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Differential effects of amisulpride and haloperidol on dopamine D_2 receptor-mediated signaling in SH-SY5Y cells

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ABSTRACT

Dopamine D_2 receptors (D_2R) are the primary target of antipsychotic drugs and have been shown to regulate Akt/glycogen synthase kinase-3β (GSK-3β) signaling through scaffolding protein β-arrestin 2. Amisulpride, an atypical antipsychotic drug, and haloperidol, a typical antipsychotic drug, are both potent D_2R antagonists, but their therapeutic effects differ. In the present study, we compared the effects of amisulpride and haloperidol on the β-arrestin 2-mediated Akt/GSK-3β pathway in SH-SY5Y cells. To determine whether these drugs affected neuronal morphology in SH-SY5Y cells, we investigated the effects of amisulpride and haloperidol on neurite outgrowth using immunostaining. We examined the effects of these drugs on Akt and GSK-3β and its well-known downstream regulators, cAMP response element-binding protein (CREB), brain-derived neurotrophic factor (BDNF), and Bcl-2 levels using Western blot analysis. Amisulpride, but not haloperidol, was found to enhance neurite outgrowth. Small interfering RNA (siRNA) for β-arrestin 2 knockdown blocked the increase in amisulpride-induced neurite outgrowth. Furthermore, amisulpride increased the levels of Akt and GSK-3β phosphorylation, while haloperidol had no effect. The elevation of Akt phosphorylation induced by amisulpride was reduced by β-arrestin 2 siRNA. Moreover, amisulpride effectively increased the levels of phospho-CREB, BDNF, and Bcl-2. However, haloperidol had no effect on the levels of these proteins. Additionally, wortmannin, a phosphatidylinositol 3-kinase (PI3 K) inhibitor, blocked the stimulatory effect of amisulpride on phosphorylated Akt. Together, these results suggest that regulation of the β-arrestin 2-dependent pathway via blockade of the D_2R in SH-SY5Y cells is one mechanism underlying the neuroprotective effect of amisulpride, but not haloperidol.

1. Introduction

Antipsychotic drugs are classified as typical and atypical based on their efficacy in alleviating schizophrenic symptoms and the incidence of extrapyramidal side effects (Andreasen, 1994; Meltzer, 1995). A growing body of evidence indicates that several atypical antipsychotic drugs have a neuroprotective effect against a variety of toxins (Bai et al., 2002; Luo et al., 2005; Wang et al., 2004; Wei et al., 2003a,b; Yulug et al., 2006). In contrast, the typical antipsychotic drug haloperidol lacks this effect (Behl et al., 1995; Noh et al., 2000). However, the molecular mechanisms underlying the differing effects of typical and atypical antipsychotic drugs have not been fully determined.

Both classes of antipsychotic drugs have an affinity for dopamine D_2 receptors (D_2R), but their therapeutic effects differ (Creese et al., 1996). D_2R belongs to the G protein-coupled receptor (GPCR) family. It couples with $G_{i/o}$ proteins to negatively regulate cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signaling, G protein-dependent mechanism (Missale et al., 1998). In addition to this canonical action, Beaulieu et al. (2004, 2005, 2006, 2007a, 2007b, 2008) recently suggested that the D_2R regulates Akt/glycogen synthase kinase-3β (GSK-3β) signaling by a G-protein-independent mechanism, involving the multifunctional scaffolding protein β-arrestin 2. D_2R stimulation induces receptor phosphorylation via the GPCR kinases (GRKs). Receptors phosphorylated by GRKs are then bound to β-arrestin 2. Subsequent β-arrestin 2 binding terminates G-protein-dependent signaling by blocking the receptor–G protein interaction causing the formation of a signaling complex, composed of

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β-arrestin 2, Akt, and the protein phosphatase2A (PP2A), which dephosphorylates (inactivates) Akt (Beaulieu et al., 2005). The formation of this complex modulates GSK-3β-mediated signaling. Thus, β-arrestin 2 may be an important mediator of Akt and GSK-3β regulation by D_2R (Masri et al., 2008). In this context, regulation of the Akt/GSK-3β pathway through β-arrestin 2 may be a key molecular mechanism modulating the action of antipsychotic drugs. However, this pathway has not been investigated. Recent studies indicate that Akt/GSK-3β signaling is a potential target for antipsychotic drugs, and alterations in this pathway have been implicated in the pathogenesis of schizophrenia (Emamian et al., 2004; Roh et al., 2007). Furthermore, we recently demonstrated that the neuroprotective effect of the atypical antipsychotic aripiprazole on human neuroblastoma SH-SY5Y cells involved inactivation of GSK-3β through brain-derived neurotrophic factor (BDNF)-mediated activation of phosphatidylinositol-3 kinase (PI3 K)/Akt signaling. The subsequent increase in transcription factor cAMP response element-binding protein (CREB) activity enhanced expression of its survival transcriptional target, Bcl-2, but the typical antipsychotic drug haloperidol did not show these effects (Park et al., 2009a).

Amisulpride, an atypical antipsychotic drug, selectively blocks dopamine D_2/D_3 receptors, but has no affinity for adrenergic, serotonergic, histaminergic, or muscarinic receptor systems (Perrault et al., 1997). This makes it unique among atypical antipsychotic drugs. Moreover, it has a dual effect that selectively blocks presynaptic D_2/D_3 dopamine autoreceptors at a low dose, enhancing dopamine transmission, and blocks postsynaptic D_2/D_3 dopamine receptors at a higher dose, inhibiting dopaminergic hyperactivity. This dual dopamine receptor-blocking effect contributes to the improvement of negative symptoms of schizophrenia at low doses and of positive symptoms at high doses (Rosenzweig et al., 2002). In contrast, haloperidol blocks D_2 receptors reduce only the positive symptoms of schizophrenia (Andreasen, 1994; Meltzer, 1995). To specifically investigate D_2R signaling, we studied amisulpride and haloperidol, which have a high affinity and selectivity for the D_2R.

Atypical antipsychotic drugs have a positive effect on neuronal viability compared with typical antipsychotic drugs (Lu and Dwyer, 2005). In the present study, we sought to investigate whether amisulpride and haloperidol affected neurite outgrowth in SH-SY5Y human neuroblastoma cells via the β-arrestin 2-dependent pathway. Next, we compared the effect of these drugs on the phosphorylation of Akt, GSK-3β, and CREB and the expression of BDNF and Bcl-2, downstream effectors of CREB, to detect changes in downstream signaling associated with the β-arrestin 2/Akt/GSK-3β pathway.

2. Methods

2.1. Drugs and reagents

Amisulpride was purchased from Sanofi-Aventis (Paris, France) and haloperidol was from Sigma (St. Louis, MO, USA). Antibodies used for immunostaining were purchased from the following sources: anti-microtubule associated protein 2 (MAP-2) from Millipore (Temecula, CA, USA); Alexa Fluor 568 goat anti-mouse IgG and Hoechst 33258 from Invitrogen (Carlsbad, CA, USA). Antibodies used for the Western blotting were purchased from the following sources: anti-phospho-ser^{473}-Akt, anti-Akt, and anti-CREB from Cell Signaling Technology (Beverly, MA, USA), anti-phospho-ser^{133}-CREB from Upstate Biotech (Lake Placid, NY, USA), anti-BDNF, anti-phospho-ser^{9}-GSK-3β, anti-GSK-3α/β, anti-Bcl-2, and anti-β-arrestin 2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-α-tubulin from Sigma, ECL anti-mouse IgG, horseradish peroxidase linked species-specific whole antibody from Amersham Pharmacia (Little Chalfont, Buckinghamshire, UK), and anti-goat and anti-rabbit IgG-horseradish peroxide conjugates from Santa Cruz Biotechnology. The PI3 K inhibitor, wortmannin, was purchased from Invitrogen.

2.2. Cell culture

SH-SY5Y human neuroblastoma cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic–antimycotic solution. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. For the neurite assay, cells were plated at a density 2×10^4 cells/well in a 12-well plate. For Western blot analysis, cells were plated at a density of 2×10^6 cells per 100 mm dish. After incubation for 24 h, the cells were treated with amisulpride (1, 10, 100 μM) or haloperidol (1, 10 μM) for 96 h. The culture media and drugs were changed every 2 days. No cell death was observed at the drug doses tested. Amisulpride and haloperidol were dissolved in dimethyl sulfoxide (DMSO) and diluted with DMEM medium to a concentration of 50 mM amisulpride and 10 mM haloperidol (final 0.5% DMSO).

2.3. Small interfering RNA-mediated knockdown of β-arrestin 2

β-arrestin 2 knockdown using small interfering RNAs (siRNAs) was performed as follows. Chemically synthesized double-stranded siRNAs were purchased from Invitrogen. The catalog numbers of the two sets of human β-arrestin 2-specific siRNA duplexes were ARRB2-HSS180981 and ARRB2-HSS180982. A nonsilencing RNA duplex (5′-AAUUCUCCGAACGUGUCACGU-3′) synthesized at Samchully Pharmaceuticals (Seoul, Korea) was used as the control (Shenoy et al., 2006). SH-SY5Y cells that were 50–60% confluent in 12-well and 6-well plates were transfected with 100 pmol and 150 pmol of siRNA (combination set), respectively for the neurite assay and Western blot analysis, using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocol. The growth medium was changed 4 h later, and amisulpride and haloperidol were treated simultaneously. The immunofluorescence assay and Western blot analysis to detect neurite outgrowth and Akt phosphorylation levels, respectively, were performed at least 3 days following siRNA transfection.

2.4. Neurite outgrowth assay

Neurites were visualized using immunostaining with a MAP2 antibody as follows. Cells were fixed for 20 min at room temperature using 4% paraformaldehyde, then permeabilized with 0.1% Triton X-100, and blocked with 4% bovine serum albumin in phosphate-buffer-saline (PBS) for 2 h to reduce non-specific binding. Cells were incubated with anti-MAP2 antibody diluted 1/200 in PBS for 2 h. Alexa Fluor 568 goat anti-mouse IgG was used as a secondary antibody and Hoechst 33258 was used for nuclear staining. Stained cells on the cover glass were observed using a fluorescent microscope (Olympus, Tokyo, Japan). Five fields were randomly selected from each sample and their images were captured using a digital camera by a person blinded to their identities. The entire procedure from neurite differentiation to image capture was performed twice. All cells in the ten fields, 300–400 in total, were included in the neurite analysis using MetaMorph, an automated image analysis program (Molecular Devices, Downingtown, PA, USA) (Klimaschewski, 2002).

2.5. Western blot analysis

Whole cell lysate preparation was conducted as follows. The cells were washed twice with ice-cold PBS. Lysis buffer (20 mM Tris–HCl, 137 mM NaCl, 10% glycerol, 1% Nonidet p-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mM EDTA, and 1 tablet complete protease inhibitor (Roche, Laval, Quebec, Canada)) were added, and the lysates were centrifuged (1000 × *g*, 10 min, 4 °C). Equal amounts of protein (20 μg) from the cell extracts for each treatment condition were separated on SDS-polyacrylamide gels and transferred electrophoretically onto polyvinylidene fluoride membranes. The blots were blocked by incubation in 5% (w/v) non-fat milk in Tris-buffered saline (TBS) with 0.15% Tween 20 (TBS-T) for 1 h. After incubation with a primary antibody (anti-phospho-ser^{473}-Akt, 1:1000, anti-Akt, 1:1000, anti-phospho-ser^{9}-GSK-3β, 1:1000, anti-GSK-3α/β, 1:1000, anti-phospho-ser^{133}-CREB, 1:1000, anti-CREB, 1:1000, anti-BDNF, 1:1000, anti-Bcl-2, 1:500, anti-β-arrestin 2, 1:250, anti-α-tubulin, 1:2000) in TBS-T at 4 °C overnight, the membranes were washed three times in TBS-T for 5 min. The membranes were subsequently incubated for 1 h in TBS-T containing the horseradish peroxidase-conjugated secondary antibody (donkey anti-goat IgG for anti-phospho-ser^{9}-GSK-3β, 1:10000, goat-anti-rabbit IgG for anti-phospho-ser^{473}-Akt, 1:1000, anti-Akt, 1:5000, anti-phospho-ser^{133}-CREB, 1:1000, anti-CREB, 1:5000, and anti-BDNF, 1:10000, ECL anti-mouse IgG for anti-GSK-3α/β, 1:2000, anti-Bcl-2, 1:500, anti-β-arrestin 2, 1:250, and anti-α-tubulin, 1:10000). Immunoreactive bands were visualized and quantified using ECL + Western blotting reagents, with chemifluorescence detected using the Las-3000 Image Reader (Fuji Film, Tokyo, Japan) software. The amount of protein was normalized based on α-tubulin, which was unaffected by the drug treatments, to adjust for protein loading variation, and the values were expressed as the percentage of vehicle control, deemed to be 100%.

2.6. Statistical analysis

Data are expressed as means ± SEM of three independent experiments. One-way analyses of variance (ANOVA) were performed and followed with *post-hoc* comparisons using Duncan's test. Values were deemed to be statistically significant at $p < 0.05$.