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Rifampicin inhibits rotenone-induced microglial inflammation via enhancement of autophagy

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ABSTRACT

Mitochondrial and autophagic dysfunction, as well as neuroinflammation, are associated with the pathophysiology of Parkinson's disease (PD). Rotenone, an inhibitor of mitochondrial complex I, has been associated as an environmental neurotoxin related to PD. Our previous studies reported that rifampicin inhibited microglia activation and production of proinflammatory mediators induced by rotenone, but the precise mechanism has not been completely elucidated. BV2 cells were pretreated for 2 h with rifampicin followed by 0.1 μM rotenone, alone or in combination with chloroquine. Here, we demonstrate that rifampicin pretreatment alleviated rotenone induced release of IL-1β and IL-6, and its effects were suppressed when autophagy was inhibited by chloroquine. Moreover, preconditioning with 50 μM rifampicin significantly increased viability of SH-SY5Y cells cocultured with rotenone-treated BV2 cells in the transwell coculture system. Chloroquine partially abolished the neuroprotective effects of rifampicin pretreatment. Rifampicin pretreatment significantly reversed rotenone-induced mitochondrial membrane potential reduction and reactive oxygen species accumulation. We suggest that the mechanism for rifampicin-mediated anti-inflammatory and antioxidant effects is the enhancement of autophagy. Indeed, the ratio of LC3-II/LC3-I in rifampicin-pretreated BV2 cells was significantly higher than that in cells without pretreatment. Fluorescence and electron microscopy analyses indicate an increase of lysosomes colocalized with mitochondria in cells pretreated with rifampicin, which confirms that the damaged mitochondria were cleared through autophagy (mitophagy). Taken together, the data provide further evidence that rifampicin exerts neuroprotection against rotenone-induced microglia inflammation, partially through the autophagy pathway. Modulation of autophagy by rifampicin is a novel therapeutic strategy for PD.

1. Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disease. The pathological hallmarks of PD include the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc) and the presence of α-synuclein-containing deposits called Lewy bodies. Currently, the precise pathogenic mechanisms in PD remain poorly understood. The interplay between genetic susceptibility and environmental factors is considered an important determinant in the risk of PD (Vance et al., 2010).

Rotenone, a widely used pesticide, is one of the environmental toxins known to play a role in the pathogenesis of PD. Rotenone can cross the blood–brain barrier (BBB) and induce mitochondrial dysfunction and reactive oxygen species (ROS) production by inhibiting complex I of the mitochondrial respiratory chain. Furthermore, rotenone induces the accumulation of α-synuclein and inflammation mediated by the activation of astrocytes and microglia. These effects are implicated in the degeneration of dopaminergic neurons (Maturana et al., 2015). Therefore, rotenone has been widely employed to model PD in cells and animals to evaluate therapeutic approaches (Johnson and Bobrovskaya, 2015).

Rifampicin, a classic antibiotic used extensively against *Mycobacterium tuberculosis* and leprosy, has recently drawn more

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and more attention because of its neuroprotective effects in acute brain injuries and chronic neurodegenerative diseases (Bi et al., 2013). Rifampicin suppresses neuronal apoptosis by activating GRP78 via the PERK-eIF2α-ATF4 pathway, and reducing α-synuclein multimers (Jing et al., 2014; Xu et al., 2007). Moreover, rifampicin treatment is beneficial to neurons injured by lipopolysaccharide (LPS)-stimulated microglia via suppressing nuclear factor-kappa B activation, phosphorylation of MAPKs and toll-like receptor-4 (TLR-4) pathway (Bi et al., 2014, 2011). Recently, we have demonstrated suppression of the NLRP3 inflammasome and interleukin-1β (IL-1β) release by rifampicin following microglial exposure to rotenone (Liang et al., 2015). Despite the above findings, the precise mechanisms by which rifampicin inhibits neuroinflammation have not yet been fully identified.

Autophagy is an evolutionarily conserved process necessary for maintaining homeostasis. Autophagy is induced during stress, and preserves cellular energy by breaking down macromolecules to recycle their components and degrading damaged proteins or organelles during stress conditions. Recent studies have provided evidence of a close relationship between autophagy and innate immunity (Keller and Lunemann, 2017; Plaza-Zabala et al., 2017). Induction of autophagy can not only enhance host protection by promoting pathogen clearance, but also prevent the damage due to excessive inflammation. Studies have demonstrated that inhibition of autophagy, especially mitophagy (mitochondrial autophagy), leads to reduced degradation of damaged mitochondria and increased generation of ROS, which in turn activates the NLRP3 inflammasome, critical in the production of active IL-1β and IL-18 (Yang et al., 2014). Similarly, suppression of the autophagic proteins LC3 B and beclin-1 can exacerbate the secretion of IL-1β and IL-18 in macrophages stimulated by LPS and adenosine triphosphate (ATP), partially though the accumulation of dysfunctional mitochondria and cytosolic translocation of mitochondrial DNA (mtDNA) (Nakahira et al., 2011).

Therefore, the modulation of autophagy contributes to the regulation of inflammatory processes in multiple diseases by controlling the immune responses to pathogenic stimuli and production of pro-inflammatory factors. The present study extends our previous work in investigating whether rifampicin as a candidate in modifying the progression of PD by inducing autophagy.

2. Materials and methods

2.1. Reagents

Rotenone (#557368) and rifampicin (#557303) were purchased from Merck KGaA (Darmstadt, Germany). Cell Counting Kit-8 (#CK04-3000T) was purchased from Dojindo (Kumamoto, Japan). JC-1 (#C2006) and DCFH staining Kit (#S0033) were purchased from Beyotime Biotechnology (Jiangsu, China). MitoTracker™ Red FM (#M22425), LysoTracker™ Green DND-26 (#L7526), and sandwich high sensitivity ELISA kits for quantitative detection of mouse IL-1β (#BMS6002) and IL-6 (#BMS603) were obtained from Thermo Fisher Scientific (Carlsbad, CA). Anti-microtubule-associated protein 1 light chain 3 (LC3) (#PM036) was from MBL international (Woburn, MA, USA). Anti-β-actin (#AP0060) was purchased from Bioworld (Jiangsu, China).

2.2. Cell lines and culture

BV2 microglia were cultured in DMEM/F12 medium (#11320-033, Thermo Fisher Scientific, Carlsbad, CA) containing 10% fetal bovine serum (#10099-141, Thermo Fisher Scientific, Carlsbad, CA) at 37 °C in 5% CO2. SH-SY5Y cells were cultured in a DMEM medium (#11965-092, Thermo Fisher Scientific, Carlsbad, CA). Rotenone was prepared in dimethyl sulfoxide (DMSO) at a stock of 1 mM and diluted with the culture medium as needed. Rifampicin was made fresh in DMSO prior to each experiment.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-1β and IL-6 in culture supernatant was measured by ELISA (Meng et al., 2017; Pan et al., 2014). Our previous study has revealed that the optimal concentration of rifampicin applied to suppress microglial inflammation is 50 μM (Liang et al., 2015). Thus, BV2 microglia were preincubated with 50 μM rifampicin for 2 h. Then, 0.1 μM rotenone was added to the wells with or without chloroquine (40 μM) for another 48 h. Following the treatments, cell supernatants were collected and stored at −80 °C. ELISA was performed to detect the concentrations of cytokines in the culture supernatants using a standard curve. The absorbance was set at 450 nm and 570 nm using a microplate reader (Tecan M200, Grodig).

2.4. Cell viability of SH-SY5Y cells co-cultured with BV2 microglial cells

We cocultured SH-SY5Y cells with BV2 microglial cells to investigate the cytotoxicity of conditional media from microglia on SH-SY5Y cells. The BV2 microglial cells were seeded in transwell inserts with 0.4 μm pore size (Corning Life Sciences, Tewksbury, MA, USA) 24 h before treatments. Then 50 μM rifampicin was added into the supernatant of BV2 cells for 2 h. The supernatant was then removed and BV2 cells were cultured in fresh medium containing 0.1 μM rotenone with or without 40 μM CQ. The inserts containing BV2 cells were then transferred onto the plates containing SH-SY5Y cells to coculture for another 24 and 48 h. The cell viability of SH-SY5Y cells was measured by CCK-8 assay. Briefly, CCK-8 reagent was added to each well according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). The culture medium was transferred to a 96-well plate and the absorbance was read at 450 nm using a micro-plate reader. Cell viability was expressed as the percentage of Abs 450 nm of vehicle control.

2.5. Measurement of ROS

For the analysis of ROS, cells were collected and resuspended in 1 × PBS. DCFH-DA (10 μM) was added and incubated with cells at 37 °C for 30 min in the dark. After washing with ice cold PBS twice, the samples were subjected to FACScan flow Cytometry. DCF fluorescence intensity was analyzed using FlowJo software version 9.3.2 (TOMY Digital Biology).

2.6. Detection of mitochondrial membrane potential using JC-1 staining

JC-1 fluorescent probe (Beyotime Biotechnology, Jiangsu, China) is widely used for the detection of mitochondrial membrane potential (MMP). When the mitochondrial membrane potential is high, JC-1 accumulates in the mitochondrial matrix and forms aggregates, which emit red fluorescence. When the mitochondrial membrane potential is low, JC-1 monomers can't aggregate in the mitochondrial matrix and will then emit green fluorescence. This is therefore a convenient tool to detect the change of mitochondrial membrane potential by detecting the change in fluorescence. Briefly, at the end of treatments, cells were collected and resuspended in culture media at a density of 1×10^6 cells/ml and incubated at 37 °C with JC-1 staining solution (5 μg/ml) for 20 min. Following staining, FACScan flow Cytometry was used to detect red fluorescence intensity of JC-1 aggregates with an excitation wavelength of 525 nm and emission wavelength of