Research article

Autophagic modulation by rosuvastatin prevents rotenone-induced neurotoxicity in an in vitro model of Parkinson’s disease

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HIGHLIGHTS

• Autophagy is seen as an important mechanism in Parkinson’s disease pathogenesis.
• Rosuvastatin revealed neuroprotective properties via modulating autophagy.
• Rosuvastatin could be a new candidate for treating Parkinson’s disease.

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ABSTRACT

Statins have been reported to have neuroprotective effects through anti-oxidant, anti-apoptotic, and anti-inflammatory mechanisms, and statin can also modulate autophagic signaling in an oxygen-toxicity models. Therefore, we investigated the effects of statin on autophagy markers and evaluated the neuroprotective effect of rosuvastatin against rotenone-induced neurotoxicity. As an in vitro model of Parkinson’s disease (PD) we adopted the rotenone-induced neurotoxicity model in SH-SY5Y cells. Cell viability was measured using the MTT assay, and to detect the expression of LC3 and α-synuclein, immunofluorescence analysis was performed. Intracellular signaling proteins associated with autophagy were explored via immunoblotting. Treatment with rosuvastatin alone increased the levels of mTOR-independent/upstream autophagy markers, including Beclin-1 and AMPK. Rosenone treatment of SH-SY5Y cells reduced their viability and α-synuclein expression; simultaneous exposure to rosuvastatin significantly restored these parameters. Rotenone enhanced mTOR expression and suppressed Beclin-1 expression, indicating suppression of the autophagic system. However, combined treatment with rosuvastatin also restored the Beclin-1 expression and decreased mTOR expression. We demonstrated the neuroprotective effect of statin in SH-SY5Y cells against rotenone-induced neurotoxicity, as well as the modulation of α-synuclein expression. The neuroprotective mechanism is likely to be associated with enhanced autophagy. The neuroprotective effect of statin on rotenone-induced dopaminergic neurotoxicity with modulation of autophagy provides a new therapeutic strategy for the treatment of PD.

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1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disease worldwide and is characterized by resting tremor, bradykinesia, rigidity, and postural imbalance [6]. Current treatment of motor symptoms of PD focuses on increasing the dopamine levels in the affected region by using medications such as L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine ago-
nists, which can produce symptomatic improvements [4]. Although symptomatic treatment is known to increase quality of life for PD patients, disease-modifying treatment that could eventually suppress or halt the disease progression is one of the greatest unmet goals in PD [14,22]. The major pathologic findings in PD include the loss of dopaminergic neurons within the substantia nigra and the presence of Lewy bodies [4]. Despite expansion of our knowledge regarding PD pathogenesis, the exact mechanism of cell loss in the substantia nigra in PD has remained unknown. It is thought that cell dysfunction and death in PD also involve several biochemical factors, such as free radicals, mitochondrial dysfunction, excitotoxicity, and inflammation. However, the final common pathway in PD pathogenesis leads to excessive deposition of toxins and to misfolding of proteins such as α-synuclein, and failure to degrade impaired protein might lead to the neuronal cell death associated with PD [14,24]. Therefore, modulating an intrinsic molecular mechanism that could neutralize or dispose of toxic misfolded proteins could be a feasible therapeutic target for developing disease-modifying agents for PD patients.

Autophagy is a self-degradative process that removes aggregated proteins, damaged organelles, and intracellular pathogens [11]. Recent studies have demonstrated dysregulation of the autophagy pathway in the brains of PD patients and in animal models of PD, suggesting a pivotal role for autophagy in the pathogenesis of PD [19]. Furthermore, there have also been several studies showing that autophagy enhancing leads to decreased levels of toxic aggregates and could show neuroprotective effects in PD models [25,29].

Statins, a class of drugs widely prescribed for reducing the levels of low-density lipoprotein, competitively inhibit HMG-CoA reductase, which is the first enzyme of the mevalonate pathway [9]. Other than their use as cholesterol lowering agents, statins have demonstrated neuroprotective effects via various mechanisms including anti-inflammatory actions, apoptosis regulation, and mitigating oxidative damage [17]. In addition, statins can also show cytoprotec
tive effects through modifying autophagy pathways. In a recent study, atorvastatin activated the autophagy pathway via AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) in mesenchymal stem cells [36]. Vilimanovich et al. also reported that inhibition of cholesterol synthesis induced autophagy and showed a cytoprotective effect in human leukemic cells [30]. Nevertheless, to the best of our knowledge, there has been no experiment showing the neuroprotective effect of statins through enhancing the autophagy pathway in a PD model. Therefore, we hypothesized that statins may decrease rotenone-induced neurotoxicity in SH-SY5Y cells as an in vitro model of PD by modulating the autophagy signaling pathway. In our previous experiments, we have demonstrated the neuroprotective effect of 1,25-dihydroxyvitamin D3 and erythropoietin on rotenone-induced neurotoxicity in SH-SY5Y cells through induction of autophagy [12,13]. In the current study, we investigated the concentration-dependent effect of a statin on autophagy markers in SH-SY5Y cells and evaluated its neuroprotective effect. Furthermore, we evaluated which levels of the autophagy signaling pathway are involved in statin-induced autophagy modulation.

2. Materials and methods

2.1. Cell culture and chemicals

We purchased the human neuroblastoma cell line SH-SY5Y from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco’s modified Eagle medium:Ham’s F12 (1:1 mixture) (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Grand Island, NY, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin (Grand Island, NY, USA) in a 5% CO2 incubator at 37 °C. Rosuvastatin was obtained from Daewoong Pharm. Co., Ltd and dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) to prepare a 10 mM stock solution. To treat the cells, rosvastatin was diluted in the culture medium to the appropriate concentration. Rotenone was obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

2.2. MTT assay

A quantitative colorimetric assay with MTT was used to measure cell viability. The MTT assay provided a sensitive measurement of the metabolic status of the cells, especially the status of the mitochondria, which reflect early redox changes. Briefly, exponentially growing cells were seeded in a 96-well plate at a density of 5 × 104 cells/well. Next, the cells were pre-treated with erythropoietin for 2 h. Rotenone was added to the culture medium to reach a final concentration of 200 nM, and the cells were incubated for 24 h. The control cells were treated with neither rosvastatin nor rotenone. After 24 h of incubation, 10 μl of the MTT assay kit reagent was added to each well, and the cells were incubated for an additional hour. The absorbance for each reaction product was measured with a microplate reader at a wavelength of 450 nm. The results are demonstrated as a percentage of the MTT absorbance of the control cells, i.e., the MTT absorbance of the control cells was set to 100%.

2.3. Immunoblot analysis

We prepared whole-cell lysates by incubating the cells in RIPA buffer (Beverly, MA, USA) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) according to the manufacturer’s instructions. The cells were harvested by centrifugation at 13,200 rpm for 5 min, and cells were washed in PBS (pH 7.2). The pellets were solubilized with the same volume of mitochondrial lysis buffer, kept on ice, vortexed for 5 min and centrifuged at 13,200 × g for 20 min at 4 °C. Equal amounts of the total lysate protein were loaded and separated on a 15% SDS–PAGE gel. The proteins were electrophoretically transferred to a PVDF membrane, which was blocked in 5% skim milk in Tris–buffered saline containing 0.1% Tween-20 (TBST) for 1 h. The membranes were incubated at 4 °C overnight with primary antibodies against one of the following proteins: LC3, Beclin-1, mTOR, AMPKα, alpha-synuclein and GAPDH from Cell Signaling (Beverly, MA, USA), LAMP1 and LAMP2 from Abcam plc. Ltd (Cambridge, UK). Next, the membranes were washed three times with TBST and probed with the corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. Probe detection was conducted using enhanced ECL Advance Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) and LAS–4000 film (Fujifilm, Tokyo, Japan).

2.4. Immunofluorescence analysis

To detect LC3 activation, alpha-synuclein and LAMP2 cells were seeded onto sterile coverslips placed in 24-well plates. The cells were treated with rosvastatin on the next day. At 24 h after treatment, the cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 30 min, and blocked with 2% bovine serum albumin (BSA) in Dulbecco’s phosphate buffered saline (DPBS) for 1 h. Cells were incubated with the primary antibodies targeting LC3B, alpha-synuclein and LAMP2 at room temperature (RT) for 1 h and with the Alexa Fluor 488 secondary antibody (Invitrogen) at RT for 1 h in the dark. Next, the samples were incubated with 1 mg/ml of 4’,6-diamidino-2-phenylindole (DAPI) at RT for 20 min in the dark. Slides were prepared with one drop of Pro-Long Gold Antifade Reagent (Invitrogen), and the
coverslips were sealed onto the slides by using clear nail lacquer. We obtained the images with a Leica TCS SP5 confocal microscope (Leica, Mannheim, Germany) and analyzed the pictures by using Image-Pro Plus 6.0 (Bethesda, MD, USA).

2.5. Statistical analysis

Data are expressed as the mean ± SD of 3 or more independent experiments. Viabilities were compared using Tukey’s test after one-way ANOVA. Levels of α-synuclein and immunoblotting results were compared using Tukey’s test after two-way ANOVA. *p < 0.05 and **p < 0.01 were considered statistically significant. Analysis was performed using SPSS for Windows (version 18.0) (SPSS, Chicago, IL, USA).

3. Results

3.1. Effects of treatment with rosuvastatin alone on SH-SY5Y cells and intracellular signaling proteins associated with autophagy

To investigate the effects of rosuvastatin mono-treatment on the viability and the autophagy system in SH-SY5Y cells, we treated the cells for 24 h with several concentrations of rosuvastatin (1, 5, 10, 20, 40, and 50 μM). Cell viability started to decrease at concentrations greater than 40 μM, and there was no cytotoxicity up to 40 μM (Supplementary Fig. 1). The levels of Beclin-1, which is a standard marker of autophagy, were significantly higher in the SH-SY5Y cells treated with various concentrations of rosuvastatin (10, 20, and 40 μM) than in the untreated controls. This effect was concentration dependent at concentrations up to 20 μM; however, the effect decreased at concentrations above 20 μM (Fig. 1). Treatment with rosuvastatin alone significantly increased the expression of AMPK, which is an upstream regulator of autophagy. The expression of LC3, a marker of autophagosome formation, and LAMP2, a marker of the lysosomal membrane, also increased after treatment with rosuvastatin alone, in a concentration-dependent manner (Fig. 1). Immunofluorescence analysis revealed that 40 μM rosuvastatin induced the expression of LC3, a marker of autophagosome formation. LC3 expression was dramatically decreased by co-treatment with 3-MA, which is an inhibitor of autophagy (Supplementary Fig. 2a, b). 3-MA specifically inhibits the early stage of autophagosome formation; therefore, this finding indicates that rosuvastatin modulates the early phase of autophagy in SH-SY5Y cells.

3.2. Effects of rosuvastatin on rotenone-induced neurotoxicity in SH-SY5Y cells

SH-SY5Y cells were incubated with 200 nM rotenone and treated simultaneously with various concentrations of rosuvastatin (0, 10, 20, and 40 μM). The viability of the cells treated with 200 nM rotenone was significantly lower than that of the controls; however, co-treatment with rosuvastatin at levels of up to 40 μM restored the cell viability in a concentration-dependent manner (Fig. 2).
3.3. Effects of rosuvastatin on levels of intracellular signaling proteins associated with autophagy in rotenone-induced neurotoxicity

Treatment of SH-SY5Y cells with 200 nM rotenone alone enhanced mTOR expression and suppressed Beclin-1 expression, indicating suppression of the autophagic system. However, treatment with the combination of rotenone + rosuvastatin (10, 20, or 40 μM) restored the expression of autophagy markers in a concentration-dependent manner (Fig. 3). The levels of mTOR decreased, and the levels of Beclin-1, a standard marker of autophagy, increased significantly in a concentration-dependent manner when treated with the combination of rotenone and rosuvastatin. Treatment with 200 nM rotenone also reduced the expression of AMPK, which is an upstream autophagy pathway marker; however, AMPK expression increased again in a concentration-dependent manner after the addition of rosuvastatin treatment (Fig. 3). The expression of LAMP2, a marker of the lysosomal membrane, significantly increased upon treatment with rotenone and 20 μM or 40 μM of rosuvastatin.

3.4. Effects of rotenone and rosuvastatin on the α-synuclein level in SH-SY5Y cells

To evaluate the neuroprotective properties of rosuvastatin, α-synuclein expression and immunoreactivity were assessed in SH-SY5Y cells. Rosuvastatin significantly increased the immunoreactivity and aggregation of α-synuclein compared with those in the control (Fig. 4a). Rosuvastatin co-treatment significantly reduced α-synuclein expression and aggregation (Fig. 4b). This finding suggests that rosuvastatin may be neuroprotective in rotenone-induced neurotoxicity.

3.5. Effects of rotenone and rosuvastatin on LAMP-2 in SH-SY5Y cells

Immunofluorescence analysis revealed that 40 μM rosuvastatin induced the expression of LAMP-2, a marker of the lysosomal membrane and a rate-limiting step of CMA (Supplementary Fig. 3). This indicates that rosuvastatin is involved in CMA in addition to the macroautophagy pathway.

4. Discussion

Through our study, we demonstrated that statin monotherapy up-regulates an autophagy marker, and thereby ameliorates rotenone induced neurotoxicity in SH-SY5Y cells.
through autophagy modulation. To the best of our knowledge, this is the first study to demonstrate the neuroprotective effect of statin through enhancement of autophagy. In previous studies, experts have reported that statin activates autophagy with various mechanisms. Zhang et al. demonstrated a protective role for autophagy in hypoxia and serum deprivation status [36]. In that study, atorvastatin activated autophagy via the AMPK-mTOR pathway in mesenchymal stem cells. In another study, simvastatin increased the protein expression of autophagy markers such as LC3B and Beclin-1 in coronary arterial myocytes [31]. Likewise, current evidence suggests that statins induce autophagy by the activation of mTOR-dependent and -independent pathway however, no study has investigated the cytoprotective or neuroprotective effect of statins associated with autophagy in dopaminergic cells. In our study, rosvastatin enhanced the expression of upstream and key markers of autophagy; thus, statin-induced autophagy occurs at multiple levels of the autophagy pathway, and we provide evidence that autophagy may be a key element of PD pathogenesis.

The primary pathogenesis of PD includes progressive degeneration of dopaminergic neurons in substantia nigra [10]. Furthermore, Lewy bodies, abnormal aggregates of protein that are primarily composed of α-synuclein, have been identified as a distinctive feature of the disorder [16,21]. Misfolded proteins in PD are degraded via the following two pathways. The ubiquitin-proteasome system (UPS) selectively degrades short-lived intracellular and plasma membrane proteins under basal metabolic conditions. In contrast, the autophagy-lysosome system (ALS) degrades long-lived, stable proteins, and this is the only pathway that can recycle large organelles such as mitochondria [24]. There is evidence indicating that the autophagy pathway is involved in the pathogenesis of PD. For instance, α-synuclein can be degraded via the chaperone-mediated autophagy (CMA) and macroautophagy pathways [18], and autophagic vacuoles are increased in mouse models of PD and in PD patients [1,23]. In addition, CMA is responsible for α-synuclein homeostasis. Overexpression of lysosomal-associated membrane protein 2a (LAMP2a), which is the rate-limiting factor of CMA, pro-

**Fig. 4.** Rosuvastatin-enhanced clearance of α-synuclein.

(a) Confocal images of α-synuclein immunostaining in SH-SYSY cells in the control, rosvastatin, rotenone, and rosvastatin + rotenone conditions, showing the aggregation of α-synuclein. The small squares in the top panels represent magnified portions of the cytoplasm. These images show that rosvastatin pretreatment decreased rotenone-induced α-synuclein aggregates, and inhibiting autophagy attenuated this phenomenon. Nuclei (blue) were stained with DAPI. (b) Protein levels of α-synuclein were determined using an immunoblotting assay. GAPDH was used as a loading control (n = 3). Asterisks indicate significant differences (**p < 0.01) from the control; hash marks indicate significant differences (#p < 0.05, ##p < 0.01) from the rotenone-treated group.
vided neuroprotection in human neuroblastoma SH-SY5Y cells by reducing total α-synuclein levels [33]. In this study, statin restored LAMP2a expression, which indicates that statin is involved in CMA in addition to the macroautophagy pathway. As CMA is considered to be a viable therapeutic target for PD [32], statins may also possibly become disease-modifying agents for PD.

In this study, statin suppressed the expression of α-synuclein, which is a 140-amino acid-long, natively unfolded protein that is encoded by the SNCA gene and is abundant in presynaptic terminals in the brain [8,15]. Although the exact functions of α-synuclein remain unclear, previous studies have suggested that it may be linked to familial and sporadic forms of PD. Point mutations in the SNCA gene were responsible for some familial forms of PD [26], and α-synuclein in Lewy bodies was found to be the main component of sporadic forms of PD. [28]. Due to genetic and environmental factors, natural α-synuclein is misfolded and converted to oligomers and fibrils and finally deposited in Lewy bodies [16]. Therefore, reducing α-synuclein synthesis or increasing clearance could become a therapeutic strategy for PD [7]. Misfolded proteins can be degraded via the UPS and ALS pathways; thus, increased autophagy may increase α-synuclein clearance and become a therapeutic possibility. The UPS is superior to the UPS in clearing misfolded proteins because large protein complexes such as oligomers of α-synuclein cannot pass through the narrow proteasome barrel in the UPS [24]. However, some evidence suggests that autophagy may induce cell death in neurons [27,34]; therefore, further investigation is needed to determine the optimal autophagy enhancement is required.

In this study, we chose to experiment with rosuvastatin over other types of statin because of its efficacy and pleiotropic effects. Although there are no studies comparing the efficacy of statins for neuroprotection, previous studies have reported outstanding efficacy of rosuvastatin for lowering the plasma low-density lipoprotein cholesterol level in comparison to other statins such as atorvastatin, simvastatin, and pravastatin [3,20]. In addition, hydrophilic characteristics dependent on the polar methyl sulfonamide group of rosuvastatin contribute to its pleiotropic properties, suggesting a potential neuroprotective role for rosuvastatin [5]. Furthermore, treatment with rosuvastatin alone revealed cytotoxicity at concentrations above 40 µM in our experiment. Considering that other types of statin have shown cytotoxicity at lower concentrations, there may be differences in the characteristics of rosuvastatin and those of other statins. Bonifaci et al. reported that simvastatin and atorvastatin were cytotoxic at 10 µM via inhibition of the AKT signaling pathway, whereas similar responses were observed for rosuvastatin at 50 µM [2]. However, in other studies, rosuvastatin-induced cytotoxicity occurred at lower concentrations [35]. Therefore, further investigation is necessary to clarify rosuvastatin-induced cytotoxicity.

There are several limitations of our study. Since this was an in vitro experimental study, the findings may not be reproducible under in vivo conditions. In addition, limited types of autophagy markers were applied for evaluating the mechanism of statin-mediated regulation of autophagy. Lastly, the rotenone-induced toxicity model of PD is not fully compatible with the microenvironment of dopaminergic cells in PD patients.

In conclusion, we demonstrated the neuroprotective effect of statin in SH-SY5Y cells against rotenone-induced neurotoxicity, as well as the modulation of α-synuclein expression. The mechanism of neuroprotection is likely to be associated with increased autophagy. The neuroprotective effect of statin on rotenone-induced dopaminergic neurotoxicity via modulation of autophagy provides a new therapeutic strategy for the treatment of PD.

**Disclosure statement**

None of the authors have anything to disclose regarding this manuscript, and all authors have no potential conflicts of interest to report concerning this article.

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The authors had full access to all the data in the study, and the corresponding author had the final responsibility to submit the manuscript for publication.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2017.01.063.

**REFERENCES**


