

Critical role of ASK1 in the 6-hydroxydopamine-induced apoptosis in human neuroblastoma SH-SY5Y cells

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Abstract

6-Hydroxydopamine (6-OHDA)-induced apoptosis in dopaminergic neuronal cells is a common cell model of Parkinson's disease (PD). The role of apoptosis signal-regulating kinase 1 (ASK1) in this model has not been well studied. We observed significant activation of ASK1, p38 and JNK, as well as apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells exposed to 6-OHDA. Over-expressing kinase-dead mutant ASK1(K709M) or knock-down of endogenous ASK1 by its small interfering RNA (siRNA) greatly suppressed activation of these kinases and apoptosis in the cells. It was found that the activation of p38 and JNK was suppressed to almost the same extent as that of ASK1 in the ASK1-knock-down cells, suggesting that activated ASK1 is almost totally responsible for activation of p38/JNK. It was also observed

that the 6-OHDA-induced cell apoptosis could be effectively prevented by over-expressing the dominant-negative mutant of p38 or p38 inhibitor SB203580, demonstrating that activation of p38/JNK signalling is required for initiating the programmed cell death. Furthermore, suppression of the 6-OHDA-generated reactive oxygen species (ROS) by pre-incubation of cells with *N*-acetyl-L-cysteine effectively inhibited the 6-OHDA-induced activation of ASK1, p38 and JNK, and protected the cells from apoptosis. This study clearly shows the route from ROS generation by 6-OHDA to initiation of p38/JNK signalling via activation of ASK1 in the studied PD model.

Keywords: apoptosis, apoptosis signal-regulating kinase 1, c-Jun N-terminal kinase, 6-hydroxydopamine, p38 mitogen-activated protein kinase, Parkinson's disease
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Parkinson's disease (PD) is one of the most common neurodegenerative disorders, affecting about 2% of the population over the age of 60 (Lo Bianco *et al.* 2004). Pathologically, PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra which leads to motor impairment, including akinesia, resting tremor, muscle rigidity, and gait and postural deficits. Current evidence suggests an involvement of both environmental and genetic factors in the progression of PD. Although the genetic contribution to the etiology of PD is indisputable in some cases, the majority of PD is sporadic, and the cause of neuronal loss in the substantia nigra is poorly understood (Xia *et al.* 2001). However, there has been an increasing body of evidence concerning dopaminergic cell death in PD. Several biochemical mechanisms have been suggested for PD pathology, including mitochondrial dysfunction (Kosel *et al.* 1999), oxidative stress (Dexter *et al.* 1989; Jenner and Olanow 1996), and apoptosis (Andersen 2001).

In PD several models have been utilized to study the biochemical and behavioural consequences of dopamine

(DA) neuron degeneration with the intent of further understanding the etiology of this disease and improving its treatment. The 6-hydroxydopamine (6-OHDA)-induced cell death in dopaminergic neurons has been a most frequently used toxin-induced PD model, as 6-OHDA selectively destroys catecholaminergic neurons (Schober 2004). 6-OHDA is a hydroxylated analogue of the natural neurotransmitter dopamine (Blum *et al.* 2001), and endogenous

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Abbreviations used: ASK1, apoptosis signal-regulating kinase 1; DA, dopamine; DCF, 2',7'-dichlorodihydrofluorescein; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; MKKn, MAP kinase kinase n; NAC, *N*-acetyl-L-cysteine; 6-OHDA, 6-hydroxydopamine; p38, p38 mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PD, Parkinson's disease; PI, propidium iodide; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA.

6-OHDA has been found accumulating in the urine of PD patients (Andrew *et al.* 1993). Today, 6-OHDA represents one of the most common neurotoxins used in degeneration models of central catecholaminergic projections, including the nigrostriatal system, *in vivo* and *in vitro* (Ungerstedt 1968; Sachs and Jonsson 1975; Ungerstedt 1976; Blum *et al.* 2001). Investigation of the molecular signalling mechanism involved in degeneration process of dopaminergic neurons should be helpful in understanding the cause of PD and searching for therapeutic agents for the treatment of PD patients. Despite 6-OHDA not inducing the formation of cytoplasmic inclusion bodies (Lewy bodies) in surviving dopaminergic neurons, which represents the most important neuropathological feature of PD, the 6-OHDA model is still useful for testing new pharmacological or cell-replacement therapies (Hirsch *et al.* 2003).

In order to understand the intracellular events leading to cell death of dopaminergic neurons, the molecular signalling mechanisms involved in 6-OHDA-induced apoptotic cell death have been considerably studied in either primary culture or some dopaminergic cell lines. The previous studies showed that 6-OHDA generates reactive oxygen species (ROS) and induces apoptosis in dopaminergic cells of the rat substantia nigra (Cohen and Heikkila 1974; He *et al.* 2000) through activation of caspase 3, NF- κ B, p53 and c-Jun transcription factors (Del Rio and Velez-Pardo 2002). An increasing body of evidence indicated that the stress-activated kinases including c-Jun N-terminal kinase (JNK) and p38 kinase play a critical role in 6-OHDA-induced degeneration process. Translocation of JNK2 to the mitochondria mediates cytochrome *c* release in PC12 cells in response to 6-OHDA (Eminel *et al.* 2004). Recently, Choi *et al.* (2004) evaluated the contribution of p38 mitogen-activated protein kinase and the events upstream/downstream of p38 leading to dopaminergic neuronal death. They found that superoxide anion and nitric oxide induced by 6-hydroxydopamine initiate the p38 signal pathway leading to activation of both mitochondrial and extramitochondrial apoptotic pathways in MN9D dopaminergic neuronal cells, another culture model of PD. However, how the ROS initiates p38/JNK signalling has not been established in these culture models of PD. According to present knowledge, as a member of the MAP kinase kinase kinase family, apoptosis signal-regulating kinase 1 (ASK1) acts upstream of JNK and p38 kinases (Ichijo *et al.* 1997). It phosphorylates and activates MKK4/MKK7 and MKK3/MKK6, which in turn induce JNK and p38 kinase activities, respectively (Davis 2000). ASK1 plays a pivotal role in cell death induced by a variety of stimuli such as TNF α , H₂O₂ and anti-cancer drugs (Ichijo *et al.* 1997; Chen *et al.* 1999; Wang *et al.* 1999; Yoon *et al.* 2002). Although the involvement of the ASK1-p38/JNK signalling cascade has been reported in anandamide- and NO-induced PC12 cell death (Sarker *et al.* 2003a,b), the H₂O₂-induced apoptosis in human pulmonary vascular

endothelial cells (Machino *et al.* 2003), it has not been identified whether ASK1 plays a key role in activation of p38/JNK signalling in the neurotoxins-induced culture models of PD, such as MPTP- and 6-OHDA-induced apoptosis in dopaminergic neuronal cells. Here, we report the study on the role of ASK1 in 6-OHDA-induced apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells and attempt to find out how critical the ASK1 is in activating p38/JNK signalling in this model.

Materials and methods

Materials

6-Hydroxydopamine (6-OHDA), myelin basic protein (MBP), SB203580, *N*-acetyl-L-cysteine (NAC) and propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). Mouse anti-p38 MAPK polyclonal antibody and the SAPK/JNK Assay Kit including anti-phospho-c-Jun (Ser63) antibody were bought from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) polyclonal antibody was bought from Chemicon (Temecula, CA, USA). Mouse anti-ASK1 monoclonal antibody, goat anti-actin polyclonal antibody and mouse anti-HA (F-7) polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) was bought from Molecular Probes (Eugene, OR, USA). G-418 sulfate (Geneticin) was from Gibco-Invitrogen Life Technologies (Rockville, MD, USA). [γ -³²P]ATP was obtained from the Furui Biotechnology Company (Beijing, China). SuperSignal west pico chemiluminescence substrate was purchased from Pierce (Rockford, IL, USA). The protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

The plasmid pcDNA3-p38 (AGF), a p38 dominant-negative mutant (p38DN), was a gift from Professor J. Han (Department of Immunology, The Scripps Research Institute, La Jolla, CA, USA). The plasmid pcDNA3-HA-tagged-ASK1(K709M), a kinase-dead ASK1 mutant, was kindly provided by Professor Hidenori Ichijo (Tokyo Medical and Dental University, Tokyo, Japan).

Cell culture, transfection and drug treatment

The SH-SY5Y cells, a human dopaminergic neuroblastoma cell line, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin in a CO₂ incubator at 37°C. The cells over-expressing the dominant-negative mutant of p38 and the kinase-dead ASK1 mutant were cloned, respectively, by stably transfecting SH-SY5Y cells with pcDNA3-p38 (AGF) and pcDNA3-HA-tagged ASK1(K709M) using transfection reagent jetPEITM (Polyplus Transfection, Biopac, France) and selection with 350 μ g/mL G418.

After being cultured in serum-reduced culture medium containing 2% FBS for 24 h, the cells were incubated in the medium containing 100 μ M 6-OHDA for 2 h, then replaced with new serum-reduced culture medium. To study the effect of NAC or SB203580, the cells were pre-incubated with 10 mM NAC for 4 h or 10 μ M SB203580 for 30 min in serum-reduced culture medium before being treated with 6-OHDA.

Detection of apoptotic DNA fragmentation

Cells were plated on 35-mm dishes and cultured to about 80% confluence. After treatment with 100 μM 6-OHDA for 2 h, cells were incubated in new serum-reduced culture medium for about 17 h. Then, both attached and detached cells were harvested, and washed twice with phosphate-buffered saline (PBS), lysed in 20 μL lysis buffer [100 mM Tris-HCl, 20 mM EDTA, 0.8% (w/v) sodium dodecyl sulfate (SDS), pH 7.8]. The lysate was incubated at 37°C for 30 min after addition of 10 μL of RnaseA (10 mg/mL; Genview, Houston, TX, USA), then further incubated for 2 h at 50°C after addition of 10 μL proteinase K (20 mg/mL; Merck, Whitehouse Station, NJ, USA). Cell lysate (8 μL) was subjected to 2% agarose gel electrophoresis in TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.0). The DNA was visualized by ultraviolet fluorescence after Ethidium Bromide (EB) staining.

Knock-down of ASK1 by synthetic siRNA

The siRNA sequence targeting ASK1, 5'-GGUAUACAUGAGUG-GAAUUTT-3', was kindly provided by Dr M. Husmann (Dersch *et al.* 2005). The siRNA and a scrambled oligonucleotide (Mock RNA) were both chemically synthesized by GenePharma (Shanghai, China). SH-SY5Y cells (8×10^5 per well) were seeded in six-well plates overnight. Cells were transfected with 4.5 μg of ASK1 siRNA (200 nM) per well using 9 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 1.5 mL of Dulbecco's modified Eagle's medium containing 10% FBS. The transfection medium was replaced with fresh culture medium 6 h later, and the expression of ASK1 was assayed by western blotting using rabbit anti-ASK1 polyclonal antibody 72 h after transfection. The cells transfected with the scrambled oligonucleotide were used as negative control.

Flow cytometric analysis of apoptotic cells

After transfection with ASK1 siRNA or the scrambled RNA for 72 h, the cells were treated with 100 μM 6-OHDA for 2 h, and incubated in fresh culture medium containing 2% FBS for another 17 h. Then, both attached and detached cells were harvested, washed once with 5 mM EDTA-PBS buffer, fixed in 70% ethanol at 4°C for 2 h, and finally centrifuged at 2000 g for 5 min. The pellet of cells was gently suspended in 400 μL of 5 mM EDTA-PBS with the addition of 10 μL of Rnase A (10 mg/mL) and incubated at 37°C for 30 min. After addition of 400 μL PI solution (100 $\mu\text{g}/\text{mL}$ PI in PBS), 3×10^4 cells of each sample were analysed on a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The portion of the apoptotic cells was calculated according to the apoptotic peak in the cell distribution against the PI fluorescence per cell.

Western blotting assay of p38 MAP kinase, phospho-p38 MAP kinase, ASK1(K709M) and phospho-c-Jun

After various treatments, the cells plated on dishes were washed with ice-cold PBS and lysed on ice for 5 min by adding ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.5). Then cell lysates were transferred into microcentrifuge tubes and sonicated on ice. The protein contents of the lysates were determined using the Bio-Rad protein assay kit. The cell lysates with equal protein content

were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto nitrocellulose membrane. The nitrocellulose blots were blocked with 5% non-fat dry milk in TBS/T buffer (20 mM Tris-HCl, 500 mM NaCl, and 0.1% Tween 20, pH 7.4), and then incubated with the desired primary antibodies. The anti-p38 MAPK antibody, anti-phospho-p38 MAPK antibody, anti-HA antibody and anti-phospho-c-Jun (Ser63) antibody were used for detection of the expression of both of p38 and p38DN, the activated p38, the expression of ASK1(K709M) and the phosphorylation of c-Jun, respectively, in the cell lysates. After three washes with TBS/T buffer, the nitrocellulose membranes were incubated with horseradish peroxidase-conjugated second antibody (1 : 5000) for 2 h. The resulting immunocomplex bands were visualized using chemiluminescence photographic detection with SuperSignal west pico chemiluminescence substrate. As an internal control, the actin contents in the samples were also assayed by the same procedure, except using anti-actin polyclonal antibody as the primary antibody.

Assay of JNK activity

The JNK activity was assayed using SAPK/JNK Assay Kit, according the manufacturer's instructions. Following 6-OHDA treatment, the cells were lysed by the same procedure as previously described. After centrifugation at 10 000 g for 15 min at 4°C, the cell lysate supernatant, containing 200 μg of total proteins, was incubated with 20 μL of immobilized c-Jun fusion protein bead with gentle rocking overnight at 4°C for selective immunoprecipitation of JNK. After centrifugation of the above reaction mixture at 14 000 g for 1 min at 4°C, the pellet was washed twice with lysis buffer and twice with kinase buffer [25 mM Tris (pH 7.5), 5 mM β -glycerolphosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4 , 10 mM MgCl_2], then suspended in 50 μL of kinase buffer supplemented with 200 μM ATP and incubated for 30 min at 30°C. The reaction was terminated by adding 25 μL 3 \times SDS sample buffer [187.5 mM Tris-HCl (pH 6.8), 6%w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromphenol blue]. After boiling for 5 min, the reaction mixture was assayed by western blot using anti-phospho-c-Jun (Ser63) antibody.

Assay of ASK1 activity by MBP phosphorylation

Following 6-OHDA treatment, the cells were lysed according to the same procedure as previously described. After centrifugation at 10 000 g for 15 min at 4°C, the cell lysate supernatant, containing 200 μg of total proteins, was incubated with 2 μg anti-ASK1 antibody with gentle rocking overnight at 4°C, and then with 20 μL protein A-sepharose (50% v/v) for an extra 3 h. Afterwards, the reaction mixture was again centrifuged at 4000 g for 3 min at 4°C, and the precipitant was obtained as a pellet. After two washes with 500 μL of cell lysis buffer and two washes with 500 μL of kinase buffer [25 mM Tris (pH 7.5), 5 mM β -glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4 , 10 mM MgCl_2], the precipitant was dissolved in 50 μL kinase buffer supplemented with 200 μM ATP, 2 μCi [γ - ^{32}P]ATP and 4 μg MBP (the substrate of ASK1), incubated at 30°C for 30 min with gentle rocking, and the reaction terminated with 25 μL 3 \times SDS sample buffer. After boiling for 5 min, the reaction mixture was separated by 15% SDS-PAGE. The gel was dried in vacuum, and the band of the ^{32}P -labelled MBP was autoradiographed on Kodak X-Omat BT

Film. The ASK1 activity was evaluated as the incorporated radioactive ^{32}P in MBP.

Fluorescence assay of the ROS generated in cells

The generation of the ROS in cells was measured as accumulated oxidized carboxy- H_2DCFDA within certain period of time as previously described (Teissier *et al.* 2004). Briefly, each of 1.5×10^6 cells were seeded in six-well plates and cultured overnight. The cells were treated with $100 \mu\text{M}$ 6-OHDA for 1 h, washed twice with PBS, and then incubated in Hank's balanced salt solution containing $10 \mu\text{M}$ carboxy- H_2DCFDA at 37°C for 45 min. After washing with Hank's balanced salt solution, the cells were lysed in 1 mL of 1 M NaOH for 5 min on ice. The lysate was centrifuged at 14 000 g for 10 min at 4°C and the fluorescence of dichlorofluorescein (DCF) in the supernatant was measured at the excitation of 480 nm and emission of 520 nm on a Fluorescence Spectrophotometer F-4500 (Hitachi).

Results

6-OHDA induces activation of ASK1 and apoptosis in SH-SY5Y cells

To know if ASK1 is activated in the 6-OHDA-stimulated cells, the activity of ASK1 was determined in wild-type SH-SY5Y cells exposed to 6-OHDA for 1 h at various

concentrations. The activation of ASK1 in the cells expressing the kinase-dead mutant of ASK1, ASK1(K709M), was also determined after exposure to 6-OHDA. As shown in Fig. 1(b), a dose-dependent activation of ASK1 was detected in the wild-type cells after exposure to various concentrations of 6-OHDA. It was observed that over-expression of ASK1(K709M) resulted in almost complete inhibition of the 6-OHDA-induced activation of ASK1.

In order to confirm the involvement of ASK1 in 6-OHDA-induced dopaminergic cell apoptotic death, the DNA fragmentation pattern was detected electrophoretically in the wild-type cells and the cells transfected with the kinase-dead mutant of ASK1, respectively, after the cells were exposed to $100 \mu\text{M}$ 6-OHDA for 2 h and then further incubated for 17 h. The choice of $100 \mu\text{M}$ for the concentration of 6-OHDA applied to all experiments was based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of survival in the cells exposed to various drug concentrations for 24 h (data are not shown here). At this concentration, nearly 50% of the 6-OHDA-treated cells died within 24 h. As shown in Fig. 1(c), 6-OHDA induced a significant DNA fragmentation in wild-type cells (see the left-hand lanes 1–4). However, almost no DNA fragmentation could be observed in the cells over-expressing the kinase-dead

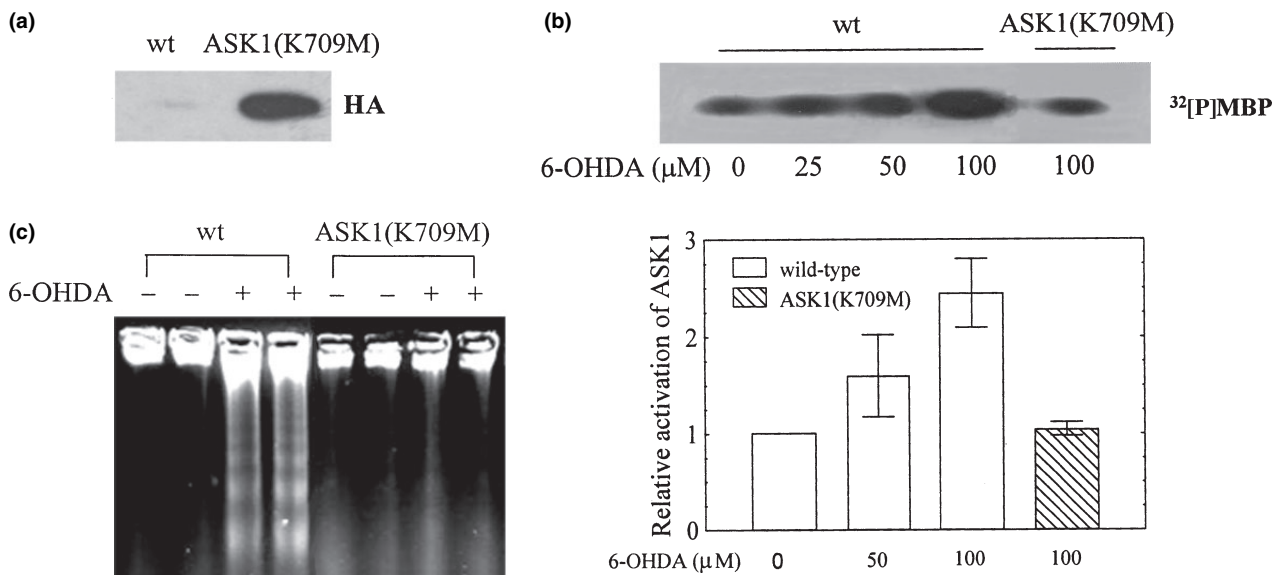


Fig. 1 6-OHDA-induced activation of ASK1 and apoptosis in wild-type SH-SY5Y cells and the cells transfected with kinase-dead mutant ASK1(K709M). The kinase activities were assayed after the cells were exposed to 6-OHDA for 1 h. (a) Expression of the kinase-dead mutant of ASK1, in the cells stably transfected with HA-tagged-ASK1(K709M). The anti-HA antibody was used for immunoblotting. (b) The ASK1 activities in wild-type and ASK1(K709M)-transfected cells after exposure to 6-OHDA of various indicated concentrations for 1 h. ^{32}P MBP stands for the phosphorylated MBP (substrate of ASK1), and the radioactivities of the bands represent the ASK1 activities in the cells.

The histogram below the blot shows the statistically analysed data from three independent measurements of the relative kinase activities under the corresponding conditions. The error bars are the SD of the measured activities. (c) The 6-OHDA induced apoptotic DNA fragmentation in wild-type cells and the cells over-expressing ASK1(K709M). The DNA fragments were determined by electrophoresis of the cell lysates after the cells were exposed to $100 \mu\text{M}$ 6-OHDA for 2 h and subsequently incubated for 17 h. The pattern of the DNA fragments is representative of three independent measurements.

ASK1 mutant (see the right-hand four lanes). The results evidently demonstrate the involvement of ASK1 activation in the 6-OHDA-induced apoptosis in the studied dopaminergic neuroblastoma cells, as inhibition of ASK1 activation by over-expressing the ASK1 mutant effectively protects the cell from apoptosis.

Activation of ASK1 leads to activation of p38 MAP kinase and JNK in 6-OHDA-stimulated SH-SY5Y cells

Because ASK1 acts as upstream of p38 kinases and JNK (Ichijo *et al.* 1997), the activation of p38 and JNK was also determined in the wild-type cells and the cells over-expressing ASK1(K709M), following exposure to 100 μ M 6-OHDA for 1 h. As shown in Fig. 2(a), significant activation of both p38 kinase and JNK were detected in the wild-type cells (comparing the left-hand three lanes with the lane fourth from the left on the western blot), but less activation of p38 and JNK in the cells over-expressing ASK1(K709M). As shown in the histograms obtained from statistical analysis of six independent measurements, the basal activity of p38 kinase and JNK in the cells over-expressing the kinase-dead mutant of ASK1 was suppressed by 61 ± 6 and $51 \pm 5\%$, respectively, while the 6-OHDA-induced activation of p38 kinase and JNK was suppressed by 55 ± 11 and $53 \pm 4\%$, respectively, in the same cells (Figs 2b and c). The results indicate that either the basal or the 6-OHDA-induced activity of p38 kinase and JNK in the cells was suppressed to almost the same extent by over-expression of the kinase-dead mutant of ASK1, indicating that ASK1 acts as the same upstream-activator of p38 kinase and JNK.

Knock-down of ASK1 results in significantly reduced activation of p38 kinase and JNK in 6-OHDA-stimulated cells

Because the dominant-negative mutant of ASK1 exerts its effect by competition with its endogenous counterpart and may affect activation of p38 kinase and JNK via interaction with MKK3/6 or MKK4/7, the siRNA technique was used to silence ASK1 gene expression and to reduce the endogenous ASK1 from the cells to a great extent. The inhibition of p38 kinase and JNK activation in the 6-OHDA-stimulated cells by knocking down ASK1 may provide more reliable information about the ASK1-dependent and ASK1-independent activation of these two kinases. At first, the expression of ASK1 in the cells transfected with various amount of ASK1 siRNA was assayed 72 h after transfection, and the expression in wild-type cells and the cells transfected with a corresponding amount of scrambled oligonucleotide were assayed as controls. As shown in Fig. 3(a), the highest efficiency for knocking ASK1 down was achieved by transfecting 200 nM of ASK1 siRNA into cells. Under this condition, the lowest ASK1 level of 0.17 was detected by western blotting in the transfected cells. The expression levels of ASK1 in both the wild-type cells and the cells transfected with the scrambled oligonucleotide were found to be almost same (see the left-hand two lanes and the right-hand lane in Fig. 3a), indicating the unspecific control siRNA was without effect.

The activation of p38 kinase and JNK in unstimulated wild-type cells, the cells transfected with ASK1 siRNA and the cells transfected with the scrambled oligonucleotide were

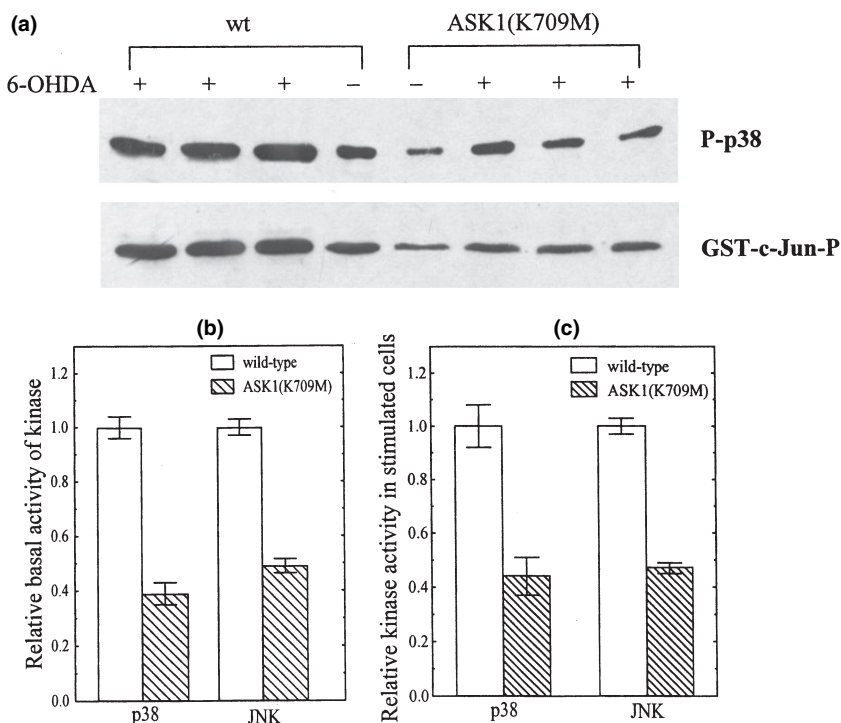


Fig. 2 6-OHDA-induced activation of p38 kinase and JNK in wild-type and ASK1(K709M)-transfected cells. (a) The p38 activity and JNK activity in wild-type and SH-SY5Y cells transfected with ASK1(K709M) after exposure to 100 μ M 6-OHDA for 1 h. (b, c) Statistically analysed data from six independent determinations on the basal activities (b) and 6-OHDA-induced activities (c) of p38 and JNK in wild-type cells and cells over-expressing ASK1(K709M). The error bars are the SD of the measured activities.

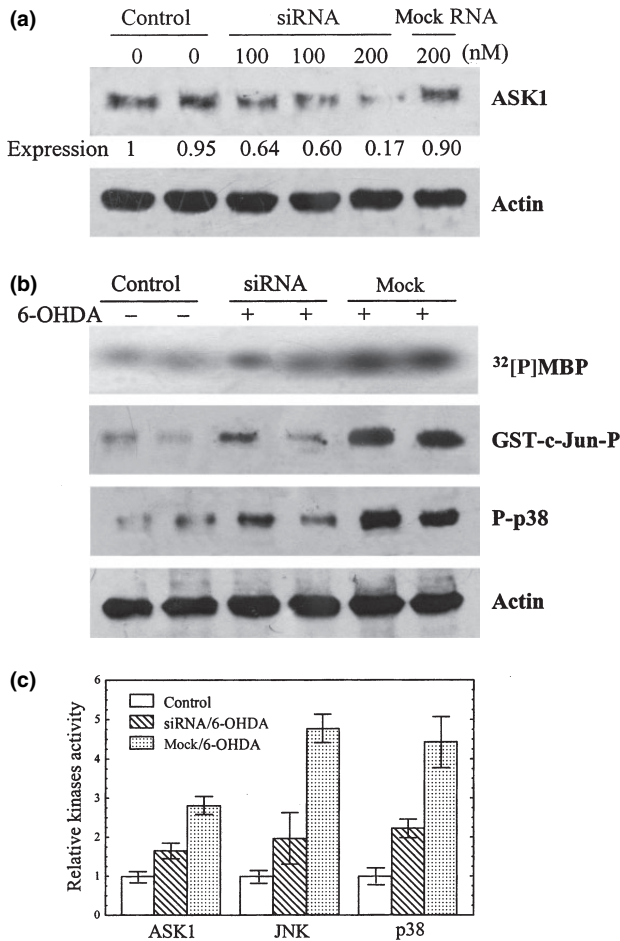


Fig. 3 Suppression of ASK1, p38 and JNK activation by knocking ASK1 down in cells. The activities of ASK1, p38 kinase and JNK were determined, respectively, in the wild-type SH-SY5Y cells, the cells transfected with ASK1 siRNA and the cells transfected with scrambled oligonucleotide after the cells were exposed to 100 μ M 6-OHDA for 1 h. (a) The expression of ASK1 in wild-type cells, the cells transfected with various concentrations of ASK1 siRNA (100 nM, 200 nM) and the cells transfected with 200 nM scrambled oligonucleotide (Mock RNA). The numbers below the bands on the western blot represent the relative expression of the kinase. The expression level of actin was used as an indication of equal loading of proteins in immunoblotting. (b) Activities of ASK1, JNK and p38 kinase in the wild-type cells (as positive control), the cells transfected with 200 nM ASK1 siRNA and the cells transfected with 200 nM Mock RNA (as negative control) determined after exposure to 100 μ M 6-OHDA for 1 h. (c) The histogram of the measured activities of these three kinases in the three types of cells specified in (b). The relative kinase activities are the means \pm SD of four independent determinations.

determined after exposure to 100 μ M 6-OHDA for 1 h. As shown in Fig. 3(b), about 80% silence of the ASK1 expression results in 48 ± 5 , 55 ± 10 and $58 \pm 7\%$ suppression of the 6-OHDA-induced activity of ASK1, p38 kinase and JNK, respectively. The fact that the activities of ASK1, p38 kinase and JNK were suppressed to almost the same

extent by knock-down of ASK1 suggests that ASK1 is almost totally responsible for the activation of p38 and JNK. It was surprising to note that the suppression of 6-OHDA-induced activation of p38 kinase and JNK in the ASK1-knock-down cells is almost as same as that observed in the cells transfected with the dominant-negative mutant of ASK1, where 55 ± 11 and $53 \pm 4\%$ suppression was obtained for p38 kinase and JNK, respectively. This observation suggests that the introduction of the kinase-dead mutant of ASK1 does not appear to bring any perceptible extra effect on the activation of p38 and JNK besides reducing ASK1 activity.

Knock-down of ASK1 prevents apoptosis in 6-OHDA-stimulated cells

It has been demonstrated that inhibition of ASK1 activation by over-expressing the kinase-dead mutant of ASK1 effectively protects the cell from apoptosis. In order to further confirm the result and exclude other possible non-specific effects of the ASK1 mutant, the 6-OHDA-induced apoptosis in wild-type cells and the cells transfected either with ASK1 siRNA or a scrambled oligonucleotide (Mock RNA) was analysed by flow cytometry using PI-staining. The distribution of cell numbers against the DNA content per cell in the wild-type cells, the cells transfected with either ASK1 siRNA or scrambled oligonucleotide (Mock RNA) was determined, respectively, after exposure to 100 μ M 6-OHDA for 2 h and further incubation for 17 h. The unstimulated wild-type cells were used as control. The results are shown in Fig. 4(a–d), respectively. The apoptotic fractions in the four types of cells are summarized as a histogram shown as Fig. 4(e). It can be seen clearly that knock-down of ASK1 greatly inhibited the 6-OHDA-induced apoptosis in SH-SY5Y cells. Two parallel determinations show that knock-down of ASK1 reduces the apoptotic fraction from 24 ± 4 to $9.6 \pm 0.2\%$ in the cells exposed to 6-OHDA, while the apoptotic fraction in the cells transfected with scrambled oligonucleotide still remained as high as $20 \pm 2\%$.

Activation of p38 is responsible for 6-OHDA-induced apoptosis in SH-SY5Y cells

Because activation of ASK1 initiates the apoptosis process in the 6-OHDA-stimulated cells through activation of p38 MAP kinase signalling and JNK signalling, it may be reasonable to further specify the role displayed by these two signalling pathways in the 6-OHDA-induced apoptosis in SH-SY5Y cells. Because a very recent report has already provided an insight into specific actions of JNK2 in 6-OHDA-induced death of PC12 cells (Eminel *et al.* 2004), the present study focused more on the role of p38 kinase in 6-OHDA-induced apoptosis in SH-SY5Y cells. Similar to the study on the role of ASK1 in the 6-OHDA-induced apoptosis of the cells, the dominant-negative mutant of p38 was transfected in the cell in order to see if inhibition of p38 activation by

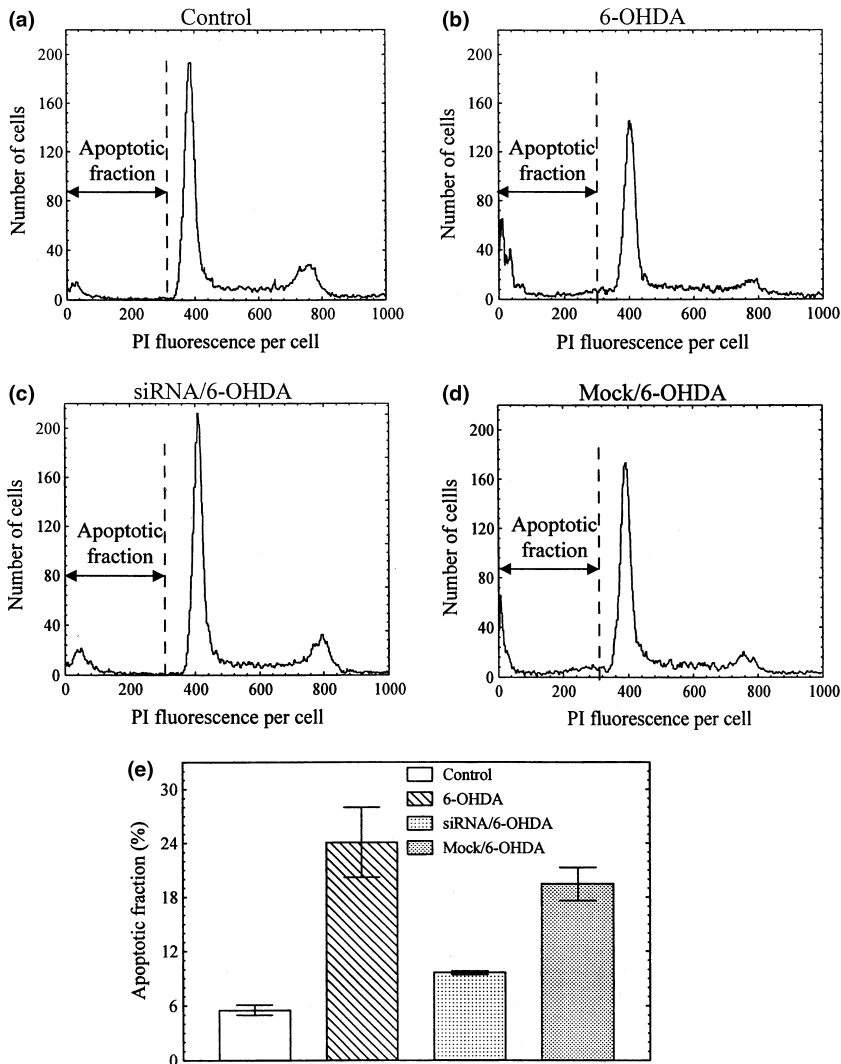


Fig. 4 Protection of the SH-SY5Y cells from 6-OHDA-induced apoptosis by knocking ASK1 down. The apoptotic fraction of the wild-type cells, the cells transfected with 200 nM ASK1 siRNA or scrambled oligonucleotide (Mock RNA) for 72 h were analysed by flow cytometry after exposure to 100 μ M 6-OHDA for 2 h and subsequently cultured for 17 h. The cell number distribution against the fluorescence per each PI-stained cell for each type of cells is shown in (b), (c) and (d), respectively. The untreated wild-type cells were used as control, and their corresponding flow cytometric analysis is shown in (a). (e) The apoptotic fraction in the above-specified cells calculated based on two parallel measurements. The bars indicate the deviation of the data obtained from two parallel determinations.

over-expressing the functional dead mutant p38DN could protect the cells from 6-OHDA-induced apoptosis. As shown in Fig. 5, a considerable activation of p38 was detected in the wild-type cells after exposure to 100 μ M 6-OHDA for 1 h, while almost no activation of p38 could be detected in the p38DN-transfected cells exposed to the same concentration of 6-OHDA (see Fig. 5a). Correspondingly, a marked DNA fragmentation was observed in the wild-type cells, but almost disappeared in the cells over-expressing the dominant-negative mutant of p38 following exposure to 6-OHDA (see Fig. 5b). The results reasonably suggest that the previously observed 6-OHDA-induced DNA fragmentation in wild-type cells but not in the cells transfected with the kinase-dead mutant of ASK1 is attributed to the p38 activation. Without activation of the p38 kinase, even activation of ASK1 could not initiate cell apoptosis.

To further confirm the involvement of p38 signalling in 6-OHDA-induced apoptosis in the SH-SY5Y cells, a selective inhibitor of p38, SB203580, was used to see if the

6-OHDA-induced apoptosis could be abrogated in the cells treated with the inhibitor. As shown in Fig. 5(c), 10 μ M SB203580 did protect the cells from 100 μ M 6-OHDA-induced apoptosis. However, it must be confirmed whether SB203580 also inhibit JNK activation in 6-OHDA-stimulated cells. For this reason, the JNK activities in the cells exposed to 100 μ M 6-OHDA for 1 h in the presence and the absence of 10 μ M SB203580 were determined as endogenous phosphorylated c-Jun in the cell by immunoblotting using anti-phospho-c-Jun (Ser63) antibody. The results showed that SB203580 did inhibit the 6-OHDA-induced JNK activation by nearly 40% (see the relative densities of the P-c-Jun bands in Fig. 5d).

ROS generation is upstream of 6-OHDA-induced activation of ASK1-p38/JNK signalling

It has been known that 6-OHDA-induced neuron degeneration involves oxidative stress. The ROS-mediated signalling pathways play an essential role in 6-OHDA-induced cell

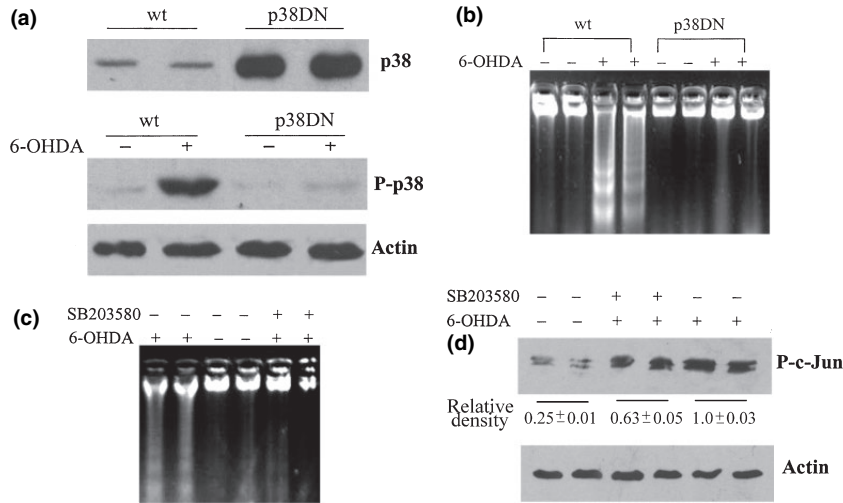


Fig. 5 Inhibition of p38 activity and the 6-OHDA-induced apoptosis in the SH-SY5Y cells by over-expressing dominant-negative mutant of p38 (p38DN) or SB203580 (the inhibitor of p38). (a) The expression of total p38 kinase and 6-OHDA-induced activated p38 kinase in wild-type cells (left-hand two lanes of the two blots) and the cells transfected with p38DN (right-hand two lanes of the two blots). The cells were exposed to 100 μ M 6-OHDA for 1 h. (b) The 6-OHDA induced apoptotic DNA fragmentation in wild-type cells and the cells over-

expressing p38DN. (c) The inhibitory effect of SB203580 (10 μ M) on the 6-OHDA-induced DNA fragmentation in wild-type cells. The DNA fragmentation pattern is a representative of three independent measurements. (d) The inhibitory effect of SB203580 (10 μ M) on the 100 μ M 6-OHDA-induced JNK activation. The JNK activity is determined as the endogenous phosphorylated level of c-Jun in cells. The numbers below the bands are the average values of relative band densities obtained from two parallel measurements.

death (Pettmann and Henderson 1998; Salinas *et al.* 2003). In the present investigation, the fluorescent indicator, carboxy-H₂DCFDA was used to display the accumulated ROS generated within 45 min in the cells immediately after exposure to 10 μ M 6-OHDA for 1 h. As a well-established antioxidant, *N*-acetyl-L-cysteine (NAC) was used to see if the activity of ASK1, p38 and JNK could be inhibited by suppressing ROS generation in the cells. The results are shown in Figs 6 and 7. Higher DCF fluorescence in the wild-type cells and its significant reduction in the cells pre-incubated with 10 mM NAC for 4 h before exposure to 6-OHDA (see Fig. 6a) demonstrate the ability of 6-OHDA in generating ROS and the capability of NAC in suppressing the generated ROS. The pre-incubation with NAC was also found to significantly reduce the activation of ASK1, p38 MAP kinase and JNK in the cells exposed to 6-OHDA (see Blots a, b and c, respectively, in Fig. 7). Statistical analysis of three independent determinations on the 6-OHDA-induced activities of these kinases in the cells pre-incubated with or without 10 mM NAC is shown in Fig. 7(c). It was found that pre-incubation with 10 mM NAC resulted in 32 ± 21 , 42 ± 13 and $37 \pm 7\%$ suppression of the activity of ASK1, JNK and p38, respectively. The suppression of activities of these kinases clearly indicates that the ROS generation is upstream of the ASK1-p38/JNK signalling. As a result of scavenging ROS, pre-incubation with NAC also inhibited the 6-OHDA-induced apoptotic DNA fragmentation in the cells (see Fig. 6b).

Discussion

Although the pathological causes of PD are still controversial, an increasing body of evidence indicates that programmed cell death, or apoptosis, is responsible for neuron loss in PD (Burke and Kholodilov 1998; Honig and Rosenberg 2000). In the present investigation, the 6-hydroxydopamine-induced apoptosis in the dopaminergic neuronal cells (SH-SY5Y cells) was used to study the molecular signalling mechanism that might be involved in the degeneration process of PD. Although some recent studies have shown that dopamine induces apoptosis in SH-SY5Y cells primarily by activation of the p38 kinase, cytochrome *c* release followed by caspase 9 and caspase 3 activation (Junn and Mouradian 2001), the ROS induced by 6-OHDA initiates the p38 signalling pathway leading to activation of both mitochondrial and extramitochondrial apoptotic pathways in MN9D dopaminergic neuronal cells (Choi *et al.* 2004), and the involvement of ASK1-p38 in anandamide- and NO-induced PC12 cell death (Sarker *et al.* 2003a,b), no precise study on the role of ASK1 in initiating p38/JNK signalling in the 6-OHDA-induced apoptosis of dopaminergic neuronal cells has been reported.

The observed disappearance of 6-OHDA-induced apoptotic DNA fragmentation in the cells over-expressing the kinase-dead mutant of ASK1 and the significant reduction of the 6-OHDA-induced apoptotic fraction in the cells with their ASK1 knocked down by ASK1 siRNA, as well as the

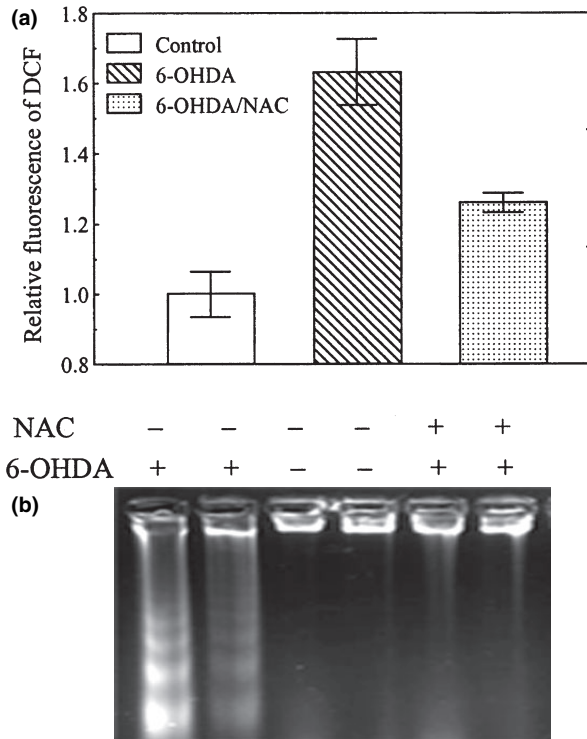


Fig. 6 Effect of NAC on 6-OHDA-induced ROS generation and apoptosis in SH-SY5Y cells. (a) The relative DCF-fluorescence intensity in lysates of the cells which were pre-incubated with or without 10 mM NAC for 4 h, exposed to 100 μ M 6-OHDA for 1 h, loaded with carboxy-H₂DCFDA and then incubated for 45 min. The carboxy-H₂DCFDA-loaded cells without 6-OHDA-treatment were used as control. Each value represents the mean SD of triplicate determinations. (b) The DNA fragmentation detected in the 6-OHDA-treated cells pre-incubated with and without 10 mM NAC for 4 h, then exposed to 100 μ M 6-OHDA for 2 h and incubated for 17 h in the presence of NAC of the same concentration.

observed 6-OHDA-induced activation of ASK1, p38 MAP kinase and JNK in wild type, but not in the cells transfected with either the kinase-dead mutant of ASK1 or the ASK1 siRNA, provide solid evidence for the involvement of ASK1, p38 and JNK in the 6-OHDA-induced apoptosis of the studied dopaminergic neuronal cells. These results also make it clear that ASK1, as upstream activator of the p38/JNK signalling cascade, initiates activation of p38 kinase and JNK. The latter kinases initiate apoptosis in the cells exposed to 6-OHDA. The observation that inhibition of p38 activity by either over-expression of the dominant-negative mutant of p38 or the selective inhibitor of the p38 kinase, SB203580, resulted in blockade of the apoptosis in the cells exposed to 6-OHDA also proved that the activated ASK1 must cause cell death through activation of the p38 kinase.

By demonstrating that 6-OHDA induces ROS generation and NAC, the inhibitor of ROS generation, suppresses the

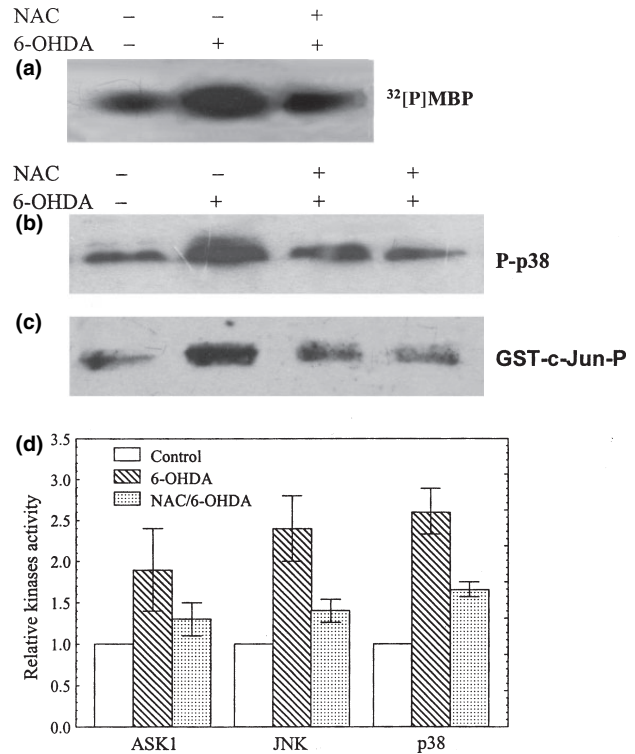


Fig. 7 The effect of NAC on 6-OHDA-induced activation of ASK1, p38 kinase and JNK in SH-SY5Y cells. The cells were pre-incubated with 10 mM NAC for 4 h, followed by exposure to 100 μ M 6-OHDA for 1 h. Untreated cells were used as control. (a) The activities of ASK1, p38 kinase and JNK in the cells pre-incubated with or without 10 mM NAC for 4 h, then exposed to 100 μ M 6-OHDA for 1 h [³²P]MBP, P-p38 and GST-c-Jun-P stand for the phosphorylated substrate of ASK1, phosphorylated p38 and the phosphorylated substrate of JNK, respectively. (b) The relative kinase activities in NAC-treated and -untreated cells calculated from the specified measurements in (a). Each value is the mean \pm SD of three independent determinations.

6-OHDA-induced apoptotic DNA fragmentation and activation of ASK1, p38 and JNK in the cells, the present investigation confirms a previous report that ASK1-p38 signalling can be activated by oxidative stress (Tobiume *et al.* 2002), and ASK1 activates the MKK3/MKK6-p38 MAP kinase pathways in various types of stress-induced apoptosis (Matsuzawa *et al.* 2002). Thus, the present study established that ROS generation initiates ASK1-p38/JNK signalling in the PD model system that uses 6-hydroxydopamine to induce cell death in dopaminergic neurons. To block the signalling at any step might prove to be beneficial for the treatment of PD disease.

It has been reported that, except for ASK1, the MAP kinase kinase kinases including MAPK/ERK kinase 4 (MEKK4; Takekawa and Saito 1998), transforming growth factor- β -activated kinase-1 (TAK-1; McDermott and O'Neill 2002), p21-activated kinase (PAK; Bagrodia *et al.* 1995), mixed lineage kinase-3 (MLK-3; Tibbles *et al.* 1996), and

neuronal symmetry-1 (NSY-1; Sagasti *et al.* 2001) are all potential upstream activators of p38 MAP kinase. Thus, one may expect that activation of some other upstream activator may partly be responsible for the 6-OHDA-induced activation of p38 kinase and JNK in the dopaminergic neuronal cells. To find out if ASK1 is totally responsible for the activation of p38 and JNK in 6-OHDA-stimulated dopaminergic neuronal cells, a possible way is to see if the 6-OHDA-induced activities of p38 and JNK are less suppressed than the activity of ASK1 in the cells by knocking down ASK1. Our results show that the three kinases were suppressed to almost the same extent (see Fig. 3c), suggesting that any possible contribution from another upstream regulator to activation of p38 and JNK could be ignored in the studied PD model.

As the kinase-dead mutant of ASK1 may interact with MKK3/6 or MKK4/7, it should be considered whether the mutant would affect the activation of p38 and JNK through any other MAPK kinase. For this reason, the suppression of the neurotoxin-induced activities of the p38 kinase and JNK in the cells over-expressing the kinase-dead mutant of ASK1 were compared with that in the cells having their ASK1 knocked down. The results show that either over-expression of the ASK1 mutant or knock-down of ASK1 leads to almost the same suppression of p38 and JNK activity. It may imply that the introduction of the kinase-dead mutant of ASK1 does not appear to make any perceptible extra effect on the activation of p38 and JNK besides reducing ASK1 activity. Considering the relatively large errors in the immunoblotting assay of kinase activity, we might conclude that p38 and JNK activation in the cells exposed to 6-OHDA very largely depends on the activation of ASK1, if a small detectable contribution from any other upstream activator could not be absolutely excluded. A previous study on the activation of JNK by axin also showed that the dominant-negative mutant of ASK1, ASK1(K709M), and TAK1, TAK1(K63W), did not appear to alter JNK activation mediated by axin, while the ATP binding-deficient, dominant-negative MEKK1, MEKK1(K1255M), reduced the axin-activated JNK by threefold (Zhang *et al.* 1999). Thus, it seems that a dominant-negative mutant may or may not affect downstream signalling through interaction with other members on the signalling pathway. It may depend on the type of mutant, the studied signalling pathway, stimuli and cell type.

Neurodegeneration is a multifactor process demanding different defence strategies. The identification of apoptosis as the loss of neurons allows the opportunity for additional preservative strategies and widens the time frame of the therapeutic intervention. Our observation that the ASK1-p38/JNK signalling pathway plays a critical role in 6-OHDA-induced apoptosis of the SH-SY5Y cells may be helpful in designing a future intervention strategy in the treatment of neuronal disorders.

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