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Echinacoside selectively rescues complex I inhibition-induced mitochondrial respiratory impairment via enhancing complex II activity

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ABSTRACT

Previous investigations have implicated mitochondrial dysfunction characterized by Complex I deficiency in the death of dopaminergic neurons in Parkinson's disease (PD). To date, there are no efficient therapeutic approaches to rescue mitochondrial respiratory impairment or prevent neurodegeneration in PD. The beneficial effects of echinacoside (ECH) on neurodegeneration have been reported in both *in vivo* and *in vitro* studies, yet the mechanisms underlying remain elusive and little has been investigated concerning the influences of ECH on mitochondrial respiratory impairment. Here, we compared the protection of ECH on cell injury and mitochondrial dysfunction induced by various inhibitors of Complex I-IV using human neuroblastoma SH-SY5Y cell line. We found that ECH selectively attenuates cell injury, reverses mitochondrial depolarization and bioenergetic failure caused by Complex I inhibitors, whereas it has little protection against Complex II-IV inhibitors. Further investigation demonstrated that ECH enhances Complex II activity and mitochondrial respiration in the cells treated with Complex I inhibitors. This suggests that ECH selectively rescues Complex I inhibition-induced mitochondrial respiratory impairment though elevating Complex II activity, and further confirms that ECH might have a promising potential in PD treatment.

1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disorder characterized by deterioration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Dickson, 2012). Mitochondrial dysfunction has long been recognized as one of the earliest events and a primary cause of neurodegeneration in PD (Langston et al., 1983). Mitochondrion is a critical organelle for ATP production by oxidative phosphorylation (OXPHOS), which is required for normal cell functions, especially in mechanically active cells such as neurons (Wallace et al., 2010). OXPHOS works by generating electron donors NADH and $FADH_2$ from the mitochondrial Krebs cycle (tricarboxylic acid, TCA) for driving electron transport chain (ETC) flux and mitochondrial ATP synthesis (Bertram et al., 2006).

ETC is consisting of four enzymes, including Complex I to IV, and Complex I is a major entry point of ETC (Ishii et al., 2013). Complex I deficiency in the SNpc have been reported, whereas Complex II-IV maintain unimpaired (Keeney et al., 2006; Mizuno et al., 1989). Besides, neurotoxins, including rotenone, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its active metabolite 1-methyl-4-phenylpyridiniuiodide (MPP^+), can induce PD models via Complex I inhibition, which represents many pathological features of the disease. Those suggest that Complex I impairment may be central to the pathogenesis of PD, and Complex I might be a potential therapeutic target for PD

Abbreviations: PD, Parkinson's disease; SNpc, substantia nigra pars compacta; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid; ETC, electron transport chain; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenyl-pyridiniuiodide; SRC, spare respiratory capacity; ECH, Echinacoside; TTFA, 2-Thenoyltrifluoroacetone; LDH, lactate dehydrogenase; CCK-8, cell counting kit-8; Δ, Ψ, m, mitochondrial membrane potential; TMRE, tetramethylrhodamine ethyl ester; OCR, oxygen consumption rate; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; CI, Complex I activity; CII, Complex II activity; CIII, Complex III activity; CIV, Complex IV activity; CS, citrate synthase activity; LC-MS/MS, Liquid chromatography tandem mass-spectrometry; SDH, succinate dehydrogenase

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treatment (Dauer and Przedborski, 2003). It has been confirmed by Marella and colleagues, reporting that exogenous expression of Ndi, the yeast alternative NADH dehydrogenase, reinstates the respiratory chain compensating for disabled Complex I (Marella et al., 2009). However, Complex I is the biggest Complex in ETC and consists of 46 subunits, only 7 of which are coded by mitochondrial DNA and the rest are coded by nuclear genes (Antonicka et al., 2003), hence it is very difficult to develop an effective gene therapy.

It has been shown that mitochondria have spare respiratory capacity (SRC) unutilized under normal conditions. SRC becomes manifest when there is an increase in energy demand, or if oxygen consumption is uncoupled from ATP production, and is crucial to cell survival under stress (Wust et al., 2015). When Complex I is inhibited in PD models, the cell vulnerability is primarily determined by SRC (Yadava and Nicholls, 2007), and increasing SRC reduces the susceptibility of cells to metabolic stress following Complex I inhibition (Kumar et al., 2003). Complex II is another entry point of ETC for reducing equivalents. Although the pathway through Complex II contributes less energy than Complex I to the overall ETC process (Berg et al., 2002), accumulating evidence indicates that Complex II is a key regulator in neuroprotection (Jodeiri Farshbaf and Kiani-Esfahani, 2018). Recently, Pfleger and colleagues demonstrated that elevating Complex II activity in rat cardiac myocytes enhances cell survival after energy deprivation via increasing SRC, whereas Complex II inhibition completely abolishes SRC, suggesting that Complex II is the main source of SRC (Pfleger et al., 2015). Therefore, Complex II might be a novel therapeutic target in neurodegenerative diseases.

Echinacoside (ECH) is a small natural compound with potent neuroprotective effects (Bao et al., 2018; Geng et al., 2007; Shiao et al., 2017; Zhang et al., 2017; Zhu et al., 2013), yet the mechanisms underlying remain elusive. Earlier studies showed that ECH is a strong antioxidant that has protective effects in both neuronal and non-neuronal tissues (Kuang et al., 2009; Wang et al., 2015; Wu et al., 2007; Zhang et al., 2007). Lately, other mechanisms have also been implicated in ECH's neuroprotection (Liu et al., 2018). It has been reported that ECH ameliorates cognitive dysfunction in dementia rats by rescuing the cholinergic neurons and promoting the recovery of cholinergic neurotransmitter levels in brain (Liu et al., 2013; Shiao et al., 2017). Zhang and colleagues found that ECH suppresses the dopaminergic neuron loss in MPTP-treated mice by regulating neuroinflammation and related signals (Zhang et al., 2017). ECH also elicits neuroprotection in *in vivo* and *in vitro* models of PD by mimicking neurotrophic functions (Zhao et al., 2010; Zhu et al., 2013). Nevertheless, much remains unknown about the effects of ECH on mitochondrial respiratory impairment. In this study, we compared the protective effects of ECH on cell injury and mitochondrial dysfunction induced by various ETC inhibitors in human neuroblastoma SH-SY5Y cell line.

2. Materials and methods

2.1. Cell culture and drug administration

Human neuroblastoma SH-SY5Y cells were obtained from American Tissue Culture Collection and cultured as previously described (Zhu et al., 2012a, 2012b, 2013). When the cells were at 80% confluence, growth medium was changed to 1% FBS containing medium before drug administration. MPP^+ (1, 3, 6 mM (Zhu et al., 2014)), Rotenone (0.2, 0.5, 1 μM (Zhu et al., 2013)), 2-Thenoyltrifluoroacetone (TTFA, 10 μM (Gonzalez-Juarbe et al., 2017)), Antimycin A (100 nM (Smith et al., 2014)) and NaN_3 (1 μM (Ziabreva et al., 2010)) were used to inhibit mitochondrial ETC. Those ETC inhibitors and succinate were all purchased from Sigma, and ECH was purchased from Chinese National Institutes for Food and Drug Control.

2.2. Cell viability assay

Cell viability assays were performed by adding cell counting kit-8 (CCK-8, Dojindo) directly into the culture medium. After incubation at 37 °C for 1 h, the absorbance was read by a spectrophotometer at a wavelength of 450 nm. Each experiment was carried out in three replicates, and the data were obtained from four independent experiments.

2.3. Lactate dehydrogenase (LDH) assay

Cell injury was detected by measuring LDH activities in the culture medium with a LDH kit (Sigma Diagnostics Procedure) and a spectrophotometer (Thermo Scientific) as described before (Zhu et al., 2013). Cells lysed by 1% Triton X-100 were used as the Positive Ctrl, while the untreated cells were assayed as Ctrl. The absorbance data were normalized to the Ctrl.

2.4. Detection of mitochondrial membrane potential (ΔΨm)

The changes in $\Delta\Psi_m$ were estimated by the fluorescence intensity of tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) as previously described (Zhu et al., 2012b). TMRE was prepared as a 100x stock solution at a concentration of 10 μM, and the cells were loaded with TMRE for 5 min and harvested with non-enzymatic cell dissociation buffer (Gibco). The fluorescence intensity of TMRE in non-quench mode was measured by immediate flow cytometry using a 561 nm laser for excitation and 585/16 nm BP filter for emission, and normalized to the Ctrl. A minimum of 20,000 cells were analyzed in each test.

2.5. Bioenergetic analysis

ATP levels were detected with an ATP Determination Kit (Invitrogen) following the instruction of the manufacturer. ADP/ATP ratio was measured by an ADP/ATP ratio assay kit (Abcam) according to manufacturer's protocol. The data were normalized to protein content measured by BCA protein assay (Pierce).

2.6. Seahorse XF24 respirometry

Oxygen consumption rate (OCR) was measured using a Seahorse Bioscience XF24 extracellular flux analyzer. Cells were seeded in XF24 cell culture plates (40000 cells/well) and allowed to attach overnight. Next, cells were treated with ECH alone or with the five ETC inhibitors for 24 h. At the end of exposure, cells were washed 3 times with pre-warmed serum-free DMEM media, incubated with XF assay medium (pH 7.4, XF base media (Seahorse Biosciences) supplemented with 2 mM L-glutamine, 10 mM glucose and 1 mM sodium pyruvate), and placed at 37 °C without CO_2 for 1 h before test. After recording 3 baseline OCR (basal respiration) measurements, 1 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was auto-injected into the experimental wells, and 3 uncoupled OCR (maximal respiration) measurements following FCCP administration were also recorded. FCCP-uncoupled respiratory capacity represents mitochondrial ETC function. Each OCR value was calculated as an average of 3 measurements and normalized to the final cell number in the same well (pmol O2/min/10 000 cells). Each experiment was carried out in three replicates and repeated 4 times with different plates.

2.7. Measurements of mitochondrial complex I-IV activities

Mitochondria were isolated from SH-SY5Y cells as described before (Zhu et al., 2012a, 2014), and purified mitochondria from every group were equally divided into two parts: one for Complex I-IV activity detection, and the other for citrate synthase activity (CS) measurement. The levels of Complex I activity (CI) were measured with a

Mitochondrial CI Colorimetric Assay Kit, Complex II activity (CII) with a Succinate Dehydrogenase Activity Colorimetric Assay Kit, Complex III activity (CIII) with Mitochondrial CIII Assay Kit, Complex IV activity (CIV) with a Cytochrome Oxidase Activity Colorimetric Assay Kit, and CS with a CS Colorimetric Assay Kit, all following the manufacturer's instructions (all from Biovision). CI and CII were measured by recording absorbance at 600 nm in kinetic mode at 30 s intervals with a spectrophotometer, CIII and CIV at 550 nm, and CS at 412 nm. As CS is commonly used as a marker enzyme for intact mitochondria, the results of CI−CIV were normalized to CS to compensate for mitochondrial enrichment in the cell samples (Birch-Machin and Turnbull, 2001). Each experiment was carried out in three replicates and repeated 4 times.

2.8. Liquid chromatography tandem mass-spectrometry (LC-MS/MS) analysis

The concentrations of succinate and fumarate in SH-SY5Y cells were detected by LC-MS/MS assay. Succinate and fumarate certified reference materials were purchased from Sigma-Aldrich for creating calibration curves. At the end of drug exposure, cells were harvested and lysed in LC/MS grade methanol. The methanol volume is calculated based on the final cell number of each group (500,000 cells/1 ml methanol). After centrifugation at 1000 × *g* for 20 min at 4 °C, 1 ml of deproteinated supernatants from each group were transferred to new glass tubes and dried. Samples were resuspended in 5% acetic acid and vortexed. Amplifex™ Keto Reagent (SCIEX) was added to each sample and incubated for 1 h. Samples were dried again, resuspended in 0.1% formic acid, and analyzed by LC20AC HPLC system (Shimadzu) coupled to an API 4000 triple-quadrupole mass spectrometer (SCIEX) operated in negative electrospray ionisation. The eluents were A: 0.1% formic acid and B: methanol/0.1% formic acid. The MS/MS reactions used for quantization were m/z 115/71 for fumarate and m/z 117/73 for succinate. Peak areas of fumarate and succinate in the sample were compared to those of the standards to calculate their concentrations in MultiQuant software (SCIEX).

2.9. Western blotting analyses

SH-SY5Y cells were collected after drug treatments. Cell lysates were prepared and ran on 8%–12% SDS-PAGE gels. Antibodies used were as follows: rabbit antibodies against SDHA (1:1000), SDHB (1:500), SDHC (1:1000) and SDHD (1:500) (all from Abcam), and mouse antibodies against β-actin (1:20,000, Sigma).

2.10. Statistical analysis

The data are exhibited as means ± SEM and were subjected to statistical analysis via one-way ANOVA, followed by Bonferroni post hoc analysis. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. ECH selectively protects SH-SY5Y cells from complex I inhibition

MPP^+ (1 mM), rotenone (0.2 μM, both selective Complex I inhibitors), TTFA (10 μM, a selective Complex II inhibitor), antimycin A (100 nM, a selective Complex III inhibitor) and NaN_3 (1 μM, a selective Complex IV inhibitor) were applied to SH-SY5Y cells to induce cell injury. To explore the protective effects of ECH in the ETC inhibition models, ECH (5, 10, 20 mg/L) was added into the cell culture medium alone or with the five ETC inhibitors. Cell viability was determined by CCK-8 kit at 24 h of drug exposure. As shown in Fig. 1A–C, cell viability was significantly reduced by each ETC inhibitor, yet it was modestly rescued by even the lowest dose of ECH in MPP^+ and rotenone models (Fig. 1A), whereas none of the three doses of ECH had any effect on the cell viability in TTFA, antimycin A or NaN_3 model. Since ECH at 10 (Figs. 1B) and 20 mg/L (Fig. 1C) provided similar protection in MPP + and rotenone models, we chose 10 mg/L ECH in the following experiments.

Next, SH-SY5Y cells were exposed to 10 mg/L ECH alone or with those five ETC inhibitors, and cell injury was detected by measuring the leakage of LDH into the culture medium at 24 h. As seen from Fig. 1D, each ETC inhibitor caused a remarkable increase in LDH release, and co-treatment with ECH completely suppressed the elevation in LDH release induced by MPP^+ and rotenone but not the other ETC inhibitors. It further confirms that ECH may selectively protect SH-SY5Y cells from Complex I inhibition.

It is noteworthy that the protective effects of ECH were weakened in the cells exposed to 3 mM MPP^+ or 0.5 μM rotenone, and hardly detectable in those treated with even higher doses of Complex I inhibitors (6 mM MPP^+ or 1 μM rotenone) (Fig. S1). As it took much shorter time for higher doses of MPP or rotenone to trigger significant cell injury, there might not be enough time for ECH to elicit its protection.

3.2. ECH selectively rescues mitochondrial depolarization and bioenergetic failure triggered by complex I inhibition

We have reported that ECH suppresses MPP^+-induced mitochondrial depolarization (Zhu et al., 2012b), however, much is unknown about the effects of ECH on the mitochondrial dysfunction in the ETC inhibition models. SH-SY5Y cells were treated with ECH alone or with the five ETC inhibitors for 24 h, $\Delta\Psi_m$ alterations were estimated by the fluorescence intensity of a $\Delta\Psi_m$-sensitive dye TMRE, which selectively localized in mitochondria. The changes in TMRE uptake in SH-SY5Y cells were measured by flow cytometry. ATP levels and ATP/ADP ratio were also determined at the same time. As shown in Fig. 2, TMRE fluorescence intensity, ATP levels and ATP/ADP ratio were all remarkably decreased by each ETC inhibitor, suggesting that each inhibitor provokes severe mitochondrial depolarization and bioenergetic failure in SH-SY5Y cells. However, TMRE fluorescence intensity, ATP levels and ATP/ADP ratio were restored to the Ctrl levels by ECH in MPP^+ and rotenone models, whereas ECH had little effect on the mitochondrial depolarization and bioenergetic failure provoked by the other ETC inhibitors. Those data indicate that ECH might selectively reverse mitochondrial dysfunction caused by Complex I inhibitors.

3.3. Mitochondrial respiration is enhanced by ECH in MPP^+ and rotenone models

In order to explore the influence of ECH on mitochondrial respiration in MPP^+ and rotenone models, SH-SY5Y cells were treated with MPP^+, rotenone or along with ECH for 24 h, and basal and maximal respiration was measured via Seahorse XF24 respirometry. SRC was calculated as the difference between maximal and basal respiration. As seen from Fig. 3A and B, basal and maximal respiration was both significantly decreased and SRC was even reduced to nearly zero by MPP^+ and rotenone. Basal respiration was restored to the Ctrl levels by ECH in MPP^+ and rotenone models. Although SRC and maximal OCR values in MPP^+ and rotenone models were magnificently enhanced by ECH, they were still much lower than the Ctrl levels. Maximal OCR values represent ETC function and mitochondrial respiratory capacity. SRC is the extra capacity to produce energy in response to stress and as such is associated with cellular survival. Therefore, the data above imply that ECH may promote mitochondrial respiration and reduce the susceptibility of SH-Y5Y cells to metabolic stress following Complex I inhibition.

3.4. Complex II activities are elevated by ECH in MPP^+ and rotenone models

To test the effects of ECH on the activities of Complex I-IV during

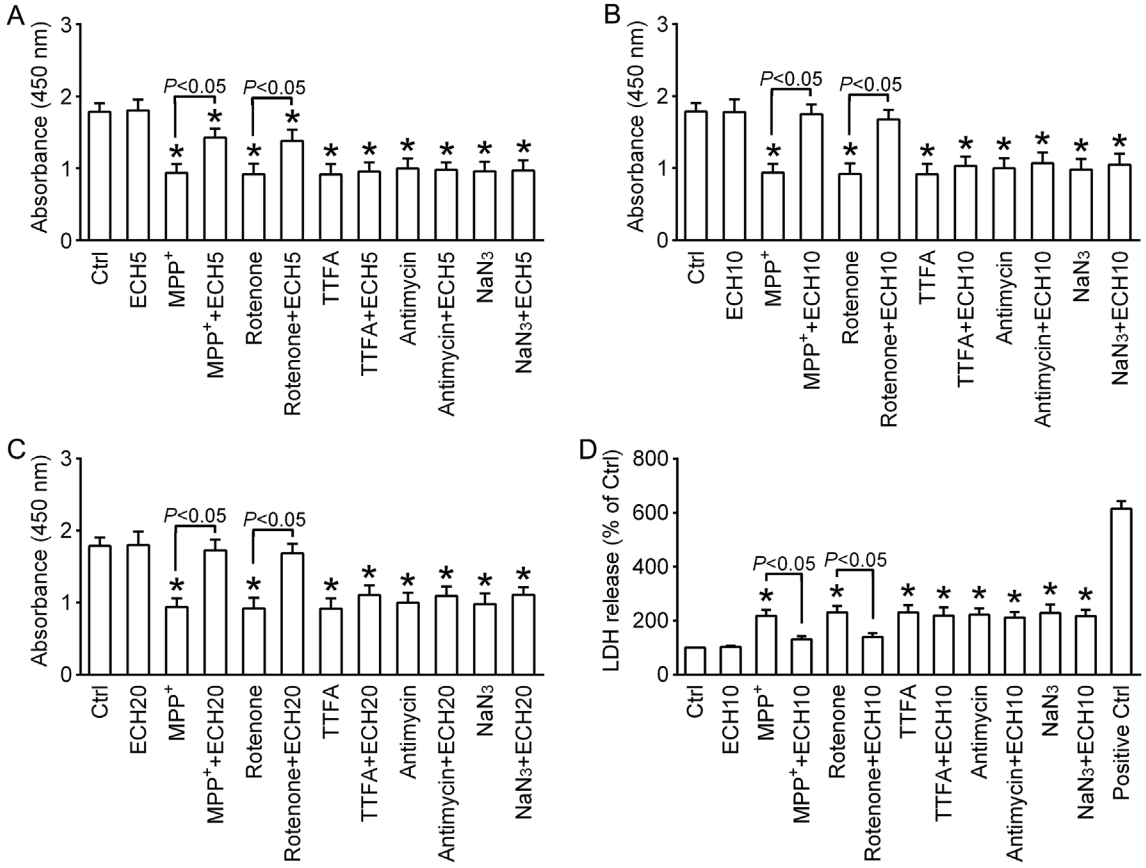

Fig. 1. ECH selectively rescues SH-SY5Y cells from Complex I inhibition. MPP^+ (1 mM), Rotenone (0.2 μM, both selective Complex I inhibitors), TTFA (10 μM, a selective Complex II inhibitor), Antimycin A (100 nM, a selective Complex III inhibitor) and NaN_3 (1 μM, a selective Complex IV inhibitor) were applied to SH-SY5Y cells. (A–C) Cell viability was determined by CCK-8 kit after echinacoside (ECH) (5 (A), 10 (B), 20 (C) mg/L) was added into the culture medium alone or with the five ETC inhibitors for 24 h. (D) Cell injury was detected by measuring the leakage of LDH into the culture medium after cells were exposed to 10 mg/L ECH alone or with ETC inhibitors. Cells lysed by 1% Triton X-100 were used as the Positive Ctrl, while the untreated cells were assayed as Ctrl. The absorbance data of LDH assay were normalized to the Ctrl. Each experiment was carried out in three replicates, and the data were obtained from four independent experiments (n = 4). ECH5, 5 mg/L ECH; ECH10, 10 mg/L ECH; ECH20, 20 mg/L ECH. *P < 0.05 versus Ctrl.

Complex I inhibition, SH-SY5Y cells were treated with MPP^+, rotenone or along with ECH. CI—CIV and CS were detected at 24 h of drug exposure. CS was hardly affected in any group (Fig. S2). As shown in Fig. 4, CI/CS was radically reduced by MPP^+ or rotenone, while CIII/CS and CIV/CS were not affected. CII/CS was slightly increased as a compensation for Complex I inhibition in the cells exposed to MPP^+ or rotenone, yet robustly elevated by co-treatment with ECH, while ECH had no further influence on the activities of Complex I, III and IV. It implies that ECH might elicit protection against Complex I inhibition by boosting Complex II activity in SH-SY5Y cells.

Complex II is also known as succinate dehydrogenase (SDH), and it is the only enzyme that participates in both the TCA cycle and the ETC. SDH oxidizes the metabolite succinate to fumarate and transfers the electrons to ubiquinone (Cecchini, 2003; Iverson, 2013). Therefore, SDH function could be assessed by measuring the concentrations of succinate and fumarate using LC-MS/MS. As shown in Fig. 5, the concentrations of succinate were barely changed in any group, whereas those of fumarate were slightly increased in MPP^+ and rotenone models but robustly elevated by co-treatment with ECH. This confirms that Complex II activity is enhanced by ECH in MPP^+ and rotenone models.

An elevation in substrate concentration would lead to increased enzyme function until all enzyme active sites are occupied. Nonetheless, the concentrations of succinate were much higher than those of fumarate in any group (Fig. 5), implying that SDH might already be saturated by its substrate. To confirm that, succinate was administrated to cells with MPP^+ or rotenone, and fumarate concentrations were determined by LC-MS/MS at 24 h. As shown in Fig. S3B, the concentrations of fumarate were barely affected in any group, suggesting that Complex II activity could not be elevated by increasing succinate concentration. More than that, succinate supplement had no protection in MPP^+ or rotenone model (Fig. S3A).

3.5. ECH enhances levels of SDHC and SDHD in the SH-SY5Y cells treated with MPP^+ or rotenone

Complex II is composed of four subunits: a flavoprotein (succinate dehydrogenase Complex subunits A, SDHA), an iron-sulphur subunit (SDHB) and two membrane anchor subunits (SDHC and SDHD) (Iverson, 2013). To determine the effects of ECH on Complex II expression, the protein levels of SDHA-SDHD were respectively determined by immunoblotting after drug treatments. As seen from Fig. 6, Complex II protein levels were hardly affected by MPP^+ or rotenone, and the levels of SDHA and SDHB were also unchanged by co-treatment with ECH; while the expression of SDHC and SDHD were significantly elevated by co-treatment with ECH, but their expression was not affected by ECH alone.

4. Discussion

Here we demonstrated that ECH selectively rescued cell injury, mitochondrial depolarization and bioenergetic failure induced by

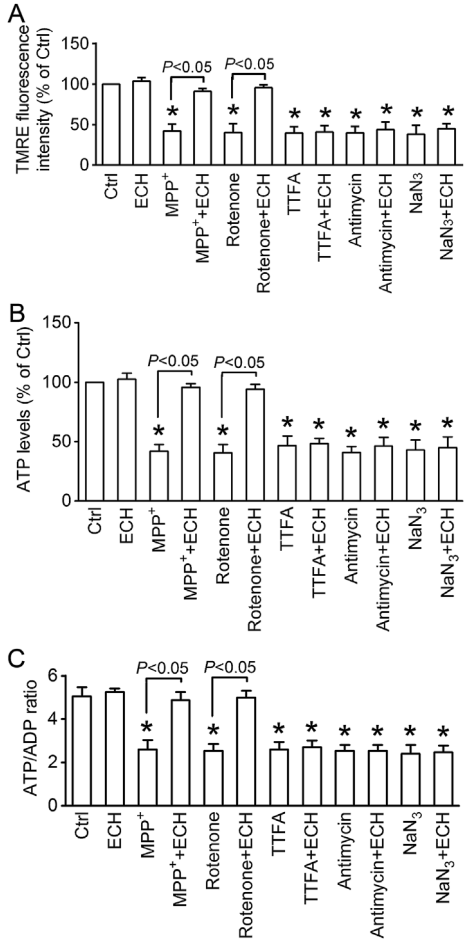

Fig. 2. ECH selectively reverses mitochondrial depolarization and bioenergetic failure in MPP^+ and rotenone models. MPP^+ (1 mM), Rotenone (0.2 μM), TTFA (10 μM), Antimycin A (100 nM) and NaN_3 (1 μM) were applied to SH-SY5Y cells alone or with ECH (10 mg/L) for 24 h. (A) Mitochondrial membrane potential ($\Delta\Psi_m$) alterations were estimated by the fluorescence intensity of a $\Delta\Psi_m$-sensitive dye tetramethylrhodamine ethyl ester (TMRE). The changes in TMRE uptake in SH-SY5Y cells were measured by flow cytometry. (B) ATP levels were measured with an ATP Determination Kit. (C) ADP/ATP ratio was measured by an ADP/ATP ratio assay kit. n = 3; *P < 0.05 versus Ctrl.

Complex I inhibitors. However, the higher doses of MPP^+ or rotenone we used, the shorter periods of time were needed to trigger significant cell injury, and the weaker ECH's protective effects became, suggesting that ECH could not trigger instant protection against mitochondrial respiratory impairment. Therefore, ECH may be more suitable for treating chronic diseases characterized by mitochondrial respiratory impairment such as PD than treating acute poisoning of Complex I inhibitors.

This is the first study providing evidence that ECH can regulate mitochondrial respiration through Complex II. Since ECH itself has no effect on Complex II function in SH-SY5Y cells, it is very possible that ECH elevates Complex II activity only when cells are under stress.

Complex II serves as a link between TCA cycle and the ETC, and provides not only electron to ETC but also precursors for many metabolites like fatty acids and sterol (Cecchini, 2003; Iverson, 2013). Previous studies showed that lipids accumulate in the brain is the prior hallmark of neurodegeneration (Shimabukuro et al., 2016), and increasing Complex II activity could possibly control lipid overloading and restore the hemostasis of fatty acids and sterol in neurodegenerative disorders, and mediate neuronal death through excitotoxicity and NMDA dependent signaling (Jodeiri Farshbaf and Kiani-Esfahani, 2018; Owen et al., 2002; Taghibiglou et al., 2009). Those indicate that besides being the main source of SRC, Complex II also regulates cell metabolism and other processes through direct and indirect ways, by which Complex II behaves as a key regulator in neuroprotection. We found here that ECH-induced elevation in Complex II activity leads to enhanced mitochondrial respiration, mitochondrial membrane potential and ATP generation, as well as increased levels of fumarate in MPP^+ and rotenone models. However, it remains unknown whether ECH has any impact on the levels of other TCA metabolites and how they mediate neuronal survival when under stress.

Several earlier investigations have implicated that succinate supplement prevents mitochondrial dysfunction and ameliorates energy deficits by enhancing Complex II activity and mitochondrial respiration (Giorgi-Coll et al., 2017; Pomytkin et al., 2007). Nevertheless, succinate's neuroprotection was reported mostly in traumatic brain injury or ischemic/hypoxic neuronal damage models but seldom in PD models. It has been demonstrated from our results above that succinate levels were much higher than fumarate in SH-SY5Y cells, and Complex II activity could not be elevated by succinate administration, implying that the enzyme active sites of Complex II may be saturated by its substrate succinate in SH-SY5Y cells. This probably explains why succinate supplement had no protection in MPP^+ or rotenone model. It is

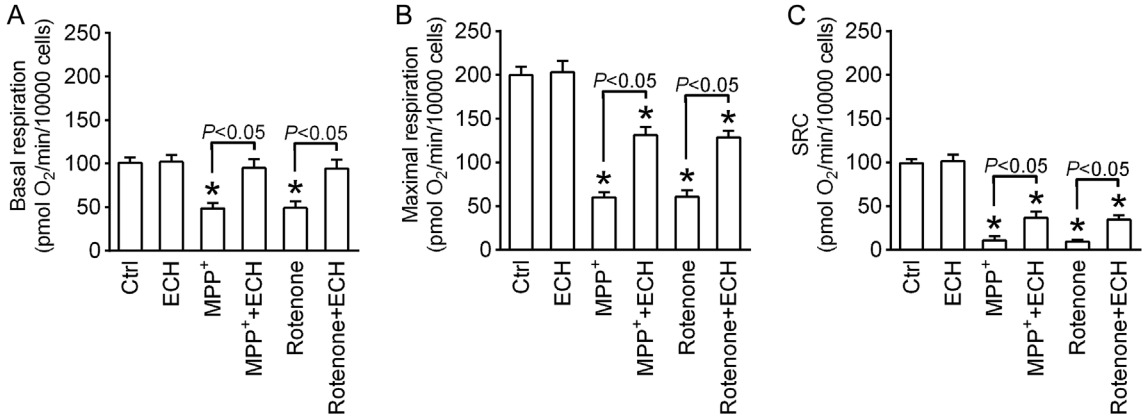

Fig. 3. ECH selectively enhances mitochondrial respiration in MPP^+ and rotenone models. SH-SY5Y cells were exposed to MPP^+ (1 mM), Rotenone (0.2 μM) alone or with ECH (10 mg/L) for 24 h. At the end of drug exposure, mitochondrial respiration was measured by detecting oxygen consumption rate (OCR) with Seahorse XF24 respirometry before and after carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) treatment. The measurements before FCCP treatment are baseline OCR (basal respiration, A), and those following FCCP treatment are FCCP-uncoupled OCR (maximal respiration, B). Spare respiratory capacity (SRC) was calculated as the difference between maximal and basal respiration (C). Each experiment was carried out in three replicates and repeated 4 times with different plates (n = 4). *P < 0.05 versus Ctrl.

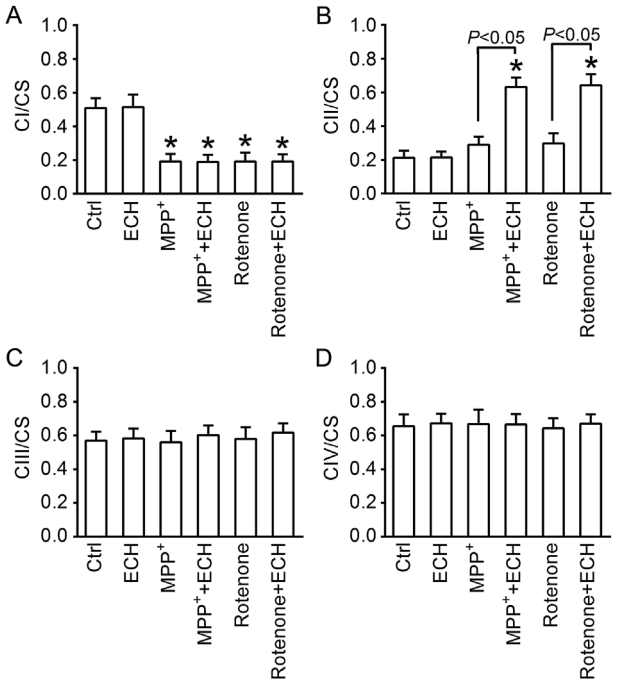

Fig. 4. Complex II activities are elevated by ECH in MPP$^+$ and rotenone models. SH-SY5Y cells were exposed to MPP$^+$ (1 mM), Rotenone (0.2 μM) alone or with ECH (10 mg/L) for 24 h. Mitochondria were isolated from the cells of each group and used for detection of Complex I-IV and Citrate Synthase activities. The levels of Complex I activity (CI) was measured with a Mitochondrial CI Colorimetric Assay Kit (A), Complex II activity (CII) with a Succinate Dehydrogenase Activity Colorimetric Assay Kit (B), Complex III activity (CIII) with Mitochondrial CIII Assay Kit (C), Complex IV activity (CIV) with a Cytochrome Oxidase Activity Colorimetric Assay Kit (D), and citrate synthase activity (CS) with a CS Colorimetric Assay Kit. The results of CI—CIV activities (Unit/ml) were normalized to CS activity (Unit/ml), a common marker enzyme for intact mitochondria. One unit of CI is the amount of enzyme that causes reduction of 1.0 μmol of Complex I dye per min at pH 7.4 at room temperature. One unit of CII is the amount of enzyme that generates 1.0 μmol of DCPI per min at pH 7.2 at room temperature. One unit of CIII is the amount of enzyme that causes reduction of 1.0 μmol of cytochrome c per min at pH 7.4 at room temperature. One unit of CIV is the amount of enzyme that oxidizes 1.0 μmol of reduced Cytochrome c per minute at pH 7.2 at room temperature. One unit of CS is the amount of enzyme that generates 1.0 μmol CoA per min at pH 7.2 at room temperature. Each experiment was carried out in three replicates and repeated 4 times (n = 4). *P < 0.05 versus Ctrl.

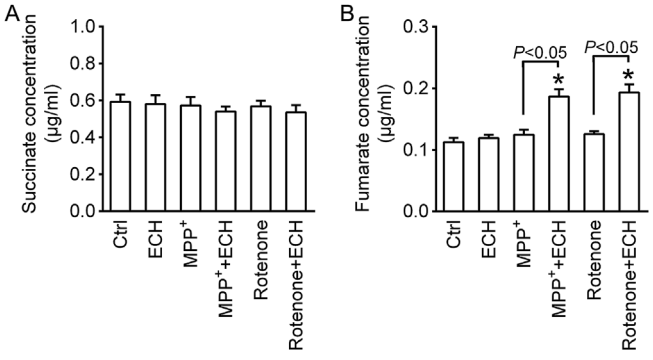

Fig. 5. The effects of ECH on the concentrations of succinate and fumarate in MPP$^+$ and rotenone models. SH-SY5Y cells were exposed to MPP$^+$ (1 mM), Rotenone (0.2 μM) alone or with ECH (10 mg/L) for 24 h. The cells were harvested at the end of drug exposure, and the concentrations of succinate and fumarate were detected by liquid chromatography tandem mass-spectrometry (LC-MS/MS) assay. n = 4; *P < 0.05 versus Ctrl.

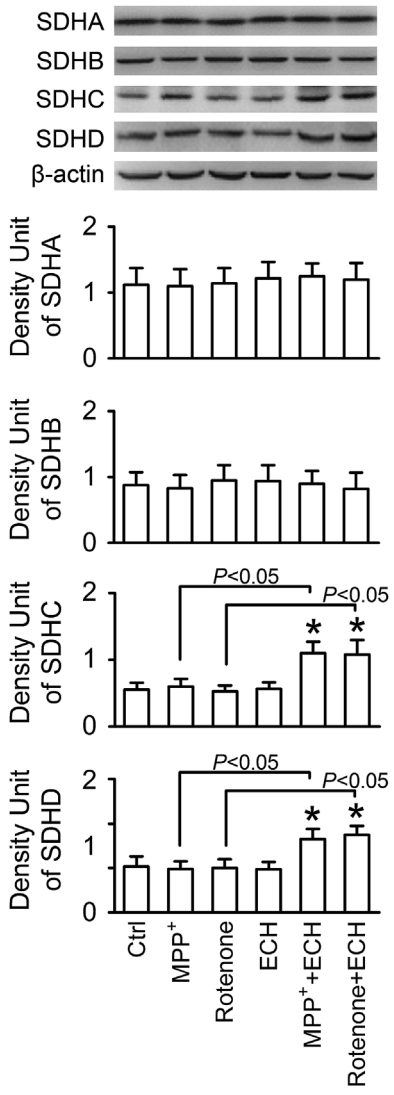

Fig. 6. ECH enhances Complex II protein levels in the SH-SY5Y cells treated with Complex I inhibitors. SH-SY5Y cells were exposed to MPP$^+$ (1 mM), Rotenone (0.2 μM) alone or with ECH (10 mg/L) for 24 h. The protein levels of Complex II subunits (SDHA-SDHD) were determined by immunoblotting respectively. n = 3; *P < 0.05 versus Ctrl.

noteworthy that succinate is not cell-permeable but transported into cells by transporters (Hering-Smith et al., 2000; Srisawang et al., 2007). Therefore, the function of exogenous succinate is very likely impacted by the actual cellular uptake after its administration, which might differ among cell types or disease models.

The structure of Complex II purified from porcine heart revealed that the hydrophobic subunits (SDHC and SDHD) have the head-tail arrangement inserted into the mitochondrial inner membrane and a short section projected into the intermembrane space; whereas the catalytic subunits (SDHA and SDHB) are protruded into the matrix (Sun et al., 2005). The overall Complex II function requires the presence of all subunits. Nakamura and colleagues performed a detailed study in E.coli and found that hydrophilic subunits are essential for the activity of catalytic subunits (Nakamura et al., 1996). Maklashina and colleagues confirmed that the unassembled SDHA of human and bacterial Complex II has impaired catalytic activity, and accumulates in the mitochondrial matrix or bacterial cytoplasm (Maklashina et al., 2018). Our data from above showed that the protein levels of SDHC and SDHD were remarkably increased by ECH in MPP$^+$ and rotenone models. Since the expression of SDHA and SDHB was barely affected by co-

treatment of ECH, it is very possible that the elevation in Complex II activity results from the increased expression of its two hydrophilic subunits, which might enhance Complex II assembly.

Recent studies have suggested that several SDH assembly factors play an essential role in the assembly of Complex II (Hao et al., 2009; Na et al., 2014; Van Vranken et al., 2014). More than that, Complex II assembly and its enzymatic activity could also be influenced by the phospholipid environment, specifically the presence of cardiolipin (Schwall et al., 2012). Therefore, further investigations are required to explore the mechanisms by which ECH enhances Complex II activity when under stress.

Our present work suggests that ECH selectively rescues Complex I inhibition-induced mitochondrial respiratory impairment by elevating Complex II activity. Furthermore, ECH could easily cross the blood-brain barrier in rats after intragastric administration (Wei et al., 2011), hence it might have a promising potential in PD treatment.

Author contribution

Wenwei Li and Min Zhu designed the study; Huihan Ma, Yang Liu, Lin Tang and Fang Song performed the research; Min Zhu, Hao Ding and Xuxia Bao analyzed the data; and Wenwei Li and Min Zhu wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.02.012.

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