Baicalein Protects against Rotenone-Induced Neurotoxicity through Induction of Autophagy

Lianghong Kuang, Xiongbin Cao, and Zuneng Lu*

Department of Neurology, Renmin Hospital of Wuhan University; No. 238, Jiefang Road, Wuhan 430060, China.
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Balicalein, a typical flavonoid compound, has neuroprotective properties in several neurological disorders. Autophagy plays a central role in maintaining the cellular homeostasis, and is involved in the pathogenesis of Parkinson's disease (PD). Recently, baicalein has been reported to induce autophagy. Therefore, the current study was designed to investigate whether baicalein could protect against rotenone-induced neurotoxicity via induction of autophagy both in SH-SY5Y cells and in a mouse model. A chronic PD mouse model was established by continuous intragastric administration of rotenone for 12 weeks. Baicalein was administrated from 7 to 12 week. Our results showed that baicalein prevented rotenone-induced behavioral deficits, dopaminergic neuronal loss, apoptosis and mitochondrial dysfunction. Furthermore, baicalein restored rotenone-impaired autophagy, and blocking the baicalein-induced autophagy using 3-methyladenine inhibited the neuroprotective effects of bacalein. Baicalein increased cell viability and restored mitochondrial function in SH-SY5Y cells. The beneficial effect of baicalein was abrogated by 3-methyladenine treatment. Furthermore, rapamycin increased autophagy and reduced the rotenone-induced neurotoxicity in SH-SY5Y cells. Collectively, these results suggest that baicalein could prevent rotenone-induced neurotoxicity via restoring autophagy.

Key words baicalein; neuroprotection; Parkinson's disease; autophagy

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by bradykinesia, rigidity, resting tremor, and postural instability. The main pathological characteristic of PD is the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta, the loss of dopamine in the striatum, and the presence of intracytoplasmic inclusions in surviving neurons known as Lewy bodies. Several biochemical mechanisms related to the pathogenesis of PD include oxidative stress, mitochondrial dysfunction, protein aggregation and misfolding, apoptosis, excitotoxicity, and neuroinflammation. Currently, none of therapies has been convincingly shown to slow down or prevent the progression of PD, and additional effective treatments for this disease are urgently needed.

Baicalein, a flavonoid, is derived from the root of the traditional Chinese herb Scutellaria baikalensis Georgi. Baicalein has been reported to have neuroprotective effects in PD model through anti-inflammatory, anti-apoptosis, and anti-oxidative actions. In vitro, baicalein protected PC12 cells against 6-hydroxydopamine (6-OHDA)- or rotenone-induced neurotoxicity, and ameliorated the 6-OHDA-induced SH-SY5Y cell apoptosis. In vivo, baicalein exerted neuroprotective effects in 6-OHDA-induced neurotoxicity in rats and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in mice. However, the exact mechanism of baicalein is poorly understood.

Autophagy is a highly conservative cellular process by which cells degrade and recycle of bulk cytosolic proteins and damaged organelles through lysosomal degradation. Importantly, autophagy plays an important role in the survival of neural cells. Furthermore, autophagy has been reported to involve in the pathogenesis of PD as indicated by impairment of autophagy pathway is observed in the brains of PD patients and in animal models of PD. Recently, Liu et al. demonstrated that baicalein attenuated liver ischemia/reperfusion (I/R) injury via induction of autophagy. However, the role of autophagy in baicalein-affected neuroprotective effects is not fully understood.

Based on these findings, we hypothesized that baicalein could reduce rotenone-induced neurotoxicity via induction of autophagy. To test this hypothesis, we investigated the neuroprotective effects of baicalein and underlying mechanisms, with a focus on autophagy both in SH-SY5Y cells and in a mouse model.

MATERIALS AND METHODS

Animals and Drug Administration Male inbred C57BL/6J mice (one-year old) were purchased from Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). All animals were housed under standard animal care conditions and had free access to water and food. Mice in vehicle group were received vehicle (4% carboxymethylcellulose and 1.25% chloroform) orally daily for 12 weeks. Rotenone (5 mg/kg rotenone suspended in vehicle, Sigma-Aldrich, St. Louis, MO, U.S.A.) was administrated orally daily for 12 weeks as described previously. Baicalein was administered (100 mg/kg, intraperitoneally (i.p.), Sigma-Aldrich) daily from 7 week to 12 week. Mice were treated with 3-Methyladenine (3-MA, 30 mg/kg, i.p., Cayman Chemical, Ann Arbor, MI, U.S.A.) from 10 to 12 week. To measure autophagic flux, chloroquine (60 mg/kg, i.p., Sigma-Aldrich) was administrated 24 h before sacrifice. All procedures were carried out according to the ethical guidelines of the Animal Care and Use Committee of Wuhan University.

Cell Culture SH-SY5Y cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). SH-SY5Y cells were cultured in Dulbec-
of 3 mM sodium heptanesulfonate, 100 mM natrium acetate, the supernatant was mixed with the HPLC mobile phase consisting used to determine the concentration of dopamine. The super - trifuged at 14000 rpm at 4°C for 20 min. The supernatant was recorded and averaged. Grid test 20: mice were hung on the vertical grid with both their fore- and hind-paws, and the time taken by each mouse to descend the grid was noted as descent latency.

Assessment of Striatal Dopamine Content Striatal dopamine level was detected by HPLC. Striatum tissue was weighed and homogenized in 0.1 mol/L HClO4 and then centrifuged at 14000 rpm at 4°C for 20 min. The supernatant was used to determine the concentration of dopamine. The supernatant was mixed with the HPLC mobile phase consisting of 3 mM sodium heptanesulfonate, 100 mM natrium acetate, 85 mM citric acid, 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 8% methanol and injected into the HPLC column under analytical conditions. Data are expressed as pg dopamine per mg tissue.

Caspase-3 Activity Assay Caspase-3 activity in striatum was determined using a caspase-3 colorimetric assay kit (Abcam, Cambridge, U.K.).

Mitochondrial Membrane Potential Analysis Mitochondrial membrane potential was detected using a JC-1 mitochondrial membrane potential assay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). Striatal mitochondria were isolated using a mitochondria isolation kit (Biyotime, Shanghai, China) according to the manufacturer’s instruction. Briefly, mice were decapitated and the striata were dissected out, washed with ice-cold phosphate-buffered saline (PBS), homogenized on ice in isolation buffer, and then centrifuged at 10000×g for 10 min at 4°C. The supernatant was collected and then centrifuged at 10000×g for 10 min at 4°C. The sediment was washed and mitochondrial protein concentration was determined using Bradford method (Thermo Fisher Scientific, Rockford, IL, U.S.A.). Mitochondria were then added to a 96-well black plate and gently mixed with JC-1 staining solution and fluorescent intensity was analyzed.

SH-SY5Y cells (5×10⁵) were cultured on a 96-well black plate. After treated with different conditions, SH-SY5Y cells were cultured with JC-1 staining solution for 20 min at 37°C. The plate was centrifuged at 400×g for 5 min at room temperature. The supernatant was discarded, and cells were washed twice by centrifugation at 400×g for 5 min at room temperature and fluorescent intensity was analyzed. The change in fluorescence at 485/530nm (green) and 535/595 nm (red) was monitored by a fluorescent plate reader (BioTeK, VT, U.S.A.). Results are expressed as the ratio of red:green fluorescence intensity.

Cell Viability Assay Cell viability was detected using the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, SH-SY5Y cells (2×10⁴) were cultured on 96-well microplates. After treatment, SH-SY5Y cells were cultured with MTT solution (20μL, 5mg/mL) for another 4 h at 37°C. Then the medium was removed and the resultant formazan crystals were dissolved in dimethyl sulfoxide (150μL). Absorbance values were measured at 490 nm using a multi-mode micro plate reader (BioTek, Winooski, VT, U.S.A.). Lactate dehydrogenase (LDH) assay was carried out using a colorimetric LDH cytotoxicity assay kit (Biovison, Milpitas, CA, U.S.A.) according to the manufacturer’s instruction. Absorbance values were measured at 490 nm with a reference wavelength at 630 nm.

Western Blot Analysis SH-SY5Y cells or striatum tissue were homogenized on ice in RIPA lysis buffer (Biowest) containing protease and phosphatase inhibitors (Roche, Indianapolis, IN, U.S.A.). Equal amount of protein (20μg) was resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat dry milk and incubated overnight with one of the following primary antibodies: primary rabbit anti-cleaved caspase-3 (1:500, Cell Signaling Technology, Beverly, MA, U.S.A.), rab-

Fig. 1. Baicalein Reverses Behavioral Deficits Induced by Rotenone

C57BL/6J mice were treated with rotenone (5mg/kg) for 6 weeks, and then co-treated with baicalein (100mg/kg) for another 6 weeks. Rotarod (A) and grid (B) tests were used to assess motor function. Data are expressed as the mean±S.D. n=10. *p<0.001 compared with the control group or the vehicle group, †p<0.01 compared with the rotenone group.
bit anti-microtubule-associated protein 1 light chain (LC3B) (1:1000, Abcam) and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:20000; Sigma-Aldrich).

**Statistical Analysis** Data are expressed as means± standard deviation (S.D.). Differences between groups were conducted with one-way ANOVA test followed by Dunnett’s test. A p-value <0.05 was considered significant.

**RESULTS**

**Baicalein Prevents Behavioral Deficits in Rotenone-Treated Mice** As shown in Fig. 1, rotenone administration induced catalepsy in mice as indicated by prolongation of descent latency of rotarod test and grid test compared to those in the vehicle control group. However, baicalein treatment significantly improved these behavioral deficits induced by rotenone toxicity. These results suggest that baicalein can reverse PD-related behavioral deficits caused by rotenone.

**Baicalein Restores Striatal Dopamine Content in Rotenone-Treated Mice** As shown in Fig. 2, rotenone administration significantly reduced striatal dopamine content compared to those in the vehicle control group (p<0.001). However, baicalein significantly increased the striatal dopamine content when compared to those in the rotenone group.

**Baicalein Suppresses Rotenone-Induced Apoptosis** To test whether baicalein could attenuate rotenone-induced apoptosis, caspase-3 activity, as well as cleaved-caspase-3 protein expression was detected. Compared to the vehicle control animals, caspase-3 activity was significantly increased in the striatum obtained from rotenone-treated mice. Baicalein treatment significantly inhibited the increase of caspase-3 activity by rotenone (Fig. 3A). Furthermore, rotenone treatment increased cleaved-caspase-3 protein expression levels, which were significantly prevented by baicalein treatment (Fig. 3B).

![Fig. 2. Baicalein Reversed Striatal Dopamine Content Reduced by Rotenone](image-url)  
C57BL/6J mice were treated with rotenone (5mg/kg) for 6 weeks, and then co-treated with baicalein (100mg/kg) for another 6 weeks. Dopamine content in the striatum was assessed by HPLC. Data are expressed as the mean±S.D. n=4. *p<0.001 compared with the control group or vehicle group, #p<0.01 compared with the rotenone group.

![Fig. 3. Baicalein Inhibited Rotenone-Induced Apoptosis and Mitochondrial Dysfunction](image-url)  
C57BL/6J mice were treated with rotenone (5mg/kg) for 6 weeks, and then co-treated with baicalein (100mg/kg) for another 6 weeks. (A) The caspase-3 activity in the striatum was measured. (B) The expression level of the cleaved-caspase-3 in the striatum was examined by Western blotting analysis. (C) Mitochondrial membrane potential was assessed by JC-1 staining. Data are expressed as the mean±S.D. n=4. *p<0.001 compared with the control group or the vehicle group, ^p<0.01 compared with the rotenone group.
Baicalein Prevents Rotenone-Induced Mitochondrial Dysfunction As shown in Fig. 3C, rotenone administration caused a decrease in mitochondrial membrane potential compared to the vehicle control animals. However, baicalein treatment significantly prevented the rotenone-induced decrease in mitochondrial membrane potential, demonstrating baicalein reduced mitochondrial dysfunction in rotenone-treated animals.

Baicalein Protects against Rotenone-Induced Neurotoxicity via Induction of Autophagy To evaluate whether baicalein could protect against rotenone neurotoxicity by modulating autophagy, autophagy activity was investigated. As shown in Fig. 4A, the expression levels of LC3B-II in rotenone-treated mice were significantly decreased compared to the vehicle control. However, baicalein significantly prevented the decrease in LC3B-II protein levels. Of note, the expression levels of LC3B-II in brains from the chloroquine+baicalein+rotenone group was higher than those in the chloroquine+rotenone group, indicating baicalein could increase autophagic flux in the rotenone-treated mice (Fig. 4B).

To determine whether the neuroprotective effect of baicalein was mediated via induction of autophagy, baicalein-induced autophagy was inhibited by a pharmacological autophagy inhibitor 3-MA. The protective role of baicalein on rotenone-induced apoptosis and mitochondrial dysfunction was blocked by 3-MA treatment (Figs. 4C, D).

Baicalein Attenuates Rotenone-Induced Neurotoxicity in SH-SY5Y Cells through Induction of Autophagy To further evaluate whether baicalein protected against rotenone-induced neurotoxicity via induction of autophagy, autophagy was inhibited by 3-MA in rotenone-induced neurotoxicity in SH-SY5Y cells. Consistent with the observation in vivo, rotenone decreased LC3B-II expression, which prevented by baicalein (Fig. 5A). Furthermore, chloroquine significantly increased LC3B-II protein levels in the baicalein-treated SH-SY5Y cells, indicating baicalein could increase autophagic flux in the rotenone-treated SH-SY5Y cells (Fig. 5B). Moreover, baicalein significantly attenuates rotenone-induced neurotoxicity in SH-SY5Y cells, as indicated by higher cell viability, lower apoptosis, and lower mitochondrial dysfunction than those in the vehicle group (Figs. 5C, D). 3-MA blocked baicalein induced autophagy (Fig. 5A). Moreover, inhibition of baicalein-induced autophagy with 3-MA resulted in decreased cell viability and increased apoptosis and mitochondrial dysfunction (Figs. 5C, D). Furthermore, rapamycin treatment increased autophagy and protected against rotenone-induced neurotoxicity, as indicated by higher cell viability and lower apoptosis and mitochondrial dysfunction (Figs. 5C, D).

DISCUSSION

It is reported that baicalein has a neuroprotective effect in PD model. However, the knowledge of the mechanism in the neuroprotective effect remains poorly understood. Therefore, the aim of the present study was to determine whether baica-
Baicalein could protect against rotenone-induced neurotoxicity via induction of autophagy. Here, we demonstrated that baicalein pretreatment reduced rotenone-induced neurotoxicity and the neuroprotective action of baicalein was mediated via induction of autophagy. Mitochondria are essential organelles that provide energy and also are involved in diverse cell signaling events in all eukaryotic cells. It is reported that mitochondrial dysfunction plays an important role in the pathogenesis of both sporadic PD and familial Parkinsonism. Mitochondrial dysfunction is observed in PD patient brain samples, in which mitochondrial complex I activity and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and PGC-1α-regulated mitochondrial genes are decreased, and high level of mitochondrial DNA deletion was observed. Patients injected with MPTP, a mitochondrial complex I inhibitor, induced PD symptoms in 1983. Furthermore, several proteins that associated with familial Parkinson’s disease are either

![Fig. 5. Baicalein Protected against Rotenone-Induced Neurotoxicity in SH-SY5Y Cells via Induction of Autophagy](image-url)
mitochondrial proteins or are associated with mitochondria.\textsuperscript{26} Li et al. demonstrated that baikalein reduced rotenone-induced apoptotic in PC12 cells \textit{via} reducing the mitochondrial dysfunction.\textsuperscript{8} In agreement with this finding, we showed that rotenone treatment caused mitochondrial dysfunction which was attenuated by baikalein. Collectively, these findings suggest that baikalein exerts neuroprotective effect, which may be \textit{via} the protective effect on mitochondria.

Autophagy has long been recognized as an adaptive response to cellular stress, however, the basal, constitutive autophagy is essential for the survival of neural cells and that its impairment leads to neurodegeneration.\textsuperscript{13,14} Very recently, these two PD associated genes, phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) and Parkin, are involved in the selective removal of damaged mitochondria through autophagy.\textsuperscript{27,28} Of note, autophagy has been reported to be involved in rotenone-mediated neurotoxicity,\textsuperscript{29} induction of autophagy decreased rotenone-induced dopaminergic neurons loss in the substantia nigra pars compacta.\textsuperscript{29–31} Rotenone has been reported to inhibit autophagy prior to inducing cell death.\textsuperscript{32} How rotenone inhibits autophagy activity remains unclear. Rotenone, an inhibitor of the mitochondrial complex I electron transport chain, induces excessive reactive oxygen species (ROS) production,\textsuperscript{13} which leads to the redistribution of cathepsin D from the lysosomal lumen to the cytosol and subsequently results in impairment of lysosomes.\textsuperscript{29} The dysfunction of lysosomes further impairs autophagic flux and leads to cell viability.\textsuperscript{34}

Baicalein is able to increase autophagy.\textsuperscript{17} It is reported that heme oxygenase-1 (HO-1) is a key mediator of autophagy.\textsuperscript{35,36} HO-1, a stress-responsive enzyme, degrades intracellular heme to free iron, carbon monoxide and biliverdin. HO-1 has anti-oxidative stress effect \textit{via} production of carbon monoxide and biliverdin and has been shown to exert protective effects in a model of ischemic stroke\textsuperscript{37} and in a rat PD model induced by 1-methyl-4-phenylpyridinium (MPP\textsuperscript{+}).\textsuperscript{38} Furthermore, HO-1 promotes mitochondrial macroautophagy and sequestration of redox-active iron in astroglia.\textsuperscript{39} Baicalein could ameliorate liver I/R injury via induction of HO-1-mediated autophagy.\textsuperscript{17} Mitochondrial dysfunction, as well as autophagic impairment is involved in the pathogenesis of PD. Mitochondrial autophagy (or mitophagy), which selectively removes damaged and dysfunctional mitochondria, has been shown to play an important role in maintaining mitochondrial homeostasis and preventing cell death.\textsuperscript{57,28} In the present study, we demonstrated that rotenone impaired autophagic activity and caused mitochondrial dysfunction. However, baikalein treatment could induce autophagy activity and reduced mitochondrial dysfunction. Inhibition of autophagy by 3-MA significantly reduced autophagy and abolished the baikalein-affected protection against rotenone-induced neurotoxicity. In contrast, induction of autophagy by rapamycin significantly increased autophagy, attenuated mitochondrial dysfunction and prevented the rotenone-induced neurotoxicity. These results imply that autophagy may be an important pathway in the baikalein-affected protection against rotenone-induced neurotoxicity.

In conclusion, the current study provides evidence that the environmental neurotoxin rotenone impaired autophagy, and baikalein could protect against rotenone-induced neurotoxicity \textit{via} induction of autophagy. Based on these observations, we can suggest that the therapeutic administration of baikalein might prevent against rotenone toxicity and autophagy is a protective molecular pathway in PD.

\textbf{Conflict of Interest} The authors declare no conflict of interest.

\section*{REFERENCES}

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