Protective effects of the p38 MAPK inhibitor SB203580 on NMDA-induced injury in primary cerebral cortical neurons

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Abstract. The p38 pathway, which is important in mitogen-activated protein kinase (MAPK) family protein signaling, leads to mitochondrial dysfunction and activation of caspase-3. B-cell lymphoma 2 (Bcl-2) family members are involved in the regulation of activities associated with the survival and death of neurons through apoptosis and have important functions in most types of apoptosis. In the present study, the effects of the p38 MAPK inhibitor SB203580 on *N*-methyl-D-aspartate (NMDA)-induced cerebral cortical neuron apoptosis were observed to further analyze the possible mechanisms of NMDA-induced neuronal death. Cultured primary cortical neurons were randomly divided into five groups: A control group, NMDA group and three SB203580 interventional groups. The lactate dehydrogenase (LDH) and MTT assays were employed to investigate the effects of the drugs on apoptosis. The morphology of apoptotic cells was observed using acridine orange/ethidium bromide (AO/EB) fluorescence staining. The expression levels of phospho-(p)-p38MAPK, Bcl-2 and Bcl-2-associated X (Bax) were assessed by immunohistochemical methods and western blot analysis to investigate the possible underlying protective mechanisms. The cell viability markedly decreased following incubation with NMDA. The protein levels of cell death repressor Bcl-2 and the levels of Bcl-2/Bax were downregulated. The protein levels of p-p38MAPK and cell death promoter Bax increased significantly in cells with NMDA treatment. However, these changes were inhibited by SB203580 treatment, particularly in the high-dose group. Neuronal death induced by NMDA in primary cortical neurons was caused in part by apoptosis, which was mediated through the activation of the p38 signaling pathway by NMDA. SB203580 has neuroprotective effects against NMDA-induced apoptosis.

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Introduction

Glutamate is involved in fast excitatory transmission and in neuronal functions, including plasticity and cognitive processes, as well as in toxic events (1). The excitotoxic effects of glutamate are largely mediated by increased Ca^{2+} influx through activated *N*-methyl-D-aspartate (NMDA) receptors (2,3). Initial neuronal death in the cerebral cortex may be due to an excessive Ca^{2+} influx through NMDA receptors (4). A number of *in vitro* studies indicated that at high concentrations, glutamate is a potent neurotoxin capable of destroying neurons (3,5).

Diverse evidence has demonstrated that among numerous signaling pathways involved in the survival and apoptosis of neurons, three of the mitogen-activated protein kinase (MAPK) signaling pathways, namely the p38 MAPK, c-Jun N-terminal kinase and extracellular-regulated kinase (ERK) pathways, have been widely studied (6,7). p38s are preferentially activated by cell stress-induced signaling in response to factors including oxidative stress, environmental stress and toxic chemical insults (2). Therefore, p38 MAPKs are considered 'stress-activated protein kinases' involved in cellular signaling, inflammation, apoptosis, carcinogenesis and in the pathogenesis of various diseases (3,5). Additionally, a large number of neurotoxic chemicals have been demonstrated to cause apoptosis in various neuronal cell preparations mediated by the activation of p38 (6-8). MAPK family members, including p38 MAPK, regulate diverse cellular functions, including response to environmental stimuli and apoptosis signaling (9). Studies also indicated that the important functions of ERK1/2 and/or p38 MAPK in response to neurotoxic insults are mediated by overstimulation of glutamate receptors in cell culture models (7) and in cerebral ischemia models (1). Glucose/oxygen-deprivation has diverse consequences in cells, including oxidative stress and excessive glutamate release reaching toxic levels (4,10). However, the specific effects of neurotoxic levels of glutamate on hippocampal slices have not been characterized, particularly the mode of cell death and possible signaling pathways involved in neurotoxicity (7-10).

The mechanisms underlying the mediation of glutamate-induced neurotoxicity or excitotoxicity have yet to

be established; however, a substantial body of evidence suggests that glutamate toxicity involves oxidative stress and apoptosis (1,2,4,11). Apoptosis is an important cell suicide program, which involves caspase-dependent and -independent apoptotic pathways, and dysregulation of apoptosis results in pathological conditions, including cancer, autoimmune diseases and neurodegenerative diseases (7). An increasing number of studies imply that NMDA-induced apoptosis is triggered by selective activation of NMDA receptors, and such a mechanism may be mediated by calcium signaling pathways or the p38 pathway, resulting in mitochondrial dysfunction and activation of caspase-3 (6,7,12). Activation by cleavage of the proteolytic enzyme caspase is a key step in the apoptotic program, and the upstream signaling pathways leading to the assembly of protein death complexes activated by caspases may or may not be dependent on mitochondria (1,10-12). Therefore, the signaling pathways upstream of the disruption of the mitochondrial membrane potential and caspase activation require further study.

In the present study, the effects of the p38 MAPK inhibitor SB203580 at different concentrations on the apoptosis of NMDA-induced cerebral cortical neurons were observed to further examine the possible mechanisms of NMDA-induced neuronal death.

Materials and methods

Cell isolation and culture. The cerebral cortical neurons were obtained from brains of newborn Sprague-Dawley rats [Experimental Animal Center of Liaoning Medical University, Jinzhou, China; Permission no. SCXK (Liao) 2003-0007] within 24 h. Neonatal brain tissues were digested with 2.5 g/l trypsin (Sigma, St. Louis, MO, USA) for 15 min at 37˚C. Following centrifugation for 5 min at 126.87 x g, the tissues were resuspended in high-glucose DMEM/F-12 (Hyclone, Logan, UT, USA) containing 10% FBS and 10% heat-inactivated horse serum (Hyclone), and triturated. The cell concentration was adjusted to $1x10^6$ /ml and the cells were seeded into plates coated with poly-L-lysine (Sigma). The cells were incubated at 37˚C in a humidified incubator containing 5% CO_2 for 24 h. The medium was replaced and the cells were incubated in serum-free neurobasal A medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 2% B27, 5 μmol/l glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Guangzhou Weijia Technology Co., Ltd., Guangzhou, Guangdong, China). On day two, the cultures were incubated with 2.5 mg/l cytosine arabinoside (Sigma-Aldrich) for 4 h to suppress the growth of glial cells. The neurons grown for 7-8 days *in vitro* (DIV) were used for further experimental observation. The study was approved by the Animal Ethics Committee of Liaoning Medical College (Jinzhou, China)

Grouping and intervention. Cortical neurons were cultured for 7 DIV prior to drug treatment. The cultured cells were randomly assigned to five groups: The control group, NMDA (50 μM) group and three groups treated with NMDA (50 μM) in combination with three different concentrations of the p38 MAPK inhibitor SB203580 (5, 10 and 20 μM). The different groups were prepared for the subsequent experiments. The control group was incubated in an equal volume of phosphate-buffered saline (PBS). Various concentrations of p38 MAPK inhibitors were added 4 h prior to NMDA treatment. NMDA at 50 μM (Sigma) was added to Mg^{2+}-free Locke's buffer in the NMDA and SB203580 (Sigma-Aldrich) groups.

Analysis of cell viability. Cell viability was determined by an MTT assay. MTT solution at 20 μl (Sigma-Aldrich), 5.0 g/l in PBS, was added to each well of the 96-well plate (containing 100 μl of medium and cells) 4 h prior to the end of incubation. The supernatant was discarded and 150 μl dimethylsulfoxide was added to dissolve the formazan, following which the culture plate was agitated. The absorbance was measured at 570/630 nm using a microplate reader (Sunrise™; Tecan, Grodig, Austria). The cell viability was calculated from the optical density (OD) using the following formula: treated group OD / control group OD x 100%.

Assessment of lactate dehydrogenase (LDH) activity. LDH released from damaged cells into the cell culture media was measured following treatment with NMDA and three different concentrations of SB203580. A colorimetric assay was used. According to this assay, the amount of formazan salt, which was formed following conversion of lactate to pyruvate and then by reduction of tetrazolium salt, was proportional to LDH activity in the sample. Cell-free culture supernatants were collected from each well and incubated with the mixture of the appropriate reagent according to the manufacturer's instructions (Cytotoxicity Detection kit; Roche Diagnostics, Mannheim, Germany) for 20 min. The intensity of the red color shown in the assay that was measured at a wavelength of 490 nm using a multilabel counter system (Perkin Elmer, Boston, MA, USA) was proportional to the LDH activity and the number of damaged cells.

Apoptosis detection. Morphological evidence of apoptosis was obtained using the acridine orange/ethidium bromide (AO/EB) staining method. Primary cortical neuronal cells were cultured in six-well flat-bottomed plates and allowed to adhere to the bottom of the wells. The neurons grown for 7 DIV were treated with SB203580 for 4 h prior to exposure to NMDA. DMEM/F12 medium (10% FCS) was used as a control for the cell lines. Following the indicated incubation times, 25 μl AO/EB mixture (Sigma-Aldrich) was added to the cells treated with or without NMDA. Then, the cells were examined using fluorescence microscopy (Nikon Optiphot; Nikon, Tokyo, Japan) and images were captured.

Immunohistochemical analysis. Cells were fixed with 4% paraformaldehyde and rinsed with 0.1 M PBS. The cells were then incubated with primary antibodies, which included phospho-p38 MAPK (p-p38MAPK; Cell Signaling Technology, Danvers, MA, USA), B-cell lymphoma 2 (Bcl-2; Abcam, Cambridge, MA, USA) and Bcl2-associated X (Bax; Millipore, Billerica, MA, USA), in the blocking buffer for 48 h at 4˚C. The cultures were rinsed and incubated with the appropriate biotinylated secondary antibody for 6 h and subsequently processed with an avidin-biotin complex kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) for another 1-2 h. Finally, the reaction product was

visualized with 0.05% 3,3'-diaminobenzidine as the chromogen. Analysis was performed under a BX-60 microscope (Olympus, Tokyo, Japan). The first antibodies were omitted in the methodological control.

Western blot analysis. The total cell lysates (20 μg each) were separated by 12.5% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore) for 1.5 h at 400 mA using a Transphor TE 62 (Hoefer, Inc., Holliston, MA, USA). The PVDF membranes were then incubated with Bcl-2 (1:200), Bax (1:300), p-p38 MAPK (1:300) and β-actin (1:2,000; Sigma-Aldrich) antibodies at 4˚C for 2 h or overnight. The membranes were washed three times in PBST (10 mM NaH_2PO_4, 130 mM NaCl and 0.05% Tween 20) and then probed with horseradish peroxidase-conjugated antibodies (1:5,000; Sigma-Aldrich) for 1 h. The signals were visualized using enhanced chemiluminesence western blot kits (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation of three independent experiments. Comparisons among groups were performed by one-way analysis of variance followed by Fisher's least significant difference, Student-Newman-Keuls or Dunnett's T3 post-hoc multiple comparisons, when appropriate. SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Neuron viability. Cultured cortical neurons were treated with or without various concentrations of SB203580 for 4 h prior to exposure to 50 μM NMDA. Apoptotic cells were assessed following 24 h using MTT assays. As illustrated in Fig. 1A, cell viability in the NMDA groups and the SB203580-treated groups at three concentrations were 44±12, 49±10, 59±9 and 72±9%, respectively. The cell viability in the NMDA groups was significantly lower than that in the control group (P<0.01), while the cell viability in the groups treated with the two highest concentrations of SB203580 was significantly different from that in the NMDA groups (P<0.05, P<0.01). The results demonstrated that the protective effect of SB203580 was dose dependent, particularly in the high-dose group.

LDH release. The exposure of cortical neurons to NMDA resulted in LDH leakage. In the NMDA group, LDH levels increased to 39.10% (P<0.01; Fig. 1B) compared with that of the control group. The cells were pretreated with different concentrations of SB203580 (5, 10 and 20 μmol/l) 4 h prior to NMDA exposure. Following cells being incubated for 24 h, the LDH release rates of the cells were reduced to 26.29, 24.59 and 21.65%, respectively, which were significantly lower than the results of the NMDA group (P<0.01; Fig. 1B).

Neuronal apoptosis. To investigate the type of cell death induced by NMDA and observe the effects of SB202580 treatment, the cells were stained with AO/EB, which allows the identification of viable, apoptotic and necrotic cells based on color and appearance. As shown in Figs. 1C and 2, the number of apoptotic neurons increased significantly (P<0.01) compared with that in the control groups. However, compared with the NMDA group, NMDA-induced neuronal apoptosis significantly decreased (P<0.01) in the SB203580 groups (5, 10 and 20 μm), particularly in the high-dose group.

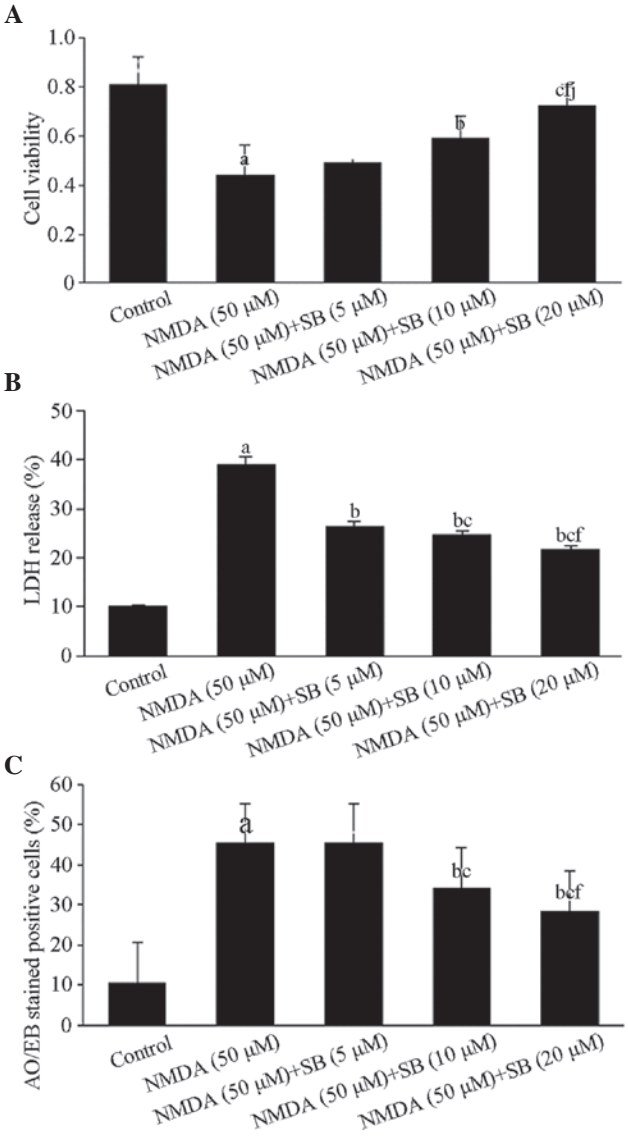

Figure 1. Effects of the p38 MAPK inhibitor SB203580 on the viability of NMDA-induced neurons, LDH release and neuronal apoptosis. (A) Cell viability following treatment with 50 μM NMDA. The values are expressed as the mean ± SD. n=6 for each group. [a]P<0.01, vs the control group; [b]P<0.05, [c]P<0.01, vs the NMDA injury group; [f]P<0.01 vs the low dose group; [j]P<0.05, vs the medium dose group. (B) LDH activity 24 h after cessation of NMDA-induced injury; the effect was dose- and time-dependent. The values are expressed as the mean ± SD. n=6 for each group. [a]P<0.01, vs the control group; [b]P<0.01, vs the NMDA injury group; [c]P<0.01, vs the low dose group; [f]P<0.01, vs the medium dose group. (C) Quantification of apoptotic cells in rats from the control group, NMDA group and SB203580 combined with NMDA groups. The values are expressed as the mean ± SD. n=6 for each group. [a]P<0.01, compared with the control group; [b]P<0.01, compared with the NMDA group; [c]P<0.01, [f]P<0.01, comparisons among SB203580 groups. MAPK, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate; LDH, lactate dehydrogenase; SD, standard deviation; AO/EB, acridine orange/ethidium bromide.

Immunohistochemical staining. Relative changes in p38 MAPK, Bcl-2 and Bax were determined by immunohistochemical

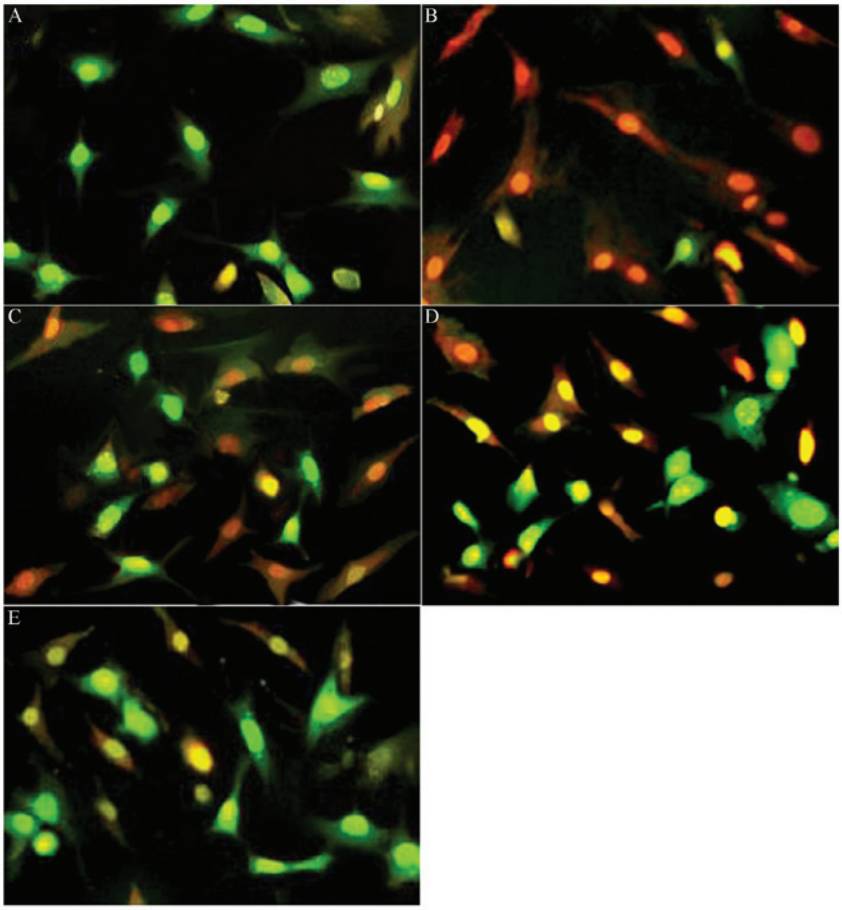

Figure 2. Morphology of neurons undergoing apoptosis induced by NMDA was evaluated by AO/EB double fluorescent staining. Representative microphotographs of neurons from the cerebral cortex under the different studied conditions. Viable cells are colored green with intact nuclei, nonviable cells had bright orange chromatin, whereas yellow staining represents early apoptotic cells, and reddish or orange staining represents late apoptotic cells. Necrotic cells appear orange with a normal nuclear structure. Scale bar, 50 μM. (A) Control group; (B) NMDA (50 μM) injury group; (C) NMDA (50 μM) + SB203580 (5 μM) group; (D) NMDA (50 μM) + SB203580 (10 μM) group; (E) NMDA (50 μM) + SB203580 (20 μM) group. NMDA, *N*-methyl-D-aspartate; AO/EB, acridine orange/ethidium bromide.

staining, and the cytoplasm of positive cells was stained brownish-yellow, as shown in Figs. 3 and 4A. NMDA treatment markedly increased the expression of p-p38 MAPK and Bax and decreased the expression of Bcl-2 compared with the control group. SB203580 treatment significantly reduced the expression of p38 MAPK and Bax in cortical neurons compared with the NMDA group (P<0.01). The expression of Bcl-2 in cortical neurons was significantly increased compared with that in the NMDA group (P<0.01), particularly in the high-dose group. The results were dose-dependent. Compared with the control group, the ratio of Bcl-2/Bax was significantly decreased in the NMDA injury group (P<0.01). Compared with the NMDA group, the Bcl-2/Bax ratio increased in the SB203580 groups at different concentrations, which was statistically significant (P<0.01).

Western blot analysis. The levels of p-p38MAPK, Bcl-2, Bax and Bcl-2/Bax were examined by western blot analysis. As shown in Figs. 4B and 5, NMDA significantly increased p-p38MAPK and Bax levels as compared with the control group (P<0.01). Bcl-2 levels decreased significantly compared with those in the control group (P<0.01). p-p38MAPK and Bax protein levels were markedly reduced and Bcl-2 was increased compared with the NMDA group (P<0.01). Comparisons among different interventional groups revealed significant differences (P<0.05, P<0.01) and the protein expression levels were dose-dependent. Compared with the control group, the ratio of Bcl-2/Bax significantly decreased in the NMDA injury group (P<0.01). Compared with the NMDA group, the ratio of Bcl-2/Bax was increased at different concentrations of SB203580, with a significant difference (P<0.01).

Discussion

Among numerous signaling pathways involved in the survival and apoptosis of neurons, MAPKs are a family of signaling molecules involved in the transduction of extracellular stimuli into intracellular responses in a wide variety of circumstances (11). p38 MAPK is important in inflammation and apoptosis and it is part of a signaling cascade that has been implicated in neuronal death associated with kainic acid (KA)-induced seizures (6). An increasing amount of evidence suggests that excitotoxicity may also have an important pathogenic function in processes that culminate in programmed cell death. Glutamate-induced neurotoxicity is mainly studied in cell cultures, in which glutamate evokes necrosis and/or apoptosis depending on experimental conditions and, at low concentrations, glutamate may induce apoptosis, however, not

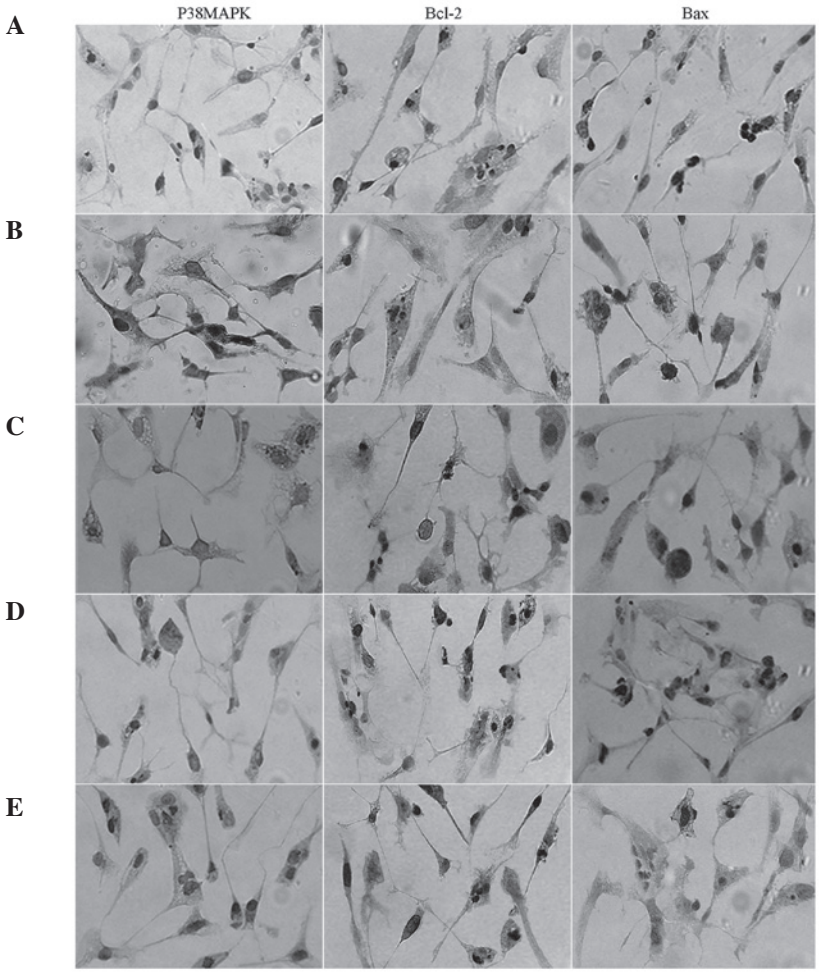

Figure 3. Expression of p-p38MAPK, Bcl-2 and Bax proteins by immunohistochemical staining. In the cytoplasm or the nuclei, brownish-yellow granules appeared in positive cells. Scale bar, 50 μM. (A) Control group; (B) NMDA (50 μM) group; (C) NMDA (50 μM) + SB203580 (5 μM) group; (D) NMDA (50 μM) + SB203580 (10 μM) group; (E) NMDA (50 μM) + SB203580 (20 μM) group. MAPK, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X.

necrosis (8,11,12). However, the contribution of p38 MAPK to NMDA-induced apoptosis has not been fully elucidated.

To evaluate the involvement of signaling pathways dependent on p38 MAPK on the NMDA-induced damage to cortical neurons, the selective inhibitor SB203580 was used. In the present study, LDH and MTT reduction assays were used to quantitatively estimate the effects of neuronal injury following NMDA exposure and SB203580 treatment. As shown in Figs. 1 and 2, cell viability significantly increased and LDH leakage markedly decreased compared with the NMDA groups. The activation of p38 MAPK under different circumstances, including degeneration induced by cerebral ischemia (5), excitotoxicity induced by glutamate in cerebellar granule neurons (3,13) and KA in the hippocampus (6-8) and methyl-mercury-induced neurotoxicity in cultured Neuro-2a cells (9), demonstrates the direct contribution of the p38 MAPK pathway to neuronal cell death. The activation of p38 MAPK may be a key step in the induction of cortical neuronal damage in response to the insult by NMDA, and SB203580 demonstrated a dose-dependent protective effect from NMDA-induced neuronal injury. Therefore, the present study confirmed that the activation of p38 MAPK is involved in NMDA-induced neuronal injury.

According to previous studies, Ca^{2+}-mediated activation of p38 MAPK causes excitotoxic neuronal death (9,14). SB203580 was revealed to have protective effects against NMDA-induced neuronal injury; however, its optimal protective condition is not known. The detailed molecular mechanisms by which NMDA induces p38 MAPK activation leading to neuronal apoptosis remain controversial and elusive. Thus, numerous mechanisms require further investigation, including those of apoptotic proteins.

Apoptotic proteins, which include anti-apoptotic Bcl-2 and pro-apoptotic Bax, may result either in the inhibition or promotion of cell death (7,13). Bcl-2 mitigates calcium entry and mitochondrial calcium overload through regulation of l-type calcium channels, and these effects may contribute to the maintenance of cell viability following injury (15,16). Pro-apoptotic Bax promotes cytochrome c release followed by the activation of caspases to induce apoptotic cell death. The protein levels of the cell death repressor Bcl-2 and cell death promoter Bax determine the threshold for neuronal cell death (4,11,16). Their expression is dynamically modulated at the onset of neurodegeneration (17). Furthermore, Bcl-2 expression and the activation of the p38 MAPK pathway are involved in neuronal apoptosis induced under neurotoxic condi-

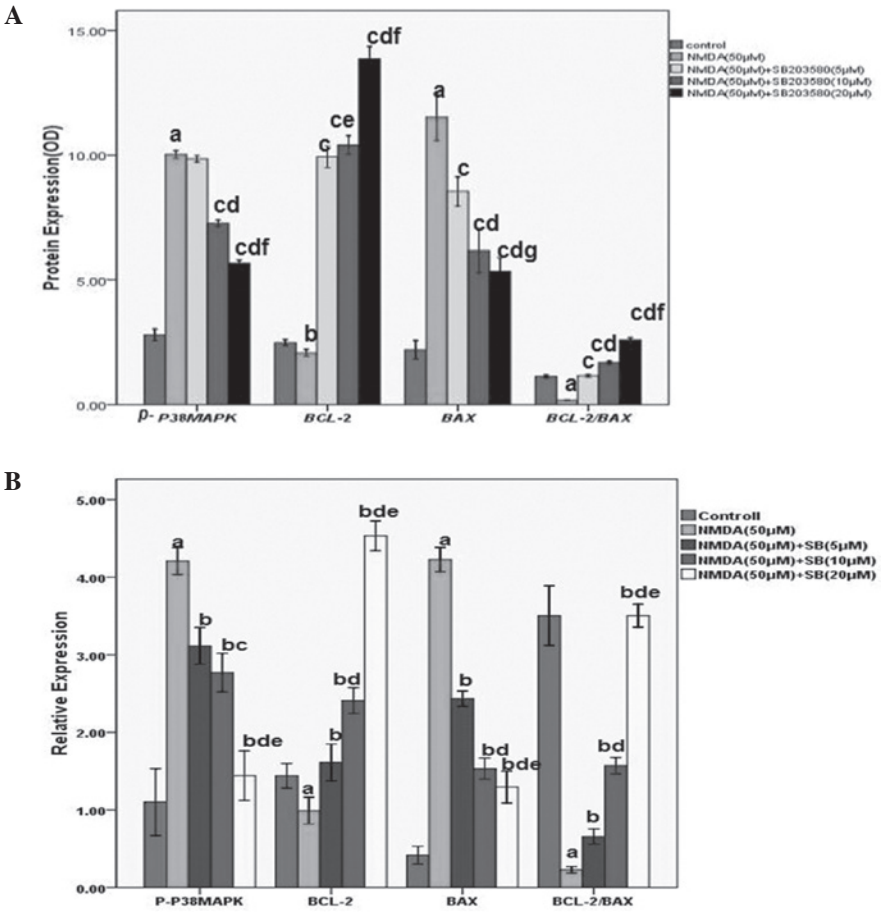

Figure 4. Quantitative analysis of relative changes in p-p38 MAPK, Bcl-2 and Bax proteins by immunohistochemical staining and western blot analysis. (A) Levels of p-p38MAPK, Bcl-2, Bax and the ratio of the Bcl-2/Bax levels in the cortical neurons with or without NMDA exposure or SB203580 immunization. The number in each column indicates the absorbance value of the detected proteins. Values (means ± SD) are expressed as the relative expression levels. [a]P<0.01, [b]P<0.05 vs the control group; [c]P<0.01, vs the NMDA injury group; [f]P<0.01, [j]P<0.05 vs the low dose group; [k]P<0.05, [l]P<0.05 vs the medium dose group. (B) Quantitative analysis of relative changes in p-p38 MAPK, Bcl-2 and Bax proteins. The values are expressed as the mean ± SD. n=6 for each group. [a]P<0.01, vs the control group; [b]P<0.01 vs the NMDA injury group; [c]P<0.05, [f]P<0.01, vs the low dose group; [k]P<0.01, vs the medium dose group. MAPK, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate; OD, optical density; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; SD, standard deviation.

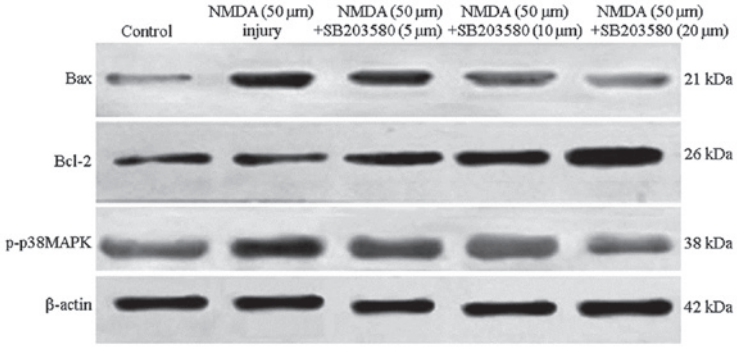

Figure 5. Assessment of p-p38MAPK, Bcl-2 and Bax in primary cultured neurons by western blot analysis. The cells were exposed to NMDA 4 h prior to pretreating them with SB203580. Treatment with SB203580 markedly inhibited the injury-induced activation of NMDA in primary neurons. MAPK, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X.

tions (5,18). The overexpression of Bcl-2 was able to attenuate the cytotoxicity of NMDA-induced apoptosis, and was able to extend cell survival time and maintain cell stability (2,12,19).

A fine balance exists between apoptosis and anti-apoptosis and this balance may be disrupted when cells are affected by external chemical stimuli, and the apoptotic process is able to be activated. The p38 MAPK signaling system for the regulation of apoptosis, whether or not there is such a focal point, requires further investigation. Studies have suggested that the mechanism of estrogen-mediated neuroprotection involves the regulation of mitochondrial Ca^{2+} and Bcl-2 expression (20,15). Exposure of glutamate has also been associated with increased cytosolic Ca^{2+} in cortical neurons (15,21) and upregulation of pro-apoptotic protein Bax in neuronal cell lines (1,22,23). In the

present study, immunohistochemical staining and western blot analyses were used to assess the expression of p-p38MAPK, Bcl-2 and Bax following NMDA treatment alone or in combination with SB203580. As shown in Figs. 4 and 5, increased levels of p-p38MAPK and Bax were observed following NMDA treatment, and the levels of Bcl-2 were downregulated. However, SB203580 was able to reverse this effect, particularly at high doses. Together with the results of the AO/EB staining, it was further verified that NMDA-induced apoptosis is mediated in part by p-p38 MAPK activation. Numerous *in vitro* studies have demonstrated that selective p38 MAPK inhibitors protect hippocampal and cortical neuron cultures from NMDA excitotoxicity (6,24), as well as cerebellar granular cell culture from glutamate-induced apoptosis (1,11,25). However, the findings of the present study agree with previous results, supporting the hypothesis that the activation of p38 MAPK may be a key step in the induction of hippocampal cell damage in response to insults by NMDA.

In conclusion, the present study provided evidence that cell death induced by NMDA in primary cortical neurons partly proceeded via apoptosis. p38 MAPK served as an external apoptosis triggering signal in neurons following NMDA treatment. Notably, inhibition of p38 MAPK by SB203580 was able to prevent neuronal cell apoptosis caused by NMDA. The results of the present study may contribute to a better understanding of the mechanisms involved in brain injury induced by NMDA. The results may also facilitate the design of an improved strategy for developing biomedical therapies for apoptosis control, as well as the control of the development of disorders that involve p-p38 MAPK, Bcl-2 and Bax, including ischemic cerebrovascular disease, epilepsy and others.

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References

1. Molz S, Decker H, Dal-Cim T, Cremonez C, Cordova FM, Leal RB and Tasca CI: Glutamate-induced toxicity in hippocampal slices involves apoptotic features and p38 MAPK signaling. Neurochem Res 33: 27-36, 2008.
2. Chen J, Errico SL and Freed WJ: Reactive oxygen species and p38 phosphorylation regulate the protective effect of Δ^9-tetrahydrocannabinol in the apoptotic response to NMDA. Neurosci Lett 389: 99-103, 2005.
3. Yang XR, Sun P, Qin HP, Si PP, Sun XF and Zhang C: Involvement of MAPK pathways in NMDA-induced apoptosis of rat cortical neurons. Sheng Li Xue Bao 64: 609-616, 2012.
4. Lu TH, Hsieh SY, Yen CC, *et al*: Involvement of oxidative stress-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic signals in methylmercury-induced neuronal cell injury. Toxicol Lett 204: 71-80, 2011.
5. Zhang R, Sun L, Hayashi Y, Liu X, Koyama S, Wu Z and Nakanishi H: Acute p38-mediated inhibition of NMDA-induced outward currents in hippocampal CA1 neurons by interleukin-1beta. Neurobiol Dis 38: 68-77, 2010.
6. Dunleavy M, Provenzano G, Henshall DC and Bozzi Y: Kainic acid-induced seizures modulate Akt (SER473) phosphorylation in the hippocampus of dopamine D2 receptor knockout mice. J Mol Neurosci 49: 202-210, 2013.
7. Giordano G, Klintworth HM, Kavanagh TJ and Costa LG: Apoptosis induced by domoic acid in mouse cerebellar granule neurons involves activation of p38 and JNK MAP kinases. Neurochem Int 52: 1100-1105, 2008.
8. Ola MS, Nawaz M and Ahsan H: Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. Mol Cell Biochem 351: 41-58, 2011.
9. Neoh CA, Wang RY, Din ZH, *et al*: Induction of apoptosis by sinulariolide from soft coral through mitochondrial-related and p38MAPK pathways on human bladder carcinoma cells. Mar Drugs 10: 2893-2911, 2012.
10. Grethe S, Ares MP, Andersson T and Pörn-Ares MI: p38 MAPK mediates TNF-induced apoptosis in endothelial cells via phosphorylation and downregulation of Bcl-x(L). Exp Cell Res 298: 632-642, 2004.
11. Liang HL, Dhar SS and Wong-Riley MT: p38 mitogen-activated protein kinase and calcium channels mediate signaling in depolarization-induced activation of peroxisome proliferator-activated receptor gamma coactivator-1alpha neurons. J Neurosci Res 88: 640-649, 2010.
12. Yan Y, Bian JC, Zhong LX, Zhang Y, Sun Y and Liu ZP: Oxidative stress and apoptotic changes of rat cerebral cortical neurons exposed to cadmium in vitro. Biomed Environ Sci 25: 172-181, 2012.
13. Sanchez A, Tripathy D, Yin X, Luo J, Martinez J and Grammas P: Pigment epithelium-derived factor (PEDF) protects cortical neurons in vitro from oxidant injury by activation of extracellular signal-regulated kinase (ERK) 1/2 and induction of Bcl-2. Neurosci Res 72: 1-8, 2012.
14. Guo RB, Wang GF, Zhao AP, Gu J, Sun XL and Hu G: Paeoniflorin protects against ischemia-induced brain damages in rats via inhibiting MAPKs/NF-kB-mediated inflammatory responses. PLoS One 7: e49701, 2012.
15. Śmiałowska M, Gołembiowska K, Kajta M, Zieba B, Dziubina A and Domin H: Selective mGluR1 antagonist EMQMCM inhibits the kainate-induced excitotoxicity in primary neuronal cultures and in the rat hippocampus. Neurotox Res 21: 379-392, 2012.
16. Tiwari M, Lopez-Cruzan M, Morgan WW and Herman B: Loss of caspase-2-dependent apoptosis induces autophagy after mitochondrial oxidative stress in primary cultures of young adult cortical neurons. J Biol Chem 286: 8493-8506, 2011.
17. Liu B, Zhang H, Xu C, *et al*: Neuroprotective effects of icariin on corticosterone-induced apoptosis in primary cultured rat hippocampal neurons. Brain Res 1375: 59-67, 2011.
18. Xu Z, Wang BR, Wang X, Kuang F, Duan XL, Jiao XY and Ju G: ERK 1/2 and p38 mitogen-activated protein kinase mediate iNOS-induced spinal neuron degeneration after acute traumatic spinal cord injury. Life Sci 79: 1895-1905, 2006.
19. Jiang W, Van Cleemput J, Sheerin AH, *et al*: Involvement of extracellular regulated kinase and p38 kinase in hippocampal seizure tolerance. J Neurosci Res 81: 581-588, 2005.
20. Segura Torres JE, Chaparro-Huerta V, Rivera Cervantres MC, Montes-González R, Flores Soto ME and Beas-Zárate C: Neuronal cell death due to glutamate excitotocity is mediated by P38 activation in the rat cerebral cortex. Neurosci Lett 403: 233-238, 2006.
21. Miloso M, Scuteri A, Foudah D and Tredici G: MAPKs as mediators of cell fate determination: an approach to neurodegenerative diseases. Curr Med Chem 15: 538-548, 2008.
22. Chen S, Xu Y, Xu B, *et al*: CaMKII is involved in cadmium activation of MAPK and mTOR pathways leading to neuronal cell death. J Neurochem 119: 1108-1118, 2011.
23. Zhang Y and Bhavnani BR: Glutamate-induced apoptosis in primary cortical neurons is inhibited by equine estrogens via down-regulation of caspase-3 and prevention of mitochondrial cytochrome c release. BMC Neurosci 6: 13, 2005.
24. Liu XW, Ma C, Xing RX, *et al*: The calmodulin-dependent protein kinase II inhibitor KN-93 protects rat cerebral cortical neurons from N-methyl-D-aspartic acid-induced injury. NRR 8: 111-120, 2013.
25. Xu B, Xu Z, Deng Y, Liu W, Yang H and Wei YG: MK-801 protects against intracellular $Ca^{(2+)}$ overloading and improves N-methyl-D-aspartate receptor expression in cerebral cortex of methylmercury-poisoned rats. J Mol Neurosci 49: 162-171, 2013.