Differential effects of aripiprazole and haloperidol on BDNF-mediated signal changes in SH-SY5Y cells

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Abstract
Recent studies have suggested that first and second generation antipsychotics (FGAs and SGAs) have different neuroprotective effects. However, the molecular mechanisms of SGAs are not fully understood, and investigations into changes in intracellular signaling related to their neuroprotective effects remain scarce. In the present study, we compared the SGA aripiprazole with the FGA haloperidol in SH-SY5Y human neuroblastoma cells via brain-derived neurotrophic factor (BDNF)-mediated signaling, notably BDNF, glycogen synthase kinase-3β (GSK-3β), and B cell lymphoma protein-2 (Bcl-2). We examined the effects of aripiprazole (five and 10 μM) and haloperidol (one and 10 μM) on BDNF gene promoter activity in SH-SY5Y cells transfected with a rat BDNF promoter fragment (−108 to +340) linked to the luciferase reporter gene. The changes in BDNF, p-GSK-3β, and Bcl-2 levels were measured by Western blot analysis. The haloperidol was not associated with a significant difference in BDNF promoter activity. In contrast, aripiprazole was associated with increased BDNF promoter activity only with a dose of 10 μM (93%, \(p<0.01\)). Treatment with aripiprazole at 10 μM increased the levels of BDNF by 85%, compared with control levels (\(p<0.01\)), whereas haloperidol had no effect. Moreover, cells treated with aripiprazole effectively increased the levels of GSK-3β phosphorylation and Bcl-2 at doses of five and 10 μM (30% and 58% and 31% and 80%, respectively, \(p<0.05\) or \(p<0.01\)). However, haloperidol had no effects on p-GSK-3β and Bcl-2 expression. This study showed that aripiprazole, but not haloperidol, appeared to offer neuroprotective effects on human neuronal cells. The actions of signaling systems associated with BDNF may represent key targets for both aripiprazole and haloperidol, but the latter may be associated with distinct effects. These differences might be related to the different therapeutic effects of FGAs and SGAs in patients with schizophrenia.

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1. Introduction

Previous studies have hypothesized that the neuroanatomical changes associated with schizophrenia might originate in problems with neurodevelopment during pregnancy or an early postnatal period (Altshuler et al., 1987; Arnold et al., 1991; Conrad and Scheibel, 1987). However, recent clinical and neuroimaging studies have shown an association between schizophrenia and such progressive abnormalities in brain morphology as ventricular enlargement, reduction in gray matter volume, and differences in thalamic volume, suggesting that neurodegeneration may occur during the pathogenesis of schizophrenia (Konick and Friedman, 2001; Wright et al., 2000).

A growing body of evidence has shown that second generation antipsychotic drugs (SGAs) might offer neuroprotective effects. Indeed, clinical studies have reported that the SGAs show better therapeutic efficacy for negative symptoms and cognitive deficits and fewer propensities to induce extrapyramidal side effects (EPS) than do first generation antipsychotics (FGAs) (Meltzer, 1995; Andreasen, 1994). Lieberman et al. (2003a, b; Yulug et al., 2006). On the other hand, haloperidol, a FGA, lacks this effect and may even cause oxidative stress, resulting in apoptotic cell death (Behl et al., 1995; Noh et al., 2004). SGAs, including olanzapine and clozapine, are known to up-regulate BDNF levels, whereas anolazapine was not. Chakos et al. (2005) also demonstrated that SGAs, rather than haloperidol, were associated with larger hippocampal volume differences in patients with schizophrenia. In addition, a growing body of in vivo and in vitro studies has indicated that several SGAs have been able to protect neuronal cells from a variety of toxicity models (Bai et al., 2002; Luo et al., 2005; Wang et al., 2004; Wei et al., 2003a, b; Yulug et al., 2006). On the other hand, haloperidol, a FGA, lacks this effect and may even cause oxidative stress, resulting in apoptotic cell death (Behl et al., 1995; Noh et al., 2000).

However, the molecular mechanisms of SGAs are not fully understood, and little has been done to investigate changes in intracellular signaling related to their neuroprotective effects.

Brain-derived neurotrophic factor (BDNF) represents a major neuroprotective protein regulating neuronal cell survival, differentiation, and synaptic strength, and morphology in the brain (Ghosh et al., 1994); abnormal regulation of BDNF plays an important role in the pathogenesis of schizophrenia (Durany and Thome, 2004). Data have been accumulating on the differential effects of antipsychotics on BDNF levels (Angelucci et al., 2005; Angelucci et al., 2000; Chlan-Fourney et al., 2002; Parikh et al., 2004). SGAs, including olanzapine and clozapine, are known to up-regulate BDNF levels, whereas haloperidol down-regulates BDNF levels in rat brains (Bai et al., 2003).

Researchers have established that BDNF inhibits glycogen synthase kinase-3β (GSK-3β) activity through increased serine phosphorylation in cerebellar granule cells and human neuroblastoma SH-SY5Y cells (Foulstone et al., 1999; Mai et al., 2002). In addition, the phosphatidylinositol-3 kinase (PI3K)/Akt pathway represents the best-understood neuronal survival pathway regulating the activity of GSK-3β. In this cascade, the activation of Akt through neurotrophins such as BDNF results in the inactivation of GSK-3β by the phosphorylation of serine9 on GSK-3β (Chen and Russo-Neustadt, 2005). Thus, suppression of neuronal apoptosis signaling must involve inhibition of GSK-3β. Recent studies have shown that alterations in GSK-3β levels contribute to the pathogenesis of schizophrenia (Kozlovsky et al., 2004; Nadri et al., 2004) and that treatments with FGAs and SGAs produce distinctive patterns of GSK-3β expression (Alimomad et al., 2005; Li et al., 2007). Because activation of GSK-3β facilitates apoptosis, inhibition of GSK-3β by SGAs supports neuroprotection.

A major antiapoptotic protein, Bcl-2, hypothesized to exert protective effects via GSK-3β-regulated signaling, contributes to the rescue of cells from apoptosis by oxidative stress. Research using H2O2 and haloperidol has demonstrated that bcl-2 prevented hippocampal cell death induced by oxidative stress (Lezoualch et al., 1996). Thus, we propose that the protective effects of SGAs might be related to the elevation of this neurotrophic and antiapoptotic factor activity.

Aripiprazole represents a novel SGA with a unique pharmacological profile including dopamine D2 partial agonism, serotonin 5-HT1A partial agonism, and 5-HT2A antagonism (Lawler et al., 1999). Clinical trials have found that aripiprazole was effective in treating the positive, negative, and cognitive symptoms of schizophrenia; showed a favorable side-effect profile in regard to extrapyramidal symptoms, weight gain, and sedation; and produced minimal changes in serum prolactin levels (Kane et al., 2002). Unlike the current SGAs such as olanzapine, clozapine, quetiapine, risperidone, and ziprasidone, aripiprazole has not been fully evaluated in regard to its neuroprotective effects.

In the present study, we determined whether aripiprazole and haloperidol differentially affected the expression of BDNF, GSK-3β, and Bcl-2 in human neuroblastoma SH-SY5Y cells.

2. Experimental procedures

2.1. Materials

Aripiprazole was supplied from Otsuka Pharmaceuticals (Tokushima, Japan) and haloperidol was purchased from Sigma (St. Louis, MO, USA). Antibodies used for Western blot analysis were purchased from the following sources: anti-BDNF, anti-phospho-ser9-GSK-3β, and anti-Bcl-2 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); α-tubulin antibodies from Sigma; and goat anti-mouse and anti-rabbit IgG-horseradish peroxide conjugates from Amersham-Pharmacia (Little Chalfont, England) and Santa Cruz Biotechnology. All other chemicals were purchased from commercial sources.

2.2. Cell culture

The SH-SY5Y human neuroblastoma cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD, USA) and was cultured in minimum essential medium (MEM)/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% CO2 at 37 °C. The culture medium was changed every two to three days.

2.3. Construction of exon III BDNF promoter plasmid

The rat DNA was isolated from rat blood using a QiAamp DNA Blood Mini Kit (Qiagen, USA). Since a cyclic AMP-responsive element...
containing the appropriate horseradish peroxidase-conjugated sec-

Subsequently, the membranes were incubated for 1 h in PBS-T

overnight, the membranes were washed three times in PBS-T for 5 min.

(PCR) with primers 5′-GAATTCGATCTAGCAGCTTG-3′ (forward primer of 22nt, containing a Kpn I) and 5′-TTTGCAA AGTAAAC GCTC- GAGGC-3′ (reverse primer of 22nt, containing a Xho I). The PCR

amplification performed in a final volume of 50 μl containing 150 ng

 genomic DNA, 1 mM dNTP, 2 mM Tris-HCl (pH 8.0), 10 mM KCl,

.01 mM EDTA, .1 mM dithiothreitol (DTT), .05% Tween20 (v/v), .05%

Nondiet P40 (V/V), 5% glycerol, TunIt Ex Taq DNA polymerase

(TAKARA, Japan), and 10 pmol of each primer. The PCR condition

was one cycle of pre-denaturation at 95 °C for 5 min, 35 cycles of

denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and

elongation at 72 °C for 1 min, and one cycle post-elongation 72 °C

for 5 min. DNA fragments of the expected size (400 base pairs) were

digested with Kpn I and Xho I and then ligated into the plasmids:

vector (Promega, USA), which has an insert coding for luciferase

se-

used as a reporter gene (−10BDNF/pGL3). The identity of the rat

exon III BDNF promoter clone was confirmed by dyeoxynucleotide

sequencing.

2.4. Transfection and luciferase assay

The cells at 60–70% confluence were transiently transfected with

−10BDNF/pGL3 plasmid using FuGENE6 reagent (Roche, Indiana-

polis, USA) according to the supplier's recommendation. In summary,

plasmids were complexed at a ratio of 1 μg DNA/3 μl FuGENE6 and

incubated at room temperature for 25 min. Twenty-four hours after

transfection, the cells were incubated with the indicated concen-

tration of antipsychotic medication for an additional 48 h. Aripip-

razole and haloperidol were dissolved in dimethyl sulfoxide (DMSO)

diluted with MEM medium to a concentration of 20 μM (final .5%

DMSO). Luciferase assay was performed using the Dual Luciferase

Reporter Assay System (Promega, WI, USA) according to the

manufacturer's instructions. Cells were lysed in Reporter Lysis Buffer

and luciferase activity was measured using Victor III (PerkinElmer,

USA). All firefly luciferase values were normalized to Renilla

luciferase in order to compare the transfection efficiencies. The

luciferase activity by transfection of pCMV-SPORT-CREB plasmid

(purchased from 21C Frontier Human Gene Bank) was effectively

increased, reflecting consistency with the results described by Shieh

and Ghosh (1999) and indicating that our cell models responded

positively to the stimulation.

2.5. Western blot analysis

Cells were plated at a density of 2 × 10⁶ cells per 100 mm dish. After 24 h

of incubation, the cells were treated with aripiprazole (five and 10 μM)

or haloperidol (one and 10 μM) for 96 h. For whole cell lysates, the cells

were washed twice with ice-cold phosphate-buffered saline (PBS).

Lysis buffer (1% Triton, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM

EDTA, one tablet complete protease inhibitor (Roche, QC, Canada),

and phosphatase inhibitor cocktail (Sigma)) was added, and the lysates

were centrifuged (1000 × g, 10 min, 4 °C). Equal amounts of protein

(10 μg) from the cell extracts for each treatment condition were

separated in 12% sodium dodecyl sulfate-polyacrylamide gels and

transferred electrophoretically onto polyvinylidene fluoride mem-

branes. The blots were blocked by incubation in 5% (w/v) non-fat milk

in PBS with .1% Tween 20 (PBS-T) for 1 h. After incubation with a

primary antibody (anti-BDNF, 1:1000; anti-phospho-ser³-GSK-3β,

1:1000; anti-Bcl-2, 1:500, anti-α-tubulin, 1:2000, in PBS-T) at 4 °C

overnight, the membranes were washed three times in PBS-T for 5 min.

Subsequently, the membranes were incubated for 1 h in PBS-T

containing the appropriate horseradish peroxidase-conjugated sec-

ondary antibody (anti-mouse IgG, 1:1000; anti-rabbit IgG, 1:1000;

respectively). The immunoreactive bands were visualized and

quantified using ECL+ Western blotting reagents, with chemilumines-

cence detected by Las-3000 Image Reader (Fuji Film, Tokyo, Japan)

software. To adjust for protein loading variation, the amounts of

protein were normalized based on α-tubulin, which was not affected

by the drug treatments, and expressed as the percentage of vehicle

control, which was deemed to be 100%.

2.6. Statistical analysis

The data are presented as the mean±S.E.M. from the three independent

experiments. One-way ANOVA was performed and Duncan's test

followed for the post hoc comparison. Value was considered

significant at p < 0.05.

3. Results

3.1. Effects of aripiprazole and haloperidol on BDNF promoter activity and protein levels in SH-SY5Y cells

Aripiprazole at the highest concentrations used (10 μM) sig-

nificantly increased the BDNF promoter activity by about 93%

(p<0.01), while haloperidol had no effects in this regard

(Fig. 1A and B). The effects of aripiprazole and haloperidol on

protein levels were also evaluated and the results clearly

showed that, similar to the effects on promoter activity, 10 μM

...
of aripiprazole induced elevation by about 85% in BDNF levels ($p<0.01$, Fig. 2A). However, haloperidol did not change the BDNF levels (Fig. 2B).

3.2. Effects of aripiprazole and haloperidol on phospho-GSK-3β and Bcl-2 levels

Treatment of cells with aripiprazole induced a dose-dependent increase in the levels of ser9-phosphorylated GSK-3β and Bcl-2 expression. Aripiprazole treatment at dose of five and 10 μM significantly increased the expression of p-GSK-3β by about 30% and 58%, respectively ($p<0.05$, Fig. 3A). Elevations of 31% and 80% in Bcl-2 levels were observed in treatment with five and 10 μM aripiprazole, respectively ($p<0.05$ or $p<0.01$, Fig. 4A). However, the expressions of p-GSK-3β and Bcl-2 were not changed in haloperidol treatment (one and 10 μM) (Figs. 3B and 4B).

4. Discussion

In this investigation of the mechanisms underlying the differential effects of the two classes of antipsychotics, we detected differences in the expression of BDNF, p-GSK-3β, and Bcl-2 as well as in BDNF promoter activity in cells treated with aripiprazole and haloperidol.

Consistent with previous research on the effect on BDNF mRNA of SGAs (Bai et al., 2003; Chlan-Fourney et al., 2002; Parikh et al., 2004), BDNF promoter activity and protein levels were significantly increased in SGA-aripiprazole-treated cells but not in FGA-haloperidol-treated cells. On the other hand, Yoshimura et al. (2008) reported that aripiprazole might not alter plasma BDNF levels at least...
after 8 weeks of treatment in patients with first-episode schizophrenia. It is still controversial in clinical studies regarding blood levels whether antipsychotics change on schizophrenia. It is still controversial in clinical studies after 8 weeks of treatment in patients with first-episode schizophrenia. It is still controversial in clinical studies.

Increased BDNF has been associated with the expression of its high affinity receptor, TrkB (Tsai, 2007). Parikh et al. (2004) have reported that olanzapine restored the reduction of BDNF and TrkB receptors produced by haloperidol in a rat hippocampus. BDNF-induced TrkB activation has been known to initiate the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) signaling pathways (Pizzorusso et al., 2000; Rodgers and Theibert, 2002). In addition, aripiprazole has stimulated the phosphorylation of MAPK (Urban et al., 2007). Therefore, up-regulation of BDNF by aripiprazole might be related to neuroprotective effects through the activation of the MAPK and PI3K pathways via its receptor.

PI3K/Akt signaling plays a principal role in regulating the balance between cell survival and apoptosis (Franke et al., 1997; Hetman et al., 1999). Phosphorylation of GSK-3β by Akt promotes cell survival by inhibiting apoptosis, whereas dephosphorylated (active) GSK-3β initiates the apoptosis pathway by inhibiting Akt (Crowder and Freeman, 2000). Lu et al. (2004) reported that olanzapine produced rapid phosphorylation of Akt, which is prevented by the addition of specific inhibitors of PI3K; inhibition of Akt blocks the mitogenic effects of olanzapine. In addition, SGA quetiapine have appeared to stimulate the phosphorylation of Akt in ways similar to the effects of olanzapine, whereas clozapine failed to activate Akt. However, such FGAs as chlorpromazine, fluphenazine and haloperidol decreased the Akt phosphorylation induced by nerve growth factor in PC12 cells (Lu and Dwyer, 2005).

Recently, we detected the differential effects of olanzapine and haloperidol on the phosphorylation of GSK-3β against serum withdrawal-induced apoptosis in SH-SY5Y cells (Kim et al., 2008). Olanzapine at all doses tested (i.e., up to 200 μM) significantly increased the level of p-GSK-3β under serum withdrawal, whereas haloperidol reduced the expression of p-GSK-3β at a 10 μM dose. A recent study demonstrated that administration of SGAs, including clozapine, olanzapine, quetiapine, and ziprasidone, rapidly increased the level of p-GSK3β in the cortex, hippocampus, striatum, and cerebellum of mice (Li et al., 2007). Therefore, inhibition of GSK-3β by activation of Akt may contribute to the neuroprotective properties of aripiprazole.

Aripiprazole treatment also strikingly enhanced Bcl-2 expression while haloperidol did not. Bcl-2 exerts neurotrophic activity as an anti-apoptotic and survival protein (Wang et al., 2006). Decreased protein levels of Bcl-2 have been implicated in the pathogenesis of schizophrenia (Jarskog et al., 2000). Our previous study showed that olanzapine accelerated the recovery of decreased Bcl-2 levels from the serum-starved conditions in SH-SY5Y cells but haloperidol did not (Kim et al., 2008). Chronic administration of olanzapine also upregulated the expression of Bcl-2 mRNA in a rat hippocampus (Bai et al., 2004) and prevented the down-regulation of Bcl-2 by methamphetamine or restraint stresses (He et al., 2004; Luo et al., 2004). Taken together, these findings suggest that SGA medications have a neuroprotective function that is regulated in part through an up-regulation of Bcl-2 protein. Up-regulation of Bcl-2 by aripiprazole may be involved in GSK-3β-induced activation of CREB via PI3K/Akt signaling. The Bcl-2 promoter contains a cyclic AMP-responsive element (CRE) to which phosphorylated CREB binds and elevates transcription (Belkhir et al., 2008). CREB represents a key transcription factor responsible for gene regulation by GSK-3β, and the activity of CREB is generally inhibited by GSK-3β (Liang and Chuang, 2006). Moreover, CREB-mediated gene expression is necessary for survival of multiple neuronal subtypes, and is involved in differentiation, synaptic plasticity, and memory (Giachino et al., 2005; Lonze and Ginty, 2002). Although originally named due to the activation of CREB by the cyclic AMP-dependent protein kinases, PI3K/Akt has been reported to promote neurotrophin-induced neuronal survival through the activation of CREB downstream (Lonze and Ginty, 2002). Eventually, aripiprazole may lead to Bcl-2 stimulation via CREB activation during PI3K/Akt/GSK-3β signaling.

The concentrations of aripiprazole required to produce positive effects, five or 10 μM, were sufficient to activate the signaling pathways discussed here in the clinical situation. The

Figure 4  The effects of aripiprazole and haloperidol on the Bcl-2 levels in SH-SY5Y cells. Cells were treated with the indicated doses of aripiprazole (A) and haloperidol (B) for 96 h. Cell lysates were analyzed by SDS-PAGE and Western blotting with an anti-Bcl-2 antibody. A representative image and quantitative analysis, normalized to the α-tubulin band, are shown. Values represent a percentage of the vehicle-treated cells (control) and are expressed as means ± S.E.M. from the three independent experiments. *p<0.05, **p<0.01 vs. control.
concentrations of aripiprazole used here (five-10 μM) are higher than the concentrations normally observed in plasma, which vary between 0.9 and 1.1 μM (Citrome et al., 2007). However, this drug can be accumulated at 10- to 30-fold higher concentrations in brain tissue (Aravagiri et al., 1999), a characteristic equally true of haloperidol (Korpi et al., 1984).

To the best of our knowledge, our in vitro data represent the first results showing the neuroprotective effects of aripiprazole on signaling systems. We suggest that the protective efficacy of aripiprazole in human neuroblastoma SH-SY5Y cells might involve inactivation of GSK-3 via the BDNF-mediated activation of PI3K/Akt signaling and the subsequent increased activation of transcription factor CREB, resulting in increased expression of its survival transcriptional target, Bcl-2.

A key finding of this study is that actions on signaling systems associated with BDNF may represent important targets for antipsychotic drugs. Significant differences between the actions of aripiprazole and haloperidol suggest that the mechanisms of the drugs might explain their differential therapeutic effects.

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Contributors
YHK designed the study, wrote the protocol, and supervised the procedures of the study. SWP organized the study and wrote the first draft of the manuscript. JGL performed the literature review and provided guidance on subsequent drafts. EKH, SMC, HYC, and MKS contributed to and have approved the final manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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