A Doctoral Dissertation

A study on the neuroprotective mechanism of nobiletin through regulation of mitochondrial membrane potential in primary cortical neurons and isolated brain mitochondria

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일차신경세포와 순수분리 미토콘드리아에서의 Nobiletin의 미토콘드리아 막전압 조절을 통한 신경보호 기전 연구

지도교수: 은 수 용

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A study on the neuroprotective mechanism of nobiletin through regulation of mitochondrial membrane potential in primary cortical neurons and isolated brain mitochondria

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LIST OF ABBREVIATIONS

5-HD	5-Hydroxydecanoate
AM	Acetoxymethyl ester
ATP	Adenosine triphosphate
$_{mito}K_{ATP}$	Mitochondrial ATP-sensitive K^+ channels
Ara-C	Arabinofuranosyl cytidine
$Ca2^+$	Calcium ion
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
FCCP	Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
CPE	*Citrus* polymethoxylated flavone
CCD	Cooled-charged device
DIV	Days in vitro
DPPH	1,1-diphenyl-2-picrylhydrazyl
EM	Electron microscopy
ETC	Electron transport chain
FBS	Fetal bovine serum
Fura-2 AM	Fura-2 acetoxymethyl ester
HT-22	Immortalized clonal mouse hippocampal cells
IPC	Ischemic pre-conditioning
MEM	Minimal essential medium
$\Delta\Psi_m$	Mitochondrial membrane potential

MCU	Mitochondrial calcium uniporter
$_{mito}$BK$_{Ca}$	Mitochondrial large-conductance Ca^{2+}-activated K^+ channels
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAC	N-acetyl cysteine
OCR	Dxygen consumption rate
PC12	Rat pheochromocytoma cells
ROS	Reactive oxygen species
Rhod-2 AM	Rhod-2 acetoxymethyl ester
SD	Sprague-Dawley
TMRE	Tetramethylrhodamine ethyl ester

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ABSTRACT

Mitochondrial calcium overload is an important event at the cellular level in determining the fate of neuronal survival and death and is regulated by a variety of channels, antiporters and pumps, as well as an electron transport chains (ETC) in the mitochondrial inner membrane. There are two motivating forces for calcium influx into the mitochondria, one of which is the calcium ion (Ca^{2+}) concentration gradient, and the other is mitochondrial membrane potential ($\Delta\Psi_m$). $\Delta\Psi_m$ is regulated within the range of -120 to -180 mV under physiological conditions. The negative charge of $\Delta\Psi_m$ has a significant effect on Ca^{2+} influx. Therefore, pharmacological manipulation of $\Delta\Psi_m$ could be a promising strategy to prevent neuronal cell death against brain insults by reducing calcium influx into the mitochondria.

Based on this hypothesis, it was investigated here whether nobiletin, a *Citrus* polymethoxylated flavone (CPE), prevents neuronal cell death by reducing mitochondrial calcium overload and oxidative stress via regulating $\Delta\Psi_m$ against neuronal insult in primary cortical neurons and brain mitochondria isolated from rat cortices. Results demonstrated that nobiletin (100 μM) treatment significantly increased cell viability against glutamate toxicity (100 μM, 20 min) in primary cortical neurons. Real-time imaging based fluorometry data proved that nobiletin evoked partial mitochondrial depolarization in these neurons. Nobiletin-induced partial mitochondrial depolarization in intact neurons was also confirmed in the isolated mitochondrial model using a fluorescence microplate reader. Nobiletin significantly reduced mitochondrial calcium overload and reactive oxygen species (ROS) generation in glutamate (100 μM)-stimulated cortical neurons. In the isolated mitochondrial model, nobiletin decreased ROS production when treated with high concentrations of Ca^{2+} (5 μM) to mimic glutamate toxicity. In experiments to determine whether nobiletin has its own ROS scavenging

ability, CPE and hesperidin exhibited considerable ROS scavenging activity, but nobiletin did not exhibit such an effect. Furthermore, nobiletin effects on basal $\Delta\Psi_m$ were completely abolished in isolated brain mitochondria maintained in K^+-free medium. To elucidate the molecular target of nobiletin, iberiotoxin 10 nM and 5-Hydroxydecanoate (5-HD) 500 μM were used to inhibit mitochondrial large-conductance Ca^{2+}-activated K^+ channels ($_{mito}BK_{Ca}$) and mitochondrial ATP-sensitive K^+ channels ($_{mito}K_{ATP}$), respectively. The results suggested that K^+ influx into the mitochondrial matrix by nobiletin is likely mediated by $_{mito}BK_{Ca}$ and $_{mito}K_{ATP}$. In addition, nobiletin markedly reduced rotenone-induced mitochondrial ROS generation, suggesting the possibility that complex I of ETC might be another mitochondrial target of nobiletin. However, more detailed studies should be conducted to determine the exact molecular targets of nobiletin in mitochondria. Taken together, results demonstrate that nobiletin-induced partial depolarization of $\Delta\Psi_m$ decreased mitochondrial Ca^{2+} overload and ROS generation, which are the important parameters of neuronal cell death. The K^+ influx into the mitochondria matrix is critically engaged in the partial mitochondrial depolarization-related neuroprotective effect of nobiletin. Nobiletin-induced mitochondrial K^+ influx is likely mediated, at least in part, by the activation of mitochondrial K^+ channels.

Keywords: nobiletin; calcium; mitochondrial calcium; mitochondrial K^+ channels; mitochondrial membrane potential

1. Introduction

The main function of mitochondria is to produce adenosine triphosphate (ATP). However, since the 20th century, many studies have focused on the regulatory function of mitochondria in determining the fate of cellular survival and death. Electron transport chains of mitochondria generate an H^+ electrochemical gradient as electrons pass through complex I, III, and IV, activating ATP synthase. The complete process is called oxidative phosphorylation. In healthy cells, the charge imbalance due to the generation of an electrochemical gradient through oxidative phosphorylation forms the basis of $\Delta\Psi_m$. Although a transient depolarization of the $\Delta\Psi_m$, through the "flickering" of one or several pores of the mitochondrial inner membrane, may occur under physiological conditions (Kroemer *et al*., 2007), a long-lasting dissipation of $\Delta\Psi_m$ is often associated with cell death. $\Delta\Psi_m$ dissipation is induced by proapoptotic stimuli such as Bax, Bak, tBid, Ca^{2+}, and cytosolic metabolites. In particular, mitochondrial calcium plays a key role in the regulatory mechanism of cell survival and death. Mitochondrial Ca^{2+} overload and oxidative stress can facilitate the opening of a high-conductance pore in the inner mitochondrial membrane. Pore opening is followed by osmotic swelling and rupture of the mitochondrial membrane that leads to the release of mitochondrial proteins including cytochrome *c*, which induces apoptosis, into the cytosol (Orrenius *et al.* 2015). Overload of mitochondrial calcium during brain insults results in oxidative stress, impaired mitochondrial function, neuronal cell death, and neuroinflammation that are implicated in the pathogenesis of many neurodegenerative diseases (Kroemer *et al*., 2007, Duchen *et al*., 2012).

Gunter (1990) described the driving forces of Ca^{2+} into mitochondria by an equation: $\Delta\mu Ca = RT \ln([Ca^{2+}]_{out}/[Ca^{2+}]_{in}) + 2F(\Psi_{out} - \Psi_{in})$. According to this equation, there are two different driving forces: i) Ca^{2+} concentration gradient between cytosolic calcium ($[Ca^{2+}]_c$) and

mitochondrial calcium ($[Ca^{2+}]_m$), ii) $\Delta\Psi_m$ gradient. Based on the correlation between $[Ca^{2+}]_m$ and $\Delta\Psi_m$, it has been proposed that pharmacological manipulation of $\Delta\Psi_m$ can be a key strategy to prevent neurotoxic $[Ca^{2+}]_m$ overload and neuronal cell death by reducing the driving forces of Ca^{2+} into the mitochondrial gradient (Ishida *et al*., 2001). Regarding these ideas, it has been reported that mild mitochondrial depolarization reduces mitochondrial calcium overload in different cell types (Sanz-Blasco *et al*., 2008, Valero *et al*., 2008).

In Wu's study (2013), the authors demonstrated that ethanolic peel extract of CPE induces mild mitochondrial depolarization intrinsically. Based on this property, CPE significantly attenuated mitochondrial calcium overload and cell death against H_2O_2 toxicity in immortalized clonal mouse hippocampal (HT-22) cells. Various flavonoid compounds in CPE (i.e., rutin, hesperidin, sinensetin, tangeretin, and nobiletin) belong to the polymethoxylated flavone (PMF) group (Wu *et al*., 2013). Nobiletin exerts several beneficial effects on improving cognitive function or motor deficits in several animal models such as cerebral ischemia (Yamamoto *et al*., 2009), Parkinson's and Alzheimer's diseases (Yabuki *et al*., 2014, Onozuka *et al*., 2008). In addition, nobiletin induced neurites outgrowth in rat pheochromocytoma (PC12) cells (Nagase *et al*., 2005) and significantly suppressed microglial activation and neuroinflammation (Cui *et al*., 2010). These results suggest that nobiletin is a promising candidate as a neuroprotective agent and warrants a thorough examination.

Based on this history, I investigated here whether nobiletin prevents glutamate toxicity induced by neurotoxic mitochondrial calcium overload and neuronal cell death through K^+ influx and $\Delta\Psi_m$ across the mitochondrial inner membrane in primary cerebrocortical neurons or isolated brain mitochondria. I also examined whether complex I is directly involved in the regulation of $\Delta\Psi_m$ by nobiletin.

2. Materials and Methods

2.1. Materials

Tetramethylrhodamine ethyl ester (TMRE), rhod-2 acetoxymethyl ester (Rhod-2 AM), MitoSOX Red, Fura-2 acetoxymethyl ester (Fura-2 AM) were purchased from Invitrogen (Carlsbad, CA, USA). 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). 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 (St Louis, MO, USA) unless otherwise indicated.

2.2 Primary culture of cortical neurons

Primary cortical neurons were prepared from cerebral cortices of postnatal 1-day-old Sprague-Dawley (SD) rats. The neonatal brain 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 fire-polished glass Pasteur pipettes. Then, cells were plated onto poly-L-lysine-coated round glass coverslips placed in 12-well plates at a density of 1.3×10^5/well. After 6 h, 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 were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere. The

cells were used after 7 days in vitro (DIV). The study was approved by Animal Care and Use Committee of Jeju National University and all experiments were performed in accordance with the guidelines.

2.3. Dual real-time imaging-based fluorometry of both cytosolic and mitochondrial calcium levels in the same cortical neurons

Fura-2 and Rhod-2 were used to measure cytosolic and mitochondrial calcium levels. Cell-permeable acetoxymethyl ester (AM) forms were used for these two probes. Fura-2 is a ratio-metric fluorescent indicator to measure $[Ca^{2+}]_c$ using the ratio of emitted fluorescence intensity at excitation wavelengths of 340 and 380 nm (Eun *et al*., 2001). Another calcium indicator Rhod-2, used for a selective $[Ca^{2+}]_m$ probe, exhibits charge-driven uptake into the mitochondria and evokes increase of fluorescence upon Ca^{2+} binding (Wu *et al*., 2013). Fura-2 and Rhod-2 were dissolved in DMSO at a concentration of 5 mM and 2 mM respectively and there are kept frozen in aliquot of 10 μL. The cortical neurons cultured on a coverslip were loaded with 10 μM Fura-2 AM and 0.1% Pluronic F127 for 45 min at 37°C. And then, cells were loaded with 2 μM Rhod-2 AM, transferred to 35 mm petri dish with media and incubated for 30 min at 4°C. After washing three times with normal Tyrode solution, a coverslip was transferred to the recording chamber. Cells were continuously superfused with normal Tyrode solution (in mM): NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1.3, HEPES 10, glucose 10, pH adjusted to 7.4 with NaOH, the osmolarity was adjusted to approximately 310 mOsm. Live-cell recording was performed with preheated normal Tyrode solution and stable perfusion at 1ml/min and the solution in the recording chamber was maintained at about 37℃. Neurons were illuminated at 340 and 380 nm with a monochromator through a T400lp dichroic mirror and an emission was monitored

through a 510/80 m filter for Fura-2. Rhod-2 fluorescence was measured using a 540/25 nm excitation filter, an AT565DC dichroic mirror and a 605/55 m emission filter (Jousset et al., 2008). High-speed filter switching device (Sutter Instrument, Lambda, DG-4) with a 175W xenon arc lamp (ILC technology, Sunnyvale, CA) as a light source was used for dual recording of Fura-2 and Rhod-2, the light source was switched at a speed of less than 2 ms to generate respective excitation wavelengths. The fluorescence images were acquired at 6 s intervals using an inverted microscope Olympus IX71 (Olympus) and a cooled-charged device (CCD) camera (Cascade, Roper Scientific). The data were analyzed using Metafluor software (Molecular Devices). The regions of selected from the initial image were converted to relative fluorescence intensity unit data. Also, the area for the background fluorescence was selected and subtracted from the measured value. All measurements were obtained after stabilization of the cell for 5 min and were displayed as a ratio of 380/340.

2.4. Real-time imaging-based fluorometry of $\Delta\Psi_m$ and mitochondrial ROS in intact cortical neurons

The cationic fluorescent probe TMRE is sequestered by mitochondria in proportion to $\Delta\Psi_m$ (Scaduto and Grotyohann., 1999). The cationic MitoSOX Red is also selectively targeted to the mitochondria. Once MitoSOX Red is oxidized by superoxide anions which are the predominant ROS in mitochondria, it exhibits red fluorescence. The cortical neurons were loaded for 15 min at 37°C with 25 nM TMRE and 5 μM MitoSOX Red for $\Delta\Psi_m$ and mitochondrial superoxide, respectively. After then, the neurons on the cover glass were washed three times with normal Tyrode solution. The cover glass was transferred to the recording chamber which was continuously superfused with normal Tyrode solution. TMRE and MitoSOx Red fluorescence

were measured using a 540/25 nm excitation filter, an AT565DC dichroic mirror and a 605/55 m emission filter. After stabilization of cells in the chamber for 5 min, the cells were treated with the reagents and fluorescence value were obtained at a single wavelength value which was subtracted background intensity. Digitized fluorescence images were acquired at 30 s intervals using an inverted microscope Olympus IX71 (Olympus) with a CCD camera (Cascade), and analyzed in a personal computer using Metafluor software (Molecular Devices). The equipment and protocols used for real-time image-based fluorescence measurements are listed in Table 1.

2.5. 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 removed from the brains of 14-16 day-old SD rats. The fragmented cortices were placed in EGTA-containing isolation buffer (IB) and homogenized in the Dounce-type tissue grinders (Kimble chase). Pestle A and B are used sequentially. The clearance of pestle A and B are 0.0035-0.0065 mm and 0.0010-0.0030 mm respectively. The IB contained (in mM): Mannitol 225, Sucrose 75, HEPES 5, ECTA 3, BSA 0.1%, titrated with KOH to pH 7.4. The homogenates were centrifuged at 600 × *g* for 10 min. The supernatant was transferred to a new tube and then centrifuged again at 600 × *g* for 10 min. Then, supernatants were centrifuged at 12,000 × *g* for 10 min. The pellets containing mitochondria were resuspended in IB buffer without EGTA and homogenized using Dounce-type tissue grinders. The clearance of pestle A and B used in 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 min. All the above procedures were carried out at 4°C. The isolated mitochondrial proteins

were quantified using Bio-Rad protein assay dye. Electron microscopy (EM) and oxygen consumption rate (OCR) measurement were conducted to evaluate morphology and metabolic activity of isolated brain mitochondria.

2.6. Measurement of $\Delta\Psi_m$ and ROS in isolated brain mitochondria using a microplate reader

After preparation of pure mitochondria isolated from rat brain cortices, an isolated brain mitochondrial model was set up. The mitochondria suspension (500 μg of protein/ml) was incubated in recording buffer for 10 min at 37°C with 25 nM TMRE and 50 μM 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) for $\Delta\Psi_m$ and mitochondrial ROS, respectively (Scaduto and Grotyohann, 1999, Blattner *et al*., 2001, Wu *et al*., 2013). The recording buffer contained (in mM): KCl 100, HEPES 20, Tris 20, NaCl 10, succinate 5, KH_2PO_4 1, EGTA 0.02, rotenone 0.002, oligomycin 0.001 and CaCl2 0.0001. The loaded mitochondria in suspension were treated with nobiletin or other reagents in 96 well plates. The mitochondrial suspension was divided into 200 μL/well, thus each well contained 100 μg of protein/ml. And then, fluorescence intensities were measured using a fluorescence microplate reader (SPECTRA FluoR, Tecan and SpectraMax i3, Molecular devices). As shown in Table 2, when measuring the $\Delta\Psi_m$ and mitochondrial ROS using SPECTRA FlioR, fluorescence was measured with excitation/emission wavelength of 485 nm/595 nm and 485 nm/535 nm for TMRE and DCF-DA, respectively. Fluorescence was measured with excitation/emission wavelength of 540 nm/595 nm and 493 nm/520 nm for TMRE and DCF-DA, respectively.

2.7. Measurement of oxygen consumption rate (OCR)

The OCR of isolated mitochondria was measured using a Seahorse XF-24 extracellular flux analyzer from Seahorse Bioscience (Santa Clara, CA, USA) following the manufacturer's protocol. Briefly, 5 μg of isolated mitochondria were suspended in 50 μL of assay medium and transferred to each well for OCR measurement. Mitochondrial assay medium contained (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), pH 7.2. One day before the experiment, sensor cartridge was placed into 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 (2 mM), oligomycin (2 μg/ml) as an inhibitor of mitochondrial ATP synthase, 2 μM carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) as an electron transport chain accelerator, rotenone/antimycin A (0.5 μM) as a complex I and III inhibitor. The OCR was recorded by sensor cartridge and analyzed using Seahorse XF-24 software.

2.8. DPPH free radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) was used to investigate free radical scavenging activity of nobiletin in the solution state, as previously described (Cui *et al*., 2015). This assay is based on redox reaction. The odd electron of N (nitrogen atom) in DPPH is reduced by receiving an H (hydrogen atom) from antioxidants. Purple-color DPPH is changed to yellow-color when it is reduced by antioxidant sample. The mixture of DPPH and nobiletin was incubated in dark at room temperature for 1 h, and the absorbance was read at 517 nm using a microplate reader (Tecan, Sunrise, AT, USA).

2.9. Cell viability assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was used to investigate cytoprotective effect of nobiletin on cell viability, as previously described (Choi *et al*., 2016). Water-soluble MTT was converted into an insoluble purple formazan by mitochondria in living cell. Primary cortical neurons were plated at a density of 7.5×10^4/well in cell culture plates. Arabinofuranosyl cytidine (Ara-C) 1 μM was treated in the cell culture well at DIV 4 to prevent growth of glial cell. Subsequently, the Ara-C concentration was kept constant at 1 μM in cell culture medium. After reagents treatment, MTT solution (2mg/ml) was added to cell culture medium for 1h at 37 ℃. The supernatant was discarded and the formazan dissolved in DMSO. Absorbance was subsequently read at 540 nm using a microplate reader (Model 550, Bio-Rad, USA).

2.10. Statistical analysis

Data were expressed as mean values ± standard error of the mean (SEM). The significance of data was analyzed using Student's t-test using the sigma plot 8.0 software. The differences between groups were considered to be significant when $p < 0.05$.

Table 1. Equipment used for real-time imaging-based fluorometry analysis in living cells

Real-time fluorescence analysis					
Indicator	**Measurement**	**Excitation filter**	**Emission filter**	**Dichroic mirror**	**Loading protocol**
Fura-2	Cytosolic calcium	ET340x/ ET380x	ET510/80m	T400lp	10μM, For 45 min at 37 ℃
Rhod-2	Mitochondrial calcium	AT540/25x	AT605/55m	AT565DC	2μM, For 45 min at 4 ℃
TMRE	Mitochondrial membrane potential	AT540/25x	AT605/55m	AT565DC	25nM, For 15 min at 37 ℃
MitoSOX Red	Mitochondrial ROS	AT540/25x	AT605/55m	AT565DC	5μM, For 15 min at 37 ℃

Table 2. Measuring protocols for $\Delta\Psi_m$ and ROS in isolated brain mitochondria

	Fluorescence indicator	**Concentration**	**Loading time / Temperature**	**Reagent**	**Ex/Em**
SPECTRA FluoR, Tecan	[1)]TMRE	25 nM	10 min/37 ℃	[2)]Nobiletin 5 min	485 nm/ 595 nm
	[3)]DCF-DA	50 μM	loading after reagent treatment/ Room temperature	[1)]Nobiletin 5 min [2)]Calcium 5μM, 15 min	485 nm/ 535 nm
SpectraMax i3, Molecular devices	[1)]TMRE	25 nM	10 min/37 ℃	[2)]Nobiletin 5 min	540 nm/ 595 nm
	[3)]DCF-DA	50 μM	loading after reagent treatment/ Room temperature	[1)]Nobiletin 5 min [2)]Calcium 5μM, 15 min	493nnm/ 520 nm

1), 2), 3) indicate the processing sequence

3. Results

3.1. Nobiletin, the key compound of CPE, exhibits neuroprotective effects based on mild mitochondrial depolarization

It was previously reported that CPE evokes partial mitochondrial depolarization in HT-22 cell, while carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 10 μM), a well-known $\Delta\Psi_m$ dissipation-inducer, evokes complete mitochondrial depolarization (Wu *et al*., 2013). Driving force of mitochondrial calcium overload can be reduced by inducing mitochondrial depolarization that blocks apoptosis. Based on this study, I investigated here whether nobiletin, an active compound of CPE, induces partial depolarization of $\Delta\Psi_m$ in resting state and reveals neuroprotective activity against glutamate toxicity in primary cortical neurons.

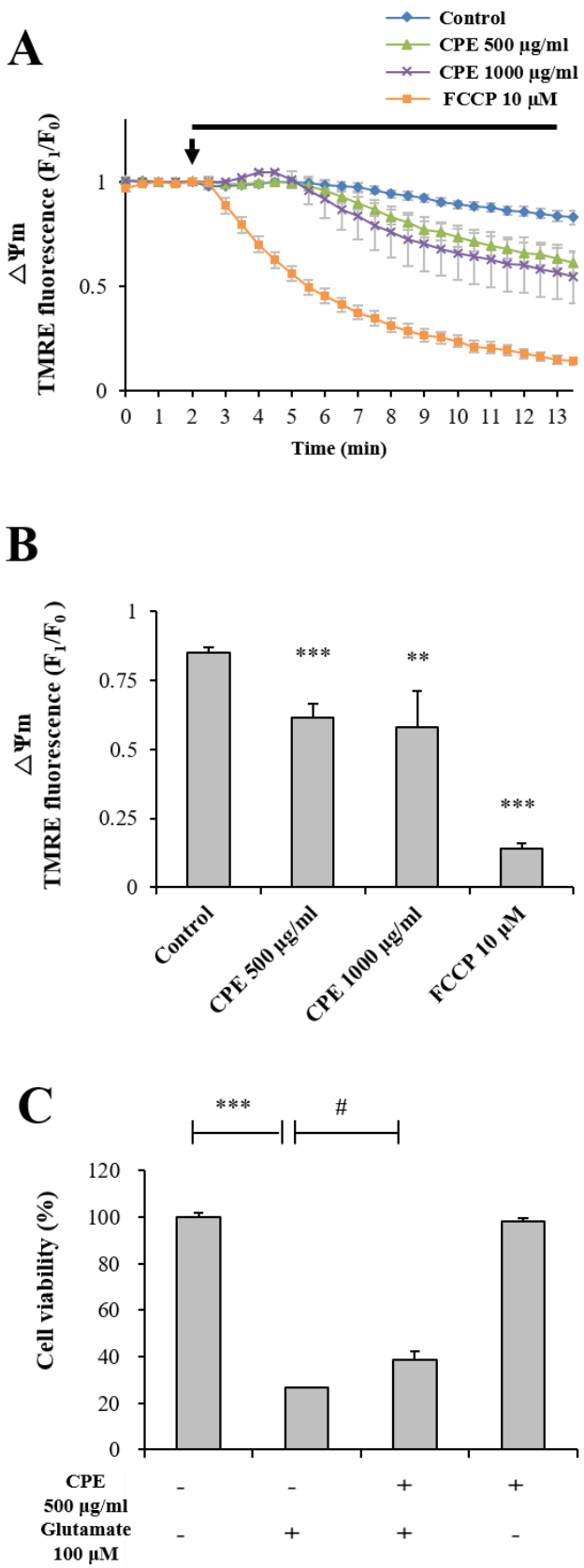
First, to determine whether the effect of CPE was maintained in primary cortical neurons, $\Delta\Psi_m$ and cell viability were measured in cortical neurons from embryonic 20-day SD rats. Fig. 1 shows the effect of CPE on $\Delta\Psi_m$ and cell viability. CPE induced mitochondrial depolarization in a dose-dependent manner (Fig.1A, B). The TMRE fluorescence values in 500 μg/ml group and 1000 μg/ml group at 13 minutes were 72.44 ± 5.67, and 68.42 ± 15.28 %, respectively (Fig.1B). 500 μg/ml CPE significantly enhanced cell viability against glutamate toxicity (100 μM, 20 min) in primary cortical neuron (Fig. 1C).

Various concentrations of nobiletin (30, 50, 100 and 200 μM) were superfused over TMRE-loaded cortical neurons on a cover slip in a recording chamber. $\Delta\Psi_m$ values were recorded and analyzed using real-time imaging-based fluorometry (See ‘Materials and Methods’ for more detailed description). TMRE fluorescence values from individual cells were normalized to values before drug treatment in Fig. 2A. Recording traces reveal the averaged recordings of

TMRE intensities obtained from individual cells. Results demonstrated that nobiletin treatment significantly evoked mild mitochondrial depolarization in dose-dependent manner. Normalized TMRE values at 13-min points were 94.71 ± 5.47%, 73.06 ± 4.79%, 61.47 ± 6.06% and 54.26 ± 10.37% in nobiletin (30, 50, 100 and 200 μM)-treated groups, respectively (Fig. 2B), compared to the control group.

It is well established that glutamate toxicity results in massive and global calcium influx into the cytosol and subsequent mitochondrial calcium overload and evokes cell death (Duchen *et al*., 2012). The neuroprotective effect of nobiletin against glutamate toxicity was examined in primary cortical neurons using MTT assay. Nobiletin (100 μM) was pretreated for 10 min and glutamate (100 μM) was added for 20 min in the presence of nobiletin. As shown in Fig. 2C, nobiletin significantly enhanced neuronal cell viability to 80.32 ± 4.80% against glutamate toxicity (100 μM, 20 min). The working concentration of nobiletin was determined as 100 μM which shows the maximum depolarization effect without cytotoxicity (Fig.3). Therefore, subsequent experiments were conducted at 100 μM of nobiletin.

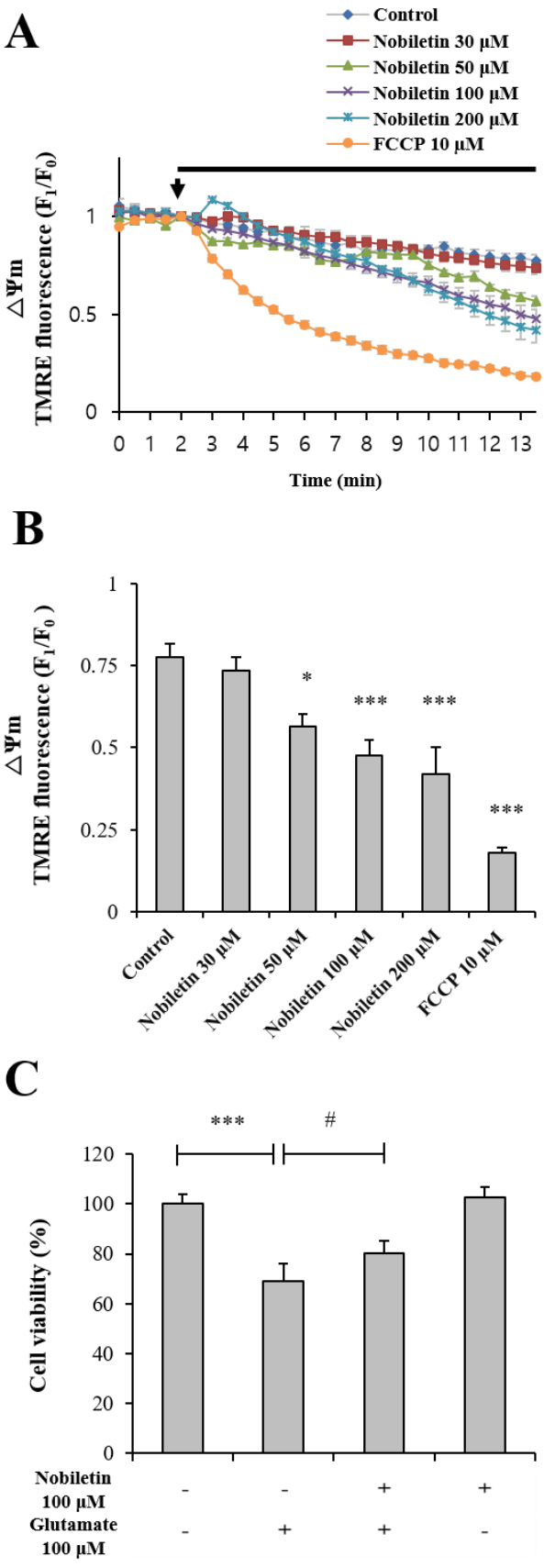
Results suggest that the effects of each active compound in the CPE, including nobiletin, can be reproduced in primary cortical neurons and nobietin-induces partial mitochondrial depolarization does not adversely affect to the physiological activity of neuronal cells at concentrations below 100 μM. The beneficial effects of CPE on $\Delta\Psi_m$ and neuronal cell viability are partially, at least, attributed to its key compound nobiletin.



[Lee JH *et al*. Korean J. Physiol. Pharmacol. 22(3):311-319]

Figure 1. The effects of *Citrus sunki* Hort. ex Tanaka (CPE) on basal $\Delta\Psi_m$ and cell viability against glutamate toxicity in primary cortical neurons.

(A) Recording traces of $\Delta\Psi_m$ using real-time imaging-based fluorometry with TMRE. After TMRE (25 nM) was loaded for 15 min, the base line was measured for 5 min. CPE (500, 1000 μg/ml) was superfused in a recording chamber from 5 min to the end of the measurement. TMRE fluorescence values from individual cells were normalized to the value at starting point of drug treatment. (B) Quantification of TMRE fluorescence at the end of the measurement, 13 min time point, was compared with among different group. (C) Neuroprotective effects of CPE on glutamate-induced neurotoxicity. Primary cortical neurons were pretreated with CPE for 10 minutes and then treated with glutamate (100 μM) for 40 minutes. The neuroprotective effects of CPE were analyzed by MTT assay. Values indicate the mean ± S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared with control group, $^{\#}p < 0.05$ as compared with glutamate alone.



[Lee JH *et al*. Korean J. Physiol. Pharmacol. 22(3):311-319]

Figure 2. The effects of nobiletin on basal $\Delta\Psi_m$ and cell viability against glutamate toxicity in primary cortical neurons.

(A) Recording traces of $\Delta\Psi_m$ using real-time imaging-based fluorometry with TMRE. Various concentrations of nobiletin were superfused over primary cortical neurons on a cover slip in a recording chamber from the arrow point. TMRE fluorescence values from individual cells were normalized to values before drug treatment shown as an arrow. (B) Quantification of $\Delta\Psi_m$ at the end of experiment. (C) Effects of nobiletin on cell viability against glutamate toxicity (100 μM, 20 min) were investigated using MTT assay. Values indicate the mean ± S.E.M. $^{*}p < 0.05$, $^{***}p < 0.001$ as compared with the control group and $^{\#}p < 0.05$ as compared with glutamate alone-treated group.

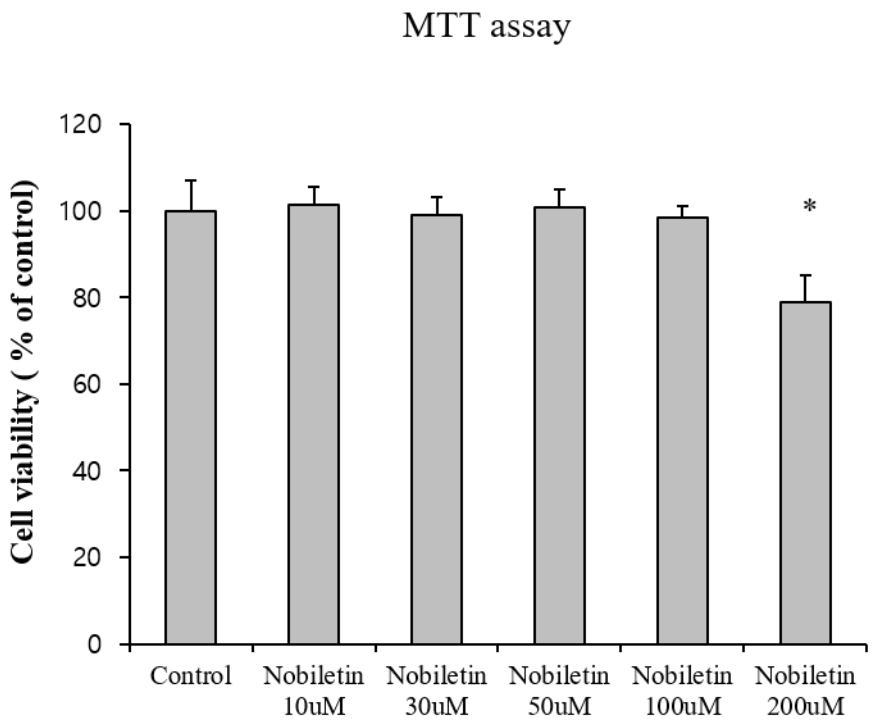


Figure 3. Dose determination of nobiletin treatment.

The toxicity of nobiletin on cortical neurons. Various concentrations of nobiletin (10, 30, 50, 100 and 200 μM) were treated for 30min. After 3 times wash out, neurobasal medium was added to cells. The cell viability was measured after 24 hours. *$p < 0.05$ as compared with untreated controls.

3.2. Nobiletin reduces glutamate-induced mitochondrial calcium overload

High concentration of glutamate induces excess Ca^{2+} influx from extracellular space to cytosol via glutamate receptor/channels and subsequently excess cytosol Ca^{2+} is moved into mitochondria (Duchen *et al*., 2012). Mild mitochondrial depolarization attenuates mitochondrial calcium overload by reducing driving force of Ca^{2+} uptake into mitochondria (Ishida *et al*., 2001, Sanz-Blasco *et al*., 2008, Valero *et al*., 2008). Therefore, I investigated here whether nobiletin attenuates mitochondrial Ca^{2+} overload against glutamate toxicity.

Cytosol and mitochondrial Ca^{2+} levels were simultaneously recorded and analyzed in both regions, cytoplasm and mitochondria, using dual real-time imaging-based fluorometry with Fura-2 AM and Rhod-2 AM (Fig. 4). Glutamate (100 μM) in the presence or absence of nobiletin (100 μM) was superfused over Fura-2 AM and Rhod-2 AM-loaded cortical neurons on a cover slip in a recording chamber. Cytosol and mitochondrial Ca^{2+} levels from individual cells, indicated as Fura-2 AM and Rhod-2 AM fluorescence values, were normalized to values before drug treatment. The cytosolic Ca^{2+} levels were increased by 2.02 ± 0.07 in glutamate group. Nobiletin markedly abolished glutamate-induced mitochondrial calcium overload in cortical neurons by 85.56 ± 4.10 % (Fig. 5). However, nobiletin did not affect glutamate-induced increase of $[Ca^{2+}]_c$ (Fig.4). Compared with the control group, nobiletin alone did not affect the intracellular Ca^{2+} level. Taken together from Fig. 4 and 5, it was demonstrated that nobiletin capable of evoking mild mitochondrial depolarization, potently blocked glutamate-induced mitochondrial Ca^{2+} overload in primary cortical neurons.

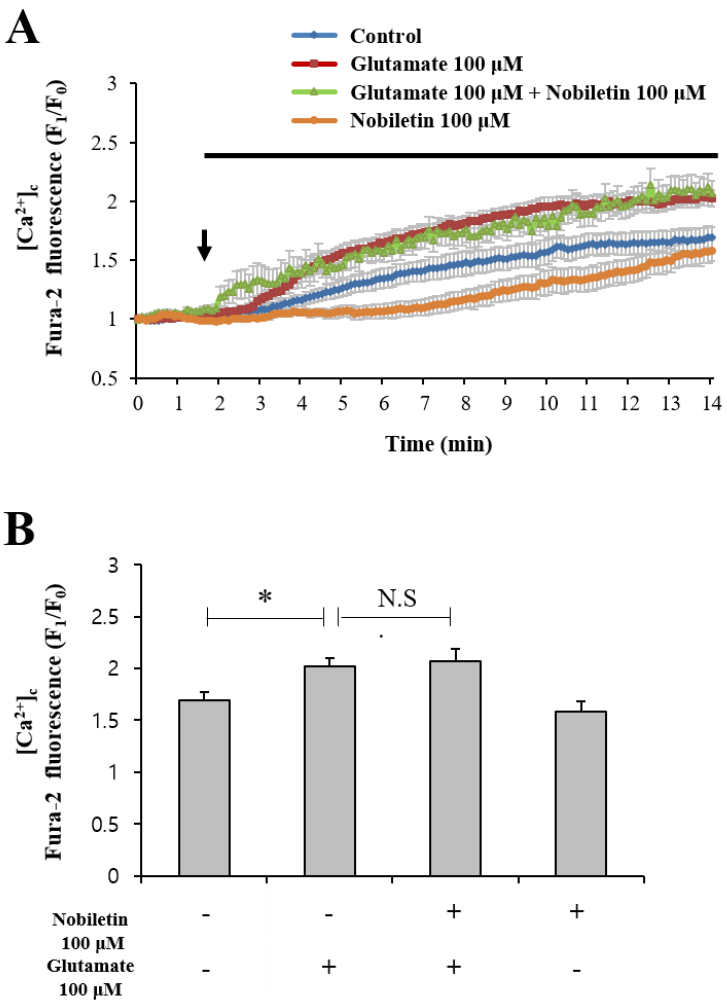


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Figure 4. The effects of nobiletin on glutamate-induced overload of cytosol calcium in primary cortical neurons.

Dual real-time imaging-based fluorometry of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were simultaneously conducted in the same neurons. (A) Fura-2 fluorescence values from individual cells were normalized to values before drug treatment. (B) Quantification of Fura-2 fluorescence values at the end of experiment. Values indicate the mean ± S.E.M. $*p < 0.05$ as compared with the control group compared. N.S., not statistically significant.

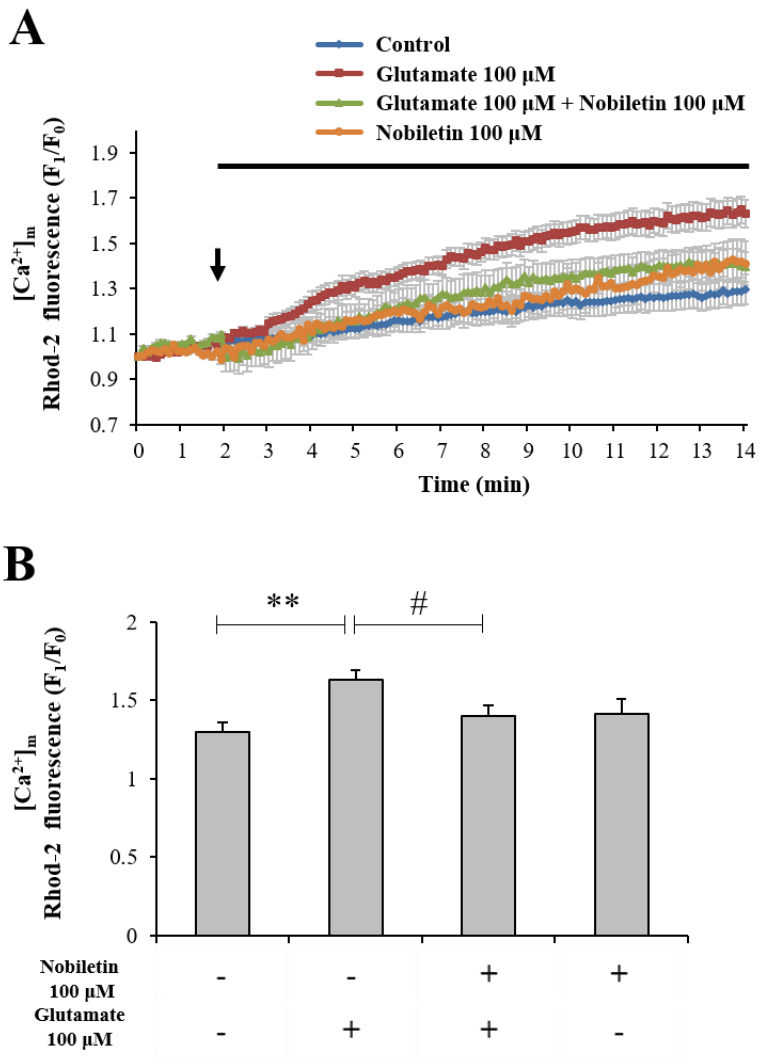

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Figure 5. The effects of nobiletin on glutamate-induced overload of mitochondrial calcium overload in primary cortical neurons.

(A) Rohd-2 fluorescence values from individual cells were normalized to values before drug treatment. (B) Quantification of Rohd-2 fluorescence values at the end of experiment. Values indicate the mean ± S.E.M. $^{**}p < 0.01$ as compared with the control group and $^{\#}p < 0.05$ as compared with glutamate alone-treated group.

3.3. Establishment of isolated mitochondrial model

The concentration of K^+ in the neuronal cytoplasm is about 140 mM, which is among the highest among intracellular ions of the resting neuron. It has been demonstrated that $_{mito}BK_{Ca}$ and $_{mito}K_{ATP}$ channel exist in mitochondria (Szewczyk et al., 2009). For this reason, I hypothesized that mitochondrial depolarization by nobiletin will be related to the influx of K^+ into mitochondria. To make sure the relationship between K^+ and nobiletin, isolated mitochondrial model was adopted. First, an experiment was conducted to confirm the physiological function of the isolated mitochondria (Fig. 6). Figure 6.A is the schematic experimental procedure for mitochondria isolation. The fragmented cortex was placed in EGTA-containing isolation buffer (IB) and homogenized. After centrifugation at 600 x g for 10 min, supernatant was transferred to a new micro-tube. This process was repeated twice. The supernatant was re-centrifuged at 12,000 x g for 10 min. The settled pellets were suspended in EGTA-free IB buffer and homogenized with dounce-type glass homogenizer.

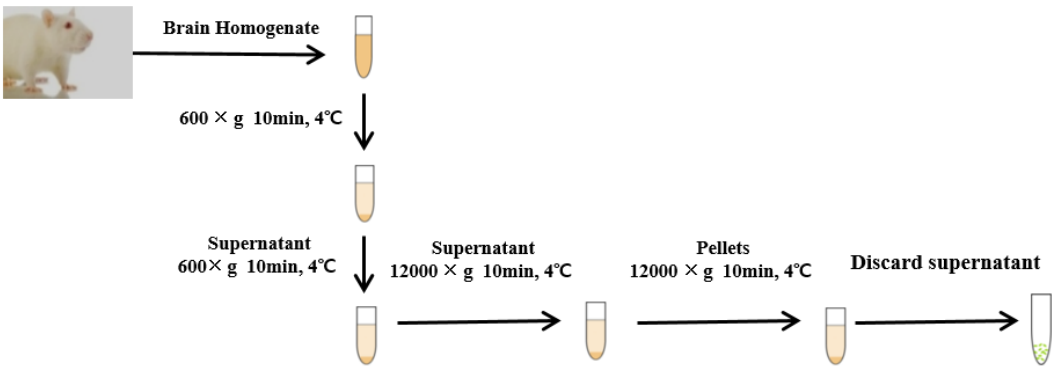
The finally obtained mitochondria are suspended in the recording buffer. In the EM results, the black arrow indicates the mitochondria and the cristae structure in isolated mitochondria is well maintained (Fig. 6B)

Afterwards, the oxygen consumption rate (OCR) was measured to test the physiological activity of isolated mitochondria (Fig. 6C). Treatment of oligomycin, an inhibitor of mitochondrial ATP synthase, reduced OCR. FCCP is generally used as protonophore and uncoupler of oxidative phosphorylation. When treated with FCCP, the OCR was increased to compensate for the electrochemical gradient. It is speculated that OCP was not elevated when treated with FCCP compared to ADP treatment because the biochemical activity of isolated mitochondria was decreased because the time required for measurement was set too long.

Rotenone and antimycin A are inhibitors of complex I and III, respectively. These reagents completely reduced the OCR due to the shutting down of the electron transport chains.

These results suggest that isolated mitochondria maintain the function of the electron transport system at least during the experiment and that the basal $\Delta\Psi_m$ is also normally formed. So isolated mitochondrial model according to the described method is appropriate for physiological experiment.

A



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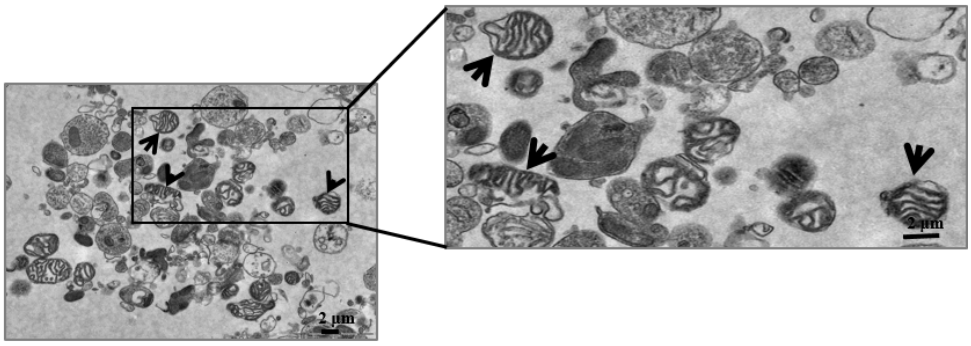

C

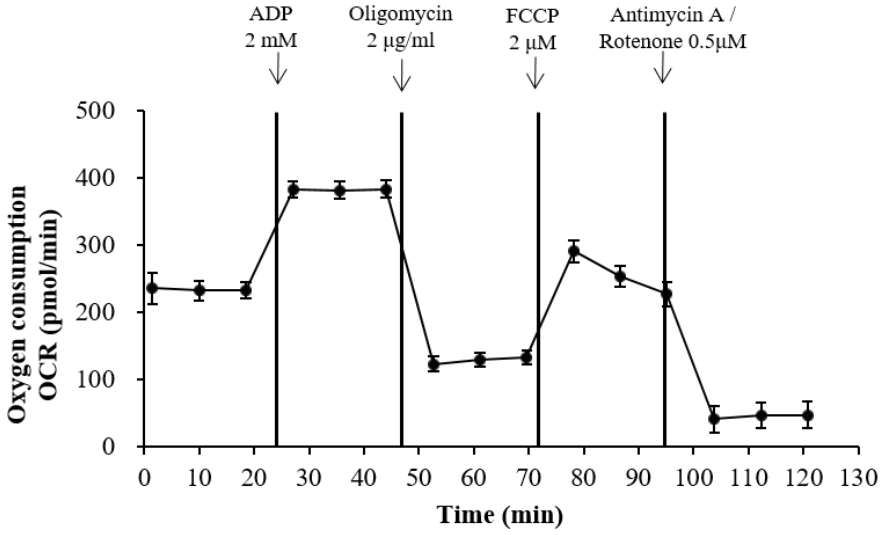

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Figure 6. Identification of physiological activity of isolated brain mitochondria.

(A) Schematic experimental procedure for mitochondria isolation from the cortices of SD rats. The fragmented cortex was homogenized using 15ml dounce-type glass tissue grinder (clearance of pestle; A is 0.0035-0.0065; B is 0.0035-0.0065). The mitochondria pellets obtained after centrifugation at 12000 × g was homogenized using 7ml dounce-type glass homogenizer (clearance of pestle; A is 0.0028-0.0047; B is 0.0008-0.0022). (B) The oxygen consumption rate (OCR) of isolated mitochondria. OCR was measured in the absence of ADP as in the other experimental conditions. Final concentrations of treated reagents were oligomycin 2 μg/ml, FCCP 2 μM, rotenone 2 μM and antimycin A 0.5 μM.

3.4. Nobiletin attenuates glutamate-induced mitochondrial ROS generation

Mitochondrial Ca^{2+} overload can lead to ROS generation and oxidative stress although Ca^{2+} has no direct effect in electron transport chains or oxidation/reduction in mitochondria (Peng et al., 2010). In this regard, I investigated whether nobiletin inhibits glutamate toxicity-induced mitochondrial ROS generation in intact primary cortical neurons. In addition, this issue was explored in isolated brain mitochondria model.

In intact cortical neurons, mitochondrial superoxide levels were recorded and analyzed using real-time imaging-based fluorometry with MitoSOX Red. Glutamate (100 μM) in the presence or absence of nobiletin (100 μM) was superfused over MitoSOX Red-loaded cortical neurons on a cover slip in a recording chamber. Mitochondrial ROS levels from individual cells were normalized to values before drug treatment. Nobiletin almost blocked glutamate-induced mitochondrial ROS in intact cortical neurons (Fig. 7A and B).

High concentration of $CaCl_2$ (5 μM) was treated to isolated mitochondria to mimic glutamate toxicity model in intact cortical neurons. Glutamate treatment did not work in isolated mitochondria since glutamate receptors were expressed in plasma membrane, not in mitochondria membrane. In isolated mitochondrial model as revealed in Fig. 8, high concentration of Ca^{2+} was added in the medium instead of glutamate. Using a fluorescence microplate reader, mitochondrial ROS was measured using DCF-DA. Nobiletin significantly attenuated ROS production in isolated brain mitochondria exposed to high concentration of Ca^{2+} in a dose (30, 50 and 100 μM)-dependent manner (Fig. 8), consistent to ROS data from intact cortical neurons.

If nobiletin has free radical scavenging activity, it becomes difficult to interpret whether the ROS reduction effect is due to $\Delta\Psi_m$ regulation or by its own ROS elimination. Therefore, an

experiment was conducted to investigate the ROS scavenging activity of nobiletin. Data from DPPH free radical scavenging assay revealed that nobiletin did not exhibit the direct free radical-scavenging activity in cell-free and mitochondria-free tube system. N-acetyl cysteine (NAC), a well-known anti-oxidant, was used as a positive control in DPPH assay (Fig. 9A). A large part of the ROS scavenging activity of CPE is due to hesperidin, which is the most abundant compound in CPE. (Fig 9 B and C).

Taken together, results suggest that nobiletin markedly reduces mitochondrial ROS production against glutamate toxicity and mitochondrial calcium overload in intact primary cortical neurons (Fig. 7A and B) and isolated brain mitochondria (Fig. 8) although it does not have free radical scavenging activity (Fig. 9A). Fig.9 implicate that the ROS reduction effect of nobiletin is based on the modulating calcium-induced ROS generation through reducing Ca^{2+} overload into the mitochondria matrix.

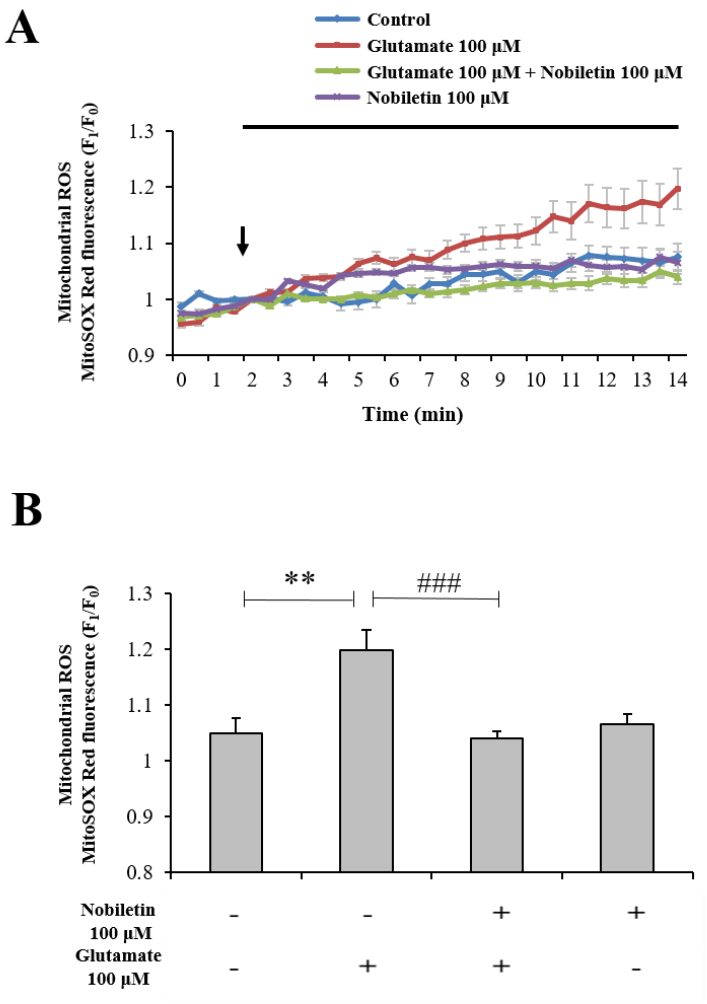

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Figure 7. The effects of nobiletin on mitochondrial ROS generation in glutamate-stimulated cortical neurons.

(A) Recording traces of mitochondrial superoxide using real-time imaging-based fluorometry with MitoSOX Red (See 'Materials and methods' for the detailed description). MitoSOX Red fluorescence values from individual cells were normalized to values before drug treatment shown as an arrow. (B) Quantification of MitoSOX Red fluorescence values at the end of experiment for panel A. Values indicate the mean ± S.E.M. $^{**}P < 0.01$ as compared with untreated controls and, $^{\#\#\#}p<0.001$ as compared with glutamate treated group.

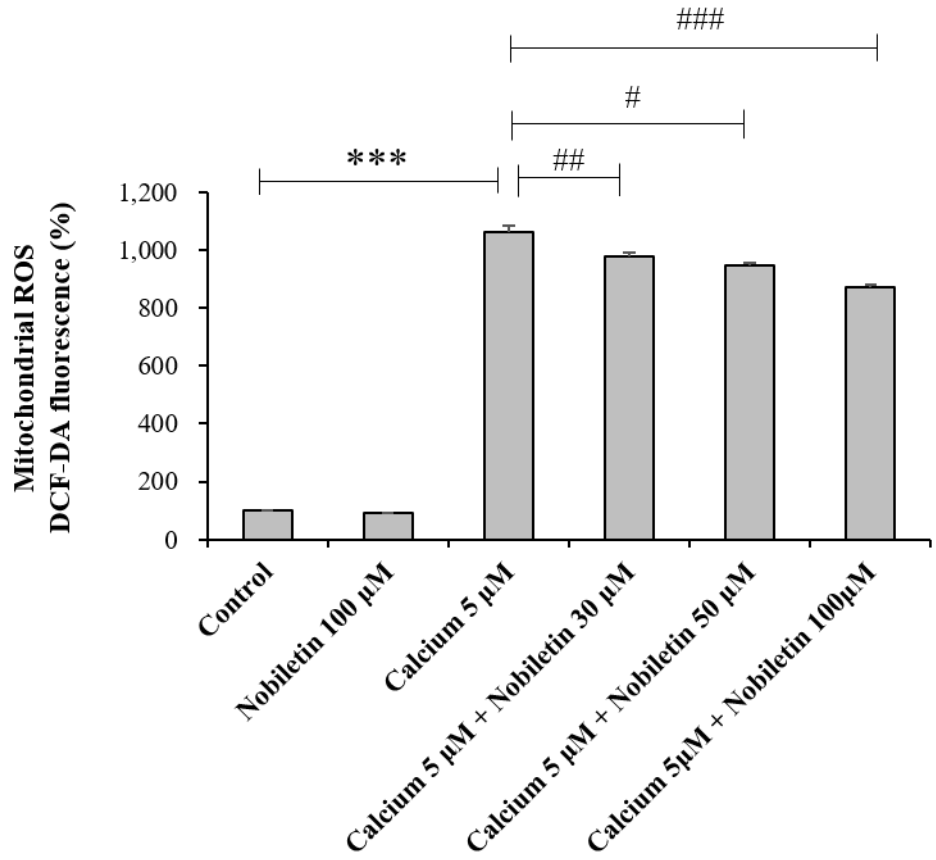

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Figure 8. The effects of nobiletin on mitochondrial ROS generation in isolated brain mitochondria exposed to high concentration of Ca^{2+}.

Effects of nobiletin on mitochondrial ROS generation were measured with DCF-DA indicator using a fluorescence microplate reader in an isolated brain mitochondrial model (See 'Materials and methods' for the detailed description). Values indicate the mean ± S.E.M. ***P < 0.001 as compared with untreated controls and, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ as compared with $CaCl_2$ (5 μM) treated group.

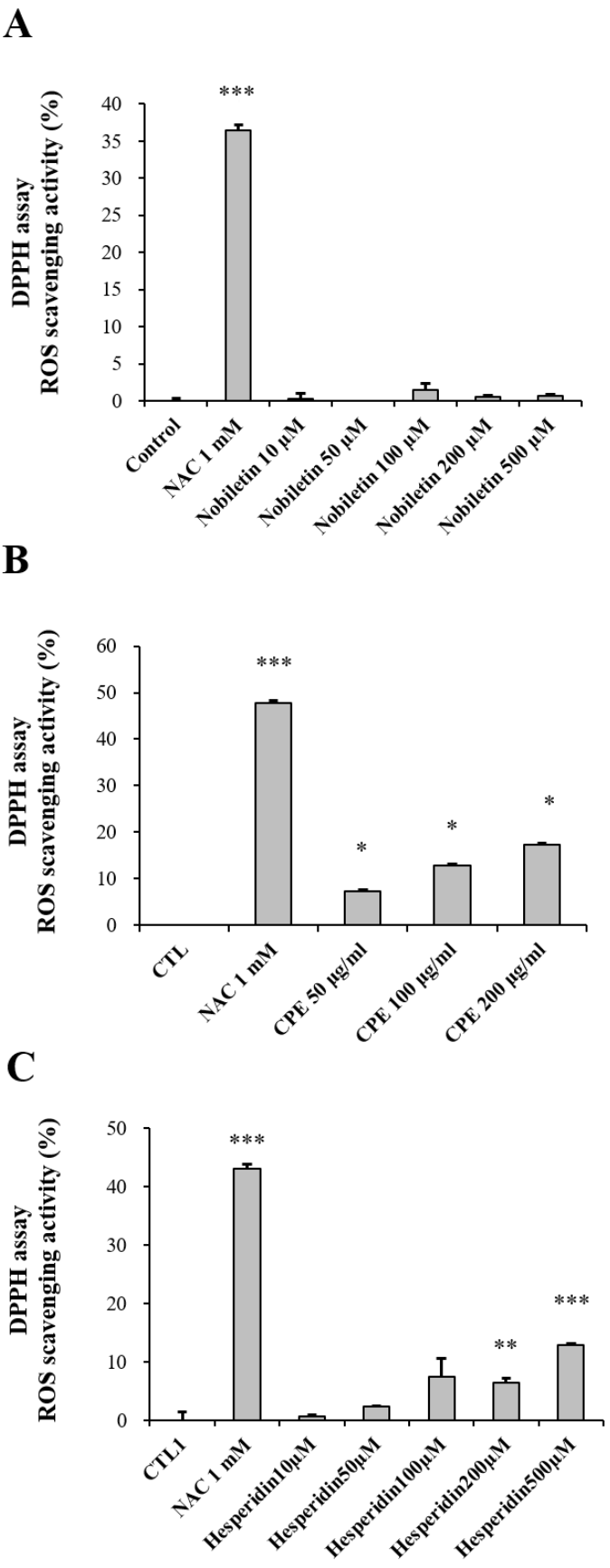

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Figure 9. The comparison of ROS scavenging activities of *Citrus* compounds in cell-free system.

(A) Antioxidant activity in various concentrations of nobiletin was measured using DPPH assay. (B), (C) Free radical scavenging activity of CPE and hesperidin in the cell-free system. Various concentrations of CPE and hesperidin were reacted with the DPPH solution for 1 hr (See 'Materials and methods' for the detailed description). Values indicate the mean ± S.E.M. $*p<0.05$, $**P < 0.01$, $***P < 0.001$ as compared with untreated controls.

3.5. Nobiletin induces partial mitochondrial depolarization through K^+ influx across mitochondrial inner membrane

Influx of K^+ from cytosol into the mitochondrial matrix induces mild uncoupling, resulting in dissipation of $\Delta\Psi_m$ (Ishida *et al*., 2001). K^+ is dominant cations among intracellular ions in resting neurons. Based on this background, I hypothesized that nobiletin-induced mild mitochondrial depolarization is mainly mediated by the influx of K^+ into mitochondria. To confirm the correlation between mitochondrial K^+ influx and nobiletin effect on $\Delta\Psi_m$, a isolated brain mitochondrial model was used. The effect of FCCP on $\Delta\Psi_m$ was investigated as positive control using a fluorescence microplate reader in isolated brain mitochondrial model. When depolarization was induced by FCCP, TMRE intensity increased in dose-dependent manner (Fig. 10A). TMRE, cationic fluorescent dye, is sequestered in mitochondria and it is released by mitochondrial depolarization (Scaduto and Grotyohann,1999). This result was due to the measurement of the total TMRE fluorescence emitted from the mitochondria into the recording buffer in a well. KCl (100 mM) was replaced with CsCl (100 mM) to remove K^+ effects in the medium and thereby K^+ dependence in nobiletin effect on $\Delta\Psi_m$ was investigated. Fig. 10B demonstrated that nobiletin effects on mild mitochondrial depolarization were not shown in the absence of K^+ in the medium when KCl (100 mM) was replaced with CsCl (100 mM) in an isolated mitochondria model. On the other hand, mitochondrial depolarization was induced by nobiletin in a dose-dependent manner in the KCl (100 mM) condition. Fig. 10C shows the difference in the change of $\Delta\Psi_m$ for 100uM nobiletin in environments with or without K^+ in time-dependent manner. In the absence of K^+, depolarization of $\Delta\Psi_m$ was not observed until 15 min.

The $_{mito}BK_{Ca}$ and $_{mito}K_{ATP}$ channel have been well known as mitochondrial K^+ channels. To

investigate the molecular target of nobiletin, I examined whether the membrane depolarization disappeared by using K^+ channel inhibitors. Iberiotoxin 10 nM and 5-HD 500 μM were used to inhibit $_{mito}BK_{Ca}$ and $_{mito}K_{ATP}$ channels, respectively. As a result, Fig. 11A demonstrated that mitochondrial depolarization by CPE was significantly inhibited by 500 μM 5-HD. In Fig. 11B, membrane depolarization by nobiletin was significantly inhibited in the group treated with 10 nM iberiotoxin in primary cortical neurons. In the group treated with 500 μM 5-HD, partial mitochondrial depolarization tended to disappear, but there was no significant difference. The $_{mito}BK_{Ca}$ channels may be closely involved in mitochondrial polarization by nobilrtin, but the association $_{mito}K_{ATP}$ channels can not be ruled out because the depolarization of CPE was disappeared by 5-HD. Results suggest that nobiletin induces partial membrane depolarization by promoting influx of K^+ across mitochondrial inner membrane. K^+ influx into mitochondrial matrix by nobiletin is probably mediated by mitochondrial K^+ channels.

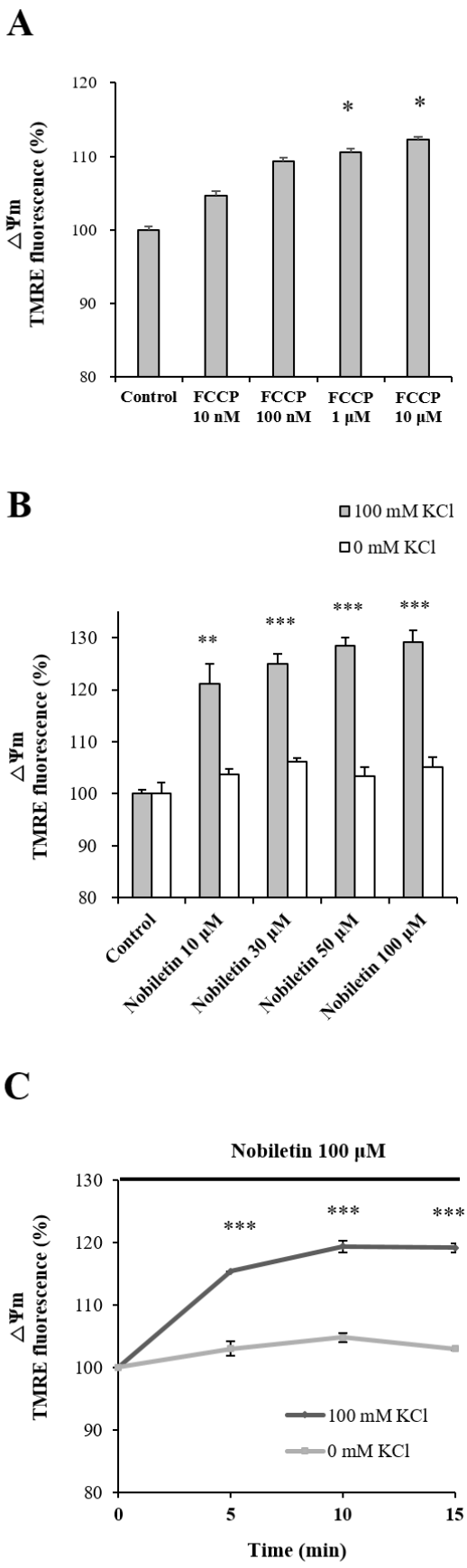

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Figure 10. The effects of K^+ influx on nobiletin-induced partial mitochondrial depolarization in isolated brain mitochondria.

(A) The effect of FCCP on $\Delta\Psi_m$ was investigated with TMRE in an isolated brain mitochondrial model (See 'Materials and methods' for the detailed description) using a fluorescence microplate reader, as a positive control. (B and C). The effect of nobiletin on $\Delta\Psi_m$ was measured with TMRE in the presence or absence of K^+ in the medium using a fluorescence microplate reader in an isolated brain mitochondrial model. To remove K^+ in the medium, KCl (100 mM) was replaced to CsCl (100 mM). Dose responses 10 min after nobiletin treatment (B) and time courses (C) were analyzed. Values indicate the mean ± S.E.M. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with the control group.

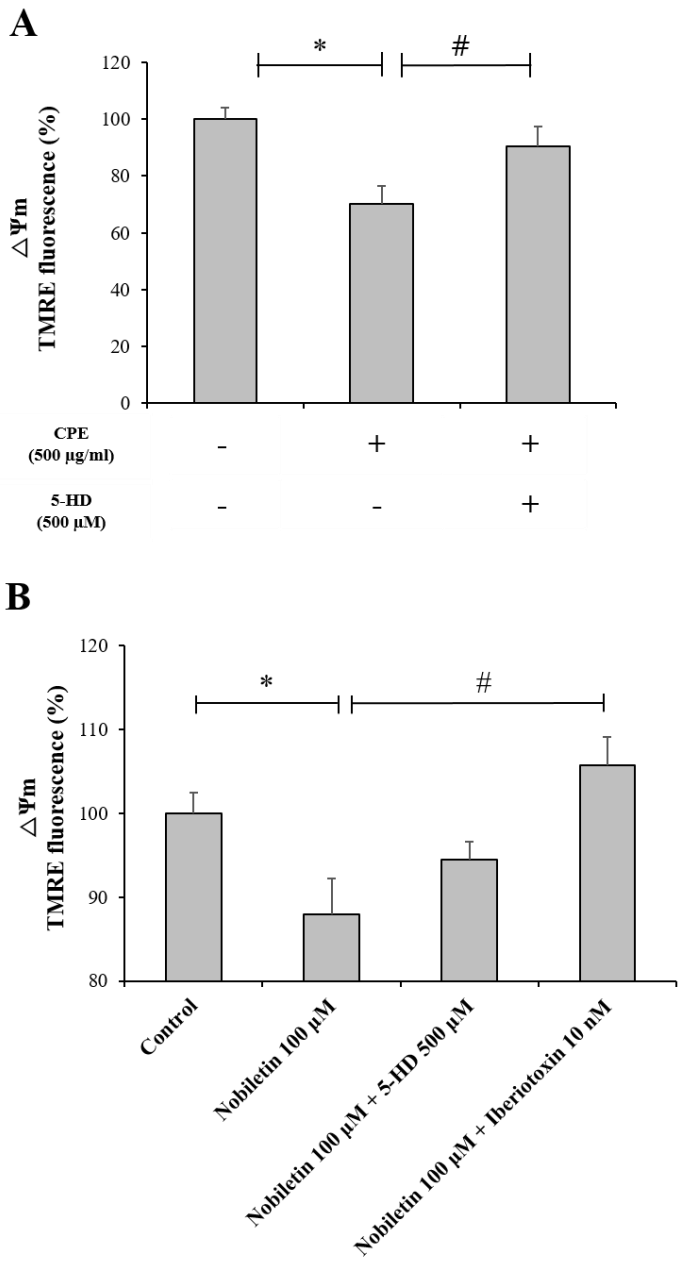

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Figure 11. The effects of mitochondrial K^+ channel inhibitors on nobiletin- induced $\Delta\Psi_m$ changes in primary cortical neurons.

(A) CPE Nobiletin was superfused with 500μM 5-HD in a recording chamber (B) Nobiletin was superfused with 10 nM iberiotoxin and 500μM 5-HD in a recording chamber, TMRE fluorescence values from individual cells were normalized to the value at starting point of drug

treatment. Quantification of TMRE fluorescence at the end of the measurement. Values indicate the mean ± S.E.M. *$p < 0.05$ as compared with untreated controls and, $^{\#}p<0.05$, as compared with nobiletin (100 μM) treated group.

3.6. Rotenone attenuate the effect of nobiletin to reducing mitochondrial calcium in glutamate toxicity model

Some studies (Sherer *et al*., 2003) have used a rotenone-induced Parkinson's model which was involved in oxidative damage of rotenone. Nobiletin has beneficial effects in improving cognitive function or motor deficits in Parkinson's diseases (Yabuki *et al*., 2014). The main source of intracellular ROS is the mitochondrial electron transport system. To investigate the possibility of nobiletin to interact with the mitochondrial electron transport system, I hypothesized that nobiletin stimulates the function of complex I as an antagonist to rotenone. To determine the effect of rotenone on $\triangle\Psi_m$, $\triangle\Psi_m$ was observed using real-time image based recording when rotenone (50 μM) and nobiletin (100 μM) was added to neuronal cells in primary neuron culture system (Fig. 12 A and B). The mitochondrial depolarization was induced more significantly in rotenone and nobiletin co-treated group. Rotenone induced mitochondrial depolarization like nobiletin but showed no significance compared with the control group. The TMRE fluorescence values in rotenone 50 μM group and rotenone 50 μM + nobiletin 100 μM group at 15 minutes were 50.36 ± 17.73, and 43.97 ± 6.18 % respectively (Fig. 12 B).

$[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were measured simultaneously in living individual neurons. Because nobiletin reduces mitochondrial calcium overload induced by glutamate, the $[Ca^{2+}]_c$ increases and the $[Ca^{2+}]_m$ decreases. However, these nobiletin effects were disappeared by rotenone, so, $[Ca^{2+}]_m$ increased and $[Ca^{2+}]_c$ decreased (Fig. 13A and C). The Fura-2 fluorescence values at the end of the measurement were 114.92 ± 5.39 %, 132.29 ± 9.64 %, 109.53 ± 10.82 % and 86.30 ± 14.70 % compared with control group respectively (Fig. 13C). The mitochondrial calcium reduced by nobiletin was increased to 146.31 ± 3.12 % by rotenone (fig. 13D).

These results show that although nobiletin does not affect $\triangle\Psi_m$ induced by inhibition of complex I, nobiletin effects on $[Ca^{2+}]_m$ were inhibited by retenone.

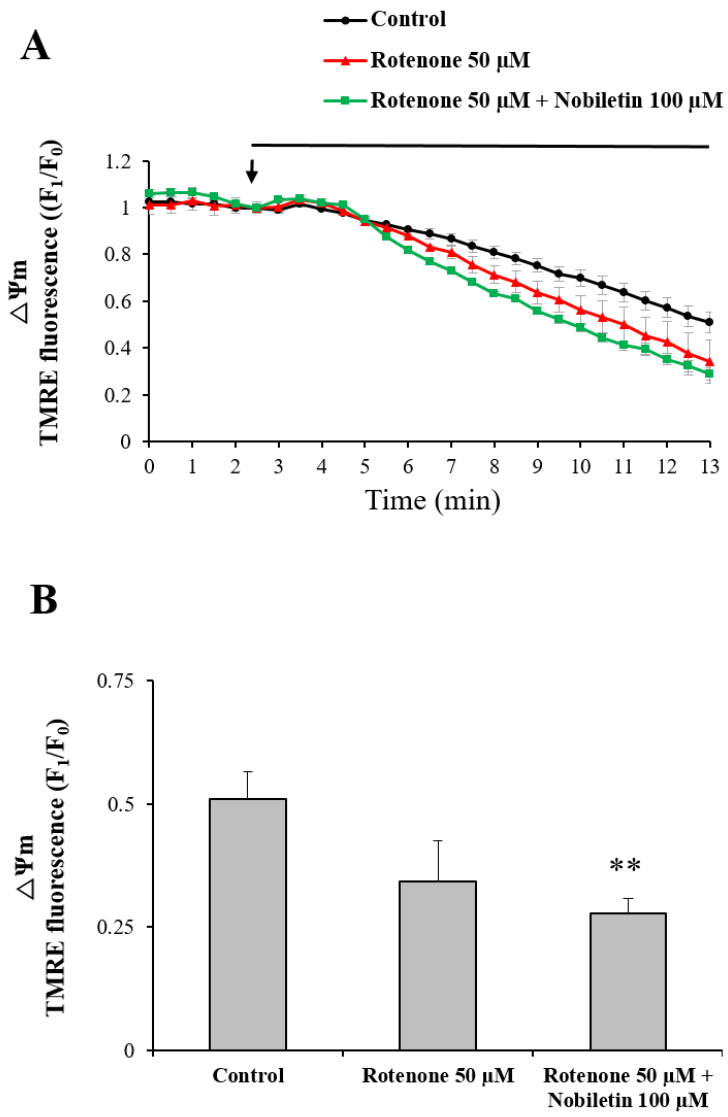

Figure 12. The effects of rotenone, a complex I inhibitor, on mitochondrial membrane potential.

(A) Effects of rotenone and nobiletin on mitochondrial membrane potential. Rotenone 50 μM and nobiletin 100 μM were superfused in a recording chamber, TMRE fluorescence values from individual cells were normalized to the value at starting point of drug treatment. (B) Quantification of TMRE fluorescence at 15 min time point was compared with among different group. Values indicate the mean ± S.E.M. $^{**}p < 0.01$ as compared with untreated controls.

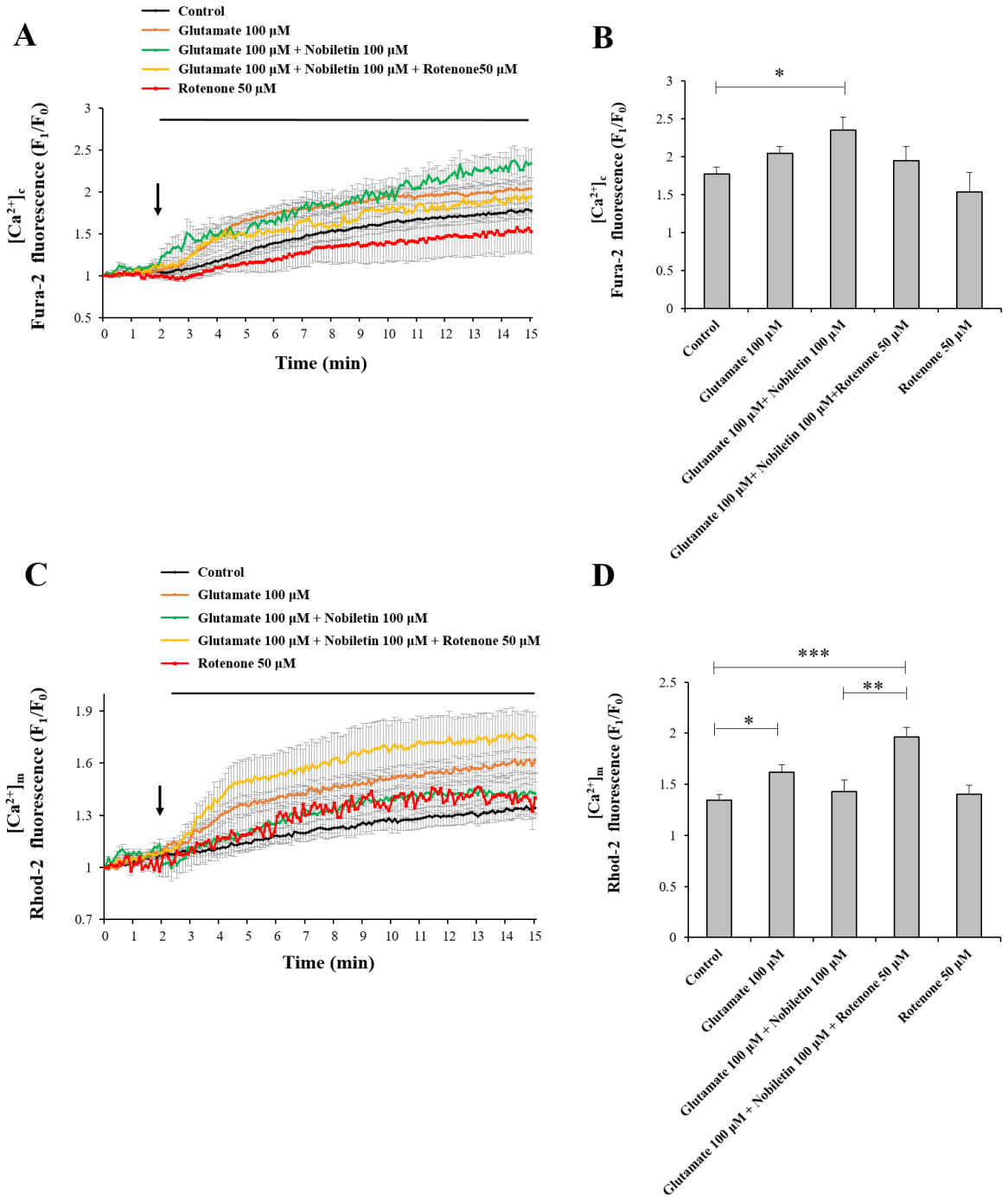

Figure 13. Rotenone inhibits the action of nobiletin, which attenuate mitochondrial calcium overload in the glutamate toxicity model.

(A and C) Dual real-time imaging-based recording of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were simultaneously measured in the same neurons. Nobiletin (100 μM), glutamate (100 μM) and rotenone (50 μM)

were superfused in a recording chamber, Fura-2 and Rhod-2 fluorescence values from individual cells were normalized to the value at starting point of baseline measurement. (B and D) Quantification of fluorescence values of Fura-2 and Rhod-2 from individual cells at the end of the measurement (15 min). Values indicate the mean ± S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3.7. Rotenone-induced mitochondrial ROS was reduced by nobiletin

It is already demonstrated that when complex I is inhibited by rotenone, ROS production is increased and apoptosis is induced. If nobiletin is antagonistic to rotenone, it may reduce ROS production caused by the inhibition of complex I. Mitochondrial ROS was measured using MitoSOX Red, mitochondrial superoxide indicator, under a real-time imaging-based recording system. When treated with rotenone alone, ROS increased to 131.83 ± 5.34 % compared to the control group. In the group treated with 50 μM rotenone and 100 μM nobiletin at the same time, ROS was decreased to 105.21 ± 3.28 % similar to the control group (Fig. 14. A and B). When 50 μM nobiletin was co-treated with rotenone, the effect of ROS generation was attenuated but no significant difference was observed compared with the rotenone alone group (Fig. 14.C and D). The MitoSOX Red fluorescence values in 50 μM rotenone group, rotenone 50 μM + nobiletin 100 μM group and nobiletin 100 μM group at the end of the measurement were 118.97 ± 3.36 %, 87.91 ± 8.00 % and 93.23 ± 7.46 % respectively. Taken together, there is a possibility that nobiletin inhibit rotenone action or promote the function of complex I because nobiletin reduced ROS from the electron transport system when complex I was inhibited. However, additional studies should be performed to determine the exact mechanism of action between nobiletin and complex I.

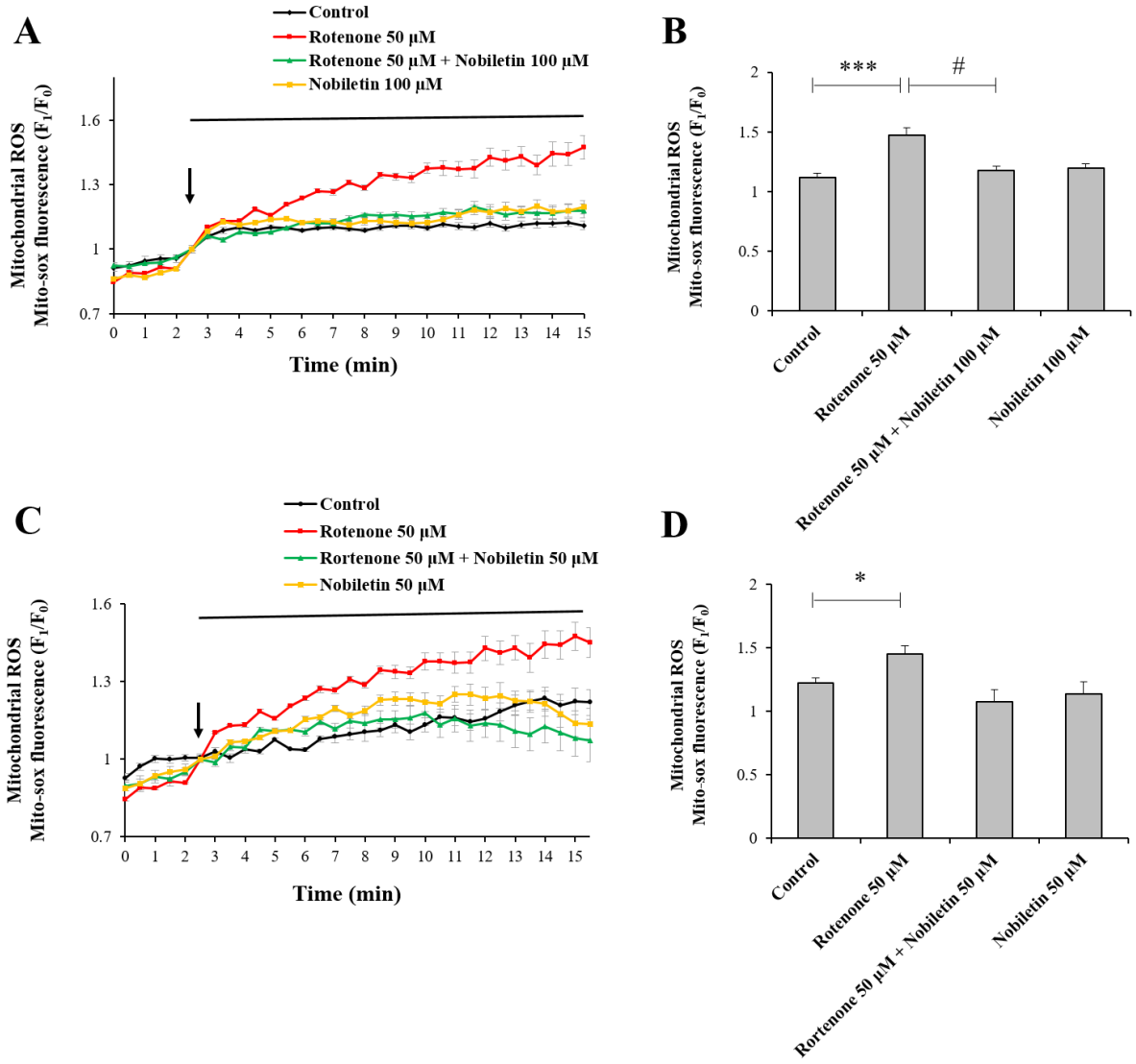

Figure 14. The effects of nobiletin on rotenone-induced mitochondrial ROS in primary cortical neurons.

(A) After MitoSOX Red (5 μM) was loaded for 15 min, base line was measured for 2 min. Nobiletin (100 μM), and rotenone (50 μM) were superfused in a recording chamber from 2min to the end of the measurement. MitoSOX Red fluorescence values from individual cells were normalized to the value at starting point of drug treatment. (B) Quantification of MitoSOX Red fluorescence at the end of the measurement, 15 min time point, was compared with among

different group. (C) Antioxidant activity of nobiletin (50 μM) in primary cortical neurons. (D) Quantification of MitoSOX Red fluorescence at the end of the measurement, 15 min time point. Nobiletin 50 μM also decreased ROS induced by rotenone, but no significant difference compared with rotenone alone treatment. Values indicate the mean ± S.E.M. *p<0.05, ***P < 0.001 as compared with untreated controls and, $^{\#}p$<0.05 as compared with rotenone 50 μM alone.

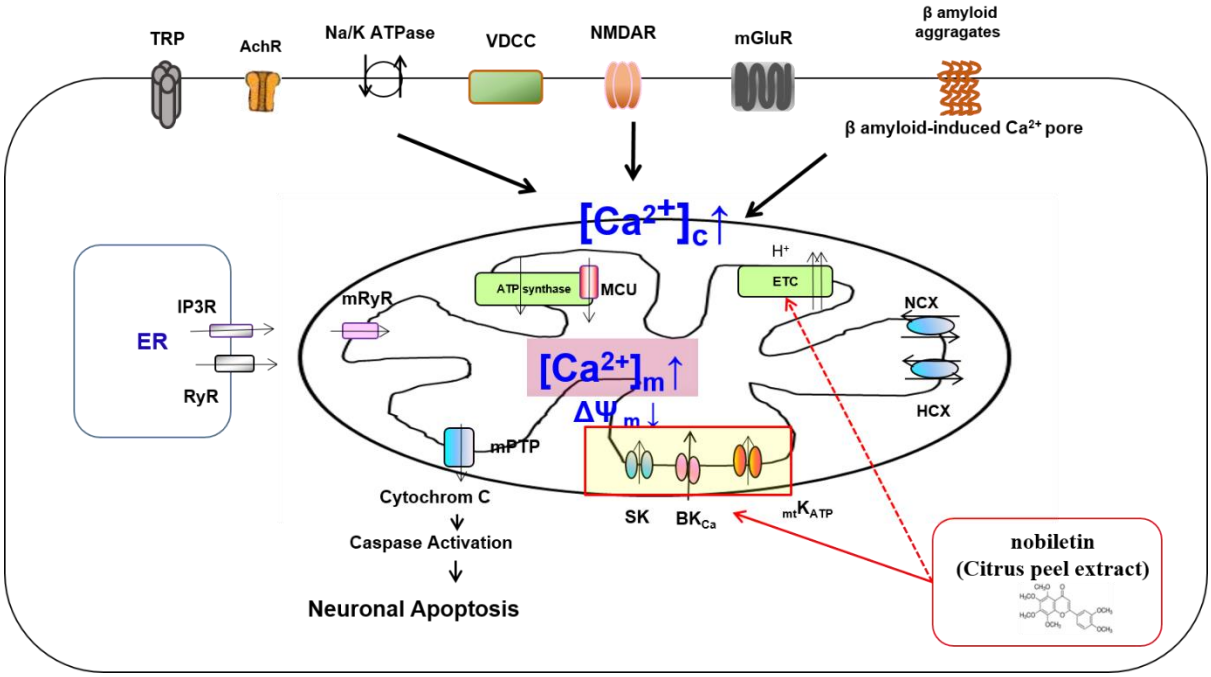

Figure 15. Schematic neuroprotective mechanism of nobiletin through mitochondrial depolarization.

Nobiletin induced mild mitochondrial membrane depolarization. This mild mitochondrial depolarization is based on regulation of K^+ channels. The blockade of mitochondrial Ca^{2+} overload via depolarization of mitochondrial membrane plays a critical role in the mechanism underlying neuroprotective effects of nobiletin. Nobiletin markedly reduce electron transport chain complex I inhibitor rotenone-induced mitochondrial ROS generation.

4. Discussion

In this study, I demonstrated in primary cultured cortical neurons and brain mitochondria isolated from rat cortices that nobiletin prevents neurotoxic mitochondrial calcium overload, excess mitochondrial ROS, and neuronal cell death through $\Delta\Psi_m$ regulation. Results demonstrated that nobiletin evoked partial mitochondrial depolarization in intact cortical neurons (Fig. 2A and B) and isolated brain mitochondria (Fig. 10B and C). Nobiletin markedly attenuated mitochondrial calcium overload and ROS generation in glutamate (100 μM)-stimulated cortical neurons (Fig. 5 and Fig. 7) and isolated brain mitochondria exposed to a high concentration of Ca^{2+} (5 μM) (Fig. 8). In neuronal cells, NMDA and AMPA receptors in cell membranes are activated by excess glutamate. This glutamate receptor activation contributes to calcium overload into the cytosol and collapse of the mitochondrial membrane potential. In particular, the accumulation of calcium in the mitochondria is an important step in the progression to cell apoptosis, promoting the loss of $\Delta\Psi_m$, and the collapse of apoptotic cell death (Abramov *et al*., 2008). Mitochondria take up Ca^{2+} electrophoretically from the cytosol through a mitochondrial calcium uniporter (MCU). Mitochondrial Ca^{2+} influx is rapidly induced by the mitochondrial membrane potential, whereas its release in exchange for protons or sodium is electroneutral. Therefore, treatment with high concentrations of glutamate results in the influx of calcium into the cytoplasm, as shown in Fig. 5, and this calcium is accumulated in the mitochondrial matrix. Fig. 2 and 5 suggest that nobiletin induces partial depolarization, which does not interfere with the normal physiological function of the cell, thereby reducing the electrophoretic force that influxes Ca^{2+} into the mitochondria..

Excess exposure to glutamate markedly evoked overload of both mitochondrial Ca^{2+} and ROS. Nobiletin treatment significantly reduced these two reciprocal parameters, as shown in Fig. 5 and 7. The exact mechanism by which mitochondrial Ca^{2+} stimulates ROS generation

inside mitochondria remains elusive. However, several plausible mechanisms have been proposed. Possible mechanisms include mitochondrial Ca^{2+}-induced increase of the metabolic rate, mitochondrial Ca^{2+}-stimulated dissociation of cytochrome *c*, and mitochondrial Ca^{2+}-stimulated opening of the mitochondrial permeability transition pore with cytochrome *c* release (Peng and Jou, 2010). Mitochondrial ROS can inversely affect Ca^{2+} dynamics and modulate Ca^{2+} surge. The reciprocal crosstalk between mitochondrial Ca^{2+} and ROS may result in a feedforward, self-amplified loop evoking subsequent cellular damage and death (Feissner *et al*., 2009).

The main finding in this study may be the regulation of mitochondrial K^+ influx by nobiletin. I demonstrated that the neuroprotective effect of nobiletin via mild mitochondrial depolarization is largely mediated by the influx of K^+ into mitochondria (Fig. 10B and C). Nobiletin effects on basal $\Delta\Psi_m$ were completely abolished in brain mitochondria isolated from rat cortices that were maintained in K^+-free medium. Results suggest that K^+ influx into the mitochondrial matrix is critically involved in the nobiletin effect on $\Delta\Psi_m$. The mitochondrial K^+ influx is likely mediated, at least in part, by the activation of mitochondrial K^+ channels. However, further detailed studies should be conducted to determine the exact molecular targets of nobiletin in mitochondria.

There are several ion channels/transporters and electron transport chains on the inner membrane of mitochondria, which are proposed as potential molecular targets of nobiletin in mitochondria. It does not seem that MCU, the major route for mitochondrial Ca^{2+} uptake, is involved in nobiletin-induced $\Delta\Psi_m$ depolarization. If nobiletin might attenuate glutamate-induced mitochondrial Ca^{2+} overload through inhibition of MCU, it should hyperpolarize the mitochondrial membrane rather than depolarize it. Several studies (Szewczyk *et al*., 2009) have revealed that K^+ channels/transporters are present in the mitochondrial inner membrane: $_{mito}K_{ATP}$, $_{mito}BK_{Ca}$, voltage-gated potassium channel Kv1.3, twin-pore domain TASK-3

potassium channels, and K^+/H^+ exchangers. The $_{mito}BK_{Ca}$ and $_{mito}K_{ATP}$ channels are major mitochondrial K^+ channels. It was demonstrated using brain mitochondria isolated from rat cortices that the neuroprotective effect of nobiletin via mild mitochondrial depolarization is largely mediated by the influx of K^+ into mitochondria (Fig. 10B and C). These results suggest that nobiletin-induced mitochondrial K^+ influx is likely mediated, at least in part, by the activation of mitochondrial K^+ channels. Based on this idea, I further explored mitochondrial targets of nobiletin. Some preliminary findings related to $_{mito}BK_{Ca}$ and $_{mito}K_{ATP}$ are shown in Fig. 11. I investigated whether nobiletin-induced $\Delta\Psi_m$ depolarization is blocked by iberiotoxin and 5-HD as they are well-known inhibitors of $_{mito}BK_{Ca}$ and $_{mito}K_{ATP}$ channels, respectively. As shown in Fig. 11, preliminary findings indicated that nobiletin-induced $\Delta\Psi_m$ depolarization was significantly inhibited in the group treated with iberiotoxin (10 nM). These results suggest that nobiletin may induce the neuroprotective $\Delta\Psi_m$ depolarization by promoting the influx of K^+ into mitochondria through the activation of $_{mito}BK_{Ca}$. However, further detailed studies should be conducted to determine the exact molecular targets of nobiletin in mitochondria.

Among flavonoids, naringenin is one of the flavanones abundant in the genus *Citrus,* such as in grapefruit and orange; it has been widely studied recently. The activation of $_{mito}BK_{Ca}$ is involved in a cardioprotective mechanism of naringenin against myocardial ischemia/reperfusion (Testai *et al*., 2013). Naringenin is proposed as one of the BK_{Ca} channel openers in vascular smooth muscle cells (Saponara *et al*., 2006). An electrophysiological study recently revealed that channel activities of $_{mito}K_{ATP}$ and $_{mito}BK_{Ca}$ were enhanced after treatment with 10 μM of naringenin in a single channel study using mitoplasts isolated from primary human dermal fibroblast cells (Bednarczyk *et al*., 2017). These recent reports focus special attention on the intracellular pathways mediated by *Citrus* flavonoids regarding their beneficial effects on several physiological and pathophysiological conditions.

In Figures 12, 13, and 14, I demonstrated the relationship between nobiletin and electron transport chain complex I, which was inhibited by rotenone. These results suggest an antagonistic relationship between nobiletin and rotenone in the regulation of $[Ca^{2+}]_m$ and ROS, but their relationship does not seem to be directly related to the regulation of $\Delta\Psi_m$. Further studies should be conducted to obtain more precise information on the interaction between nobiletin and complex I.

In conclusion, I reveal here that nobiletin evokes partial mitochondrial depolarization in intact cortical neurons as well as isolated brain mitochondria, and thereby prevents neurotoxic mitochondrial calcium overload, excess mitochondrial ROS, and neuronal cell death. Furthermore, K^+ influx into the mitochondrial matrix is critically involved in the nobiletin effect on $\Delta\Psi_m$. In addition, results suggest that the mitochondrial K^+ influx is likely mediated, at least in part, by the activation of mitochondrial K^+ channels. All these findings reveal a beneficial role for nobiletin-induced partial mitochondrial depolarization in neuroprotection, which is similar to the ischemic pre-conditioning (IPC) in the endogenous mechanism of cardioprotection. Therefore, pharmacological manipulation of $\Delta\Psi_m$ through novel substances (i.e., nobiletin) could be a promising strategy to prevent neuronal cell death during brain insults.

References

1. **Abramov AY, Duchen MR. 2008**. Mechanisms underlying the loss of mitochondrial membrane potential in glutamate excitotoxicity. Biochim. Biophys. Acta. 1777 : 953–964

2. **Bednarczyk P, Kicinska A, Jarmuszkiewicz W, Debowska R, Szewczyk A. 2017.** Flavonoids as natural modulators of mitochondrial potassium channel. Biophys. J. 112:405a-406a.

3. **Blattner JR, He L, Lemasters JJ. 2001.** Screening assays for the mitochondrial permeability transition using a fluorescence multiwell plate reader, Anal. Biochem. 295:220-226.

4. **Choi SY, Kim JY, Jo Y, Kim HD, Song M, Moon C, Kim CH, Kim K, Sesaki H, Rhyu IJ, Kim H, Sun W. 2016.** Constriction of the mitochondrial inner compartment is a priming event for mitochondrial division. Nat. Comm. 8:15754-15770.

5. **Choi SY, Hwang JH, Ko HC, Park JG, Kim SJ. 2007.** Nobiletin from citrus fruit peel inhibits the DNA-binding activity of NF-kappaB and ROS production in LPS-activated RAW 264.7 cells. J. Ethnopharmacol. 113:149-155.

6. **Cui Y, Wu J, Jung SC, Park DB, Maeng YH, Hong JY, Kim SJ, Lee SR, Kim SJ, Kim SJ, Eun SY. 2010.** Anti-neuroinflammatory activity of nobiletin on suppression of microglial activation. Biol. Pharm. Bull. 33:1814-1821.

7. **Cui Y, Park JY, Wu J, Lee JH, Yang YS, Kang MS, Jung SC, Park JM, Yoo ES, Kim SH, Ahn JS, Suk K, Eun SY. 2015.** Dieckol Attenuates Microglia-mediated Neuronal Cell Death via ERK, Akt and NADPH Oxidase-mediated Pathways. Korean J. Physiol. Pharmacol. 19:219-228.

8. **Duchen MR. 2012.** Mitochondria, calcium-dependent neuronal death and neurodegenerative disease. Pflugers. Arch. 464:111–121.

9. **Eun SY, Jung SJ, Park YK, Kwak J, Kim SJ, Kim J. 2001.** Effects of capsaicin on Ca^{2+} release from the intracellular Ca^{2+} stores in the dorsal root ganglion cells of adult rats. Biochem. Biophy. Res. Commun. 285:1114-1120.

10. **Feissner RF, Skalska J, Gaum WE, Sheu SS. 2009.** Crosstalk signaling between mitochondrial Ca^{2+} and ROS. Front. Biosci. 14:1197-1218.

11. **Gunter TE, Pfeiffer DR. 1990.** Mechanisms by which mitochondria transport calcium. Am. J. Physiol. Cell. Physiol. 258:C755-C786.

12. **Iglesias-González J, Sánchez-Iglesias S, Beiras-Iglesias A, Soto-Otero R, Méndez-Álvarez E. 2013.** A simple method for isolating rat brain mitochondria with high metabolic activity: Effects of EDTA and EGTA. J. Neurosci. Methods. 213: 39– 42.

13. **Ishida H, Hirota Y, Genka C, Nakazawa H, Nakaya H, Sato T. 2001.** Opening of mitochondrial K_{ATP} channels attenuates the ouabain-induced calcium overload in mitochondria. Circ. Res. 89:856-858.

14. **Jousset H, Malli R, Girardin N, Graier W.F, Demaurex N, Frieden M. 2008**. Evidence for a receptor-activated Ca^{2+} entry pathway independent from Ca^{2+} store depletion in endothelial cells. Cell Calcium. 43:83–94.

15. **Kroemer G, Galluzzi L, Brenner C. 2007.** Mitochondrial membrane permeabilization in cell Death. Physiol. Rev. 87:99-163.

16. **Lee JH, Amarsanaa1 K, Wu JJ, Sang-Chan Jeon SC, Cui YJ, Jung SC6, Park BD, Kim SJ, Han SH, Kim HW, Rhyu IJ, Eun SY.** 2018. Nobiletin attenuates neurotoxic mitochondrial calcium overload through K+ influx and $\Delta\Psi_m$ across mitochondrial inner membrane. Korean J. Physiol. Pharmacol. 22(3):311-319.

17. **Nagase H, Omae N, Omori A, Nakagawasai O, Tadano T, Yokosuka A, Sashida Y, Mimaki Y, Yamakuni T, Ohizumi Y. 2005.** Nobiletin and its related flavonoids with CRE-dependent transcription-stimulating and neuritegenic activities, Biochem. Biophy. Res. Commun. 337:1330-1336.

18. **Onozuka H, Nakajima A, Matsuzaki K, Shin RW, Ogino K, Saigusa D, Tetsu N, Yokosuka A, Sashida Y, Mimaki Y, Yamakuni T, Ohizumi Y. 2008.** Nobiletin, a citrus flavonoid, improves memory impairment and Aβ pathology in a transgenic mouse model of Alzheimer's disease. J. Pharmacol. Exp. Ther. 326:739-744.

19. **Peng TI, Jou MJ. 2010.** Oxidative stress caused by mitochondrial calcium overload. Ann. N. Y. Acad. Sci. 1201:183–188.

20. **Sanz-Blasco S, Valero RA, Rodríguez-Crespo I, Villalobos C, Núñez L. 2008.** Mitochondrial Ca^{2+} overload underlies Aβ oligomers neurotoxicity providing an unexpected mechanism of neuroprotection by NSAIDs. PLoS One. 3:e2718.

21. **Saponara S, Testai L, Iozzi D, Martinotti E, Martelli A, Chericoni S, Sgaragli G, Fusi F, Calderone V. 2006.** (+/-)-Naringenin as large conductance Ca^{2+}-activated K^+ (BK_{ca}) channel opener in vascular smooth muscle cells. Br. J. Pharmacol. 149:1013-1021.

22. **Scaduto RC Jr, Grotyohann LW. 1999.** Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys. J. 76:469-477.

23. **Sherer TB, Betarbet R, Testa CM, Seo BB, Richardson JR, Kim JH, Miller GW, Yagi T, Matsuno-Yagi A, Greenamyre JT. 2003.** Mechanism of toxicity in rotenone models of Parkinson's disease. J. Neurosci. 23(34):10756– 10764.

24. **Szewczyk A, Jarmuszkiewicz W, Kunz WS. 2009.** Mitochondrial potassium channels. IUBMB Life. 61:134–143.

25. **Testai L, Martelli A, Marino A, D'Antongiovanni V, Ciregia F, Giusti L, Lucacchini A, Chericoni S, Breschi MC, Calderone V. 2013.** The activation of mitochondrial BK potassium channels contributes to the protective effects of naringenin against myocardial ischemia/reperfusion injury. Biochem. Pharmacol. 85:1634-1643.

26. **Valero RA, Senovilla L, Núñez L, Villalobos C. 2008.** The role of mitochondrial potential in control of calcium signals involved in cell proliferation. Cell Calcium. 44:259–269.

27. **Wu J, Cui Y, Yang YS, Jung SC, Hyun JW, Maeng YH, Park DB, Lee SR, Kim SJ, Eun SY. 2013.** Mild Mitochondrial Depolarization is Involved in a Neuroprotective mechanism of citrus sunki peel extract. Phytother. Res. 27:564–571.

28. **Yabuki Y, Ohizumi Y, Yokosuka A, Mimaki Y, Fukunaga K. 2014.** Nobiletin treatment improves motor and cognitive deficits seen in MPTP-induced Parkinson model mice. Neuroscience. 259:126-141.

29. **Yamamoto Y, Shioda N, Han F, Moriguchi S, Nakajima A, Yokosuka A, Mimaki Y, Sashida Y, Yamakuni T, Ohizumi Y, Fukunaga K. 2009.** Nobiletin improves brain ischemia-induced learning and memory deficits through stimulation of CaMKII and CREB phosphorylation. Brain Res. 129:218-229.

국문 초록

미토콘드리아로의 과도한 칼슘 유입은 신경 퇴행성 질환의 발병 기전에서 중요한 신경세포 생존과 사멸을 결정하는 데 결정적인 요소이며 이 과정은 다양한 채널, 역방향 수송체와 펌프뿐만 아니라 미토콘드리아 내막의 전자전달사슬이 조절한다. 미토콘드리아 내로 칼슘 유입을 일으키는 원동력으로 두 가지가 있는데 하나는 세포질과 미토콘드리아 기질간 칼슘 이온농도 차이와 다른 하나는 미토콘드리아 막전압의 분극정도가 제시되었다. 생리학적인 조건에서 미토콘드리아 막전압은 -120에서 -180 mV 사이의 음전하를 나타내는데 칼슘 이온은 양이온이기 때문에 미토콘드리아 막전압이 칼슘 이온을 유입시키는 중요한 원동력으로 작용한다. 따라서, 미토콘드리아 막전압의 약리학적 조절은 신경독성, 뇌 손상에 따른 신경세포의 사멸을 방지하고 신경세포를 보호할 수 있는 유력한 전략이 될 수 있다. 본 연구에서는 일차 대뇌피질 신경세포와 쥐의 대뇌피질로부터 분리한 순수한 미토콘드리아에 글루타메이트(glutamate) 독성이나 세포 내 칼슘과부하가 가해졌을 때 이에 대항하여 시트러스 폴리메톡실레이티드 플라본(*Citrus* polymethoxylated flavone)인 노빌레틴(nobiletin)이 미토콘드리아 막전압을 조절함으로서 신경독성적인 미토콘드리아 칼슘 유입과 산화적 스트레스를 막을 수 있는지를 연구하였다. 본 연구결과에 의하면, 일차 대뇌피질 신경세포에 100 μM의 글루타메이트를 20분간 처리 했을 때 노빌레틴이 신경세포의 생존력을 현저하게 증가

시켰다. 실시간 영상 기반의 형광 측정을 통해 노빌레틴이 뉴런에서 부분적인 미토콘드리아의 탈분극을 유발하는 것을 확인하였다. 노빌레틴이 유발하는 부분적인 미토콘드리아 탈분극을 일차 대뇌피질 신경세포에서뿐만 아니라 쥐의 뇌에서 분리한 미토콘드리아에서도 형광 마이크로플레이트 판독기를 사용하여 증명하였다. 노빌레틴은 일차 대뇌피질신경세포에 100 μM의 글루타메이트를 처리했을 때 발생하는 미토콘드리아로의 과도한 칼슘유입과 활성산소의 생성을 현저하게 감소시켰다. 뇌조직에서 분리한 미토콘드리아에 글루타메이트 독성을 모방하기 위해 고농도칼슘이온 5μM을 처리하였을 때 노빌레틴이 활성산소의 생성을 현저하게 감소시켰다. 노빌레틴이 그 자체적으로 활성산소 소거능을 나타내는지 알아보기 위한 실험에서 시트러스 폴리메톡실레이티드 플라본과 헤스페리딘 (hesperidin)은 활성산소 소거능을 보였지만 노빌레틴은 그 자체만으로는 활성산소 소거능을 보이지 않았다. 이에 더하여 포타슘이온이 없는 배지에서 실험할 경우 미토콘드리아의 기저 막전압에 미치는 노빌레틴의 효과가 완전히 사라지는 것으로 나타났다. 노빌레틴의 미토콘드리아내 분자 표적을 규명하기 위하여 $_{mito}BK_{Ca}$와 $_{mito}K_{ATP}$와 같은 주요한 미토콘드리아 포타슘 채널의 억제제인 5-HD와 iberiotoxin을 사용하여 일차 대뇌피질 신경세포에서 노빌레틴에 의한 부분적인 미토콘드리아 탈분극 현상에 대한 영향을 조사하였다. Iberiotoxin은 노빌레틴에 의한 미토콘드리아 탈분극을 유의성 있게 억제시켰다. 5-HD는 노빌레틴에 의한 막전압 탈분극을 억제하는 경향성을 보였다. 이러한 결과들은 노빌레틴에의한 미토콘드리아

기질로의 포타슘이온 유입에 $_{mito}BK_{Ca}$와 $_{mito}K_{ATP}$ 가 관여됨을 시사한다. 또한 노빌레틴은 로테논 (Rotenone)에 의해 발생한 활성산소를 확연하게 감소시켰다. 이는 미토콘드리아 전자 전달계의 복합체 Ⅰ이 노빌레틴과 상호작용하는 또 다른 표적이 될 수 있음을 시사한다. 그러나 노빌레틴의 정확한 미토콘드리아에서 분자 표적을 규명하기 위해서는 더 상세한 연구가 수행되어야 한다.

정리하면, 노빌레틴에 의해 유도되는 부분적인 미토콘드리아 막전압 탈분극은 세포사멸의 중요한 변수인 미토콘드리아로 과한 칼슘이온 유입과 활성산소의 생성을 감소시킨다. 미토콘드리아 기질로 포타슘이온의 유입은 부분적인 미토콘드리아 탈분극과 관련된 노빌레틴의 신경보호 효과에 중요하게 관여하며 이 과정에 미토콘드리아의 포타슘 채널 활성화가 관여할 것이다

감사의 글

제주도에 와서 공부한지도 어느덧 7년이 지났습니다. 연구과정과 임상공부를 병행하다 보니 7년의 시간이 정신 없이 지나간 것 같습니다. 학위과정을 돌아보면 많은 도움과 격려를 주신 분들이 계시기에 짧은 글로나마 감사의 인사를 드리고자 합니다.

우선 지난 7년동안 항상 저를 믿고 많은 가르침을 주신 은수용 교수님께 깊은 감사의 말씀을 드립니다. 작은 발표에서부터 논문에 이르기까지 꼼꼼하게 지도해 주시고 때로는 엄하게 또 때로는 어머니처럼 따듯하게 가르침을 주셔서 지금의 제가 있을 수 있는 것 같습니다. 그리고 가까이에서 늘 많은 조언과 응원을 해주신 정성철 교수님께 진심으로 감사 드립니다. 교수님께서 계셔서 미숙하고 어렸던 제가 더 성장할 수 있었습니다. 또 바쁘신 와중에 저의 학위논문 심사를 맡아주시고 학위과정에 많은 애정과 관심을 보여주신 박덕배 교수님, 윤상필 교수님, 김진우 교수님께도 감사 드립니다.

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생리학 교실의 선배로서 많은 실험 기법과 실험생활에 대해 알려준 금희 언니, 윤실이, 문석 오빠, 연희 언니 그리고 누구보다 가까이서 가장 많은 시간을 함께 지내며 도와준 지연이, 홀란에게 감사의 마음을 전하며 더불어 자신의 길을 열심히 걸어가고 있는 유리와 쳉겔에게도 고마운 마음을 전하고자 합니다.

저보다 앞서 낯선 길을 걸어가며 좋은 본보기가 되어주고 조언을 해주는 은정 선배, 복합 학위 과정 후배이자 같은 생리학 교실의 멤버로 의지가 된 상찬이, 약리학 교실과 생화학 교실에서 열심히 연구하고 있는 성민씨와 혜란이 그리고 이제 연구과정에 첫발을 시작한 창민이 모두에게 감사의 마음을 전하며 많은 시간 공부하고 노력한 만큼 각자가 꾸는 꿈과 목표가 이루어지길 바랍니다.

그리고 조교선생님이자 친한 동생으로 학위과정에 관련된 행정을 신경 써준 상언이와 언니보다 씩씩하게 자기 일을 잘 하고 언제나 큰 위로가 되어 주는 해부학교실의 하나, 3학년에 복학하여 힘들 때, 논문과 실습을 병행하기에 너무 바빠서 잠도 못 자고 힘들어 할 때 발벗고 나서서 도와준 동생 동휘에게 고마운 마음을 전합니다.

마지막으로 이날까지 공부하는 딸을 믿고 지지해주고 힘들게 뒷바라지 해주신 우리 엄마, 아빠. 행여나 부담될까 공부하는데 방해 될까 늘 공부하는 며느리부터 배려해 주시고 걱정해 주시는 어머님, 아버님. 그리고 한결같이 내 곁을 지키며 내 편이 되어주는 신랑 채호씨. 동생 경무와 철호 아주버님까지... 가족들이 있었기에 여기까지 올 수 있었어요. 앞으로도 발전해 가는 모습 보여드릴게요. 고맙고 사랑합니다. 늘 건강하세요.

짧은 글에 다 담을 수 없지만 따듯한 격려와 지지를 보내 주신 많은 교수님, 동료, 친구들에게 다시 한 번 감사의 말씀을 전하며, 기초도 임상도 마무리가 아닌 이제 시작임을 알고 있기에 앞으로도 부족한 부분을 채워나가며 연구하는 의사가 되어가도록 하겠습니다. 감사합니다.