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Glycogen synthase kinase-3β activation mediates rotenone-induced cytotoxicity with the involvement of microtubule destabilization

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ABSTRACT

Rotenone, a mitochondrial complex I inhibitor, has been used to generate animal and cell culture models of Parkinson's disease. Recent studies suggest that microtubule destabilization causes selective dopaminergic neuronal loss. In this study, we investigated glycogen synthase kinase-3β (GSK3β) involvement in rotenone-induced microtubule destabilization. Rotenone-induced cytotoxicity in SH-SY5Y cells was attenuated by the GSK3β inhibitor SB216763. Tau, a microtubule-associated protein and substrate for GSK3β, has been implicated in the pathogenesis of tauopathies such as Alzheimer's disease. Rotenone induced an increase in phosphorylated tau, the effect of which was attenuated by concomitant treatment with SB216763. Rotenone treatment also decreased tau expression in the microtubule fraction and increased tau expression in the cytosol fraction. These effects were suppressed by SB216763, which suggests that rotenone reduces the capacity of tau to bind microtubules. Rotenone treatment increased the amount of free tubulin and reduced the amount of polymerized tubulin, indicating that rotenone destabilizes microtubules. Rotenone-induced microtubule destabilization was suppressed by SB216763 and taxol, a microtubule stabilizer. Taxol prevented rotenone-induced cytotoxicity and morphological changes. Taken together, these results suggest that rotenone-induced cytotoxicity is mediated by microtubule destabilization via GSK3β activation, and that microtubule destabilization is caused by reduction in the binding capacity of tau to microtubules, which is a result of tau phosphorylation via GSK3β activation.

1. Introduction

The pathological features of Parkinson's disease (PD) include dopaminergic neuron loss and the presence of abnormal intracytoplasmic inclusions, i.e., Lewy bodies [1]. Mitochondrial complex I inhibitors such as rotenone and MPP^+ induced loss of dopaminergic neurons in experimental models [2,3]. Recently, it has been proposed that microtubule destabilization plays a role in dopaminergic neuron loss [4]. Microtubule destabilization could also cause neurodegeneration because intact microtubules are required for axonal transport and normal neuronal functions. Previously, it has been reported that rotenone could directly bind to microtubules in cell-free systems [5–7]. Thus, the direct binding of rotenone to microtubules is thought to be a mechanism associated with microtubule destabilization. However, there is no evidence supporting this belief using a cell-based system.

It has been reported that the tau phosphorylation increased after intravenous injections of rotenone in rats [8] and that rotenone induced tau phosphorylation in SH-SY5Y cells [9]. Tau contributes to microtubule stabilization by directly binding to these microtubules. It is also known that tau hyperphosphorylation reduces the ability of tau to bind microtubules, thereby resulting in microtubule destabilization. Phosphorylated tau is also a component of Lewy bodies. Although the role of phosphorylated tau in the pathology of PD is unknown, phosphorylated tau and glycogen synthase kinase-3β (GSK3β), which is known as the tau kinase, have been observed in the autopsied brain tissues of PD patients [10].

Based on the aforementioned studies, we hypothesized that rotenone could induce increased tau phosphorylation via GSK3β activation and that tau phosphorylation resulted in microtubule destabilization. To test this hypothesis, we investigated the

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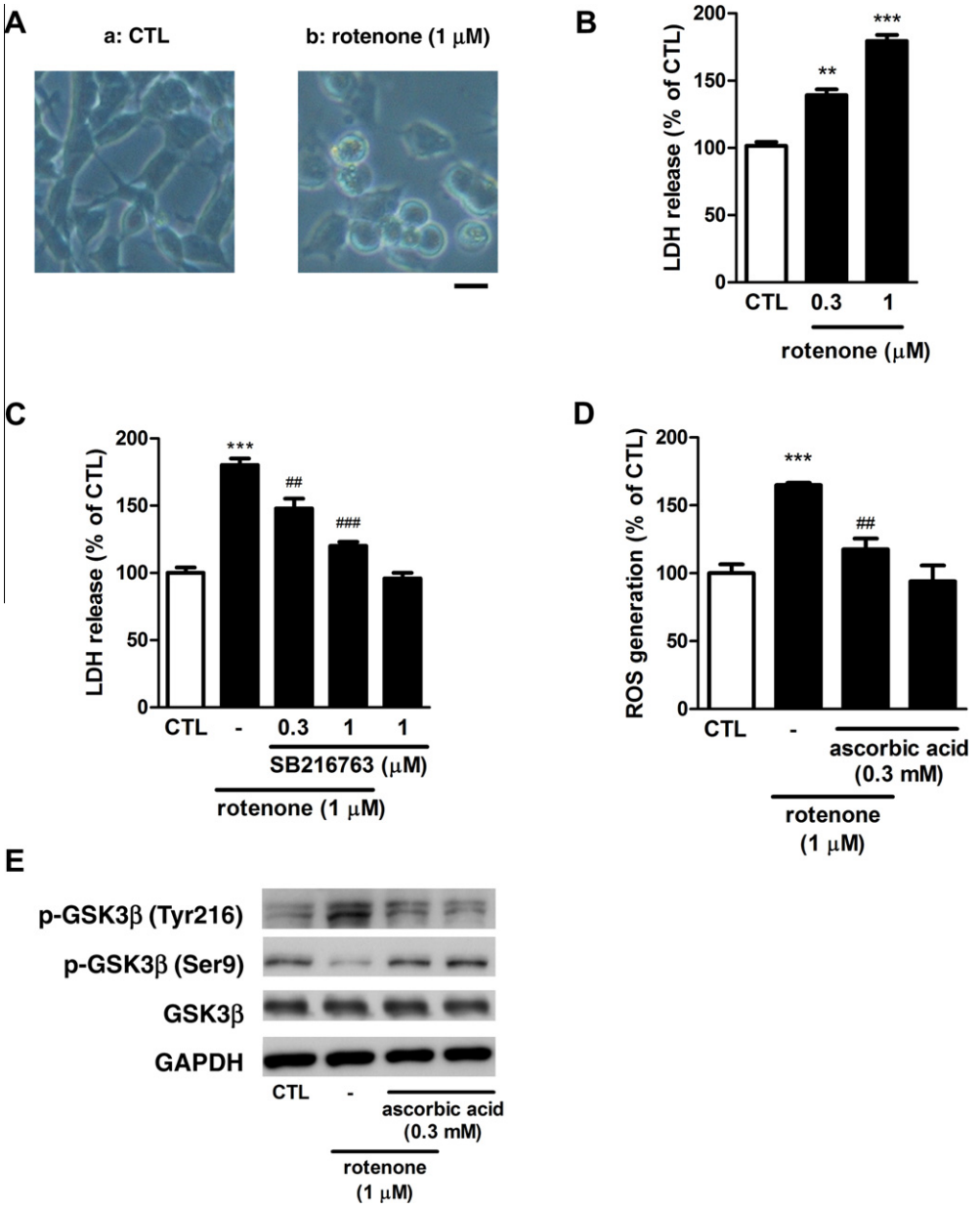

Fig. 1. Rotenone-induced cytotoxicity is mediated via ROS generation followed by GSK3β activation. (A) Representative phase-contrast imaging showed the rotenone-induced cytotoxicity in SH-SY5Y cells. Panel a, control (CTL); panel b, rotenone (1 μM). Scale bar = 10 μm. (B) Rotenone treatment significantly increased LDH release from SH-SY5Y cells in a concentration-dependent manner. Cells were exposed to rotenone for 24 h. Data are expressed as mean ± SEM, $n = 4$. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control (CTL). (C) SB216763, a GSK3β inhibitor, significantly suppressed rotenone (1 μM)-induced LDH release in a concentration-dependent manner. ROS generation was evaluated using CM-H_2-DCFDA. Data are expressed as mean ± SEM, $n = 4$. $^{***}p < 0.001$, compared with control (CTL), $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with rotenone (1 μM). (D) Ascorbic acid, an antioxidant, significantly suppressed rotenone (1 μM)-induced ROS generation. Data are expressed as mean ± SEM, $n = 4$. $^{***}p < 0.001$ compared with control (CTL), $^{\#\#}p < 0.01$, compared with rotenone (1 μM). (E) Rotenone treatment decreased phosphorylated GSK3β (Ser9) and increased phosphorylated GSK3β (Tyr216). Simultaneous administration of ascorbic acid suppressed rotenone (1 μM)-induced changes of phosphorylation states of GSK3β. SH-SY5Y cells were exposed to rotenone for 4.5 h. Phosphorylation state of GSK3β was evaluated by western blotting.

mechanisms associated with rotenone-induced microtubule destabilization using SH-SY5Y human neuroblastoma cells.

2. Materials and methods

2.1. Reagents

The drugs and other materials used in these experiments were obtained from the following sources: rotenone (Sigma, MO, USA); SB216763 (Tocris, Bristol, UK); taxol (Wako, Osaka, Japan); Opti-MEM and anti-phosphorylated tau (Ser396) antibody (Invitrogen, CA, USA); anti-tau antibody (EPITMICS, CA, USA); fetal bovine serum (FBS) (JRH Biosciences, Kansas, USA); MTX-LDH (Kyokuto, Tokyo, Japan); anti-GAPDH antibody (Ambion, TX, USA); and anti-α/β-tubulin antibody (Cell Signaling, MA, USA).

2.2. Cell culture

SH-SY5Y human neuroblastoma cells were maintained in Opti-MEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere. The medium was replaced every 2 days. The SH-SY5Y cells

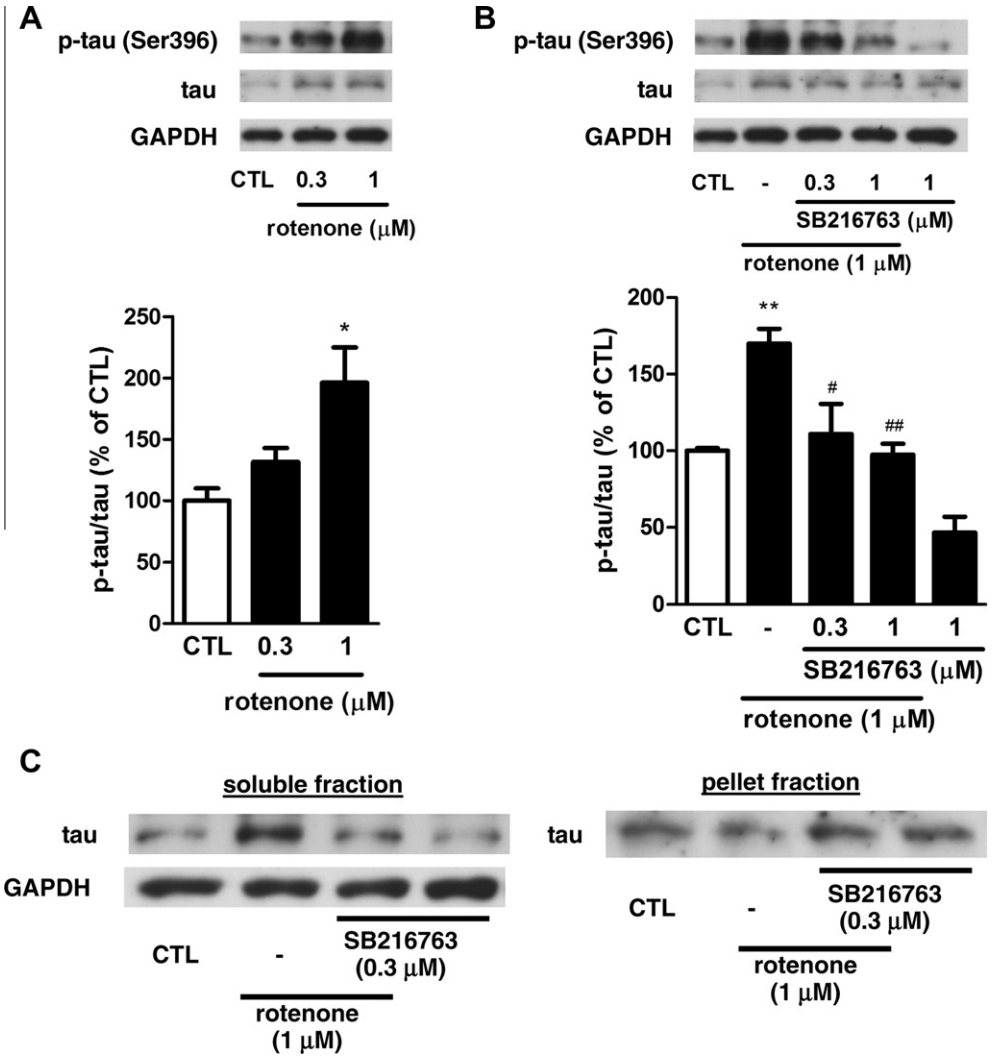

Fig. 2. Rotenone-induced tau phosphorylation decreases tau binding to microtubule (A) Rotenone treatment significantly increased phosphorylated tau (Ser396) in a concentration-dependent manner. SH-SY5Y cells were exposed to rotenone for 4.5 h. Phosphorylation state of tau was evaluated by western blotting. Data are expressed as mean ± SEM, $n = 3$. $^{*}p < 0.05$, compared with control (CTL). (B) SB216763, a GSK3β inhibitor, significantly suppressed rotenone (1 μM)-induced increase of phosphorylated tau. Data are expressed as mean ± SEM, $n = 4$. $^{**}p < 0.01$, compared with control (CTL), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with rotenone (1 μM). (C) SH-SY5Y cells were exposed to rotenone (1 μM), harvested and separated into soluble and pellet fractions by centrifugation as described under "Section 2". Administration of rotenone for 4.5 h increased tau in soluble fraction and decreased tau in pellet fraction, respectively. SB216763, a GSK3β inhibitor, suppressed these phenomenons.

were plated on culture plates. One day after plating, the culture medium was replaced with the same medium containing several drugs such as rotenone, SB216763, or taxol.

2.3. Assessment of cell viability

The amount of lactate dehydrogenase (LDH) released from SH-SY5Y cells damaged because of drug treatment was determined using an MTX-LDH assay kit according to manufacturer's instructions. In brief, 50 μl medium was collected 24 h after treatment, added to the wells of a 96-well plate, and incubated with 50 μl of the reaction solution. After incubation for 30 min at 37 °C, the reaction was terminated by adding 100 μl of stop solution. LDH level was spectrophotometrically determined at 570 nm using a Bio-Rad microplate reader (Hercules, CA, USA). The LDH activities of the control and experimental groups were normalized by subtracting the basal LDH activity in medium. LDH activity for an experimental condition was expressed as the percentage of the control.

$$\%\text{LDH} = [\text{LDH (experimental)} - \text{LDH (basal)}]/[\text{LDH (control)} - \text{LDH (basal)}] \times 100\%.$$

2.4. Assay for reactive oxygen species (ROS)

Intracellular ROS generation was detected using a sensitive fluorescent probe, 5-(and-6)-chloromethyl- 2′,7′-dichlorodihydrofluorescein diacetate and acetyl ester (CM-H_2DCFDA), according to manufacturer's instructions. In brief, cells cultured in a 96-well black-walled plate were incubated with CM-H_2DCFDA (5 μM) diluted in EBSS in a humidified atmosphere of 5% CO_2–95% air at 37 °C for 1 h. After washing twice with the same buffer without CM-H_2DCFDA, cell cultures were recovered in a phenol red-free EMEM without FBS for 15 min. Fluorescence intensity was determined (excitation, 485 nm; emission, 538 nm) using a WALLAC ARVO SXFL 1420 multi-label counter (Perkin Elmer. Life Sciences, MA, USA). ROS generation for the control and experimental groups was normalized by subtracting basal ROS in medium. ROS generation for an experimental condition was expressed as the percentage of the control.

$$\%\text{ROS} = [\text{ROS (experimental)} - \text{ROS (basal)}]/[\text{ROS (control)} - \text{ROS (basal)}] \times 100\%.$$

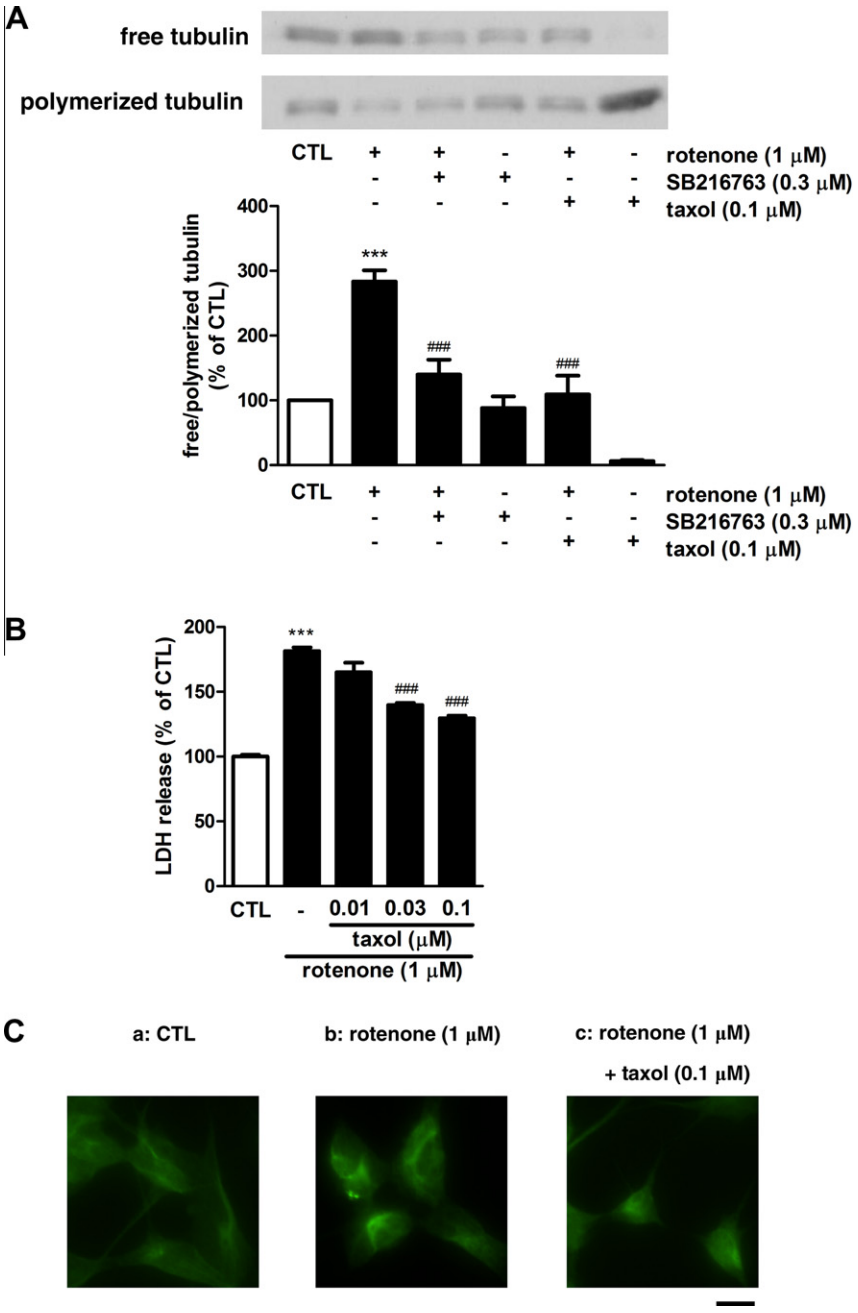

Fig. 3. Taxol, a microtubule stabilizer, attenuated rotenone-induced cytotoxicity. (A) Rotenone induced the increase of free tubulin and the decrease of polymerized tubulin, respectively. SB216763, a GSK3β inhibitor, or taxol, a microtubule stabilizer, attenuated these phenomenons. SH-SY5Y cells were treated with rotenone (1 μM) for 4.5 h. Data are expressed as mean ± SEM, $n = 4$. $^{***}p < 0.001$, compared with control (CTL), $^{###}p < 0.001$, compared with rotenone (1 μM). (B) Rotenone treatment for 24 h significantly increased LDH release in SH-SY5Y cells. Taxol, a microtubule stabilizer, significantly suppressed rotenone (1 μM)-induced LDH release. Cytotoxicity was evaluated by LDH assay. Data are expressed as mean ± SEM, $n = 4$. $^{***}p < 0.001$, compared with control (CTL), $^{##}p < 0.01$, $^{###}p < 0.001$ compared with rotenone (1 μM). (C) Fluorescence microscopic images of α/β-tubulin in SH-SY5Y cells. Panel a, control (CTL); panel b, rotenone (1 μM); panel c, rotenone (1 μM) + taxol (0.1 μM). Scale bar = 10 μm.

2.5. Western blot analysis

After each treatment, cells were solubilized in lysis buffer containing 20 mM Tris (pH 7.0), 2 mM EGTA, 25 mM 2-glycerophosphate, 2 mM dithiothreitol, 1% Triton X-100, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktail on ice for 30 min. Next, the cell lysates were centrifuged at 16,000×*g* for 15 min at 4 °C. Total protein concentrations in the supernatants were determined for each sample using the Bio-Rad Protein Assay Kit. These samples were stored at −20 °C until analysis. Cell lysates (10 μL) from each sample fraction were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. To block non-specific binding, the membranes were incubated in a buffer containing 2% bovine serum albumin at room temperature for 1 h and then incubated with a primary antibody diluted in TBS–Tween buffer at 4 °C overnight. The membranes were then incubated with an HRP-conjugated secondary antibody. Specific immunoreactive protein bands were visualized with an enhanced chemiluminescence detection system and exposed to an X-ray film. Densitometric analysis was performed using ImageJ software.

2.6. Determinations of free and polymerized tubulin

Free and polymerized tubulins were extracted from SH-SY5Y cells using standard protocols [4]. In brief, the SH-SY5Y cells were washed twice with 1 ml of buffer A containing 0.1 M MES (pH 6.75), 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA, and 4 M glycerol at 37 °C. After incubating the cells at 37 °C for 5 min in 400 μl of free tubulin extraction buffer (buffer A plus 0.1% Triton X-100 and protease inhibitors), the extracts were centrifuged at 37 °C for 2 min at 16,000×*g*. The supernatant fractions contained free tubulin that had been extracted from the cytosol. The cell pellet and lysed cells in a culture dish were dissolved in 400 μl of 25 mM Tris (pH 6.8) with 0.5% SDS, which contained tubulin in its original polymerized state (i.e., microtubules). Equal amounts of total proteins from the free and polymerized tubulin fractions were analyzed by Western blot analysis using an anti-α/β-tubulin antibody.

2.7. Microtubule binding assay

We used a standard protocol to determine the ability of tau to bind microtubules *in vitro* [11]. In brief, SH-SY5Y cells were harvested and placed in 400 μl of microtubule-stabilizing buffer (80 mM PIPES, pH 6.8, 1 mM GTP, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 30% glycerol, and 1× complete protease inhibitors) in the presence of taxol (10 μM) at 37 °C. Next, the cell debris was removed by centrifugation (10,000×*g* for 10 min) at 37 °C and supernatants were collected and re centrifuged at 100,000×*g* for 1 h at 37 °C. The resultant pellets were washed twice with and resuspended in microtubule-stabilizing buffer (400 μl), sonicated for 10 s, and then subjected along with 100,000×*g* supernatants to SDS–PAGE and Western blot analysis as described above.

2.8. Immunocytochemistry

For immunofluorescent staining, cells were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 15 min. The fixed cells were rinsed three times with PBS, blocked with 5% goat serum in PBS with 0.1% Triton X-100 for 30 min, and then incubated overnight at 4 °C with anti-α/β-tubulin (1:1000) antibody diluted in PBS containing 1% goat serum and 0.1% Triton X-100. On next day, the cells were washed three times and then incubated for 1 h at room temperature with Cy2-AffiniPure goat anti-rabbit IgG (1:2000). Cultures were washed with PBS and mounted on glass coverslips in FluorSave Reagent (Calbiochem, Germany). After being dried, cultures were examined under an Olympus photomicroscope equipped for epifluorescence. Digital images were obtained using a CCD camera.

2.9. Statistical analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Group comparisons were made by one-way ANOVA followed by Turkey's test using GraphPad Prism software 4.03. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. ROS generation mediates rotenone-induced cytotoxicity followed by GSK3β activation

Consistent with previous results, rotenone (1 μM) induced changes in SH-SY5Y cell morphology (Fig. 1A) and significantly increased the release of LDH from the cells (Fig. 1B). To investigate the involvement of GSK3β in rotenone toxicity, cells were simultaneously treated with rotenone and SB216763 (0.3 and 1 μM), a known GSK3β inhibitor. SB216763 significantly attenuated the rotenone-induced release of LDH (Fig. 1C).

Previously, it was reported that rotenone induced ROS generation and oxidative stress in neuronal cells [12]. Thus, we investigated the effect of rotenone on ROS production. Rotenone (1 μM) administration for 1 h significantly increased ROS generation. Ascorbic acid (0.3 mM), an antioxidant, attenuated rotenone-induced ROS generation (Fig. 1D).

GSK3β activity is negatively regulated by serine 9 (Ser9) phosphorylation and positively regulated by tyrosine 216 (Tyr216) phosphorylation [13]. To investigate the effect of rotenone on GSK3β activation, we examined the phosphorylation state of GSK3β by Western blot analysis. Rotenone treatment resulted in decreased GSK3β phosphorylation at Ser9 and increased GSK3β phosphorylation at Tyr216 without affecting the total expression of GSK3β (Fig. 1E).

Previous reports indicated that oxidative stress changed the phosphorylation state of GSK3β, followed by GSK3β activation [14]. We evaluated the effects of ascorbic acid to investigate the involvement of oxidative stress in rotenone-induced GSK3β activation. Simultaneous administration of ascorbic acid significantly suppressed the rotenone-induced changes in GSK3β phosphorylation (Fig. 1E).

3.2. Rotenone-induced tau phosphorylation decreases tau binding to microtubules

Tau is a known substrate for GSK3β and contributes to microtubule stability. We investigated the effect of rotenone on tau phosphorylation using anti-phosphorylated tau (Ser396) antibody. When cells were exposed to rotenone (0.3 and 1 μM) for 4.5 h, tau phosphorylation was significantly increased (Fig. 2A). To investigate the possible involvement of GSK3β in rotenone-induced tau phosphorylation, cells were simultaneously treated with SB216763 (0.3 and 1 μM) and rotenone. This resulted in significantly reduced rotenone-induced tau phosphorylation (Fig. 2B).

It is known that tau phosphorylation reduces the affinity of tau for microtubules, which causes microtubule destabilization [15]. We investigated the effect of rotenone on the binding capacity of tau to microtubule using the subcellular fractionation experiment. Rotenone (1 μM) increased the amount of tau protein in the soluble (cytoplasmic) fraction and decreased the amount in the pellet (microtubule) fraction (Fig. 2C).

Next, we examined the effect of SB216763 on the subcellular localization of tau. SB216763 (1 μM) abrogated rotenone-induced fractioned changes in tau.

3.3. Taxol, a microtubule stabilizer, attenuates rotenone-induced cytotoxicity

To evaluate the effects of rotenone on microtubule stability, we determined the amounts of free and polymerized tubulin. Rotenone (1 μM) treatment increased the amounts of free tubulin and decreased the amounts of polymerized tubulin (Fig. 3A). SB216763 inhibited these rotenone-induced changes in tubulin (Fig. 3A). Similarly, taxol, a microtubule stabilizer, significantly attenuated these rotenone (1 μM)-induced changes in tubulin.

To investigate the involvement of microtubule destabilization in rotenone cytotoxicity, cells were simultaneously treated with taxol (0.01–0.1 μM) and rotenone. Taxol significantly attenuated the rotenone-induced LDH release (Fig. 3B). Next, we performed immunocytochemical analysis using anti-α/β-tubulin antibody. Rotenone caused cell morphological changes such as retraction of cell processes (Fig. 3B). However, taxol attenuated this rotenone-induced retraction (Fig. 3C).

4. Discussion

In the present study, we demonstrated that rotenone-induced microtubule destabilization in SH-SY5Y cells was attenuated by GSK3β inhibition. GSK3β inhibition and microtubule stabilization prevented rotenone-induced cytotoxicity. These results suggested that rotenone-induced cytotoxicity was a result of microtubule destabilization via GSK3β activation.

As shown in Fig. 1, rotenone-induced toxicity was inhibited by SB216763, a GSK3β inhibitor. It has been reported that rotenone caused cell toxicity via GSK3β activation [13,16,17]. Moreover, it has been reported that MPP^+ caused cell toxicity via GSK3β activation [18]. Rotenone and MPP^+ can induce mitochondrial dysfunction by inhibiting mitochondrial complex I, and GSK3β activation is believed to be downstream of mitochondrial dysfunction [19]. Mitochondrial dysfunction reduces ATP production and promotes ROS generation [12]. Moreover, treatment with hydrogen peroxide activates GSK3β and induces PC12 cell death [14]. Therefore, it is assumed that rotenone activates GSK3β via ROS generation, which is a result of mitochondrial dysfunction.

We showed that rotenone could induce an increase in tau phosphorylation via GSK3β (Fig. 2). Mitochondrial complex I inhibitors such as MPTP and MPP^+ induced tau phosphorylation via GSK3β activation [9,18]. This suggested that tau phosphorylation occurred downstream of mitochondrial dysfunction. Previous reports also showed that tau phosphorylation reduced its affinity for microtubules and caused microtubule destabilization [20]. As shown in Fig. 3B, rotenone-induced cytotoxicity was inhibited by taxol. This suggests that rotenone-induced cytotoxicity was partly caused by microtubule destabilization.

Two pathways have been considered to be mechanistically involved in the toxicity caused by increased tau phosphorylation. The first pathway involves a "loss of function." The binding capacity of tau for microtubules decreased with increased tau phosphorylation. Because microtubules are involved in cytoskeletal formation, cells cannot maintain their morphologies as a result of loss of function. Microtubules also play a role in the transport of intracellular organelles such as mitochondria. Thus, it is believed that microtubule destabilization inhibits intercellular transport, which would cause cell death. It has been reported that dopaminergic neuron loss caused by microtubule destabilization may be a result of impaired transport of dopamine via synaptic vesicles [4].

The second pathway involves a "gain of function". Phosphorylated tau forms aggregates, and accumulation of these aggregates exerts neurotoxic effects [21]. In this study, we did not detect an increase in tau protein aggregates after rotenone treatment by Western blot analysis (data not shown). Therefore, tau protein aggregates are probably not involved in rotenone-induced cytotoxicity. In our study, rotenone-induced microtubule destabilization was mostly inhibited by SB216763. This suggested that GSK3β had an important role in rotenone-induced microtubule destabilization. However, we cannot exclude the possibility that rotenone induces microtubule destabilization by directly binding to tubulin.

In conclusion, rotenone could induce cytotoxicity in SH-SY5Y cells because of microtubule destabilization via GSK3β activation. Microtubule destabilization was believed to be caused by a reduced binding capacity of tau for microtubules, which was a result of GSK3β-mediated tau phosphorylation.

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