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ORIGINAL INVESTIGATION

Neuroprotection of paliperidone on SH-SY5Y cells against β-amyloid peptide$_{25-35}$, *N*-methyl-4-phenylpyridinium ion, and hydrogen peroxide-induced cell death

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Abstract
Rationale Antipsychotic drugs (APDs) were widely used in treating schizophrenia. Some APDs were reported to have neuroprotective effects against neurotoxicants in the cell level.
Objectives Thus, one typical APD (haloperidol) and three atypical APDs (paliperidone, olanzapine, and risperidone) were tested whether they provide neuroprotection against stressor-induced cell death of SH-SY5Y.
Methods Hydrogen peroxide, *N*-methyl-4-phenylpyridinium ion, and β-amyloid peptide were used to treat cells with or without preconditioning by APDs; cell survival and indicators of oxidative stress were measured, respectively.
Results Paliperidone has the lowest baseline cytotoxicity compared with other APDs at 24 h; in addition, the paliperidone group showed a better survival than the other APD groups ($P<0.05$). In stressor challenging, with a fixed concentration of stressors, olanzapine provided the best neuroprotection at 100 μM against Aβ_{25-35} and MPP^+ ($P<0.05$). In contrast, paliperidone works finely at low concentrations (10 and 50 μM) against Aβ_{25-35} and MPP^+ and solely protected SH-SY5Y from hydrogen peroxide. At 100 μM, paliperidone completely diminished cell reduction induced by different stressors, regardless of their dosages. Paliperidone was demonstrated with a higher oxidative stress-scavenging properties than other APDs in several aspects, such as generated bulk glutathione, low HNE, and protein carbonyl productions. Contradictorily, olanzapine, at 24 h, also enhanced HNE and protein carbonyl productions, which may underlie its induced cytotoxicity.
Conclusions Different APDs exhibit variations against different stressors. Paliperidone might be useful not only in alleviating oxidative stress induced by Aβ_{25-35} and MPP^+ but also in providing neuroprotection against hydrogen peroxide.

Keywords Atypical antipsychotics · β-amyloid · *N*-methyl-4-phenylpyridinium ion · Hydrogen peroxide · Cell death · Neuroprotection

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Introduction

Schizophrenia is a severe chronic debilitating disease that affects approximately 1% of the world's population (Frangou and Murray 1996). It may be a heterogeneous disorder with considerable variations in symptoms; the disease etiology has been debated up to this day (Tandon et al. 2008). Medication for schizophrenia relies on antipsychotic drugs (APDs) which are simply divided into two groups, typical and atypical, based on the characteristics of D2 receptor occupancy (Altar et al. 1986; Scherer et al. 1994; Goyer et al. 1996). Although the classic neuroleptics, such as haloperidol, produce a marked reduction in the

positive symptoms of schizophrenia, they do not improve the negative symptoms, such as apathy, confusion, and social withdrawal, nor do they alter the progressive deterioration in the mental abilities of the patient (Lindenmayer et al. 2007). On the other hand, the atypical APDs are reported to have a superior therapeutic efficacy in treating negative symptoms/treatment-resistant schizophrenia and have a low incidence of extrapyramidal side effects (EPS) and a reduced capacity to induce catalepsy in a rodent model (Arnt 1995; Kinon and Lieberman 1996; Shadach et al. 2000). In clinical practice, early and prolonged intervention with atypical APDs is reported to improve the long-term outcome of schizophrenia (Wyatt and Henter 1998); however, the mechanisms of their action are uncertain. Therefore, determining the genetic factors that contribute to the different therapeutic actions of these APDs is important for antipsychotic medication. To date, the different drug responses of APDs have been documented to link to the function of the cytochrome gene (Alenius et al. 2008), neurotransmitter receptors (Mossaheb et al. 2010), and P-glycoprotein (multiple-drug-resistant genes; Wang et al. 2004). Moreover, the neuroprotective effect of APDs is also believed to be important in determining their therapeutic actions.

A growing body of evidence suggests that oxidative stress may play a role in the neurodegeneration and disease progression of schizophrenia (Carter 2006; Fendri et al. 2006; Ng et al. 2008). It has been suggested that in patients with schizophrenia, plasma antioxidants (albumin, bilirubin, uric acid) are reduced, indicating a perturbation in the antioxidant defense system (Reddy et al. 2003). On the other hand, the neuroprotection of APDs is suspected to be linked, in part, to the attenuation of oxidative stress. It has been demonstrated that haloperidol causes oxidative stress via the induction of intracellular peroxide accumulation followed by the depletion of intracellular glutathione in clonal hippocampal HT22 cells and that vitamin E could protect against haloperidol excitoxicity in cultured cells (Behl et al. 1995). In contrast to haloperidol, the atypical APDs clozapine, quetiapine, and risperidone protect PC12 cells from death induced by serum withdrawal via regulating the expression of copper/zinc superoxide dismutase (SOD1) and p75 neurotrophin receptor (p75NTR) mRNA (Bai et al. 2002). Besides, some studies have demonstrated that clozapine, olanzapine, and quetiapine can protect PC12 from some types of cell death induced by hydrogen peroxide, β-amyloid peptide, and MPP^+ (Wei et al. 2003a; Wang et al. 2005; Magliaro and Saldanha 2009; Qing et al. 2003). Recently, clozapine was shown to protect PC-12 cells from death due to oxidative stress induced by hydrogen peroxide in a cell type-specific manner and through the inhibition of extracellular signal-regulated kinase (ERK) phosphorylation. Only clozapine exhibits a cell type-specific protective effect against oxidative stress in PC12, but not in HEK293 kidney epithelial cells, SH-SY5Y neuroblastoma cells, and rat primary cortical neurons. One possible reason for this effect is that, in addition to improving positive and negative symptoms, atypical APDs provide some degree of neuroprotection, specifically in terms of resisting oxidative stress, by mechanisms that have begun to be unraveled but still largely remain unknown (Krebs et al. 2006). Among the APDs, paliperidone was reported to have better efficacy and tolerability than other atypical APDs in the acute and long-term treatment of schizophrenia (Fowler et al. 2008). However, paliperidone has not been determined for its neuroprotective effect on neuronal cell via cell base assay. A recent study showed that paliperidone enhanced the cell survival of SH-SY5Y and U937 cells (Schmidt et al. 2010), two cell lines separately representing neural and immune cell types which are usually involved in the pathogenesis of schizophrenia. In an ex vivo study through a proteomic approach, paliperidone was proposed to have a model of action similar to lithium and valproic acid due to stimulated similar protein expression profile in treating the synaptoneurosomal-enriched prefrontal cortex. These findings concluded that the pathways affected in common by paliperidone and lithium were oxidative phosphorylation, electron transport, carbohydrate metabolism, and postsynaptic cytokinesis, implicating the effect of these drugs involved in signaling pathways, energy metabolism, and synaptic plasticity (Corena-McLeod Mdel et al. 2008).

Therefore, we decided to use one typical APD, haloperidol, and three atypical APDs, comprising olanzapine, risperidone, and paliperidone, in a test of their neuroprotective effects against different stressors (MPP^+, $A\beta_{25\text{-}35}$, and hydrogen peroxide) in SH-SY5Y cells. The results may provide further information regarding which APD exerts the best neuroprotective effect on SH-SY5Y under stressor stimuli aimed at mimicking several models of psychosis. The results may also contribute to antipsychotic therapy and provide further understanding of the different actions of currently used APDs.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, and fetal bovine serum were purchased from Gibco-BRL, Life Technologies (Grand Island, NY, USA). DMEM/F12 media, non-essential amino acids, and penicillin/streptomycin were purchased from Invitrogen; 30% H_2O_2 stabilized in water (8.82 M) from Acros Organic (#41188-5000); MPP^+ iodide (100 mm in sterile saline) from Sigma-Aldrich (St. Louis, MO, USA), and the $A\beta_{25\text{-}35}$ from Oncogene Research Products (La Jolla, CA, USA). The WST-1 reagent

was obtained from Roche (Mannheim, Germany). All other chemicals were purchased from commercial sources.

Cell culture and treatments

A SH-SY5Y cell line (human dopaminergic, neuroplastoma) was purchased from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute (BCRC, Hsinchu, Taiwan) and was cultured in a minimum essential medium/nutrient mixture, Ham's F-12 (1:1) medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 Ag/ml), and L-glutamine (2 mM). The cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

To determine the protective effect of APDs, cells were initiated for culturing in the presence or absence of olanzapine (10, 50, 100, or 200 μM); paliperidone (10, 50, or 100 μM); risperidone (10, 50, or 100 μM); or haloperidol (10, 50, or 100 μM) for 24 h. Afterward, to test the effects of different stressors, DMEM was replaced with a serum-free DMEM/high-glucose medium containing various concentrations (0, 0.01, 0.1, 1, 10, 20, 40 μM) of $A\beta_{25\text{-}35}$, MPP^+ (5, 12.5, 25, 50, or 100 μM) and hydrogen peroxide (0, 100, 200, or 400 μM), and the cells were cultured for another 24 h. Control cells were cultured for 48 h with neither different stressors nor APDs. The different stressors were prepared in distilled water and the stock solution (1 mM) stored in a −80°C freezer for further use. Cell survival was determined by the WST-1 assay. To determine gene expression consequent to stressor challenging, SH-SY5Y cells were seeded in 10-mm^2 Petri dishes and treated and/or untreated with various concentrations of atypical APDs prior to stressor exposure. The cultures were harvested at 48 h and also assayed for cell survival.

Cell viability assay

SH-SY5Y cells were pre-incubated with various concentrations (0–100 μM) of APDs (haloperidol, risperidone, paliperidone, and olanzapine) for 24 h and then were exposed to various concentrations of $A\beta_{25\text{-}35}$, superoxide, and MPP^+ in the presence of APDs for 24 h. All APDs were dissolved in 20% acetic acid and diluted with 9 volumes of DMEM to a concentration of 5 mM, respectively. Immediately before use, the solutions were diluted into various concentrations with DMEM. The cell viability assay was carried out using a WST-1 reagent to identify the activation of mitochondrial dehydrogenase in SH-SY5Y cells. For the experiments of establishing 96-h survival curve, the cells were seeded at the density of 2.5×10^4 cells/well in six-well clustered plates and harvested at the 48-, 72-, and 96-h time points, which had been pre-incubated with various concentrations (0, 50, and 100 mM) of APDs (haloperidol, risperidone, paliperidone, and olanzapine). For stressor challenging experiments, the cells were seeded at the density of 2.5×10^4 cells/well in six-well clustered plates and harvested for WST-1 assay at the 0-, 8-, 12-, and 24-h time points, into which 10 μl/well of the WST-1 cell proliferation reagent (Roche Applied Science, Mannheim, Germany) was added and further incubated for an additional 1–2 h. The formazan colorimetric signal was measured using an Infinite M200 microplate reader (TECAN, Durham, NC, USA) at a wavelength of 440 nm with a reference wavelength of 600 nm. Moreover, the samples' OD values were normalized by that of the controls and indicated as percentages. The control was defined as cells not exposed to any stressor and preconditioned with APDs.

Glutathione, HNE, and protein carbonyl assay

For detecting the baseline level of oxidative stress indicators triggered by each APD, SH-SY5Y cells were pre-incubated with 100 μM of APDs (haloperidol, risperidone, paliperidone, and olanzapine) for 24 h and then assayed for glutathione, HNE, and protein carbonyls directly. For clarification of the abilities of each APD in modulating the level of oxidative stress indicators after treatments with different stressors, the cells were preconditioned with APDs for 24 h and then were exposed to various concentrations of $A\beta_{25\text{-}35}$, superoxide, and MPP^+ in the presence of APDs for another 24 h. Finally, the cells were harvested for detecting the cellular level of each oxidative stress indicator, respectively. The total glutathione assay was carried out using a glutathione assay reagent (Sigma-Aldrich) which measured the reduced GSH level using a kinetic assay. For detecting the glutathione content of the sample, the sample is first deproteinized with the 5% 5-sulfosalicylic acid solution and then the kinetic assay in which catalytic amounts of glutathione cause a continuous reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid to 5-thio-2-nitrobenzoic acid (TNB). The oxidized glutathione formed is recycled by glutathione reductase and NADPH. The product, TNB, is assayed colorimetrically at 412 nm. The HNE assay was performed using Oxiselect HNE-His Adduct ELISA kit (Cell Biolabs Inc., San Diego, CA, USA), an enzyme immunoassay, in which the quantity of the HNE-His adduct in protein samples is determined by comparing its absorbance at 450 nm with that of a known HNE–bovine serum albumin (BSA) standard curve. The protein carbonyl assay was done using an Oxiselect protein carbonyl ELISA kit (Cell Biolabs Inc.) in which the quantity of protein carbonyls in protein samples is determined by comparing its absorbance at 450 nm with that of a known reduced/oxidized BSA standard curve. All procedures of detecting the oxidative stress-related molecule within cells were according to the manufacturer's protocol.

Statistical analysis

All quantitative results were expressed as means±SEM. For two-group comparisons with continuous data, the Student's *t* test or nonparametric Mann–Whitney *U* test was used to discriminate the significant intergroup differences. For multiple group comparisons, the significance of differences was determined by one- or two-way ANOVA, followed by Dunn's (with the Bonferroni correction) post hoc test for multiple comparisons. The Generalized Equation Estimation-I was applied for analyzing the repeat measurement data. A values of P <0.05 was regarded as statistically significant.

Results

The neuroprotective effects of haloperidol, paliperidone, olanzapine, and risperidone in concentrations of 0, 10, 50, and 100 μM on the cell survival of a human neuroblastoma SH-SY5Y cell line were examined using different stressors, including MPP^+, $A\beta_{25\text{-}35}$, and hydrogen peroxide. At first, the cytotoxicity of four APDs were determined by time course (up to 96 h) treating SH-SY5Y with different concentrations of APDs (0–100 μM). As per the results shown in Fig. 1, at 24 h, palipleridone did show a minor cytotoxicity against SH-SY5Y, whereas haloperidol exhibited a higher cytotoxicity across different concentrations compared with other APDs (P<0.05). At 10 μM, paliperidone demonstrated a low cytotoxicity, followed by risperidone, olanzapine, and haloperidol (Fig. 1a). At 50 μM, olanzapine caused less cell reduction compared with the other APDs, followed by paliperidone, risperidone, and haloperidol (Fig. 1b). Contradictorily, at 100 μM, olanzapine was dramatically down as to cause an almost half cell death, and in this concentration point, the paliperidone group showed the best cell viability, followed by risperidone, olanzapine, and haloperidol (Fig. 1c). Basically, all APDs retain cytotoxicity against SH-SY5Y cells, gradually enhanced by concentrations of the drugs; however, the cytotoxicity may be counteracted by several unknown mechanisms, which resulted in re-growth back to the original seeded levels. After preconditioning SH-SY5Y cells with four APDs for 48 h, the variations on cell viability with respect to each paired APD were less distinguishable; therefore, we choose 24 h as a time point to assay the neuropotection of APDs against stressor-induced cell death without ignoring the baseline cytotoxicity of each APD. At 48 h, risperidone somehow has a minor cytoxicity to SH-SY5Y cells, followed by palperidone, haloperidol, and olanzapine (P<0.05), especially at concentrations of 50 and 100 μM in the treatment group (P<0.001; Fig. 1b, c). However, at 96 h, the cell survival of risperidone was significantly lower than that of the other APD groups. Cells treated with paliperidone almost re-grow to the original seeding number of the cells at concentrations of 10 and 50 μM (97.3–101.6%); in addition, cell survival was better than the other groups (P<0.05; Fig. 1a, b), although the trends of cytotoxicity of four APDs did not significantly vary with the concentration change. However, at 48 h, APDs, except olanzapine, all demonstrated a slight better survival corresponding to increased concentrations. Thus, the concentration of APDs for preconditioning was determined as 100 μM. All APDs, in addition to their cytotoxicity, probably exert a neurotrophic effect to the SH-SY5Y, even though this effect differs in each APD. Subsequently, the cytotoxicity of the three different stressors toward SH-SY5Y was determined in a dose-dependent manner. The cytotoxicity was enhanced, causing 37.9–49.2% of cell death, corresponding to concentrations of 0.01–40 μM of $A\beta_{25\text{-}35}$ (Fig. 2a). Approximately 52.1–64% of cell death corresponded to concentrations of 5–100 μM MPP^+ (Fig. 2b). Besides, a 32.3–73.1% cell death could be triggered by adding 100–400 μM hydrogen peroxide into the culturing medium (Fig. 2c). In brief, the cytotoxicity of hydrogen peroxide was highest at a concentration of 400 μM (which killed 73.1% of cells), followed by MPP^+ at 100 μM (killing 64% of cells), and $A\beta_{25\text{-}35}$ at 40 μM (killing 49.2% of cells; Fig. 2). The three stressors could cause high to modest cell death of SH-SY5Y, and no significant difference could be found between the survival curves of any two stressors (P>0.05, data not shown). Given the results of the cytotoxicity assay, we chose the highest concentration of each stressor for some further neuroprotection experiments in which 40 μM of $A\beta_{25\text{-}35}$, 100 μM of MPP^+, and 400 μM of hydrogen peroxide were used. However, the other concentration groups of the three different stressors still performed synchronously.

In the WST-1 assay, paliperidone and haloperidol showed significant neuroprotection at a low concentration (10 μM) against $A\beta_{25\text{-}35}$-induced cell death compared with the vehicle control group (pal: P<0.003, hal: P<0.0001). When the concentration of APDs was adjusted up to 50 and 100 μM, all four APDs were observed to have a neuroprotective effect on SH-SY5Y under $A\beta_{25\text{-}35}$ stimuli (P< 0.05). In the $A\beta_{25\text{-}35}$ challenging model, olanzapine and resperidone provided neuroprotection in a concentration-dependent manner; however, paliperidone and haloperidol also revealed a mild and relatively strong cytotoxic effect on SH-SY5Y, respectively (Fig. 3a). The effects of $A\beta_{25\text{-}35}$ on cell viability were completely reversed by paliperidone at 10 μM, which increased cell viability to a maximum of about 30% for the original seeded cells and about 156% for the vehicle control, where the maximum for haloperidol at 10 μM was about 114% of the vehicle control. In contrast, both olanzapine and risperidone worked best at 100 μM, which diminished the toxic effect of $A\beta_{25\text{-}35}$ and restored cell viability up to 151% and 104% of the vehicle control, respectively. In the MPP^+ challenging model, paliperidone,

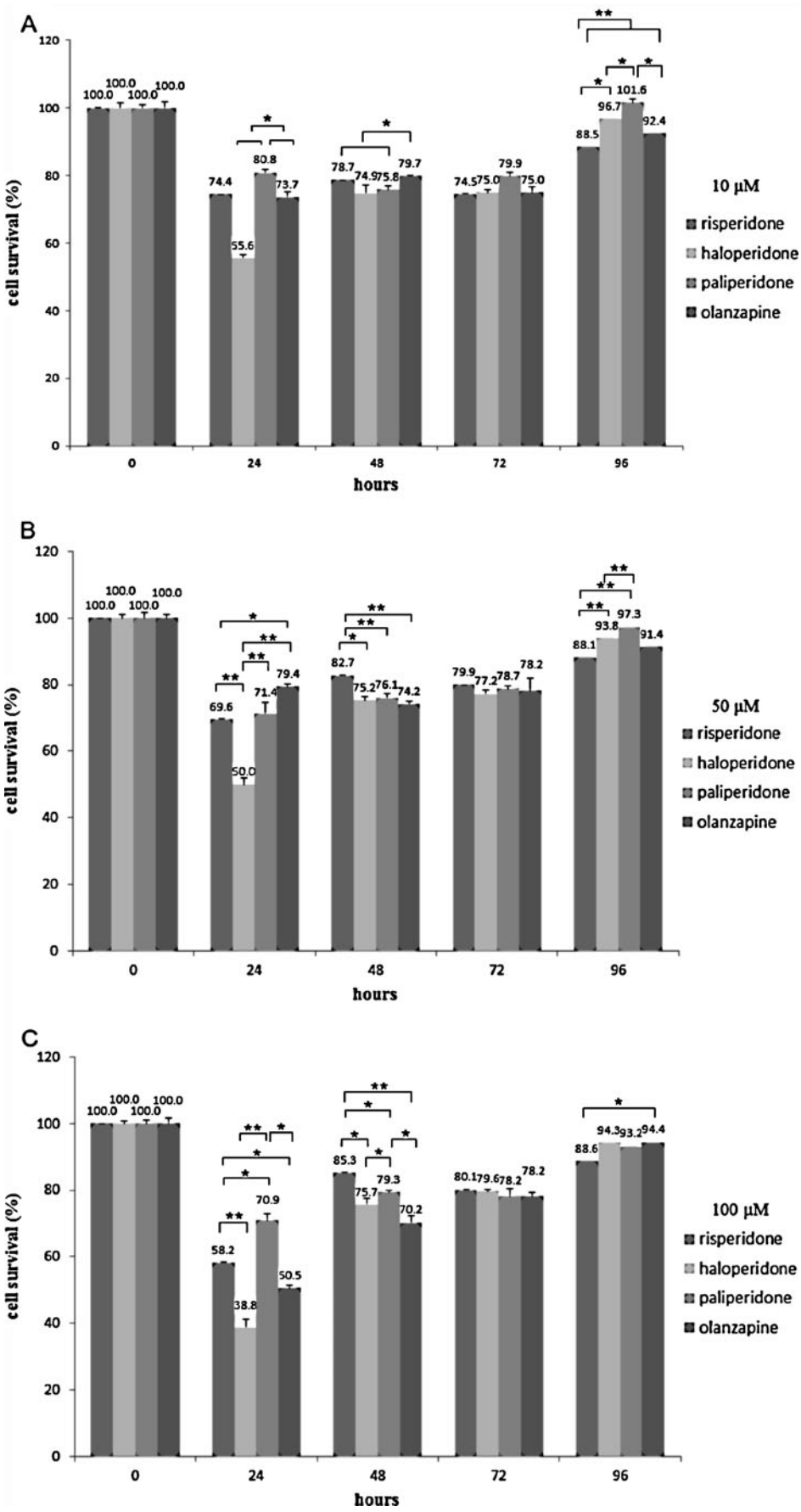

Fig. 1 Cytotoxicity of four antipsychotics to SH-SY5Y cells. The 96-h survival curve of SH-SY5Y under exposure to the four APDs at the concentration of 10 μM (**a**), at the concentration of 50 μM (**b**), and at the concentration of 100 μM (**c**). Data are presented as means± SEM. *$P<0.05$; **$P<0.01$. The *Y*-axis indicates the percentage of survival of cells

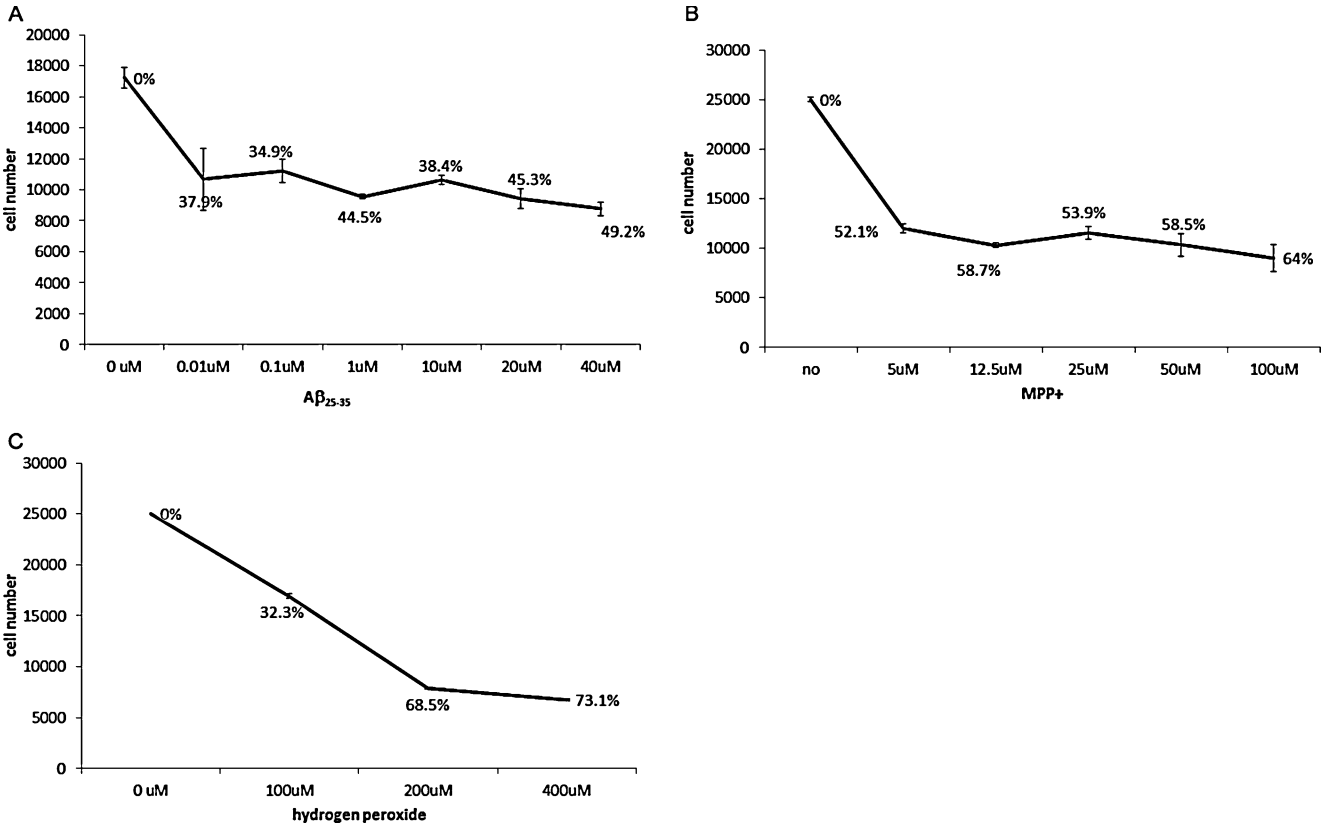

Fig. 2 The cytotoxicity of three kinds of stressors, including $A\beta_{25\text{-}35}$, MPP^+, and hydrogen peroxide, toward SH-SY5Y was increased in a dose-dependent manner. **a** $A\beta_{25\text{-}35}$ caused less cell death compared with MPP^+ and hydrogen peroxide, which induced approximately 49.2% cell reduction at the concentration of 40 μM. **b** MPP^+ induced a 64% reduction in cell viability at the concentration of 100 μM. **c** Hydrogen peroxide killed 73.1% of cells at a concentration of 400 μM. All three stressors had a similar pattern in the cell viability test, and the trend of the curve was not significantly different between them ($P>0.05$). Data are presented as means±SEM. The percentage of reduced cells is also indicated in the survival curves

olanzapine, and haloperidol showed significant neuroprotection at low concentrations (10 μM) against MPP^+-induced cell death compared with the vehicle control group (pal: $P<0.009$, ola: $P<0.025$, hal: $P<0.011$). When the concentration of APDs was adjusted up to 50 μM, all four APDs were observed to have a neuroprotective effect on SH-SY5Y under MPP^+ stimuli ($P<0.05$). It should be noted that most of the APDs, except resperidone, were found to have good neuroprotective effects at 100 μM. In this model, only olanzapine provided neuroprotection in a concentration-dependent manner; however, when the dosage was increased, palperidone, risperidone, and haloperidol, respectively, also had a mild cytotoxic effect on SH-SY5Y (Fig. 3b). The effect of MPP^+ on cell viability was completely reversed by paliperidone at 10 μM, which increased cell viability to a maximum of about 94% of the original seeded cells and about 161% of the vehicle control. The maximum for olanzapine at 10 μM was about 120% of the vehicle control, whereas that of haloperidol at 10 μM was about 111% of the vehicle control. Overall, both paliperidone and haloperidol worked well at a low concentration (10 μM). The ability of risperidone to restore cell viability was poor; it rescued only 82% of the vehicle control at the concentration of 50 μM. Olanzapine worked best at 100 μM, which diminished the toxic effect of MPP^+ and restored cell viability up to 162% of the vehicle control. In the hydrogen peroxide challenging model, only paliperidone had a significant neuroprotective effect on the SH-SY5Y cells against 400 μM hydrogen peroxide at all three conditioning concentrations (10, 50, and 100 μM; $P=0.014$, $P=0.004$, $P=0.003$); the other three APDs did not protect cells from death induced by hydrogen peroxide ($P>0.05$), as shown in Fig. 3c. At 10 μM, paliperidone restored 91.5% of the vehicle control and worked best at 50 μM, which increased viability up to 177% of the vehicle control; paliperidone still exhibited a mild cytotoxicity at 100 μM.

In further examining the differences among the four different APDs in terms of neuroprotection of SH-SY5Y against stressors, cell treatment was carried out by fixing the dosage of APDs at the highest concentration for cell preconditioning and cells were challenged with stressors at different dosages. In the $A\beta_{25\text{-}35}$ model, 100 μM of all four

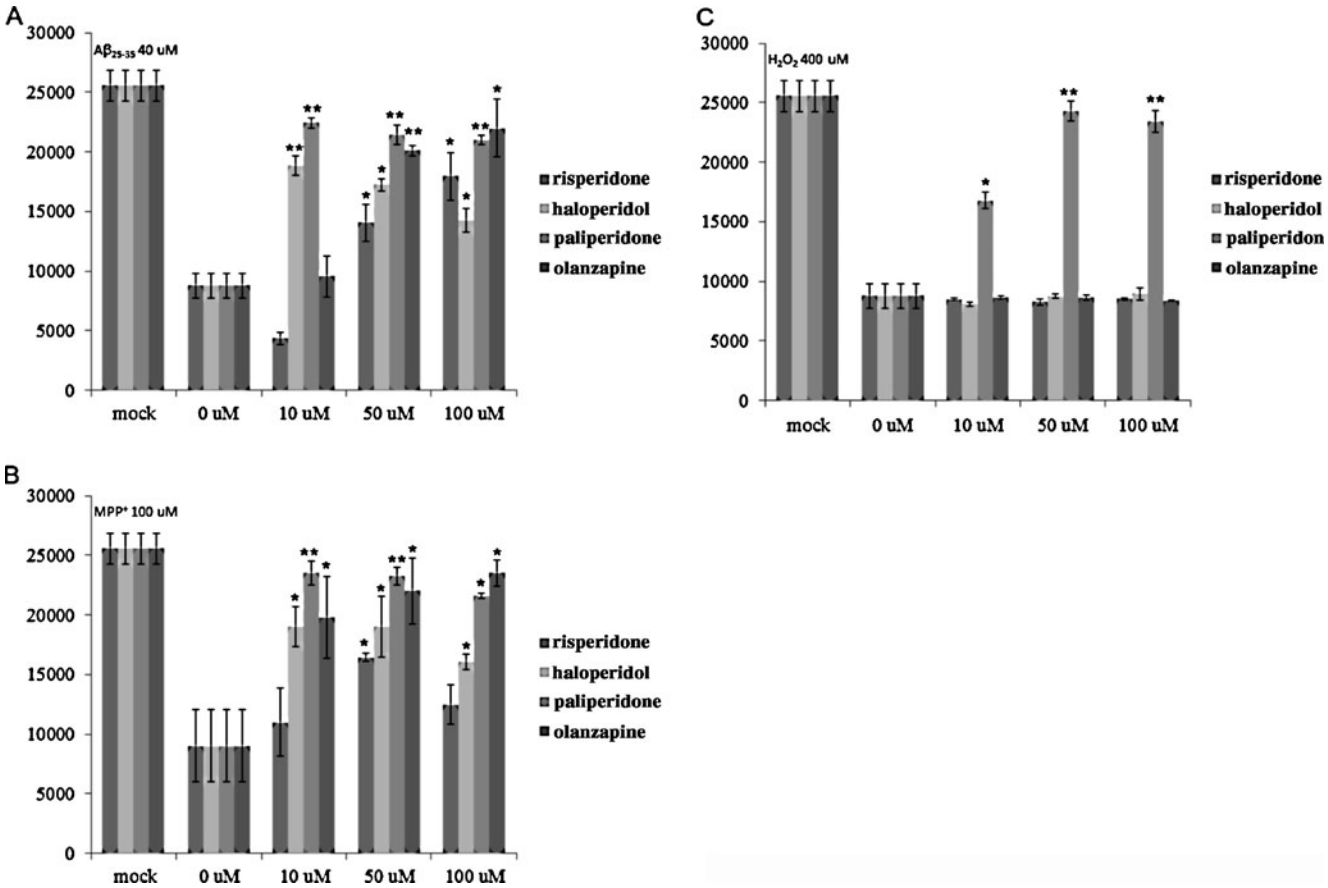

Fig. 3 The neuroprotective effect varied with different APDs in different stressor-induced cell death models using SH-SY5Y cells. SH-SY5Y cells were pretreated with various concentrations (0–100 μM) of olanzapine, palperidone, risperidone, and haloperidol for 24 h, followed by exposure to different concentrations of $A\beta_{25\text{-}35}$, MPP^+, or hydrogen peroxide for another 24 h. Then, cell viability was measured by the WST-1 assay as described in "Materials and methods." **a**, **b** Olanzapine demonstrated the best neuroprotective effect at a concentration of 100 μM against $A\beta_{25\text{-}35}$ and MPP^+-induced cell death, followed by palperidone, haloperidol, and risperidone. In contrast, paliperidone exerted the best neuroprotective effect of the four APDs against cell death at concentrations of 10 and 50 μM. **c** Only paliperidone had a neuroprotective effect on SH-SY5Y under hydrogen peroxide stimuli. Data are presented as means±SEM. *$P<0.05$, **$P<0.01$ compared with the APD-free group. The *Y*-axis indicates the cell number. *Mock*, the group with no APD preconditioned and no stressor

APDs was sufficient to protect the cells from $A\beta_{25\text{-}35}$-induced cell death, and there was no significant variation in cell numbers between the high concentration groups and the vehicle control. In this cell base assay, paliperidone was the best APD, followed by haloperidol, olanzapine, and risperidone. The cell numbers in the palperidone, haloperidol, and olanzapine treatment groups were significantly different from the cell numbers with risperidone, respectively ($P<0.001$, Fig. 4a). The situation in the MPP^+ model was similar to that of the $A\beta_{25\text{-}35}$ model in which paliperidone worked best compared with the other APDs and risperidone offered a significantly poor protection of SH-SY5Y cells against MPP^+-induced death ($P<0.001$, Fig. 4b). In the hydrogen peroxide model, paliperidone best protected against cell death at 100 μM of hydrogen peroxide, followed by olanzapine, resperidone, and haloperidol. However, only paliperidone had a significant neuroprotective effect compared with haloperidol when the cells were under hydrogen peroxide stimuli ($P<0.001$). Olanzapine and risperidone worked even better at low concentrations of hydrogen peroxide challenge (100 μM) than haloperidol; however, they were useless in protecting cells from high concentrations of hydrogen peroxide. As the results show in Fig. 4c, olanzapine and risperidone showed no significant difference in neuroprotection from that of haloperidol against hydrogen peroxide, respectively (olan: $P=0.08$, res: $P=0.33$).

To evaluate the brief mechanism of oxidative stress scavenging which underlies the neuroprotection of each APD, SH-SY5Y cells were harvested to determine the cellular level of total glutathione, HNE, and protein carbonyls, respectively. As per the result shown in Fig. 5a, without the presence of different stressors, paliperidone and risperidone showed a higher production

of total glutathione, probably as antioxidant, compared with the other two APDs. These two APDs generated significantly high total glutathione than that of the mock group ($P<0.05$, $P<0.001$), but olanzapine produced lesser antioxidant than the mock group ($P<0.05$). In stressor challenging experiments, the total glutathione level of the olanzapine group was higher than that of the mock group when treated with $A\beta_{25\text{-}35}$ and MPP^+; however, no significant difference was found for the other APD groups. In contrast, when cells are treated with hydrogen peroxide, except the olanzapine group, all APD groups showed a significantly high level of total glutathione compared to the mock group ($P<0.05$). Regarding the baseline HNE level of each APD, only one intergroup difference was found between the paliperidone and olanzapine groups, as shown in Fig. 5b ($P<0.001$). When treated with $A\beta_{25\text{-}35}$, only the haloperidol group exhibited a higher HNE production than the mock group ($P<0.05$). In contrast, when treated with MPP^+, except the paliperidone group, all APDs caused a significantly different level of HNE compared to the mock group ($P<0.001$). Olanzapine was unfavorable, which generated relatively high HNE levels against MPP^+ in SH-SY5Y cells, linking to a status of high activity of lipid peroxidation. In the hydrogen peroxide challenging model, palperidone group noticeably caused the lowest HNE level compared with the other APDs ($P<0.05$). A 34–42% reduction of HNE level of the paliperidone group was observed when compared with that of the other APD groups (Fig. 5b). In the protein carbonyl assay, olanzapine group showed an extremely higher baseline protein carbonyl production than the mock group ($P<0.001$), as well as higher than other APDs (Fig. 5c). Unlike that of the olanzapine group, all the other APD caused a lower level of protein carbonyls than the mock group ($P<0.05$). Moreover, in three stressor-induced cell death experiments, only olanzapine group produced higher protein carbonyls than the mock group ($P<0.001$), but not the other APD groups, as shown in Fig. 5c.

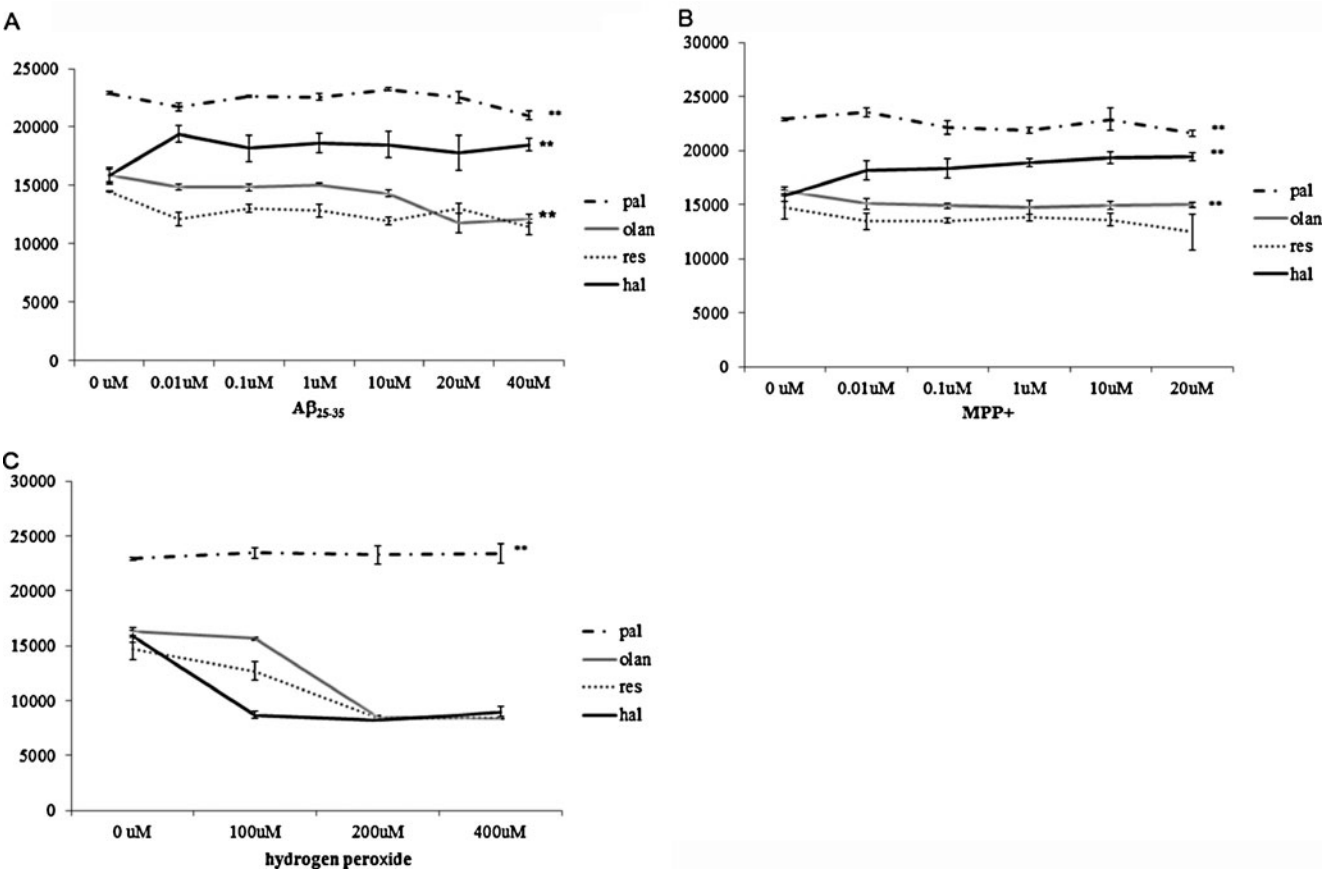

Fig. 4 The neuroprotective effect of the APDs varied with the different APDs used, the stressor type, and their concentration in SH-SY5Y cells. SH-SY5Y cells were pretreated with 100 μM of olanzapine, palperidone, resperidone, and haloperidol for 24 h, followed by exposure to different concentrations of $A\beta_{25\text{-}35}$, MPP^+, or hydrogen peroxide for another 24 h. Then, cell viability was measured by the WST-1 assay, as described in "Materials and methods." **a**–**c** Paliperidone demonstrated not only better neuroprotection but also less cytotoxicity against the SH-SY5Y cells under exposure to different concentrations of stressors. **c** No difference was found between haloperidol, olanzapine, and risperidone in neuroprotection against hydrogen peroxide challenging, which indicated that all three APDs were unable to combat the oxidative stress produced by hydrogen peroxide. Data are presented as means±SEM. $^*P<0.05$, $^{**}P<0.01$ compared with the values of risperidone. The *Y*-axis indicates the cell number. *pal* palperidone, *hal* haloperidol, *ola* olanzapine, *ris* risperidone

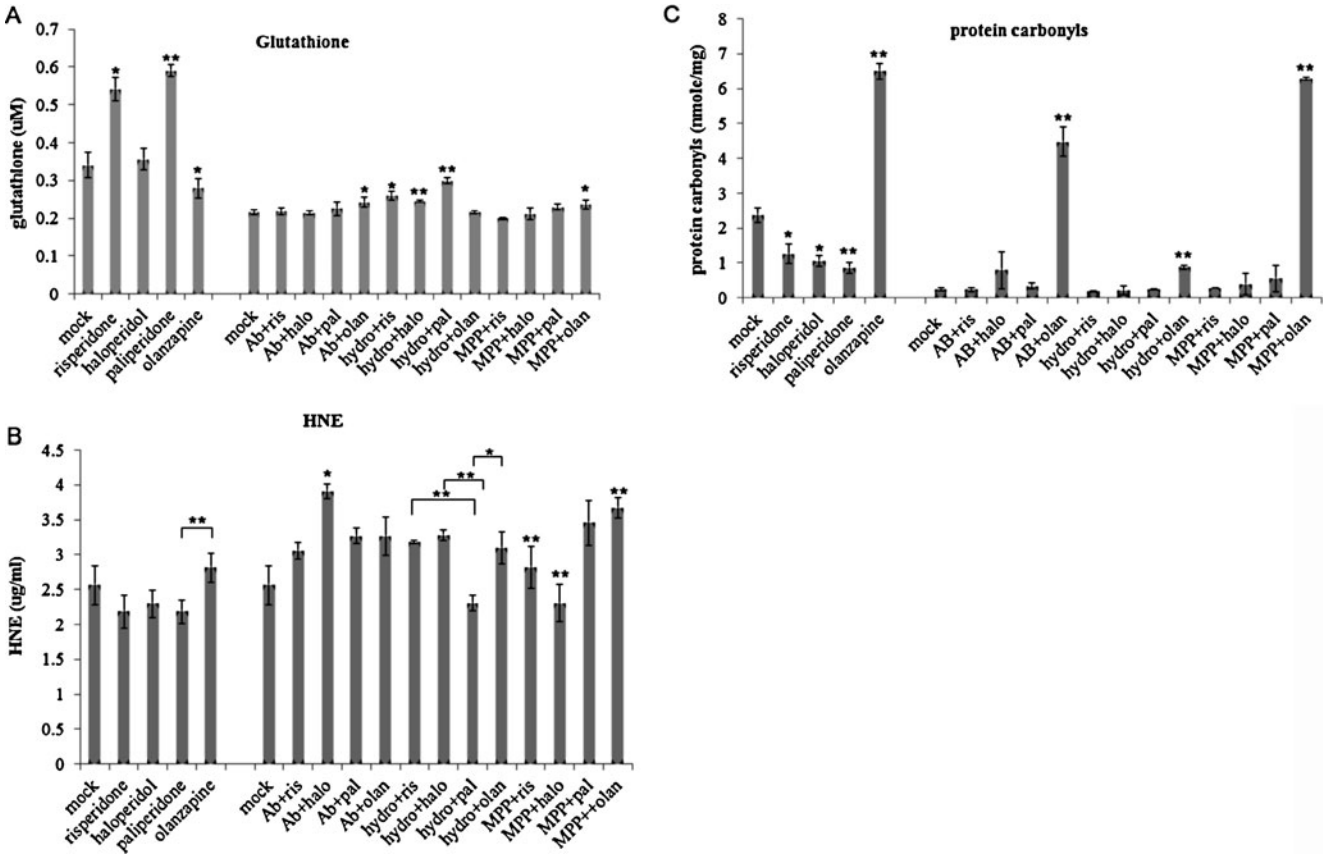

Fig. 5 Assays of antioxidant metabolites resulted from lipid and protein oxidation caused by either APDs or stressors in SH-SY5Y. SH-SY5Y cells were pretreated with 100 μM of olanzapine, palperidone, resperidone, and haloperidol for 24 h, then assayed for glutathione, HNE, and protein carbonyls directly. Alternatively, in stressor challenging models, after preconditioning with different APDs, cells were followed by exposure to the highest concentrations of $A\beta_{25\text{-}35}$, MPP^+, or hydrogen peroxide for another 24 h. Then, cells were measured for those indicators of oxidative stress as described in "Materials and methods." **a** Paliperidone generated bulk glutathione than the other APDs. **b** Paliperidone also has less HNE production than the other APDs in treating SH-SY5Y cells, especially under stimuli of hydrogen peroxide. **c** Olanzapine showed the highest quantities of protein carbonyls, which may represent a mechanism underlying its cytotoxicity. Data are presented as means±SEM. $*P<0.05$, $**P<0.01$ compared with the values of the mock group or values achieved by comparisons of two groups. The *Y*-axis indicates quantities of oxidative stress indicators. *pal* palperidone, *hal* haloperidol, *ola* olanzapine, *ris* risperidone, *Ab* β-amyloid $_{25\text{-}35}$, *hydro* hydrogen peroxide

Discussion

In this study, different APDs were tested for their neuroprotection effects against different stressors; as a result, paliperidone did show a better neuroprotective effect, especially in combating hydrogen peroxide. Olanzapine also exhibited a nice neuroprotection to the SH-SY5Y cells at the high concentration while treated with $A\beta_{25\text{-}35}$ and MPP^+. Risperidone even has a relatively low cytotoxicity, but its neuroprotection was not as good as the other APDs in this study. Haloperidol was unexpected to be the worst one in protecting SH-SY5Y cells (Reinke et al. 2004; Schmidt et al. 2009), even though it showed a significantly high baseline cytotoxicity at 24 h in a dose-dependent fashion. Against $A\beta_{25\text{-}35}$ and MPP^+ challenge, haloperidol still provided neuroprotection rather than cytotoxicity; however, it has no effect against hydrogen peroxide which, at 100 μM, already killed almost 50% of the cells. Overall, paliperidone had the best at concentrations of 10 and 50 μM, even though it retained a mild cytotoxicity when the high dosage was used. This finding is partly consistent with a recent report from Schmidt et al. (2010) in which they also showed that incubation for 24 h with risperidone and paliperidone (9-hydroxy risperidone) led to a significantly enhanced cell survival in SH-SY5Y cells in the lower concentrations. Higher concentrations exerted in part cytotoxic effects, with the exception of quetiapine (Schmidt et al. 2010). Measurement of ATP contents in SH-SY5Y cell line showed significantly increased levels after a 24-h treatment with 25 μM risperidone and paliperidone (9-hydroxy risperidone), which may represent a high effectiveness in excitement of ATP-dependent cellular activities by these two drugs. In this study, paliperidone completely diminished cell reduction induced by different stressors in the concentration of 100 μM. Moreover, paliperidone generated bulk total glutathione and produced less HNE

and protein carbonyls than the other APDs, especially against hydrogen peroxide. In contrast, olanzapine, even not as good as paliperidone and risperidone in producing antioxidant and/or scavenging the oxidative stress-related molecules, still provided neuroprotection in combating $A\beta_{25\text{-}35}$- and MPP^+-induced cell death. On the other hand, risperidone exhibited a modest oxidative stress-relieving ability, but without benefits for its overall neuroprotective action. Therefore, according to such findings, we suspected that the neuroprotection of paliperidone was more likely attributed to not only the oxidative stress scavenging but also the possible neurotrophic effects.

Regarding oxidative stress, in the human brain, the neuronal membrane contains a high proportion of polyunsaturated fatty acid and is, such as the mitochondria, the site of oxidative stress. Oxidative stress is closely associated with a number of diseases including Parkinson's disease (PD), Alzheimer-type dementia, and Huntington's chorea. In patients with schizophrenia, the reduced plasma antioxidants possibly lead to oxidative stress, which occurs early in the course and is independent of treatment effects (Reddy et al. 2003). A previous study had suggested that some antioxidant enzyme levels such as decreased glutathione were considered to be a biological indicator corresponding to the severity of schizophrenic symptoms (Raffa et al. 2009). Besides, in patients with schizophrenia, transcriptional alteration of genes involved in energy metabolism and oxidative stress was evident; subsequently, the molecular signature comprising these genes was reported to discriminate 90% of schizophrenia from normal subjects (Prabakaran et al. 2004). As per the aforementioned reasons, the attenuation of neuronal oxidative stress might, in part, underlie the neuroprotective effects of APDs. In fact, in addition to regulate oxidative stress, APDs have multiplicity of roles involved in lipid peroxidation, SOD, and ascorbic acid metabolism (Dakhale et al. 2004, 2005). It has been demonstrated that in primary cultures of hippocampal neurons, C6 glioma cells and NCB20 cells, haloperidol induced the bulk of free radicals and lead to severe cytotoxicity (Behl et al. 1995). Moreover, in cortical neuron, the cytotoxicity of haloperidol also has been linked to the activation of apoptosis triggered by the p38 and JNK MAPK signaling pathways, a well-known signaling cascade sensitive to cycloheximide and insulin treatment (Noh et al. 2000). Sustaining the known therapeutic effects of APDs, clinical studies have indicated that typical and atypical APDs exactly have different effects on the antioxidant defense system in patients with schizophrenia. For example, haloperidol could not return the changed oxidant and antioxidant defense systems to their normal states in schizophrenic patients. However, atypical APDs could, and they improved the results on the Brief Psychiatric Rating Scale for schizophrenia. In an ex vivo study, haloperidol was observed to have a higher oxidative stress induction capability in the rat brain compared with clozapine; however, no oxidative damage was found as a result of olanzapine injection (Reinke et al. 2004). Some known side effects of haloperidol, such as EPS and tardive dyskinesia, have been proposed to be associated with overproduction of reactive oxygen species (ROS) and subsequently cause neuronal damage, especially in the striatum (Cadet and Kahler 1994). In contrast, the atypical antipsychotics were not frequently found to induce high levels of oxidative stress in the rat brain (Reinke et al. 2004) by persistently changing the oxidative enzyme activity and membrane lipid peroxidation (Parikh et al. 2003). Taken together, current evidences supporting the superior therapeutic effects of atypical APDs to haloperidol were more likely due to, in part, the enhancement of oxidative stress scavenging. In fact, our major findings about the APDs against oxidative stress-producing stressor also agree with the oxidative scavenging ability which may, in part, underlie the neuroprotective actions of different APDs. As per the results, paliperidone provided higher neuroprotection compared with the other three APDs, especially against hydrogen peroxide. This effect may be due to generating bulk total glutathione as well as reducing cellular HNE and protein carbonyls levels.

In this study, we introduced three different stressors to evaluate the neuroprotection of APDs against oxidative stress in a cell base assay. First, the β-amyloid peptide, a hallmark of Alzheimer's disease (AD) and $A\beta_{25\text{-}35}$, has usually been used as a stressor to mimic the pathological microenvironment of the AD brain at the cell level. In previous studies, $A\beta_{25\text{-}35}$ was demonstrated to have a high level of cytotoxicity to PC12 cells, which decreases cell viability and causes apoptosis. Besides, it was also mentioned that $A\beta_{25\text{-}35}$ produced ROS in different cultured cell systems, such as PC12, rat cortical neurons, and hippocampal cells. The ROS induced by $A\beta_{25\text{-}35}$ was clarified as a hallmark that is associated with mitochondria membrane breakdown; thus, the displaced ROS in cytoplasm may lead to oxidative stress and trigger apoptotic events via activating the signal-regulating kinase 1 cascade. More recently, $A\beta_{25\text{-}35}$ was characterized as increasing SOD activity and was rationalized to be linked to the actions of catalyzing dismutation of the superoxide anion radical to hydrogen peroxide, indicating a compensatory event of cells balancing the cellular amount of redox molecules. Treatment of SH-SY5Y cells with $A\beta_{25\text{-}35}$ has been described as leading to a decrease in cell viability, release of lactate dehydrogenase, morphological alterations, neuronal DNA condensation, and the cleavage of poly (ADP-ribose) polymerase by activated caspase-3, which are all hallmarks of neural apoptosis. Second, MPP^+, a metabolite of neurotoxin MPTP, can induce PD-like clinical

symptomatology and neuropathological destruction and has been used in cell base assays to study PD. According to a previous study, MPP^+ inhibited NADH dehydrogenase activity in SH-SY5Y when concentrations exceeded 10 μM (Song et al. 1997). It is known that apoptosis is the mode of cell death induced in SH-SY5Y cells by MPTP and MPP^+, and this has been confirmed with nick-end labeling and bisbenzimide staining. Through electron microscopic observations, MPP^+-induced cytotoxicity strongly suggested links to mitochondrial dysfunction and missegregation of chromosome (Sheehan et al. 1997). In the following, SH-SY5Y cells exposed to MPP^+, likewise to $A\beta_{25\text{-}35}$, showed elevated oxygen free radical production and antioxidant enzyme activities, which may reflect a self-protection of cells against oxidative stress (Cassarino et al. 1997). However, it was proposed that MPP^+-induced cytotoxicity was not primarily caused by oxygen free radicals; alternatively, accumulated oxidative stress in cells played a role in increasing the vulnerability of cells to oxidative stress (Lee et al. 2000). With MPP^+-induced cytotoxicity toward SH-SY5Y at 100 μM of MPP^+, the cells were found to have a decreased mitochondria potential, slightly increased cytoplasmic cytochrome c and caspase-9 activation, and increased VDVADase and DEVDase activities. These findings led to the simple conclusion that MPP^+ can induce caspase-mediated apoptosis in SH-SY5Y cells (Gomez et al. 2001). In reviewing the molecules participating in MPP^+-induced cytotoxicity, B cell lymphoma protein-2 (Bcl-2), Bax, JNK, and the caspases are thought to be involved in this neurotoxic effect (Nicotra and Parvez 2002). Third, it has been reported that hydrogen peroxide may induce excessive oxidative stress in SH-SY5Y and cause apoptosis-like cell death. Some APDs, such as clozapine, haloperidol, and quetiapine, have been described as having a cytoprotective effect in SH-SY5Y. Haloperidol and quetiapine have demonstrated a significantly reduced expression of genes encoded for antioxidant enzymes (e.g., glutathione-S-transferase), which may underlie the molecular mechanism of their cytotoxicity (Schmidt et al. 2009). Clozapine did not have a neuroprotective effect against hydrogen peroxide on SH-SY5Y, but had a protective effect toward PC12 (Magliaro and Saldanha 2009) under hydrogen peroxide treatment. Similarly, olanzapine, an APD very close to clozapine, was also observed to have a protective effect on PC12 against hydrogen peroxide (Wei et al. 2003a). Our findings on olanzapine also showed that it provides no neuroprotection at 100 μM in treatment with 400 μM hydrogen peroxide. These findings basically indicated that the neuroprotective effects of different APDs against hydrogen peroxide vary; in addition, the gross effect of cytoprotection from APDs also differs by cell type.

Among the three stressors used in this study, as per the aforementioned properties, both MPP^+ and $A\beta_{25\text{-}35}$ have been identified as causing oxidative stress and oxidative damage in vivo and in vitro (Xiao et al. 2002; Sharma et al. 2003; Miwa et al. 2004; Zeng et al. 2004; Wang et al. 2005). In a previous study, atypical APDs protected PC12 cells from MPP^+-induced apoptosis (Qing et al. 2003). Furthermore, it has been reported that olanzapine, but not quetiapine, increased the SOD activity of PC12 cells, suggesting that these two drugs do not share the same molecular mechanism of protecting PC12 cells against the cytotoxicity of $A\beta_{25\text{-}35}$. It is likely that olanzapine prevented $A\beta_{25\text{-}35}$-induced oxidative stress via inhibiting the overproduction of ROS and increasing the dismutation of the superoxide anion radical. In our data, olanzapine did exhibit a modest neuroprotection toward SH-SY5Y cells at concentrations >50 μM when the cells were treated with MPP^+ and $A\beta_{25\text{-}35}$. However, the gross effect of olanzapine on SH-SY5Y was unlikely compared with that of paliperidone, which is outstanding in combating hydrogen peroxide and worked best at low concentrations against both MPP^+ and $A\beta_{25\text{-}35}$. Given the hints from previous studies, the neuroprotective effects of some APDs may be attributed, in part, to their oxidative stress-scavenging activities; in our study, the superior actions of paliperidone may not only have included some oxidative stress-scavenging events but also contributed to some underlying molecular mechanisms, such as activation of the survival signaling pathways and induced cell proliferation and neurotrophic effects. Several examples of the molecular mechanism, except attenuation of oxidative stress, results in the neuroprotection of APDs can be given; for instance, atypical APDs had been documented to stimulate neurogenesis in adult rat brain (Wakade et al. 2002; Kodama et al. 2004), up-regulate the expression of brain-derived neurotrophic factor mRNA in rat hippocampus (Bai et al. 2003), and increase the levels of both mRNA and protein of Bcl-2 in the rat frontal cortex and hippocampus (Bai et al. 2004). Moreover, atypical APDs modulated Bax and Bcl-XL/S expression and localization (Wei et al. 2003b). Enhanced phosphorylation of the ERK signaling pathway by clozapine against hydrogen peroxide in PC12 also indicated that other molecular events outside of attenuating free radicals may contribute to the neuroprotection of clozapine (Magliaro and Saldanha 2009). Taken together, different APDs exhibited different therapeutic effects via regulating convergent or distinct signaling pathways. To date, the intracellular signaling cascades of PKA, DARPP-32, MAPK, Akt/GSK-3, and β arrestin-2 have been discussed as being crucial for post-receptor mechanisms of neuroplasticity and cellular resilience induced by APDs (Molteni et al. 2009). Also, the neurotrophic factors and the glutamatergic system are important mediators for antipsy-

chotic drug-induced neuroplasticity. Recently, evidence from a study aimed at differentiating signaling molecules involved in the underlying mechanism of the post-receptor actions of APDs highlights that these signaling pathways will eventually be promising targets for the development of novel drugs that, through their impact on neuroplasticity, may contribute to the improved treatment of patients diagnosed with schizophrenia (Molteni et al. 2009).

In clinical practice, antioxidants such as *N*-acetylcysteine are reported to be helpful in treating schizophrenia patients who have a poor response to antipsychotic medication (Bulut et al. 2009). Another material identified as an antioxidant, Ginkgo, is proposed as an add-on therapy to ameliorate the symptoms of chronic schizophrenia (Singh et al. 2010). These findings may indicate that antioxidative stress is an important issue in the treatment of schizophrenia, especially in terms of improving negative symptoms. Nevertheless, other than oxidative stress reduction, the gross neuroprotective effects attributed to APDs against different stressors is more likely the result of a convergence of several complex mechanisms. One issue is that the APD treatment duration determines its ability to attenuate oxidative stress and lipid peroxidation (Al-Chalabi et al. 2009). Sometimes, acute and chronic administration of APDs in schizophrenia brings adverse outcomes. Another issue is that the effective concentrations of olanzapine used in the present study against MPP^+ and $A\beta_{25\text{-}35}$ were different; in MPP^+ treatment, when using olanzapine, the concentration of 10 μM was much closer to the reported effective concentrations of this drug in the sera of patients with schizophrenia (>23.2 μM; Perry et al. 2001). Nevertheless, in SH-SY5Y cells, increased dosage to 100 μM inversely caused higher baseline cytotoxicity compared with the other two concentrations. Contradictorily, it was noticed that high dosage of olanzapine provides considerable neuroprotection against MPP^+ and $A\beta_{25\text{-}35}$. These findings suggest that the clinical uses of olanzapine in patients may be different from the cell-based assay which involves a more complex mechanism, and/or a different dosage is required for the treatment of different CNS diseases (AD disease vs. PD), necessitating further studies to clarify these aspects. Moreover, early medical intervention with APDs also brings different outcomes. In patients, the earlier the treatment with atypical APDs begins, the less the degeneration and disease progression observed (Lieberman et al. 2001; Schaffner and McGorry 2001; Mahadik et al. 2006).

In summary, MPP^+, $A\beta_{25\text{-}35}$, and hydrogen peroxide, as expected, cause the cell death of SH-SY5Y, possibly due to oxidative stress and the ensuing damage and apoptosis. Paliperidone worked well against hydrogen peroxide treatment, which might be due, in part, to good ability to both scavenge the oxidative stress-related molecules and enhance the production of antioxidant. All APDs exerted protective effects toward cells in combating $A\beta_{25\text{-}35}$ treatment. Haloperidol and the atypical APDs, except risperidone, diminished MPP^+-induced cell death. Taken together, these results provide us with new insights into the superior therapeutic effects and the lower levels of untoward side effects of some APDs, such as paliperidone and olanzapine, in patients with schizophrenia. Nevertheless, current results just represent single neuron-like cell and may be insufficient to illustrate fully the actions of APDs in the CNS environment of patients with schizophrenia. Therefore, further studies using PC12 cells, neuron-microglia co-culture system, and animal models will help delineate the molecular mechanism of neuroprotetion of APDs.

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Conflict of interest All of the authors declare that we have no affiliation with or financial involvement in any organization or conflict of interest.

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