Full Paper

Amyloid- β -Induced Cytotoxicity of PC-12 Cell Was Attenuated by *Shengmai-san* Through Redox Regulation and Outgrowth InductionHiroshi Nishida¹, Megumi Kushida¹, Yuki Nakajima¹, Yoshihiro Ogawa¹, Naoto Tatewaki¹, Shinji Sato¹, and Tetsuya Konishi^{1,*}¹Department of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata 956-8063, Japan

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Abstract. Neurodegenerative brain disorders such as Alzheimer's disease (AD) have been well investigated. However, significant methods for the treatment of the promotion and progression of AD are unavailable to date. Recent studies suggested that the redox imbalance and the accumulation of amyloid- β (A β) peptide occurring in the brain of AD patients lead to oxidatively-induced apoptotic cell death. Here, we show the effects of *Shengmai-san* (SMS) on A β -induced cytotoxicity in PC-12 cells. SMS dose-dependently attenuated the cytotoxicity by A β incubation and also prevented the morphological damage in neurites of the PC-12 cells. Heme-oxygenase-1 and glutathione peroxidase-1 expressions were increased by SMS pretreatment. SMS decreased the phosphorylation level of *c-jun* amino-terminal kinase (JNK) and the activity of caspase-3, which were enhanced by A β incubation. Of importance, SMS treatment promoted neurite outgrowth. These data demonstrated dual roles of SMS in PC-12 cells. SMS prevents the apoptosis through the enhancement of anti-oxidant enzymes and inhibition of the JNK signaling pathway with the promotion of nerve cell maturation, thus suggesting benefits of SMS for the treating of neurodegenerative diseases. It may also be beneficial not only for the treatment of brain disorders but also for other diseases caused by oxidative stress.

Keywords: Alzheimer's disease, apoptosis, mitogen-activated protein kinase (MAPK), neurite outgrowth, *Shengmai-san*

Introduction

Alzheimer disease (AD) is a serious and complex neurodegenerative disorder that affects approximately 15 million people worldwide (1). Recent studies have suggested that intracellular amyloid- β (A β) peptide and its aggregates are a cause of AD as a neurotoxic agent (2–4), and the association of A β accumulation with the redox imbalance in the brain was revealed (5–8). However, significant challenges to prevent the promotion and/or progression of AD through the modulation of redox status have not been well performed yet. Basically, it is assumed that there are two ways to avoid the oxidative injury caused by reactive oxygen species (ROS) in brain tissue and cells: One is to improve the anti-oxidative capacity by some treatment

of tissue and cells through the induction of anti-oxidant enzymes such as glutathione peroxidase (GPx), heme-oxygenase (HO-1), Mn-superoxide dismutase (Mn-SOD), and so on. Such enzymes are thought to be candidates for the elimination or the attenuation of cytotoxicity by intracellular generated ROS. The other is to modulate pathways that lead to cell death including the apoptotic signaling pathway. Mitogen-activated protein kinases (MAPKs) have a critical role in apoptotic cell death (9, 10). Among the main stress signaling pathways or central mediators activated in response to oxidant injury are MAPK signaling cascades implicated as extra-cellular regulated kinases (ERK) (11, 12), *c-jun* amino-terminal kinase (JNK) (13, 14), and p38 MAPK (15). Importantly, these MAPKs not only work as transducers of cell death leading to apoptosis but also are associated with various kinds of events occurring in cells such as proliferation and differentiation (9, 10). Therefore, some of the MAPK pathways could be expected to play a role in neuronal-regeneration and maturation in neuro-

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degenerative disorders.

Shengmai-san (SMS) is a traditional Chinese medicine commonly used for its effect on oxidative damage in heart, brain, and other tissues (16, 17). Although SMS has been applied for heart and blood diseases, routinely and prophylactically for thousands years in China, little available data is currently provided to prove the molecular based usability of SMS treatment. In our previous studies, we demonstrated that SMS treatment of ischemia-reperfusion model rat (18) and PC-12 cells (19) decreased oxidative injury such as the accumulation of lipid oxidation and protein carbonyl in brain and cells, respectively. These observations were presumably due to the improvement of anti-oxidative capacities and inhibition of apoptotic signaling cascades both of rat and cultured cells. Here, we demonstrate that the effect of SMS on the expression of anti-oxidant enzymes, whereas the influence of SMS on MAPK signaling and neurite maturation of PC-12 cells during the incubation with $A\beta$.

Materials and Methods

Cell culture

PC-12 cells, a rat pheochromocytoma, were obtained from Riken cell bank (Ibaraki) and maintained in Dulbecco's modified Eagle medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 50 units/ml penicillin (Invitrogen, Carlsbad, CA, USA) and 100 mg/ml streptomycin (Invitrogen). The cells were seeded in ϕ 60 mm dishes (Nalge Nunc Int., Rochester, NY, USA) at a density of 1×10^4 cells/cm². All cultures were kept in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. PC-12 cells were incubated and differentiated by the addition of nerve growth factor (NGF) for 48 h before all the experiments performed in the present study.

Chemicals and preparation of SMS

$A\beta$ was purchased from Sigma as 25 to 35 of peptide fragment. The three composite dry herbs of SMS, *Ginseng* (48 g), *Ophiopogon japonicus* (48 g), and *Schisandra chinensis* (24 g) were kindly provided by Kotaro Kanpo Co., Osaka and were kept in a -80°C freezer until used. The specimen numbers of herbs (*Ginseng*: 06100501, *Ophiopogon japonicus*: 06190101, *Schisandra chinensis*: 06020301) for this study were recorded and stored for 10 years at Niigata University of Applied Life Sciences. The herbs were suspended in 1,200 ml distilled water, soaked for 1 h, and then boiled for 1 h in each experiment. After the filtration, the extracts were used for the addition to the culture (18). The concentration of SMS was calculated and indicated

as weight per volume (w/v) of dry herb into medium. To estimate the pharmacological effect of SMS, α -tocopherol (Sigma) was compared as a reference (positive control) in the present study (Fig. 1C).

HPLC analysis

SMS solution was centrifuged at 10,000 rpm for 10 min, and stock solutions of Schisandrin and Gomisin-A were each diluted to an appropriate concentration range (0–50 μ g/ml) for standard curves. The supernatant of SMS and standard solutions were analyzed by HPLC. HPLC was carried out by using a Shimadzu HPLC system (Kyoto) equipped with a photodiode array detector (SPD-M10Avp). The following conditions were used: Mightysil RP-18 column (250 $\times$ 4.6 mm i.d., 5- μ m particle size), linear gradient elution with CH₃CN and aqueous solution, and flow rate at 0.8 ml/min. Aliquots (20 μ l each) of SMS and standard solutions were injected to the column. Temperatures of the column oven and auto-sampler tray were set at 30°C and 15°C, respectively. UV detection was set at 254 nm.

Cell viability

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as described previously (20). The cells were cultured in growth medium for 24 h in 96-well multi plates and then pre-incubated with or without SMS for 24 h following incubation with $A\beta$ for another 24 h. After replacing the medium for 0.05% MTT with GM, cells were incubated for 4 h at 37°C. The cells were lysed by the addition of lysing buffer [20% SDS, 50% *N,N*-dimethyl formamide (DMF), pH 4.7] and applied to a microplate reader at 570 nm.

Microscopic observation

For the microscopic observation, PC-12 cells were seeded onto 60-mm dishes. After the incubation with or without SMS for 24 h, the cultures were incubated in the presence or absence of $A\beta$ in medium. Cells were photographed under a microscope (Zeiss, Jena, Germany). Bars indicate 50 μ m in each field.

Neurite outgrowth

PC-12 cells were treated with 50 ng/ml NGF (Sigma) for 72 h. For the microscopic observation of neurite outgrowth, SMS was added in the last 24 h with NGF treatment. The percentage of cells with neurites extending longer than 1 and 2 diameters of the cell bodies was counted as "Medium" and "Long", respectively. The proliferating cells were also counted as shorter than 1 diameter of the cell body.

Protein preparation

Cells were harvested by scraping into ice cold phosphate-buffered saline (PBS). After washing twice with cold PBS, proteins were extracted twice from the cell pellets into UTB-buffer (8 mM urea, 150 mM 2-mercaptoethanol, 50 mM Tris, pH 7.5) for immunoblot analysis. Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA, USA).

Immunoblot analysis

For immunoblot analysis, 30 μ g proteins were loaded and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane by electro-blotting. The membrane was blocked by 5% skim milk in Tris-buffered saline containing 0.1% Triton X-100 (TBS-T) for 1 h at room temperature. Immunoblots were performed with appropriate antibodies: primary antibodies for HO-1 (Stressgen, Ann Arbor, MI, USA), GPx-1 (Biogenesis, Kingston, NH, USA), phospho-JNK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), JNK (Cell Signaling Technologies, Danvers, MA, USA), phospho-p38 (Santa Cruz Biotechnology), p38 (Santa Cruz Biotechnology), phospho-ERK (Santa Cruz Biotechnology), ERK (Santa Cruz Biotechnology), caspase-3 (Cell Signaling Technologies), Bcl-2 (Cell Signaling Technologies), tubulin (Sigma) were incubated with membrane for 16 h at 4°C; secondary antibodies conjugated with horse radish peroxidase (HRP) (Zymed Laboratories, San Francisco, CA, USA) were incubated for 1 h at room temperature. Target proteins were visualized by an ECL reaction kit (Amersham, Piscataway, NJ, USA) and chemiluminescence film (Amersham).

Statistics

Statistical analysis was performed by the General Linear Model procedure of SAS (1995) in conjugation with the Duncan's multiple range test. The level of statistical significance was taken as $P < 0.05$.

Results

The effect of SMS on cytotoxicity of A β treatment in PC-12 cells

The viability of PC12 cells was determined by MTT assay in order to visualize the effect of SMS on A β -induced cytotoxicity. A 24-h incubation with A β induced cell death occurring in a dose-dependent manner up to 4 μ M (Fig. 1A). The cell viability was decreased to approximately 60% at 4 μ M, with no further decrease being observed at A β concentrations greater than 4 μ M. In this culture system, the toxic effect

of A β was saturated in these ranges of A β concentration. The pretreatment with SMS for 24 h prior to A β incubation produced dose-dependent attenuation of the A β toxicity in PC-12 cells, and the protective effects of SMS were obvious at A β concentrations of 4–20 μ M, increasing cell viability to 70%–80%. Significant differences were seen in all SMS-treated cells at the A β concentrations over 0.4 μ M.

To investigate how A β decreased the cell viability, we studied the time-dependent effect of A β (20 μ M) on PC-12 cells with or without SMS pretreatment (0.2%) (Fig. 1B). In the absence of SMS, the viability of PC-12 cells was gradually decreased with the time of A β incubation to 70%. SMS pretreatment significantly attenuated the toxicity of A β in all tested periods in this study. These results suggested that SMS pretreatment exerts cellular protection against the cytotoxicity of A β in the differentiated PC-12 cells. In addition, the preventive effect of SMS against A β cytotoxicity was compared to that of α -tocopherol, a positive control (Fig. 1C). Although the treatment with α -tocopherol significantly attenuated the cell death induced by A β , its efficacy was less than that of SMS. By our quality control of SMS, we can assure the reproducibility of SMS's preventive effect against cytotoxicity in A β -treated PC-12 cells. The SMS extraction was performed under the specific and controlled conditions described in the Materials and Methods section. The presence of both Schisandrin and Gomisins-A (Fig. 1D), the ingredients of SMS, was confirmed by obtaining the HPLC profile of the extract. The typical HPLC profile chromatogram showed that Schisandrin and Gomisins-A appeared at 32.3 and 34.3 min of retention time without any interfering signals. Among these, the concentration of Schisandrin and Gomisins-A in SMS were consistently monitored for each extraction to control the quality of the SMS solution.

Morphological observation of SMS- and A β -treated cell

Microscopic observation confirmed the differentiated PC-12 cells with matured neurites in our culture system after 48-h pre-incubation with NGF (Fig. 2A, control). In the A β -incubated cells, many dispirited cells with atrophied or fragmented neurites were observed (Fig. 2A, arrows in panel A β). However, no marked difference was seen in SMS-pretreated cells either in the presence or absence of A β (Fig. 2A, SMS and SMS + A β); thus the protective effect of SMS against A β toxicity in PC-12 cells was morphologically confirmed.

The effect of SMS on DNA fragmentation induced by A β

FACS analysis was performed to estimate DNA

fragmentation that occurred in individual cells as an index of apoptosis. Incubation of cells with A β for 24 h dramatically increased DNA fragments in the PC-12 cells (Fig. 2B). SMS pretreatment, however, dramatically reduced the percentage of DNA fragments induced by A β incubation. Since the DNA fragmentation in an individual cell indicates the occurrence of apoptotic cell death, this observation suggested that SMS prevented a certain apoptotic signaling pathway leading PC-12 cells to apoptosis after the incubation with A β . This was further confirmed by agarose gel electrophoresis showing A β -induced fragmentation of DNA (data not shown).

The induction of antioxidant enzymes by SMS

To examine the improvement effect of SMS on antioxidative capacity of cells, we estimated the intracellular protein level of HO-1 and GPx-1. SMS pretreatment increased the expression level of both HO-1 (4.3-fold) and GPx-1 (1.2-fold vs CT) after the 24 h incubation with SMS (Fig. 3). The expression level of HO-1 was also enhanced by A β incubation for 24 h (4.1-fold) and GPx (1.1-fold). The stability of HO-1 and GPx-1 expression in SMS-pretreated cells was not continued by subsequent incubation of A β for 24 h. In another experiment, identically, we observed that SMS stimulated the mRNA expression of Mn-SOD in PC-12 cells (data not shown).

Apoptosis-related signal modulation by SMS

To examine whether SMS inhibits the apoptotic signals induced by A β , we studied the effects of SMS on MAPK and related signals in PC-12 cells. Although the phosphorylation level of p38 and ERK were relatively higher than that of a non-differentiated culture

Fig. 1. SMS prevented cell death of PC-12 cells induced by A β . **A:** Dose-dependent effect of A β on cell viability of PC-12 cells. After the pre-incubation with or without various concentrations of SMS for 24 h, cells were treated with different concentrations of A β as indicated for 24 h. Each value represents the mean $\pm$ S.D. obtained from at least quadruplicate determinations. **B:** Time-dependent change of cell viability of PC-12 cells by A β treatment. The cells were pre-incubated with or without 0.2% SMS (w/v) prior to the 20 μ M A β -treatment. Each value represents the mean $\pm$ S.D. obtained from at least quadruplicate determinations (* P <0.01). **C:** The effect of SMS on viability of PC-12 cells. The efficacy of SMS on cell viability was compared to that of α -tocopherol-treated cells. After the pre-incubation with SMS (0.2%) or α -tocopherol (50 mM in ethanol) for 24 h, cells were treated with A β (20 μ M) for 24 h. Each value represents a mean $\pm$ S.D. obtained from at least quadruplicate determinations (* P <0.01). **D:** A typical HPLC chromatogram of SMS extract. UV spectrum was obtained at 254 nm with linear gradient elution with CH₃CN and aqueous solution. Schisandrin and Gomisin-A appeared at 32.3 and 34.3 min, respectively.

Fig. 2. SMS protected against the morphological damage and sub-G1 accumulation caused by A β . **A:** After the pre-incubation with or without 0.2% SMS (w/v) for 24 h, PC-12 cells were treated with or without 20 μ M A β and observed by phase-contrast microscopy. The arrows indicate damaged and atrophied neurites. The bar indicates 50 μ m. **B:** The cell cycle analysis was performed by a flow cytometry system. The cells treated with or without SMS and/or A β were harvested and fixed before staining with propidium iodide and analysis by flow cytometry. Red fluorescence intensity was measured and histograms of DNA content generated. The numbers in each panel are the percentage of cell cycle phases as G1/G0, S, G2/M, and Sub-G1. **C:** The percentage of Sub-G1, DNA fragmentation. Each value represents a mean $\pm$ S.D. obtained from triplicate determinations (* $P < 0.01$).

(data not shown), A β increased the phosphorylation levels of JNK and p38 but not that of ERK compared to the control and also activated caspase-3 but decreased Bcl-2 in PC-12 cells (Fig. 4). In contrast, SMS pretreatment dramatically enhanced the p38 and ERK phospho-

rylation in PC-12 cells. Notably, the treatment of SMS and A β did not show any synergic effects in any of the MAPK activities, although p38 and ERK kinase signals were increased compared to those of the control. SMS pretreatment eventually prevented the activation of

Fig. 3. SMS enhanced the expression of antioxidant enzymes. A: The expression of anti-oxidant enzymes was determined by immunoblotting analysis. After the pre-incubation with or without 0.2% SMS (w/v) for 24 h, PC-12 cells were treated with or without 20 μ M A β and then lysed for immunoblotting to detect the protein expression of hemoxygenase-1 (HO-1) and glutathione peroxidase-1 (GPx-1). B: The expression levels of HO-1 and GPx-1 were digitized by using NIH-image software and standardized by GAPDH.

Fig. 4. SMS modulated apoptosis-related signals. A: The phosphorylation levels of MAPKs (JNK, p38, and ERK) and the expression level of the other apoptosis signal-related proteins were determined by immunoblotting analysis. After the pre-incubation with or without 0.2% SMS (w/v) for 24 h, PC-12 cells were treated with or without 20 μ M A β and lysed for immunoblotting. B: The phosphorylation or expression levels were digitized by using NIH-image software and standardized by GAPDH.

caspase-3 and the decrease of Bcl-2 in A β -treated cells.

The effect of SMS on neurite outgrowth

In order to understand the relationship between MAPK activation and nerve cell maturation by the treatment of SMS in PC-12 cells, we measured the neurite outgrowth of PC-12 cells under the condition with NGF in the medium because recent reports revealed the strong connection of p38 and ERK to neurite maturation/extension (9, 21). NGF treatment itself induced the differentiation of PC-12 cells, which was achieved to about 70% of total, and approximately 50%

of the differentiated cells as matured neurites described as "Long" were observed in Fig. 5. The accelerated differentiation (82%) and the dramatic extension of neurite outgrowth were observed in the presence of SMS. The SMS-dependent neurite extension was not observed without NGF (data not shown). Thus SMS promoted the nerve growth in collaboration with NGF in the culture.

Discussion

We previously showed that SMS pretreatment sup-

Fig. 5. SMS-induced neurite outgrowth of PC-12 cells. After the pre-incubation with or without 0.2% SMS (w/v) for 24 h, PC-12 cells were treated with or without 20 μ M A β , and neurite outgrowth of PC-12 cells was estimated by using a phase-contrast microscope. The percentage of cells with neurites extending longer than 2, 1, or 1 diameters of the cell bodies was counted as “Long”, “Medium”, or “Proliferating”, respectively. Each value is the mean $\pm$ S.D. of over 200 cells obtained from three independent experiments (* P <0.01).

pressed the cerebral oxidative stress in rats after ischemia-reperfusion (18). In addition, SMS prevented oxidative damage of protein, lipids, and DNA caused by H₂O₂ in PC-12 cells (19). SMS may have great potential as a new method to treat AD. Here, we clarified the further preventive mechanisms of SMS in A β -treated PC-12 cells, a typical model of AD in a culture system. Since PC-12 cells were incubated with NGF (22) for 48 h before the start of all the experiments, we could confirm the well-differentiated cells (75%, Fig. 5) exhibiting outgrowth.

A β treatment of differentiated PC-12 cells decreased the cell viability to 60% to 70%, and concomitantly the atrophies and fragmentations of neurites occurred everywhere in the culture as observed morphologically (Fig. 2A). Notably, we demonstrated the preventive effect of SMS on A β -induced cell death (Fig. 1A) and morphological changes (Fig. 2A). Furthermore, SMS dramatically decreased the percentage of DNA fragmentation produced by A β (Fig. 2B), which means the treatment by A β induced the typical apoptotic pathway involving the eventual activation of caspase-3, and the cytotoxic effects of A β were prevented by SMS pretreatment of PC-12 cells. We previously demonstrated that SMS pretreatment of animals and cells effectively decreased the oxidative damage as evaluated by lipid oxidation and protein carbonyl formation both in ischemia-reperfusion model rat (18) and A β -treated

PC-12 cells (19); therefore, the present results implicating that SMS prevented A β 's cytotoxicity in PC-12 cells may be due to the improvement of anti-oxidative capacity or the inhibitory effect of the apoptotic pathway of cells by SMS.

In addition, α -tocopherol prevents the progression of AD through anti-oxidant activity by itself (23, 24). The preventive effect of SMS on the cytotoxicity of A β was superior to that observed in α -tocopherol-treated cells in the present study (Fig. 1C), although α -tocopherol has more potent anti-oxidant activity. The enhancement of anti-oxidant potential by SMS was more effective than that by α -tocopherol, at least in this culture system. Since the supplement of SMS to the medium was performed only before the damage induction, there is no residual SMS together with A β , so that the possibility of direct reaction between SMS and A β is excluded in our culture system. Hence, it suggests that SMS is capable of modulating the cellular signals related to the stimulation of anti-oxidative capacity and/or the apoptotic pathway.

To clarify the inhibitory effect of SMS on cytotoxicity of A β , we performed the immunoblot analysis of HO-1 and GPx (Fig. 3A) as anti-oxidant enzymes. As predicted, SMS increased the protein expression of HO-1 and GPx-1 by 24-h incubation prior to A β abuse. In the mean time, the mRNA level was also increased in the PC-12 cells (data not shown). These results suggest that SMS may modulate the expression of anti-oxidant enzyme through the stimulation of common transcriptional factors such as AP-1 during the 24-h pretreatment because SMS's effect on protein expression was not only seen in one particular member of the anti-oxidant enzyme family but in all tested members. On the other hand, these enzyme expressions were also up-regulated by A β and were followed by the decreased expression in SMS + A β cells. This might be due to the negative feedback mechanisms by the redox sensing system (25, 26) that was exerted by subsequent accumulation of ROS by A β treatment. It was suggested that pre-increased anti-oxidant enzymes by SMS contributed to the attenuation of the oxidative stress that was induced by A β treatment, and thus the increased expression of these enzymes by A β was passive induction during the oxidative stress rather than these induction by SMS treatment. The enhancement of anti-oxidant enzymes improves the redox status and then protects the cells prior to oxidative stress not only in the brain but also in other tissues through the elimination of ROS (27, 28), so that such a strategy will be advantageous for the many kinds of diseases and aging caused by ROS accumulation (29).

The cytotoxic agents induce the sequential signaling

from MAPKKK, MAPKK, and MAPK to the transcriptional factors and thus induce cleavage of caspase-3 leading to DNA fragmentation and apoptosis (30). JNK is playing an important role in the apoptotic signaling pathway compared to the other MAPK during the above cascade (31). On the other hand, p38 is activated during the promotion of neurite extension of PC-12 cells (21), and the activation of the ERK pathway is required for the differentiation of PC-12 cells (32, 33). There are different cross-talks of MAPK in each other (34). Here, we observed the marked increase of JNK phosphorylation in $A\beta$ -treated PC-12 cells, but not in SMS-pretreated cells followed by $A\beta$ addition (Fig. 4). This means that SMS inhibited the MAPK pathway that leads to caspase-3 activation and thus apoptosis through the prevention of JNK phosphorylation during the $A\beta$ -induced damage in PC-12 cells. In contrast, other MAPKs, p38 and ERK, were not phosphorylated by $A\beta$, although there is no evidence whether SMS is involved in this pathway directly or indirectly. Since the selective inhibition of the ERK or p38 MAPK pathways by their specific inhibitors had no effect on the extent of oxidative stress-induced DNA fragmentation or caspase activation in several cell types (30), JNK is more critical in apoptotic pathways than other MAPKs. Hence, it was strongly indicated that SMS preferentially inhibited the JNK-dependent pathway in apoptosis. Akt, phosphatidylinositol 3-kinase/AKT, is well known to prevent the cell death through the inhibition of the apoptosis signal-regulating kinase-1 (ASK1) – JNK pathway (35, 36). Our data suggest that Akt activity might be up-regulated by SMS prior to the $A\beta$ abuse. These results are interesting because SMS treatment influenced not only a specific pathway involved in apoptosis but also the regulation of other molecules such as anti-oxidant enzymes that have modulatory modulation effects on cellular redox status.

SMS significantly promoted the differentiation of PC-12 cells with matured outgrowth compare to the control culture without SMS (Fig. 5). The requirements of MAPK, especially p38 and ERK, for neuronal maturation were reported (9, 21, 37). Figure 4 showed SMS treatment increased the phosphorylation levels of both p38 and ERK, which could lead to the neuronal maturation of PC-12 cells. Differentiated PC12 cells have also been reported to be more resistant towards oxidative stress than proliferating cells (10, 38). Therefore, our present results implicate that the maturation of neurites by SMS facilitates the anti-oxidative potential of the PC-12 cells against $A\beta$ exposure and was due to the intracellular activation of p38 and ERK in these cells. SMS seems to have multiple effects not only on the expression of anti-oxidant enzymes and JNK

inhibition but also affects p38/ERK activations.

In summary, these findings indicate that SMS prevented the $A\beta$ -induced apoptosis of PC-12 cells through the enhancement of anti-oxidant enzymes and the inhibition of JNK signaling in the apoptotic pathway with the promotion of nerve cell maturation.

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