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HALOPERIDOL AND CLOZAPINE BLOCK FORMATION OF AUTOPHAGOLYSOSOMES IN RAT PRIMARY NEURONS

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Abstract—Early intervention and maintenance treatment for schizophrenia patients may prolong the duration of exposure to antipsychotic agents; however, there have been few studies on the neurotoxicity of these agents. Here, we investigated the effects of antipsychotics on cell viability and autophagy in rat primary neurons. Cultured cortical neurons obtained from rat embryos were treated with various concentrations of haloperidol and clozapine, and the neuronal toxicity was assessed by measuring lactate dehydrogenase (LDH) activity and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Autophagosomes were quantitated by measuring the level of microtubule-associated protein 1A/1B-light chain 3 (LC3-II) by Western blot and immunofluorescence staining. Autophagic flux was assayed using bafilomycin A1 and GFP-mCherry-LC3 transfection. Haloperidol and clozapine decreased the viability of neurons *in vitro* in a concentration- and time-dependent manner. We also observed increased accumulation of autophagosomes after antipsychotic treatment. Using bafilomycin A1 and GFP-mCherry-LC3 transfection, we discovered that haloperidol and clozapine inhibited autophagosome turnover resulting in a dysfunctional autophagic process, including impaired lysosomal fusion. Together, these results suggest that haloperidol and clozapine negatively affect neuronal viability, possibly by blocking autophagolysosome formation.

Key words: autophagy, clozapine, haloperidol, neurotoxicity, schizophrenia.

Schizophrenia is a severe neuropsychiatric disorder characterized by delusions, social withdrawal, anhedonia, and memory deficits, which are usually grouped into positive/negative symptoms and memory deficits (Crow, 1980). Antipsychotic medications are the cornerstone for current schizophrenia treatment. The key pharmacologic property of all neuroleptics with antipsychotic activity is their ability to block dopamine-2 (D2) receptors, and their clinical antipsychotic potency correlates with their affinity for these receptors (Meltzer, 1991). Most schizophrenic patients take antipsychotic agents for a substantial portion of their lifetimes, often for decades. Furthermore, early intervention has advanced the age when patients are first exposed to antipsychotics, further prolonging their treatment duration.

Haloperidol is a typical first-generation antipsychotic (FGA) that is widely utilized clinically. In addition to blocking D2 receptors, it also inhibits alpha-1 adrenergic receptors, but has little affinity for muscarinic cholinergic and histamine receptors (Niemegeers and Laduron, 1976; Gelders, 1986). Although haloperidol has been reported to have neuroprotective effects against NMDA-induced neurotoxicity (Zhuravliova et al., 2007; Gardoni et al., 2008) and ketamine-induced cell death (Keilhoff et al., 2010), it has also been shown to be cytotoxic to neurons in a concentration-dependent manner and to induce cell death via oxidative stress and apoptosis (Reinke et al., 2004; Agostinho et al., 2007). Haloperidol has been shown to induce apoptosis by reducing cell survival signaling, which may contribute to its differential therapeutic efficacy and side effects in patients with schizophrenia (Mitchell et al., 2002; Reinke et al., 2004; Zhuravliova et al., 2007; Abekawa et al., 2011).

Clozapine was the first atypical or "second-generation" antipsychotic (SGA) introduced into clinical practice. It has been demonstrated to be more effective than FGAs in treatment-refractory patients and was associated with a reduction in suicidal tendencies (Lieberman et al., 1989). Although the exact pharmacological mechanisms of action of clozapine are not fully understood, it has a strong affinity for dopamine receptors and a robust serotonin blocking effect (Aitchison et al., 2000). Its tendency to cause agranulocytosis substantially limited its utilization until the 1990s (Lieberman et al., 1988), and research has reported contradictory effects on cell viability, indicating that it is both neuroprotective against oxidative stress (Magliaro and Saldanha, 2009) and neurotoxic (Dwyer et al., 2003).

Protein synthesis and degradation are carefully balanced in all cells to avoid the accumulation of misfolded proteins. The removal of excess proteins is especially critical in postmitotic cells, such as neurons. Indeed, most neurodegenerative diseases are characterized by the intraneuronal accumulation of specific cellular proteins (Ling and Salvaterra, 2009). Autophagy and the ubiquitin-proteasome system are the two major intracellular catabolic

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Abbreviations: HDAC, histone deacetylase; LC3-II, 1A/1B-light chain 3; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; TTBS, Tris-buffered saline Tween 20.

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systems responsible for protein clearance. Importantly, the accumulation of autophagic intermediates has been associated with neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's diseases (Chu, 2006; Nixon, 2007; Spassieva et al., 2009). Moreover, mice with neuron-specific autophagy defects were found to have intracellular inclusions in the brain and developed neurodegenerative symptoms despite the absence of any disease-causing, aggregation-prone proteins (Hara et al., 2006; Komatsu et al., 2006). Autophagy is reported to be constitutively active in healthy neurons, which may aid neuron survival (Boland and Nixon, 2006).

Determining antipsychotic drug mechanisms of action could enhance our understanding of schizophrenia. No reports have demonstrated the effects of antipsychotic drugs on autophagy. Thus, we assessed the effects of antipsychotic agents haloperidol and clozapine on neuronal viability and autophagy. A gene expression study demonstrated that schizophrenics had greater expression of genes involved in processes mediating cell adhesion, synaptic contact, cytoskeletal remodeling, and apoptosis (Barnes et al., 2011). Thus, the disruption of many common pathways and processes underpinning synaptic plasticity, including autophagic process in schizophrenia, were likely mediated by the disease. In particular, we analyzed the effects of these agents on autophagic changes associated with psychiatric disorders to provide clues regarding the role of autophagy in schizophrenia treatment.

EXPERIMENTAL PROCEDURES

Neuron cultures

Primary cortical neuron cultures were prepared from the brains of embryonic day 16 rat pups, as previously described (Kim et al., 2010a). Briefly, the cerebral cortices were dissected in calcium- and magnesium-free Hanks' balanced salt solution and incubated with 0.125% trypsin for 10 min at 37 °C. The trypsin was inactivated with Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal bovine serum, and the cortical tissue was further dissociated by serial trituration using a Pasteur pipette. The resulting cell suspensions were diluted in neurobasal medium supplemented with B27 components (Gibco-BRL, Grand Island, NY, USA) and plated onto poly-D-lysine- (Sigma-Aldrich, St. Louis, MO, USA, 50 mg/ml) and laminin-coated (Gibco-BRL, 1 mg/ml) 48-well plates at a density of 5×10^4 cells per well. The neurons were maintained at 37 °C in a 5% CO_2 atmosphere for 12 days.

Drug treatment

Neurons were incubated for 4 or 24 h with clozapine (Sigma) or haloperidol (Sigma) at concentrations of 400 nM, 2, 10 μM, or 25 μM with 25 nM of bafilomycin A1 (Sigma), rapamycin (Sigma), or with medium alone. Bafilomycin A1 was characterized initially by its selective inhibition of vacuolar type-ATPase (V-ATPase), which maintains the low pH of acidic vesicles by regulating proton pumping (Yoshimoto et al., 2009). By inhibiting V-ATPase, bafilomycin A1 increases intravesicular pH (Isidoro et al., 2009), thus acting as a lysosomal inhibitor.

Lactate dehydrogenase (LDH) assay

LDH activity in the medium of neuron cultures was assessed quantitatively using a kit (Cytotox 96, Promega, Madison, WI, USA), as previously described (Lim et al., 2010).

MTS assay

Neuronal cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction assay (Cell Titer kit, Promega) according to the manufacturer's instructions. The absorbance was measured spectrophotometrically at 490 nm.

Western blot analysis

Cell cultures were harvested with a cell scraper and centrifuged at 400 g for 5 min. The cell pellets were then resuspended in a protein extraction solution (Pro-prep, Intron, Seongnam, Gyeonggi, Korea), incubated at −20 °C for 20 min, and centrifuged at 13,400 g (4 °C) for 5 min. The supernatants were transferred to 1.5-ml tubes, and protein concentrations were measured using the Bradford method. Equal amounts of protein were mixed with a sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 2.5% glycerol, 0.5% 2-h-mercaptoethanol, and Bromophenol Blue), boiled at 100 °C for 5 min, and stored at −20 °C until use. The proteins were subjected to SDS-polyacrylamide gel electrophoresis at constant voltage (130 V) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (pore size, 0.2 mm, Bio-Rad Laboratories, Hercules, CA, USA) at 110 V for 2 h. The blots were incubated for 1 h in a blocking Tris-buffered saline Tween 20 (TTBS) buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) containing 2% bovine serum albumin (BSA), and then at 4 °C for 16 h in TTBS with 2% normal horse serum and a rabbit anti-LC3B antibody (1:1000, Sigma). Finally, the blots were washed with TTBS buffer, incubated with an HRP-labeled anti-IgG (Vector, 1:2000), and visualized using enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL, USA) and X-ray film. The bands were quantified densitometrically using ImageJ (Image processing and Analysis in Java) software (http://rsbweb.nih.gov/ij/).

Lysotracker staining

Lysotracker staining was performed as previously described (Yoon et al., 2008). Briefly, cells grown on glass coverslips were incubated with 50 nM LysoTracker-Red DND-99 (#L-7528, Molecular Probes, Carlsbad, CA, USA) in cell culture medium at 37 °C for 2 h.

Immunocytochemistry

Cells grown on glass coverslips were fixed for 30 min at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer. The cell membranes were permeabilized by incubation for 30 min in 0.05 M Tris buffer (pH 7.4) containing 0.1% Triton X-100, 2% BSA, and 2% normal horse serum. The cultures were incubated overnight at 4 °C with a rabbit anti-LC3B antibody (1:200, Sigma) or rabbit anti-cathepsin D antibody (1:200, Dakocytomation, Sure-Medical, Seoul, Korea), washed, and incubated with FITC- or Cy3-labeled anti-rabbit or anti-mouse secondary antiserum (1:300, Jackson Laboratories, ORIENT BIO, Seongnam, Gyeonggi, Korea).

LC3 gene plasmid transfection

Primary cortical neurons were transfected with plasmids encoding GPF-LC3 and mCherry-GFP-LC3 using mixtures of 0.8 μg plasmid per 2 μl Lipofectamine 2000 (#11668-027, Invitrogen, Carlsbad, CA, USA) in Opti-MEM medium (Invitrogen #31985). After incubation for 2–3 h, the supernatants were removed, and the cells were cultured in regular complete media for 1 day. Haloperidol or clozapine was added on the second day, and the incubation was continued.