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Perphenazine and trifluoperazine induce mitochondria-mediated cell death in SH-SY5Y cells

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Drug-induced parkinsonism has been associated with an increased risk for Parkinson's disease. Antipsychotic drugs have long been known to cause parkinsonian symptoms. However, it remains unclear whether antipsychotics can directly damage the nigrostriatal pathway. In the present study, we investigated the toxicity mechanism of two typical antipsychotics, perphenazine and trifluoperazine, in a human dopaminergic cell line, SH-SY5Y. Perphenazine and trifluoperazine induced mitochondrial damage as evidenced by fragmentation of mitochondria, activation of Bax, cytochrome *c* release and a decrease in cellular ATP level. In addition, activation of caspase-3 and apoptotic nuclei were observed following the drug treatment. However, pan-caspase inhibitor did not suppress the cell death induced by the antipsychotics, suggesting that the initiated apoptosis was possibly shifted to necrosis upon caspase inhibition. Damaged mitochondria may have induced oxidative stress since the drug-induced cell death was partially suppressed by an antioxidant. Taken together, our results suggest that perphenazine and trifluoperazine can induce apoptotic cell death in a dopaminergic cell line via mitochondrial damage accompanied by oxidative stress.

Keywords: perphenazine; trifluoperazine; antipsychotics; parkinsonism; mitochondria

1. Introduction

Many of the psychotropic drugs such as antipsychotics and antidepressants are known to have side effects of extrapyramidal or parkinsonian symptoms. This type of drug-induced parkinsonism (DIP) usually disappears following the discontinuation of the drugs. In some cases, however, the DIP persists and develops into progressive parkinsonism (Mena and de Yébenes 2006). It is possible that a patient with unrecognized parkinsonian symptoms has taken psychotropic drugs and is later diagnosed to have Parkinson's disease (PD) independently of DIP. However, the possibility cannot be ruled out that the long-term use of psychotropic drugs may have direct neurotoxicity on the nigrostriatal pathway. Neurotoxicity of atypical antipsychotics and antidepressants has been reported in cell culture models (Gil-Ad et al. 2001, 2006; Lirk et al. 2006). However, it remains to be investigated thoroughly whether the direct neurotoxicity of antipsychotics or antidepressants can be a causative factor for the pathogenesis of PD.

Perphenazine and trifluoperazine are typical antipsychotics of phenothiazine compounds used for the treatment of schizophrenia, psychosis, and anxiety (Nikam and Awasthi 2008). There have been opposing reports regarding the effect of these antipsychotics on the viability of cells in culture. It has been reported that trifluoperazine can inhibit MPT at lower concentrations (Broekemeier and Pfeiffer 1989). A possible cytoprotective effect of trifluoperazine via MPT inhibition has been demonstrated in cells damaged by oxidative stress or mitochondrial toxin (Lee et al. 2005; Tang et al. 2005). However, perphenazine and trifluoperazine at higher concentration have been shown to induce cell death in neuronal cell lines and primary cultured neurons (Gil-Ad et al. 2001, 2006). Trifluoperazine has been shown to induce mitochondrial permeability transition (MPT) and cytochrome *c* release from the isolated mitochondria (Cruz et al. 2010). In patients, antipsychotics have been reported to exert direct toxicities on many organs or cells, including liver and blood cells, in addition to the indirect systemic side effects such as endocrine disturbances, hypotension and seizures (Lader 1999; Flanagan and Dunk 2008). This suggests that the clinical dose of these drugs is high enough to exert cytotoxicity. More recently, Ho et al. (2011) reported that there is a correlation between long-term antipsychotic treatment and a decrease in brain tissue volume independently of brain changes due to schizophrenia. These reports validate the question of the possibility of direct neurotoxicity of antipsychotics leading to nigrostriatal damage.

Mitochondrial dysregulation is known as a common mechanism of death in both apoptosis and necrosis (Lemasters et al. 1998). Although the causal relationship between MPT and mitochondrial fragmentation

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remains rather unclear, mitochondrial fragmentation is often observed in both mitochondrial damage-induced apoptosis and necrosis (Cho et al. 2010). Although mitochondria continuously undergo fusion and fission even in a healthy state, perturbation of the balance in the dynamic remodeling of the mitochondria ultimately leads to compromise of their bioenergetic function (Cho et al. 2010). Mitochondrial dysregulation, including mitochondrial fragmentation and the resulting autophagic clearance, has been frequently observed in neurodegenerative disease such as Alzheimer's disease and PD (Cho et al. 2010). In cell culture and animal models of PD, both PD-related gene mutations and neurotoxins are known to induce mitochondrial damage (Büeler 2009).

Neurotoxins implicated in sporadic cases and animal models of PD include 1-methyl-4-phenylpyridinium (MPTP), 6-hydroxydopamine, paraquat, rotenone and maneb (Büeler et al. 2009). Use of pesticides has been associated with an increased risk factor for PD and its toxicity mechanism has been much studied in cell culture and animal models (Cicchetti et al. 2009). However, a recent study reported that the correlation between the history of psychotropic drug use and a later PD diagnosis is higher than that between pesticide exposure and PD (Dick et al. 2007). Therefore, it is necessary to examine whether psychotropic drugs such as antipsychotics or antidepressant can be a causative factor for the pathogenesis of PD. It has been shown that antipsychotics such as perphenazine and trifluoperazine induce cell death in cultured neurons (Gil-Ad et al. 2001, 2006). However, the mechanism or mode of cell death induced by these phenothiazine compounds is still unclear.

In the present study, we investigated how perphenazine and trifluoperazine induce cell death in SH-SY5Y human dopaminergic neuroblastoma. We present evidence that these drugs induce an apoptotic form of cell death via mitochondrial damage.

2. Materials and methods

2.1. Antibodies and reagents

Anti-active Bax and anti-active caspase-3 antibodies were purchased from Cell Signaling, anti-cytochrome *c* antibody was from Chemicon. Perphenazine and trifluoperazine were purchased from Sigma. MitoTracker Red 580 was from Invitrogen. Carbobenzoxy-valyl-alanyl-aspartyl[*O*-methyl]fluoromethylketone (zVAD-fmk) was from Bachem and DPQ (3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinoline) was from Calbiochem. All other reagents were purchased from Sigma unless stated otherwise.

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells were maintained in 100 mm diameter plastic cell culture dishes containing 10 ml of culture medium consisting of MEM and Ham's F12-K (JBI) supplemented with 10% fetal bovine serum (JBI) and 1% antibiotics plus antimycotics (JBI) in a 37°C CO_2 (5%) incubator. Medium was changed every 2 or 3 days and the cells were subcultured when the confluency reached 70–80%.

2.3. Cell viability assay

To measure the viability, cells were plated on 96-well plates and treated with drugs for various time periods. Then the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay reagent (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) for 1 hr according to the manufacturer's protocol. The plates were then read at 490 nm using a microplate reader (Bio-rad).

2.4. Immunocytochemistry

Immunocytochemistry was performed as described by Lim et al. (2008). For mitoTracker staining, cells in culture were incubated with mitoTracker Red 580 (100 nM) for 30 min. After washing, the cells were fixed with 4% paraformaldehyde. The immunostained or mitoTracker-stained cells were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Slowfade Gold antifade reagent with DAPI, Invitrogen). The samples were examined under a fluorescence microscope (Axioplan 2, Zeiss) or a confocal microscope (Leica TCS SP5, Leica).

2.5. Measurement of ATP level

Cellular ATP levels were measured using the Luminescence ATP Detection Assay System (PerkinElmer). The cells were incubated with or without drugs for various time points. Then 100 μl of the ATPlite 1 step reagent was added to the wells and the cell plates were orbitally shaken for 3 min. The plates were read for the emitted luminescence using a microplate luminometer (GloMax 9100-100, Promega).

2.6. Statistics

For the statistical analysis, all the experiments were repeated at least three times. The results were expressed as means $\pm$ SD of at least three independent experiments, unless stated otherwise. Paired data were evaluated by ANOVA.

3. Results

3.1. Perphenazine and trifluoperazine induced cell death and mitochondrial damage

To examine whether perphenazine and trifluoperazine induce cytotoxicity in human dopaminergic neuroblastoma SH-SY5Y, the cells were incubated with varying concentrations (10–100 μM) of the phenothiazine antipsychotic drugs and the cell viability was measured by MTS assay at 48 hr after the incubation. As shown in Figure 1A, both of the drugs induced about 80% cell death at a concentration of 25 μM.

To gain insight into the modality of cell death induced by perphenazine and trifluoperazine, mitochondrial morphology was examined following the drug treatment. As shown in Figure 1B, the cells damaged by perphenazine or trifluoperazine showed fragmented mitochondria as early as 4 hr after the drug treatment when the cell death was not evident. Many of the fragmented mitochondria were doughnut-shaped. This result suggests that perphenazine and trifluoperazine induced mitochondrial damage at an early phase of their action to induce cell death. Mitochondrial insertion and activation of Bax has been shown to be an event common to caspase-dependent and caspase-independent cell death (Lemasters et al. 1998). Since we observed massive mitochondrial fragmentation in the early phase of the damage, we examined whether Bax activation follows after the antipsychotic treatment. As shown in Figure 1C, an active form of Bax began to be detected in the damaged cells at 8 hr after perphenazine or trifluoperazine treatment. It has been well documented that mitochondrial fragmentation results in the release of mitochondrial intermembrane proteins including cytochrome *c* to induce caspase-dependent apoptosis (Lemasters et al. 1998). To monitor the possible change in the subcellular localization of cytochrome *c*, the cells were incubated with perphenazine or trifluoperazine for various time points (4, 8, 16 and 32 hr) and then the immunostaining for cytochrome *c* was performed. As shown in Figure 1D, cytoplasmic immunoreactivity to cytochrome *c* was detected in the cells treated with perphenazine or trifluoperazine at 16 hr after the incubation. These results suggest that the antipsychotics induced mitochondrial damage and permeabilization of the mitochondrial outer membranes.

3.2. Perphenazine and trifluoperazine induced caspase-3 activation and a decrease in cellular ATP level

Since cytochrome *c* release was detected following the drug treatment, it is highly possible that the drugs induce caspase-dependent apoptosis. To examine this possibility, the cells treated with the two antipsychotics were immunostained with antibodies detecting the activated form of caspase-3. As shown in Figure 2A and B, both perphenazine and trifluoperazine induced activation of caspase-3 as evidenced by immunoreactivity against active caspase-3. As shown in the inset in Figure 2A, many of the active caspase-3-positive cells showed fragmented or condensed nuclei of typical apoptotic morphology.

Since we observed signs of mitochondrial damage such as mitochondrial fragmentation, Bax activation and cytochrome *c* release, we monitored the level of cellular ATP following the drug treatment. As shown in Figure 2C, the level of cellular ATP began to decrease at 8 hr and dropped to about 50% at 16 hr after the drug treatment. These results suggest that perphenazine or trifluoperazine induced mitochondrial damage, leading to the decrease in cellular ATP level.

3.3. The cell death induced by perphenazine or trifluoperazine was partially suppressed by antioxidant but not by pan-caspase inhibitor

To examine whether the cell death induced by perphenazine or trifluoperazine is dependent on caspases, the cells were preincubated with the irreversible pan-caspase inhibitor zVAD-fmk, followed by incubation with the antipsychotics. Interestingly, the pan-caspase inhibitor could not suppress the cell death induced by perphenazine or trifluoperazine (Figure 3A). A potent inhibitor of poly(ADP-ribose) polymerase-1 did not suppress the cell death either (Figure 3A). This result suggests that perphenazine or trifluoperazine induced caspase-mediated apoptotic cell death but apoptosis may have been shifted to a necrotic form of cell death upon caspase inhibition.

It has been well documented that damaged mitochondria can produce toxic levels of reactive oxygen species and induce oxidative stress (Büeler 2009). Thus we examined whether the cell death induced by perphenazine or trifluoperazine can be suppressed by antioxidant. As shown in Figure 3B, the antioxidant Trolox, a water-soluble derivative of vitamin E, partially suppressed the cell death induced by perphenazine or trifluoperazine. This result suggests that the mitochondrial damage induced by the antipsychotics results in oxidative stress, which can contribute to the cell death induced by these drugs.

4. Discussion

In the present study, we show that typical antipsychotics of phenothiazine compounds, perphenazine and trifluoperazine, induced mitochondria-mediated apoptotic cell death in SH-SY5Y. Although the drugs induced extensive mitochondrial fragmentation, activation of Bax, cytochrome *c* release, and caspase-3 activation, the

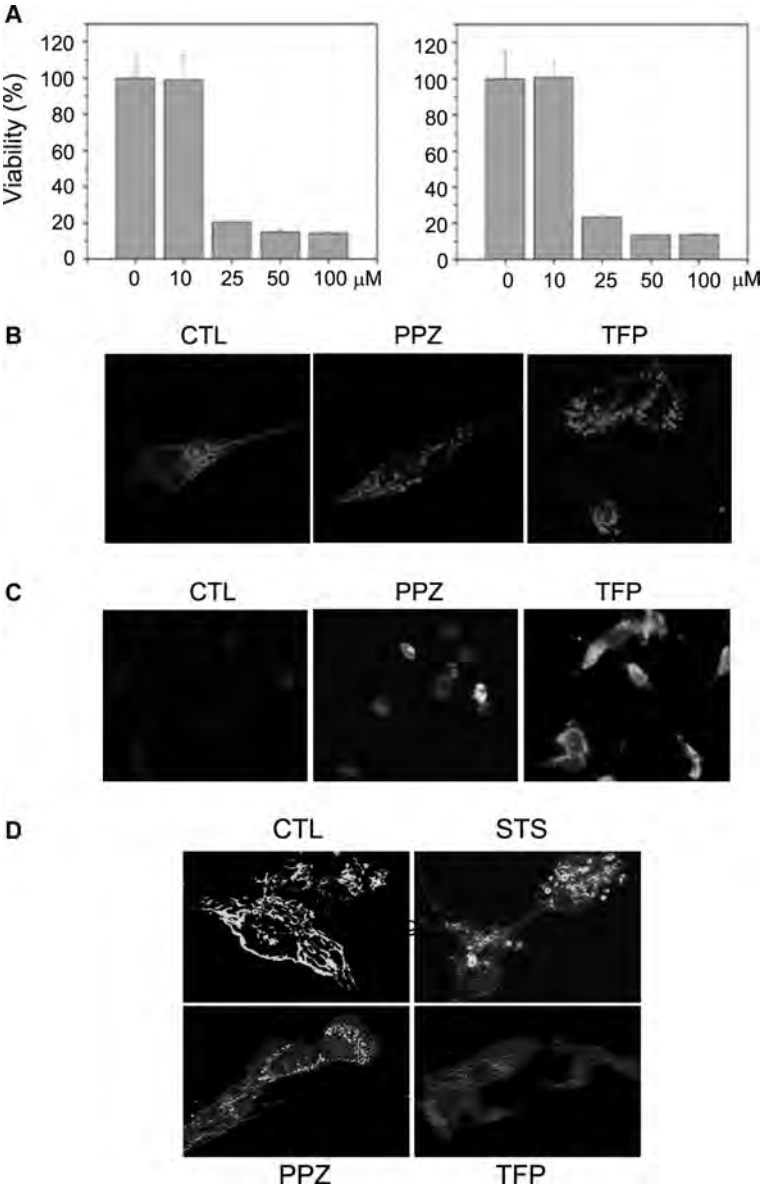

Figure 1. Perphenazine and trifluoperazine induced cell death and mitochondrial damage. (A) Cell viability was examined by MTS assay following a treatment of the SH-SY5Y cells with perphenazine (PPZ) or trifluoperazine (TFP) at the indicated concentrations for 48 hr ($n = 4$). (B–D) To examine whether perphenazine or trifluoperazine induces mitochondrial damage, the cells were first incubated with the indicated drugs (25 μM) or vehicle (control, CTL) for various time points (4, 8, 16, and 32 hr). (B) To examine morphological changes in mitochondria, the cells treated with the compounds were further incubated with mitoTracker Red 580 for 20 min. The cells incubated with the drugs for 4 hr are shown. (C) The cells incubated with the indicated drugs were fixed and processed for active Bax immunostaining. The cells were mounted and examined under a conventional fluorescence microscope. The cells at 8 hr incubation are shown. (D) The cells were treated with the drugs and then processed for immunostaining for cytochrome *c*. The cells at 16 hr incubation are shown. The cells incubated with staurosporine (STS, 200 nM) for 8 hr were also stained for cytochrome *c* as a positive control. The stained cells were examined under a confocal microscope.

cell death induced by these drugs was not suppressed by potent and selective inhibitors of caspases. Our results imply that perphenazine and trifluoperazine can induce apoptotic cell death via mitochondrial damage, but inhibition of caspases possibly shifted the cell death mode to necrosis.

Previously, it was reported that trifluoperazine can inhibit MPT at a concentration range of 0.1–1 μM (Broekemeier and Pfeiffer 1989). In this relatively low concentration range trifluoperazine inhibited MPT, possibly by acting as a calmodulin antagonist (Lee et al. 2005) or mitochondrial phospholipase A2 (PLA2)

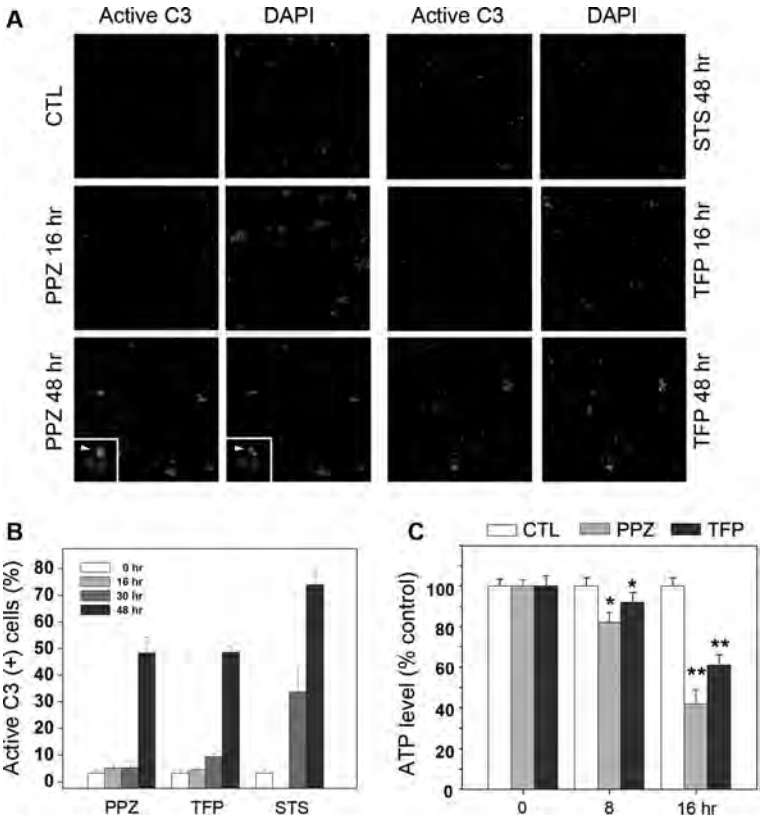

Figure 2. Perphenazine and trifluoperazine induced caspase-3 activation and a decrease in cellular ATP level. (A) To examine the mode of cell death, SH-SY5Y cells were incubated with perphenazine (PPZ, 25 μM) or trifluoperazine (TFP, 25 μM) for 16, 30, and 48 hr and then immunostained for active caspase-3 (C3). The cells at 16 and 48 hr treatment are shown. The cells treated with staurosporine (STS, 200 nM) were included as a positive control. Insets show typical apoptotic nuclei of the cells treated with the antipsychotics. (B) The rate of active caspase-3-positive cells was determined from the experiments shown in A. The rate was calculated by counting the number of active caspase-3-positive cells over the number of total cells from five different fields. (C) The cellular level of ATP was measured from the cells incubated with the drugs for 8 and 16 hr by ATPlite assay. Data are presented as means ± SD of three independent experiments, each performed in duplicate (Control vs. PPZ or TFP, $*P < 0.05$; $**P < 0.001$).

inhibitor (Broekemeier et al. 1985). Therefore, trifluoperazine at this concentration range has been shown to partially protect cells from oxidative damage (Liu et al. 2011). In contrast, it has been recently shown that a higher concentration of trifluoperazine can induce MPT in isolated mitochondria and apoptosis of melanoma cells (Gil-Ad et al. 2006; Cruz et al. 2010). In the report by Cruz et al. (2010), it has been shown that phenothiazine derivatives such as trifluoperazine and thioridazine induced swelling of isolated mitochondria and cytochrome *c* release. Concordantly, we observed neurotoxicity at a similar concentration range and we could detect cytochrome *c* release following perphenazine or trifluoperazine treatment in the cultured cells. Earlier studies showed that the trifluoperazine can induce cell death in cultured neurons (Zhelev et al. 2004; Gil-Ad et al. 2006). However, the mode of cell death induced by perphenazine or trifluoperazine was not thoroughly investigated in the previous studies. In our observations, the two antipsychotics induced typical mitochondria-mediated apoptosis. Interestingly, however, the cell death induced by trifluoperazine or perphenazine was not inhibited by a pan-caspase inhibitor. It is possible that perphenazine or trifluoperazine initiated apoptotic cascades but inhibition of caspases by zVAD-fmk switched the apoptosis to necrosis. Another possibility is that the drugs induced a mixed form of cell death, i.e. apoptosis plus necrosis initially, but the caspase inhibition shifted the mode of cell death to necrosis. The decrease in cellular ATP level and a possible oxidative stress might have made it easier to shift the cell death program towards necrotic cell death.

In addition to inducing MPT by inhibiting calmodulin or PLA2, trifluoperazine was shown to induce autophagy (Zhang et al. 2007). However, it has not

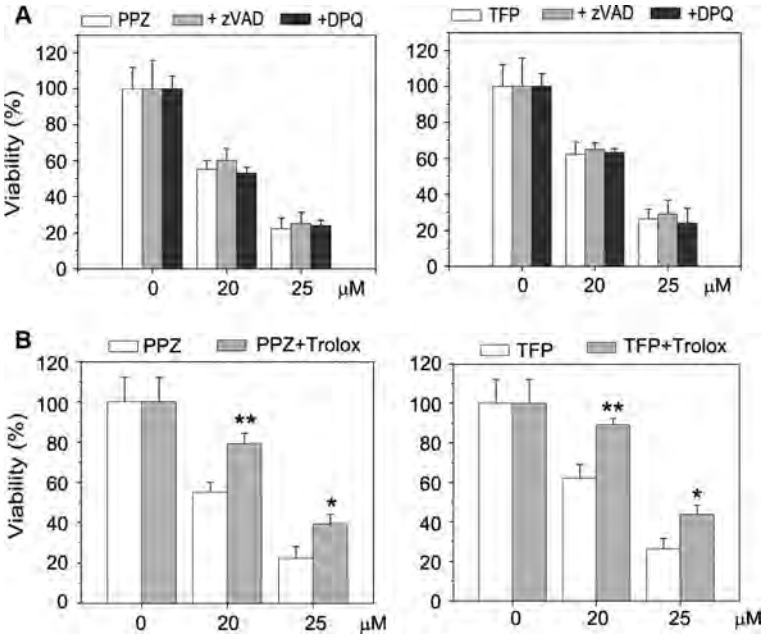

Figure 3. The cell death induced by perphenazine or trifluoperazine was partially suppressed by antioxidant but not by pan-caspase inhibitor. (A) To examine whether the cell death induced by perphenazine or trifluoperazine can be suppressed by caspases inhibition, the cells were pretreated for 1 hr with pan-caspase inhibitor zVAD-fmk (50 μM) or poly(ADP-ribose) polymerase-1 inhibitor DPQ (40 μM) and then incubated with perphenazine (PPZ) or trifluoperazine (TFP) at the indicated concentrations. Cell viability was measured by MTS assay at 48 hr after the incubation. (B) To examine whether antioxidants can suppress the cell death induced by the antipsychotics, SH-SY5Y cells were preincubated for 30 min with Trolox (100 μM) and then treated with the drugs at the indicated concentrations for 48 hr. Then the viability of the cells was measured by MTS assay (PPZ or TFP vs. plus Trolox, $*P < 0.05$; $**P < 0.001$).

been reported whether autophagy itself induced by trifluoperazine can be a causative factor for the cytotoxicity. It has been well documented that mitochondrial fission can induce autophagy, i.e. mitophagy (Cho et al. 2010). Since we observed massive mitochondrial fragmentation at a very early phase of the antipsychotic-induced toxicity, it is highly plausible that autophagy follows the mitochondrial fragmentation. In earlier reports, the PD neurotoxin 6-hydroxydopaine was shown to induce mitophagy (Gomez-Lazaro et al. 2008). However, inhibition of autophagy in the cells with fragmented mitochondria exacerbated the cytotoxicity induced by the mutations of the PD-related genes (Dagda et al. 2009). Therefore, it is unlikely that the autophagy itself is a causative factor for the cytotoxicity induced by trifluoperazine.

Mitochondrial dysfunction has been considered as a common pathogenic mechanism in both neurotoxin-induced and inherited PD. Mitochondrial and oxidative toxins such as MPTP, 6-hydroxydopamine, paraquat, rotenone and maneb reproduce important features of PD in rodent and cell culture models (Büeler 2009). Accumulating evidence suggests that exposure to pesticides such as paraquat, rotenone and maneb is causally related to the pathogenesis of PD (Büeler 2009). In addition to the pesticides, use of psychotropic drugs such as antidepressant, hypnotic or anxiolytic drugs has been recently shown to be associated with increasing risk of PD (Dick et al. 2007). Although the correlation between risk of PD and use of antipsychotics has not been studied, antipsychotics are well known to show DIP (Mena and de Yébenes 2006). Considering the incidence with which DIP turns into permanent and progressive parkinsonism, the toxicity of antipsychotics towards the nigrostriatal pathway needs more thorough investigation. Our present result shows that two typical phenothiazineantipsychotics, perphenazine and trifluoperazine, can induce cell death via mitochondrial damage in the cultured dopaminergic cell line. This raises the possibility that extended use of these antipsychotics may result in permanent damage to the nigrostriatal pathway. Moreover, it may make the dopaminergic neurons affected in PD more vulnerable to other toxic insults. Further studies using animal models are necessary to answer the question regarding the causal relationship between the use of psychotropic drugs and the pathogenesis of PD.

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