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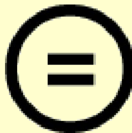

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Master's Thesis

A study on neuroprotective effects of methyl lucidone on glutamate-induced neuronal cell death in HT-22 neurons

Jee-Yun Park

Department of Biomedicine & Drug Development

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

February, 2014

글루탐산염에 의해 유도된 신경세포 사멸에 대한 methyl lucidone의 신경보호효과에 관한 연구

지도교수 은 수 용

박 지 연

이 논문을 이학 석사학위 논문으로 제출함

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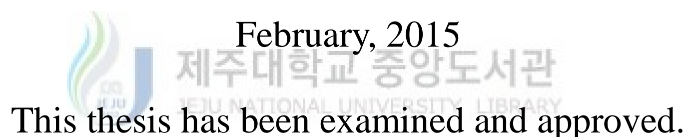
2015년 2월

A study on neuroprotective effects of methyl lucidone on glutamate-induced neuronal cell death in HT-22 neurons

Jee-Yun Park
(Supervised by professor **Su-Yong Eun**)

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

February, 2015



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ABSTRACT

Oxidative stress elicits neuronal cell death in many neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and ischemia. Therefore, it has been evaluated as the effective treatment strategy against neurodegenerative disorders to reduce neuronal cell death by decreasing intracellular reactive oxygen species (ROS). Methyl lucidone (MLC) was previously reported to suppress neuroinflammation in microglia. In the present study, the neuroprotective mechanism of MLC was investigated in HT-22 neuronal cells against glutamate-induced oxidative neurotoxicity.

Pretreatment of MLC (0.1-5 μM) increased neuronal cell viability against glutamate-induced neuronal cell death. Glutamate-induced ROS production was decreased by MLC, although MLC did not show any free radical scavenging activity in cell-free condition, as shown in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The expression of heme oxygenase 1 (HO-1) as an intracellular antioxidant enzyme was up-regulated by MLC treatment in HT-22 neurons. Nuclear translocation of NF-E2-related factor 2 (Nrf-2) known as a transcription factor of HO-1 was also up-regulated by MLC treatment. Furthermore, pretreatment of PD98059 as a pharmacological inhibitor of extracellular signal-regulated kinase (ERK) or SB203580 as p38 mitogen-activated protein kinase (p38) inhibitor did not affect the MLC-induced neuroprotection. To address whether phosphatidylinositol 3-kinase (PI3K) pathway is involved in the upstream of Nrf-2, pretreatment of an inhibitor of phosphatidylinositol 3-kinase (PI3K), LY294002, suppressed the MLC-increased HO-1 induction and neuroprotection against glutamate-induced neuronal cell death.

These results suggest that MLC significantly protects HT-22 neurons against glutamate-induced oxidative neurotoxicity by inducing the expression of the antioxidant enzyme HO-1 via PI3K signaling pathway.

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Key words: Methyl lucidone; Glutamate toxicity; Oxidative stress; HO-1; PI3K; ROS

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I. INTRODUCTION

Glutamate-induced neurotoxicity can lead to neuronal cell death in neurodegenerative diseases, such as Alzheimer's disease, ischemia and Parkinson's disease (Beal, 1995; Coyle and Puttfarcken, 1993; Jenner, 1994). The extracellular glutamate in the central nervous system (CNS) induces lower glutathione levels inhibiting cystine/glutamate transporter, which cause accumulation of reactive oxygen species (ROS) and the death of neuronal cell, called oxidative glutamate toxicity (Murphy et al., 1989). The mouse hippocampal cell line, HT-22, is a commonly used cell line for oxidative glutamate toxicity. This cell line is deficient in functional ionotropic glutamate receptors and responds to oxidative glutamate toxicity via non-receptor mediated pathway (Davis and Maher, 1994).

Oxidative stress can be caused by accumulation of ROS, such as hydroxyl radical, hydrogen peroxide and superoxide anions. This ROS damages the cellular components including DNA, proteins and lipids, which cause apoptosis (Thannickal and Fanburg, 2000). Mammalian cells have an antioxidant system to prevent ROS formation or enhance ROS degradation (Blokhina et al., 2003). Among the various antioxidant enzymes, the heme oxygenase (HO)-1 is important enzyme of the cellular antioxidant system. HO-1 catalyzes the rate-limiting step in the conversion of heme to biliverdin while producing carbon monoxide (CO) and iron (Ryter et al., 2006). The activity of HO-1 is mainly regulated by transcriptional level of nuclear transcription factor-E2-related factor 2 (Nrf-2). In normal state, Nrf-2 is coupled with Kelch-like ECH-associated protein1 (Keap1) which inhibits nuclear translocation of Nrf-2. During oxidative stress, Nrf-2 is detached from Keap1 and translocated into nucleus. And it binds to antioxidant response element (ARE) to activate the promoter region of many genes encoding phase II detoxification enzymes and antioxidants, including HO-1 (Surh, 2003). The dissociation of Nrf-2 from Keap1 is induced phosphorylation of Nrf-2, which is mediated via diverse kinases including mitogen-activated

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protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K).

Linderaerythrocarpa Makino (Lauraceae), a deciduous shrub, is broadly distributed in Taiwan, Korea, Japan and China. The fruit of *L. erythrocarpa* is used in traditional medicines as analgesic, antibacterial, antidote, digestive and diuretic (Ichino et al., 1988). The extract of the fruit was separated into four cyclopentenediones, including linderone, lucidone, methyl linderone and methyl lucidone (MLC). It was reported that lucidone inhibited human farnesyl protein transferase (FPTase) activity (Oh et al., 2005). Also it was demonstrated that lucidone and MLC suppressed NO production (Wang et al., 2008). Our previous studies reported that MLC had neuroprotective effect through inhibition of microglia-mediated neurotoxicity (Cui et al., 2012). However, the direct effect of MLC on neurons was not still investigated. Therefore, we examined whether MLC shows directly neuroprotective effects on gluatamate-induced neurotoxicity in HT-22 neurons.

II. MATERIALS AND METHODS

1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Antibodies against c-jun NH_2-terminal kinase (JNK), phospho-JNK, p38 and phospho-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against extracellular signal-regulated kinase (ERK), ERK and nuclear transcription factor-E2-related factor 2 (Nrf-2) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibody against heme oxygenase-1 (HO-1) was purchased from Millipore (Temecula, CA, USA). Antibody against TATA binding protein (TBP) was purchased from Abcam (Cambridge, UK). Antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). 2',7'-Dichlorofluorescin diacetate (DCF-DA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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2. Extraction and isolation of MLC

The dried fruits (175.18 g) of *L. erythrocarpa* were extracted with MeOH (2×1 L) for 48 h at room temperature. After filtration, the extract was concentrated and the residue weighed 11.31 g. The MeOH extract was partitioned between H_2O and EtOAc (1:1, v/v) to obtain a EtOAc-soluble fraction(9.91 g). The EtOAc fraction showed FPTase inhibition activity and was partitioned again with MeOH to give MeOH-soluble fraction (8.5 g) and –insoluble fraction (1.26 g). The MeOH-soluble fraction was concentrated, and then the residue was chromatographed on a silica gel (350 g) column, eluted with a gradient of n-hexane/EtOAc (19:1, 18:2, 17:3, 16:4, 14:6, 1:1, each 1.5 L) to provide 45 fractions. Active

fractions were collected and concentrated to yield 7.3 g. The active fraction was resubjected to a C-18 column, and it was eluted with a gradient of MeOH/H_2O (6:4, 7:3,8:2, MeOH, each about 3 L) to provide methyl lucidone (1450 mg)(Oh et al., 2005).

3. Cell culture

HT-22 neurons, an immortalized hippocampal neuronal cell line (Breyer et al., 2007), were a generous gift from Dr. B. H. Lee (Gachon University of Medicine and Science, South Korea). HT-22 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and incubated at 37℃under 5% CO_2.

4. Measurement of cell viability

For determining cell viability, MTT assay was performed. HT-22 cells were seeded at a density of 5×10^4 cells/well in a 24-well plate. After 12 h, these cells were treated with various concentrations of MLC for 1 h. The cells were washed with DMEM and treated with 5 mM glutamate for 12 h. And then, 100 μl of MTT solution (2 mg/ml) was added to each well and cells were incubated at 37℃ for 2 h. The supernatant was removed, and the MTT formazan crystals were dissolved with 300 μl of DMSO. The absorbance was measured at 550 nm using a microplate reader (Model 550, Bio-Rad, USA).

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5. Measurement of intracellular ROS level

The intracellular reactive oxygen species (ROS) level was measured using 2',7'-dichlorofluorescin diacetate. The HT-22 cells were seeded at a density of 5×10^4 cells/well in a 24-well plate and incubated for 12 h. MLC was treated for 1 h in indicated concentrations. The cells were washed with DMEM and treated with 5 mM glutamate for 12 h. And then, the cells were loaded with 50 μM DCF-DA for 15 min. The fluorescence intensity was detected on a fluorescence reader (Spectra Fluor, Tecan, Austria) at an

excitation wavelength of 485 nm and an emission wavelength of 535 nm.

6. Measurement of free radical scavenging effect

2,2-Diphenyl-1-picrylhydrazyl(DPPH), a purple-colored, is reduced into diphenylpicryl hydrazine, a yellow-colored. To measure free radical scavenging activity of MLC, DPPH assay was performed. 10 μl of MLC was added to 190 μl DPPH (0.15 mM) in each well (96-well plate) and mixed vigorously. The mixture was incubated at room temperature for 1hin the dark covered with aluminum foil. Absorbance was detected at 517 nm using microplate reader (VersaMax, Molecular devices, USA).

7. Preparation of cytoplasmic and nuclear protein

Preparation of cytoplasmic and nuclear protein was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagets (Invitrogen, USA) according manufacturer's protocol. HT-22 cells were seeded at a density of 1×10^6 cells/dish in 100 mm dish. These cells were treated with MLC indicated time, and then washed twice and collected with cold PBS. After centrifuging, cell pellet was resuspended in cytoplasmic extraction reagent. After centrifuging, the supernatant cytoplasmic extract was transferred to a new tube. And nuclear pellet was resuspended in Nuclear extraction reagent, and centrifuged. The supernatant nuclear protein extract was transferred to a new tube and stored at -80°C until usage.

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8. Western blot analysis

Cell extracts were separated with 10-12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, CA, USA). After blocking with 5% skim milk in TBS (25 mM Tris, pH 7.4, 150 mM NaCl), the membrane was probed with anti-Nrf2, anti-HO-1, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, anti-p38, anti-phospho-JNK, anti-JNK, anti-β-actin and anti-TBP. Blots were washed three times

with TTBS and incubated with HRP-conjugated anti-mouse or anti-rabbit antibody. Then the blots were detected using an enhanced chemiluminescence reagent according to the manufacturer's protocol. Optical densities of the band were quantified with an Image J program.

9. Statistics

The data were presented as the mean ±S.E.M. at least 3 independent experiments. Statistical analysis was performed using the *t*-test and one-way ANOVA. The differences between groups were considered to be statistically significant when $p<0.05$, $p<0.01$ or $p<0.001$.

III. RESULTS

1. The protective effect of MLC on glutamate-induced neuronal cell death in HT-22 neurons.

To investigate whether MLC protects HT-22 neurons against glutamate-induced cell death and MLC has the cytotoxicity in the cells, we examined the ctyotoxic effects of glutamate (5 mM) on HT-22 cells with or without MLC pretreatment. The treatment of MLC did not show any cytotoxic effect at concentrations less than 5 μM (Figure 1). Glutamate (5 mM) decreased cell viability of HT-22 to 75.3 % compared to the non-treated group. Pretreatment of MLC (0.5 - 5 μM) dose-dependently increased cell viability of HT-22 compared to the glutamate-treated group. These results indicate that MLC protectedthe cells against glutamate-induced cytotoxicity (Figure 1).

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2. MLC inhibits intracellular ROS production by glutamate in HT-22 neurons.

To investigate whether MLC suppresses the glutamate-induced ROS in HT-22 cells, the intracellular ROS levels were monitored by DCF-DA assay. Glutamate (5 mM) increased intracellular ROS level up to 136 ± 4.3% (Figure 2A). The pretreatment of MLC (5 μM) significantly reduced intracellular ROS compared to the glutamate-treated group. To evaluate the antioxidant effect of MLC, DPPH assay was performed with MLC and N-acetyl cysteine (NAC). MLC did not show the ROS scavenging effect (Figure 2B). These results indicate that MLC plays a role to reduce the glutamate-induced ROS. It is estimated that MLC is involved in the expression of antioxidant enzyme, albeit MLC is not antioxidant.

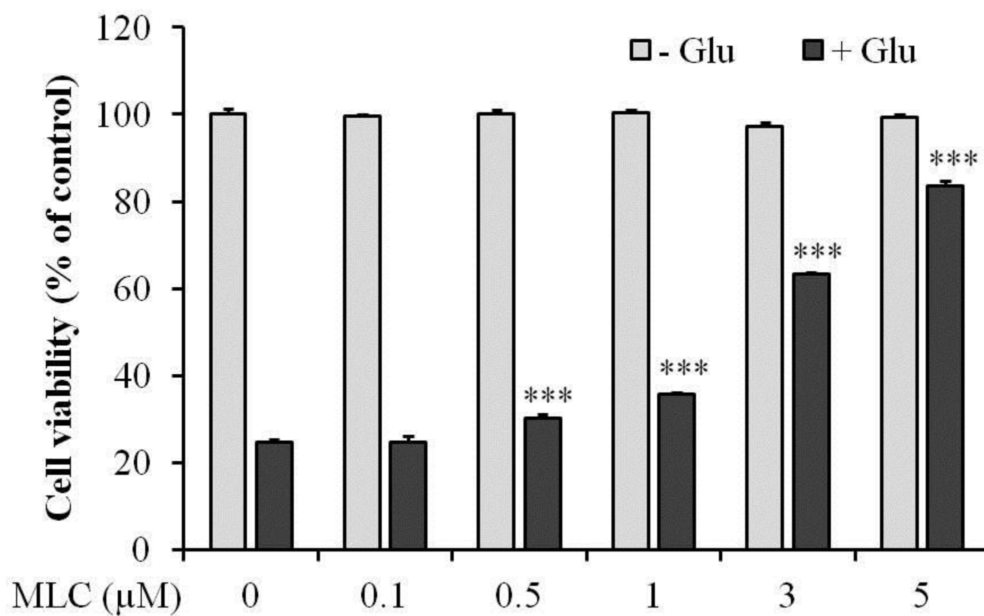

Figure 1. MLC protects HT-22 neurons against glutamate-induced neuronal cell death. HT-22 cells were pretreated with MLC for 1 h. Then these cells were washed and incubated with glutamate (5 mM) of fresh media for 12 h in the absence of MLC. The cell viability was examined by MTT assay. The values were represented mean ±S.E.M. (n=3). ***$p < 0.001$ as compared to the group treated with glutamate alone.

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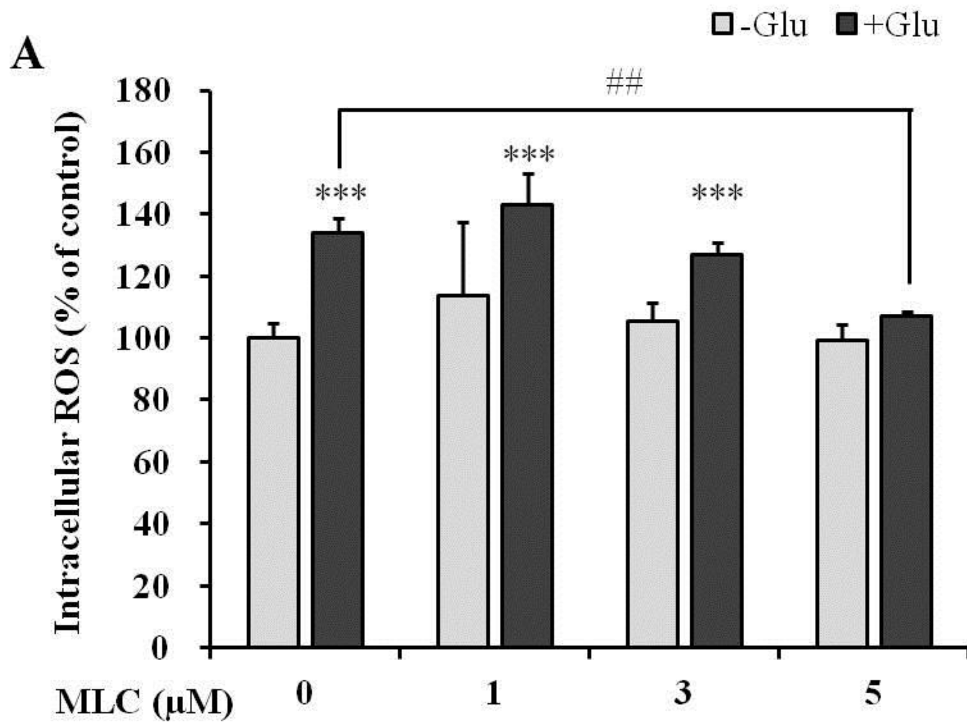

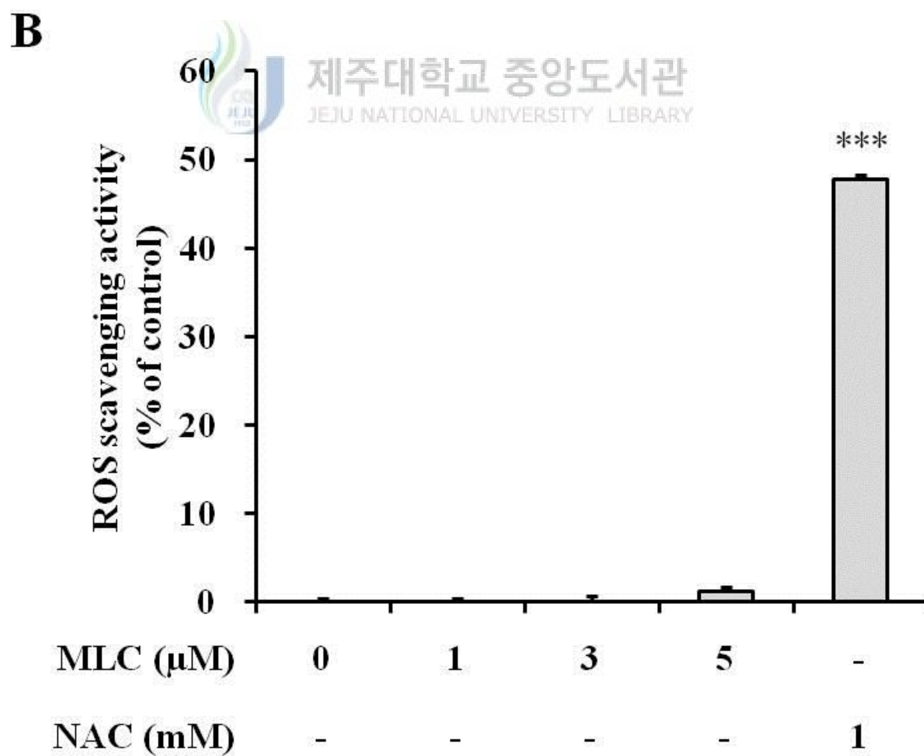

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Figure 2. Glutamate-induced ROS generation was suppressed by MLC. (A) HT-22 cells were pretreated with MLC (1, 3, 5 μM) for 1 h. Then these cells were washed and incubated

with glutamate (5 mM) for 12 h in the absence of MLC. Intracellular ROS levels were measured using ROS-sensitive fluorescent DCF-DA. The values were represented mean ± SEM (n=3). ***p < 0.001 as compared to the untreated control and ##p < 0.01 as compared to the group treated with glutamate alone. (B) DPPH assay was perforemed to measure an antioxidative effect of MLC. Free radical scavenging activities were indicated as % inhibition. The values were represented mean ±S.E.M. (n=3). ***p < 0.001 as compared to the untreated control

3. MLC activates Nrf-2/HO-1 signaling transduction in HT-22 neurons.

To determine that MLC regulate expression of HO-1, Western blot analysis was performed. When MLC was applied in HT-22 cells, the induction of HO-1 was observed at 3 h and highly increased at 6 h (Figure 3). As the translocation of Nrf-2 to nucleus can induce expression of HO-1, we measured protein levels of Nrf-2 in nuclear fraction. Protein levels of Nrf-2 were increased to a maximum at 30 min (Figure 4). These results indicate that MLC induces the expression of HO-1 by Nrf-2 translocation. Accordingly, MLC has the antioxidant effect on glutamate-induced oxidative stress.

4. The role of MAPKs pathway in protective effects of MLC.

To evaluate which kinase of MAPKs was involved in Nrf-2 accumulation to nucleus, phosphorylation rates of ERK, p38 and JNK were examined by Western blot analysis. The treatment of MLC increased the phosphorylation of ERK compare to control group (Figure 5). In p38 and JNK, however, the significant increase of phosphorylation was not observed (Figure 6, 7). Thus, we confirmed the involvement of ERK activation in neuroprotection of MLC. Cell viability that was increased by MLC on glutamate-induced cell death was not affected by PD98059, an ERK inhibitor (Figure 8). These findings were suggested that the activation of ERK was not involved in MLC effects, although the phosphorylation of ERK was increased by MLC.

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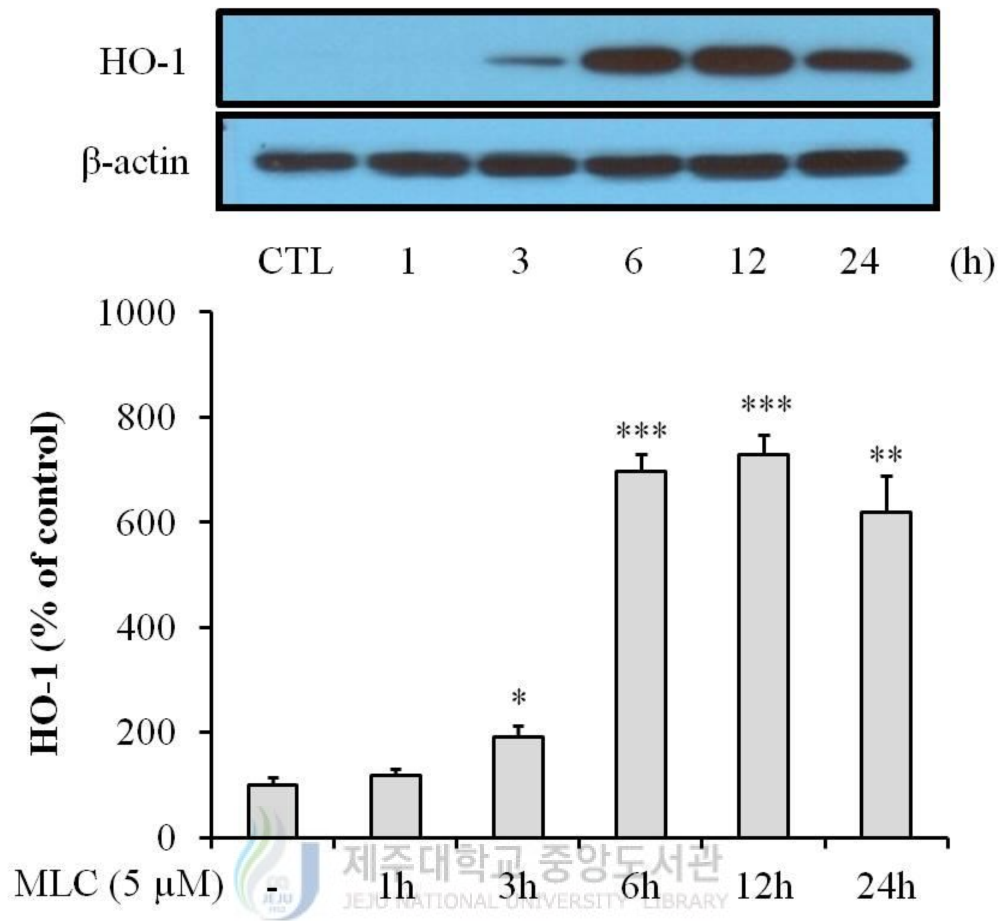

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Figure 3. MLC up-regulated HO-1 expression in HT-22 neurons. MLC was treated for 1 h in HT-22 cells. Then these cells were washed and further incubated in fresh media for 2 h, 5 h, 11 h and 23 h. The expression of HO-1 was examined by Western blot analysis. The values were represented mean ± S.E.M. (n=3). *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$ as compared to the untreated control.

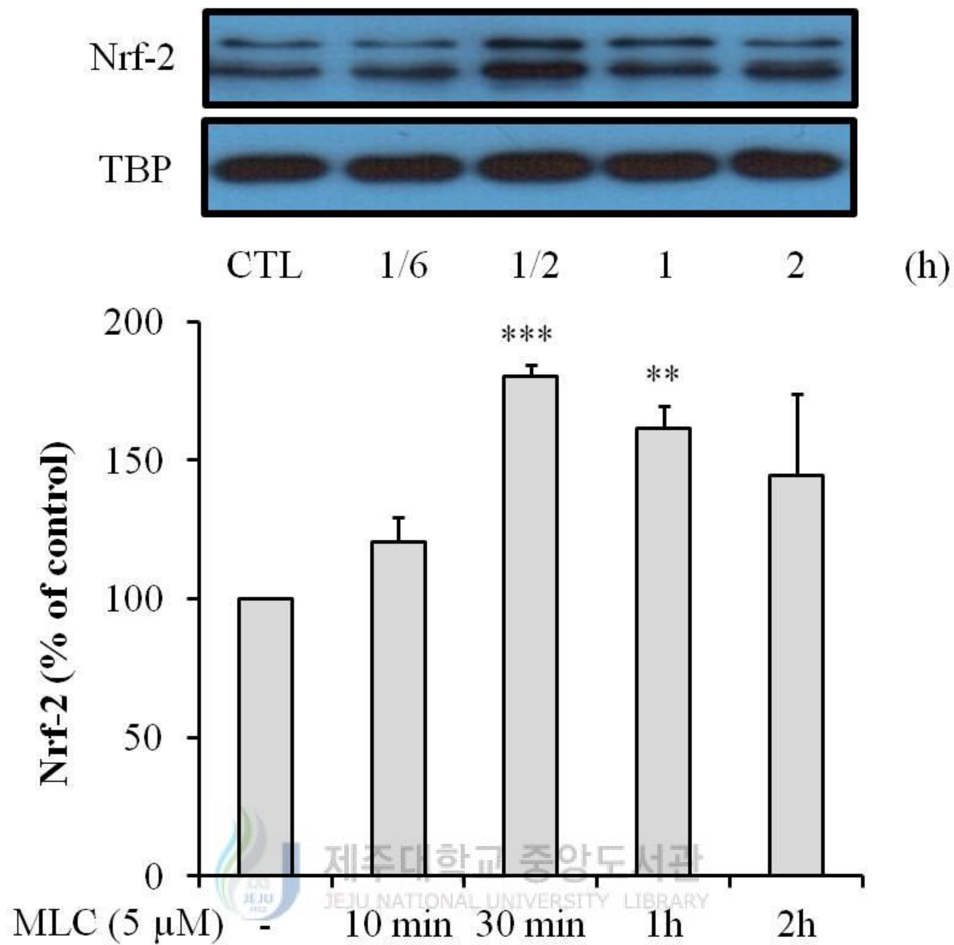

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Figure 4. MLC induced Nrf-2 translocation to nucleus in HT-22 neurons. MLC was treated for 10 min, 30 min, 1 h and 2 h in HT-22 cells. The nuclei were fractionated from the cytosol using NE-PER Nuclear and Cytoplasmic Extraction Reagets as described in Materials and methods. Nrf-2 proteins were detected by Western blot analysis. The values were represented mean ± S.E.M. (n=3). **p < 0.01 or ***p < 0.001 as compared to the untreated control.

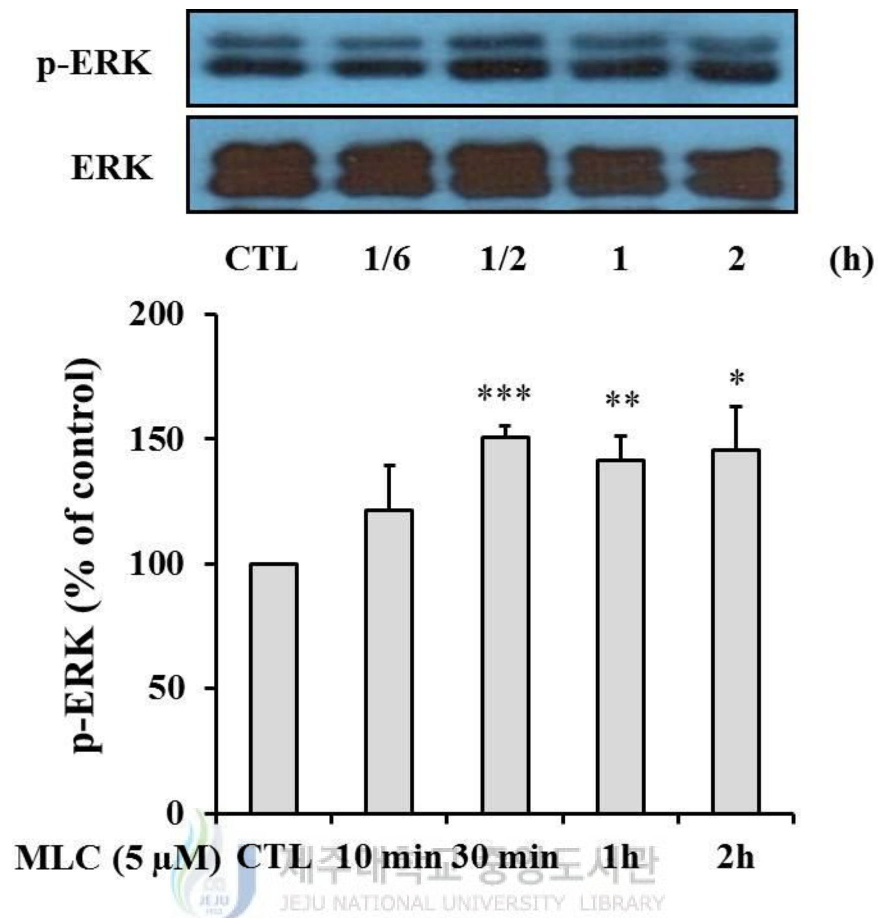

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Figure 5. MLC regulated the phosphorylation of ERK. HT-22 cells were incubated with MLC for the indicated times. The cells were lysed and the p-ERK and ERK proteins were detected by Wetsern blot analysis. The values were represented mean ± S.E.M. (n=3). $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$ as compared to the untreated control.

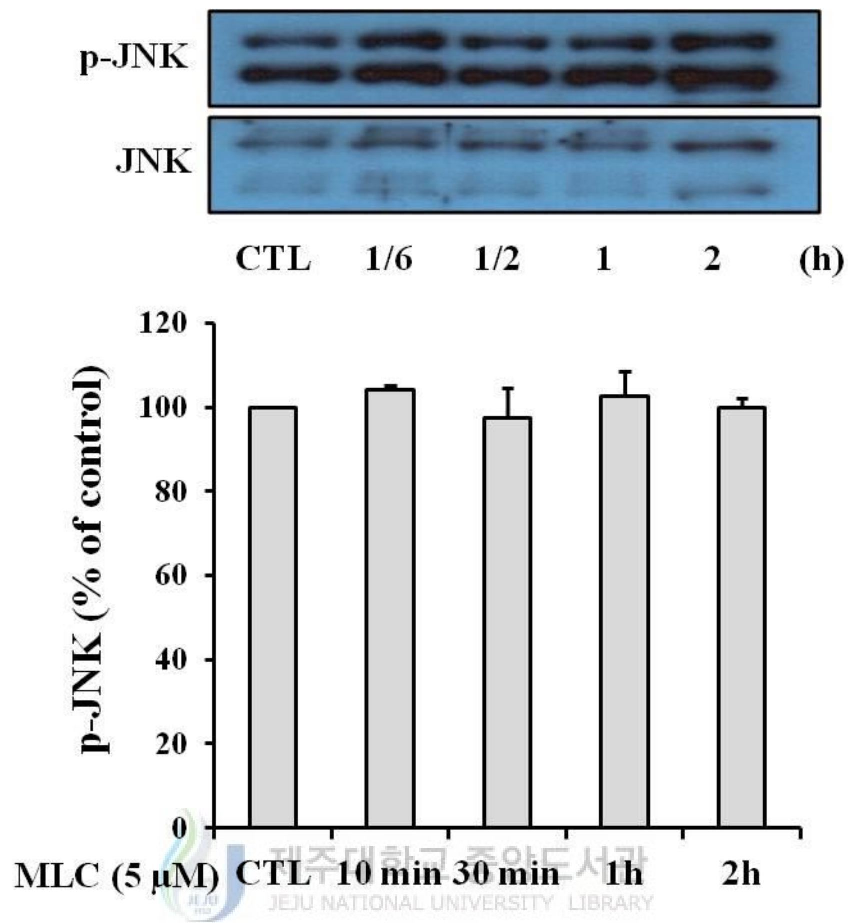

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Figure 6. Effect of MLC on the phosphorylation of JNK. HT-22 cells were incubated with MLC for the indicated times. These cells were lysed and the p-JNK and JNK proteins were detected by Wetsern blot analysis. The values were represented mean ± S.E.M. (n=3).

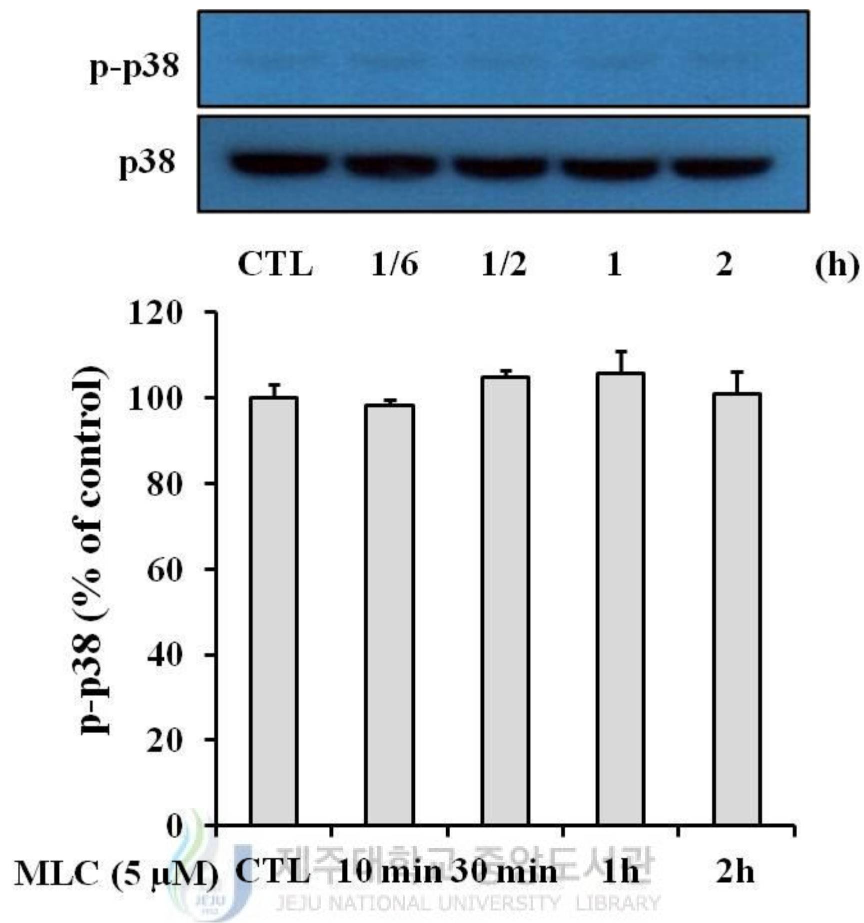

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Figure 7. Effect of MLC on the phosphorylation of p38. HT-22 cells were incubated with MLC for the indicated times. These cells were lysed and the p-p38 and p38 proteins were detected by Wetsern blot analysis. The values were represented mean ± S.E.M. (n=3).

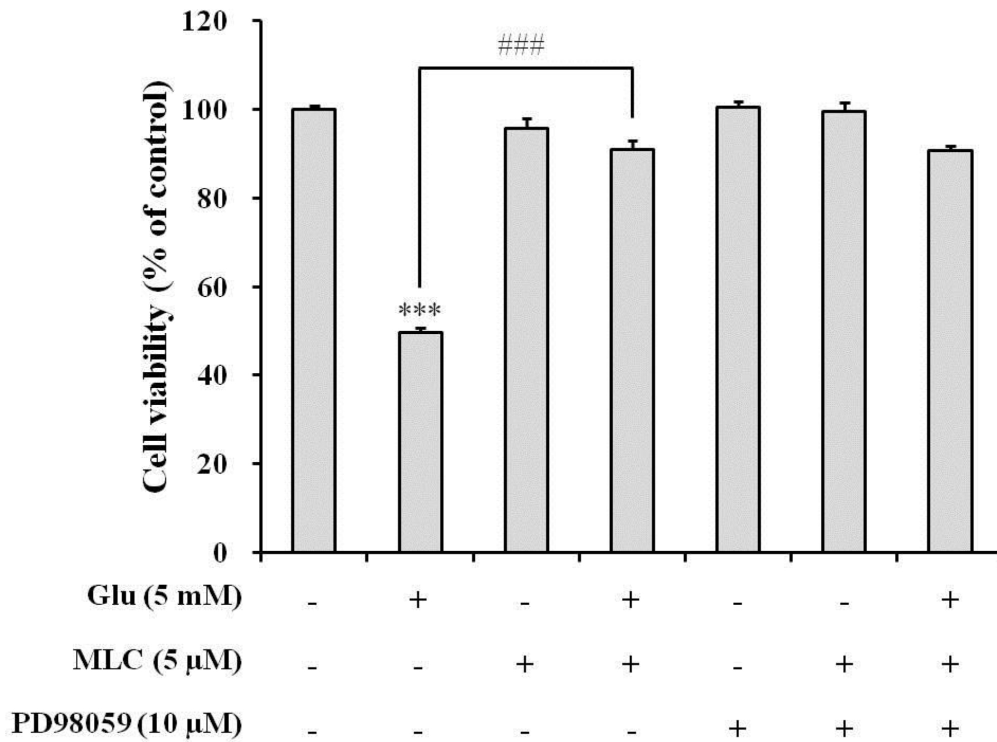

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Figure 8. Inhibition of ERK did not affect protective effects of MLC in HT-22 neurons. PD98059 (10 μM) was treated for 1h prior to treatment of MLC. The cells were incubated with MLC for 1 h. Then these cells were washed and incubated with glutamate (5 mM) for 12 h in the presence of PD98059. The values were represented mean ± S.E.M. (n=3). ***$p < 0.001$ as compared to the untreated control and ###$p < 0.001$ as compared to the group treated with glutamate alone.

5. The PI3K/Akt pathway was involved in protective effects of MLC.

PI3K/Akt signaling pathway is involved in the activation of HO-1. Thus, we examined the role of PI3K/Akt in protective effects of MLC. MLC-increased protein levels of HO-1 were decreased by LY924002, a PI3K inhibitor (Figure 9). Also, the protective effect of MLC was suppressed by LY294002 (Figure 10). These results indicate that PI3K/Akt signaling was involved in protective effects of MLC through HO-1.

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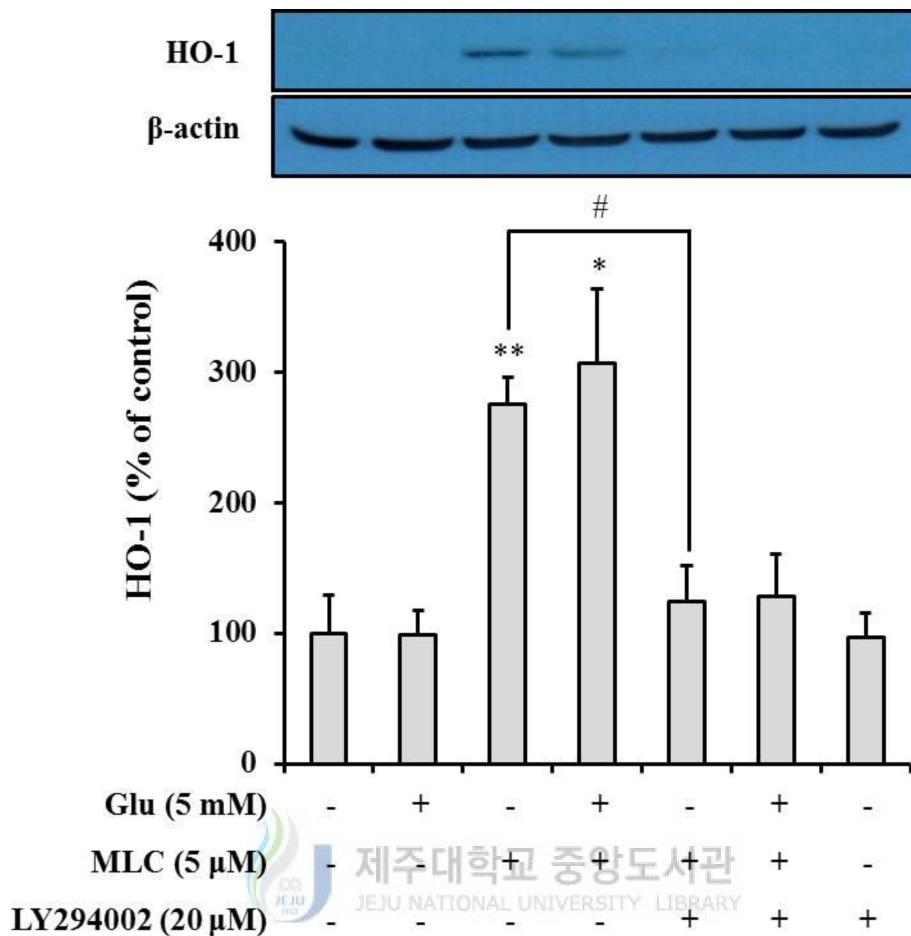

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Figure 9. The effect of PI3K inhibitor on MLC-induced HO-1 expression in HT-22 neurons. LY294002 (20 μM) was treated for 1h prior to treatment of MLC. These cells were incubated with MLC (5 μM) for 1 h. Then these cells were washed and further incubated with glutamate (5 mM) for 5 h in the absence of MLC and the presence of LY294002. The cells were lysed and HO-1 proteins were detected by Western blot analysis. The values were represented mean ± S.E.M. (n=3). *$p < 0.05$ or **$p < 0.01$ as compared to the untreated control and #$p < 0.05$ as compared to the group treated with MLC alone.

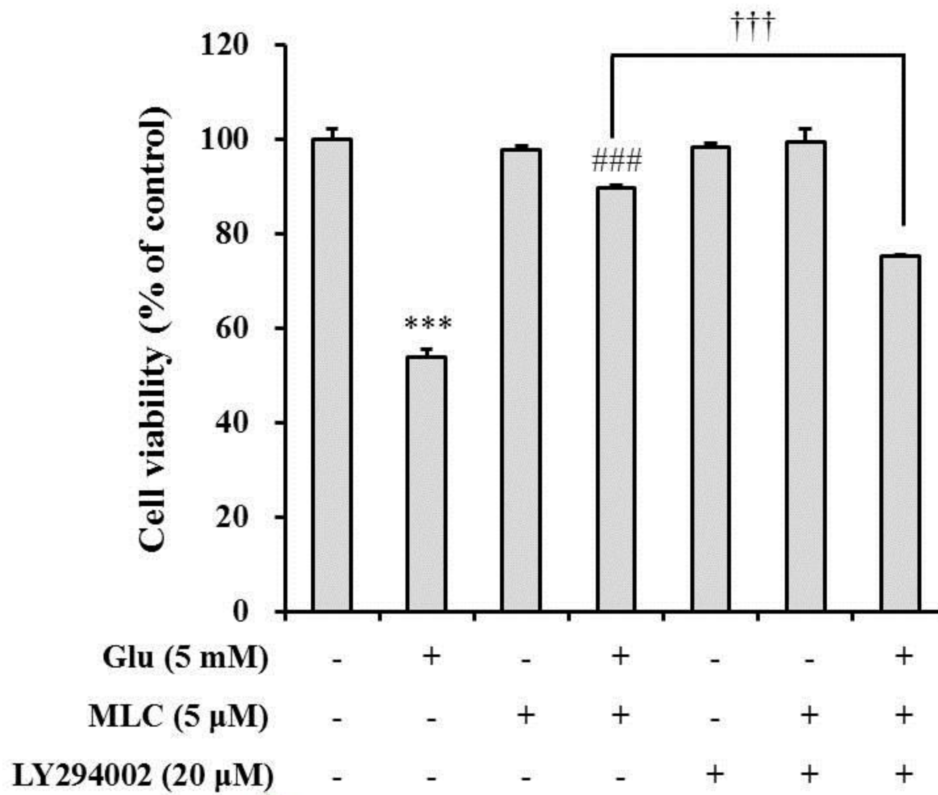

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Figure 10. PI3K involved in MLC-induced neuroprotection. LY294002 (20 μM) was treated for 1h prior to treatment of MLC. These cells were incubated with MLC (5 μM) for 1 h. Then these cells were washed and incubated with glutamate (5 mM) for 12 h in the absence of MLC and the presence of LY294002. The cell viability was measured by MTT assay. The values were represented mean ± S.E.M. (n=3). ***$p < 0.001$ as compared to the untreated control, ###$p < 0.001$ as compared to the group treated with glutamate alone and †††$p<0.001$ as compared to the group treated with glutamate and MLC.

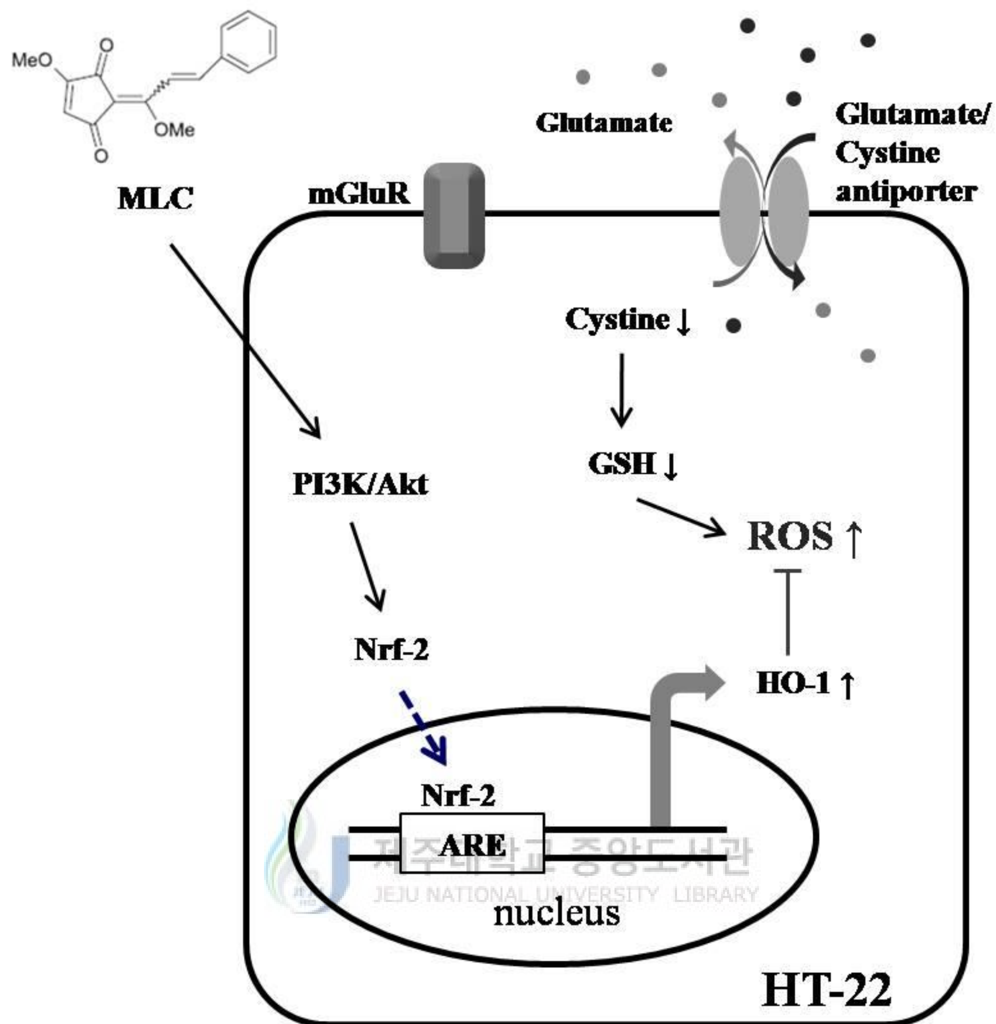

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Figure 11. A neuroprotective mechanism of MLC on glutamate-induced toxicity in HT-22 neurons.

IV. DISCUSSION

The excessive intracellular ROS causes various diseases by damaging cellular components, such as DNA, lipids and proteins. In CNS, the high level of ROS results in neuronal cell death, which contributes to neurodegenerative diseases. Oxidative glutamate toxicity has been described in primary neuronal cell cultures (Murphy et al., 1989; Oka et al., 1993), neuronal cell line (Davis and Maher, 1994; Miyamoto et al., 1989) and tissue slices (Vornov and Coyle, 1991). Here I used the mouse hippocampal cell line, HT-22, because it is a commonly used cell line for oxidative glutamate toxicity. The high level of extracellular glutamate suppresses cystine uptake through the inhibition of glutamate/cystine antiporter, which induces oxidative stress and neuronal cell death. In this process, HO-1 as antioxidant enzyme is one of candidates to reduce oxidative stress. The present study investigated whether MLC could inhibit oxidative glutamate toxicity.

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Although MLC was not antioxidant, it could suppress the glutamate-induced ROS generation (Figure 2). In some reports, it has been shown that phytochemicals had the protective effect by activating some cytoprotective proteins including HO-1 (Hwang and Jeong, 2008; Senthil Kumar et al., 2012; Zhang et al., 2012). The pretreatment of MLC increases the expression of HO-1 and induces the nuclear translocation of Nrf-2 (Figure 3 and 4). Thus, it is suggested that the induction of HO-1 by MLC may inhibit the glutamate-induced oxidative stress.

It was reported that the activation of Nrf-2 was controlled by its upstream regulators, such as ERK, JNK, p38 and PI3K (Surh, 2003). First, MAPKs were targeted as on upstream of Nrf-2 phosphorylation. It was observed that the treatment of MLC increased the phosphorylation of ERK (Figure 5), but one inhibitor of ERK (PD98059) did not suppress MLC-increased cell viability (Figure 8). So it was confirmed that MAPKs were not involved in the activation of Nrf-2. Additional studies about change of ERK phosphorylation by MLC

are required. Next, it was examined that PI3K acts as an upstream regulator of Nrf-2. The pretreatment of PI3K inhibitor (LY294002) reduced the expression of HO-1 that was increased by MLC (Figure 9). Also MLC-induced neuroprotective effect was reduced by the pretreatment of LY294002 (Figure 10). Therefore, it was suggested that MLC protected HT-22 neurons against glutamate-induced oxidative stress via PI3K signaling pathway.

In conclusion, these findings demonstrated that MLC induced Nrf-2 activation through PI3K signaling transduction and protected HT-22 neurons by suppressing glutamate-induced ROS production by HO-1, which is regulated by Nrf-2 (Figure 11).

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ABSTRACT IN KOREAN

산화적 스트레스는 신경세포 사멸을 유발시키는 주요한 원인 중의 하나로서 알쯔하이머 병, 파킨슨 병, 뇌졸중/허혈과 같은 신경 퇴행성 질환의 발병 및 진행과 관련이 있다고 알려져 있다. 그러므로 신경세포 내 활성산소가 급격하게 증가하는 병적 상태에서 이를 조절하여 신경세포의 사멸을 줄이는 것은 신경 퇴행성 질환에 있어서 매우 중요한 전략으로 평가되고 있다. 식물로부터 추출된 화합물이 여러 세포 종에서 세포 사멸에 대해 보호효과를 가진다는 연구가 많이 이루어 지고 있다. 비목나무 추출물에 포함된 화합물 중 하나인 methyl lucidone (MLC)이 미세신경아교세포(microglia)에서 신경염증을 억제하는 것이 이전에 보고되었다. 이번 연구에서는 신경세포에서 글루탐산염에 의해 유도된 산화적 스트레스에서 MLC의 신경보호 기전을 조사하였다.

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MLC (0.1~5 μM)를 처리하였을 때, 글루탐산염 신경독성 모델에서 글루탐산염에 의해 유도된 신경세포 사멸이 유의적으로 억제되는 것을 확인하였다. 산화적 글루탐산염 독성 상황에서 세포 내 활성산소의 양이 증가되는 것이 이미 알려져 있다. 이러한 상황에서 MLC는 글루탐산염에 의해 증가된 활성산소의 양을 억제하였다. 세포 내 활성산소의 감소가 MLC의 자체적인 항산화 활성에 의한 것인지 알아본 결과, MLC는 항산화 작용이 없는 것으로 확인 되었다. 따라서, MLC에 의한 세포 내 활성산소 생성의 억제는 세포 내 항산화 효소의 발현에 관여하는 것으로 생각되었고, 그 중 하나인 heme oxygenase (HO-1)를 조사하여 보았다. MLC는 HO-1의 발현을 증가시켰고 그의 상위 신호분자인 NF-E2-related factor 2 (Nrf-2)가 핵으로 이동하는 것을 증가시켰다. 이러한 과정에서 Nrf-2의 상위 신호전달을

조절한다고 알려진 분자들 중 어떤 것이 관여하는 지 알아보았다. 각각의 억제제를 사용하여 실험한 결과, mitogen-activated protein kinases (MAPKs) 억제제는 MLC의 보호효과에 대해 아무런 변화를 보이지 않았지만 phosphatidylinositol 3-kinase (PI3K) 억제제는 MLC에 의해 유도된 HO-1의 발현과 보호효과를 감소시켰다. 이는 Nrf-2의 상위에 PI3K가 관여한다는 것을 의미한다.

결론적으로 MLC는 PI3K를 통해 Nrf-2의 핵으로 이동을 증가시키고 그에 따라 HO-1의 발현을 증가시킨다. 글루탐산염에 의해 증가된 세포 내 활성산소는 MLC에 의해 유도된 HO-1에 의해 억제되어 HT-22 신경세포에서 보호작용을 나타내는 것으로 확인되었다. 따라서 이번 연구는 MLC의 신경 퇴행성 질환에 대한 예방 및 치료제로의 활용 가능성에 대한 기초 자료를 제공하였다고 본다.

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실험실 생활 하나도 모르던 저를 가르쳐 주고 이끌어 준 오금희 언니, 최연희 언니, 양윤실 언니, 강문석 오빠에게 진심으로 감사 드리고, 저와 같이 밤 늦게까지 실험실에 남아 공부하며 고생한 이지형 언니에게 감사 드립니다. 그리고 김선희 선생님, 실험실 후배 홀란, 유리에게도 감사의 마음을 전합니다.

그리고 대학원 동기 차지원 언니, 가영이, 새벽이에게도 우리 모두 수고했다는 말과 고맙다는 말을 전합니다.

세상에서 가장 사랑하는 우리 가족들에게 타지에서 고생한다고 걱정만 많이 끼쳤는데 이제 돌아가서 의젓한 딸, 누나 역할 하겠습니다. 2년 동안 믿고

기다려주셔서 정말 감사합니다. 그리고 힘들다고 투정만 부렸는데도 이야기 잘 들어주고 다독여 준 내 친구들에게도 고맙다는 인사를 전합니다.

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