Sertindole, a Potent Antagonist at Dopamine D2 Receptors, Induces Autophagy by Increasing Reactive Oxygen Species in SH-SY5Y Neuroblastoma Cells

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Autophagy is associated with cell survival and cell death. Autophagy is implicated in the pathophysiology of various human diseases. In order to identify autophagy regulatory molecules, we screened a chemical drug library in SH-SY5Y cells and selected Sertindole as a potent autophagy inducer. Sertindole was developed as an antipsychotic drug for Schizophrenia. Sertindole treatment highly induced the formation of autophagosomes as well as LC3 conversion. Subsequently, Sertindole-induced autophagy was efficiently suppressed by down regulation of ATG5. Sertindole also increased reactive oxygen species (ROS) production, which contributes to autophagy-associated cell death in neuroblastoma cells. ROS scavengers such as N-acetylcysteine and Trolox suppressed not only ROS generation but also autophagy activation by Sertindole. These results suggest Sertindole induces autophagy and autophagy-associated cell death by ROS production in neuroblastoma cells.

Key words Sertindole; autophagy; ATG5; reactive oxygen species; SH-SY5Y cell

Autophagy refers to a highly conserved lysosome-dependent degradation mechanism that involves the removal of cytoplasmic proteins and organelles.1,2) Autophagy plays an important role in development, immune defense, and cell death. The fundamental role of autophagy helps cell survival under stressful conditions by enhancing metabolic homeostasis. However, autophagy may also contribute to non-apoptotic cell death processes under some conditions.3–6) Indeed, autophagy has been implicated in many pathophysiological conditions such as neurodegenerative diseases such as cancer, type 2 diabetes, infectious diseases, cardiomyopathies, and innate immune diseases.3–6) Therefore, understanding the molecular mechanism of autophagy may help to develop new therapeutic strategies for those diseases. Based on this notion, several groups reported on small molecules that modulate autophagy and can be used as potential therapeutic drugs.7,8) For example, Rapamycin, an autophagy inducer, enhances clearance of intracellular aggregate proteins associated with Huntington’s disease.9,10) In addition, some autophagy regulators sensitize tumor cells to anticancer therapy.11) However, the precise mechanisms of autophagy in pathological conditions are not fully understood.

Schizophrenia (SZ) is a complex mental disorder often associated with cognitive impairment and depressive symptoms that affects about 1% of the population. SZ is caused by environmental and genetic risk factors or their interactions.12) Although, several genome wide association studies have identified SZ-related genes, the molecular mechanism is still largely unknown.13–15) The primary treatment for SZ is antipsychotic medications.16,17) Sertindole, a phenylindole derivative was developed as an antipsychotic drug. It is a potent antagonist at dopamine D2, serotonin 5-HT2, and α1-adrenergic receptors with high affinity.18,19) Additionally, Sertindole suppresses potassium channel in the brain and heart.20,21) In contrast to other antipsychotics, Sertindole is not associated with sedative effects; sedation may add to the cognitive problems inherent in SZ. However, both the effect of Sertindole on autophagy and the role of autophagy in SZ are completely unknown.

In this study, we identified Sertindole as an autophagy inducer from a chemical library (Prestwick chemical library) screening. Sertindole treatment efficiently induced autophagy in SH-SY5Y neuroblastoma cells. We also investigated reactive oxygen species (ROS) production involved in Sertindole-mediated autophagic cell death. Our data suggest that Sertindole induces both autophagy and ROS generation in neuroblastoma cells.

MATERIALS AND METHODS

Cell Culture SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC). m5-7 mouse embryonic fibroblast cells (ATG5 Tet-off system by doxycycline) were kindly provided by Noboru Mizushima (Tokyo Medical and Dental University, Japan). All cells were cultured at 37°C in a 5% CO2 incubator and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, U.S.A.).

Reagents The expression plasmid green fluorescent protein microtubule-associated protein 1 light chain 3 (pGFP-LC3) was a gift from Noboru Mizushima (Tokyo Medical and Dental University, Japan). Sertindole, 3-methyladenine (3MA), Rapamycin, Thapsigargin, N-acetylcysteine (NAC), Trolox, and H2O2 were purchased from Sigma (St. Louis, MO U.S.A.). ARPI01 was purchased from TOCRIS (St. Louis, MO, U.S.A.). The short interfering RNA (siRNA) targeting ATG5 (5'-CAGGUAAGUCAAGCCUACA-3'; #2: 5'-CAGUAUCAGACACGAGAUCAU-3') and negative scrambled siRNA (5'-CCUACGCCCAUUAUCGU-3') were synthesized from Bioneer (Daejeon, Korea).

Stable Cell Line and Image-Based Chemical Screening To generate stable cell line (SY5Y/GFP-LC3), SH-SY5Y cells were transfected with pEGFP-LC3 using Lipofectamin

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according to manufacturer’s protocol (Invitrogen, Carlsbad, CA, U.S.A.). Then, stable transfectants were selected by growth in selection medium containing 1 mg/mL of G418 for 10 d. After single cell cloning, the stable clones were selected under fluorescence microscope. For the image-based chemical library screening, SY5Y/GFP-LC3 (3×10^3) cells were seeded in 96-well plate. Thirty hours later after seeding, each chemical of Prestwick library was added to each the well to a final concentration about ca. 10 µM. And the cells were cultured for 24 h then, cell with activated autophagy were observed under fluorescence microscope (IX71, Olympus, Japan). Rapamycin and Thapsigargin were used as positive control. The experiments were repeated two times with consistent results.

Fluorescence Microscopy SY5Y/GFP-LC3 cells were seeded on glass bottom well plate. After 24 h, the cells were treated with Sertindole (10 µM) for 24 h and washed with phosphate buffered saline (PBS), then fixed with 4% paraformaldehyde (PFA) for 20 min. Autophagy punctuates with GFP-LC3 was captured with fluorescence microscope (IX71, Olympus, Japan). Rapamycin and Thapsigargin were used as positive control. The experiments were repeated twice with consistent results.

Cell Viability Assay and Autophagy Analysis Cell viability was measured with NucleoCounter NC-3000 (Chemometec, Denmark). Cells were loaded to the Pi-Cassette™ (Chemometec) that immobilized with Propidium iodide fluorescent dye. Then, the fluorescence images were recorded and measured. The most widely used method for autophagy detection is observation and quantification of GFP-LC3 puncta by fluorescence microscopy. SY5Y/GFP-LC3 cells were treated with either Sertindole or ARP101 (10 µM) for 24 h and then subjected into Western blot analysis with anti-LC3 or anti-GFP. Actin was used as an internal loading control (E). Data represent ± standard error of the mean (S.E.M.) from three independent experiments (n=3, *** p<0.01).
After blocking with 4% skim milk in TBST (25 mM Tris, 3 mM 140 mM NaCl, 0.05% Tween 20), the membranes were incubated over-night with specific primary antibodies at 4°C. Anti-ATG5 (Ab54033, 1:2000) was from Abcam (Cambridge, U.K.); anti-LC3 (NB100-2220, 1:10000) antibody was from NOVUS Biologicals (Littleton, CO, U.S.A.); p62 (Sc-28359, 1:1000) antibody and GFP (Sc-8334–1:5000) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); anti-Acin (MAB1501, 1:10000) antibody was from Millipore (Temecula, CA, U.S.A.). For protein detection, the membranes were incubated with HRP-conjugated secondary antibodies (Pierce, Rockford, IL, U.S.A.).

**RESULTS**

**Sertindole, an Antipsychotic Drug, Induces Autophagy in Neuroblastoma Cells**

Autophagy dysfunctions are emerging as a theme in neurodegenerative diseases in which aggregated proteins accumulate. Recently, it has been shown that Rapamycin, an autophagy inducer, accelerates clearance of toxic aggregated proteins in cells. To identify autophagy regulatory molecules, we established an autophagy-screening assay system in SH-SY5Y neuroblastoma cells. GFP-LC3, a molecular marker of autophagosome formation was stably expressed in SH-SY5Y cells (SY5Y/GFP-LC3). Using this assay, we performed cell based-screening of the Prestwick chemical library (a collection of 1120 chemicals with 85% market drugs). From the screening, we selected Sertindole for further analysis. Sertindole is an antipsychotic medicine, and the effect of Sertindole on autophagy has not been investigated yet.

At first, to confirm the screening result, the SY5Y/GFP-LC3 cells were treated with Sertindole, and the cells were observed under both fluorescence microscopy and electron microscopy. Sertindole treatment induced formation of punctate dot structure by GFP-LC3 protein (Fig. 1A). Electron microscopy analysis also showed increased autophagosomes following...
Sertindole treatment (Fig. 1B). This autophagy activation was increased in a dose- and time-dependent manner (Fig. 1C). Sertindole treatment increased the number of punctate GFP-LC3 per cell as well as the number of autophagic cells (Fig. 1D). The conversion of LC3I to LC3II is considered to be another autophagy marker. According to the previous results, Sertindole treatment highly enhanced LC3 protein conversion (Fig. 1E). We previously showed that ARP101 is a strong autophagy inducer. Thus, APR101 was used as a positive control. Several phosphoinositide 3 (PI3) kinase inhibitors including 3-methyladenine (3MA), could inhibit the early step of autophagy activation. Thus, we next examined the effect of an autophagy inhibitor on Sertindole-induced autophagy. SY5Y/GFP-LC3 cells pretreated with 3MA were incubated with Sertindole for 24h. 3MA treatment significantly reduced the number of puncta in cells as well as LC3 conversion in SY5Y/GFP-LC3 cells respectively (Figs. 2A–C).

Autophagy is a cellular degradation process by which target substrates are delivered and degraded in the lysosome. Besides autophagy induction, autophagosomes could be accumulated due to trouble with autophagy flux, in the fusion process of autophagosomes to lysosomes. Thus, we next examined autophagic flux. The p62 protein is known to incorporate into autophagosome and selectively degraded in lysosome. Thus, we next investigated lysosome activation and p62 protein degradation following Sertindole treatment. SH-SY5Y cells were incubated with Sertindole for 24h and then stained with both LysoTracker (red) and Hoechst nuclear dye (blue). Stimulation by Sertindole showed enhanced lysosome staining compared to control (Fig. 2D). Moreover, the p62 protein level was also reduced following Sertindole treatment (Fig. 2E). These results suggest that Sertindole is a potent autophagy inducer in neuroblastoma cells.

**Suppression of ATG5 Inhibits both Sertindole-Mediated Autophagy and Cell Death**

ATG5 is an essential regulator of autophagosome formation during autophagy. In order to examine the effect of ATG5 on Sertindole-mediated autophagy, we employed specific siRNA against ATG5 (siATG5#1 and siATG5#2). SY5Y/GFP-LC3 cells were transfected with either siATG5 or scrambled siRNA. After 3d, the cells were exposed to Sertindole to induce autophagy. Down regulation of ATG5 resulted in suppressed formation of autophagosomes compared to control cells (Fig. 3A). Autophagy plays dual functions on cell survival and cell death. Thus, we also investigated the effect of Sertindole-mediated autophagy on cell viability. Inhibition of ATG5 slightly recovered Sertindole-induced cytotoxicity in SH-SY5Y cells, indicating that Sertindole-mediated autophagy contributes to cytotoxicity in neuroblastoma cells.
The effect of ATG5 on Sertindole-mediated autophagy was further examined in mouse embryonic fibroblast (MEF) cells (m5-7) that are regulated by the Tet-off system. In the presence of doxycycline (Dox), ATG5 expression was completely blocked, and autophagy was also inhibited in these cells. Sertindole failed to induce autophagy in m5-7 MEF cells when Dox was added, while it dramatically induced LC3II conversion in the control cells (Fig. 3D). Moreover, inhibition of autophagy by adding Dox significantly suppressed Sertindole-mediated cell death (Fig. 3E). Taken together, these results suggest that ATG5 plays an important role in the induction of Sertindole-mediated autophagy.

Increased ROS Involved in Sertindole-Mediated Autophagy and Autophagic Cell Death

Sertindole is a potent antagonist at dopamine D2, serotonin 5-HT2, and α1-adrenergic receptors. Dopamine has contracting effect on ROS which are important intracellular signaling molecules that play in autophagy and cell death. To evaluate the mechanism associated with Sertindole-mediated autophagy and cell death, we analyzed the relationship of these phenomena with ROS. The results showed that treatment of Sertindole increased ROS generation in SH-SY5Y cells (Figs. 4A, B). Thus, we next examined the effect of ROS scavengers (NAC and Trolox) on Sertindole-mediated autophagy. SY5Y/GFP-LC3 cells were incubated with Sertindole in the presence or absence of ROS scavengers. These inhibitors significantly suppressed Sertindole-mediated autophagy (Fig. 4C). More interestingly, ROS inhibition also significantly reduced Sertindole-mediated cytotoxicity in neuroblastoma cell (Fig. 4D). These results indicate that production of ROS triggers Sertindole-mediated autophagy and cell death.

**DISCUSSION**

Autophagy is involved in many pathophysiological conditions including cancer and neurological disorders. We previously showed some autophagy regulators in cancer cells. In the present study, we identified Sertindole as a potent autophagy inducer in neuroblastoma cells. From the screening, we also discovered several known autophagy regulator not only cyclosporin A, niclosamide but also some dopamine receptor antagonists such as Pimozide. And Trifluoperazine. Sertindole is a second-generation antipsychotic medicine for the treatment of SZ. Although, the relationship between autophagy and SZ is almost not elucidated, some recent evidences suggested the role of autophagy in SZ. For example, expression of some autophagy regulatory genes such as PI3K and ATG3 is altered in both Alzheimer’s and SZ disease.
and ApoL1, associated with SZ susceptibility could induce autophagy dependent cell death.\textsuperscript{27} Glutamine metabolism is also altered in SZ, and ammonia. A metabolic product of glutamine is a diffusible regulator of autophagy.\textsuperscript{28,29} However the role of autophagy in SZ is still largely unknown. In this study, we showed that Sertindole enhances ROS production as well as autophagy activation.

Dopamine signaling in the nervous system is mediated by dopamine receptors. Sertindole has higher binding affinity to dopamine D\(_2\) receptors and weaker affinity for dopamine D\(_1\) receptors.\textsuperscript{30} Accumulating studies suggest that dopamine has a neurotoxic effect. Indeed, dopamine can induce both apoptotic and autophagy-associated cell death in neuronal cells.\textsuperscript{30–32} In addition, inactivation of dopamine D\(_2\) receptors could also prevent dopaminergic neurotoxicity.\textsuperscript{33} However, the roles of agonists and antagonists to dopamine receptors in neuronal cell death are debatable. For instance, many agonists to dopamine D\(_2\) receptor mediate neuroprotective signaling.\textsuperscript{34,35} Dopamine itself also could protect striatal neurons against glutamate toxicity.\textsuperscript{36}

Furthermore, dopamine signaling has dual contrasting effect on ROS generation. Excessive stimulation of dopamine D\(_2\)-like receptors could enhance ROS production. But previous studies suggest that dopamine-D3-like and D\(_2\)-like receptors acts as antioxidants in various cells.\textsuperscript{37} Additionally, dopamine D\(_2\) agonist showed antioxidant activity by free radical scavenging.\textsuperscript{38} More importantly, Armando et al. showed that dysfunciton of dopamine D\(_2\) receptor increased ROS production and oxidative stress.\textsuperscript{39} ROS were recently shown to activate autophagy and autophagy-associated cell death.\textsuperscript{40,41} In this study, we found that inhibition of ROS suppressed Sertindole-induced formation of autophagosomes and cell death. These results imply that ROS is also key player in autophagy regulation by Sertindole.

Here, we also showed that Sertindole significantly increased autophagy-associated cell death by ATG5 dependent manner in neuroblastoma cells. In addition, Sertindole also shows high affinity for serotonin 5-HT\(_{2}\), and \(\alpha\)-adrenergic receptors. Therefore, additional studies regarding the role of Sertindoles in these receptor's signaling and autophagy are needed. The screening results showed that several antipsychotic medicines including Sertindole, Pimozide, Triflurromazine, Chlorpromazine, and Fluphenazine could induce autophagy. Thus, further studies about the role of autophagy in antipsychotic drugs will help to increase understanding and providing new therapeutic strategies in SZ.

In conclusion, Sertindole induces autophagy and autophagy-associated with cell death by ROS production in SH-SYSY cells.

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