to be secreted. These factors participated in the pain modulation of spinal cord<sup>[1]</sup>. What will be the change of p38MAPK, CREB and NF- $\kappa$  B in the activation of astrocytes from spinal cord in vitro? What will be the change regulation of their active expression form? And, how p38MAPK, CREB and NF- $\kappa$  B affect on other inflammatory factors? All these questions need further study. In this study, astrocytes of the spinal cord were isolated and cultured from SPF rat. Astrocytes were activated by SP. The change of p-p38 (phosphorylation activated p38MAPK), CREB, NF- $\kappa$  B, TNF- $\alpha$ , IL-1 $\beta$  were detected. The relationship of p38MAPK activity with astrocyte activation was also studied in this paper.

## 1 Materials and methods

### 1.1 Major equipments and reagents

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DMEM/F12 culture medium and calf serum were purchased from American Hyclone Corporation. p-p38MAPK, CREB, NF- $\kappa$  B rabbit-anti-rat antibody were purchased from Wuhan Boster Biological Technology Co.Ltd. The 2nd antibody from sheep was purchased from American Santa Cruz Corporation. The chemiluminescence used in WB experiment was purchased from American Singaling Corporation. Tripure TM Isolation Reagent used in RNA extraction was purchased from Germany Boehringer Mannheim Company. Primer synthesis and RT-PCR kits were purchased from Shanghai Sangon Biological Engineering Technology & Services Co.Ltd. TNF- $\alpha$  ELISA kits was purchased from American Diaclone Corporation. The IL-1 $\beta$  detection kits were purchased from Beijing Bangding Taike biological reagents company.

### 1.2 Culture and identification of astrocytes from spinal cord of rats

Five SPF rats of 2~3 days, Supplied by the experimental animal center of Third Military Medical University, were used in the experiment. The superficial skin was disinfected. Then the spinal cord was taken out by cutting open the vertebral canal in surgery. After spinal meninges had been washed and stripped, the myeloid tissue was broken. Astrocytes were digested by 0.125% trypsin repeatedly and were collected by centrifugalization. Astrocytes put in DMEM /F12 including 10% calf serum were cultured in 5% CO<sub>2</sub> incubator at 37°C. The medium was exchanged every 3 days. Cells cultured for 7~9 days, set at 37°C constant temperature rocking bed, were shaken overnight at 200 r/min for about 12 hours.

The supernatant mainly having oligodendrocyte cells and microglia was discarded. The cells were rinsed for 1 time. The obtained cells adherent to the wall firmly were astrocytes. After trypsinization, astrocytes were inoculated to culture flask or 6-hole board according to the cell density needed for experiment. Astrocytes of the third generation were selected to be used in the experiment. Conventional method immunofluorescence of GFAP was used to identify the astrocytes from spinal cord (labeled with FITC).

1.3 Experimental groups and methods

Astrocytes of  $1 \times 10^6/\text{ml}$  were inoculated to the 6 hole board and cultured in serum-free medium for 24 hours. There were 4 groups in this study: 1) the normal control group (the normal group), 2) the SP stimulus group (the SP group,  $10^{-7}\text{mol/L}$ ), 3) the SP stimulus and the SB ( $10\mu\text{mol/L}$ ) blocking group (the SP+SB group). 4) the SP stimulus and the PD98059 ( $10\mu\text{mol/L}$ ) blocking group (the SP+PD group). 5) the SP stimulus and the SN50 ( $10\mu\text{mol/L}$ ) blocking group (the SP+SN group). The final concentration of sp was  $10^{-7}\text{mol/L}$ , and the final concentration of SB203580/ PD98059/SN50 was  $10\mu\text{mol/L}$ . The action time of SP and SB203580 was 12 hours. The observation time were 12 and 24 hours after the end of the action SP and/or SB203580/ PD98059/SN50.

1.4 Detection of p-p38MAPK,p-CREB,NF-κBp65 levels by Western blot

Astrocytes of the third generation with  $1 \times 10^6/\text{ml}$  were fully inoculated to 6 hole board. The different observation point holes were set as at 12<sup>th</sup> and 24<sup>th</sup> hour at random. After the experimental time had been reached, astrocytes were washed with cold Tris TBS. The culture was put on ice. Then  $500\mu\text{l}$  cell lysis  $1 \times \text{RIPA}$  buffer was added in each hole, incubating for 10 minutes. Astrocytes were scraped and transferred to a 1.5 ml centrifuge tube. Ultrasonication (500 W, 5s,  $\times 5$  times) was performed. Then centrifugalization of 10000 r/min was performed too. The supernatant was chosen to be frozen. Folin phenol method was used for protein quantitation. SDS-PAGE electrophoresis was done. After transmembrane, the 1st antibody (1:1000) and the 2nd antibody (1:2000) were added in turn and sealed. The ends were shown by chemiluminescence coloration and analyzed by computerized gel imaging system. The above process was repeated three times. Results were shown by light density ratio expression (OD ratio, objective fragment /GAPDH ).

1.5 Detection of GFAP expression by immunofluorescence

Conventional immunofluorescence method was used to detect GFAP expression of endothelial cells. 1:200 GFAP antibody was incubated with at 4 °C overnight. Then FITC labeled IgG were incubated for 30min. The results was observed and photographed in fluorescence microscope.

1.6 Detection of TNF-α and IL-1β with ELISA kit

All procedure was carried out according to the kit description. The reagents were mixed to be used. The blank and the positive control hole were set up.  $100\mu\text{l}$  standard preparation and sample were added in the hole in turn.  $50\mu\text{l}$  diluted biotin labeled antibodies were added to each hole, and then blocked.  $100\mu\text{l}$  diluted streptavidin labeled HRP were added in each hole with the good chain mildew compatible element mark HRP and blocked and incubated again. The board was washed. Substrate solution was added and incubated in the aluminum box of which out of light.  $100\mu\text{l}$  sulphuric acid was added in each hole to terminate the reaction. Standard curve was drawn and the concentration of measured factor was gained according to the results by the enzyme mark instrument with 450nm dominant wavelength. The unit of the TNF-α detection was pg/ml.

1.7 Statistical analysis

The data collected were expressed in mean ± variance. Student t test was used accordingly by using SPSS11.0, with  $P < 0.01$  as statistical significance.

2 Results

2.1 Results of detection of p-p38,p-CREB,NF-κBp65 levels by Western blot

p-p38, p-CREB, NF-κ Bp65 of spinal cord astrocytes in the SP group increased significantly. Compared with the SP group, p-p38, p-CREB, NF-κ Bp65 of SP + SB group were significantly lower after P38MAPK pathway was blocked by SB203580. p-p38, NF-κ Bp65 of SP+PD group had not significant changes, but p-CREB became lower after CREB pathway was blocked by PD98059. p-p38, p-CREB of SP+SN group had not significant changes, but NF-κ Bp65 became lower after NF-κ B was blocked by SN50. Which is shown in the Figure 1, Table 1-3.

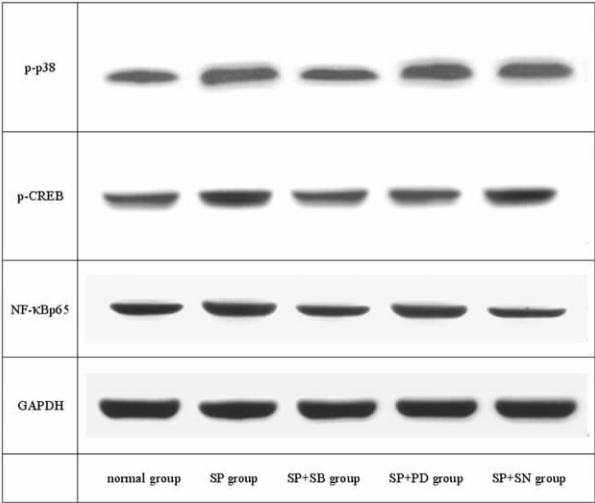


Figure 1 Western blot test results of p-p38, p-CREB, NF-κ Bp65 levels of astrocytes (24h )

Table 1 p-p38 levels of spinal cord astrocytes (IOD / GAPDH, n = 3)

Group	Time	
	12 h	24 h
normal group	0.224± 0.017	0.219± 0.018
SP group	0.387± 0.031*	0.370± 0.028*
SP+SB group	0.235± 0.018▲	0.224± 0.018▲
SP+PD group	0.370± 0.030▲	0.357± 0.027▲
SP+SN group	0.383± 0.029▲	0.365± 0.027▲

Note: \* P<0.01 compared with normal group, ▲ P<0.01 compared with SP group, ▲ P<0.01 compared with SP+SB group

Table 2 p-CREB levels of spinal cord astrocytes (IOD / GAPDH, n = 3)

Group	Time	
	12 h	24 h
normal group	0.254± 0.021	0.268± 0.022
SP group	0.354± 0.029*	0.356± 0.027*
SP+SB group	0.283± 0.021▲	0.268± 0.022▲
SP+PD group	0.273± 0.022▲	0.274± 0.020▲
SP+SN group	0.364± 0.027*▲	0.366± 0.027*▲

Note: \* P<0.01 compared with normal group, ▲ P<0.01 compared with SP group, ▲ P<0.01 compared with SP+SB group

Table 3 NF-κ Bp65 levels of spinal cord astrocytes (IOD / GAPDH, n = 3)

Group	Time	
	12 h	24 h
normal group	0.297± 0.024	0.288± 0.023
SP group	0.452± 0.037*	0.445± 0.033*
SP+SB group	0.321± 0.024▲	0.310± 0.025▲
SP+PD group	0.440± 0.036*▲	0.441± 0.033*▲
SP+SN group	0.329± 0.025▲	0.323± 0.024▲

Note: \* P<0.01 compared with normal group, ▲ P<0.01 compared with SP group, ▲ P<0.01 compared with SP+SB group

2.2 Results of GFAP of the spinal cord astrocytes

GFAP of spinal cord astrocytes in the SP group increased significantly. Compared with the SP group, GFAP of SP + SB group were significantly lower after P38MAPK pathway was blocked by SB203580. GFAP of SP+PD group decreased after CREB pathway was blocked by PD98059. GFAP of SP+SN group became lower after NF-κ B was blocked by SN50 too. Which is shown in the Figure 2, Table 4.

2.3 Results of TNF-a and IL-1β sereted by the spinal cord astrocytes

TNF-a and IL-1β of spinal cord astrocytes in the SP group increased significantly. Compared with the SP group, TNF-a and IL-1β of SP + SB group were significantly lower after P38MAPK pathway was blocked by SB203580. TNF-a and IL-1β of SP+PD group decreased after CREB pathway was blocked by PD98059. TNF-a and IL-1β of SP+SN group became lower after NF-κ B was blocked by SN50 too. Which is shown in the Table 5-6.

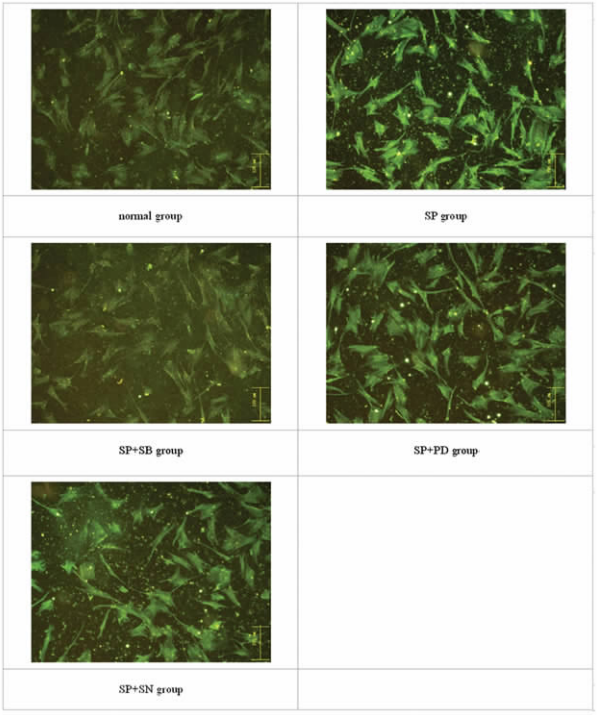


Figure 2 GFAP levels of spinal cord astrocytes (24 h)

Table 4 results of GFAP levels of spinal cord astrocytes (IOD, n = 5)

Group	Time	
	12 h	24 h
normal group	4.36± 0.54	4.29± 0.54
SP group	14.41± 2.03*	12.85± 1.81*
SP+SB group	5.04± 0.55 <sup>▲</sup>	4.72± 0.63 <sup>▲</sup>
SP+PD group	9.33± 1.01* <sup>▲▲</sup>	8.30± 0.90* <sup>▲▲</sup>
SP+SN group	10.49± 1.26* <sup>▲▲</sup>	8.92± 1.14* <sup>▲▲</sup>

Note: \* P<0.01 compared with normal group, <sup>▲</sup> P<0.01 compared with SP group, <sup>▲▲</sup> P<0.01 compared with SP+SB group

Table 5 TNF-a levels of spinal cord astrocytes (pg/ml, n=5, $\bar{x}\pm s$ )

Group	Time	
	12 h	24 h
normal group	128.3± 12.9	129.7± 13.1
SP group	245.1± 29.2*	235.3± 28.0*
SP+SB group	171.4± 17.3* <sup>▲</sup>	168.3± 17.0* <sup>▲</sup>
SP+PD group	195.4± 21.5* <sup>▲▲</sup>	186.1± 20.5* <sup>▲▲</sup>
SP+SN group	201.5± 21.4* <sup>▲▲▲▲</sup>	196.8± 20.9* <sup>▲▲▲▲</sup>

Note: \* P<0.01 compared with normal group, <sup>▲</sup> P<0.01, <sup>▲▲</sup> P<0.05 compared with SP group, <sup>▲▲</sup> P<0.01, <sup>▲▲▲</sup> P<0.05 compared with SP+SB group

Table 6 IL-1 $\beta$  levels of spinal cord astrocytes (pg/ml, n=5, $\bar{x}\pm s$ )

Group	Time	
	12 h	24 h
normal group	22.2± 2.2	21.8± 2.2
SP group	104.4± 12.4*	95.1± 11.3*
SP+SB group	34.1± 3.4* <sup>▲</sup>	33.1± 3.3* <sup>▲</sup>
SP+PD group	61.1± 6.7* <sup>▲▲</sup>	56.4± 6.2* <sup>▲▲</sup>
SP+SN group	60.2± 6.4* <sup>▲▲</sup>	55.6± 5.9* <sup>▲▲</sup>

Note: \* P<0.01 compared with normal group, <sup>▲</sup> P<0.01 compared with SP group, <sup>▲▲</sup> P<0.01 compared with SP+SB group

Discussion

Astrocytes are the most widely distributed glial cells in the central nervous system. They not only have insulation, nutrition, protection and support role on the neurons, but also have important roles in the damage and repair process of central nervous system [2,3]. In this study, astrocytes from spinal cord of SPF rats were primarily cultured and purified. On account of the different biological characteristics of nerve cells, astrocytes, oligodendrocytes cells and microglia in the cell suspension, pure astrocytes were got through separation and purification. According to the identification, the purity was more than 95 percent. All these provided a good basis for the follow-up experiment.

The mitogen activated protein kinase (MAPK) is a class of the serine / threonine protein kinase within cells. MAPK is the most important signal system of eukaryotic cells transducing extra-cellular signal to the interior of cells to cause cellular response[4]. The mainly effect of MAPK is on the different stimulation from the exterior of cells and the basic physiological processes of cells. The p38 mitogen-activated protein kinase (p38MAPK) signal

pathway participates in cellular growth and the intercellular space function synchronization and many other kinds of physiological processes. The p38MAPK is closely related with inflammation, stress response regulation and considered as the common pathway of the cellular information transfer [5,6]. The p38MAPK, similar to JNK/SAPK pathway, may be activated by inflammation, stress and damage. After being activated, corresponding proteins are produced through transcription factors. It mainly carries on the conduction of the inflammatory cell factors and stress signals of many kinds of cells. Furthermore, p38MAPK has relationship with cell apoptosis and is the main pathway of the external stimulus signals transduced from the exterior of cells[7,8]. Researches discovered that p38MAPK could participate in the inflammation process and the stress response directly, or indirectly through the mediation of some cell factors such as tumor necrosis factor Alpha (TNF- $\alpha$ ). After the macrophages of mice had been stimulated by LPS, the phosphorylation of p38MAPK in the macrophages could occur. If the phosphorylation was inhibited, the formation of TNF- $\alpha$  of



macrophages was reduced or even completely blocked<sup>[9]</sup>. The biological effect of p38MAKP can be inhibited by SB203580, SB216995, SB220025 or VK199, which are pyrrole imidazole compounds. These compounds can clearly inhibit the role of p38MAKP in the corresponding pathophysiology activities<sup>[10]</sup>. The mechanisms of the p38MAKP inhibitors are inhibiting the transcription factors or the further activation of protease of the lower reaches through their high-affinity with p38MAPK. The results of this study indicate that SP stimulation of the spinal cord astrocytes could cause marked phosphorylation of p38MAKP.

cAMP response element binding protein (CREB), involved in the regulation of gene expression of a variety of biological molecules, belongs to basic amino acid leucine zipper (bZIP) transcription factor family. Nuclear factor-kappa b (NF-κ B), regulating variety of inflammatory and immune gene expression, is an important factor in transcriptional regulation. There is evidence that these factors play a role in the pain mechanisms. Research on the biological characteristics of these factors and the regulation mechanism of glial cell activation of spinal cord in the chronic prostate pain contributes to a better understanding of the pathogenesis of chronic prostate pain and the promotion of effective development of anti-chronic prostate pain drug.

In this study, p-p38, p-CREB, NF-κ Bp65 of spinal cord astrocytes in the SP group increased significantly. Compared with the SP group, p-p38, p-CREB, NF-κ Bp65 of SP + SB group were significantly lower after P38MAPK pathway was blocked by SB203580. p-p38, NF-κ Bp65 of SP+PD group had not significant changes, but p-CREB became lower after CREB pathway was blocked by PD98059. p-p38, p-CREB of SP+SN group had not significant changes, but NF-κ Bp65 became lower after NF-κ B was blocked by SN50.

The main inflammatory pain factors secreted by nerve center were TNF-α, IL-6, IL-1, free radicals, nitric oxide, which are closely related to the conduction, maintenance and generalization of pain because of the increasing of astrocytes proliferation and inflammatory factors in an animal model of neuropathic pain<sup>[11]</sup>. TNF-α is one of the most important inflammatory cytokines. High concentration of TNF-α in the central nerve was considered as a toxic peptide. Serious inflammation, encephalopathy and neurodegenerative diseases could happen in transgenic mice with high expressed TNF-α. TNF-α, mainly through the binding with cell receptor of p55, activates phosphatidylcholine specific phospholipase C, releases diacylglycerol (DAG) and plays a variety of biological effects, such as promotion of NF-κ B activation, induction of cells to produce oxygen free radicals, etc<sup>[12]</sup>. In this study, TNF-α and IL-1β of spinal cord astrocytes in the SP group increased significantly. Compared with the SP group, TNF-α and IL-1β of SP + SB group were significantly lower after P38MAPK pathway was blocked by SB203580. TNF-α and IL-1β of SP+PD

group decreased after CREB pathway was blocked by PD98059. TNF-α and IL-1β of SP+SN group became lower after NF-κ B was blocked by SN50 too.

In short, SP stimulation can cause significant activation of the spinal cord astrocytes. The p-p38 could participate in the activation process and the p38MAKP signaling pathway blockers can reduce the increasing of inflammatory factors secreted by the spinal cord astrocytes. To block the signaling pathway of p38MAKP may be contributed to the treatment of locally inflammatory neuralgia, especially the generalization and continuity of it.

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CREB 和 NF-κ B 在 p38MAPK 所致脊髓星形胶质细胞活化中的作用

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摘要 目的:探讨 CREB 和 NF-κ B 在 p38MAPK 所致脊髓星形胶质细胞活化中的作用,明确脊髓星形胶质细胞活化中 p38MAPK 细胞信号转导途径的作用。方法:分离培养 SPF 大鼠脊髓星形胶质细胞,设正常组、SP 刺激组 (SP 组,10<sup>-7</sup> mol/L)、SP 刺激 +SB203580(10 μ mol/L) 阻断 p38MAPK 组(SP+SB 组)、SP 刺激 + PD98059(10 μ mol/L)阻断 CREB 组(SP+PD 组)、SP 刺激 + SN50 (10 μ mol/L) 阻断 NF-κ B (SP+SN 组)。WB 法、免疫荧光法、ELISA 法检测 12 h 和 24 h 时 p-p38、p-CREB、NF-κ Bp65 水平及 GFAP、TNF-、IL-1β 水平变化。结果:SP 组脊髓星形胶质细胞 p-p38、p-CREB、NF-κ Bp65 显著升高,GFAP 水平显著增高,同时 TNF- 和 IL-1β 水平显著增高。与 SP 组比较,用 SB203580 阻断 p38MAPK 通路后,SP+SB 组 p-p38、p-CREB、NF-κ Bp65 显著降低,GFAP、TNF- 和 IL-1β 水平显著降低。用 PD98059 阻断 CREB 通路后,SP+PD 组 p-p38、NF-κ Bp65 无显著变化,p-CREB 显著降低,GFAP 水平降低,同时 TNF- 和 IL-1β 水平降低。用 SN50 阻断 NF-κ B 通路后,SP+SN 组 p-p38、p-CREB 无显著变化,NF-κ Bp65 显著降低,GFAP 水平降低,同时 TNF- 和 IL-1β 水平降低。结论:体外培养中,SP 刺激后脊髓星形胶质细胞显著活化,p38MAPK 活化后通过 CREB 及 NF-κ B 信号途径导致胶质细胞炎症因子水平显著升高。

关键词:星形胶质细胞;活化;p38MAPK;CREB;NF-κ B

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