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SP-8203 reduces oxidative stress via SOD activity and behavioral deficit in cerebral ischemia

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ABSTRACT

Both oxidative stress and excessive activation of glutamate receptors are implicated as major causes of ischemic brain injury. However, the existing *N*-methyl-D-aspartate (NMDA) receptor antagonists have not exerted good clinical outcome, most likely because they do not protect neurons against oxidative stress. Thus, more effective glutamate antagonists and antioxidants are needed for the treatment of ischemic stroke. In previous study, SP-8203, derived from earth worms, showed the blocking effect of NMDA receptor. We provided evidence that SP-8203 could also suppress the oxidative stress in this study. *In vitro*, 250 μM H_2O_2 was treated to SH-SY5Y cells after the pre-treatment of SP-8203 (2, 20 and 200 μM). SP-8203 significantly suppressed H_2O_2-induced cell death and reactive oxygen species production. In addition, we investigated the effects of SP-8203 in middle cerebral artery (MCA) occluded rat model. SP-8203 (5 and 10 mg/kg) was administered intraperitoneally to rats before and after the MCA occlusion and was injected daily for 10 days. After 10 days, SP-8203 remarkably reduced brain infarct volume and lipid peroxidation products in the MCA-occluded rats but MK-801 didn't. Moreover, SP-8203 significantly improved neurological deficits such as shortening of latency time in Rota rod performance. However, MK-801 didn't improve behavioral deficits. Therefore, SP-8203 may be more effective for multiple-target mechanisms of ischemic stroke.

1. Introduction

Oxidative stress is a major cause of neuronal cell death, including ischemic brain injury (Ozkul et al., 2007). Cell culture models and animal experiments indicated that reactive oxygen species such as O_2^- (superoxide anion), ·OH (hydroxyl radical), H_2O_2 (hydrogen peroxide) are produced during neuronal cell death, and they attack lipids, protein and DNA of neurons in ischemia brain tissues. The neuronal cell membrane and the mitochondrial membrane constitute with high levels of polyunsaturated fatty acids which can be easily attacked by the ROS. Increased ROS coupled with a reduced antioxidant defense has been postulated to play a pivotal role in brain injury (Hall and Braughler, 1989; McCord, 1985; Xu et al., 1999).

It was reported that the ROS is also related to glutamate-induced cell death (Coyle and Puttfarcken, 1993; Schulz et al., 1995). Glutamate, the excitatory neurotransmitter in the CNS, is thought to mediate neuronal injuries as a result of excessive stimulation of *N*-methyl-D-aspartate (NMDA) receptors. The mechanisms of cell death through NMDA receptor stimulation include increased ROS formation (Gunasekar et al., 1995; Sun et al., 2010). Thus, oxidative stress plays a critical role in excitotoxicity (Bondy and Lee, 1993). However the NMDA receptor antagonists such as MK-801 cannot protect neurons against oxidative stress and furthermore cannot exert a good outcome in clinical trials for treatment of ischemic stroke (Shohami et al., 1997; Weinberger, 2006).

SP-8203 (N-[3-(2,4-Dioxo-1,4-dihydro-2 H-quinazolin-3-yl)propyl]-N-{4-[3-(2,4-dioxo-1,4-dihydro-2H-quinazolin-3-yl)ropylamino]butyl} acetamide) is the compound found from the earth worms, *Eisenia andrei*, and then organically synthesized (not published). Earth worms extract, a kind of traditional medicine, has been used for treatment for inflammation, pain, fever and stroke. It is also reported that the protease fractionated from the earth worms, *Eisenia andrei*, appeared to be very stable and showed greater antithrombotic activity than other currently used antithrombotics (Lee et al., 2007). In addition anti-thrombotic activity, SP-8203 shows neuroprotective effects as a competitive NMDA antagonist (not published).

In this study, we provided evidence that SP-8203 can suppress the oxidative stress and protect neurons against cell death and attenuate neurological deficit in cerebral ischemia rat model.

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2. Materials and methods

2.1. Chemicals

SP-8203 was purified from the coelomic fluid of the live earth worm, *Eisenia Andrei*, after electric shock, and then organically synthesized as previously reported (not published). SP-8203 was dissolved in phosphate-buffered saline (PBS). All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). Synthetic (±)-α-tocopherol (Sigma T-3251) was used in this study.

2.2. Cell culture

SH-SY5Y cells were maintained in DMED (Thermo Scientific HyClone, Fisher, UK) supplemented with 10% FBS (GIBCO BRL, Gainthersburg, MD) and 100 U/ml/100 μg/ml of penicillin/streptomycin (Invitrogen) at 37 °C in 5% CO_2.

2.3. Cell viability test

WST-1 was used to evaluate cell viability according to the manufacturer's instructions (Roche, IN, USA). SH-SY5Y cells were plated in a 96-well plate at a density of 5×10^3 cells/well. Cells were allowed to adhere to plates for 24 h and then pretreated with SP-8203 (2, 20 or 200 μM), MK-801 (10 μM) or vitamin E (50 μM) for 4 h before the treatment with 1 mM glutamate or 250 μM H_2O_2. This colorimetric assay measures the metabolic activity of viable cells. Briefly, after incubating cells treated with various reagents, 10 μl WST-1 was added to the culture media. The culture was incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 1 h. The absorbance of the reaction product was measured with an ELISA reader (Bio-Rad, Germany) at a 450 nm.

2.4. Measurement of ROS generation

Intracellular ROS in SH-SY5Y cells were detected using dye 2′,7′-dichlorofluoroscein diacetate (DCFH-DA; Molecular Probes, Eugene, OR). Cells were washed with HEPES-buffered saline (HBS) and incubated in the dark for 1 h in HBS containing 100 μM of DCFH-DA. On incubation, DCFH-DA is taken up by cells where intracellular esterase cleaves the molecule to DCFH, which is oxidized to DCF in the presence of H_2O_2. The total fluorescence was measured using spectrofluorometer (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 488 nm and an emission wavelength of 524 nm.

2.5. Measurement of SOD activity

The activities of SOD were all determined by using commercially available kit (Dojindo, Japan). All procedures completely complied with the manufacturer's instructions. The activities of enzymes were expressed as units per 1 mg protein. The assay of SOD activity was based on its ability to inhibit the oxidation of hydroxylamine by O^{2-} produced from the xanthine–xanthineoxidase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 450 nm by 50%.

2.6. Animals

Nine-week-old male Wistar rats weighing 280–310 g (Central Laboratory Animal Incorporation) were used in this study. Animals were allowed to acclimatize for at least 5 days prior to experimentation. Animals were housed in groups of two or three in clear Plexiglas cages containing sawdust at a room temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%. Animals were maintained on a 12-h light/dark cycle (light on at 8:00 a.m.) and given food and water *ad libitum*. All experiments were performed in accordance with the Guidelines for Animal Experiments of Ethics Committee of Seoul National University (Publication No. 1653, revised 2008).

2.7. Transient middle cerebral artery occlusion

Transient middle cerebral artery (MCA) occlusion was induced by using a suture-occlusion technique (Nagasawa and Kogure, 1989) to rats. Briefly, after pentobarbital injection (3 mg/kg, i.p.), the left common carotid artery was exposed after a midline cervical incision. The external carotid artery and the extra-cranial branch of the internal carotid artery were ligated. The origin of MCA was then occluded by introducing 4–0 monofilament nylon suture with a tip rounded by heating through the stump of the external carotid artery. The nylon filament was secured in place with a ligature, and the wound was closed. After 1 h of occlusion, the nylon filament was removed to restore blood flow.

2.8. Administration of SP-8203

One of MCA-occluded rats was injected intraperitoneally with 5 or 10 mg/kg of SP-8203 30 min before and an hour after MCA-occlusion. Each concentration of SP-8203 was injected daily during 10 days. Normal saline or 0.5 mg/kg of MK-801 were injected with the same volume of SP-8203 injection in the other group.

2.9. TTC staining

Animals were sacrificed 10 days after MCA-occlusion operation and infarct volume of each focal ischemia model rat was analyzed. After anesthesia (i.p.) with pentobarbital, the brain of each rat was isolated, coronally sectioned into 1 mm thick, and placed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 30 min and immersion-fixed in a 4% paraformaldehyde. TTC-stained sections were photographed and the digital images were analyzed using image analysis software (ImageJ 1.42q). The lesion volume was calculated by multiplying the area by the thickness of slices. To compensate for the effect of brain edema, the corrected infarct volume was calculated by the following equation: corrected infarct area = contralateral hemisphere area − (ipsilateral hemisphere − infarction area) (Swanson et al., 1990).

2.10. Rota rod

The Rota rod test was used to assess motor coordination and balance. The Rota rod tests was performed by measuring the time animals remained on a Rota rod (Ugo Basile, Italy) that was using constant speed (9 rpm) over 300 s at 2 day, 5 day, 10 day after MCAO in rats. It was measured 6 times at each test, and data are presented as the mean percent of 6 Rota rod measurements.

2.11. Measurement of lipid peroxidation

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) and 4-hydroxynonenal (HNE) levels using the Lipid peroxidation assay kit from Calbiochem (LaJolla, CA, USA) in accordance with the protocol supplied by the manufacturer. Briefly, brain tissues were homogenized in 4 vol. of ice-cold 20 mM phosphate-buffered saline (pH 7.4) containing 5 mM butylated hydroxytoluene. Homogenates were centrifuged at 3000 *g* for 10 min at 4 °C, and the supernatant (200 μl) was used for each assay. For each reaction, 650 μl of diluted R1 reagent (1:3 of methanol:N-methyl-2-phenylindole) were added, and mixed with 150 μl of R2 reagent. Each reactions was incubated at 45 °C for 60 min and centrifuged at 10,000 *g* for 10 min. The

supernatant was measured at 586 nm. The data were normalized versus the protein concentration.

2.12. Statistical analysis

Data were expressed as mean ± S.E. value or as percent of control value ± S.E. One-way ANOVA test were applied to study the relationship between the different variables. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and $\#p<0.001$were considered to be significant.

3. Results

3.1. SP-8203 exerts neuroprotective effects against glutamate or H_2O_2 in SH-SY5Y Cells

We studied the effects of SP-8203 on neuronal cell death induced by glutamate or H_2O_2. As measured by WST-1 assay, glutamate significantly decreased cell viability compared to vehicle-treated control in SH-SY5Y cells at 24 h after treatment with glutamate (83.73 ± 2.26% vs. vehicle treated control cells) or H_2O_2 only (61.64 ± 3.98%), respectively (Fig. 1A and B). Pretreatment with SP-8203, however, significantly attenuated cell death compared to the glutamate-treated (2 μM SP-8203, 97.89 ± 3.72%; 20 μM SP-8203, 97.15 ± 5.37%; 200 μM SP-8203, 96.63 ± 4.67%) or H_2O_2-treated cells (2 μM SP-8203, 88.63 ± 1.69%; 20 μM SP-8203, 85.93 ± 3.38%; 200 μM SP-8203, 88.08 ± 1.66%). Pretreatment with 10 μM MK-801 or 50 μM vitamin E also significantly attenuated cell death induced by glutamate (97.98 ± 4.3%) or H_2O_2 (88.63 ± 1.69%), respectively. However MK-801, an NMDA antagonist, could not block H_2O_2-induced cell death (69.16 ± 8.13%).

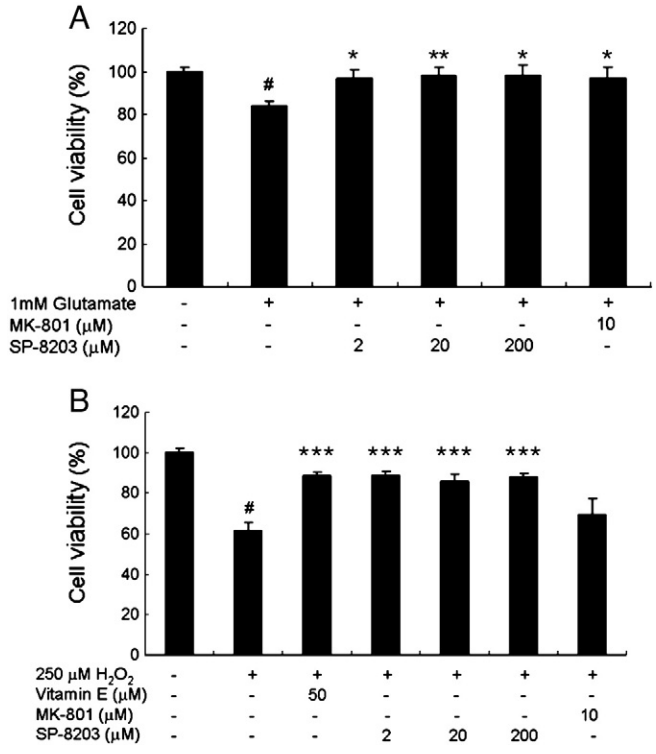

Fig. 1. SP-8203 protects neuronal cells against glutamate or H_2O_2 in SH-SY5Y cells. SH-SY5Y cells were plated in a 96-well plate at a density of 5×10^3 cells/well. SP-8203 was introduced into the media of SH-SY5Y cells 4 h before treatment with 1 mM glutamate (A), 250 μM H_2O_2 (B) and incubated for 24 h, and then measured cell viability using WST-1 reagent. Data were expressed as the percent of vehicle control value ± SEM. At least two experiments were carried out in triplicate. $\#p<0.001$, $^{*}p<0.05$ and $^{**}p<0.01$ compared to glutamate-, or H_2O_2-treated group, one-way ANOVA.

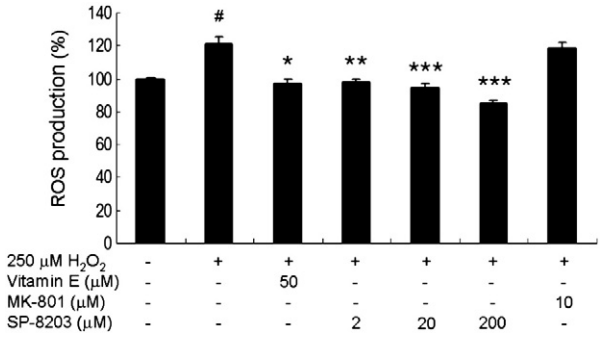

Fig. 2. SP-8203 reduces the production of ROS induced by H_2O_2 in SH-SY5Y cells. The intracellular level of ROS was measured using DCFH-DA. SP-8203 (2, 20 or 200 μM) was pretreated 4 h before treatment with H_2O_2. Data were expressed as the percent of vehicle control value ± SEM. At least two experiments were carried out in triplicate. $\#p<0.001$, $^{***}p<0.001$ compared to H_2O_2-treated group, one-way ANOVA.

3.2. SP-8203 Reduces the ROS accumulation by H_2O_2 treatment in SH-SY5Y Cells

To examine whether SP-8203 could reduce ROS accumulation, we measured ROS levels using DCFH-DA. Pretreatment with SP-8203 (2 μM, 20 μM or 200 μM) for 4 h before treatment with H_2O_2 significantly decreases ROS production (Fig. 2; 2 μM SP-8203, 98.19 ± 2.31%; 20 μM SP-8203, 94.74 ± 2.06%; 200 μM SP-8203, 84.77 ± 1.64% vs. vehicle-treated H_2O_2 treated cells). Pretreatment with 50 μM vitamin E significantly recovered ROS production (97.42 ± 2.31%). MK-801, however, could not suppress ROS production in H_2O_2-treated SH-SY5Y cells (118.89 ± 3.37%).

3.3. SP-8203 Increases SOD activity in H_2O_2-treated SH-SY5Y Cells

To know how SP-8203 could reduce cell death and ROS production induced by H_2O_2, we performed mechanism study on ROS. To investigate whether SP-8203 could directly scavenge ROS, hydroxyl radical was measured by electron spin resonance spectrometer (ESR) using 5,5-dimethyl-1-pyrroline N-oxide (DMPO). SP-8203 could not directly scavenge ROS such as hydroxyl radical (Supplementary Figure).

ROS can be also scavenged by endogenous antioxidant enzymes such as superoxide dismutase (SOD) (Dinkova-Kostova and Talalay, 2008), we performed superoxide dismutase (SOD) activity test. Pretreatment with SP-8203 (2 μM, 20 μM or 200 μM) for 4 h before treatment with H_2O_2 significantly increases SOD activity in SH-SY5Y cells (Fig. 3; 2 μM, 29.85 ± 8.11 unit/ mg protein; 20 μM, 31.35 ± 5.59 unit/mg protein; 200 μM, 58.53 ± 6.92 unit/mg protein). Interestingly, MK-801 could not affect the SOD activity in H_2O_2-treated SH-SY5Y cells (32.10 ± 3.50 unit/mg protein). To check whether SP-8203 could

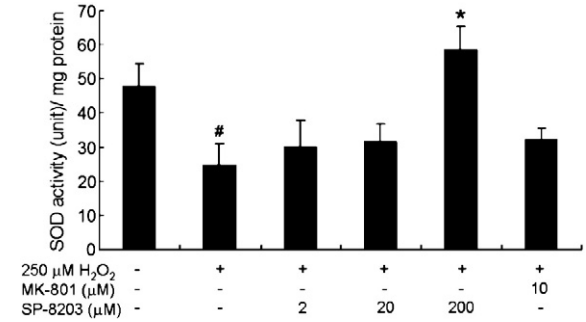

Fig. 3. SP-8203 increases SOD activity in H_2O_2-treated SH-SY5Y cells. SP-8203 (2, 20 or 200 μM) was pretreated 4 h before treatment with H_2O_2. Data were expressed as unit per 1 mg of protein ± SEM. At least two experiments were carried out in triplicate. $\#p<0.05$ and $^{*}p<0.05$ compared to H_2O_2-treated group, one-way ANOVA.

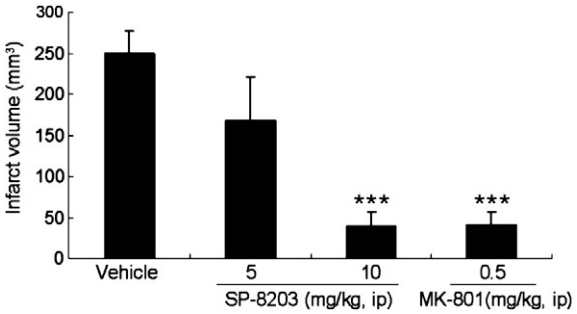

Fig. 4. SP-8203 reduces the infarct volume in MCAO rats. SP-8203 (5 and 10 mg/kg), MK-801 (0.5 mg/kg) or the same volume of saline was administered intraperitoneally to rats before and after the MCA occlusion and injected daily. At 14 days, infarct volume was measured by TTC staining. Data represent mean ± SEM (n = 4). ***p<0.001 compared to vehicle control, one-way ANOVA.

increase SOD activity directly, we also performed cell-free SOD activity test. As a result, SP-8203 also increased SOD activity directly but MK-801 did not (data not shown).

We wondered the effects of SP-8203 on other ROS enzymes, catalase and glutathione reductase. However, neither SP-8203 nor MK-801 showed increased activity in catalase and glutathione reductase in both cell-free assay and neuronal cell culture (data not shown).

3.4. SP-8203 prevents neuronal cells from ischemic brain injury in vivo

We check the effects of SP-8203 on ischemic brain injury *in vivo* using TTC staining. Animals were sacrificed at day 10 after MCA-occlusion operation and infarct volume of each focal ischemia model rat was analyzed. Infarct volume of 10 days administration of 10 mg/kg SP-8203 group (Fig. 4; 39.88 ± 16.82 mm^3; p<0.001) or 0.5 mg/kg MK-801 group (41.41 ± 14.36 mm^3; p<0.001) was greatly diminished compared to vehicle-treated group (249.94 ± 27.93 mm^3).

3.5. SP-8203 attenuates impairment of stroke-induced motor function

To assess the effect of SP-8203 on motor function after experimental stroke, we performed a rotor rod test in the 5 mg/kg SP-8203-treated, 10 mg/kg SP-8203-treated, 0.5 mg/kg MK-801-treated and vehicle-treated rats at 2 day, 5 day, 10 day after experimental stroke and sham-surgery (Fig. 5). The riding time of vehicle control group, which was significantly shortened by MCAO (2 day, 6.56 ± 0.63%; 5 day, 5.73 ± 0.67%; 10 day, 13.92 ± 3.15%), was significantly recovered almost to that of sham-control group (2 day, 29.833 ± 4.74%; 5 day, 73.31 ± 8.87%; 10 day, 78.40 ± 9.92%) by administration of 10 mg/kg SP-8203 in a time-dependent manner (2 day, 19.53 ± 3.42%; 5 day, 34.67 ± 5.30%; 10 day, 63.44 ± 11.12%; p<0.001). MK-801, however, could not restore

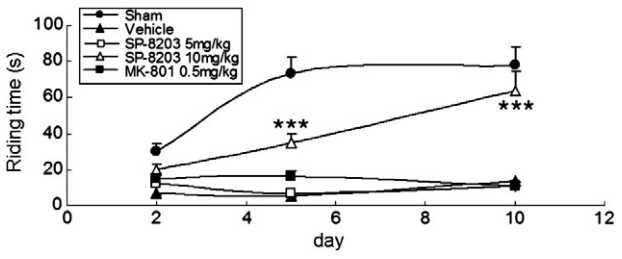

Fig. 5. SP-8203 ameliorates neurological impairments in MCAO rats. SP-8203 (5 and 10 mg/kg), MK-801 (0.5 mg/kg) or the same volume of saline was administered intraperitoneally to rats before and after the MCA occlusion and injected daily. Latency time was measured using Rota rod with constant speed (9 rpm) over 300 seconds at 2 day, 5 day, 10 day after surgical operation. Data represent mean ± SEM (sham, n = 8; vehicle, n = 8; SP-8203 5 mg/kg, n = 6; SP-8203 10 mg/kg, n = 6; MK-801 0.5 mg/kg, n = 8). ***p<0.001 compared to vehicle control, one-way ANOVA.

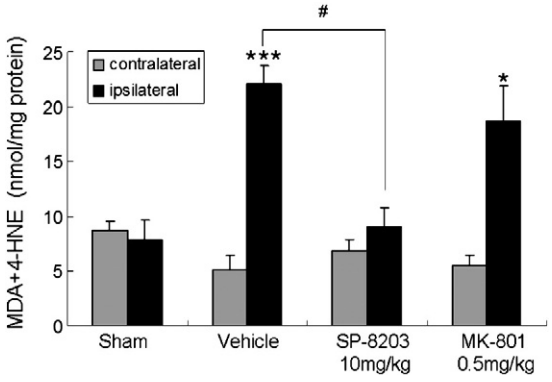

Fig. 6. SP-8203 reduces the accumulation of lipid peroxidation products in MCAO rats. SP-8203 (5 and 10 mg/kg), MK-801 (0.5 mg/kg) or the same volume of saline was administered intraperitoneally to rats before and after the MCA occlusion and injected daily. MDA and 4-HNE levels, lipid peroxidation products, were measured using the Lipid peroxidation assay kit from Calbiochem (LaJolla, CA, USA) in according to the manufacturer's instruction. Data represent mean ± SEM (sham, n = 3; vehicle, n = 5; SP-8203 10 mg/kg, n = 5; MK-801 0.5 mg/kg, n = 6). *p<0.05 and ***p<0.001 compared to contralateral hemisphere and #p<0.001 compared to vehicle control, one-way ANOVA.

the motor function after MCAO (2 day, 15.29 ± 2.23%; 5 day, 16.71 ± 2.67%; 10 day, 10.90 ± 1.87%).

3.6. SP-8203 ameliorates the oxidative stress from ischemic brain injury in vivo

To investigate whether SP-8203 could reduce oxidative stress *in vivo* model of experimental stroke, we conducted lipid peroxidation assay. MCAO for 1 h in rats dramatically increased lipid peroxidation levels, suggesting high oxidative stress is produced in experimental stroke (Fig. 6; contralateral, 4.99 ± 1.86%; ipsilateral, 22.07 ± 1.64%). Moreover, this increased level of lipid peroxidation in MCAO rats was significantly suppressed by daily administration of 10 mg/kg of SP-8203 (contralateral, 6.84 ± 1.00%; ipsilateral, 10.16 ± 1.89%), not by administration of MK-801 (contralateral, 4.68 ± 0.70%; ipsilateral, 18.63 ± 3.31%), suggesting that SP-8203 could reduce oxidative stress *in vivo* model of experimental stroke.

4. Discussion

In the present study, we evaluated anti-oxidant effects of SP-8203 in a rat model of focal cerebral ischemia. The major findings of the present study were that SP-8203 significantly suppressed ROS levels and the accumulation of lipid peroxidation products. SP-8203 also increased SOD activity. Moreover, these changes were associated with functional improvement in Rota rod performances.

Acute brain insults such as cerebral ischemia and traumatic brain injury have traditionally been the focus of excitotoxicity research (Lau and Tymianski, 2010), and MK-801, non-competitive antagonist of NMDA receptor, has been frequently using as a comparative material in many screening studies on therapeutic agents for stroke (Foster et al., 2009; Regan et al., 2009; Zhao et al., 2009). MK-801, however, have not improved neurological deficits in cerebral ischemia, most likely because not protect neurons against other toxic factors including oxidative stress (Shohami et al., 1997; Weinberger, 2006). In this study, the antioxidant effects of SP-8203 were compared to those of MK-801. Interestingly, SP-8203 significantly inhibited H_2O_2-induced cell death and ROS production, but MK-801 did not block neuronal death and ROS levels induced by H_2O_2. Moreover, SP-8203 significantly improved neurological deficit and blocked lipid peroxidation but MK-801 did not suppress neurological deficit and lipid peroxidation in MCA-occluded rats. Because of the synergism between excitotoxicity and oxidative stress, therapeutic dual

modalities such as glutamate antagonists and antioxidants, could be more effective than monotherapy (Gwag et al., 2007; Kempski, 1994). Therefore SP-8203 may be more effective for multiple-target mechanisms of ischemic stroke than other drugs.

ROS can be scavenged by the direct effect of drug or indirect effects via endogenous antioxidant enzymes such as superoxide dismutase (SOD) (Dinkova-Kostova and Talalay, 2008). If ROS is directly scavenged, it could be measured by ESR. To measure ROS such as hydroxyl radical, we performed fenton reaction and detected ROS using DMPO. But, SP-8203 could not directly scavenge ROS such as hydroxyl radical. So we focused on endogenous antioxidant enzymes such as SOD, catalase and glutathione reductase. SP-8203 increased SOD activity, but MK-801 did not. SOD activity of SP-8203-treated cells was also more significantly increased than that of H_2O_2-treated neuronal cells. However, neither SP-8203 nor MK-801 showed increased activity in catalase and glutathione reductase in both cell-free assay and neuronal cell culture. Detailed antioxidant mechanisms of SP-8203 should be clarified further in future. Overall, these data suggest that SP-8203 attenuates oxidative stress through increasing of SOD activity.

Supplementary materials related to this article can be found online at doi:10.1016/j.pbb.2010.12.014.

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