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Salviae Miltiorrhizae BGE *Radix* Increases Rat Striatal K^+-Stimulated Dopamine Release and Activates the Dopamine Release with Protection Against Hydrogen Peroxide-Induced Injury in Rat Pheochromocytoma PC12 Cells

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The present study investigated the effect of the medicinal plant *Salviae miltiorrhizae radix* (SMR) on dopaminergic neurotransmission in comparison with amphetamine. The effect of SM (0.1 g/ml) on K^+ (20 mM)-stimulated dopamine (DA) release from rat striatal slices was compared with amphetamine (10^{-4} M). Amphetamine and SMR significantly increased K^+-stimulated DA release ($P < 0.001$) from rat striatal slices when compared with K^+-stimulated alone. On the other hand, to examine whether *in vitro* SMR treatment induces DA release in PC12 cells, the role of protein kinases has been investigated in the induction of the SMR-mediated events by using inhibitors of protein kinase C (PKC), mitogen activated protein kinase (MAP kinase) or protein kinase A (PKA). PKC inhibitors chelerythrine (50 and 100 nM), Ro31-8220 (100 nM) and the MAP kinase inhibitor, PD98059 (20 μM) inhibited the ability of SMR to elicit the SMR-stimulated DA release. The direct-acting PKC activator, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA, 100 nM) mimicked the ability of SMR to elicit DA release. On the contrary, a selective PKA inhibitor, 50 μM Rp-8-Br-cAMP, blocked the development of SMR-stimulated DA release. The results demonstrated that SMR may stimulate DA release and that SMR-induced increases in MAP kinase and PKC are important for induction of the enhancement in transporter-mediated DA release and PKA was also required for the enhancement in SMR-stimulated DA release. SMR treatment (0.1–10 μg/ml) to the hydrogen peroxide (H_2O_2)-treated PC12 cells activated the enzyme activities such as catalase, superoxide dismutase and glutathione peroxidase, and decreased the malondialdehyde level, indicating that SMR has also protective effects against free radical-induced

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Abbreviations: SMR, *Salviae miltiorrahizae* radix extract; H_2O_2, Hydrogenperoxide; MDA, malondialdehyde; AD, Alzheimer's disease; Aβ, β-amyloide peptide; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; GST, glutathione-S-transferase; AChE, acetylcholinesterse; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; MTT, (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide.

cell toxicity. Therefore, the mechanism by which SMR induces the enhancement in SMR-stimulated DA release is apparent. It remains to be determined whether the effect of SMR on DA function is important in its therapeutic use in the treatment of drug addiction.

KEY WORDS: *Salviae miltiorrhizae radix*; dopamine; amphetamine; PKC, MAP kinase; PKA; free radicals; hydrogen peroxide; glutathione peroxidase; catalase; superoxide dismutase; PC12 cells; malondialdehyde.

INTRODUCTION

Salviae miltiorrhizae radix (SMR) is herbal medicine used as an antidote for several poisonous agents in Korean traditional medicine (1). Recently, it has been reported that extracts of SMR leaves have a protective effect on ethanol-induced hepatotoxicity using hepatic lipid peroxidation, blood ethanol concentration as well as alcohol dehydrogenase and aldehyde dehydrogenase activity as indicators (2). There are no published clinical trials on the use of SMR in the treatment of drug addiction but this plant is used extensively as components of mixtures of crude extracts to treat patients with drug addiction.

The PC12 cell can be a useful single-cell model in which to investigate molecular mechanisms of SMR, although the PC12 cells are not neurons. These cells contain endogenous dopamine (DA) which can be released in response to plasmalemmal norepinephrine transporter (NET) (3). In addition, PC12 cells characteristically differentiate in response to trophic factors such as nerve growth factor (4). Protein kinase-dependent signal transduction is a factor in the action of stimulants (5). Protein kinase C (PKC), a Ca^{2+}/lipid-dependent serine and threonine kinase, plays a pivotal role in cellular neurite outgrowth (6). PKC activation is involved in SMR-induced outward transport of DA through both DA transporter (DAT) (Kim et al., unpublished results). Mitogen activated protein kinase (MAP kinase) is also involved in the behavioral sensitization in animals to psychostimulants and DA transporter function (7). Protein kinase A (PKA) also contributes to psychostimulant sensitization (8). All three of these protein kinases, PKA (9,10), PKC (11) and MAP kinase (4) play a role in neurite outgrowth in PC12 cells.

In the presence of oxygen, free radicals can react with polyunsaturated fatty acids, resulting in highly reactive peroxyl free radicals. Peroxyl free radicals can further propagate the peroxidation of lipids or compromise the integrity of cell membranes, therefore this is thought to be involved toxic actions of some chemicals (12,13). However, living systems are protected from activated oxygen species by enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione-*S*-transferase (GST): they also could be protected by non-enzymatic, antioxidative nutrients, the so-called vitamins (A, C and E), β-carotene and antioxidative materials including methionine and glutathione (14). Recently, much attention has been focused on natural antioxidants, in particular it was reported that SMR may exert an anti-aging and sexual-reinforcing actions in experimental *in vivo* system (15,16).

Our recent preliminary results showed that the SMR treatment concurrently induce an enhancement in DA release in PC12 cells that had characteristics analogous to those found in striatum and nucleus accumbens (17). We found that neurite outgrowth and enhanced SMR-mediated DA release occur in rat pheochromocytoma PC12 cells. The fact that SMR can elicit the neuroadaptation in the PC12 cells demonstrates that the response does not require an intact neuroanatomy. It also suggests that PC12 cell is an appropriate model in which to investigate the mechanism of induction of the neuroadaptation by SMR.

Therefore, this study is the first to investigate the effect of SMR on endogenous DA release from rat striatal slices and compares its effect with those of amphetamine. In addition, we investigated whether PKC, MAP kinase or PKA is involved in the induction of SMR-enhanced DA release in PC12 cells after treatment with SMR. Furthermore, we also determined whether the SMR-stimulated DA release is induced through activation of identical signaling pathways. To investigate the effect of SMR on lipid peroxidation and antioxidative enzyme activities in H_2O_2-treated conditions, we examined PC12 cell viability and oxidative-antioxidant system induced by hydroxyl free radicals by direct application of hydrogen peroxide (H_2O_2).

MATERIALS AND METHODS

Materials. 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and forskolin were purchased from Sigma (St. Louis, MO, USA). Chelerythrine, Ro31-8220, Rp-8-Br-cAMPS, PD98059 and H-89 were purchased from Calbiochem (La Jolla, CA, USA). Male Wistar rats weighing 200–250 g (the National Laboratory's Animal Center, Dongguk University, Kyungju, Korea) were used. They were housed in groups of five in a temperature controlled room with a 12 h light/12 h dark cycle (07:00–19:00 h light on), with access to food and water *ad libitum*. Rats were killed by decapitation, the brains were quickly removed and the striatum was dissected out. The striatum was sliced using a manually operated brain tissue slicer which produces slices (0.35×0.35 mm) suitable for the measurement of endogenous DA release (18). The slices were placed in a flask containing 10 ml nominally Ca^{2+}-free Krebs–Henseleit buffer (KHB) and pre-washed three times with 10 ml Ca^{2+}-free KHB. The flask was filled with 10 ml Ca^{2+}-free KHB, gassed, capped and placed in a shaking water bath for 60 min at 37°C. The Ca^{2+}-free KHB was changed three times (every 20 min) during the pre-incubation. At the end of the pre-incubation, the incubation medium was discarded and the suspension of packed striatal slices was dispensed using a HandyStep (Brand, Germany) onto the columns (50 µl packed slices/column). An initial 3 ml of normal KHB were washed through the slices on the columns by gravity flow leaving the washed striatal slices on the top of the column, and the eluate was discarded. The columns containing the striatal slices were capped and incubated in a water bath (37°C) for 10 min after the addition of 300 µl of normal KHB (basal release). The columns were removed from the water bath, 1 ml of normal KHB was added to the columns, and the total 1300 µl eluate was collected into microtubes containing 130 µl of an antioxidant solution (0.3% sodium metabisulfite and 0.1 M perchloric acid). After collection of the basal sample, a further 300 µl of KHB containing high $[K^+]$ (20 mM) was added (K^+-stimulated release), and columns were incubated at 37°C for an additional 10 min with the eluate collected as described above using 1 ml of normal KHB to wash out the columns. The samples were snap frozen in liquid nitrogen and kept at −70°C until analyzed. The crude extract of SMR (0.1 g/ml) and amphetamine (10^{-4} M), with amphetamine (10^{-4} M) were added to high $[K^+]$ KHB so that the effects on K^+-stimulated release were measured.

SMR Extract Preparation and Content of Sal B in SMR. Dried SMR is obtained from Dongguk University Herbal Garden, Korea. The water extracts of SMR was prepared immediately before use by boiling 10 g of dried leaves in 50 ml ultrapure water for 15 min. The boiled materials were filtered and boiled until the final volume was equal to 1 ml. The high $[K^+]$ KHB (100 ml) was then added to obtain the final concentration of 0.1 g/ml SMR. This concentration was selected on the basis of preliminary data. For the quality control, the major constituent, Salvianolic acid B (Sal B), a water-soluble polyphenolic antioxidant, was isolated from the roots of this plant and quantitatively analyzed during the extraction, as follows: The dry roots of *S. miltiorrhiza* were extracted with a mixture of water and ethanol (4:1, v/v) at room temperature for 24 h. After evaporation of solvent under reduced pressure, the SMR was stored under nitrogen at 4°C before use. The content of Sal B in SMR was determined by reversed-phase HPLC. The column (5C18, 4.6×250 mm) was eluted with 25% aqueous methanol at 1.0 ml/min for 6 min. The volume percentage of methanol was increased to 35% linearly in the next 4 min and maintained isocratically for 5 min. It was further increased to 40% linearly in the next 5 min and kept constant for 20 min. During the entire elution, a low percentage of acetic acid was added to suppress ionization of phenolic acids. Sal B was eluted at a retention time of 24.9 min. Quantification of Sal B was based on peak area at 290 nm (15).

Cell Culture. Clonal rat pheochromocytoma line PC12 cells provide useful model system for the investigation of neuronal injury. PC12 cells were high passages from ATCC (American Type Culture Collection) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. PC12 cells were maintained in a 75-cm^2 tissue culture flask in growth medium composed of Dulbecco's modified Eagle's medium from BioWhittaker (Walkersville, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 5% heat-inactivated horse serum, 2 mm L-glutamine, 100 µg/ml of streptomycin and 100 U/ml of penicillin (Gibco, Rockville, MD, USA) and were incubated at 10% CO_2. Cells (5×10^4 cells/ml) were plated in a 75-cm^2 tissue culture flask at 10% CO_2 in the same growth medium. PC12 cells were treated with 50 µg/ml SMR for 5 min a day for 5 days. After a 10-day drug-free period (withdrawal), cells were analyzed for SMR-stimulated DA release. To block the activity of protein kinases, the inhibitors were added to the media for either 30 min (chelerythrine, Ro31-8220, PD98059 or H-89) or 1 h (Rp-8-Br-cAMPS) before the SMR. To activate PKC or PKA, PC12 cells were treated with 100 nM TPA or 10 µM forskolin for 5 min a day for 5 days in the same manner as with SMR.

DA Release Method. DA release *in vitro* was measured using a semi-superfusion method (19) adapted from the column method previously described to measure synaptosomal release (20). Small plastic columns (Poly-Prep columns, Bio-Rad Laboratories Ltd.) were fixed onto a stand (12 cm height). KHB was freshly prepared on the day of the experiment and contained 123 mM NaCl, 1.3 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 1.185 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM D-glucose, and 2 mM $CaCl_2{\cdot}2H_2O$. Ca^{2+}-free KHB was identical to normal KHB with the $CaCl_2{\cdot}2H_2O$ omitted. For the K^+-stimulation experiment, KHB was prepared containing the same concentration of $MgSO_4{\cdot}7H_2O$, D-glucose and $CaCl_2{\cdot}2H_2O$ as normal KHB but the concentrations of $NaHCO_3$, NaCl, KCl, and KH_2PO_4 were adjusted to give final concentrations of 20 mM $[K^+]$ without changing the osmolarity of the buffer solution. All solutions used in this study were prepared using ultrapure water (Milli-Q, Millipore, France). The solutions were gassed with 95% O_2/5% CO_2 and kept in a water bath (37°C) throughout the experiment.

Measurement. Samples were removed from storage at −70°C, thawed, and filtered through 0.45 µm filters (Gelman Sciences) prior to injection (20 µl) into a high-performance liquid chromatography system with electrochemical detection (HPLC-ECD; ANTEC). The mobile phase was prepared using ultrapure water containing 150 mM $NaH_2PO_4{\cdot}2H_2O$, 0.5 mM diaminoethanetetraacetic acid, 0.5 mM sodium octyl sulfate, and 14% methanol with pH adjusted to 3.8 with 1 M perchloric acid. The mobile phase was pumped through the column (Hypersil ODS 3 µm, Phenomenex) at a flow rate of 0.3 ml/min using a HPLC pump (Waters). The dual glassy carbon electrode was held at a potential of +0.65 V against an Ag/AgCl reference electrode and the current produced by oxidation of DA measured. The DA level in each sample was compared with standard DA (2 pmol/20 µl injection loop, Sigma-Aldrich Co., USA), and the data were presented as DA release

(pmol/μl tissue). The limit of detection for DA was 10 fmol. All data are presented as means ± SEM. The data were analyzed using a one-way analysis of variance test (Newman–Keuls Multiple Comparison test) with $P < 0.05$ considered significant.

Superfusion and DA Release Assays. Cells were harvested by washing the flasks with Kreb's Ringer buffer containing 125 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 24.9 mM $NaHCO_3$ and 0.25 mm ascorbic acid and oxygenated by 95% O_2 and 5% CO_2 for 1 h. DA release was measured in the superfused cells by HPLC with electrochemical detection as described previously (21).

PKC Assay. Cells were washed in serum-free media and resuspended in the same media and incubated at 37°C with or without 50 μg/ml SMR for 5 min. Cold phosphate-buffered saline was added to the samples and the cells were centrifuged at 800×*g* for 3 min. Cells were washed twice with cold phosphate-buffered saline and lysed in an extraction buffer containing 25 mm Tris–HCl (pH 7.4), 0.5 mM EGTA, 0.5 mM EDTA, 10 mM dithiothreitol and protease inhibitor cocktail, by sonication for 60 s in an ice bath. Lysates were centrifuged at 800×*g* to remove cell debris and then centrifuged at 100,000×*g* for 1 h. The supernatant was designated as the cytosolic fraction. The pellet was then extracted with extraction buffer containing 0.25% Triton X-100 at 4°C for 1 h and centrifuged at 100,000×*g* for 30 min. The solubilized material was designated as the membrane fraction. Both the cytosolic and membrane fractions were loaded on 0.5 ml DE-52 columns pre-equilibrated with their respective extraction buffers. Columns were washed with five column volumes of buffer and then eluted with buffer containing 0.2 M NaCl. Eluates were assayed for PKC activity.

PKC activity was assayed using SigmaTECT PKC assay system from Promega (Madison, WI, USA). Briefly, extracts were assayed for PKC activity using a biotinylated substrate peptide, corresponding to the PKC phosphorylation site in neurogranin. The assay (20 μl volume) contained 10 mM $MgCl_2$, 0.4 mM $CaCl_2$, 100 μM substrate, and 100 μM ATP (containing 0.05 μCi of [γ-^{32}P]ATP/assay) in the presence and absence of 0.30 mg/ml phosphatidyl serine and 0.032 mg/ml diacylglycerol. Reactions were started by adding the sample and incubating at 30°C for 5 min. Reactions were terminated by adding 7.5 M guanidine hydrochloride and 10 μl of the reaction was spotted on a special biotin capture membrane. The membrane was washed sequentially four times in 2 ml NaCl followed by four times in 2 ml NaCl in 1% phosphoric acid and dried and counted by liquid scintillation. Enzyme activity is expressed as pmols of ^{32}P transferred to the substrate per min per mg protein.

PKA Assay. PC12 cells were washed with serum-free media and resuspended in the same media. Cells were then incubated at 37°C and treated without or with 30 or 50 μg/ml SMR for 5 min. At the end of the incubation, 0.5 ml of cold phosphate-buffered saline was added to the cells and they were centrifuged at 800 *g* for 3 min. Cells were washed twice with cold phosphate-buffered saline and lysed in 0.2 ml of lysis buffer (10 mm potassium phosphate, 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol containing protease inhibitor cocktail) by sonication for 5 min in an ice bath. The lysate was centrifuged at 10,000× *g* for 15 min in a micro-centrifuge and the supernatant was used for the assay of PKA activity. PKA activity was assayed in a volume of 50 μl in a buffer containing 10 mM HEPES, pH 7.4, 10 mM dithiothreitol, 5 mM sodium fluoride, 10 mM magnesium chloride, 0.5 mM IBMX and 0.2 mM ATP using 30 μM kemptide as substrate in the presence and absence of 30 μM cAMP. The reaction was allowed to proceed for 5 min at 30°C and stopped by spotting 25 μl of the reaction mix on a P81 strip. The strips were washed four times in 75 mM phosphoric acid, dried and counted using a Beckman LS5800 liquid scintillation counter. The cAMP-dependent activity of PKA is defined as pmols of radioactive phosphate transferred to the substrate per min per mg protein.

Treatment of SMR for H_2O_2-Induced Cell Damage. Cells were seeded into multiwell plates (Nunc) at a density of 2×10^4 cells per ml in phenolsulfonphthalein (phenol red) free RPMI 1640 medium (Gibco), supplemented with 10% heat-inactivated bovine calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. All experiments were carried out 24–48 h after cells were seeded. Hydrogen peroxide (H_2O_2, 8.8 μM solution) was stored at 4°C until 100 mM stock solutions were prepared in phosphate-buffered saline (PBS) on the day of application to cultures. The 100 mM H_2O_2 was further diluted for addition to the cultures. SMR was dissolved and diluted with PBS. The PC12 cells were pre-incubated with SMR 2 h before the H_2O_2 was added. Assays for cell viability, lipid peroxidation and antioxidant enzyme activities were performed 6 h after H_2O_2 was added.

Toxicity Assay and Cell Viability. Cell were cultured in polyethylenimine-coated 96 well culture plates at a density of 1×10^4 cells per well for lactate dehydrogenase (LDH) assay. LDH activities in the medium were measured by a Cytotox 96 non-radioactive cytotoxicity assay kit (Promega) according to the manufacturer's instructions. The results were expressed as percentages of peak LDH release on complete cell lysis (control) (22). For all findings, each condition represents five separate wells per experiment and is repeated in two or five independent experiments. Cell survival was evaluated by two different methods: morphological observation with phase-contrast microscope (Nikon) and MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide, Sigma) reduction (23). For assay of antioxidant enzymes and lipid peroxide, the cultures were washed with ice cold PBS and then pooled in 0.1 μM PBS-0.05 mM EDTA buffered solution and homogenized. The homogenate was centrifuged for 1 h at 10,000×*g* at 4°C. The supernatants were used in the assay.

Enzyme Assays. All antioxidative enzyme activities were determined spectrophotometrically using a Giford Response Spectrophotometer. Glutathione peroxidase (GSH-Px) activity was analyzed by Mills's procedure (24). An enzyme unit of activity was defined as a decrease in 1 μM GSH per minute after the decrease in 1 μM/min GSH of non-enzymatic reaction was subtracted. The assay of catalase was based on the consumable rate of H_2O_2 measured at $\lambda = 240$ nm by spectrophotometer (25). The content of malondialdehyde (MDA), a compound that is produced during lipid peroxidation, was determined by using the thiobarbituric acid method (26). SOD activity was measured (Superoxide Dismutase Assay Kit; Calbiochem) in 40 μl of the supernatant and the results were read spectrophotometrically at 525 nm.

Data Analysis. Statistical significance between two groups was determined using a two-tailed Student's *t*-test. Analysis among three or more groups was conducted using one-way analysis of variants (Anova) with post-test Tukey–Kramer multiple comparison analysis. Data were also evaluated for statistical significance with one-way ANOVA followed by Duncan's multiple range test by using a computerized statistical package.

Table I. Effects of Amphetamine and SMR on *In Vitro* Endogenous DA Release from Rat Striatal Slices[a,b]

Group	DA release (pmol/μl)	
	Basal	20 mM [K^+]
K^+ (20 mM)	0.98 ± 0.12	3.12 ± 0.29
Amphetamine (10^{-4} M) + K^+ (20 mM)	1.01 ± 0.14	4.72 ± 0.54[c]
SMR (0.1g/ml) + K^+ (20 mM)	1.01 ± 0.12	5.46 ± 0.72[c,d]
SMR (0.1g/ml) + Amphetamine (10^{-4} M) + K^+ (20 mM)	0.91 ± 0.10	4.35 ± 0.51[c]

[a]Slices are incubated for 10 min in normal K^+ (1.3 mM; basal release) and then in the presence of high K^+ (20 mM) with or without drugs.
[b]Data are presented as means ± SEM.
[c]$P < 0.001$ compared with K^+ (20 mM) alone.
[d]$P < 0.001$ compared with amphetamine in K^+ (20 mM), $n = 10$ in all groups.

RESULTS

Potential Effects of Amphetamine and SMR on K^+-Stimulated DA Release

High K^+ (20 mM) induced a significant increase in endogenous DA release from 0.98 ± 0.12 to 3.12 ± 0.29 pmol/μl (about 150%) when compared with basal release (Table I). The concentration of 20 mM [K^+] was used in all subsequent studies as SMR is concentration has been shown to produce a submaximal increase in DA release using the present methodology (27). Amphetamine (10^{-4} M) significantly ($P < 0.001$) increased K^+-stimulated DA release from 1.01 ± 0.14 to 4.72 ± 0.54 pmol/l (data not shown), while SMR (0.1 g/ml) produced a significant ($P < 0.001$) increase of the K^+-stimulated endogenous DA release from 1.01 ± 0.12 to 5.46 ± 0.72 pmol/l when compared with K^+-stimulated release alone. The percentage change of endogenous DA release from control is shown in Fig. 1. While SMR (0.1 g/ml) + Amphetamine (10^{-4} M) produced a significant ($P < 0.001$) increase of the K^+-stimulated endogenous DA release from 0.91 ± 0.10 to 4.35 ± 0.51 pmol/l when compared with K^+-stimulated release alone.

SMR-Stimulated DA Release Depending on PKC in PC12 Cells

To investigate the role of PKC in SMR-induced DA release, PC12 cells were pre-treated with a PKC inhibitor, either chelerythrine or Ro31-8220, for 30 min before each daily SMR treatment. These drugs are structurally dissimilar and have been shown to block SMR-stimulated DA release in PC12 cells. SMR treatment of the PC12 cells results in an enhancement of DA release to a subsequent challenge of SMR in the perfusion. To investigate whether the induction of the enhanced DA release is also dependent on PKC activity, the PC12 cells were pre-treated with chelerythrine or Ro31-8220 before the daily SMR treatment as described above. After the 10 drug-free days, SMR-mediated DA release was measured. Pre-treatment of the PC12 cells with the PKC inhibitors chelerythrine and Ro31-8220 before each SMR treatment effectively inhibited the induction of enhanced DA release to a challenge dose of SMR (Fig. 2a, b). The drugs had no effect on basal DA release. To confirm that an activation of PKC by SMR results in the enhancement in transporter-mediated DA release, the PC12 cells were treated with 100 nm TPA instead of 50 μg/ml SMR for each of 5 days and given 10 drug-free days. As shown in Fig. 2c, the repeated TPA treatment mimicked the ability of SMR to induce the enhancement in SMR-stimulated DA release.

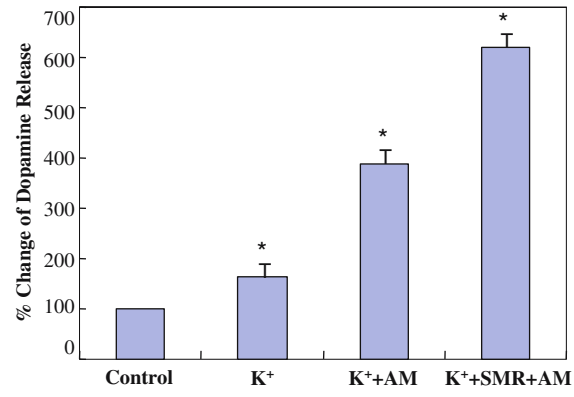

Fig. 1. Percentage change of endogenous DA release from control. K^+, 20 mM [K^+]; K^+ + Am, amphetamine (10^{-4} M) in 20 mM [K^+]; K^+ + (SMR), (SMR) (0.1 g/ml) in 20 mM [K^+]; and K^+ + (SMR) + Am, (SMR) (0.1 g/ml) and amphetamine (10^{-4} M) in 20 mM [K^+]. Data are presented as means ± SEM. *$P < 0.001$ compared with K^+ (20 mM) alone.

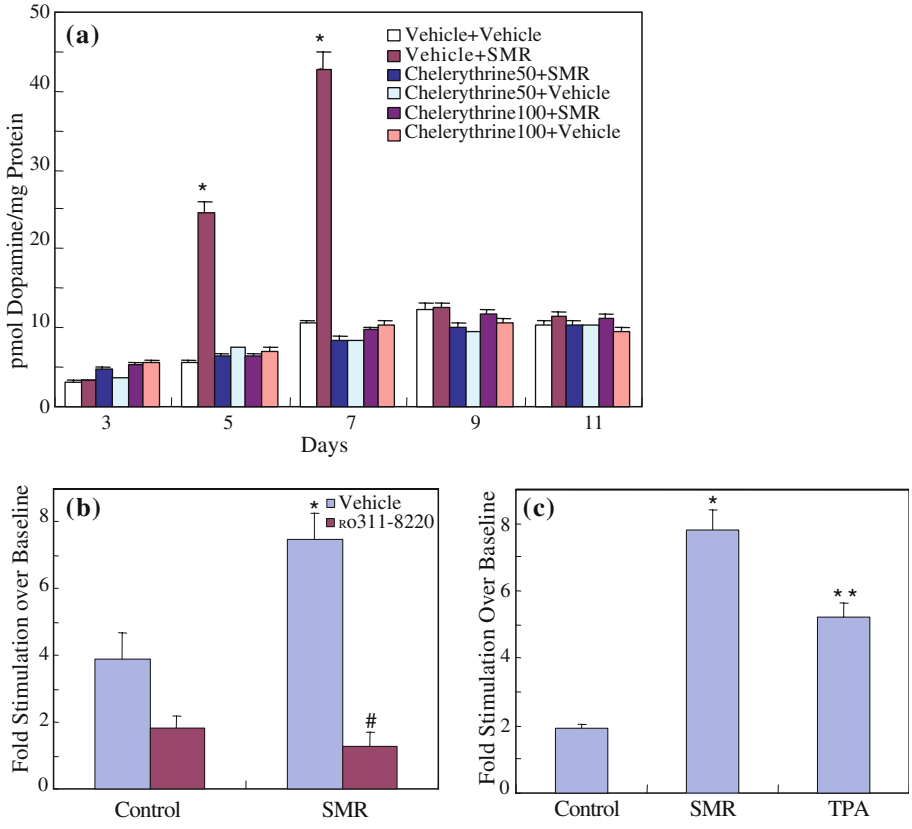

Fig. 2. Effect of PKC inhibitors and TPA on the induction of SMR-stimulated DA release. (a) PC12 cells were treated for 5 min a day for 5 days with 50 μg/ml SMR or vehicle preceded by a 30-min pre-treatment with 50 nM or 100 nM chelerythrine (CH). The legend indicates the pre-treatment + the inducing treatment (vehicle or SMR, 30 min later). After 10 drug-free days, PC12 cells were harvested and perfused as described in Methods and Materials. All samples received a bolus of 50 μg/ml SMR for 2.5 min at fraction 4. Due to the length of tubing and rate of perfusion, the DA elutes at fraction 7. Results are given in pmol DA/mg protein ± SEM. $n = 5$. For fraction 7, Anova $P < 0.001$. In post hoc Tukey analysis $^{*}P < 0.05$, $^{**}P < 0.01$ vs. A + CH50 and A + CH100 (SMR and chelerythrine 50 or 100 nM treatment). (b) PC12 cells were treated with vehicle or 100 nM Ro31-8220 followed 30 min later by 5 min of 50 μg/ml SMR (SMR, *x*-axis) or vehicle (control, *x*-axis) for 5 days (pre-treatments are given in the legend in the Figure). Following 10 drug-free days, SMR-mediated DA release was measured in the perfusion as described in part (a). Results are expressed as the fold stimulation of the DA in fraction #7 over baseline. $^{*}P < 0.05$ compared to control-vehicle, $^{\#}P < 0.05$ compared to SMR-vehicle, by two-tailed Student's *t*-test. (c) PC12 cells were treated for 5 min a day for 5 days with vehicle (control), 50 μg/ml SMR or 100 nm TPA, given 10 drug-free days, harvested and perfused. Results are given in fold-stimulation of the DA in fraction #7 over baseline. SMR and TPA significantly differed from vehicle at $^{*}P < 0.05$ or $^{**}P < 0.001$, respectively, by two-tailed Student's *t*-test.

The MEK Inhibitor, PD98059 Blocks SMR-Stimulated DA Release in PC12 Cells

We also investigated whether MAP kinase activation is similarly necessary for the induction of enhanced SMR-stimulated DA release after SMR treatment. To investigate whether MAP kinase activity is required for the induction of SMR-induced DA release, PC12 cells were treated with 50 μg/ml SMR for 5 min a day for 5 days in the absence or presence of 20 μM of the MEK inhibitor, PD98059. After 10 drug-free days, DA release was measured. After 10 drug-free days, PC12 cells were challenged with SMR and DA release was measured by HPLC. Pre-treatment with the MEK inhibitor, PD98059, inhibited the induction of SMR-stimulated enhanced DA release demonstrating that the neuroadaptation require the activation of MAP kinase (Fig. 3).

The PKA Inhibitor Rp-8-Br-cAMP Inhibits SMR-Stimulated DA Release in PC12 Cells

To determine whether PKA contributes to SMR-induced DA release, the PKA inhibitors H-89 and Rp-8-Br-cAMP were used. Pre-treatment with 100 nM H-89, given 30 min before each repeated SMR treatment, blocked the induction of DA

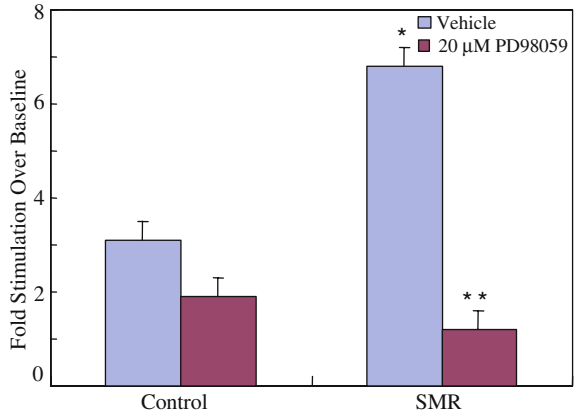

Fig. 3. Effect of the MEK inhibitor, PD98059 (30 μM), on the ability of SMR-stimulated DA release. PC12 cells were treated for 5 min a day for 5 days with 50 μg/ml SMR (*x*-axis) or vehicle (control, *x*-axis) following a 30-min pre-treatment with 20 μM PD98059 or vehicle (legend in Figure). After 10 days of withdrawal, DA release in response to 50 μg/ml SMR were measured as described in Material and methods. Results are given in fold-stimulation of DA in fraction #7 over baseline. Values represent means ± SEM. $n = 5$. Anova, $P < 0.0001$. In post hoc Tukey–Kramer analysis, $^{*}P < 0.001$ for vehicle-control vs. vehicle-SM; $^{**}P < 0.001$ for PD98059-SM vs. vehicle-SMR.

release in response to SMR treatment (Fig. 4). To further examine the mechanism of induction of DA release, the role of PKA in enhanced SMR-stimulated DA release was investigated. Either Rp-8-Br-cAMP or H-89 was added to the media before each

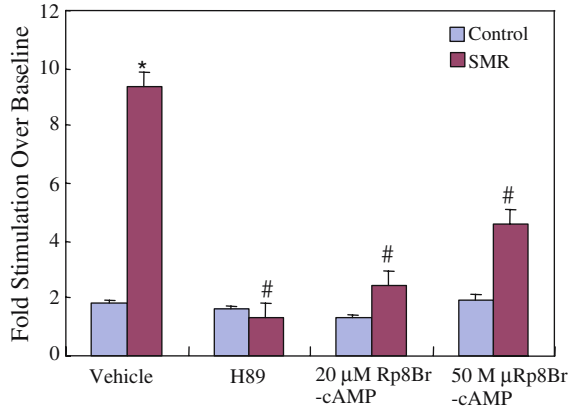

Fig. 4. Effect of PKA on SMR-induced DA release in PC12 cells. Effect of H-89 and Rp-8-Br-cAMP on the induction of enhanced SMR-stimulated DA release. PC12 cells were treated for 5 min a day for 5 days with 50 μg/ml SMR (*x*-axis) or vehicle (control, *x*-axis) with or without pre-treatment with vehicle, 20 μM or 50 μM Rp-8-Br-cAMP (1 h) or 100 nM H-89 (30 min). After 10 drug-free days, PC12 cells were harvested and perfused and DA release was measured as described in Methods and materials. Results are given in fold-stimulation of DA in fraction #7 over baseline ± SEM. $n = 5$. Anova, $P < 0.0001$. In post hoc Tukey–Kramer analysis, $^{*}P < 0.001$ vs. vehicle-control, $^{\#}P < 0.001$ vs. vehicle-SMR.

of the five SMR treatments. After 10 drug-free days, DA release in response to a challenge of 50 μg/ml SMR was measured in perfused cells. Both Rp-8-Br-cAMP and H-89 pre-treatment significantly blocked the ability of repeated SMR to induce an enhancement in SMR-stimulated DA release as compared to vehicle (Fig. 4). The drugs had no effect on basal DA release. To determine whether direct activation of PKA can mimic SMR in the induction of enhanced DA release, PC12 cells were treated with 100 μm forskolin for 5 min a day for 5 days. After 10 drug-free days, SMR-stimulated DA release was measured. In contrast to direct PKC activation, forskolin did not mimic the ability of SMR to elicit enhanced SMR-stimulated DA release (fold stimulation by 50 μg/ml SMR after repeated forskolin = 1.84 ± 0.12, $n = 6$, not different from vehicle).

Microscopic Observation and Effect on H_2O_2-Induced Cell Cytotoxicity in Cultured Cells

As shown in Fig. 5, there was a significant decrease in cell number and most cells lost neurites and demonstrated round shape and some of which were lysed or replaced by debris following 6 h exposure of the cells to 200 μM H_2O_2. In contrast, cultures exposure to the same amount of H_2O_2 in the presence of SMR appeared remarkably preserved, indicating SMR has protective effects against H_2O_2 insult. The toxicities of H_2O_2 (100 μM) were assessed by LDH assay. The H_2O_2 increased LDH release by 67.4 ± 4.6% of the maximal value at 100 μM concentration (Table II). For effects of SMR treatment, cells were treated with H_2O_2 for 2 h, thereafter added SMR at the indicated concentration, LDH activities in the conditioned medium of cultured cells were assayed at 48 h after treatment with SMR. LDH releases were severely decreased from the cells, indicating that SMR treatment reduced the cell injury and protected the cells against H_2O_2-induced cytotoxicity (Table II).

Effects of SMR on Superoxide-Scavening Enzymes and SOD Activity

Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly to the living cell number. Cell viability as determined by MTT reduction was markedly decreased after PC12 cultures were exposed to 200 μM

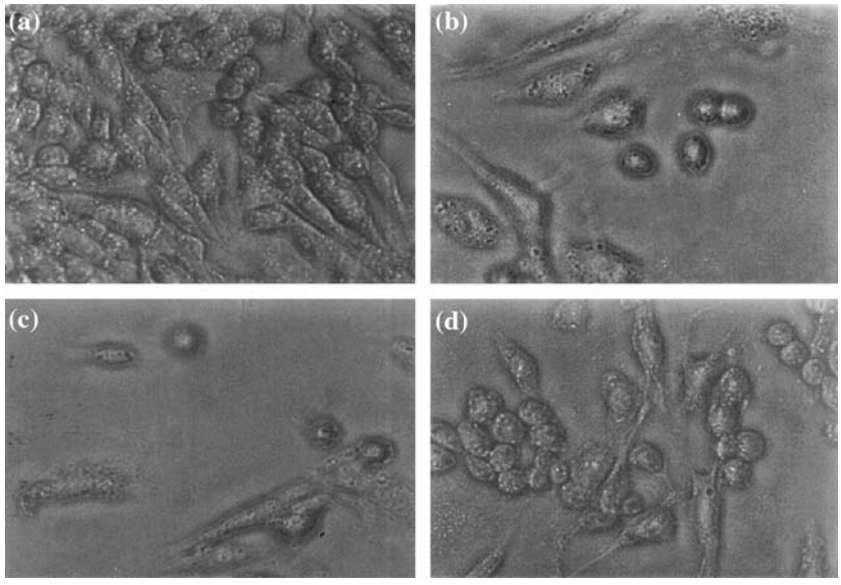

Fig. 5. Effects of SMR on PC12 cell injury induced by H_2O_2. (a) PC12 control cells. (c) PC12 cells exposed to 200 μM H_2O_2 for 6 h. There is a significant decrease in cell number and most of the cells lose neurites and demonstrate round shape. (b,d) PC12 cells were pre-incubated with 0.1 and 10 μg/ml SMR, respectively and exposed to 200 μM H_2O_2 for 6 h. Scale bar, 10 μm.

Table II. LDH Activity in Cultured Cells after Treatment with H_2O_2 and SMR

	LDH (% of maximal release)	
Concentration (μM)	H_2O_2	H_2O_2 + SMR
Control	2.7 ± 0.2	2.8 ± 0.3
100	67.4 ± 4.6	22.4 ± 1.7**

The results are expressed as percentage of maximal LDH release that was obtained on complete cell lysis. For effects of SMR treatment, cells were treated with H_2O_2 (100 μM) for 2 h and further treated with 10 μg/ml of SMR extract. LDH activities in the culture medium of cultured cells were assayed at 48 h after treatment with indicated concentrations of SMR. Data are expressed as mean ± SD values obtained from five culture wells per experiment, determined in three to five independent experiments.
**Significantly different, $P < 0.001$.

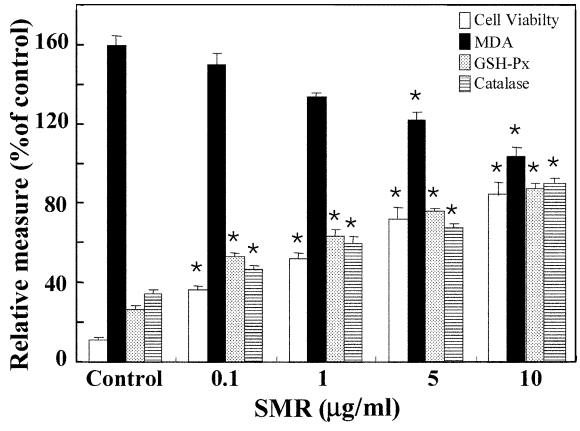

Fig. 6. Effects of SMR on cell viability, MDA level, GSH-Px and catalase activities in PC12 cells. Cells were incubated with 200 μM H_2O_2 for 6 h. SMR was added to the culture 2 h prior to H_2O_2 addition. All data were expressed as percent of control value. Statistical comparison was made using ANOVA followed by Dunnett's test. At least two independent experiments were carried out in triplicates. *$P < 0.05$ vs. H_2O_2 group (control).

H_2O_2 for 6 h (Fig. 6), suggesting that PC12 cells were very sensitive to H_2O_2 induced cell injury. Both GSH-Px and catalase are enzymes that hydrolyse H_2O_2. In our experiments, GSH-Px and catalase activities in PC12 cells were 9.6 ± 1.7 and 19.4 ± 3.1 (units/mg protein), respectively. Exogenous H_2O_2 (200 μM) reduced the activities of GSH-Px and catalase by 68.6% and 62.2%, respectively (Fig. 6). Pre-treatment with SMR at concentrations higher than 0.1 μg/ml significantly attenuated the decrease in GSH-Px and catalase activities in H_2O_2-treated PC12 cells ($P < 0.05$ vs. H_2O_2 group). The toxicity caused by H_2O_2 accompanied by increased lipid peroxides. As shown in Fig. 6, intracellular MDA, a product of lipid peroxidation, was raised by 67% 6 h after the cultures exposed to 200 μM H_2O_2 ($P < 0.01$ vs. control). However, increase in MDA level induced by 200 μM H_2O_2 was markedly attenuated when the cells were pre-treated with SMR (0.1–10.0 μg/ml). To assess the defense mechanisms involved in the 200 μM H_2O_2-induced oxidative stress, the activity of SOD was determined. The data showed that 200 μM H_2O_2 potently decreased SOD ($P < 0.05$) compared

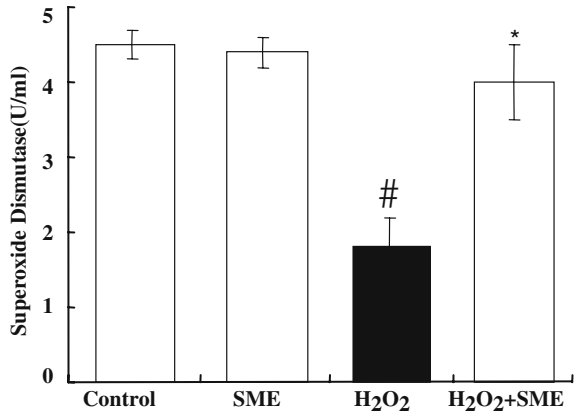

Fig. 7. Effects of CLE on activity of SOD in 200 μM H_2O_2-treated mice. Cells were received saline (control) and cells were incubated with 200 μM H_2O_2 for 6 h (H_2O_2). SMR was added to the culture 2 h prior to H_2O_2 addition (H_2O_2 + SMR). All data were expressed as percent of control value. Statistical comparison was made using ANOVA followed by Dunnett's test. At least two independent experiments were carried out in triplicates. $^*P < 0.05$ vs. H_2O_2 group. $^{\#}P < 0.05$ vs. (control).

with the Saline groups. However, SMR significantly reversed the alterations of SOD induced by 200 μM H_2O_2 (all $P < 0.05$) (Fig. 7).

DISCUSSION

The results show that SMR is a potent stimulator of K^+-stimulated endogenous DA release from striatal slices, in this experimental protocol. It remains to be determined whether the increase in DA release by SMR is significant in the therapeutic use of the extracts in patients with drug addiction. Psychostimulants such as cocaine and amphetamine activate the mesolimbic DA system (28). DA release in the nucleus accumbens has been implicated in the rewarding effects of stimulant drugs. In this study, we used striatal instead of accumbens slices due to the size limitation of the latter region for *in vitro* release studies. Further experiments are required to show whether the effect on DA release produced by SMR occurs in vivo, is dose related and to compare the effects on mesolimbic and striatal DA systems.

SMR is a herbal medicine used for a variety of symptoms related to complications arising from cerebrovascular diseases (1). Recent investigations have revealed an etiologic relation between oxidative damage and above pathologic conditions and suggested that SMR act on active oxygen species (29–40). The aqueous extract of *S. miltiorrhiza* contains phenolic compounds that are effective in protecting liver microsomes, hepatocytes, and erythrocytes against oxidative damage (37,38). It was also reported that SMR extracted from the Chinese herb, *S. miltiorrhiza* can improve cognitive impairment in a broad range of animal models of memory deficiency (39) and in AD patients (40). In the previous study (41), therefore, we evaluated the effect of this drug on superoxide production by microglias. Then, we at first investigated the effect of SMR extracts on endogenous dopamine release from rat striatal slices and compares its effect with those of amphetamine. The flavonoids found in SMR were reported to act as antioxidants by scavenging reactive oxygen species (ROS) and/or chelating metal iron which is responsible for the generation of ROS (42). There are no reports concerning a relationship between these compounds and the mechanisms involved in drug addiction. Further studies need to be conducted to identify the active compounds in the extract that may be involved in the reported effects of SMR. Such studies may lead to the identification of novel compounds useful in the treatment of addiction.

Some drugs of addiction, such as cocaine, exert their effect by inhibiting DA uptake. Phentermine is reported to reduce craving for alcohol and cocaine and to prevent relapse by reducing cocaine-induced increases in extracellular DA using the *in vivo* microdialysis method (43,44). It remains to be determined whether the increase in K^+-stimulated release of DA by SMR is due to an effect on the DA uptake system. The DA D3 receptor has been proposed as a possible target in the treatment of drug addiction (45). Activation of D3 receptors reduces DA release and causes hypoactivity (46). It is possible that the plants used in the present study may exert their reported effects on disrupting drug-seeking behavior by antagonism of DA D3 receptors in a manner similar to 1-(4-(2-Naphthoylamino)butyl)-4-(2-methoxyphenyl)-1A-piperazine HCl (BP 897) which has recently been reported to have potential in the treatment of addiction and withdrawal (46).

We have also investigated the role of protein kinases in the mechanism of induction of the known neuroadaptations following SMR treatment: enhanced SMR-stimulated DA release. Our study is the first to demonstrate that induction of the neuroadaptation results from divergent downstream signaling pathways. PKC is clearly important in the induction of the neuroadaptation. This conclusion is based on the fact that two structurally dissimilar PKC inhibitors, chelerythrine and Ro31-8220, blocked the induction of the neuroadaptation and that direct

activation of PKC by TPA induced the neuroadaptation. The demonstration of activation of PKC in response to SMR further confirms that SMR elicits a rapid activation of PKC that initiates induction of the event. MAP kinase plays a critical role in the induction of the enhancement in SMR-mediated DA release. This was confirmed by the fact that the MEK inhibitor, PD98059, blocked the induction of the neuroadaptations. This conclusion is further supported by the fact that a short treatment of the cells with SMR did not activate PKA and that direct activation of PKA did not mimic SMR-induced DA release.

A role for PKC in the induction of the neuroadaptation in response to SMR treatment might be expected based on the following results: Inhibitors of PKC block SMR-stimulated DA release whereas PKC activators increase DA release through the plasmalemmal transporter in PC12 cells. Similarly, PKC inhibitors block the expression of the enhanced SMR-stimulated DA release in following SMR treatment. MEK inhibitor PD98059 blocked the ability of SMR to induce SMR-mediated DA release. PD98059 did not, however, block acute SMR-stimulated DA release (data not shown), indicating that SMR can activate PKC followed by MAP kinase activation. In contrast to the ability of PKC and MAP kinase inhibitors to block the neuroadaptation, PKA inhibition blocked the induction of SMR-stimulated DA release in PC12 cells. This indicates the mechanism by which SMR induces DA release. The role of PKA in the induction of SMR-stimulated DA release is confirmed by the use of two different PKA inhibitors: H-89, a competitive inhibitor at the ATP substrate site, and Rp-8-Br-cAMPS, which binds to the regulatory site of PKA and blocks enzyme activation. Rp-8-Br-cAMPS blocked the induction of the SMR-stimulated DA release, even at the lower dose of 30 μg/ml, demonstrating that it was active at both the 30 μg/ml and 50 μg/ml concentrations. cAMP and PKA activation appear to have some role in the induction of behavioral sensitization to SMR but manipulation of this system neither totally mimic nor block the full range of responses obtained with SMR. Our results in the PC 12 cell showing that PKA activation is necessary in the induction of neuroadaptation resulting from SMR.

Oxygen free radicals, formed as by-products of respiration and oxidative metabolism, are of particular interest. The generation of free radical molecules can lead to damage or destruction of a variety of tissues. Consequences of excessive reactive oxygen species are lipid peroxidation, oxidation of proteins and damage of DNA (47). The major reactive oxidants in cells are O_2^- (superoxide) and the more detrimental hydroxyl radical, the latter being derived from H_2O_2. However, cells are often equipped with several antioxidants. GSH-Px, catalase and SOD, along with other non-enzymatic antioxidants, such as α-tocopherol, ascorbate, GSH and cysteine, serve as detoxifying system to prevent damage caused by reactive oxygen species and among these, antioxidant enzymes play a pivotal role. SOD maintains a very low steady-state intracellular O_2^-. The H_2O_2 thus formed is removed by catalase, which is active only against H_2O_2 and by GSH-Px, which can also act on lipid hydroperoxides (48). The combined action of these two enzymes provides a repair mechanism for oxidized membrane components. The previous studies showed a rapid decline in cell viability after treated with 0.5 mM H_2O_2 in PC12 cells (49). The results of present study also demonstrated that SMR has significant protective effects against H_2O_2-induced injury on PC12 cells. It also prevented the decrease in GSH-Px, SOD and catalase activities and inhibited overproduction of MDA caused by exogenous H_2O_2. When PC12 cells were pre-incubated with SMR, an elevation in activities of catalase and GSH-Px as well as the enhanced cell survival were observed, suggesting that cytoprotective effects of SMR involves in a stimulation of antioxidant enzymes. The stimulation of antioxidant enzymes seems to attribute more to the anti-peroxidation effect of SMR according to our studies in direct free radical generative system.

In summary, induction of DA release after SMR treatment is dependent on PKC and MAP kinase activation in PC12 cells. The cAMP-dependent signaling pathway also contributes to SMR-induced enhanced DA release in PC12 cells, suggesting that DA release has different mechanism of induction by SMR. Understanding the regulation of the DA release in PC12 cells after treatment with SMR will give greater insights into the neurite. In addition, the results indicated that SMR has protective effects against free radical-induced cell toxicity and SMR could be a useful neuroprotective agent that mitigates the oxidative stress.

ACKNOWLEDGMENT

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