Acta Physiologica Sinica, December 25, 2012, 64(6): 609–616
http://www.actaps.com.cn

Research Paper

Involvement of MAPK pathways in NMDA-induced apoptosis of rat cortical neurons

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Abstract: NMDA-induced excitotoxicity cause severe neuronal damage including apoptosis and necrosis. The present study was aimed to evaluate the proportion of NMDA-induced apoptosis of rat cortical neurons and discover signal transduction mechanism. Caspase inhibitor and lactate dehydrogenase (LDH) assay were used to study the NMDA-induced apoptosis. To explore the involved signal pathways, the primary culture of rat cortical neurons were pretreated by the inhibitors of three MAPK pathways, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. With 2 h of NMDA treatment, cellular apoptosis was measured by caspase-3 activity, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) and Annexin V staining. The results showed that: (1) Caspase-dependent apoptosis accounted for 22.49% in NMDA-induced neuronal death; (2) Pretreatment with p38 MAPK inhibitor SB203580 (10 μmol/L) significantly decreased NMDA-mediated caspase-3 activity by 30.43% ($P < 0.05$). However, ERK inhibitor PD98059 (20 μmol/L) or JNK inhibitor SP600125 (20 μmol/L) did not influence caspase-3 activity; (3) Pretreatment with SB203580 significantly reduced the number of NMDA-induced TUNEL-positive cells by 33.10% ($P < 0.05$). PD98059 (20 μmol/L) or SP600125 (20 μmol/L) did not show obvious effect; (4) Pretreatment with SB203580 (10 μmol/L) significantly reduced the number of NMDA-induced early apoptotic neurons by 55.56% ($P < 0.05$). Also, SP600125 (20 μmol/L) significantly decreased the amount of late apoptotic/dead cells by 67.59% ($P < 0.05$). There was no effect of PD98059 (20 μmol/L). These results indicate that: (1) NMDA induces neuronal apoptosis besides necrosis; (2) p38 MAPK, but not JNK and ERK, is involved in NMDA-induced neuronal apoptosis, and inhibition of the apoptotic signaling pathway contributes to neuroprotection; (3) JNK activation might contribute to NMDA-induced neuronal necrosis rather than apoptosis.

Key words: NMDA; excitotoxicity; apoptosis; MAPKs

MAPKs介导NMDA诱导的大鼠皮层神经元凋亡

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摘 要：NMDA诱导兴奋毒造成的神经损伤，包括细胞的凋亡和坏死。本研究旨在探讨神经元凋亡在NMDA兴奋毒所致大鼠皮层神经元死亡中的所占比例，并分析了NMDA致神经元凋亡的信号通路机制。通过使用Caspase抑制剂和测定乳酸脱氢酶活性，研究NMDA (100 μmol/L, 2 h)兴奋毒所致的神经元凋亡；并使用MAPKs选择性抑制剂，分别采用Caspase-3活性检测，TUNEL和Annexin V染色方法，进一步观察MAPKs通路中细胞外信号调节激酶(ERK)、c-Jun N-末端激酶(JNK)和p38 MAPK三条不同途径在NMDA所致神经元凋亡中的作用。结果显示：(1) Caspase依赖的凋亡占NMDA所致细胞死亡总数的22.49%；(2) p38 MAPK抑制剂SB203580 (10 μmol/L)使NMDA诱导的caspase-3活性降低30.43% ($P < 0.05$)；而ERK抑制剂PD98059 (20 μmol/L)和JNK抑制剂SP600125 (20 μmol/L)不影响caspase-3的活性；(3) SB203580 (10 μmol/L)使NMDA所致的TUNEL阳性细

Received 2012-03-10 Accepted 2012-05-28

This work was supported by the National Natural Science Foundation of China (No. 31000481), the Natural Science Foundation of Shanxi Province, China (No. 2011011040-2), the Scientific and Technological Innovation Programs of Higher Education Institutions in Shanxi Province, China (No. 20091110) and the Innovative Talents Support Programs of Higher Education Institutions in Shanxi Province, China (2011).

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胞数减少33.10% ($P < 0.05$)；而PD98059 (20 μmol/L)或SP600125 (20 μmol/L)都没有作用；(4) Annexin V 染色结果显示，SB203580 (10 μmol/L)使NMDA所致的早期凋亡细胞减少55.56% ($P < 0.05$)；SP600125 (20 μmol/L)使NMDA所致的晚期凋亡/死亡细胞减少67.59% ($P < 0.05$)；PD98059 (20 μmol/L)对细胞凋亡/死亡没有明显作用。以上结果表明，NMDA介导的大鼠皮层神经元死亡除坏死外，还包含有一小部分神经元凋亡；p38 MAPK途径，而非JNK和ERK途径，介导了NMDA诱导的神经元凋亡，抑制与此相关的凋亡信号通路可发挥神经保护作用；JNK途径可能介导了NMDA所致的神经元坏死而非凋亡。

关键词：NMDA；兴奋性神经毒；凋亡；MAPKs
中图分类号：R338

Over-activation of NMDA receptors leads to neuronal damage and even cell death including necrosis and apoptosis, which is called excitotoxicity. Previous studies mainly focused on NMDA-induced neuronal necrosis, however, increasing evidence suggests that excitotoxicity may also play an important role in induction of apoptosis [1], which may occur in many pathogenic disorders [2].

Among many signaling pathways involved in survival and apoptosis of neurons, three of the MAPK signaling pathways, p38 MAPK, JNK and ERK1/2, have been widely studied. JNK and p38 MAPK pathways are considered as "stress-activated protein kinases" and play an essential role in inflammation and apoptosis [3]. ERK pathway is important in differentiation and proliferation, as well as in cell survival [4]. Cell survival is usually controlled by the co-effects of different MAPK signaling pathways rather than by any single one.

NMDA-induced excitotoxicity is a useful tool to evaluate neurotoxicity in isolated cells and the optimal model of nerve injury that mimics closely the situation *in vivo*. Our previous studies have found that JNK and p38 MAPK were involved in cortical neuronal cell death triggered by NMDA[5]. However, the contribution of MAPKs to NMDA-induced apoptosis has not been elucidated. The aim of this study was to examine the mode of cell death and the possible involvement of MAPK signaling pathways in the NMDA-dependent apoptosis.

1 MATERIALS AND METHODS

1.1 *Chemicals*

DMEM/F-12 and fetal bovine serum (FBS) were from Gibco-BRL. Both the DeadEnd™ Fluorometric terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) System and Caspase inhibitor Z-VAD-FMK were from Promega. SP600125, SB203580, and PD98059, which are inhibitors for JNK, p38 MAPK and ERK, respectively, were purchased from Cell Signaling, dissolved in dimethylsulfoxide (DMSO) and diluted with PBS to their final concentrations. Other agents were from Sigma.

1.2 *Primary cortical neuronal cultures*

Primary cortical cells were isolated from 1–3-day-old Wistar rats and were cultured as previously described [6]. In brief, cortical neurons from rats anesthetized with ketamine (intraperitoneal injection, 100 mg/kg, 3 min) were dissected and incubated in 0.025% trypsin solution, followed by centrifugation at 1 000 r/min for 5 min. Cells were resuspended in DMEM/F-12 with 15% FBS medium, and cultured at 37 °C in 5% CO_2 incubator. Cytosine arabinofuranoside (10 μmol/L) was added at 24 h after plating. After 10–12 d of primary culture, the neurons were used for further experimental observation.

1.3 *Grouping and intervention*

Cultured cells were randomly assigned to several groups: control, NMDA, NMDA + Z-VAD-FMK, NMDA + MAPK inhibitors (PD98059, SP600125 or SB203580), and NMDA + MK-801. The control group was incubated in drug-free Mg^{2+}-free Locke's buffer. Cells from the NMDA group were exposed to NMDA-containing Mg^{2+}-free Locke's buffer at a final concentration of 100 μmol/L. Caspase inhibitor Z-VAD-FMK was added to cultures at 4 h prior to NMDA induction. MAPK inhibitors were added 4 h before NMDA treatment. MK-801 and NMDA were simultaneously added to Mg^{2+}-free Locke's buffer in the NMDA + MK-801 group. After incubating cells for 2 h with drug-free or NMDA-containing Mg^{2+}-free Locke's buffer [7], the different groups were ready for the following experiments.

1.4 *Lactate dehydrogenase (LDH) assay*

LDH is released from cells into culture medium upon cell lysis. The cells were plated in 24-well plate. At 24 h after NMDA exposure, supernatant was collected to measure LDH release according to instructions. LDH leakage due to membrane damage was assessed by measuring LDH activity in culture medium as previ-

ously described with some modifications [6].

1.5 *Caspase-3 activity assay*

In brief, the cells were plated in 96-well plate. Caspase-3 activity was assayed at 20 h after NMDA exposure. The Caspase-3 Colorimetric Protease Assay Kits provide a simple and convenient mean for assaying the activity of caspases that recognize the sequence DEVD. The cells were first rinsed in PBS and then were collected. After 10 min of incubation with 50 μL of chilled Cell Lysis Buffer. The buffer was centrifuged for 5 min in a microcentrifuge (10 000 *g*). Supernatant (cytosolic extract) was transferred to a fresh tube and put on ice. Protein concentration was assayed. The protein (50–200 μg) was diluted to 50 μL Cell Lysis Buffer for each assay. Fifty μL of 2 × Reaction Buffer (containing 10 mmol/L DTT) was added to each sample. At last, 5 μL of the 4 mmol/L DEVD-pNA substrate was added, and the reaction system was incubated at 37 °C for 1.5 h. Optical density value was detected using a microplate reader at 400–405 nm.

1.6 *In situ TUNEL*

The cells were plated on glass coverslips in six-well plate. The TUNEL assay was performed at 24 h after NMDA treatment with a fluorescein apoptosis detection system (Promega), according to the manufacturer's instructions. Confocal microscope (Olympus, FV-1000) was used to detect localized green fluorescence of TUNEL-positive cells (fluorescein-12-dUTP) in a red (PI) background. The average number of TUNEL-positive cells was obtained from three sections from each group.

1.7 *Apoptosis assay by Annexin-V labeling*

The cells were plated in 96-well plate. At 24 h after NMDA exposure, the cells were harvested, washed twice with cold PBS and resuspended in binding buffer to reach 1×10^5 cells/mL. A suspension of 100 μL was taken and incubated with 100 μL Guava Nexin Reagent in the dark for 20 min at room temperature. Finally, cell samples were acquired on a Guava System. The percentages of distribution of viable (Annexin V^-/7-AAD^-), early apoptotic (Annexin V^+/7-AAD^-), late apoptotic/dead cells (Annexin V^+/7-AAD^+) and nuclear debris (Annexin V^-/7-AAD^+) were calculated by Guava Nexin software.

1.8 *Statistical analysis*

The data are presented as bar graphs showing the mean ± SEM from at least three independent experiments. The differences between groups were evaluated by one-way ANOVA followed by LSD or S-N-K *post hoc* multiple comparisons. The statistical significance level was set at $P < 0.05$.

2 RESULTS

2.1 Effects of caspase inhibitor on NMDA-induced LDH release

Exposure of cortical neurons to NMDA resulted in LDH leakage. In NMDA group, LDH levels rose by 118.77% ($P < 0.05$) compared with that of the control group (Fig. 1). NMDA-induced LDH release was fully blocked by the noncompetitive NMDA receptor antagonist, MK-801 (10 μmol/L), which suggested that the toxic effects were induced by NMDA. Pretreatment with general caspase inhibitor Z-VAD-FMK (100 μmol/L) 4 h before NMDA exposure reduced NMDA-induced LDH release by 22.49% ($P < 0.05$), which suggested that caspase-dependent apoptosis accounted for 22.49% in NMDA-induced neuronal death.

2.2 Effects of MAPKs on NMDA-induced increase of caspase-3 activity

In preliminary experiments, caspase-3 activity peaked at 20 h after NMDA exposure (data not shown). Therefore, the effects of MAPK inhibitors on NMDA-induced caspase-3 activity were observed at the same time point (20 h) after NMDA challenge. As shown in Fig. 2, caspase-3 activity increased to 0.47 ± 0.03 after NMDA treatment ($P < 0.05$), compared with that of the control group (0.18 ± 0.02). It means that NMDA increased caspase-3 activity and induced neuronal apoptosis. MK-801 administration completely inhibited

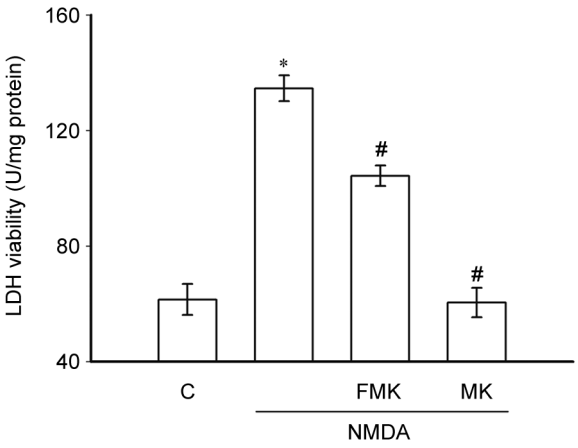

Fig. 1. Reduction of NMDA-induced LDH release by Z-VAD-FMK. C: control, FMK: Z-VAD-FMK, MK: MK-801. $^*P < 0.05$ *vs* control group; $^\#P < 0.05$ *vs* NMDA group.

NMDA-induced caspase-3 activation. Pretreatment with SB203580 (10 μmol/L), a p38 MAPK inhibitor, significantly rescued NMDA-induced cell apoptosis by 30.43% ($P < 0.05$), whereas ERK inhibitor PD98059 (20 μmol/L) or JNK inhibitor SP600125 (20 μmol/L) had no effects on NMDA-induced caspase-3 activation. None of the MAPK inhibitors alone resulted in significant effects on neuronal cultures from the rat cerebral cortex (data not shown).

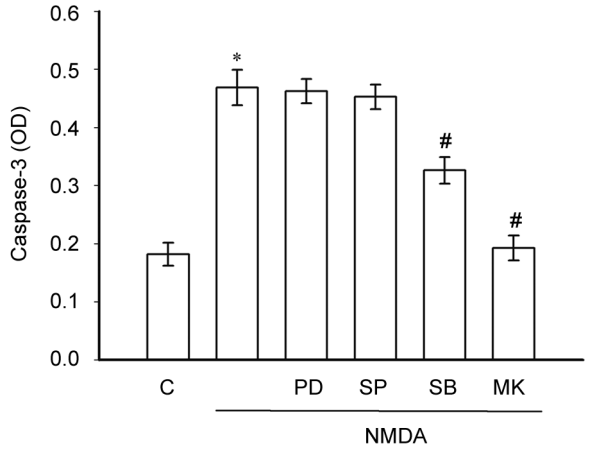

Fig. 2. Decrease of NMDA-induced caspase-3 activity by SB203580. C: control, PD: PD98059, SP: SP600125, SB: SB203580, MK: MK-801. $^{*}P < 0.05$ *vs* control group; $^{\#}P < 0.05$ *vs* NMDA group.

2.3 Effects of MAPKs on NMDA-induced apoptosis detected by TUNEL assay

As shown in Fig. 3, NMDA-induced apoptosis increased to 33.63% ± 2.20% ($P < 0.05$) compared with control values (1.76% ± 1.01%). MK-801 also inhibited NMDA-induced apoptosis. NMDA in combination with pre-treatment of p38 MAPK inhibitor SB203580 (10 μmol/L) significantly reduced NMDA-induced TUNEL-positive cells by 33.10% ($P < 0.05$). However, ERK inhibitor PD98059 (20 μmol/L) or JNK inhibitor SP600125 (20 μmol/L) administration did not suppress NMDA-induced apoptosis.

2.4 Effects of MAPKs on NMDA-induced apoptosis by Annexin V assay

As shown in Fig. 4, Compared with the control group,

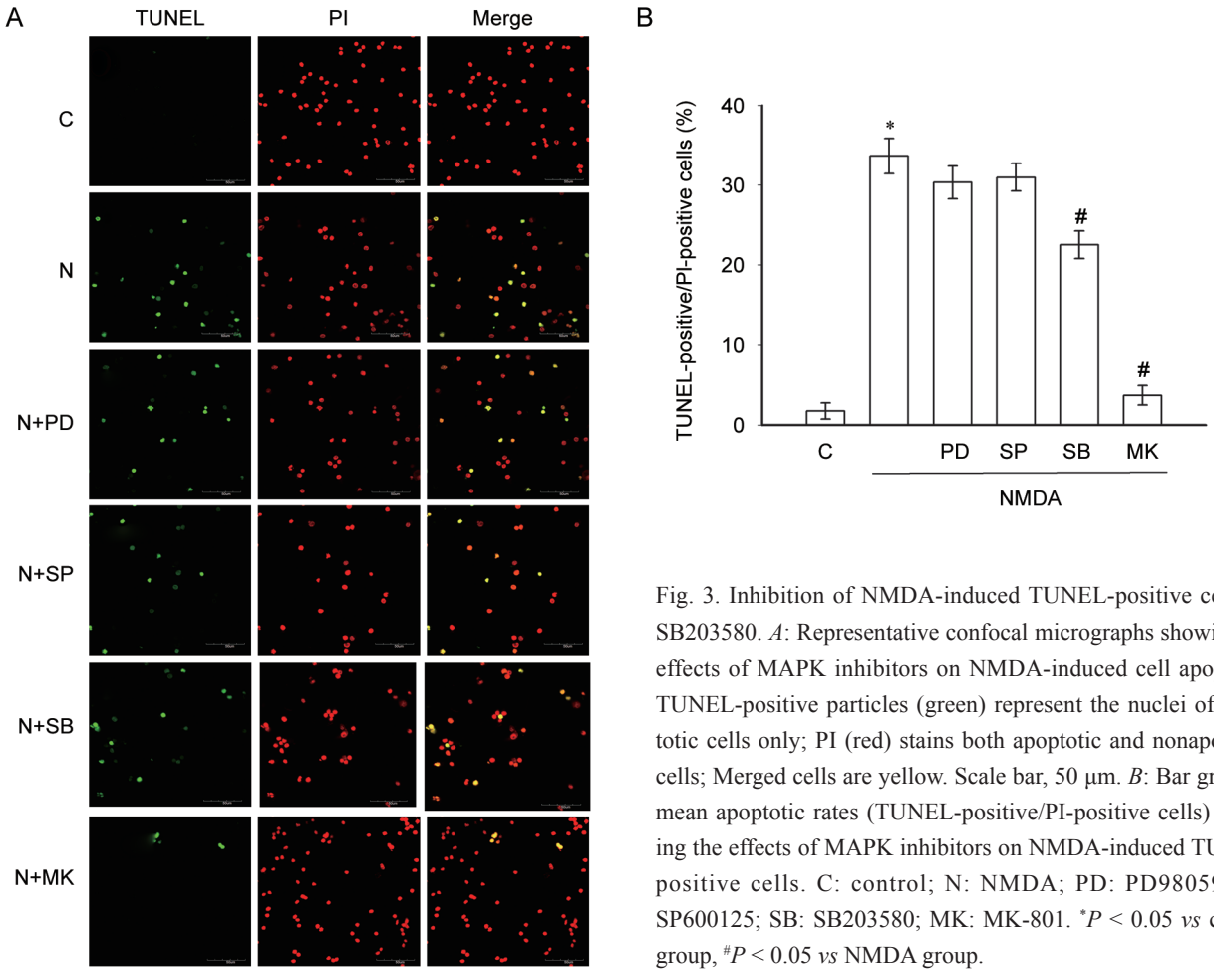

Fig. 3. Inhibition of NMDA-induced TUNEL-positive cells by SB203580. *A*: Representative confocal micrographs showing the effects of MAPK inhibitors on NMDA-induced cell apoptosis. TUNEL-positive particles (green) represent the nuclei of apoptotic cells only; PI (red) stains both apoptotic and nonapoptotic cells; Merged cells are yellow. Scale bar, 50 μm. *B*: Bar graph of mean apoptotic rates (TUNEL-positive/PI-positive cells) showing the effects of MAPK inhibitors on NMDA-induced TUNEL-positive cells. C: control; N: NMDA; PD: PD98059; SP: SP600125; SB: SB203580; MK: MK-801. $^{*}P < 0.05$ *vs* control group, $^{\#}P < 0.05$ *vs* NMDA group.

NMDA group showed increased early apoptosis (20.77% ± 1.63%) and apoptotic/dead rates (10.53% ± 1.64%) ($P < 0.05$). Administration of MK-801 completely inhibited NMDA-induced cell death. Pretreatment with SB203580 (10 μmol/L) significantly reduced NMDA-induced early apoptosis by 55.56% ($P < 0.05$).

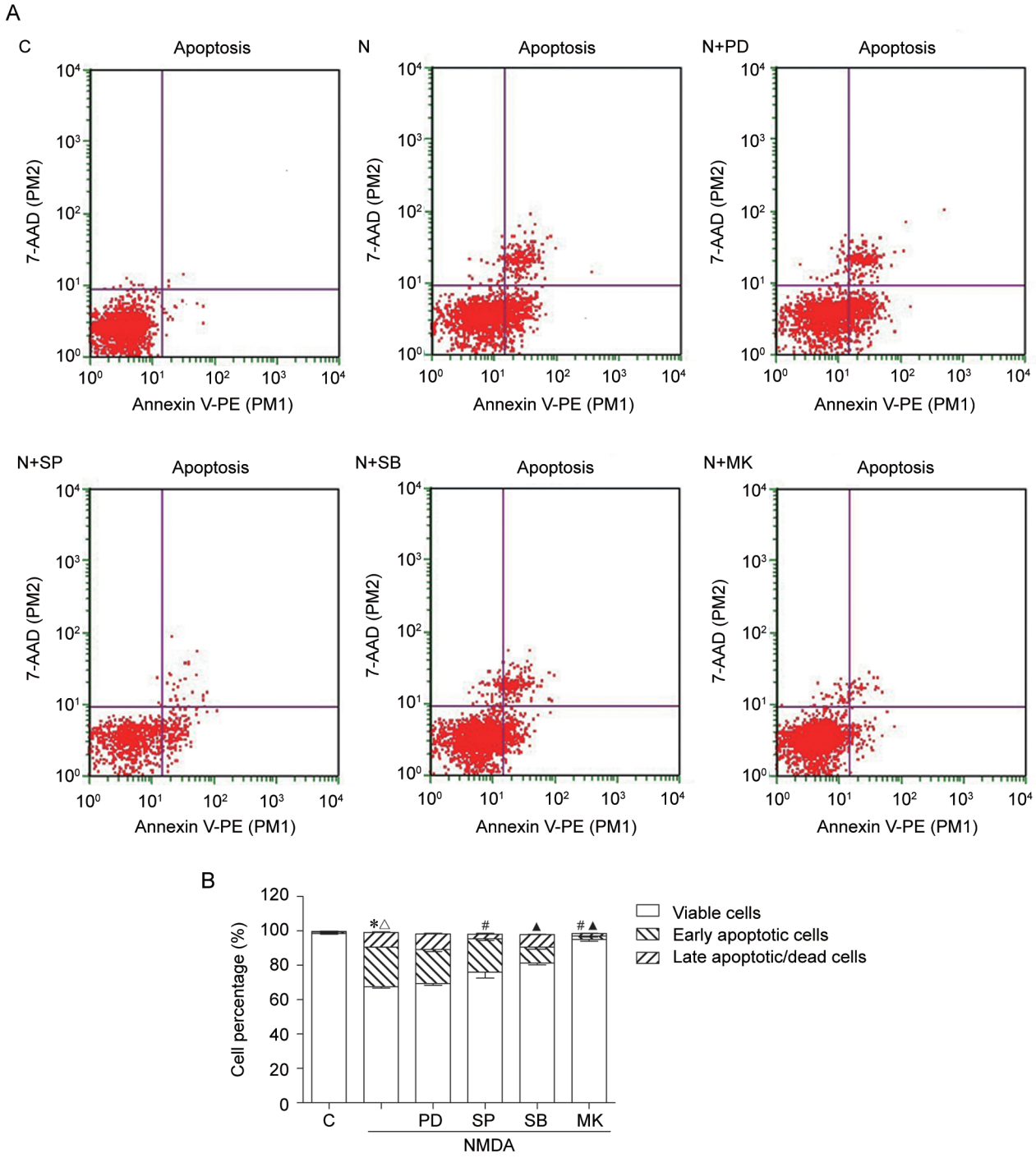

Fig. 4. Reduction of NMDA-induced apoptosis by SB203580. *A*: Representative Annexin V staining graphs showing the effects of MAPK inhibitors on NMDA-induced cell apoptosis. *B*: Percentages of viable, early apoptotic and late apoptotic/dead cells. C: control; N: NMDA; PD: PD98059; SP: SP600125; SB: SB203580; MK: MK-801. $^{\triangle}P < 0.05$ *vs* control group (early apoptotic cells), $^{*}P < 0.05$ *vs* control group (late apoptotic/dead cells), $^{\blacktriangle}P < 0.05$ *vs* NMDA group (early apoptotic cells), $^{\#}P < 0.05$ *vs* NMDA group (late apoptotic/dead cells).

Also, SP600125 (20 μmol/L) administration significantly decreased the amount of late apoptotic/dead cells by 67.59% ($P < 0.05$). PD98059 (20 μmol/L) did not show any obvious effects on NMDA-induced apoptosis.

3 DISCUSSION

Glutamate is the major excitatory neurotransmitter in the central nervous system. Activation of glutamate receptors plays a critical role in normal situations [8]. In addition, glutamate/NMDA is also a potent neurotoxin by overstimulation of postsynaptic receptors known as excitotoxicity. A growing body of evidence has implicated excitotoxicity as a mechanism of neuronal death in acute and chronic brain tissue injury diseases, such as stroke/ischemia, and certain neurodegenerative disorders [9].

NMDA-induced excitotoxic neuronal damage includes apoptosis and necrosis [10]. Apoptosis is an important cell suicide program which involves caspase-dependent and caspase-independent apoptotic pathways [11], whereas necrosis is a caspase-independent process [12]. In addition, it has been previously established that LDH release correlates linearly with the number of damaged neurons after toxic insult [13]. Therefore, the broad caspase inhibitor Z-VAD-FMK did not influence LDH release by NMDA-mediated necrosis. The present study showed that NMDA-induced LDH release decreased significantly with the exposure to Z-VAD-FMK, which demonstrated the existence of caspase-dependent apoptosis in NMDA-induced neuronal death. This was also confirmed by caspase-3 activity assay, TUNEL and Annexin V staining. It was reported that NMDA-mediated excitotoxic neuronal death can show features of apoptosis, necrosis or a combination of these two types [10], depending on the duration and intensity of the insult and the model used [2, 14, 15]. Some evidence suggested glutamate at low concentrations might induce apoptosis but not necrosis [2, 16–18]. It has been shown that the reports *in vitro* and *in vivo* are discordant with regard to whether excitotoxic neuronal death is apoptotic or necrotic [9, 19, 20]. Our results suggest that pretreatment with NMDA at 100 μmol/L concentration for 2 h induced apoptosis in cultured cortical neurons.

To further evaluate the involvement of MAPK signaling pathways in NMDA-induced neuronal apoptosis, the effect of MAPK inhibitors on cortical neurons were analyzed following NMDA exposure. The results showed that preincubation of cortical neurons with SB203580 provided potent protection against cell apoptosis induced by NMDA, as indicated by the results of caspase-3 activity assay, TUNEL and Annexin V staining. Indeed, the activation of p38 MAPK in different circumstances such as the degeneration induced by cerebral ischemia [21], the excitotoxicity induced by glutamate in cerebellar granule neurons [22] and methylmercury-induced neurotoxicity in cultured Neuro-2a cells [23], evidences the direct contribution of this p38 MAPK pathway to neuronal cell death. Consistent with these observations, the activation of p38 MAPK may be a key step to provoke cortical neurons damage in response to NMDA insult. Moreover, we observed that SB203580 inhibited the activation of caspase-3 and subsequent NMDA-induced apoptosis. The finding suggested that p38 MAPK contributes to caspase-3-dependent apoptotic pathway and may be an upstream effector of caspase-3. This finding has also been supported that p38 MAPK pathway regulated caspase-3 expression and induced apoptosis in the substania nigra of the mouse model of Parkinson's disease [24].

It is very interesting that NMDA-mediated cell apoptosis was not affected by JNK signaling, however, inhibition of JNK pathway reduced late apoptotic/dead cells by apoptotic or necrotic mechanisms. In addition, our previous study demonstrated that the JNK pathway contributed to NMDA-induced cortical neuronal insults [5]. Thus, it was indicated that the JNK pathway may be involved in NMDA-induced cortical neuronal necrosis rather than apoptosis. Although most of the current evidence points to the proapoptotic function of JNK [25], there are reports showing the importance of ROS-mediated JNK activation in non-apoptotic or necrotic cell death [26]. For instance, JNK activation has been shown to be involved in TNFα-induced necrotic cell death [27, 28] and nonapoptotic cell death elicited by exogenously applied H_2O_2 in MEF cells [29]. In the present study, JNK inhibitor was supposed to prevent necrotic cell death, indicating that activation of JNK may result in necrosis in an *in vitro* model of excitotoxic neuronal death.

The contribution of ERK pathway to neural injury *in vivo* and *in vitro* models is still in debate. For example, ERK1/2 is activated in neuronal and renal epithelial cells upon exposure to oxidative stress and toxicants and deprivation of growth factors, and inhibition of the ERK pathway blocks apoptosis [30], while it is also suggested that inhibition of ERK induces apoptosis and facilitation of ERK activation prevents apoptosis in re-

sponse to glutamate excitotoxicity in SCN2.2 cells [31]. The signaling of cell survival or apoptosis by ERK seems to be dependent on the model system and injury paradigm [30]. Stimulation of either synaptic or extrasynaptic NMDA receptors is linked to the activation of ERK1/2, which appear to play a dual role as prosurvival [32] and prodeath kinases [33]. In the present study, the inhibition of ERK did not prevent cell apoptosis or alter the cytotoxicity by NMDA, which showed that the ERK pathway is not directly involved in the NMDA neurotoxicity in primary cultured cortical neurons. Thus the inhibition of ERK1/2 pathway by pharmacological treatment cannot prevent NMDA-mediated cell death, pointing out the different role of ERK1/2 in comparison with JNK and p38 MAPK in NMDA-induced neuronal degeneration.

In NMDA-mediated cell death, the increase in intracellular Ca^{2+} concentration or overloading of Ca^{2+} is thought to be the key event [9]. Our previous studies have also observed NMDA-induced elevation of intracellular Ca^{2+} and a consequent excitotoxic neuronal death [6]. Additionally, the activation of MAPK has been well documented to depend on the NMDA receptor-mediated Ca^{2+} influx [22, 23]. This suggested that Ca^{2+}-mediated activation of MAPK causes excitotoxic neuronal death. However, the detailed molecular mechanisms through which Ca^{2+} induces MAPK activation that leads to neuronal cell death remain unclear.

In summary, we found that NMDA-induced apoptosis of rat cortical cells was partially dependent on the activation of p38 MAPK. It is well known that NMDA-induced neuronal cell death is a well-established and useful nerve injured model, which is relevant to the pathogenesis of many degenerative diseases. Therefore, the results suggest that p38 MAPK might be one of the novel approaches to develop new therapies for these diseases.

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