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Involvement of reactive oxygen species and stress-activated MAPKs in satratoxin H-induced apoptosis

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Abstract

Satratoxins, members of the trichothecene mycotoxin family, have been known to be harmful to health. However, the mechanisms underlying the toxicity still remain unclear. The present study is undertaken to elucidate the mechanisms of the satratoxin H-induced cytotoxicity in PC12 cells. Satratoxin H caused cytotoxicity, which was reflected from apoptosis determined by chromatin staining and flow cytometry. Satratoxin H stimulated the phosphorylation of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK). Pre-incubation with SB203580, a p38 MAPK inhibitor, or SP600125, a JNK inhibitor, but not PD98059, an ERK inhibitor, reduced satratoxin-induced cytotoxicity. Co-incubation of cells with glutathione, *N*-acetyl-L-cysteine or glutathione reductase inhibited cytotoxicity and the phosphorylation of p38 MAPK induced by satratoxin H. Our data suggest that satratoxin H-induced apoptosis in PC12 cells is dependent on the activation of p38 MAPK/JNK and the increase in reactive oxygen species.

Keywords: Satratoxin H; Reactive oxygen species; Apoptosis; Glutathione; *N*-Acetyl-L-cysteine; Mitogen-activated protein kinase

1. Introduction

The fungus *Stachybotrys* species and their associated trichothecene mycotoxins have been recognized as one of potential etiologic agents in outbreaks of sick building syndromes which clinical signs include nasal drainage and congestion, allergies, watering eyes, airway infection, headaches, cough, fatigue, and dizziness (Cooley et al., 1998; Mahmoudi and Gershwin, 2000). Purified macrocyclic trichothecenes, especially satratoxin H, potently inhibit protein synthesis and thymocyte proliferation (Ehrlich and Daigle, 1987; Sorenson et al., 1987). Satratoxin H also causes diseases such as an immune dysfunction (Johanning et al., 1996) and idiopathic pulmonary hemorrhage in infants (Mahmoudi and Gershwin, 2000). As well as the potent toxicity on the immune system, recent reports suggest that a possible relationship between trichothecenes and disorders of central nervous system including severe neuronal death (Croft et al., 2002; Johanning et al., 1999). Despite of the possible harmful-

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ness, the mechanisms of action of trichothecene mycotoxins on central nervous system still remain unclear.

In myeloid cells, trichothecenes including satratoxin H have been shown to induce apoptosis and activate mitogen-activated protein kinases (MAPKs) (Shifrin and Anderson, 1999; Yang et al., 2000). The MAPK family includes c-Jun N-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK), which has fundamental roles in both the maintenance of cell survival and the induction of cell death. However, the role of MAPKs in apoptosis has not been established. While JNK and/or p38 MAPK mediate cellular response to stress and induce apoptosis in some experimental conditions (Assefa et al., 2000; De Zutter and Davis, 2001; Sarker et al., 2003), the study in HeLa cells showed that JNK1 and p38 MAPK protected cells from apoptosis induced by hypericin (Assefa et al., 1999). Although ERK is mainly activated by growth factors and known to be associated with cell proliferation and differentiation, its activation is also necessary for Zn^{2+}-induced apoptosis in differentiated PC12 cells (Seo et al., 2001).

Interestingly, MAPKs participate in cell death induced by reactive oxygen species such as superoxide anion, hydrogen peroxides and hydroxyl radicals (Lee et al., 2002). The oxidative stress induced by 3-nitropropionic acid increases the activity of JNK and p38 MAPK in liver extracts from young mice (Hsieh et al., 2003). The tumor necrosis factor-related apoptosis-inducing ligand has also been reported to induce reactive oxygen species accumulation, p38 MAPK activation and apoptosis, and the pretreatment of antioxidants such as glutathione or estrogen attenuated these responses in HeLa cells (Lee et al., 2002). *N*-Acetyl-L-cysteine, a precursor of glutathione, have been reported to inhibit the phosphorylation of p38 MAPK and apoptosis induced by dopamine in neuroblastoma cells (Junn and Mouradian, 2001). The generation of reactive oxygen species appears to be upstream of p38 MAPK and/or JNK activation in these conditions. The production of reactive oxygen species exceeding the capacity of internal antioxidants leads to DNA damages, cellular injury and cell death. Oral administration of trichothecene mycotoxins caused lipid peroxidation in liver, which was prevented by feeding supplement of antioxidants such as vitamin C or E (Rizzo et al., 1994). The another trichothecene mycotoxin 4-acetyl-12,13-epoxyl-9-trichothecene-3,15-diol induced the formation of intracellular reactive oxygen species and the depletion of glutathione, which were blocked by *N*-acetyl-L-cysteine (Pae et al., 2003). Thus, it is possible that the reactive oxygen species and MAPKs may interact with trichothecene-induced dysfunction of neural cells.

In the present study, we investigated the cytotoxicity and signaling transduction mechanism of satratoxin H in undifferentiated PC12 cells, a cell line of rat pheochromocytoma as a model of neural cells.

2. Materials and methods

2.1. Materials

Satratoxin H was extracted from a fungus *Podostroma cornu-damae* and purified by chromatography. Dulbecco's modified Eagle's medium was purchased from Nissui Pharmaceutical, Japan. Fetal bovine serum, horse serum, chromatin dye bisbenzimide (Hoechst 33342), reduced form of glutathione, *N*-acetyl-L-cysteine, glutathione reductase, poly-L-lysine, and 2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD98059) were obtained from Sigma-Aldrich (St. Louis, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was from Research Organics (Cleveland, OH, USA). Anthra(1,9-*cd*)pyrazol-6(2*H*)-one (SP600125) was from Biomol Research Laboratories (Plymouth Meeting, PA) and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580) were from Wako (Osaka, Japan). Satratoxin H and all inhibitors were dissolved in dimethylsulfoxide, and were subsequently diluted with culture medium. After appropriate dilutions, the final concentration of dimethylsulfoxide in culture medium was kept less than 0.5%, which has no effect on the cell viability. Antibodies specific for JNK, phospho-JNK, p38 MAPK, phospho-p38 MAPK, ERK, phospho-ERK and secondary anti-rabbit Ig G (H&L, horseradish peroxidase-linked) were from Cell Signaling Technology (MA, USA). The enhanced chemiluminescence Western blotting detection reagent was purchased from Amersham Biosciences, USA. All other chemicals used were commercially available reagents or analytical reagent quality.

2.2. Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5% horse serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were passaged for every 2–3 days.

2.3. Cell viability measurement

PC12 cells were seeded at a density of 5×10^4 cells/ml in 96-well plates. Cell viability was assessed by a dye staining method using MTT reduction assay (Hansen et al., 1989). Briefly, the cells were incubated with MTT solution at a final concentration of 1 mg/ml in a 96-well plate for 4 h at 37 °C. Viable cells with active mitochondria cause cleavage of the tetrazolium ring into a visible dark blue product through formazan reaction, whereas dead cells remained uncolored. Subsequently, the reaction was stopped by adding 100 μl of cell lysis buffer containing 50% (v/v) *N*,*N*-dimethyl formamide and 20% sodium dodecyl sulfate (pH 4.7). The terminated reaction mixture was maintained overnight at 37 °C and the optical density at an absorbance

of 595 nm was quantified using a micro-plate reader (Sunrise Classic, Tecan, Austria).

Before the experiments using enzyme inhibitors or antioxidants, cells were incubated in serum-free medium for 24 h. The cells were treated with each inhibitor for 1 h, then further incubated with 50 nM of satratoxin H for 48 h. The antioxidant, glutathione, *N*-acetyl-L-cysteine or glutathione reductase, was added immediately before the application of satratoxin H. The cells were washed out with serum-free medium before adding MTT solution because these antioxidants themselves induce the formazan reaction when used in higher concentrations.

2.4. Flow cytometric analysis of apoptotic cells

After the incubation in serum-free medium for 24 h, PC12 cells were treated with satratoxin H at a concentration of 50 nM. The cells were detached and washed with phosphate-buffered saline, then re-suspended in 1 ml of cold 70% ethanol and stored at 4 °C for 24 h. After washing with phosphate-buffered saline, the cells were stained with 100 μg/ml of propidium iodide in phosphate-buffered saline at 4 °C for 30 min in dark condition. The cells were washed and subjected to flow cytometric analysis of DNA content by determining 600-nm fluorescence at an excitation wavelength at 488 nm. Over 10,000 cells per sample was analyzed. The number of cells with subdiploid content of DNA, representing apoptotic cells with partly fragmented DNA, was expressed as percentage of total cells.

2.5. Hoechst 33342 assay

Hoechst 33342 assay was performed as described previously (McKeague et al., 2003). In brief, after the incubation in serum-free medium for 18 h, PC12 cells were exposed to satratoxin H at a concentration of 50 nM for 24 h. They were stained with 1 μg/ml of Hoechst 33342 for 15 min in the dark at 37 °C. The morphological change in nuclear was visualized with a fluorescence microscope (model IX70, Olympus, Tokyo, Japan). The cells with brightly stained condensed chromatin, nuclear fragmentation or apoptotic bodies were considered as apoptotic cells.

2.6. Western blotting analysis

PC12 cells were seeded in 6-well plates and the medium was changed to serum-free medium for 18 h at confluence. At indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline and solubilized in 90 μl of cold lysis buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1% NP-40, 0.02% NaN_3 with protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin) to prevent proteolysis and/or dephosphorylation (Datta and Long, 2002). Lysates were briefly sonicated to reduce viscosity. Protein concentrations in the samples were determined by Bradford method. Whole cell extracts were added by half volume of 3× Laemmli loading buffer (225 mM Tris–HCl, pH 6.8, 6% sodium dodecyl sulfate, 30% glycerol, 9% 2-mercaptoethanol and 0.009% bromophenol blue), boiled for 5 min to solubilize protein, and stored at −20 °C until use. Equal amounts of protein (50–75 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide and then transferred onto polyvinylidene difluoride membranes using a semi-dry blotting system. The membranes were blocked with 5% skimmed milk in Tris-buffered saline solution (10 mM Tris, 0.1 M NaCl and 0.01% Tween 20) for 2 h at room temperature and then incubated with a primary

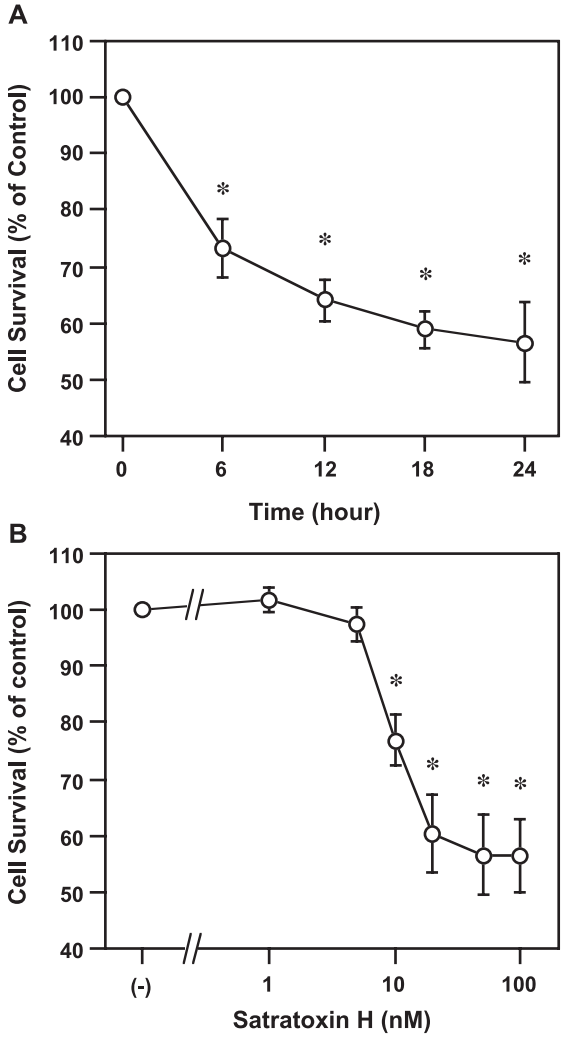

Fig. 1. Satratoxin H-induced cytotoxicity of PC12 cells. (A) Time course of satratoxin H (50 nM)-induced cytotoxicity. (B) Concentration-dependency of satratoxin H-induced cytotoxicity in the cells incubated for 24 h. Data were expressed as % of control cell survival measured by MTT assay. Each point represents the mean±S.E.M. of four different experiments, each performed in triplicate. *P<0.05 compare to the cells without satratoxin H.

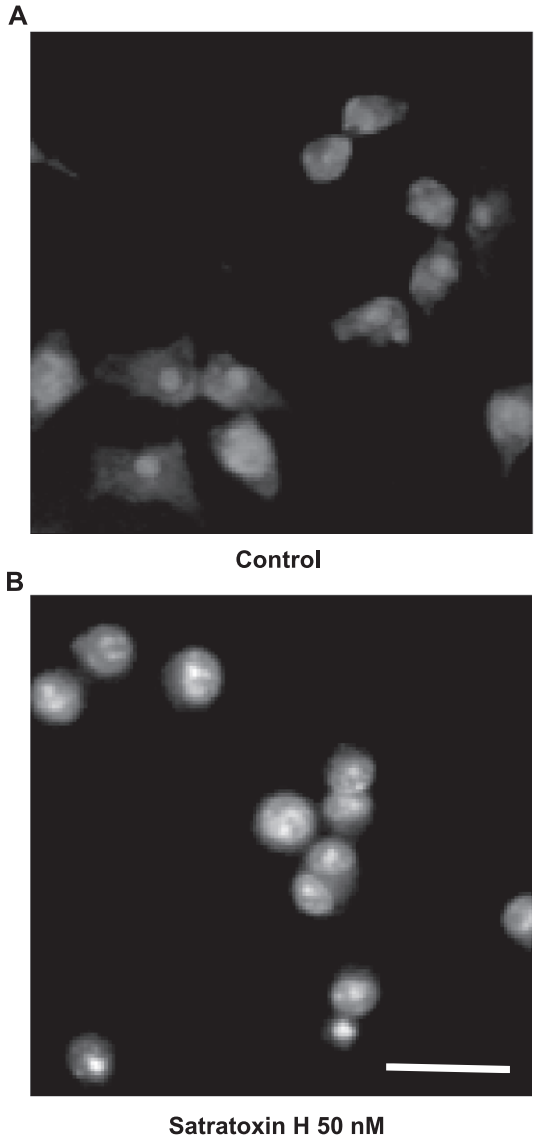

Fig. 2. Morphological analysis of nuclear chromatin in PC12 cells treated with satratoxin H. The cells were treated with vehicle (A) or 50 nM satratoxin H (B) for 6 h. Scale bar=100 μm.

antibody to MAPKs with 1:500 dilution in Tris-buffered saline solution containing 2% skimmed milk at 4 °C overnight. After washing with Tris-buffered saline solution, the blots were incubated with a secondary antibody conjugated with horseradish peroxidase at 3000 times dilution for 2 h at room temperature. The antibody-labeled proteins were rinsed with Tris-buffered saline solution and visualized using an enhanced chemiluminescence detection reagent.

2.7. Statistical analysis

All values are expressed as means±S.E.M. Time- and concentration-dependency were analyzed by one-way analysis of variance followed by Dunnett's test. All the other data were evaluated by two-way analysis of variance and Scheffé's test for multiple comparisons of means. Results with $P<0.05$ were considered statistically significant.

3. Results

3.1. Effect of satratoxin H on viability of PC12 cells

To examine the satratoxin H-induced cytotoxicity, cell viability was determined in PC12 cells by MTT assay. The exposure of PC12 cells to 50 nM satratoxin H resulted in a time-dependent decrease of viable cells (Fig. 1A). Satratoxin H reduced the viable cells 24 h after its treatment in a concentration-dependent manner with a range from 10 to 100 nM (Fig. 1B).

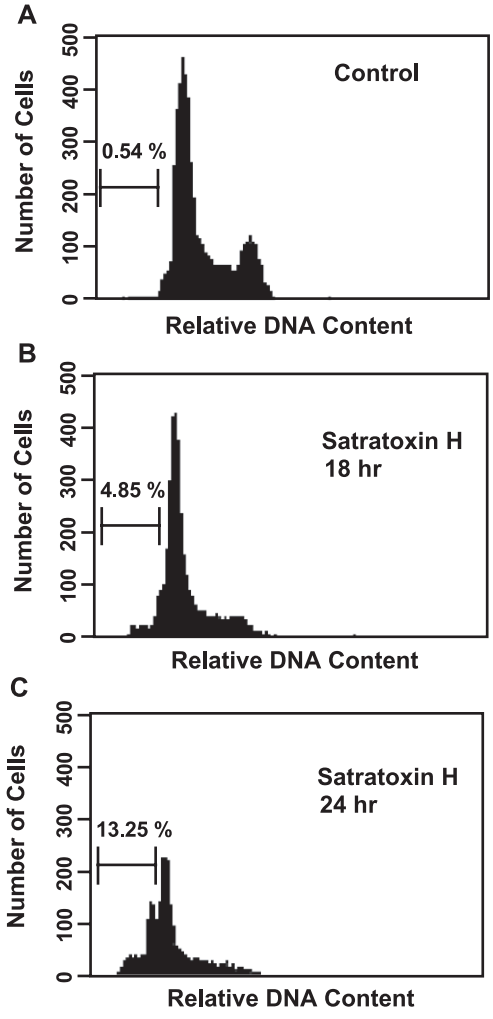

Fig. 3. Flow cytometric analysis showing effects of satratoxin H-induced apoptosis in PC12 cells. Satratoxin H at a concentration of 50 nM was incubated for 0 (A) 18 (B) or 24 h (C) after serum starvation for 24 h. The number above line indicates a percentage of apoptotic cells in each pre-G_0/G_1 peak.

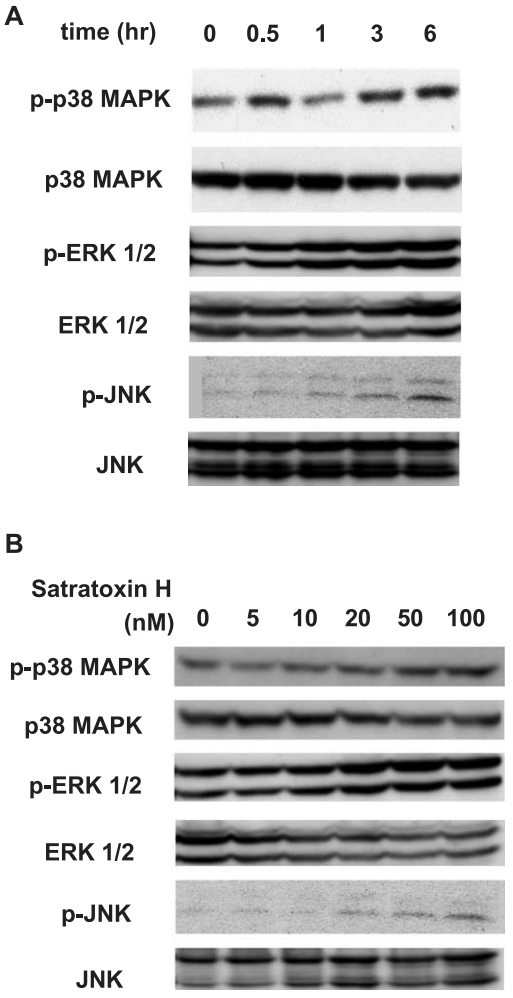

Fig. 4. Satratoxin H-induced phosphorylation of p38 MAPK, ERK1/2 and JNK in PC12 cells. (A) Time course of phosphorylation of these MAPKs after an exposure to 50 nM satratoxin H for indicated time. (B) Concentration-dependency of satratoxin H-induced phosphorylation of MAPKs in the cells incubated with satratoxin H for 3 h. Cell lysates (50 μg protein/lane) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Immunoblotting was performed using antibodies for non-phosphorylated or phosphorylated (p-) MAPKs. Representative result is shown, and data represent similar results in three separate experiments.

3.2. Effect of satratoxin H on apoptosis in PC12 cells

To investigate whether the reduction in cell viability was due to apoptosis, morphological observation of the cells and cytometric analysis were performed.

PC12 cells were treated with 50 nM satratoxin H for 24 h. The control cells were not reactive to Hoechst 33342 and exhibited the normal shape of living cells. In contrast, the most cells treated with satratoxin H for 24 h were stained with Hoechst 33342 (Fig. 2), which binds to chromatin. They exhibited the typical morphological changes of apoptosis, namely nuclear condensation, nuclear cleavage and apoptotic bodies. However, some cells were also stained with trypan blue (data not shown), suggesting that satratoxin H could cause necrosis as well.

The satratoxin H-induced apoptosis in PC12 cells was also investigated using a flow cytometer. After serum starvation for 24 h, the cells were exposed to 50 nM satratoxin H for 18 or 24 h. Satratoxin H increased the percentage of the pre G_0/G_1 phase, indicating the increase in apoptotic cells (Fig. 3).

3.3. Satratoxin H-induced phosphorylation of p38 MAPK, ERK1/2 and JNK

To clarify the involvement of MAPKs in satratoxin H-induced cell death of PC12 cells, we studied the time- and concentration-dependent effects of satratoxin H on the phosphorylation of p38 MAPK, ERK1/2 and JNK. Satratoxin H increased the phosphorylation of p38 MAPK and ERK1/2 0.5 to 6 h after its addition (Fig. 4A). The weak phosphorylation of JNK was also induced by satratoxin H. The treatment of the cells with satratoxin H for 3 h resulted in the increase in phosphorylation of p38 MAPK, ERK1/2 and JNK in a concentration-dependent manner (Fig. 4B). Therefore, the activations of p38 MAPK, ERK1/2 and JNK might be involved in the responses of PC12 cells to satratoxin H.

3.4. Effects of MAPK inhibitors on satratoxin H-induced cell death

To examine whether p38 MAPK, ERK1/2 or JNK is involved in the satratoxin H-induced cell death, PC12 cells were pretreated with inhibitors of MAPKs 1 h before the exposure of 50 nM satratoxin H, and the cell viability was

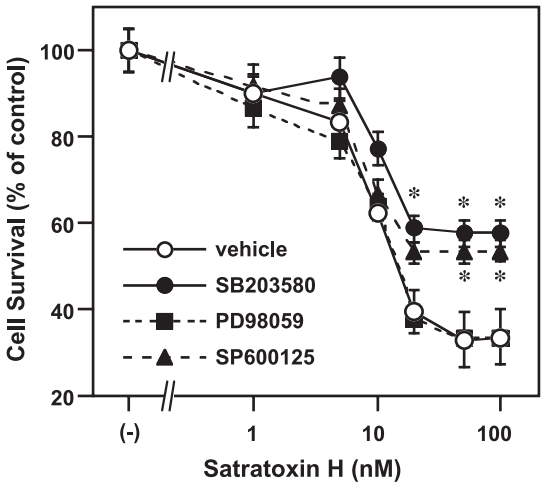

Fig. 5. Effects of SB203580, PD98059 and SP600125 on satratoxin H-induced cytotoxicity. Inhibitors were added 1 h before exposure to various concentrations of satratoxin H, then the cells were further incubated for 48 h. Each point represents the mean±S.E.M. of three independent experiments, each performed in triplicate. *$P<0.05$ compare to the cells exposed to satratoxin H alone (vehicle).

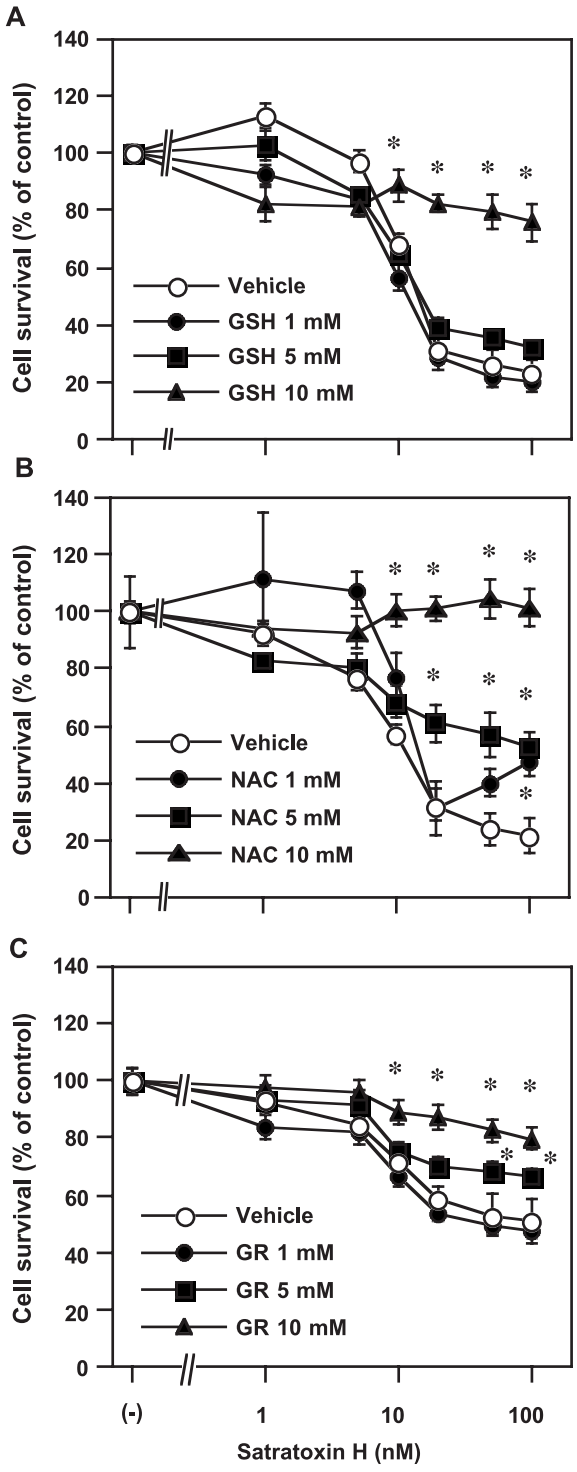

Fig. 6. Protective effects of glutathione, *N*-acetyl-L-cysteine and glutathione reductase on satratoxin H-induced cytotoxicity in PC12 cells. After incubation in serum-free medium for 24 h, cells were treated with various concentrations of reduced glutathione (A), *N*-acetyl-L-cysteine (B) or glutathione reductase (C) immediately before the exposure to satratoxin H at indicated concentrations and then they were further incubated for 24 h. Cell viability was determined by MTT assay. Data are expressed as a percentage of the control value. Each point represents mean±S.E.M. of independent three experiments, each performed in triplicate. *P<0.05 compared to the cells exposed to satratoxin H alone (vehicle).

determined after incubation for 48 h. SB203580 at 30 μM or SP600125 at 10 μM alone decreased the viability of cells slightly. SB203580 or SP600125 partially inhibited satratoxin H-induced cell death, while PD98059 at 30 μM did not affect the cytotoxicity (Fig. 5). These results indicate that p38 MAPK and JNK may contribute to satratoxin H-induced cell death in undifferentiated PC12 cells.

3.5. Protective effects of glutathione, N-acetyl-L-cysteine and glutathione reductase on satratoxin H-induced cytotoxicity in PC12 cells

Glutathione (10 mM) or *N*-acetyl-L-cysteine (5 or 10 mM) significantly reduced the cytotoxicity induced by various concentrations of satratoxin H (Fig. 6). Glutathione reductase (5 or 10 mM) also showed the preventive effect on satratoxin H-induced cytotoxicity after 48-h incubation. Higher concentrations of these antioxidants inhibited satratoxin-induced cell death almost completely.

3.6. Effects of glutathione, N-acetyl-L-cysteine and glutathione reductase on satratoxin H-induced p38 MAPK phosphorylation

Since the phosphorylation of p38 MAPK was clearly observed in the cells treated with satratoxin H (Fig. 4), p38 MAPK was selected for further analysis. Treatment of PC12 cells with glutathione, *N*-acetyl-L-cysteine or glutathione reductase at indicated concentration immediately before the treatment with satratoxin H (50 nM) resulted in a decrease in p38 MAPK phosphorylation, in comparison with cells treated with satratoxin H alone (Fig. 7). Taken together with MTT assay, these results indicated that the antioxidants protected cell death at least partially through the decrease in phosphorylation of p38 MAPK.

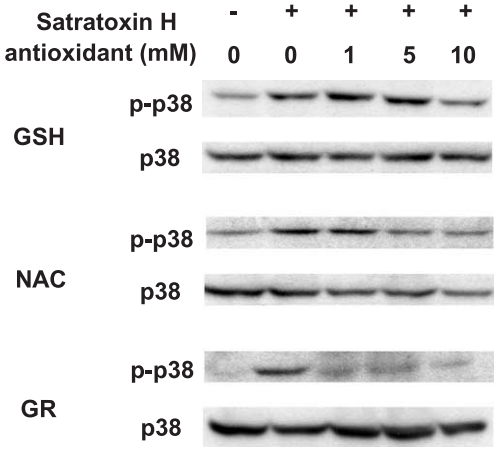

Fig. 7. Effects of antioxidants, reduced glutathione, *N*-acetyl-L-cysteine and glutathione reductase on satratoxin H-induced phosphorylation of p38 MAPK. Cells were treated with various concentrations of antioxidants immediately before adding satratoxin H (50 nM) and they were further incubated for 6 h.

4. Discussion

In the present study, we showed the involvement of reactive oxygen species and the activations of p38 MAPK and JNK in satratoxin H-induced apoptosis in PC12 cells. The cell death induced by satratoxin H was accompanied by time- and concentration-dependent phosphorylations of p38 MAPK and JNK. The specific inhibitors for these MAPKs reduced the satratoxin H-induced cell death. Furthermore, the antioxidant glutathione, *N*-acetyl-L-cysteine and glutathione reductase prevented the apoptosis with the concomitant inhibition of the phosphorylation of p38 MAPK.

Satratoxin H, one of trichothecene mycotoxins, induces apoptosis in PC12 cells in the concentration range from 10 to 100 nM. The decrease in vital cell numbers was accompanied by the chromatin condensation and nuclear cleavage, which are typical morphological features of apoptotic cells. DNA content analysis by flow cytometry showed that satratoxin H induced an accumulation of cells in the pre G_0/G_1 phase. Because satratoxin H caused cytotoxicity in PC12 cells, a cell line originated from pheochromocytoma, as well as in the previously reported myeloid cell lines (Nagase et al., 2002; Yang et al., 2000), it will injure neural cells and induce disorders of central nervous system.

The molecular mechanism of satratoxin-induced cell death has been remained unclear, although the activity of MAPKs have been previously reported in satratoxin-treated cells (Yang et al., 2000). As indicated by Western blotting analysis in the present study, p38 MAPK and JNK were continuously phosphorylated by satratoxin H in PC12 cells. The functional significance of these responses was revealed by the finding that SB203580, a p38 MAPK-specific inhibitor, or SP600125, a specific inhibitor of JNK, increased cell survival rate after 48-h exposure of satratoxin H. Therefore, satratoxin H can be thought to induce apoptosis through the activation of p38 MAPK or JNK. In contrast to these two MAPKs, ERK 1/2 showed no apparent role in satratoxin H-induced apoptosis, because a specific MAPK/ERK kinase inhibitor, PD98059, did not affect cell survival. Although the phosphorylation of ERK 1/2 was increased by satratoxin H, the less and slower phosphorylation of ERK 1/2 may not relate to satratoxin H-induced apoptosis. Yang et al. (2000) have shown the protective role of ERK 1/2 in trichothecene-induced apoptosis in murine macrophage cells.

Accumulated evidence showed that cellular oxidative stress plays an important role in regulating p38 MAPK and JNK. In our knowledge, however, there is no previous study showing the participation of reactive oxygen species in the satratoxin-induced cell death. The results presented in this paper suggest that satratoxin H stimulates intracellular stress signaling pathway including p38 MAPK through reactive oxygen species generation. *N*-Acetyl-L-cysteine and reduced form of glutathione are known to modulate cell viability as the radical scavengers by removing hydrogen peroxide, hydroxyl radicals and superoxide anions. These antioxidants inhibited potently the cell death and p38 MAPK phosphorylation induced by satratoxin H in the present study. The cytotoxicity of satratoxin H was also reduced by glutathione reductase with the concomitant prevention of p38 MAPK phosphorylation. Glutathione reductase changes glutathione from oxidized form to reduced form and play an important role in detoxification of reactive oxygen species. Our results suggest that intracellular redox state of glutathione and *N*-acetyl-L-cysteine have a crucial role in activation of p38 MAPK by satratoxin H. Unlike antioxidants, p38 MAPK or JNK inhibitor provided a partial recovery of satratoxin H-induced cell death. Although ASK-1-induced activation of p38 MAPK/JNK is thought to be the main pathway of apoptosis induced by reactive oxygen species, recent studies have suggested the participation of alternative ways in oxidative stress-induced apoptosis (Chen et al., 2003; Filomeni et al., 2003; Sarker et al., 2003). One possible pathway might be direct or indirect injury of mitochondrial membrane by reactive oxygen species, followed by an increase in cytosolic cytochrome *c* (Nagase et al., 2002). Thus, we cannot exclude a possibility that a reactive oxygen species-dependent but p38 MAPK/JNK-independent pathway is also responsible for the apoptosis induced by satratoxin H. Although we do not know how reactive oxygen species are generated by satratoxin H so far, our results suggest that they are essential mediators of satratoxin H-induced apoptosis in PC12 cells.

In conclusion, a trichothecene mycotoxin satratoxin H induces apoptosis in PC12 cells in a time- and concentration-dependent manner. The generation of reactive oxygen species and the activation of p38 MAPK/JNK are involved in satratoxin H-induced apoptosis.

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