A Doctoral Dissertation

A study on antioxidant effects of nobiletin in neurons by regulating electron transport chain of mitochondria

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Nobiletin의 미토콘드리아 전자전달계 조절을 통한 신경세포에서의 항산화 효과에 대한 연구

지도교수 정성철

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이 논문을 이학 박사학위 논문으로 제출함

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A study on antioxidant effects of nobiletin in neurons by regulating electron transport chain of mitochondria

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(Supervised by Professor Sung-Cherl Jung)

A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Science

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This thesis has been examined and approved.

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ABSTRACT

Mitochondrial electron transport chain (ETC) is the major source of reactive oxygen species (ROS) in both physiological and pathological conditions. Particularly, mitochondrial calcium overload and subsequent excessive generation of ROS are critical factors in the pathological conditions of neurons. High level of mitochondrial ROS also is the primary cause of macromolecular damage in neurons. It is well known that mitochondria have eleven separate ROS production sites. Among them, complex I (CI) and complex III (CIII) are dominant sites for mitochondrial ROS production. Therefore, neuroprotective mechanisms correlatd with the regulation of mitochondrial ETC CI may also play an important role to determine neuronal survival rates.

Nobiletin, a polymethoxylated flavone from *Citrus sunki* Hort. ex Tanaka, significantly protected primary cortical neurons against neurotoxicity induced by rotenone, the mitochondrial ETC CI inhibitor. It has already been reported that nobiletin showed a neuroprotective effect through K_+ channel regulation in our previous study. Additional to those data, nobiletin significantly reduced rotenone-induced mitochondrial ROS and increased cell viability. In contrast, nobiletin did not show any effects to reduce ROS elevation in pure isolated mitochondria or to increase the survival rate of primary cortical neurons of Sprague-Dawley (SD) rats under the treatment of antimycine A, a CIII inhibitor. The effects of nobiletin to increase CI activity as

well as ATP production were dose-dependent. These results indicate that nobiletin can prevent mitochondrial dysfunction especially correlated with the inhibition of ETC CI, although the action mechanism should be addressed in further studies. Taken together, this study strongly suggests a possibility that nobiletin may be a promising neuroprotective agent against neurodegenerative diseases and neuroinflammation, regulating mitochondrial K^+ channels as well as their metabolic functions specific to ETC CI.

Keywords: Mitochondria; ETC; Complex I; Rotenone; ROS, Cell death; Nobiletin

CONTENTS

LIST OF ABBREVATIONS

AIF	Apoptosis-inducing factor
ATP	Adenosine triphosphate
AM	Acetoxymethyl esther
Ca^{2+}	Calcium ion
CI	Complex I
CIII	Complex III
DMEM	Dulbecco's Modified Eagle Medium
DIV	Days in vitro
DCF-DA	2′,7′-Dichlorofluorescin diacetate
ETC	Electron transport chain
FBS	Fetal bovine serum
HT-22	Immortalized clonal mouse hippocampal cells
HO-1	Heme oxygenase-1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

$\Delta\Psi_m$	Mitochondrial membrane potential
Nrf-2	Nuclear factor erythroid 2-related factor 2
OCR	Oxygen consumption rate
ROS	Reactive oxygen species
SD	Sprague-Dawley
TBP	TATA binding protein

LIST OF FIGURES

1. Introduction

Mitochondria play multi-functional roles in most types of cells, and many studies have focused on regulatory functions of mitochondria of determining the survival or death of cells (Czabotar *et al*., 2014; Giampazolias *et al*., 2016; Kroemer *et al*., 1998; Ong *et al*., 2012; Segawa *et al*., 2014; Smaili *et al*., 2003; Wei *et al*., 2001). In the aspect of cellular fate, their functions are crucially correlated with ATP generation, calcium storation, reactive oxygen species (ROS) generation and even detoxification. In mitochondria, electron transport chains (ETC) generate the electrochemical gradient of H^+ ions as they are passing through complex I, II, III and IV, activating the mechanism of ATP synthase (Dallner *et al*., 2000; Rhoda *et al*., 2017). This process is generally called as the oxidative phosphorylation. Mitochondrial complex I (CI) is a large enzyme complex of over 40 subunits directly embedding the inner mitochondrial membrane where the first oxidative phosphorylation occurs (Gonzalez-Halphen *et al*., 2011). Additionally, CI dysfunction in mitochondira is the predominant cause leading to cell death (Bhatti *et al*., 2017).

Nobiletin known as 5,6,7,8,3´,4´-hexamethoxyflavone is a polymethoxylated flavonoid found in citrus. It has multifunctional pharmacological agent and various pharmacological activities of nobieltin include neuroprotection, cardiovascular protection, antimetabolic disorder, anticancer, anti-inflammation, and antioxidation (Huang *et al*., 2016). In previous study, it was demonstrated

that, by regulating K^+ influx and ($\Delta\Psi_m$) across mitochondrial inner membrane, nobiletin prevents glutamate-induced neurotoxicity, in which glutamate exhibited mitochondrial calcium overload and neuronal cell death (Lee *et al*., 2018). Nobiletin was also reported to exert several beneficial effects to improve cognitive function and to effectively recover motor deficits in several animal models under pathological conditions, such as cerebral ischemia or Parkinson's and Alzheimer's diseases (Onozuka *et al*., 2008; Yabuki *et al*., 2014; Yamamoto *et al*., 2009). In addition to, nobiletin enhanced the outgrowth of neurites in PC12 cells and significantly suppressed microglial activation as well as neuroinflammation *in-vitro* ((Nagase *et al*., 2005; Cui *et al*., 2010).

Also, nobiletin has been studied to increase metabolism by changing the mitochondrial matrix substrate (Jojua *et al*., 2015). This previous report strongly suggests that it is necessary to study if and how nobiletin directly affects ETC system. However, it is still unclear yet. Therefore, in this study, I wanted to determine if nobiletin might have additional neuroprotective effects via regulating mitochondrial ETC system. I tried to focus on ETC functions specific to both CI and CIII, because they are dominant sites for mitochondrial metabolism. To observe the neuroprotective effects of nobiletin, experiments were designed to inhibit CI of ETC by rotenone or 6-hydroxydopamine (6-OHDA), or to inhibit CIII of ETC by antimycin A, in primary cortical cells, HT-22 and pure mitochondria isolated from Sprague-Dawley (SD) rats. The nobiletin changed activities of ETC complex were also observed in this study. In brief, nobiletin

significantly reduced mitochondrial ROS production under the condition of CI inhibition, but not under the CIII inhibition. It also increased ATP production. Although it was not clear how nobiletin exerted a neuroprotective effect under mitochondrial dysfunction specific to ETC CI inhibition, it was confirmed in this study that nobiletin directly enhanced the activity of CI. These results strongly suggest that nobiletin may exhibit a neuroprotective effect through activating the regulatory function correlated with ETC CI.

Together with the previous study reporting the neuroprotective effect to regulate mitochondrial depolarization through K^+ channels, these results provide evidence that nobiletin can act as a major substance to suppress apoptotic signaling processes in neurodegenerative/neurological diseases or neuroinflammation, and there is a possibility as a new drug to treat neuropathological conditions.

2. Materials and Methods

2.1 Materials

Dulbecco's modified Eagle's medium (DMEM), Minimal essential medium (MEM), Neurobasal medium, fetal bovine serum (FBS), B-27 serum-free supplement, glutamine and penicillin/streptomycin were purchased from Gibco BRL (Gland Island, NY, USA). Antibodies against NF-E2–related factor 2 (Nrf-2) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). The antibodies against heme oxygenase-1 (HO-1) and TATA binding protein (TBP) were purchased from Millipore (Temecula, CA, USA) and Abcam (Cambridge, UK), respectively. The antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Amresco (Solon, OH, USA). 2′,7′-Dichlorofluorescin diacetate (DCF-DA) was purchased from Sigma-Aldrich. Nobiletin was isolated and purified from the peel of Citrus sunki Hort. ex Tanaka in Jeju National University, Department of Biology as described previously (Choi *et al*., 2007). All other reagents were obtained from Sigma-Aldrich unless otherwise indicated.

2.2 Primary culture of cortical neurons

Primary cortical neurons were prepared from cerebral cortices of postnatal 1-day-old SD rats. The neonatal brains were dissected, and the cortices were transferred to plating medium (containing MEM supplemented with 10% FBS, 25 mM glucose, 1 mM sodium pyruvate, 25 mM glutamine and 1 % penicillin/streptomycin) and dissociated by trituration using pipettes. Then, cells were plated onto poly-L-lysine-coated 24-well plates at a density of 1×10^5/well. After 6 hours, plating medium was replaced to Neurobasal media supplemented with 2 % B-27, 50 mM glutamine and 1 % penicillin/streptomycin, and a half of culture medium was replaced every four days. Cultured neurons incubated at 37 °C in a humidified 5 % CO_2 / 95 % O_2 air atmosphere. The cells were used after 7-8 days in vitro (DIV). All experiments were conducted in accordance with the Jeju University Animal Care and Use Committee and guidelines.

2.3 Culture of HT-22 neurons

HT-22 neurons, an immortalized hippocampal neuronal cell line, were a generous gift from Dr. B.H. Lee (Gachon University of Medicine and Science, South Korea). The cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin and incubated at 37 °C in a humidified 5 % CO_2 / 95 % O_2. Cells thawed were the passage number 3 and used up to passage 9. Cultured cells were added in 100 mm dish at a density 4×10^4 cells/dish, grown for 48 hours,

and then used for western blotting experiment.

2.4 Preparation of pure mitochondria isolated from rat brain cortices

The isolated mitochondria were obtained from rat brain cortices, as previously described (Iglesias-González *et al.*, 2013). In short, the cortices were separated from brains of 14 ~ 17 day-old rats and placed in EGTA-containing isolation buffer (IB) and homogenized in the Dounce-type tissue grinders (Kimble chase). Pestle A and B were used sequentially, and their clearances were 0.0035 - 0.0065 mm and 0.0010 - 0.0030 mm, respectively. The IB was containing (in mM): Mannitol 225, Sucrose 75, HEPES 5, ECTA 3, BSA 0.1 %, adjusted at pH 7.4 with KOH (1N). The homogenates were centrifuged at 600 × *g* for 10 minuntes, and then, the supernatant was transferred to a new tube and then centrifuged at 600 × *g* for 10 minutes again. Next, supernatant was centrifuged at 12,000 × *g* for 10 minutes. The pellet containing mitochondria was resuspended in IB without EGTA and homogenized using Dounce-type tissue grinders. The clearances of pestle A and B at this stage were 0.0028 - 0.0047 mm and 0.0008 - 0.0022 mm, respectively. The homogenates were centrifuged at 12,000 × *g* for 10 minutes one more. All the above procedures were carried out at 4 °C. The isolated mitochondrial proteins were quantified by using Bio-Rad protein assay dye. Electron microscopy and oxygen consumption rate (OCR)

measurement were hired to evaluate the morphology and metabolic activity of isolated mitochondria.

2.5 Measurement of mitochondrial viability and ROS in pure isolated mitochondria

After finishing the isolation of pure mitochondria from rat brain cortices, the mitochondria suspension (0, 100, 250, 500, 1000 μg of protein/ml) was incubated in mitochondrial assay buffer for 10 minutes at 37 °C with 0.4 mg/ml [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) and 2′, 7′-Dichlorofluorescin Diacetate (DCF-DA) 25 μM for measuring mitochondrial viability and ROS, respectively, as previously discribed (Garcia-Ruiz C *et al*., 1997; Puntel RL *et al.,* 2010). The mitochondrial assay buffer was containing (in mM): mannitol 220, sucrose 70, KH_2PO_4 10, $MgCl_2$ 5, HEPES 2, EGTA 1 and fatty acid-free BSA 0.2 % (w/v) and pH was set at 7.2. The mitochondria loaded in suspension were treated with nobiletin or other reagents in 96 well plates. The mitochondrial suspension was divided into 200 μL/well, so each well contained 100 μg/ml of protein. And then, fluorescence intensities were measured by using a fluorescence microplate reader (SpectraMax i3, Molecular devices Spectramax i3, Sunnyvale, CA, USA) at the excitation/emission wavelengthes of 485/535 nm for DCF-DA, respectively. The absorbance wavelengths to measure the protein quantification and mitochondrial viability

were 550 nm on a multifunctional plate reader and compared with the untreated control cells.

2.6 Measurement of Oxygen consumption rate (OCR) in pure isolated mitochondria

The OCR of pure isolated mitochondria was measured by using a Seahorse XF-24 extracellular flux analyzer (Seahorse Bioscience, Inc, North Billerica, MA, USA) as the manufacturer's protocol. Briefly, 5 μg of isolated mitochondria was suspended in 50 μL assay medium and transferred to each well for OCR measurement. Mitochondrial assay medium was containing (in mM): mannitol 220, sucrose 70, KH_2PO_4 10, $MgCl_2$ 5, HEPES 2, EGTA 1 and fatty acid-free BSA 0.2 % (w/v), and pH was set at 7.2. One day before the experiment, a sensor cartridge was placed into the calibration buffer and incubated overnight in a non-CO_2 condition at 37 °C. The reagents listed below were added sequentially according to the manufacturer's protocol: ADP (1 mM), oligomycin (2 μg/ml) as an inhibitor of mitochondrial ATP synthase, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP 2 μM) as an ETC accelerator, and rotenone or antimycin A (0.5 μM) as a CI or CIII inhibitor, respectively. The OCR data recorded by sensor cartridge were analyzed by using Seahorse XF-24 software.

2.7 Preparations of cytoplasmic and nuclear proteins

Preparations of cytoplasmic and nuclear protein were performed by using NE-PER nuclear and cytoplasmic extraction reagets (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For this experiment, HT-22 cells were seeded at a density of 1×10^6 cells/dish in 100 mm dish. These cells were treated with nobiletin and other reagents at the indicated time and washed out twice and collected with cold phosphate-buffered saline (PBS). After centrifuging (3,000 rpm), cell pellets were resuspended in the cytoplasmic extraction reagent. Next, after centrifuging (16,000 × *g*) again, the supernatant cytoplasmic extract was transferred to a new tube. Nuclear pellets were resuspended in the nuclear extraction reagent, and centrifuged (16,000 × *g*). The supernatant nuclear protein extract was transferred to a new tube and stored at - 80 °C until used.

2.8 Western blotting analysis

HT-22 cells were washed three times with PBS (pH 7.4) and lysed with the modified RIPA buffer (10 mM TrisHCl, 150 mM NaCl, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 1 μg/mL aprotinin, and 1 μg/mL leupeptin, pH 7.4). Proteins (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred onto a

polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA) using Towbin transfer buffer (192 mM glycine, 25 mM Tris, and 20 % methanol, pH 8.3). The blots were incubated with 5 % skim milk in TTBS (25 mM Tris, 150 mM NaCl, pH 7.4, containing 0.1 % Tween 20) for 1 hour at a room temperature to block nonspecific binding. Subsequently, the membranes were incubated overnight at 4 °C with anti-Nrf-2, anti-TBP, anti-HO-1, anti-AIF and anti-β-actin antibodies. The blots were washed three times with TTBS and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at a room temperature. After several washes, the blots were developed using enhanced chemiluminescence detection reagents (Intron Biotechnology, Sungnam, Korea) according to the manufacturer's instruction. Optical densities of bands were quantified with an Image J analyzer (http://rsb.info.nih.gov/ij/) and normalized with those of β-actin and nuclear trans.

2.9 Measurement of Cell viability

MTT was used to investigate the cytoprotective effect of nobiletin on cell viability, as previously described (Choi *et al*., 2016). This protocol is based on the phenomenon that water-soluble MTT is converted into an insoluble purple formazan by mitochondria in living cells. Primary cortical neurons (DIV 4) were plated at a density of 15 X 10^4/ ml and arabinofuranosyl cytidine (Ara-C) 1 μM was added in each culture well to block the growth of glial cells.

Subsequently, Ara-C concentration was consistantly kept at 1 μM in cell culture medium (4 days). After that, MTT solution (0.4mg/ml) was added to the cell culture medium for 1 hour at 37 ℃, and then, the supernatant was discarded, and the formazan was dissolved in DMSO. The absorbance was read at 550 nm by using a microplate reader (Model 550, Bio-Rad, USA).

2.10. Quantification of ATP level

Using mitochondria isolated from brain tissues, ATP was quantified by using luciferase/luciferin ATP determination kit (Molecular Probes, Eugene, OR), according to the kit's manual.

2.11 Mitochondrial Respiratory Complex I Activity Assay

The activity of mitochondrial respiratory CI was determined by using the MitoCheck Complex I Activity Assay Kit (Cayman Chemical Company®, Ann Arbor, MI, USA), according to the manufacturer's instructions. For the determination of CI activity, 20 μg of mitochondrial protein was used and the absorbance of all samples was measured at 340 nm for 15 minutes using a microplate reader (SpectraMax i3). The amount of CI activity was quantifiedby the increase of absorbance per minute, and three trials were hired for statistical analysis.

2.12 Statistical analysis

All data were indicated as the mean value ± standard error of the mean (SEM), and the statistical analysis was performed by Student's t-test using the sigma plot 8.0 software. The significance of statistical differencesbetween groups was considered when $p < 0.05$ or 0.01

3. Results

3.1 The experimental processes of isolated mitochondria and to measure mitochondrial physiological function.

Figure 1 shows the schematics of experimental processes how to isolate mitochondria and to measure OCR. Using this protocol, the level of functional mitochondria was measurable and comparable through MTT colorimetric assay (Figure 2). This is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH) dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye, showing that MTT to its insoluble formazan, represents a purple color.

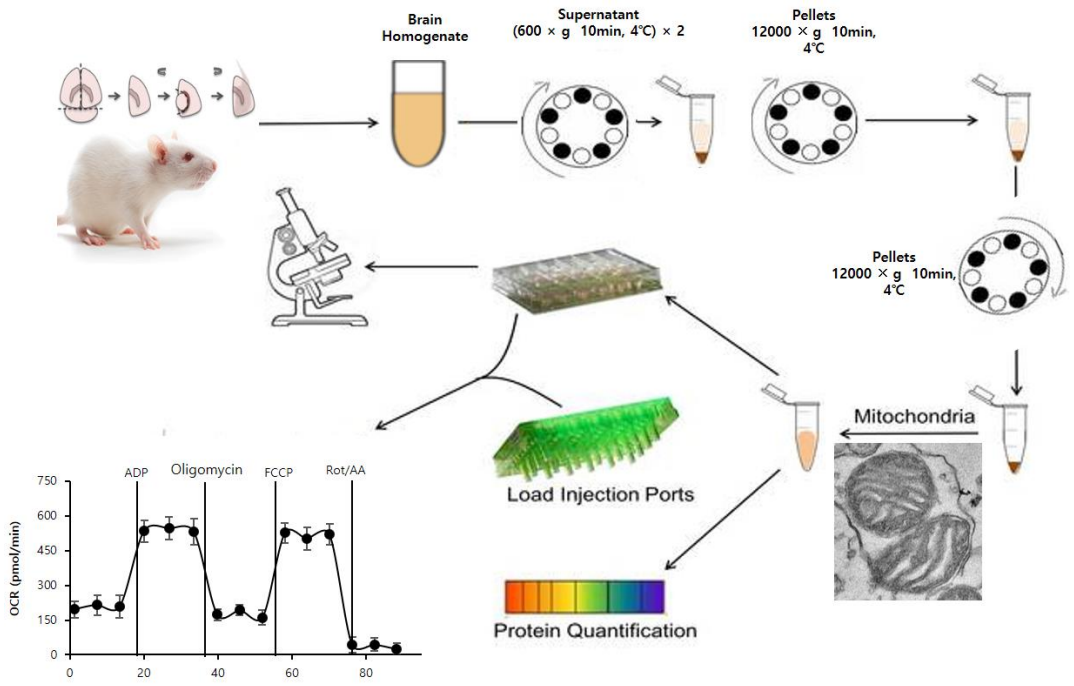

Figure 1. The schematics of experimental processes to isolate mitochondria and to measure OCR.

The brain hemispheres were extracted from SD rats by a surgical blade and immediatelyimmersed into ice-cold IB in the dounce-type tissue grinder. Tissues were transferred into a 1.5-ml microcentrifuge tube. The brain homogenate was centrifuged at 600 × *g* for 10 minutes in a refrigerated (4 °C) table-top centrifuge. The supernatant was transfered to a new tube and centrifuged again at 600 × *g* for 10 minutes. The supernatant was removed and pellet was combined with IB-EGTA free, and then centrifuged at 12,000 × *g* for 10 minutes. The mitochondria-enriched fraction was collected at the bottom of the tube and the final mitochondrial pellet was resuspended in IB-EGTA free solution (1:1) and stored on ice. The

electron microscope was used to confirm the mitochondrial morphology and condition. The protein amount of mitochondrial sample was quantified and 50 μg/ml of mitochondria was used for the exiperment of OCR measurement.

A

B

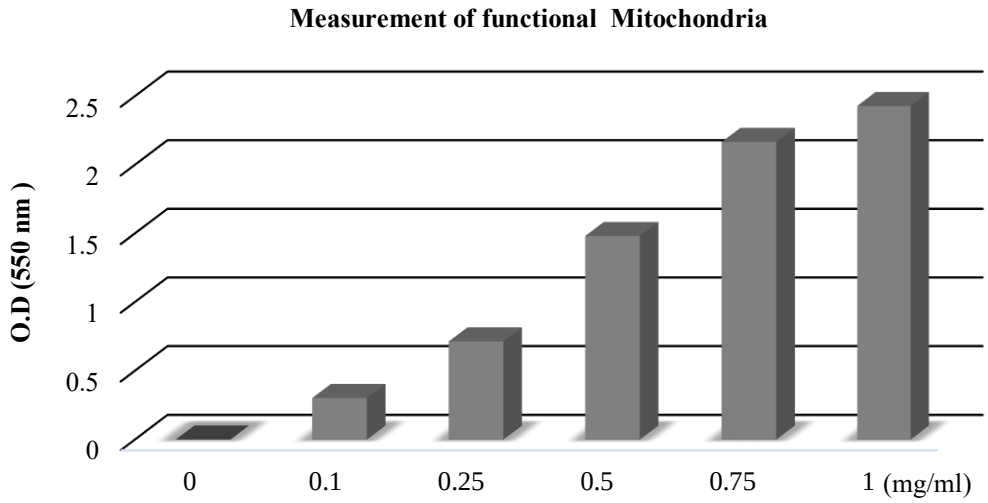

Figure 2. The measurement of mitochondrial viability or metabolic activity with MTT colorimetric assay.

The various concentrations of pure isolated mitochondrial samples incubated with 0.4 mg/ml MTT at 37 ℃ for 10 minutes. After 'A' procedure, microtubes were spinned down and the supernatant was removed. DMSO was added to the pellets, and the optical density of samples was measured at 550 nm with a microplate reader.

3.2 Mitochondrial ROS was significantly increased by ETC inhibition.

Intracellular ROS plays important roles as a second messenger in cellular system under physiological conditions, and CI and CIII of mitochondrial ETC are the major sites for ROS generation (Barja *et al*., 1998; Turrens *et al*., 1980; Turrens *et al*., 1982). However, in pathological conditions, the excessive production of ROS has been observed in a number of types of cells and can induce cellular damage or death (Dickinson *et al*., 2011; Halliwell *et al*., 2011; Kaminskyy *et al*., 2014; Pallepati *et al*., 2012). The enhanced production of ROS is related with the development of pathologies such as diabetes, cancer, ischemia, stroke, arthritis, and most types of neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (Brieger *et al*., 2012; Ghezzi *et al*., 2016; Kehrer *et al*., 2015). For exhibiting neuroprotective effects of nobiletin, the exessive production of ROS was induced by inhibiting ETC system in pure mitochondria isolated from cortices of SD rats. For blocking CI or CIII, rotenone, 6-OHDA or antimycin A was used, and their effects were determined by measuring the level of ROS in pure isolated mitochondria (500 μg/ml from brain cortices of 14-16 day old SD rats). Figure 3 and 4 show effects of mitochondrial CI inhibitors, rotenone and 6-OHDA, on mitochondrial ROS production. Both CI inhibitors excessively elevated mitochondrial ROS production in manners of dose- and time-dependences (Fig. 3, ROT 0.5 μM = 130.5 ± 3.6 %, n = 8, $p < 0.01$ compared with the

control; Fig. 4, 6-OHDA 10 μM = 177.7 ± 5.4 %, n = 3, $p < 0.01$, compared with the control). Additionally, antimycin A, a mitochondrial CIII inhibitor, exhibited the significant effect to increase mitochondrial ROS production, as shown in Figure 5A and B. These results indicate that both CI and CIII of ETC are major sites to regulate ROS production in mitochondria of neurons.

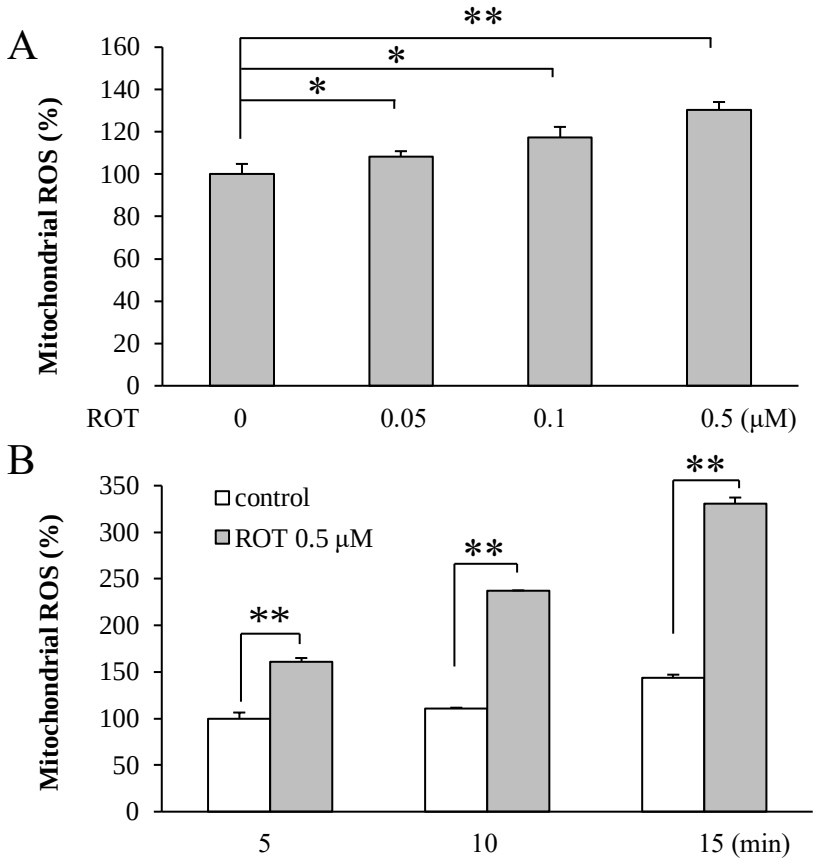

Figure 3. Rotenone-increased mitochondrial ROS production in pure isolated mitochondria.

The effects of rotenone on mitochondrial ROS generation were measured with the DCF-DA indicator using a fluorescence microplate reader. DCF-DA (25 μM) was loaded to isolated mitochondria for 10 minutes, then rotenone (ROT, 0.05, 0.1 or 0.5 μM) was added for 5, 10 or 15 minutes. Rotenone significantly increased mitochondrial ROS in manners of dose- and time-dependences. Dose responses of totenone (A) and 5 minutes after treatment time courses (B) were analyzed. Data represent the mean ± S.E.M, and $p < 0.05$ (*), $p < 0.01$ (**), compared with the control group.

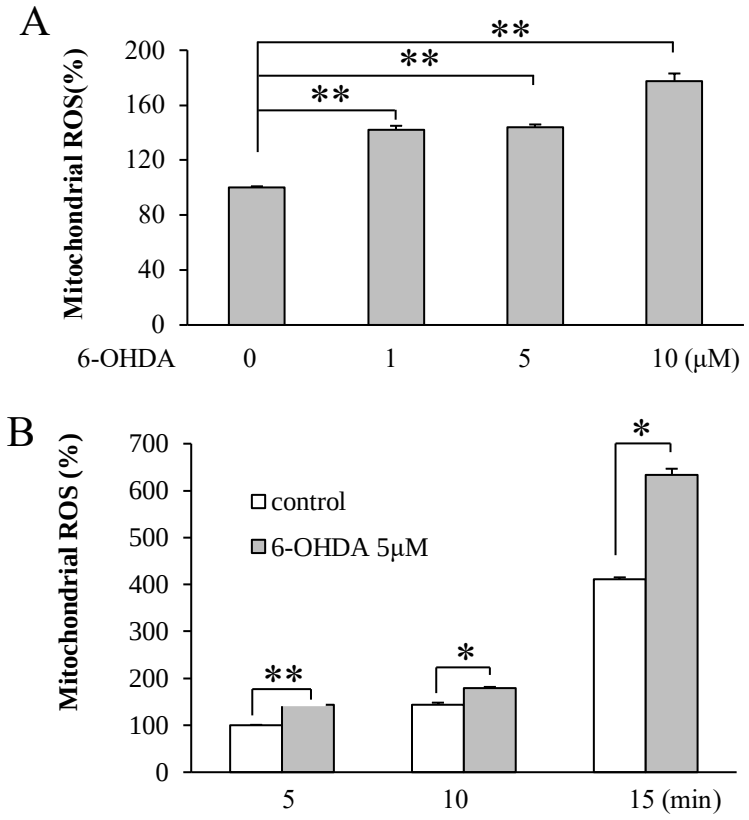

Figure 4. 6-OHDA-increased mitochondrial ROS production in pure isolated mitochondria.

(A) The effects of 6-OHDA on mitochondrial ROS generation were measured with DCF-DA indicator using a fluorescence microplate reader. Mitochondrial sample 500 μg/ml was loaded with DCF-DA (25 μM) for 10 minutes, then 6-OHDA (1, 5 or 10 μM) was added for 5, 10 or 15 minutes. 6-OHDA significantly increased mitochondrial ROS in manners of dose- and time-dependences. Dose responses of 6-OHDA (A) and 5 minutes after treatment time courses (B) were analyzed. Data represent the mean ± S.E.M, and $p < 0.05$ (*), $p < 0.01$ (**), compared with the control group

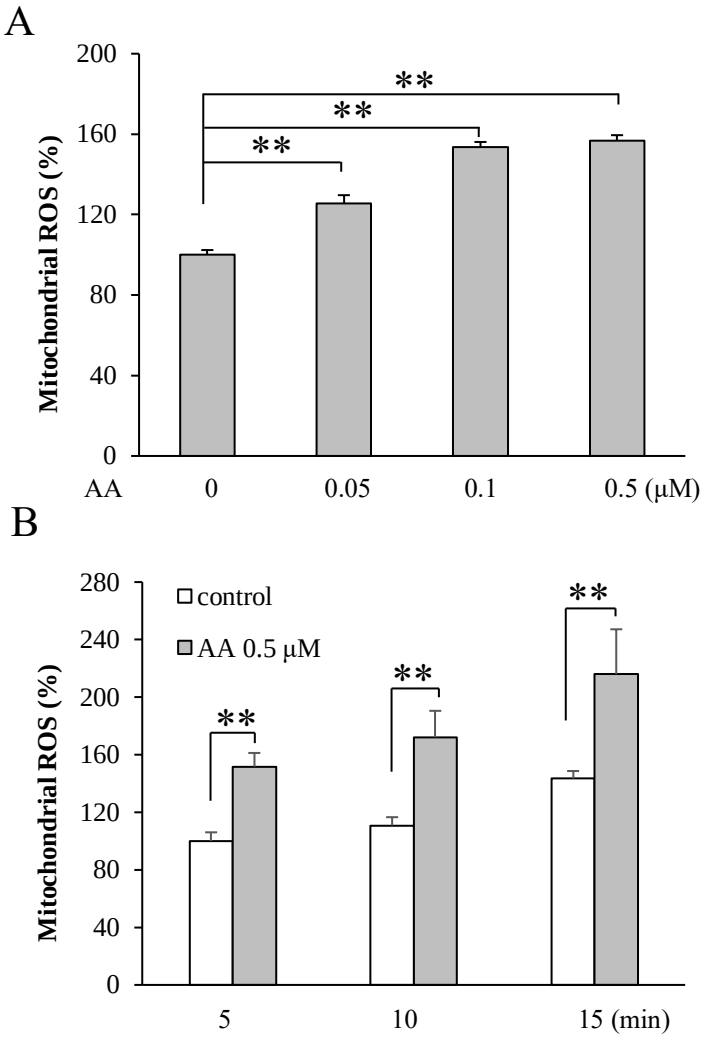

Figure 5. Antimycin A-increased mitochondrial ROS production in pure isolated mitochondria.

(A) The effects of CIII inhibitor on mitochondrial ROS generation were measured with DCF-DA indicator using a fluorescence microplate reader. Mitochondrial sample 500 μg/ml was loaded DCF-DA (25 μM) for 10 minutes, then antimycin A (AA, 0.05, 0.1 or 0.5 μM) added for 5, 10 or 15 minutes. Antimycin A significantly increased mitochondrial ROS in manners of dose and time-

dependences. Dose responses of antimycin A (A) and 5 minutes after time courses (B) were analyzed the treatment. Data represent the mean ± S.E.M, and $p < 0.01$ (**), compared with the control group.

3.3 The neuroprotective effects of nobiletin regulating mitochondrial ROS production.

The mitochondrial dysfunction can induce neuronal damage or death, so it is necessary to figure out if nobiletin might exhibit neuroprotive and antioxidant effects by regulating mitochondrial ROS production. Previous study showed that nobiletin did not show the ROS scavenging activity even though it revealed antioxidant effects in neurons (Lee et al., 2018). In this study, it was confirmed that nobiletin significantly reduced mitochondrial ROS and this effect seemed to be based on the regulation of ETC complexes. In figure 6, pure isolated mitochondria were loaded with the DCF-DA fluorescence indicator for 10 minutes, and then pretreated with nobiletin (30 μM) for 5 minutes. After that, ECT complex inhibitors, rotenone (0.5 μM, Fig. 6A), 6-OHDA (10 μM, Fig. 6B) or antimycin A (0.5 μM. Fig. 6C) was added for 5 minutes to enhance ROS production. Figure 4A and B show that the pretreatment of nobiletin markedly blocked the increase of ROS production which was induced by adding a CI inhibitor, rotenone or 6-OHDA (Fig. 6A, ROT = 131.7 ± 5.4 % $p < 0.01$, n = 7, compared with the control; ROT + NOB = 101.0 ± 2.1 %, n = 6, $p < 0.01$ compared with the ROT; Fig. 6B, 6-OHDA = 113.4 ± 5.4 %, n = 6, p < 0.01, compared with the control; 6-OHDA + NOB = 102.3 ± 3.8 %, n = 5, $p < 0.05$ compared with the 6-OHDA). However, nobiletin showed no effects to reduce or block antimycin A-induced ROS enhancement (Fig. 6C). This result indicates that the antioxidant effect of nobiletin might be

correlated with the mitochondrial dysfunction specific to the inhibition of ETC CI.

Next, it was tested if the antioxidant effect of nobiletin against rotenone or 6-OHDA was also participated in the neuroprotective mechanism to preserve the survival rate of neurons by performing MTT assays. In the Figure 5, DIV-8 primary cortical neurons of SD rats were treated with rotenone or antimycin A (0.5 μM) for 24 hours under the presence or absence of nobiletin. The pretreatment of nobiletin (30 μM) was performed for 1 hour prior to the addition of rotenone or antimycin A. The nobiletin pretreatment remarkably increased the viability of cortical neurons against rotenone showing the effect to reduce the cortical survival rate under the absence of nobiletin (Fig. 7A, ROT = 70.5 ± 1.4 %, n = 6, $p < 0.05$, compared with the control; ROT + NOB = 91.4 ± 1.6 %, n = 6, $p < 0.01$ compared with the ROT). However, nobiletin pretreatment did not show neuroprotective effects on antimycin A-reduced cell viability (Fig. 7B, AA = 75.9 ± 4.6 %, n = 4, $p < 0.01$, compared with the control; AA + NOB = 76.2 ± 0.8 %, n = 4, n.s, compared with the AA). The nobiletin alone did not exhibit any cytotoxicity at concentrations below 30 μM in primary cortical neurons. This indicates that nobiletin may exhibit neuroprotective effects via activating antioxidant signaling mediated to ETC CI in neuronal mitochondria.

Excessive mitochondrial ROS can activate calpain, which induces the translocation of an apoptosis inducing factor (AIF) from mitochondria where AIF is localized under the physiological condition, to the nucleus (Bano *et al*., 2018; Sevrioukova *et al*., 2011). During the activation of

apoptotic signaling, the translocation of AIF from mitochondria to cytosol and nucleus is well known to cause DNA fragmentation. In this study, nobiletin blocked AIF translocation to nucleus, which was induced by the addition of rotenone in HT-22 cells (Fig. 6). Using Western blotting analysis, it was observed that the translocation of rotenone-induced AIF expression was remarkably increased both in nucleus and cytosol (Fig. 8A, ROT = 146.2 ± 8.7 %, n = 3, $p < 0.01$ compared with the control; Fig. 8B, ROT = 168.5 ± 6.5 %, n = 3, $p < 0.01$, compared with the control). And, nobiletin significantly reduced nuclear AIF translocation and also as well as cytosolic AIF. Taken together, these results suggest that nobiletin may activate neuroprotective mechanisms mediated to mitochondrial antioxidant signaling, blocking apoptotic processing. This neuroproctective effect of nobiletin is possibly based on the mitochondrial regulation correlated with ETC-CI in neurons.

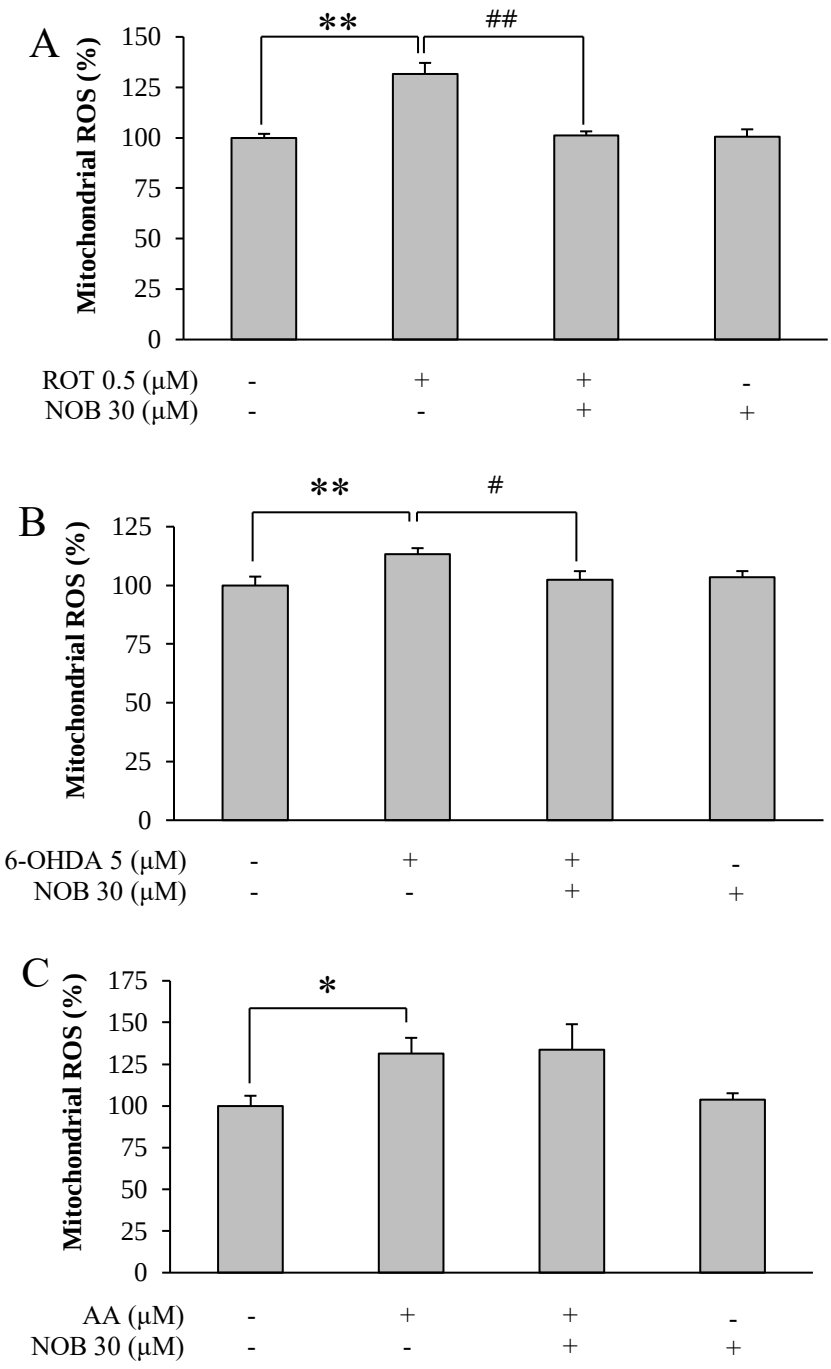

Figure 6. The ETC-mediated antioxidant effects of nobiletin on ROS generation in mitochondria isolated from rat cortices.

Isolated mitochondria were pretreated with nobiletin (NOB, 30 μM) for 5 minutes, and then added

with complex inhibitors for 5 minutes. (A and B) Nobiletin was significantly reduced ROS generation which was increased by CI inhibition (ROT + NOB, 6-OHDA + NOB). However, ROS elevation by antimycin A, CIII inhibitor, was not affected by the pretreatment of nobiletin (C, AA + NOB). ROS level was determined by measuring the intensities of DCF-DA fluorescence, and compared between each group. Data represent the mean ± S.E.M, and $p < 0.05$ (*), $p < 0.01$ (**) compared with the control group, $p < 0.05$ ($^{\#}$), $p < 0.01$ ($^{\#\#}$) compared with the inhibitor alone.

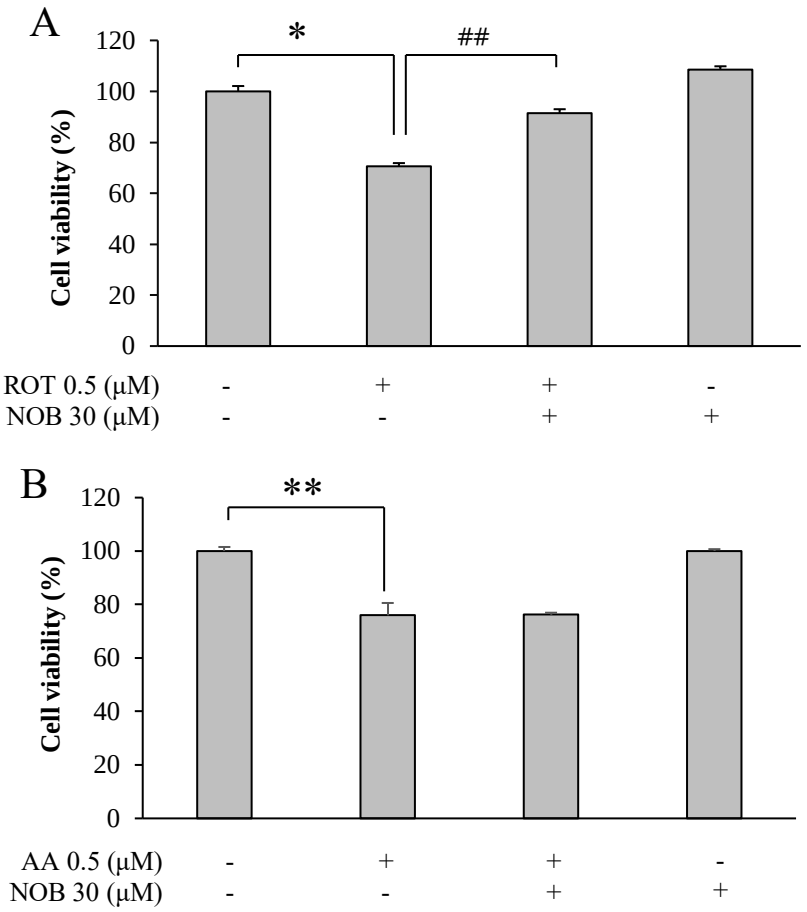

Figure 7. The neuroprotective effects of nobiletin on the viability of primary cortical neurons dissociated from SD rats

(A and B) Cultured primary cortical neurons were pretreated with/without nobiletin (NOB) for 1 hour and then incubated with the addition of rotenone (ROT, 0.5 μM) or antimycin A (AA, 0.5 μM) for 24 hours. Cell viability was measured by using MTT assay. Here, nobiletin showed an effect of reducing cell death only against rotenone. Data represent the mean ± S.E.M, and $p < 0.05$ (*), $p < 0.01$ (**) compared with the control group, $p < 0.05$ ($^{\#}$) as not significant compared with the inhibitor alone.

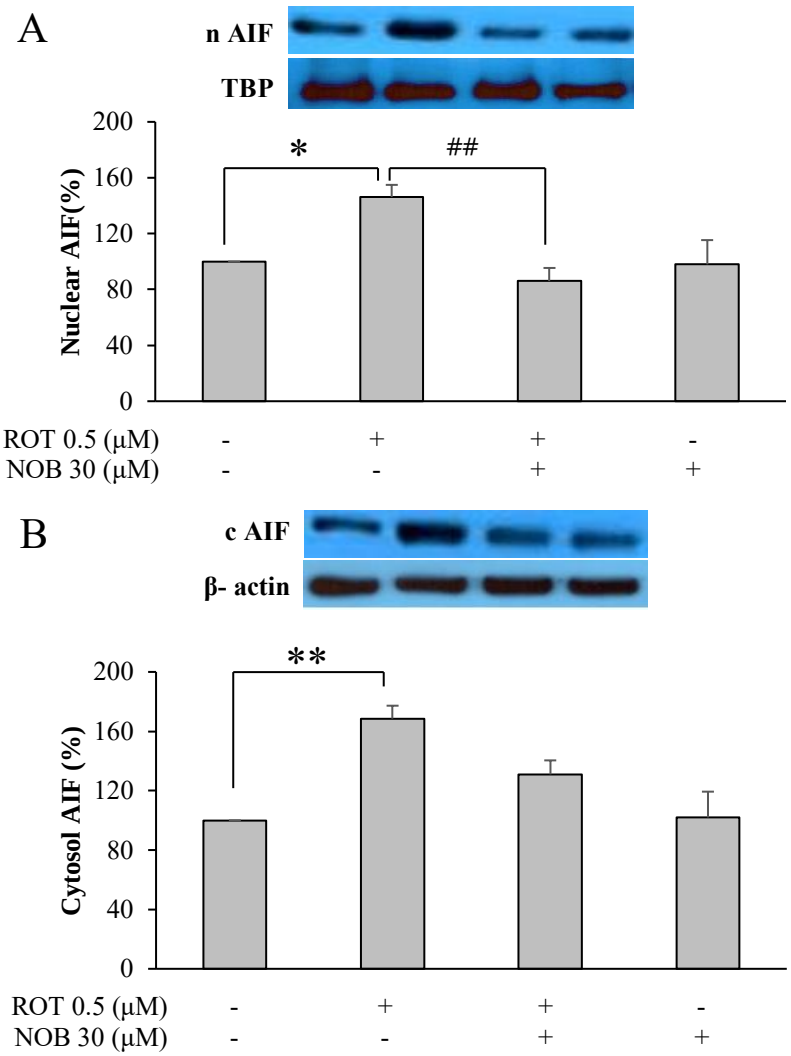

Figure 8. The effect of nobiletin on levels of AIF protein expressed in nucleus or cytosol in HT-22 cells

HT-22 neurons were pretreated with/without nobiletin for 1 hour and then incubated with rotenone (ROT, 0.5 μM) for 6 hours. Nuclear fractions were isolated and Western blotting analysis was performed using anti-AIF antibody. Nobiletin (NOB) significantly reduced both cytosolic and nuclear AIF levels which were increased by adding rotenone. Data represent the mean ± S.E.M, and $p < 0.05$ (*), $p < 0.01$ (**) compared with the control group, $p < 0.01$ ($^{\#\#}$) compared with the

rotenone.

3.4. The effects of nobiletin on physiological mitochondrial functional.

The primary role of mitochondria is to produce ATP for physiological conditions, even though they can also act as calcium stores and ROS regulators. Therefore, it was necessary to figure out if nobiletin regulated metabolic functions of mitochondria which are correlated with oxygen consumption. Figure 9 and 10 show the measurable parameters about mitochondrial respiration by Mito Stress assay, representing the functioning of pure isolated mitochondria. This provides evidence that isolated mitochondria are suitable for observing their metabolic activity in an *in-vitro* condition.

In Figure 11, nobiletin directly enhanced mitochondrial viability under the physiological condition (control, NOB, 1, 10, or 30 μM, 126.8 ± 0.7 %, 108.7 ± 1.5 %, 105.6 ± 1.3 %, n = 4, $p < 0.05$, $p < 0.01$, compared with the control). This nobiletin effect seemed to be mediated to the inhibition of ETC CI, because, under CI substrate-free condition, the mitochondrial viability was not affected by nobiletin treatment (CI inhibition, NOB, 1, 10, or 30 μM 82.7 ± 1.1 %, 84.0 ± 1 %, 77.9 ± 1.3 %, n = 4, n.s, compared with the CI inhibition alone). Unexpectedly, nobiletin effect on the enhancement of mitochondrial viability was more remarkable at low concentrations. This may be due to the effect of nobiletin to induce the mild depolarization of mitochondrial membrane

in a dose-dependent manner (Lee *et al*., 2018). This is also the reason why nobiletin showed the effect to slightly enhance mitochondrial ROS in this study (data not shown). Next, it was tested if nobiletin exhibited the effect to influence mitochondrial OCR (Fig. 12). The various concentrations of nobiletin slightly but significantly increased OCR in pure isolated mitochondria. After adding nobiletin, the level of mitochondrial OCR-linked ATP was also dose-dependently increased but not significant (Fig. 13A, NOB, 1, 10, or 30 μM, 346.3 ± 24.4, 360.2 ± 33.7, 403.4 ± 16.5, n = 6, n.s, compared with the control). Here, the increment of OCR-linked ATP was determined as following;

[Increment of OCR-linked ATP] =

[The last rate of OCR measured right before oligomycin addition]

– [the minimal rate of OCR measured after oligomycin addtion].

There is another protocol to improve the mitochondrial function to produce ATP per each mitochondrion by targeting the processes underlying ATP supply. The previous report demonstrated that coupling oxidation to phosphorylation can enhance ATP generation per each O_2 uptake (i.e., mitochondrial coupling efficiency, Conley., 2016). In this study, nobiletin also slightly increased coupling efficiency in a dose-dependent manner (Fig. 13B, NOB, 1, 10, or 30 μM, 181.3 ± 21.7, 207.3 ± 38.8, 207.9 ± 29.0, n = 6, n.s, compared with the control). Although the statistical significance was not revealed in changes of OCR-linked ATP and coupling

efficiency, this clearly provides evidence that nobiletin may directly regulate the ATP production via enhancing OCR.

Figure 14 represents the effects of nobiletin on mitochondrial OCR under the CI substrate free condition. In this experiment, CI substrate free condition significantly reduced the mitochondrial OCR (Fig. 14A). After injecting nobiletin under the same condition, OCR was significantly enhanced, compared with the absence of nobiletin (Fig. 14B). In Figure 15, OCR-linked ATP and coupling efficiency were also observed to be significantly reduced under the substrate free condition. However, nobiletin slightly and, in some case significantly increased levels of OCR-linked ATP (CI inhibition, 524.2 ± 16.1, n = 4, $p < 0.01$, compared with the control; CI inhibition + NOB, 573.1 ± 9.9, n = 4, $p < 0.05$, compared with the CI inhibited group) and coupling efficiency (CI inhibition, 154.4 ± 7.9, n = 4, $p < 0.01$, compared with the control; CI inhibition + NOB, 171.7 ± 1.3, n = 4, n.s, compared with the CI inhibited group). This strongly suggests a possibility that nobiletin effect to enhance ATP production may be correlated with mitochondrial functions specific to ETC CI. Furthermore, it was confirmed that nobiletin dose-dependently increased ATP synthases (Fig. 16, NOB, 1, 10, or 30 μM, 100 ± 2.4 %, 123 ± 3.4 %, 124 ± 2.0 %, 130 ± 1.2 %, n = 4, $p < 0.01$, compared with the control) and CI activity (Fig. 17, NOB 0.1, 0.5, 1, 10 or 30 μM, 100 ± 0.8 %, 109.5 ± 0.7 %, 110.1 ± 0.7 %, 177.1 ± 1.4 %, 219.7 ± 1.5 % and 253 ± 1 %, n = 7, $p < 0.01$, compared with the control) in mitochondria isolated from rat cortices

under a normal condition. Taken together, these results may suggest that nobiletin can regulate ATP synthase via contributing to the metabolic function of mitochondria specific to ETC CI in neurons.

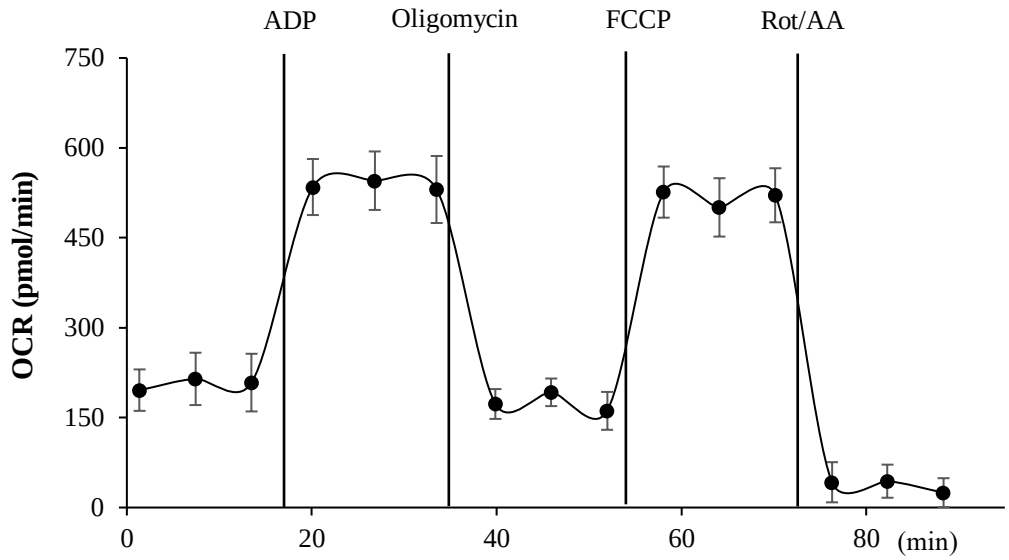

Figure 9. The measurement of mitochondrial respiration.

The normal OCR of pure isolated mitochondria was measured by using a Seahorse XF-24 extracellular flux analyzer (Seahorse Bioscience Agilent Technologies). Adenosine diphosphate (ADP), Oligomycin (O), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). For more details, see 'Materials and method'.

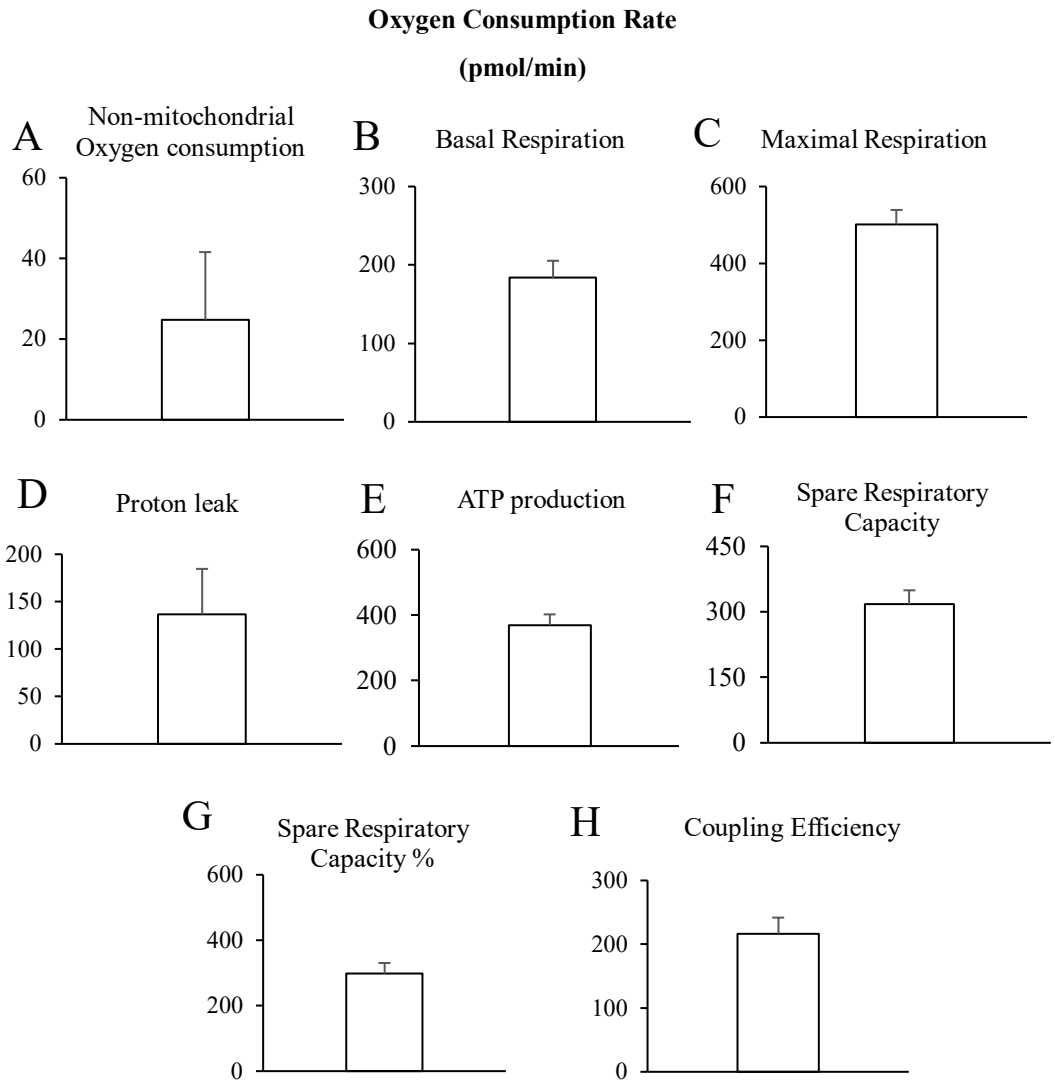

Figure 10. The measurements of detail parameters about mitochondrial respiration.

(A) Non-mitochondrial oxygen consumption calculated as the minimum rate measured after adding rotenone/antimycin A. (B) Basal respiration determined by [the last rate measured before the first injection] – [non-mitochondrial respiration rate]. (C) Maximal respiration calculated by [the maximal rate measured after FCCP injection] – [Non-mitochondrial respiration rate]. (D) H^+

(proton) leak calculated by [the minimal rate measured after oligomycin injection] – [Non-mitochondrial respiration rate]. (E) ATP production calculated by [the last rate measured before oligomycin injection] – [the minimal rate measured after oligomycin injection]. (F) Spare respiratory capacity calculated by [the maximal respiration] / [the basal respiration]. (G) Spare respiratory capacity as % [the maximal respiration] / [the basal respiration]×100. (I) Coupling efficiency calculated by [ATP production rate] / [the basal respiration].

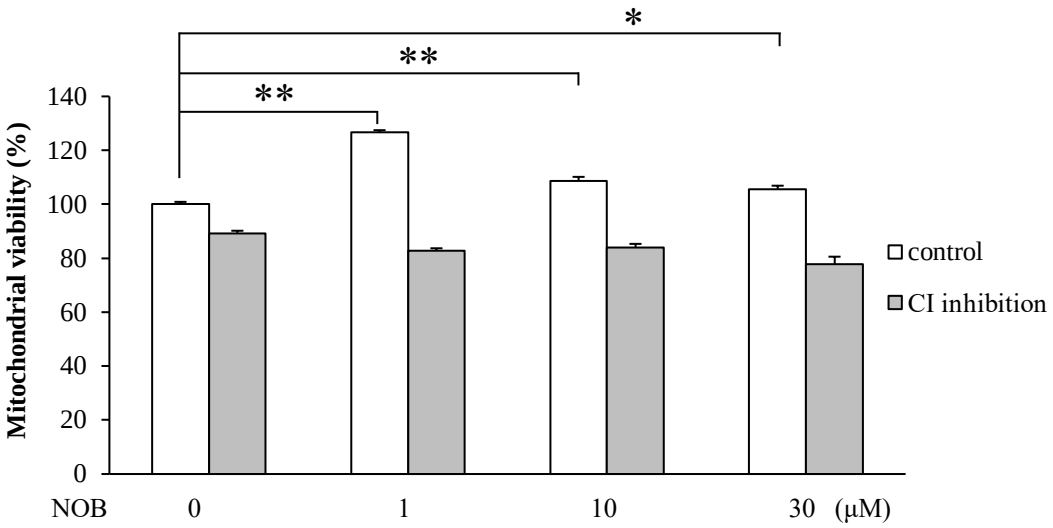

Figure 11. The effects of nobiletin on mitochondrial viability under normal and CI substrate free conditions

The effects of nobiletin on mitochondrial viability in CI substrate free and normal conditions. Nobiletin effect (NOB in control) on the enhancement of mitochondrial viability was more remarkable at low than high concentrations, but its effect was not observed under the absence of a CI substrate (NOB in CI inhibition). Additionally, rotenone used as positive control did not alter any mitochondrial viability in this experiment. Data represent the mean ± S.E.M, and $p < 0.05$ (*), $p < 0.01$ (**) compared with the control group.

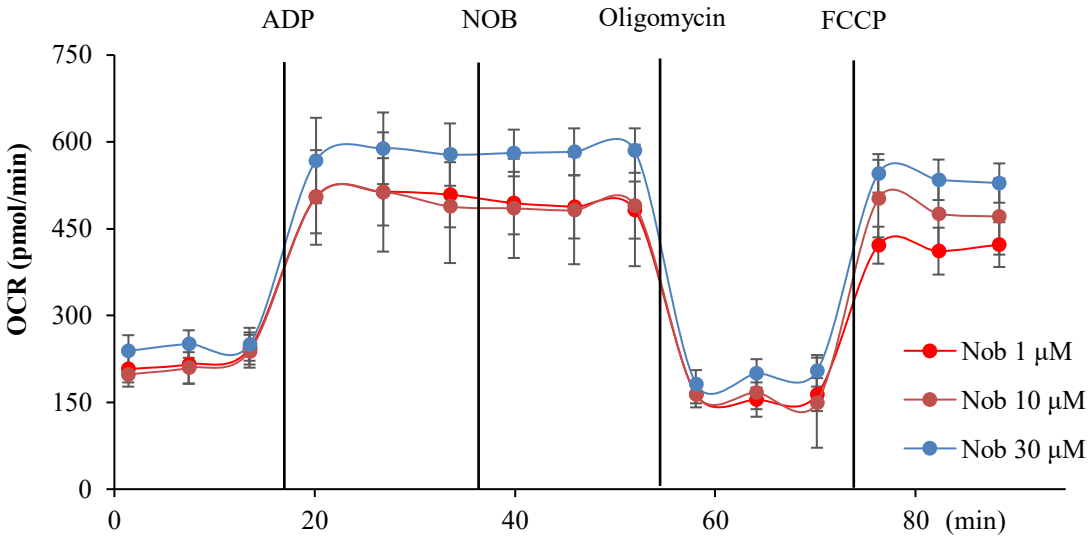

Figure 12. The effects of nobiletin on mitochondrial OCR.

The various concentrations of nobiletin remarkably increased OCR in pure isolated mitochondria.

Data represent the mean ± S.E.M

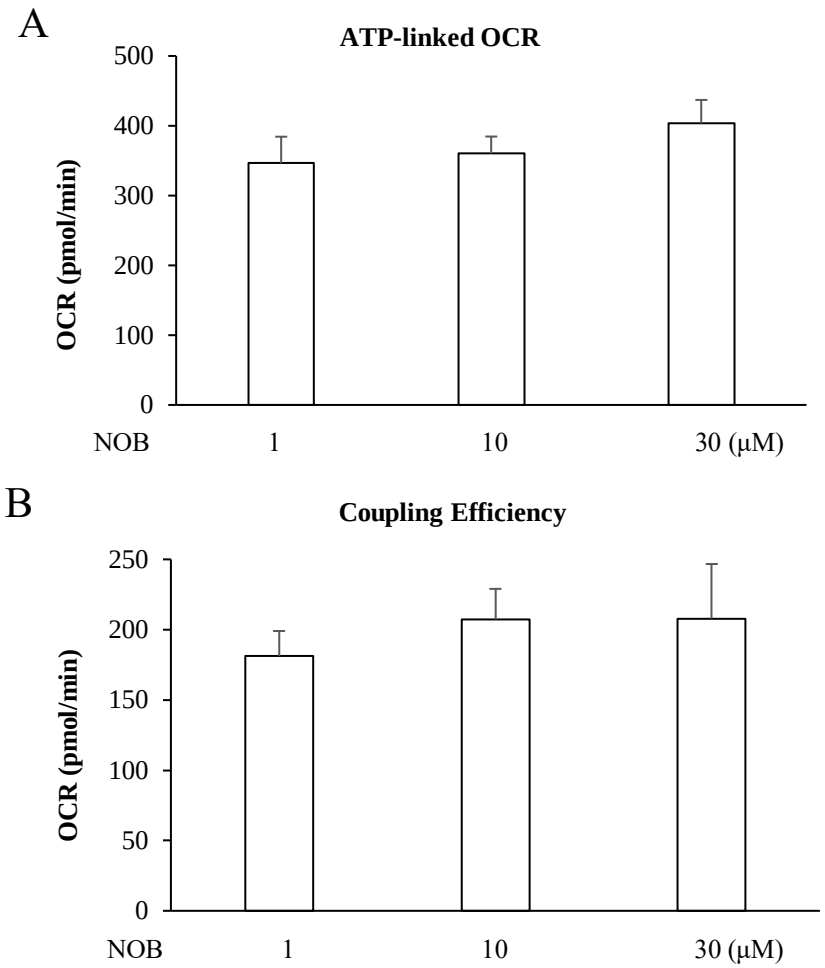

Figure 13. **The effects of nobiletin on mitochondrial efficiency.**

(A) The various concentrations of nobiletin (NOB) slightly increased the level of [the last rate measured before oligomycin injection] – [the minimal rate measured after oligomycin injection]. (B) Nobiletin also slightly increased the coupling efficiency but not significant. Data represent the mean ± S.E.M.

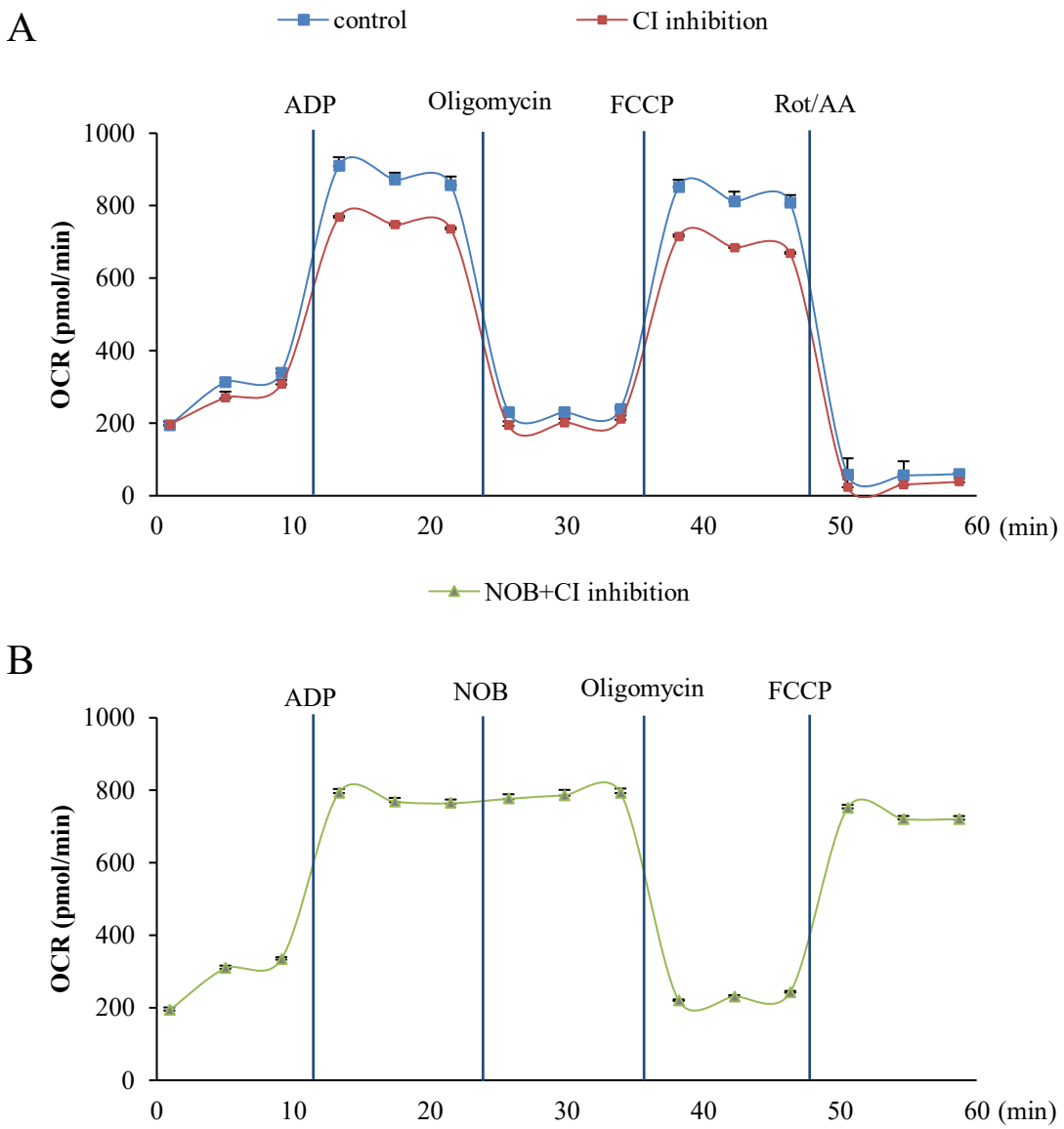

Figure 14. The effects of nobiletin on mitochondrial OCR under CI substrate free condition.

(A) Normal OCR levels were reduced in the absence of CI substrate. (B) The effects of nobiletin restored mitochondrial OCR, wich was reduced under CI substrate free condition.

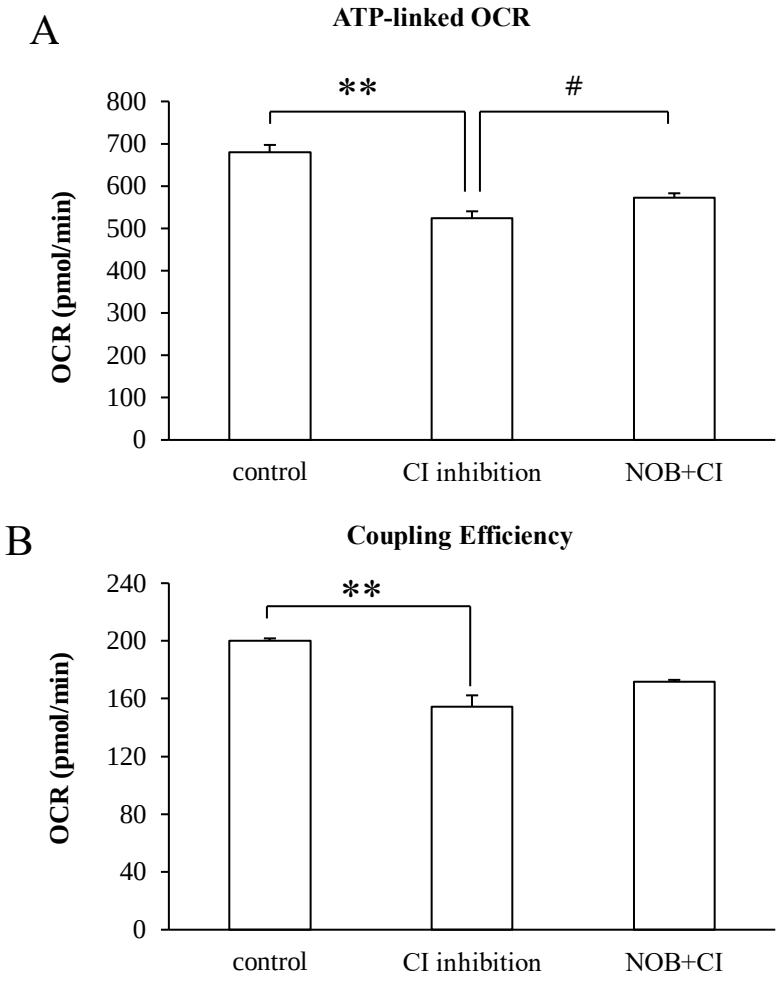

Figure 15. **The effects of nobiletin on OCR-linked ATP and mitochondrial efficiency under CI substrate free condition.**

The CI substrate free condition significantly reduced both OCR-linked ATP (A) and coupling efficiency (B). Nobiletin (NOB+CI, NOB, 30 μM) slightly but significantly increased the level of OCR-linked ATP (A). Also, nobiletin slightly increased the coupling efficiency but not significant. Data represent the mean ± S.E.M, and $p < 0.01$ (**) compared with the control group, $p < 0.05$ ($^{\#}$) compared with the CI inhibited group.

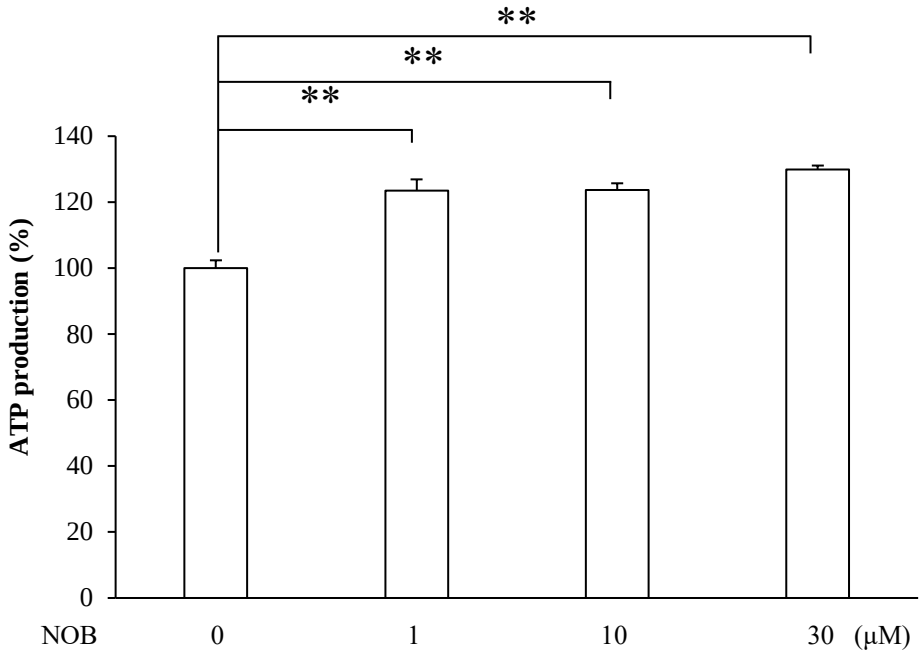

Figure 16. Nobiletin increases ATP production in pure isolated mitochondria.

The level of mitochondrial ATP was directly measured by using a luciferase/luciferin ATP determination kit. ATP production was dose-dependently increased by nobiletin (NOB). This means that nobiletin can enhance metabolitic functions of mitochondria under the normal condition. Data represent the mean ± S.E.M, and $p < 0.01$ (**) compared with the control group.

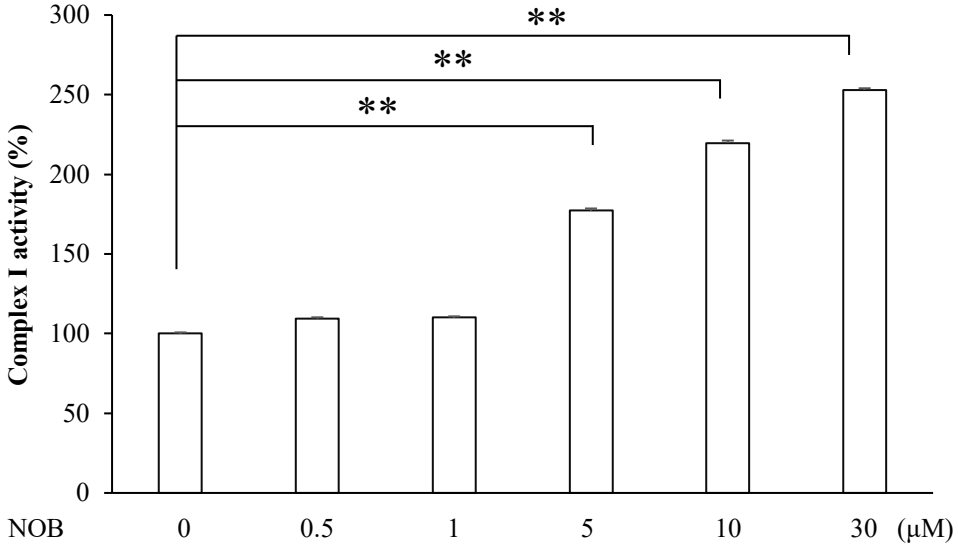

Figure 17. Nobiletin increases the activity of ETC CI.

Using 20 μg of mitochondrial protein, the activity of ETC CI was directly determined. The absorbance of all samples was measured at 340 nm for 15 minutes (MitoCheck Complex I Activity Assay Kit). Nobiletin (NOB) dose-dependently increased the activity of ETC CI in pure mitochondria isolated from rat cortices. Data represent the mean ± S.E.M, and $p < 0.01$ (**) compared with the control group.

3.5. The effect of nobiletin on the antioxidant enzyme expression.

It was confirmed that nobiletin induced antioxidant effect under the mitochondrial dysfunction mediated to the inhibition of ETC CI and restored the survival level of neurons under oxidative stress. Therefore, it was necessary to test if nobilein also regulated the expression of antioxidant enzymes. Nrf-2 is one of important transcription factors that regulate the gene expression of HO-1, an antioxidant enzyme. In the experiment using Western blotting analysis, nobiletin significantly and dose-dependently upregulated the expression of HO-1 via enhancing the translocation of Nrf-2 to nucleus in HT-22 cells, which were treated with nobiletin for 6 hours (Fig. 18). This clearly indicates that the neuroprotective effect of nobiletin under mitochondrial dysfunction specific to the inhibition of ETC CI is also mediated to the antioxidant signaling cascade in neurons.

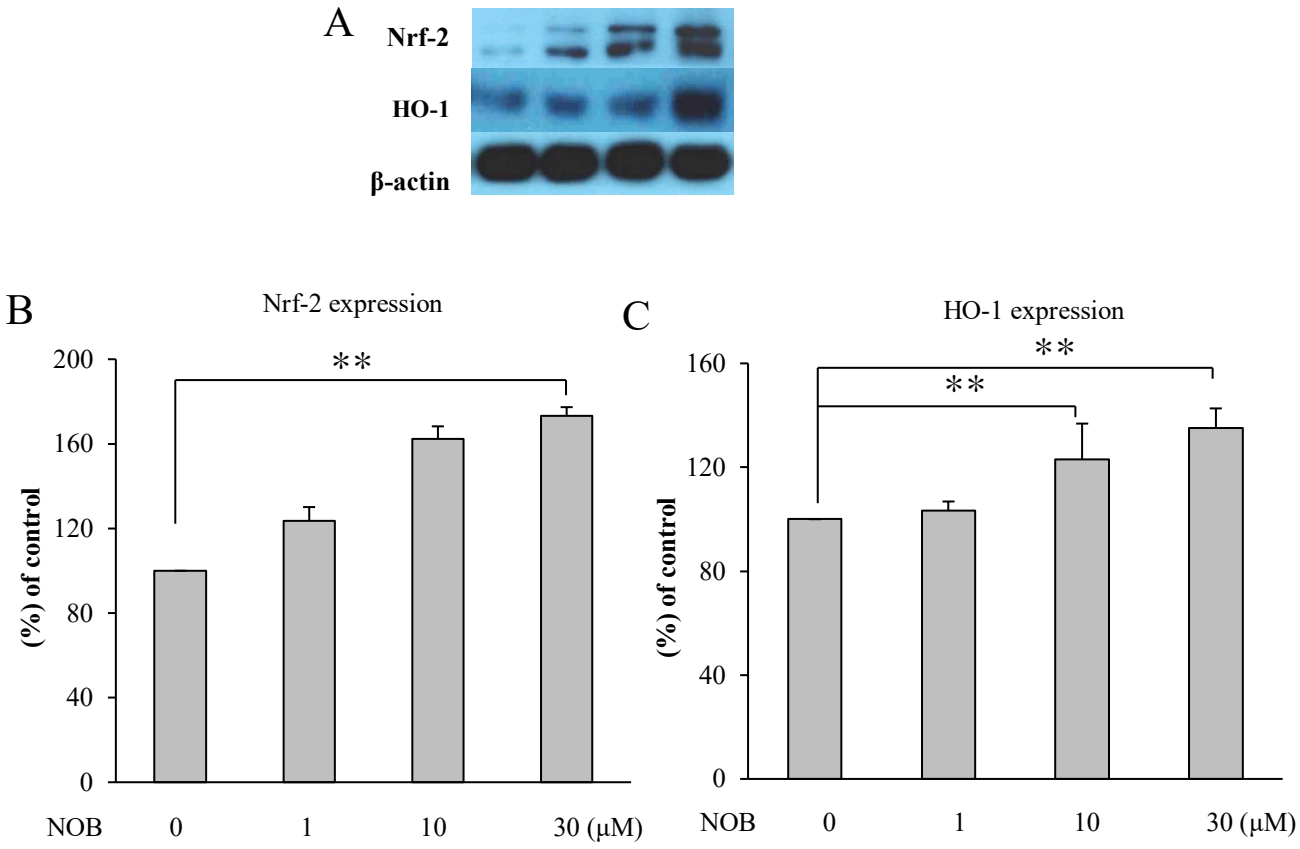

Figure 18. Effects of nobiletin on antioxidant enzyme levels in neurons.

(A-C) HT-22 cells were exposed to nobiletin (1, 10 and 30 μM) for 6 hours, total proteins were isolated and Western blotting analysis was performed using anti-HO-1 antibody and anti-Nrf-2. Data represent the mean ± S.E.M, and $p < 0.01$ (**) compared with the control group.

DISCUSSION

In neurons of mammalian nervous system, oxidative stresses induced by the excessive ROS production of mitochondria or the impairment of the antioxidant defense system result in serious mitochondrial dysfunctions to trigger the irreversible cell death. Overproduction of mitochondrial ROS and alteration in mitochondrial redox homeostasis seem to be involved in both a number of neuropathological conditions and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Alzheimer's Association. 2016; Grimm *et al*., 2016; Ibáñez *et al*., 2004; Indo *et al*., 2015; Kishida *et al*., 2007; Rego *et al*., 2003).

Mitochondria are crucial organelles that produce ATP in aerobic eukaryotic organisms and contribute to other processes of metabolic and cellular signalings. In early studies, mitochondria were thought to produce ROS as a by-product resulted from aerobic metabolism, but recently, mitochondrial ROS has been often reported to mediate the intracellular signal transduction (Castro *et al*., 2001; Schieber *et al*., 2014). Mitochondrial contribution to the total level of intracellular ROS appears to be dynamic according to species, organs, tissues and mitochondrial subpopulations. Mitochondria are well known to have at least 11 sites to produce ROS (Brand, 2016; Mailloux, 2015). Of them, ETC CI and CIII are major sources for the production of mitochondrial ROS as well as metabolic ATP. It has been reported that CIII is the dominant site

to produce ROS in circulatory and respiratory systems, whereas ETC CI is responsible for most ROS produced in nervous system (Barja *et al*., 1998; Turrens *et al*., 1980; Turrens *et al*., 1982). According to their mechanistic processes, two complexes exhibit different sites to produce ROS, demonstrating that CI (and CII) generates ROS within the mitochondrial matrix but CIII produces ROS in both matrix and the intermembrane space (Brand, 2010; Muller *et al*., 2004). The original view was that superoxide generated in the intermembrane space was released into the cytoplasm through voltage-dependent anion channels (Han *et al*., 2003).

Nobiletin is the multifunctional agent that reported to have various pharmacological activities including neuroprotection, cardiovascular protection, antimetabolic disorder, anticancer, anti-inflammation, and antioxidation (Huang *et al*., 2016). When ROS is abnormally produced under pathological conditions, nobiletin promotes protein synthase, showing antioxidant effects. Also, its protective effects may be mediated to functions upregulating Nrf-2 and HO-1, downregulating NF-κB expression. (Chen *et al*., 2012; Zhang *et al*., 2016). Recently, it has been reported that nobiletin activates the matrix substrate level of phosphorylation (Jojua *et al*., 2015). In this study, I demonstrated that nobiletin effectively blocked rotenone-induced excessive production of ROS and cell death, showing the specificity to the inhibition of ETC CI rather than CIII in both neurons and pure mitochondria isolated from rat cortices.

The significant accumulation of ROS was observed during CI inhibition induced by rotenone

in pure isolated mitochondria, and nobiletin completely abolished the effect of rotenone (Fig. 6A). However, its antioxidant effect was not observed in the case of ROS increase during the inhibition of CIII, which was induced by antimycin A (Fig. 6C). Both CI and CIII inhibitions are well-known to trigger mitochondrial dysfunction, increasing ROS production (Guzy *et al*., 2005; Greenamyre *et al*., 2001; Lenaz, 2001; Murphy et al., 1999; Piskemik *et al*., 2008; Turrens, 1997). Consistent with these reports, it was observed that ROS level was significantly increased under both CI and CIII inhibition in this study. However, the fact that nobiletin exhibited the effect specific to CI inhibition indicates that it might have an antioxidant mechanism to act under specific conditions. Additionally, it was also observed that nobiletin significantly reduced the neuronal death which was induced by rotenone in primary cortical neurons dissociated from the rat brain (Fig. 7A). If nobiletin had an ability to restore the survival rate of neurons through reducing ROS level, antimycin A-induced cell death also should be restored by nobiletin treatment. However, nobiletin did not show the neuroprotective effect to restore the cell death induced by antimycin A in primary cortical neurons (Fig. 7B). This indicates that neuroprotective effects of nobiletin might be mediated to the CI inhibition-specific dysfunction of mitochondria in neurons, consistent with the results observed in pure mitochondria isolated from rat cortices. In this study, it has not been addressed how nobiletin exerts neuroprotective effects specific to the mitochondrial dysfunction and apoptotic processes which are correlated with ETC CI inhibition,

or where it is acting to. However, for the production of ATP through oxidative phosphorylation, it is required to allow electrons to pass through electron transport. The electrons required for oxidative phosphorylation come from electric carriers such as nicotinamide adenine dinucleotide (NADH) and (flavin adenine dinucleotide) $FADH_2$, which are activated from the tricarboxylic acid cycle (TCA cycle) in mitochondrial matrix space (Martínez-Reyes et al., 2020). CI is known to convert NAD from its reduced form, NADH to its oxidized form, NAD^+ and makes ATP synthases. Jojua et al. previously reported that nobiletin can recover the reduced ATP metabolism during hypothyroidism in the hippocampal mitochondria and regulate the activation of mitochondrial substrate phosphorylation (Jojua *et al*., 2015). This indicates that nobiletin may directly or inderctly regulate CI.

Under the CI inhibition inducing excessive oxidative stress, the loss of mitochondrial integrity can release apoptotic-mediated molecules such as cytochrome c and AIF from the inter-membrane space of mitochondrial into the cytosol (Green *et al*., 1998). In particular, the activation of AIF, a 67kDa favoprotein, which contributes to the caspase-independent apoptotic signaling pathway, represents as a hallmark of cell death in the previous study (Joza et al., 2001). When AIF is released from the mitochondrial inter-membrane space and translocated to the nucleus, it generally induces DNA fragmentation, nuclear pyknosis, and finally neuronal cell death. In this study, CI inhibition induced by rotenone was confirmed to mediate to the significant enhancement

of AIF in both nuclear and cytosol of HT-22 cells (Fig. 8). In this experiment, nobiletin effectively blocked rotenone-induced AIF accumulation in both nuclear and cytosol. This indicates that nobiletin may contribute to the regulation of apoptotic signaling during mitochondrial dysfunction induced by CI inhibition. This effect of nobiletin might be based on the activation of antioxidant signaling against the apoptotic processing, because the expression levels of Nrf-2 and HO-1 were significantly enhanced by nobiletin (Fig.18). Nrf2 is a basic leucine zipper transcription factor, and under normal conditions, usually sequestered by its inhibitory partner, kelch-like protein 1 (Keap1) in the cytosol. However, under the activation of antioxidant signaling, Nrf2 is released from Keap1 and translocated to the nucleus where it activates the antioxidant response element (ARE) of phase 2 genes such as HO-1 and accelerates their transcription. And then, HO-1 plays an important role for the detoxification by forming biliverdin and bilirubin during heme degradation, which can serve as ROS scavengers (Origassa *et al*., 2013). Therefore, the result that nobiletin enhanced Nrf-2 and HO-1 expression, strongly suggests that its neuroprotective effect to block apoptotic signaling may mediate the activation of antioxidant signaling cascade under abnormal conditions specific to CI inhibition. The possible signaling cascade activated by nobiletin was summarized in Figure 19.

Nobiletin showing antioxidant and neuroprotective effects in neurons, also strongly regulated the normal function of mitochondria in this study. In the experiment using a luciferase/luciferin

ATP determination kit to measure the level of ATP production, nobiletin significantly increased ATP production in pure mitochondria, indicating the existence of its modulatory function in neuronal mitochondria (Fig.16). However, it is not sure that its effect to affect ATP production is related to ETC CI activation, although nobiletin enhanced the level of ETC CI activation in Figure 17. It should be addressed in the further study where nobiletin acts at and how it regulates mitochondrial functions and activates the antioxidant signaling.

In summary, nobiletin clearly exerted neuroprotective effects specific to the oxidative stress induced by CI inhibition in both pure isolated mitochondria and cortical neurons of rats. It may be mediated with the regulation of mitochondria, inhibition of apoptotic signaling and activation of antioxidant signaling cascades. Taken together, nobiletin has the potential to be an agent capable of treating the oxidative stress-induced neurological and neurodegenerative diseases.

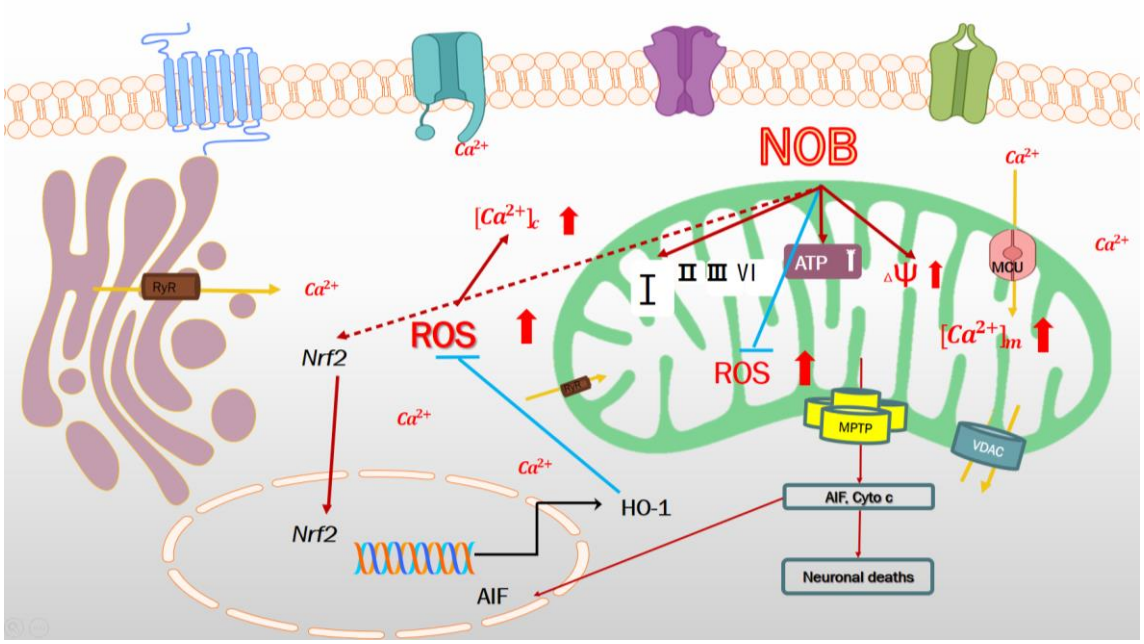

Figure 19. Ideal mechanism of nobiletin in neurons.

In our previous study, nobiletin exhibited neuroprotective effects in neurons under glutamate-induced clacium overload by mildly increasing mitochondria depolarization through K^+ channels. In this study, nobiletin additionally showed antioxidant and neuroprotective effects specific to the oxidative condition of CI inhibition, reducing mitochondrial ROS production, preventing the translocation of AIF from mitochondria to nucleus and cytosol, which is activated by apoptosis induction. Also nobiletin might activate the activities of antioxidant enzymes and increase cell metabolism as well as CI activity, thereby leading a neuroprotective mechanism mediated to antioxidant signaling.

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ABSTRACT IN KOREAN

미토콘드리아는 세포 사멸과 밀접하게 관련되어 있는 신호전달 기전들을 수행하며, 미토콘드리아 내막에 존재하는 전자전달계를 통해 세포 생존에 중요한 에너지원인 ATP를 만들어낸다. 전자전달계는 I~V까지의 총 5개의 단백질 복합체로 구성되어 각각의 복합체에서 연달아 전자를 전달하는 과정을 통해 ATP를 생산한다. 이 과정은 전자가 전달되는 과정에서 수소이온을 배출해 생성된 막전위(membrane potential) 차를 이용하여 ADP을 ATP로 만들어 주는 산화적 인산화과정(Oxidative phosphorylation)을 형성함으로써 우리 몸에 필요한 에너지원인 ATP 생성 과정을 완성한다. 또한, 전자전달계의 Complex I과 Ⅲ은 생리학적 및 병리학적 상황에서 reactive oxygen species (ROS) 생성의 주공급원의 하나로 알려져 있는데 미토콘드리아의 과도한 활성산소 생성 및 많은 양의 칼슘유입은 신경퇴행성질환의 발병 원인과 밀접한 관련이 있는 것으로 알려져 있다. 따라서 미토콘드리아 전자전달계 Complex I 조절을 통한 신경 보호 기전을 밝히는 것은 신경세포 생존 및 사멸을 결정하는 중요한 기전의 일부가 될 가능성이 있으며, 미토콘드리아 Complex I 조절을 통하여 미토콘드리아 신호전달을 조절할 수 있다면 퇴행성 뇌질환과 심혈관 질환 및 대사질환에서 다양한 질병치료에 응용할 수 있을 것으로 예상된다. 본 연구에서는, primary cortical neuron과 Sprague-Dawley (SD) 흰 쥐의 대뇌피질로부터 분리한 순수한 미토콘드리아에서 Complex I의 억제제로 알려진 로테논(rotenone)이 작용했을 때 발

생한 활성산소 독성을 노빌레틴(nobiletin) 처리를 통해 조절함으로써 신경독성 활성을 줄이고 세포사멸에 있어 신경세포 보호효과를 나타내는지 연구하였다. 연구결과에서, 노빌레틴이 primary cortical neuron에서 미토콘드리아 ETC Complex I 억제제 로테논에 의해 유도된 신경독성을 감소시키는 보호작용을 유도했는데, 노빌레틴이 로테논에 의해 유도된 미토콘드리아 ROS를 유의하게 줄이고 세포 생존능력을 증가시켰다. 이러한 결과는 이전에 보고된 K^+ 채널 조절을 통한 노빌레틴의 신경보호 효과와 같은 결과를 보여주는 것이다. 또한, 본 연구에서는, 노빌레틴이 직접 농도의 존적으로 ATP 생산뿐만 아니라 Complex I 활성을 증가시키는 효과가 있다는 것도 확인하였다.

이와는 대조적으로, 노빌레틴은 Complex III 억제제인 안티마이신 에이 (antimycin A)를 처리한 흰쥐의 primary cortical neurons과 순수하게 분리된 미토콘드리아에서 ROS 양을 감소시키거나 세포의 생존율을 증가시키는 효과는 나타내지 못하였다.

노빌레틴은 HT-22 신경세포에서 Nuclear factor erythroid 2-related factor 2 (Nrf-2)와 Heme oxygenase-1 (HO-1)의 발현양을 증가시켜, 직접 항산화 신호전달을 조절할 가능성도 있었으며, 이러한 결과는 노빌레틴이 전자전달계 Complex I 억제와 관련된 미토콘드리아 기능장애를 예방할 수 있는 효과가 있고, 그 효과가 항산화 신호전달과도 관련성이 있음을 의미한다. 결론적으로, 본 연구는 노빌레틴이

전자전달계 Complex I 대사기능 조절을 통해 퇴행성 신경질환과 신경염증 등의 발병에 있어 효과적인 신경보호제로서 사용될 수 있음을 의미한다.

키워드: 미토콘드리아; 복합체 I; 로테논; ROS, 세포 사멸; 노빌레틴