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An Inhibitor of p38 MAPK Prevents Apoptosis of Cultured Cerebellar Granule Neurons via Inhibiting the Activity of JNK

LI Ming-tao, WANG Wen-ya, Sun Juan, TANG Xiao-li, SU Xing-wen, QIU Peng-xin, YAN Guang-mei

(Department of Pharmacology, Sun Yat-sen University of Medical Sciences, Guangzhou 510089, China)

Abstract: 【Objective】 To study the effect of the specific p38 mitogen-activated protein kinase(p38 MAPK) inhibitor SB203580 on apoptosis of cerebellar granule neurons induced by low potassium. 【Methods】 Apoptosis was induced by switching the cultured cerebellar granule neurons from a culture medium containing K^+ 25 mmol·L^{-1} to a medium containing K^+ 5 mmol·L^{-1}(cLK). Fragmentation of DNA was analyzed using agarose gel eletrophoresis. SAPK/JNK activity was measured by SAPK/JNK assay kit. 【Results】 Low potassium resulted in apoptosis as characterized by morphological and biochemical features, but the specific p38 MAPK inhibitor SB203580 improved the survival of cerebellar granule neurons cultured in cLK medium by blocking apoptosis in a concentration-dependent manner. The expression and phosphorylation of c-Jun increased and the activity of c-Jun N-terminal protein kinase (JNK) elevated when cerebellar granule neurons were cultured in cLK medium. But when the cerebellar granule neurons cultured in cLK medium were exposed to 25 μmol·L^{-1} SB203580, the expression and phosphorylation of c-Jun and the activity of JNK were both decreased evidently. 【Conclusions】 These results indicate that SB203580 inhibits the activation of JNK and phosphorylation of c-Jun, and therefore protects granule neurons from apoptosis induced by low potassium.

Key words: apoptosis/drug effects; cerebellar granule neuron; protein kinase MAPK inhibitor/pharmacology

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p38 MAPK 抑制剂通过抑制 JNK 活性抑制培养的小脑颗粒神经元凋亡

黎明涛, 王文雅, 孙 娟, 唐孝礼, 苏兴文, 邱鹏新, 颜光美

(中山医科大学药理教研室, 广东 广州 510089)

摘 要: 【目的】 研究特异性 p38 分裂原激活的蛋白激酶(MAPK)抑制剂 SB203580 对低钾诱导的小脑颗粒神经元凋亡的作用。【方法】 把体外培养的小脑颗粒神经元从含去极化浓度钾离子(KCl 25 mmol·L^{-1})的培养基中转移至低钾培养基(KCl 5 mmol·L^{-1})中诱导神经元凋亡。凝胶电泳分析 DNA 片段, SAPK/JNK 分析盒测定 c-Jun N-末端蛋白激酶(JNK)活性。【结果】 低钾诱导小脑颗粒神经元的具有典型形态学和生化特征的凋亡。特异性的 p38 MAPK 抑制剂 SB203580 通过抑制细胞凋亡, 促进低钾环境中培养的小脑颗粒神经元存活。这种保护作用具有浓度依赖性。培养于低钾环境中的颗粒神经元, c-Jun 表达和磷酸化水平升高了, 且激活了 JNK 活性。当小脑颗粒神经元生长在含 SB203580 25μmol·L^{-1}的低钾培养基中, c-Jun 表达、磷酸化水平和 JNK 活性都明显降低。【结论】 SB203580 抑制 JNK 活性, 降低 c-Jun 的磷酸化而对低钾培养的小脑颗粒神经元具有保护作用。

关键词: 凋亡/药物作用; 小脑颗粒神经元; 蛋白激酶(MAPK)抑制剂/药理

We and others have shown that depolarizing concentration of K^+ (25 mmol·L^{-1}) improved the survival of cultured cerebellar granule neurons by blocking their cell death via apoptosis[1,2]. The death of neurons induced by nondepolarizing conditions is characterized by all of the morphological and bio-

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Biography: Li Ming-tao(1957-), male, Jiangxi Province, Associate Professor of Pharmacology.

chemical features of apoptosis, including cytoplasmic blebing, condensation and aggregation of nuclear chromation and internucleosomal DNA fragmentation[2]. Due to their relative homogeneity, primary cultures of cerebellar granule neurons represent an ideal *in vitro* model system for studying the cellular and molecular events underlying apoptosis of mammalian central nervous system (CNS) neurons. Mammalian mitogen-activated protein kinases (MAPK, including the extracellular signal-regulated protein kinase, the c-Jun amino-terminal kinase, the p38 sustained activation of Jun kinase) have been shown to precede apoptosis of PC12 pheochromicytoma cells induced by withdrawal of trophic factors. The pyridinyl imidazole compound SB203580, a selective p38 inhibitor promoted the survival of PC12 cells in which trophic factors have been removed. This indicated that p38 inhibitor-sensitive pathways may be involved in apoptosis of neurotrophic factor-deprived primary neurons[3]. Whether the specific p38 inhibitor SB203580 prevents cerebellar granule neurons from apoptosis induced by nondepolarizing conditions and how it affects the survival of cerebellar neurons cultured in low K^+ medium remain unknown.

1 Materials and Methods

1.1 Materials

SB203580, Hoechst 33258 were obtained from Sigma Chemical Co. SAPK/JNK assay kit (LumiGLO) was obtained from New England Biolabs, Inc. Seven or eight-day-old Sprague-Dawley rats (15 g ±2 g, clean) of either sex were obtained from Sun Yat-sen University of Medical Sciences.

1.2 Cell culture and cell viability assay

Cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley rat pups as described previously[4]. Cell viability assays and morphological methods used were the same as before[5].

1.3 Detection of DNA fragmentation

Fragmentation of DNA was analyzed as described previously[6].

1.4 Immunoblotting

Cells were fixed in 30 g/L paraformaldehyde for 20 min at 4 ℃, permeabilized with phosphate-buffered saline (PBS) solution for three times, and then were blocked with 50 g/L goat serum in PBST for 1 h. After washing three times with PBST, the polyclonal phospho-c-Jun rat antibody was used at a dilution of 1∶1000 overnight at 4 ℃. After washing 3 times with PBST, cells were incubated with goat-anti-rat monoclonal antibody. The primary and secondary antibodies were diluted in 20 g/L.

1.5 Protein kinase assays

JNK activity was measured using SAPK/JNK assay kit, as described by Guo Yan-Lin[7].

1.6 Statistical analysis

Results were presented as $\bar{x} \pm s$. Statistical analysis was performed with t test.

2 Results

2.1 SB203580 blocked neuronal death induced by lowering K^+ in a dose-dependent manner

When fully differentiated granule neurons were switched from depolarizing medium (KCl 25 mmol·L^{-1}, cHK) to low potassium-medium (KCl 5 mmol·L^{-1}, cLK), they died by apoptosis. But SB203580, a specific p38 MAPK inhibitor, was capable of maintaining survival of granule neurons in the absence of elevated K^+. At 24 h, while over 60% of the K^+ deprived, neurons were dead. Those treated with SB203580 (25 μmol·L^{-1}) displayed viability that was comparable with cells maintained in high K^+. The survival effect was observed at doses $\geqslant$5 μmol·L^{-1}, and the maximal effect was observed at 25 μmol·L^{-1} (Table 1). Although SB203580 effectively prevented the death at 24~48 h after lowering K^+, it was much less effective at 72 h.

2.2 SB203580 prevented neurons from morphological features of apoptosis

We examined the morphological changes in cultured cerebellar granule neurons induced by cLK medium using Hoechst 33258 staining, which specifically labeled nuclear chromatin. Cerebellar granule

Table 1　SB203580 protects cerebellar granule neurons from apoptosis induced by cLK　($n=3$)

SB203580	Neuronal Survival
$c/\mu mol \cdot L^{-1}$	$\bar{x} \pm s$, %
0	38.4±5.7
1	39.5±5.9 1)
5	46.8±6.4 2)
10	57.6±6.9 2)
15	85.1±8.3 2)
20	93.1±9.5 2)
25	97.6±8.8 2)

After 8 days, cultures were switched to different concentrations of SB203580 in 5 $mmol \cdot L^{-1}$ KCl medium for 24 h. Control neurons were maintained in 25 $mmol \cdot L^{-1}$ KCl medium and the numbers of survival cells were taken as 100 % [(1.29±0.01)× $10^9 \cdot L^{-1}$]. Compared with 0 $\mu mol \cdot L^{-1}$ SB203580, 1) $P<0.05$, 2) $P<0.01$

neurons cultured in cHK medium for 24 h maintained normal nuclear morphology (Fig. 1A). Switching cerebellar granule neurons from cHK medium to cLK medium for 24 h resulted in the typical apoptosis changes in neuronal nuclei, including a characteristic condensation of nuclear chromatin and heterochromatic clumping (Fig. 1B). Addition of SB203580 (25 $\mu mol \cdot L^{-1}$) to cLK medium rescued most of the neurons and maintained normal nuclear morphology (Fig. 1C)

2.3　SB203580 prevented internucleosomal DNA fragmentation of cerebellar granule neurons induced by cLK

Apoptotic cell is often associated with internucleosomal DNA fragmentation resulting in nucleosome size DNA and multiples thereof. DNA was extracted from cultured cerebellar granule neurons after treatment with SB203580 for up to 24 h and analyzed by agraose gel electrophoresis. Agarose gel eletrophoresis of DNA extracted from neurons cultured in cHK medium showed a wide band (Fig. 2, lane 2). When the neurons were switched from cHK medium to cLK medium, agarose gel eletrophoresis of neuron DNA revealed that cLK medium induced typical apoptotic DNA fragmentation characterized by oligonucleosome-length DNA of about 185 base pairs (Fig. 2, lane 3). Exposure of cultured cerebellar granule neurons to 25 $\mu mol \cdot L^{-1}$ SB203580 prevented the DNA fragmentation induced by cLK medium (Fig. 2, lane 4).

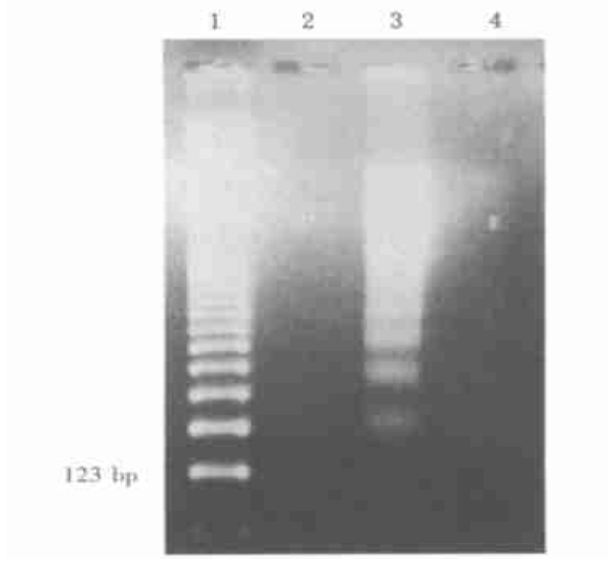

Fig. 2　Agarose gel eletrophoresis for detecting DNA fragmentation induced by cLK

lane 1, DNA size marker ladder; lane 2, in cHK medium for 24 h (control); lane 3, in cLK medium for 24 h; lane 4, 25 $\mu mol \cdot L^{-1}$ SB203580 in cLK medium for 24 h. Note that 185-base pair ladder characteristic of the DNA degradation that occurs in apoptotic cells is detected in cLK medium

2.4　SB203580 inhibited the overexpression of c-Jun induced by cLK

When cerebellar granule neurons were switched from cHK medium to cLK medium, the death-related nuclear transcription factor overexpressed. Exposure of cultured cerebellar neurons to 25 $\mu mol \cdot L^{-1}$ SB203580 inhibited the overexpression of c-Jun induced by cLK (Fig. 3).

Fig. 3　Pattern of expression of c-Jun in cerebellar granule neurons treated with or without 25 $\mu mol \cdot L^{-1}$ SB203580 for 24 h

Lane 1, control in cHK medium; Lane 2, in cLK medium; Lane 3, 10 $\mu mol \cdot L^{-1}$ SB203580 in cLK medium; Lane 4, 25 $\mu mol \cdot L^{-1}$ SB203580 in cLK medium

2.5　SB203580 inhibited the phosphorylation of c-Jun induced by cLK

We analyzed the phosphorylation of c-Jun by using the phospho-c-Jun antibody in immunofluorescence experiments with cerebellar granule neurons. Cerebellar granule neurons cultured in cHK medium shows little phosphorylated c-Jun (Fig. 4A). When neurons were switched to cLK medium, phosphorylated c-Jun increased (Fig. 4B). Exposure of cultured neurons to SB203580 25 $\mu mol \cdot L^{-1}$ suppresses the phosphorylation of c-Jun induced by cLK (Fig. 4C).

2.6 SB203580 inhibited the elevated activity of JNK induced by cLK

By using the phospho-c-Jun antibody in immunofluorescence experiments with cerebellar granule neurons, JNK activation was observed after removal of KCl from primary cerebellar granule neurons (Fig. 5, lane1). Exposure of cerebellar granule neurons to SB203580 suppressed the elevated JNK activity induced by cLK medium in a dose-dependent manner (Fig. 5, lane 2~4).

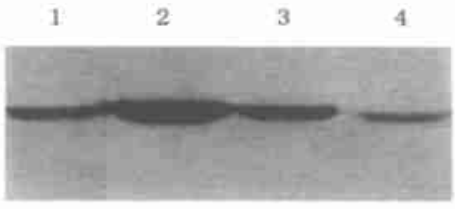

Fig. 5 Analysis of JNK activity of cLK by Western blot

Lane 1, control, in cHK medium for 24 h; Lane 2, in cLK medium for 24 h; Lane 3, plus 10 $\mu mol \cdot L^{-1}$ SB203580; Lane 4, plus 25 $\mu mol \cdot L^{-1}$ SB203580

3 Discussion

Chronic depolarization induced by elevated extracellular K^+ can substitute for neurotrophic factors in maintaining survival of several neuronal types in culture. We have shown that SB203580, a pyridinylimidazole compound, rescues cerebellar granule neurons from apoptosis induced by nondepolarization. When cerebellar granule neurons cultured in cHK medium are switched to cLK medium, the features characteristic of apoptosis occur, such as chromatin condensation, pyknosis, and nucleosomal DNA fragmentation. However, if the neurons cultured in cHK medium are switched to cLK medium containing 25 $\mu mol \cdot L^{-1}$ SB203580, most of neurons survived.

The c-Jun N-terminal protein kinase (JNK) pathway modulates AP-1 activity and is involved in apoptosis in response to stress or withdrawal of survival signals in neuronal cells[8]. The JNK signaling cascade is one of important neuronal models of cell death. The nuclear transcription factor c-Jun is an endogenous substrate of JNK. A role for JNK in the induction of apoptosis has also been examined in non-neuronal systems. Inhibiting JNK or kinase upstream of JNK protects different cell types from death induced by a variety of stimuli such as camptothecin, thermal shock, cis-platinum, and ceramide[9]. Although many other signaling events, in addition to JNK/c-Jun activation, may be involved in committing neurons to a death pathway, inhibition of c-Jun expression and suppression of elevated JNK activity can rescue cerebellar granule neurons from apoptosis induced by non-depolarization.

Our results suggest that the p38 inhibitor SB203580 block cerebellar granule neurons against apoptosis induced by lowering K^+, the mechanisms of which may include inhibiting the expression and phosphorylation of c-Jun and suppressing the elevated activity of JNK. In summary, we have shown that SB203580 can improve survival of mature granule neurons. Aberrant death of mature neurons, presumably by apoptosis, occurs in several neurodegenerative diseases. Studying the precise mechanisms by which SB203580 promotes neuronal survival may have important clinical implications in the prevention of neuronal death in neurodegenerative diseases.

(Fig. 1, 4 see in inside back cover)

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(continued from page 164)

designated m1-m5. Which mAChR subtype is responsible for the protective effect and its molecular mechanism should be further studied.

(Fig. 2, 3 see in inside front cover)

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p38MAPK 抑制剂通过抑制 JNK 活性抑制培养的小脑颗粒神经元凋亡 （正文见第 165 页）

An Inhibitor of p38 MAPK Prevents Apoptosis of Cultured Cerebellar Granule Neurons via Inhibiting the Activity of JNK (Text in page 165)

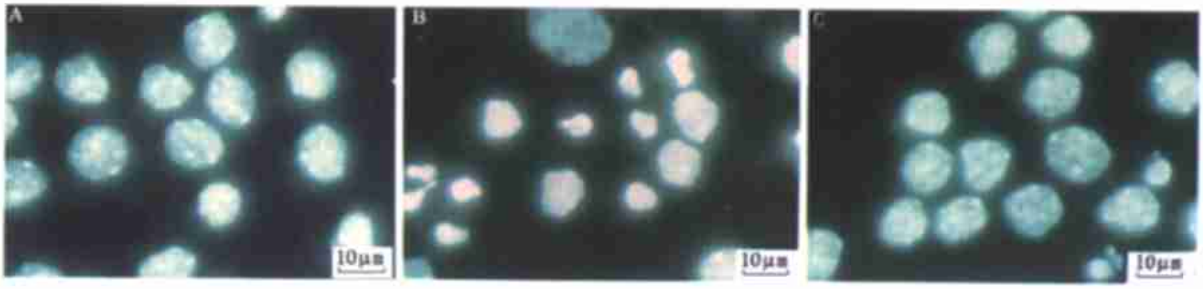

图 1　低钾诱导的小脑颗粒神经元凋亡的形态学特征

Fig. 1　Morphological features of apoptosis induced by low potassium in cultured cerebellar granule neurons

Note the typical apoptotic morphology: nuclear condensation and heterochramatin clumping in neurons in B, but not in A and C. Black arrow, apoptotic neurons (Hoechst 33258 stained, ×1000)

图 4　经 cLK 处理的小脑颗粒神经元中 c－Jun 磷酸化

Fig. 4　c－Jun becomes phosphorylated after cerebellar granule neurous were treated by cLK

Cerebellar granule neurons cultured on glass coverslips were maintained in serum－free medium. (A) control, in cHK medium for 24 h; (B) in cLK medium for 24 h. Nuclear phospho－c－Jun staining was clearly visible; (C) treated with SB203580 25 μ mol · L^{-1} in cLK medium for 24 h (phospho－c－Jun－specitic antibody and FITC stained, ×1000)

散发性早发帕金森病 parkin 基因 1、2 号外显子突变的研究 （正文见第 209 页）

Mutation Detection on Exon 1 and 2 of Parkin Gene in Sporadic Early－onset Parkinson's Disease (Text in page 209)

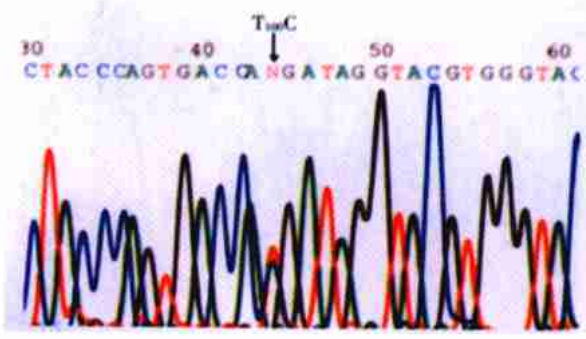

图 4　1 号外显子突变测序图

Fig. 4　Mutation sequencing map of exon 1

↓: mutation position

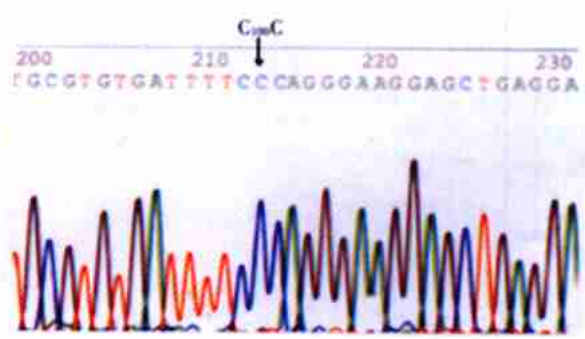

图 5　2 号外显子突变测序图

Fig. 5　Mutation sequencing map of exon 2

↓: mutation position