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의학석사 학위논문

Glutamate에 유도된 SH-SY5Y 세포에서의 미토콘드리아 손상, 세포 자연사 그리고 NLRP3 inflammasome 활성에 대한 KHG21834의 억제 효과

KHG21834 attenuates glutamate-induced mitochondrial damage, apoptosis, and NLRP3 inflammasome activation in SH-SY5Y human neuroblastoma cells

울산대학교 대학원

의학과

한아름

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이 논문을 의학석사 학위 논문으로 제출함

2020년 02월

울산대학교 대학원

의학과

한아름

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2020년 02월

Abstract

New compounds were screened to develop effective drugs against glutamate-induced toxicity. The present study assessed the effects of the novel thiazole derivative KHG21834 against glutamate-induced toxicity in human neuroblastoma SH-SY5Y cell cultures. Treatment of SH-SY5Y cells with KHG21834 significantly protected cells against glutamate-induced toxicity in a dose-dependent manner, with an optimum concentration of 50 μM. KHG21834 protected SH-SY5Y cells against glutamate toxicity by suppressing glutamate-induced oxidative stress by 50%. KHG21834 also attenuated glutamate-induced intracellular Ca^{2+} influx, mitochondrial membrane potential and ATP level reductions. Furthermore, KHG21834 efficiently reduced glutamate-induced ER stress and NLRP3 inflammasome activation (59% and 65% of glutamate group, respectively). In addition, KHG21834 effectively attenuated glutamate-induced levels of Bax, Bcl-2, cleaved caspase-3, p-p38, p-JNK proteins, and TUNEL positive cells. To our knowledge, this is the first study showing that KHG21834 can effectively protect SH-SY5Y cells against glutamate toxicity,

suggesting that this compound may be a valuable therapeutic agent for the treatment of glutamate toxicity.

Keywords: Glutamate, KHG21834, oxidative stress, mitochondrial dysfunction, NLRP3 inflammasome, ER stress

List of abbreviations

ATF6 : Activating transcription factor-6

ATP : Adenosine triphosphate

BAK : Back-2 agonist of killer

BAX : Bcl-2-associated X protein

Bcl-2 : B-cell leukemia/lymphoma 2

Bip/GRP78 : Binding immunoglobulin protein/Glucose-regulated protein 78 kDa

CHOP : C/EBP Homologous protein

CNS : Central nervous system

eIF2α : Eukaryotic translation initiation factor 2 subunit α

ER : Endoplasmic reticulum

ERK : Extracellular signal-regulated kinase

GSH : Glutathione

GSH-Px : Glutathione peroxidase

GSK : Glycogen synthase kinase-3β

IRE1 : Inositol-requiring enzyme 1

JNK : Jun N-terminal Kinase

LDH : lactate dehydrogenase

MAPK : Mitogen-activated protein kinase

MDA : Malondialdehyde

NF-κB : Nuclear Factor kappa-light-chain-enhancer of activated B cells

NLRP3 : nucleotide-binding and oligomerization domain-like receptor containing a pyrin domain 3

NMDAR : N-methyl-D-aspartate receptor

PERK : PRK (Protein kinase R)-like ER protein kinase

SOD : Superoxide dismutase

TNF : Tumor necrosis factor

TUNEL : Terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labeling

Contents

Figures

Introduction

Glutamate is a major excitatory neurotransmitter in the vertebrate central nervous system that accounts for up to 40% of all synapses.[1)] Glutamate is extensively distributed throughout the CNS and plays important role in learning, memory. But excessive concentration of glutamate leads to uncontrolled continuous depolarization of neurons, called excitotoxicity.[2)] Extracellular overloaded glutamate binds and activates N-methyl-D-aspartate glutamatergic receptors (NMDARs). NMDAR is a glutamate receptor and ion channel highly permeable to Na^+, K^+ and Ca^{2+} ions. At normal physiological membrane potential, Mg^{2+} ions bind to the pores on the NMDAR blocking the passage of other cations through the receptor. At depolarization, Mg^{2+} ions are released from the pores, allowing a voltage-dependent flow of Na^+ and Ca^{2+} ions into the cell and leading to postsynaptic depolarization and action potential in the postsynaptic neuron. Normally, NMDARs-mediated Ca^{2+} influx regulates physiological processes such as cell growth, differentiation, and synaptic activity and higher cognitive functions like learning and memory. But when neuron get ischemic or traumatic injury, it

releases excessive dose of glutamate causing postsynaptic neuron continuously depolarized. Persistent depolarization results in excessively high intracellular calcium concentrations leading to neuronal cell death which is called glutamate excitotoxicity. Chronic glutamate excitotoxicity eventually causes neurodegenerative disease such as Alzheimer's disease, Parkinson's disease and Huntington's disease. And preventing glutamate excitotoxicity can prevent the progression of neurodegenerative disease. Memantine approved by the FDA in 2003 as a treatment of Alzheimer's disease is an uncompetitive NMDA receptor antagonist that inhibits glutamate overactivation.

How glutamate toxicity causes CNS disorders is not fully understood and complex. Excessive influx of Ca^{2+} through glutamate receptors induces rising of the Ca^{2+} concentration in the sensitive organelles like mitochondria promoting mitochondrial swelling and depolarizing its membrane.[2] Mitochondrial dysfunction leads cytotoxic cascades to produce reactive oxygen species (ROS), which induces neuronal cell apoptosis and/or necrosis [3),4)]

And it also has been reported that endoplasmic reticulum (ER) stress is closely related with neuronal cell injury.[5] ER is crucial cell organelle where protein translocation, folding, and post-translational modification occur. And ER also stores calcium and control its concentration. Unfolded proteins and calcium disequilibrium termed as ER stress disturb ER functions and activate signaling an adaptive pathway to restore protein homeostasis.[6] Chaperone Bip/Grp78 (Bip) sensed ER dysfunction and it activates ER sensor proteins such as PRK (RNA-dependent protein kinase)-like ER protein kinase (PERK), eukaryotic translation initiation factor 2 subunit α (eIF2α) kinase, activating transcription factor-6 (ATF6), and the inositol-requiring enzyme 1 (IRE1), which in turn activate signaling cascades mediating the ER stress response. ER stress response promotes expression of chaperones, ER-mediated degradation and inhibits protein translation.[7] However despite these efforts, uncompensated severe stress causes cell apoptotic signaling.[8] Because glutamate excitotoxicity stimulating ER stress can causes neuronal cell injury, ER stress may be one of causes of CNS disorders by glutamate excitotoxicity. [9] And also, inhibition of ER stress caused by glutamate receptor

overstimulation had been reported to have neuro-protective effects.[5)]

Recently, researchers have been interested in subcellular multiprotein complexes known as inflammasomes. Among them, the nucleotide-binding and oligomerization domain-like receptor containing a pyrin domain 3 (NLRP3) inflammasome attracted their attention. It is abundant in the CNS and mediate innate immunity. It recognizes various stimuli like Aβ and makes multiprotein complex with active caspase-1, leading to releasing of interleukin-1β (IL-1β) and IL-18. [10),11)] And they in turn promotes T cell differentiation, releasing many inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF).[12)] And persistent inflammation eventually leads to neuronal cell dysfunction and apoptosis.[13)] This way, NLRP3 inflammasome mediated signaling can promote neurodegenerative disease. Some reports have demonstrated that high level of plasma IL-1β have been found in neurodegenerative disease. [14)] And also pharmacological interference with persistent NLRP3 inflammasome activation may be a therapeutic strategy of treating glutamate-induced toxicity in neurodegenerative diseases.

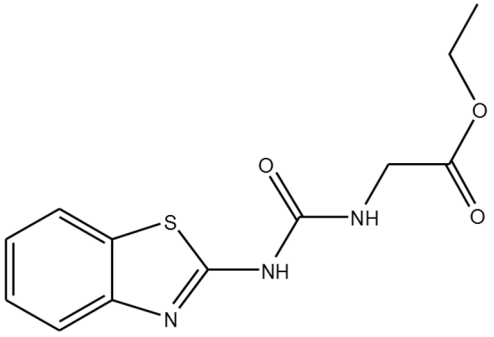
Previous studies have reported that thiazole analogues acts as a glutamate antagonist due to its high affinity to glutamate receptors in addition to its anti-inflammatory effect.[15),16)] Furthermore, the drug-like physical properties of thiazole analogues suggest their possible use as a novel class of orally active mGluR1 antagonists.[17),18)] However, the detailed mechanism of action of thiazole analogues has not yet been elucidated. We synthesized a novel thiazole derivative, KHG21834, and showed that this compound suppressed oxidative stress and inflammation caused by amyloid β in neuronal cells.[19) 20)] This study assessed the effects of KHG21834 on glutamate-induced toxicity in cultured SH-SY5Y cells and investigated the mechanisms underlying an effective strategy for the treatment of glutamate-related neurodegenerative diseases.

Materials and methods

2.1. Materials

Glutamate, penicillin, streptomycin, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), $CaCl_2$, and Fura-2 AM were purchased from Invitrogen (Carlsbad, CA, USA). Anti-CHOP, anti-GRP78, anti-NLRP3, anti-cleaved caspase-1, anti-Bcl2, anti-Bax, anti-caspase-3, anti-p38, anti-p-p38, anti-JNK, anti-p-JNK, and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). KHG21834, a benzothiazole derivative, was synthesized as described (Fig. 1) .[19] The chemical properties of KHG21834 are follows; mp 282°C, ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.21 (t, J = 7.10 Hz, 3H, ethyl-CH_3), 3.96 (d, J = 5.8 Hz, CH_2), 4.11 (q, J = 7.10 Hz, 2H, ethyl-CH_2), 6.95 (br s. 1H, NH), 7.08–7.89 (m, 4H ArH), 11.1 (br s. 1H, NH). The purity of the compound was over 98% based on

the NMR spectral and melting point data. All other commercial reagents were of the highest available purity.



[Fig. 1] Chemical structure of KHG21834

2.2. Cell culture and drug treatment

Human neuroblastoma SH-SY5Y cells were cultured in DMEM containing 10% FBS and 100 units/ml penicillin/streptomycin/glutamine at 37 °C in a humidified environment with 5% CO_2 as described.[21] Cells were passaged using a solution containing 0.05% trypsin in 0.02% ethylenediaminetetraacetic acid (EDTA). Confluent cells were washed with DMEM and cultured in serum-free medium for 48 h. These cells were subsequently pretreated with

different concentrations of KHG21834 for 2 h, followed by 10 mM glutamate for 24 h. Glutamate concentration, 10 mM, was chosen to test protection by KHG21834 on the basis of previous studies, where 10 mM glutamate was used to examine the effect of tanshinone IIA on glutamate toxicity in SH-SY5Y cells[22] and the effect of berberine against glutamate-induced oxidative stress and apoptosis in PC12 cells.[23] KHG21834 was dissolved as a stock solution (10 mM) in DMSO and diluted to the desired concentrations with treatment medium. Equivalent volumes of DMSO were used for controls and glutamate-treated cells throughout the study.

2.3. Determination of cell viability

SH-SY5Y cells were seeded at a density of 5 x 10^5 cells/mm^2 in 96-well plates and cell viability was determined by the MTT assay. The absorbance of each well at 595 nm was measured using a microplate reader (Molecular Devices Corp., Sunnyvale, CA) as described.[20] The level of

lactate dehydrogenase (LDH) released from damaged cells into the medium was measured using an LDH assay kit (Roche, Mannheim, Germany), as described.[19)]

2.4. Measurement of reactive oxygen species and MDA contents

The generation of reactive oxygen species was monitored using 2′,7′-dichlorofluorescin diacetate (DCF-DA), which is oxidized to the green fluorescent compound dichlorofluorescein (DCF) by reactive oxygen species.[20)] Fluorescence intensity was measured in the dark using a SpectraMax GEMINI XS fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Malondialdehyde (MDA) level, an index of lipid peroxidation, was measured as described with a slight modification.[24)] Briefly, samples (500 μl) were suspended in a reaction mixture containing trichloroacetic acid (15%, 400 μl) and 2-thiobarbituric acid (TBA) 0.67%/butylated hydroxytoluene 0.01% (800 μl). MDA reacts with TBA to form a fluorescent adduct. The

mixture was heated for 30 min at 95°C and centrifuged at 4000×g for 10 min at 4°C. The supernatant was transferred to a 96-well plate and fluorescence intensity was measured at excitation and emission wavelengths of 430 and 550 nm, respectively.

2.5. Measurement of enzyme activities and GSH levels

Superoxide dismutase (SOD) activity was determined by monitoring inhibition of the autoxidation of pyrogallol.[25] Briefly, the reaction was initiated by 0.2 mM pyrogallol in Tris-HCl buffer (pH 8.2, 50 mM) and 1 mM EDTA, and the decrease in absorbance at 420 nm was spectrophotometrically recorded. Under these conditions, 1 unit of activity was defined as the amount of SOD required to inhibit the rate of pyrogallol auto-oxidation by 50%.

Glutathione peroxidase (GPx) activity was measured by a coupled reaction with glutathione reductase (GR).[20] Cell lysates were added to the GPx-detection working solution (10 mM NADPH, 84 mM GSH, GR, 15 mM t-Bu-OOH) in a 96-well plate, and the absorbance at 340

nm was measured using a microplate reader (Molecular Devices Corp., Sunnyvale, CA).

Reduced glutathione (GSH) concentration was assayed as described with minor modifications.[20] The reaction mixture contained 100 μl of 5′,5′-dithio-bis(2-nitrobenzoic acid) (6 mM), 25 μl of protein-free extracts, 875 μl of NADPH (0.3 mM), and 10 μl of GSH reductase (10 U/ml), and the change in absorbance at 412 nm was measured spectrophotometrically.

The activity of caspase-4 was determined using commercially available caspase-4 assay kit (Abcam, Cambridge, MA) according to the manufacturer's instructions, based on spectrophotometric detection of the chromophore p-nitroanilide (p-NA) after cleavage from the labeled substrate LEVD-p-NA using a micro plate reader at 405 nm.

2.6. Measurement of mitochondrial membrane potential, ATP level, Ca^{2+} influx, and cytokines

Mitochondrial membrane potential change was determined as described, using the

mitochondrial membrane potential-sensitive fluorescence dye JC-1 at an excitation wavelength of 480 nm and emission wavelengths of 530 nm and 590 nm[20]. The ratio of the intensity of green fluorescent monomers (measured at 530 nm) to the intensity of fluorescent aggregates of JC-1 (measured at 590 nm) is directly related to mitochondrial membrane potential.

ATP content was assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, MA, USA) and by measuring the absorbance at 570 nm, according to the manufacturer's instructions. [21] The ATP concentration of each sample was calculated from a calibration curve made with ATP standards and normalized to total protein content.

The content of intracellular Ca^{2+} was measured using Fura-2 AM. [4),26)] Briefly, cells were loaded with Fura-2 AM by incubation in HBSS containing 5 mM $CaCl_2$ and 2 μM Fura-2 AM at 37°C for 30 min, followed by three washes in HBSS solution containing 5 mM $CaCl_2$. Fura-

2 AM loaded cells were resuspended in 200 μl HBSS solution containing 5 mM $CaCl_2$. Intracellular Ca^{2+} level was determined by measuring Ca^{2+}-dependent fluorescence intensity at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm.

Levels of IL-1β and IL-6 were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.[21)]

2.7. Western blotting analysis

Cell lysates were analyzed by western blotting with the appropriate primary antibodies to determine the levels of CHOP, GRP78, NLRP3, cleaved caspase-1, Bcl2, Bax, caspase-3, p38, p-p38, JNK, and p-JNK.[20)] Protein concentration was determined using bovine serum albumin as the standard and all blots were normalized against anti-β-actin antibody to confirm equal protein loading. Immunoreactive bands were detected using an enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham Bioscience, Piscataway, NJ, USA).

2.8. TUNEL staining

TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay was performed in accordance with manufacturer specifications with the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI, USA). The TUNEL positive cells with green fluorescent staining were defined as apoptotic cell death. For cell counts, TUNEL positive cells were counted manually in three different images of each of three coverslips by three individuals blinded to the experiment.

2.9. Statistical analysis

Statistical comparisons were performed by single-factor ANOVA, followed by Tukey's *post hoc* test, to control for Type I errors. A P value less than 0.01 was considered statistically significant. Data from three independent experiments were averaged and reported as means ± standard deviation (S.D.).

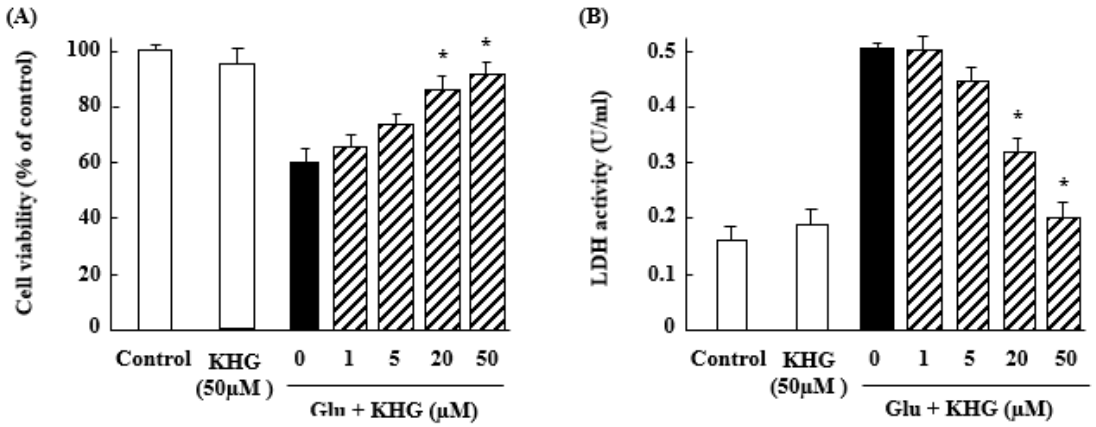
Results

3.1. Effects of KHG21834 on glutamate-induced toxicity in cultured SH-SY5Y cells

To study the effects of KHG21834 on glutamate-induced toxicity, SH-SY5Y cells were pretreated with various concentrations of KHG21834 for 2 h before exposure to 10 mM glutamate for 24 h, and cell viability was determined by MTT assays. Incubation with glutamate reduced cell viability to 60% of control (Fig. 2A); however, pretreatment with KHG21834 dose-dependently attenuated the glutamate-induced reduction of cell viability. Cells pretreated with 50 μM KHG21834 showed 92% viability compared with control cells (Fig. 2A). Moreover, treatment with KHG21834 alone, at concentrations up to 50 μM, had no effect on cell viability.

LDH release is an indicator of cellular damage. Exposure of SH-SY5Y cells to 10 mM glutamate for 24 h increased LDH release up to 3-fold compared with control cells (Fig. 2B). Pretreatment of SH-SY5Y cells with KHG21834 dose-dependently decreased glutamate

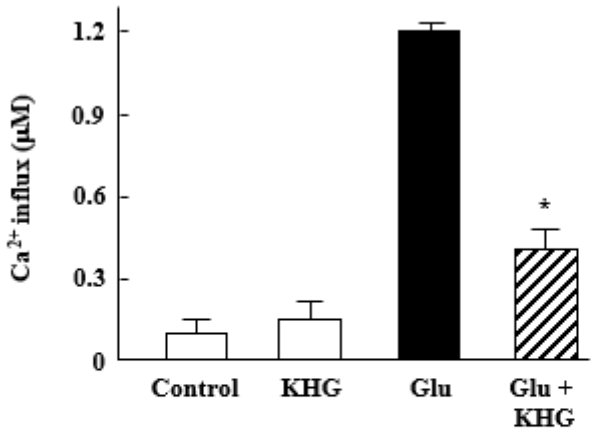
toxicity, as shown by reduced LDH leakage. For example, cells pretreated with 50 μM KHG21834 significantly reduced LDH leakage by approximately 60% (Fig. 2B). These results suggest that KHG21834 effectively prevents glutamate-induced toxicity in SH-SY5Y cells.



[Fig. 2] Effects of KHG21834 on glutamate-induced toxicity in cultured SH-SY5Y cells. (A) Relative viability of SH-SY5Y cells by MTT assays. (B) Relative LDH activity of SH-SY5Y cells. Data represent the means ± S.D. of three independent experiments. $^{*}P < 0.01$ versus glutamate-treated cells.

3.2 *KHG21834 reduced glutamate-induced intracellular calcium ion concentration in SH-SY5Y cells*

Glutamate-induced toxicity has been reported to result in a loss of Ca^{2+} homeostasis. Intracellular high Ca^{2+} concentration promotes dysfunction of mitochondria and endoplasmic reticulum. We therefore measured glutamate-induced intracellular Ca^{2+} levels using a calcium-specific indicator dye, Fura-2 AM, in the absence and presence of KHG21834. Ca^{2+} influx was markedly higher in glutamate-treated than in control cells, an increase significantly suppressed by KHG21834 treatment (Fig. 3), suggesting that the protective effects of KHG21834 may be closely related with its regulation of glutamate-induced Ca^{2+} influx.

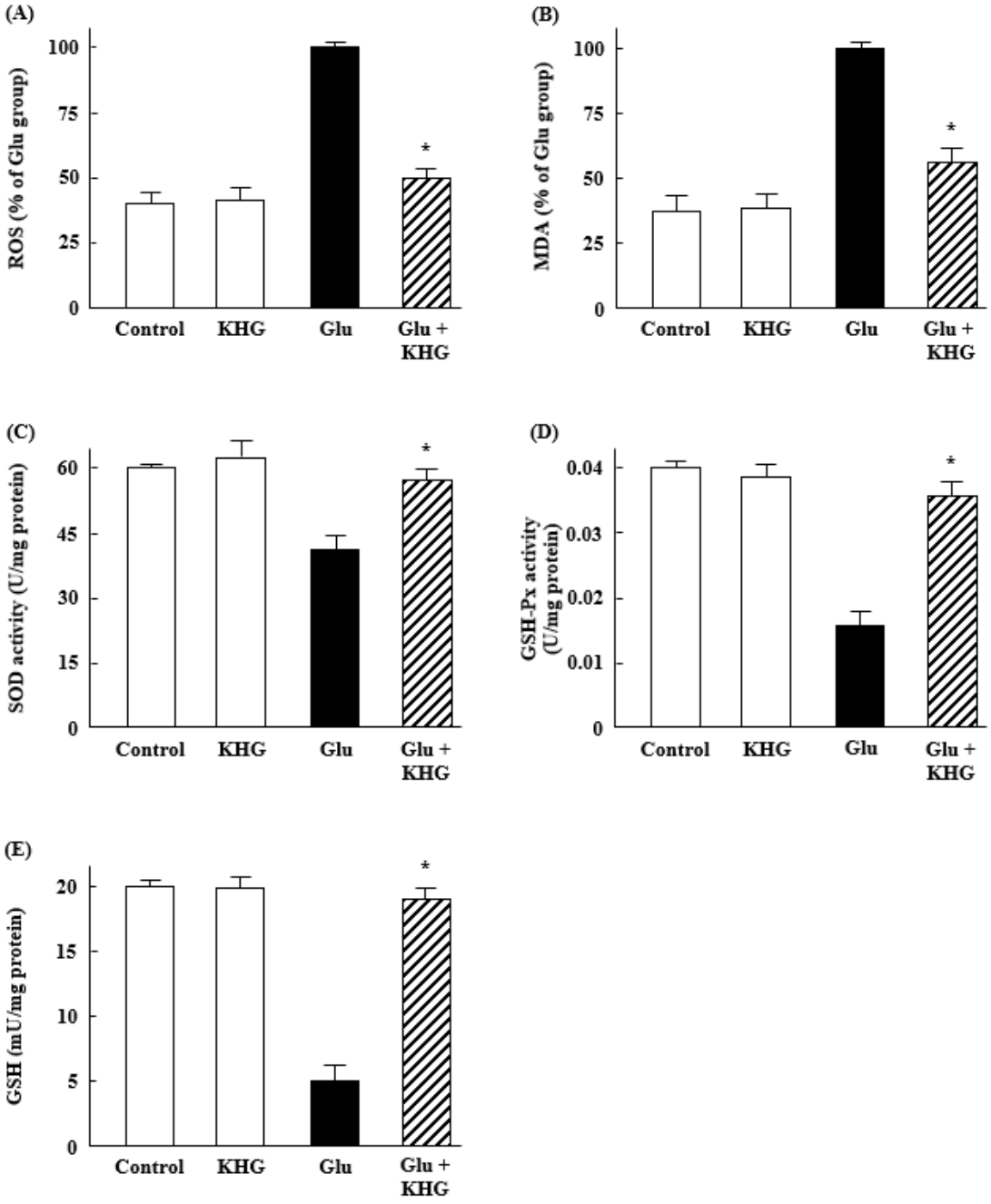


[Fig. 3] Effects of KHG21834 on glutamate-induced intracellular Ca^{2+} influx in SH-SY5Y cells. Data represent the means ± S.D. of three independent experiments. $^{*}P < 0.01$ versus glutamate-treated cells.

3.3. KHG21834 suppresses glutamate-induced oxidative stress in SH-SY5Y cells

The pathological condition induced by glutamate is associated with oxidative stress and accompanied by lipid peroxidation.[23] Therefore, we examined whether the protective effect of KHG21834 against glutamate-induced toxicity was associated with suppression of excessive production of reactive oxygen species, a cause of oxidative stress, and MDA, a major lipid peroxide involved in reactive oxygen species-mediated oxidative stress. Treatment of H_2DCF-DA-loaded SH-SY5Y cells with glutamate increased fluorescence intensity up to 3-fold, suggesting enhanced reactive oxygen species generation (Fig. 4A). Glutamate-exposed cells also showed an increase in MDA content compared with control cells (Fig. 4B). Pretreatment with KHG21834, however, significantly attenuated the upregulated levels of both reactive oxygen species and MDA (Fig. 4A-B), demonstrating that KHG21834 can attenuate glutamate-induced oxidative stress by reducing the generation of reactive oxygen species and lipid peroxidation.

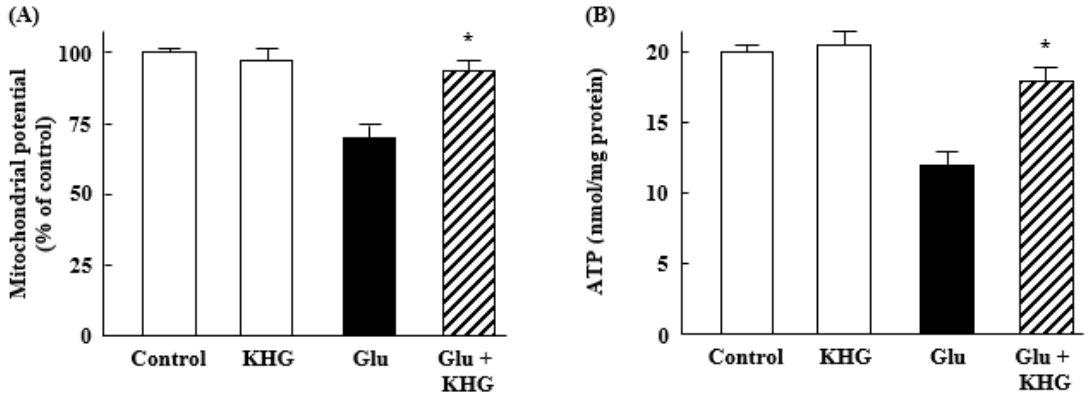
Antioxidant enzymes such as SOD and GSH-Px, which degrade reactive oxygen species directly, are central components in antioxidant defenses against reactive oxygen species production.[27] In addition, GSH is a substrate for various peroxidases and plays important roles in cellular defenses against reactive oxygen species overproduction.[28),29] Treatment of SH-SY5Y cells with glutamate reduced the activities of SOD and GSH-Px as well as GSH levels (Fig. 4C-E), suggesting that glutamate-induced reactive oxygen species overproduction and lipid peroxidation is closely accompanied by changes in antioxidant enzyme activities and GSH level. Pretreatment of these cells with KHG21834, however, attenuated the marked reductions in SOD and GSH-Px activities (Fig. 4C-D) and GSH levels (Fig. 4E), indicating that KHG21834 possesses potent antioxidant activities in SH-SY5Y cells exposed to glutamate.



[Fig. 4] Effects of KHG21834 on glutamate-induced oxidative stress in SH-SY5Y cells. Levels of reactive oxygen species (A), MDA level (B), SOD activity (C), GSH-Px activity (D), and GSH (E) were measured as described in Materials and Methods. Data represent the means ± S.D. of three independent experiments. $^{*}P < 0.01$ versus glutamate-treated cells.

3.4. Effects of KHG21834 on glutamate-induced mitochondrial potential and ATP level in SH-SY5Y cells

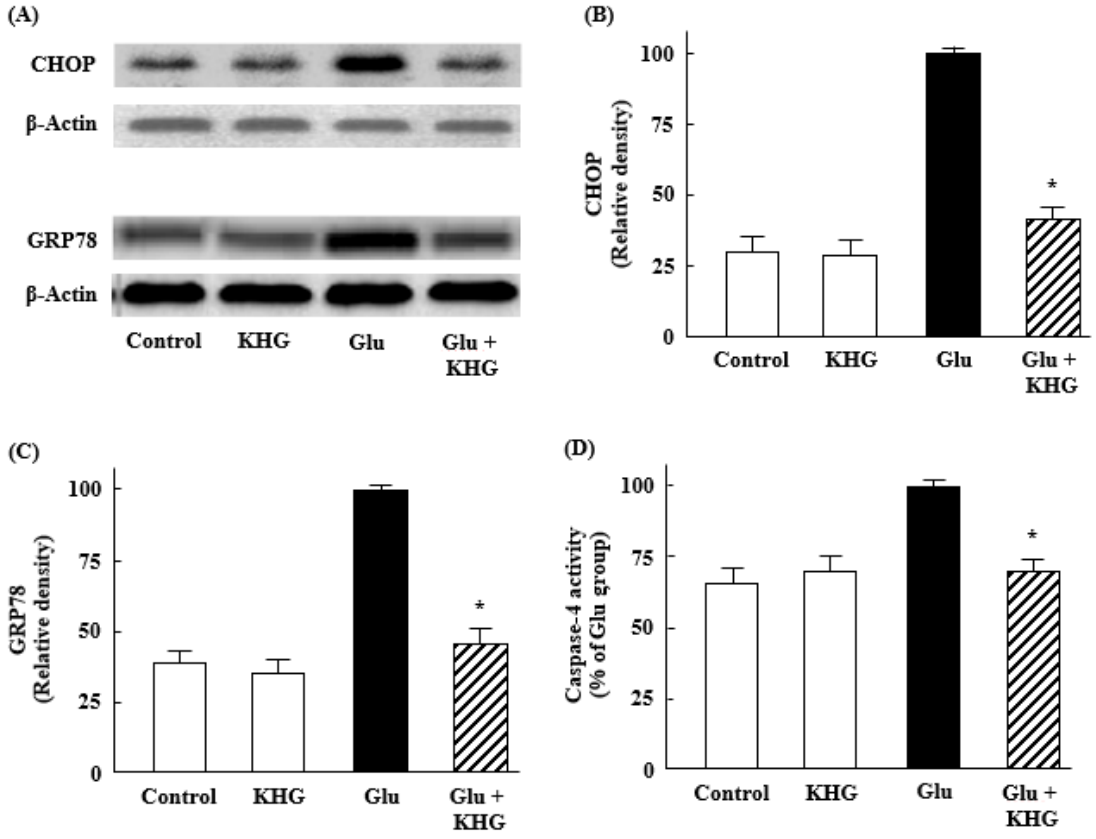
High glutamate levels have been reported to reduce mitochondrial membrane potential representing the collapse of mitochondrial membrane function.[30] In addition, the NLRP3 inflammasome-mediated release of IL-1β is closely related to overall mitochondrial function.[13),21] Treatment of SH-SY5Y cells with glutamate reduced mitochondrial membrane potential up to 70% compared with control cells, but KHG21834 treatment resulted in almost complete recovery of mitochondrial membrane potential (Fig. 5A). Because mitochondrial dysfunction can lead to defects in ATP synthesis, we also assessed the effect of KHG21834 on glutamate altered ATP levels. Treatment with glutamate reduced ATP content by approximately 40% compared with control cells, a reduction restored by pretreatment with KHG21834 (Fig. 5B). Taken together, these results indicate that KHG21834 can maintain mitochondrial function under conditions of glutamate-induced toxicity.



[Fig. 5] Effects of KHG21834 on glutamate-induced mitochondrial membrane potential (A), ATP level (B) in SH-SY5Y cells. Data represent the means ± S.D. of three independent experiments. $^{*}P < 0.01$ versus glutamate-treated cells.

3.5. Effects of KHG21834 on glutamate-induced ER stress in SH-SY5Y cells

ER stress has been associated with glutamate-induced neuronal cell death.[31),32)] CHOP is a leucine zipper transcription factor and GRP78 is an ER molecular chaperone protein[33)], both of which are upregulated during ER stress and are therefore important components of ER-induced cell death.[34),35)] We therefore investigated the effects of KHG21834 on glutamate-induced ER stress by measuring the expression of these ER stress-related proteins. Western blotting showed that glutamate increased the levels of expression of CHOP and GRP78 up to 3.3-fold and 2.7-fold, respectively, compared with control cells (Fig. 6A-C). However, KHG21834 treatment significantly attenuated these glutamate-induced increases in CHOP and GRP78 proteins by approximately 60% and 55%, respectively (Fig. 6A-C). Activation of caspase-4 has been found to lead to ER stress-induced apoptosis of human neuronal cells. Treatment with glutamate increased caspase-4 activity, whereas KHG21834 treatment reduced the increase of caspase-4 activity almost to control levels (Fig. 4D). These results suggested that KHG21834 treatment effectively regulates glutamate-induced ER stress in SH-SY5Y cells.



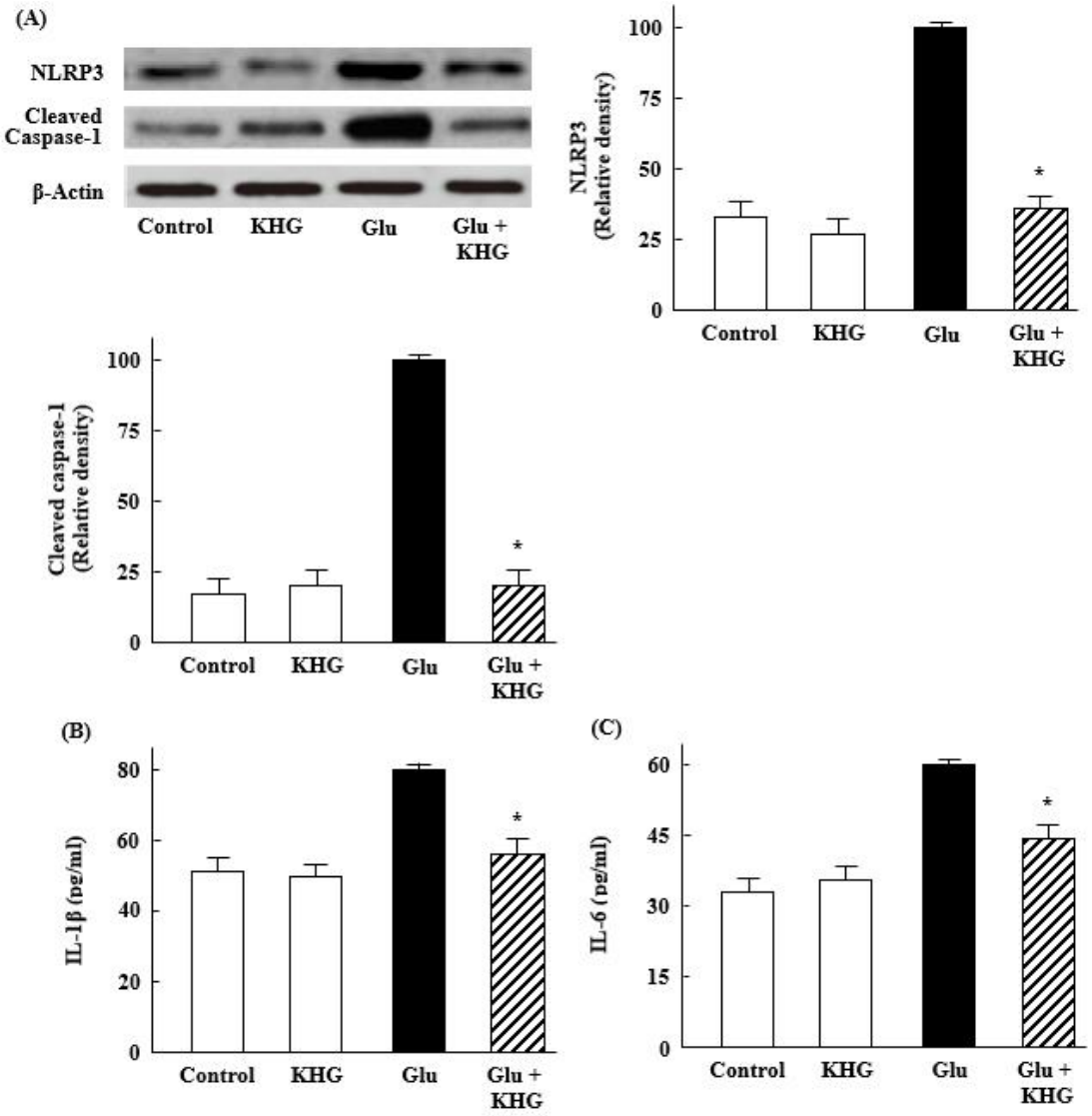
[Fig. 6] Effects of KHG21834 on glutamate-induced ER stress in SH-SY5Y cells. Protein levels of CHOP and GRP78 (A–C) were determined by Western blot analyses. The intensities of the bands were quantified by densitometric analyses and normalized to the expression of β-actin in the same samples. (D) Caspase 4 activity in total cell lysates. Data represent the means ± S.D. of three independent experiments. $^{*}P < 0.01$ versus glutamate-treated cells.

3.6. KHG21834 suppressed glutamate-induced NLRP3 inflammasome activation in SH-SY5Y cells

Glutamate-induced toxicity is closely associated with neuroinflammatory pathologies, with activation of NLRP3 inflammasomes mediating IL-1β and IL-6 production in a manner dependent on caspase-1 cleavage.[21),36)] Therefore, NLRP3 inflammasome activation may be an important pathway by which glutamate induces toxicity in neuronal cells.[13),36)] The effects of KHG21834 on the NLRP3 inflammasome-mediated signaling pathway associated with glutamate-induced toxicity was therefore assessed in SH-SY5Y cells. Western blotting analysis showed that the expression of NLRP3 protein was markedly increased in response to glutamate stimulation, an increase attenuated by treatment with KHG21834 (Fig. 7A). Similarly, glutamate upregulated the expression of cleaved caspase-1, an increase attenuated by KHG21834 to a level statistically indistinguishable from that of control cells (Fig. 7A).

KHG21834 also suppressed glutamate-induced increases in the production of NLRP3

inflammasome, IL-1β and its downstream target, IL-6 (Fig. 7B-C), suggesting that the mechanisms underlying the effects of KHG21834 on glutamate-induced toxicity may involve mediators of inflammation. Because KHG21834 alone did not display significant toxicity toward SH-SY5Y cells, the anti-inflammatory effects of KHG21834 were not attributable to drug-induced cell death. Taken together, these results indicate that KHG21834 protected neuronal cells against glutamate toxicity by reducing ER stress and subsequently suppressing NLRP3 inflammasome activation.



[Fig. 7] KHG21834 suppressed glutamate-induced NLRP3 inflammasome activation in SH-SY5Y cells. Protein levels of NLRP3 and cleaved caspase-1 (A) were determined by Western blot analyses. The intensities of the bands were quantified by densitometric analyses and normalized to the expression of β-actin in the same samples. IL-1β (B) and IL-6 (C) levels in medium were determined by ELISA. Data represent the means ± S.D. of three independent experiments. $^{*}P < 0.01$ versus glutamate-treated cells.

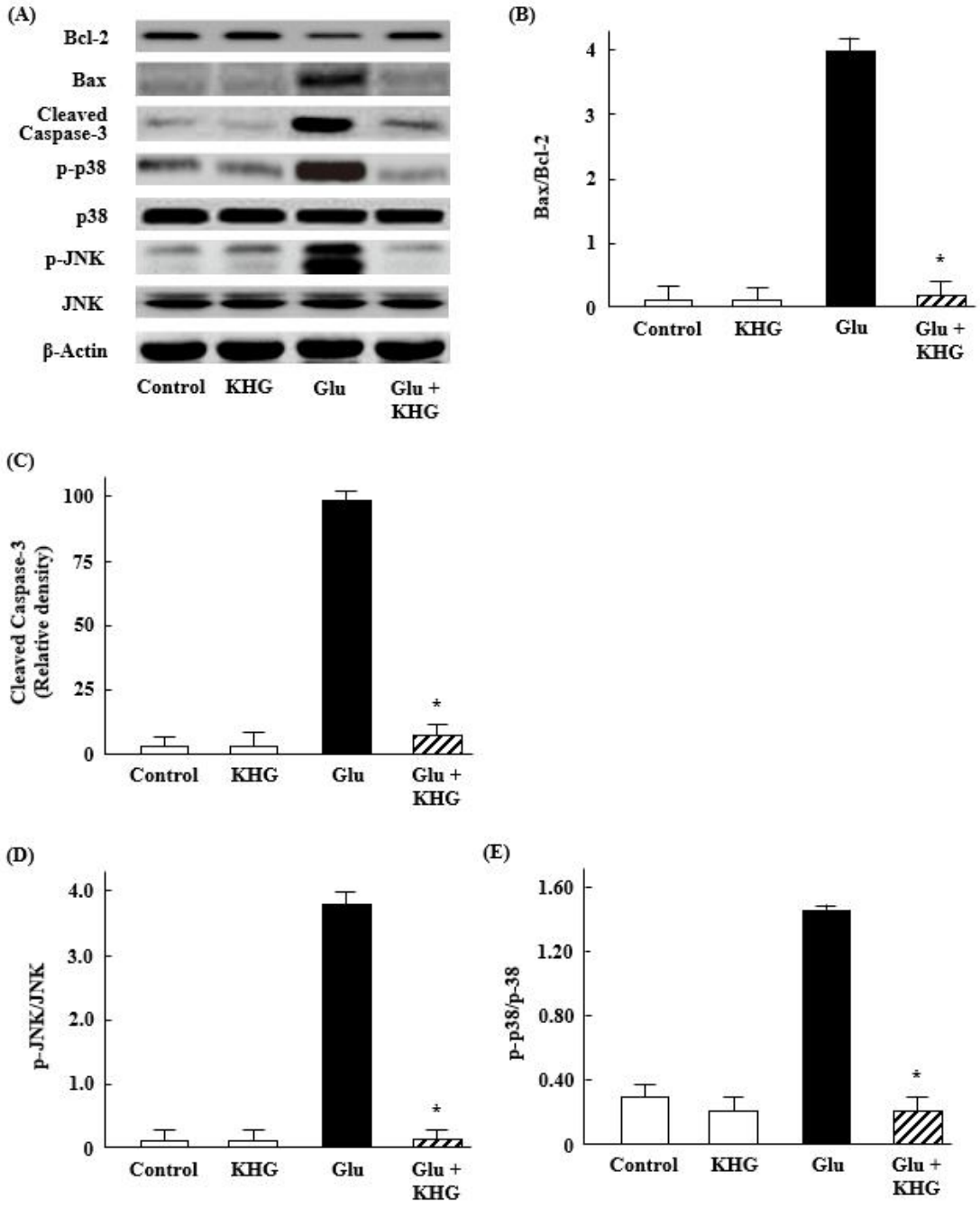
3.7. Effects of KHG21834 on apoptosis and apoptosis-related proteins in glutamate-treated SH-SY5Y cells

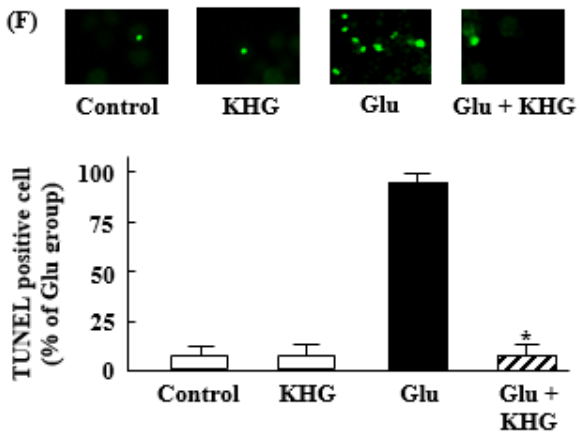
Because caspase family proteases and Bcl-2 family proteins play important roles in ER stress and mitochondria-dependent apoptosis, the effect of KHG21834 on glutamate-induced apoptosis was investigated by measuring the expression of apoptosis-related proteins. Western blotting analysis showed that glutamate markedly upregulated the Bax/Bcl-2 ratio, cleaved caspase-3 levels compared with control cells (Fig. 8A-C). However, treatment with KHG21834 significantly reversed these effects of glutamate, suggesting that KHG21834 can prevent glutamate-induced apoptosis by regulating apoptotic signals, such as the expression of Bcl-2 family proteins and caspase family proteases.

Glutamate has been reported to induce neuronal apoptosis by activating MAPK, a factor associated with various stress signaling pathways.[37] We found that treatment with 10 mM glutamate for 24 h increased the levels of the phosphorylated MAPK forms p-p38 MAPK and

p-JNK, but had no effect on the protein levels of p38 MAPK and JNK (Fig. 8A, D-E). Interestingly, KHG21834 significantly reduced the levels of p-p38 MAPK and p-JNK increased by glutamate. These results demonstrate the glutamate-induced activation of MAPK was effectively attenuated by KHG21834.

Finally, the effects of KHG21834 on the glutamate-induced apoptosis were also confirmed by TUNEL assay. Our results showed that glutamate caused widespread TUNEL staining compared to that in the control group, showing apoptotic cell death by glutamate (Fig. 8F). Once again, KHG21834 treatment significantly attenuated the glutamate-induced TUNEL staining (Fig. 8F), indicating its regulatory function for the apoptotic process induced by glutamate.


(A)
Bcl-2
Bax
Cleaved Caspase-3
p-p38
p38
p-JNK
JNK
β-Actin
Control
KHG
Glu
Glu + KHG
(B)
Bax/Bcl-2
0
1
2
3
4
*
Control
KHG
Glu
Glu + KHG
(C)
Cleaved Caspase-3 (Relative density)
0
25
50
75
100
*
Control
KHG
Glu
Glu + KHG
(D)
p-JNK/JNK
0
1.0
2.0
3.0
4.0
*
Control
KHG
Glu
Glu + KHG
(E)
p-p38/p-38
0
0.40
0.80
1.20
1.60
*
Control
KHG
Glu
Glu + KHG



[Fig. 8] Effects of KHG21834 on apoptosis and apoptosis-related proteins in glutamate-treated SH-SY5Y cells. Western blot analysis of the effects of KHG21834 on the protein levels of Bcl-2, Bax. cleaved caspase-3, p-p38, and p-JNK in glutamate-treated SH-SY5Y cells. (A) Western blot analysis was performed using antibodies against Bcl-2, Bax, cleaved caspase-3, p-p38, and p-JNK, with β-actin used as a loading control. (B–E) Relative protein levels were quantified by densitometry and normalized relative to the expression of β-actin in the same samples. (F) TUNEL assay. Data represent the means ± S.D. of three independent experiments. $^{*}P < 0.01$ versus glutamate-treated cells.

Discussion

Compelling evidence supports the hypothesis that an glutamate excitotoxicity is closely associated with the pathological processes of various neurological disorders.[3),38)] However, no consensus molecular targets or promising strategies to alleviate glutamate toxicity are currently available. We previously reported that KHG21834 suppressed oxidative stress and inflammation induced by amyloid β in neuronal cells.[19),20)] We therefore further investigated the ability of KHG21834 to protect against glutamate-induced toxicity in cultured SH-SY5Y cells and to elucidate its underlying mechanism of action. The present study showed that treatment with 10 mM glutamate reduced the viability of SH-SY5Y cells while enhancing cell apoptosis and LDH release, demonstrating that glutamate induces toxicity in neuroblastoma cells. By contrast, KHG21834, at concentrations up to 50 μM, did not affect the viability of SH-SY5Y cells. Cell viability was higher in the KHG-treated group than in the no KHG-treated group, and the amount was proportional to the drug concentration. It indicates that KHG21834 can protect neuroblastoma cells against glutamate-associated toxicity and

KHG21834 may have therapeutic potential in glutamate-related neurodegenerative diseases.

Glutamate excitotoxicity followed by excessive intracellular Ca^{2+} concentration may induce dysfunction of mitochondria and endoplasmic reticulum resulting in metabolic failure, ROS production and finally cell death. In this study, we observed that glutamate dramatically increased intracellular Ca^{2+} levels, whereas KHG21834 significantly reduced these glutamate-induced increases in Ca^{2+} levels, indicating that KHG21834 may have protective effect against glutamate excitotoxicity by suppressing glutamate-induced Ca^{2+} influx.

High intracellular Ca^{2+} level leads mitochondrial dysfunction by reducing mitochondrial membrane potential.[30] In addition, the NLRP3 inflammasome-mediated release of IL-1β is closely related to overall mitochondrial function.[13),21] So we examined the mitochondrial membrane potential and ATP level of glutamate and KHG21834 treated cells as well as of only glutamate treated ones. Interestingly, we found that KHG21834 could restore the glutamate-induced mitochondrial dysfunction, as indicated by increases in both membrane potential and

ATP level. These findings indicated that KHG21834 can maintain and restore mitochondrial function under conditions of glutamate excitotoxicity.

Glutamate excitotoxicity induces oxidative stress and in turn can induce depletion of GSH.[39),40)] The production of reactive oxygen species is an important feature of lipid peroxidation and mitochondrial dysfunction in glutamate-induced neuronal damage.[41),42)] The present study found that glutamate increased reactive oxygen species and MDA levels in SH-SY5Y cells, resulting in an oxidant-antioxidant imbalance that made cells vulnerable to oxidative stress. Such oxidative stress can be scavenged by activating endogenous antioxidant defense systems, including antioxidant enzymes like SOD and GSH-Px, to maintain cellular homeostasis. In addition, the non-enzymatic antioxidant GSH is indicative of oxidative stress and plays an important role in removing free radicals as well as in regulating of the cellular redox state.[43)] Therefore, regulating oxidative stress may a promising strategy to counteract glutamate-induced toxicity. We found that KHG21834 could suppress glutamate-induced increases in reactive oxygen species level, MDA level, and enhance glutamate-attenuated activities of SOD,

GSH-Px and GSH level, suggesting that KHG21834 has antioxidant properties.

Glutamate excitotoxicity-induced cell apoptosis is partly caused via ER dysfunction. Unfolded proteins and Ca^{2+} disequilibrium promote ER stress, triggering apoptotic signaling.[9),44)] Several molecules are related to cell apoptosis. In ER stress condition, one of ER molecular chaperone proteins - GRP78 are upregulated to maintain ER homeostasis.[45)] Interestingly, overexpression of GRP78 was found to be accompanied by cell death induced by oxidative stress and Ca^{2+} disturbances.[46)] Other important component of ER-induced cell apoptosis is C/EBP homologous protein (CHOP). CHOP is a basic leucine zipper transcription factor. It is interspersed in cells at low concentrations and expression is increased by ER stress. Overexpressed CHOP by ER stress results in promoting apoptotic signaling by directly affecting cell death genes and inhibiting the antiapoptotic protein B-cell leukemia/lymphoma 2 (Bcl-2). In this report, we observed that glutamate elevated the levels of expression of GRP78 and CHOP and also found that KHG21834 reduced the glutamate-enhanced levels of GRP78 and CHOP expression in SH-SY5Y cells, indicating that KHG21834 efficiently inhibits

glutamate-induced ER stress. One of the mechanisms by which KHG21834 protects glutamate-treated SH-SY5Y cells may therefore be prevention of ER stress.

NLRP3 inflammasomes detect pathogenic substances and mediate innate immunity, making multiprotein complex with active caspase-1. And this multiprotein complex releases IL-1β and IL-18, in turn IL-6. We observed that glutamate upregulated NLRP3 inflammasomes, enhancing the production of IL-1β and IL-6, in SH-SY5Y cell cultures. Interestingly, KHG21834 significantly attenuated the glutamate-induced enhancement of NLRP3 and cleaved caspase-1 expression, as well as suppressing IL-1β and IL-6 production. These results indicated that KHG21834 inhibited NLRP3 inflammasome activation under glutamate-induced ER stress conditions.

In ER stress, other components like Bcl-2 family and caspases mediate mitochondrial-dependent cell apoptosis. Bcl-2 family plays an important role in the mitochondria to trigger cytochrome c release and apoptosome assembly. Bcl-2 family is subdivided into pro-apoptotic

members such as BAX/BAK and anti-apoptotic members. ER stress makes IRE1α – ER stress sense protein - autophosphorylation with forming a protein complex with BAX/BAK. BAX/BAK activation subsequently promote caspase-8-mediated activation of IL-1β, NLRP3 inflammasome-mediated caspase-1-dependent IL-1β maturation and apoptotic effector caspases (Caspase-3,-7).[47] And then these processes subsequently promote cell apoptosis by increasing ER membrane permeability and triggering cytochrome c release from mitochondria. IRE1α activation also controls the activation of stress responses involving JNK (*J*un *N*-terminal *K*inase), ERK (*E*xtracellular signal-*r*egulated *k*inase), p38MAPK, NF-κB (*N*uclear *F*actor *k*appa-light-chain-enhancer of activated *B* cells).[5),48)] Activation of caspase-4 has also been found to lead to ER stress-induced apoptosis of human neuronal cells.[49),50)] We observed that KHG21834 increases the expressions of Bcl-2 and reduces TUNEL positive cells and proapoptotic proteins such as cleaved caspase-3,-4 and BAX in glutamate-treated SH-SY5Y cells. In addition, we showed that KHG21834 attenuated glutamate-enhanced phosphorylation levels of p38 and JNK. Therefore, KHG21834 has antiapoptotic properties in regulating Bcl-

2, caspase-3,44, BAX and downstream p38, JNK pathways.

However, further studies are necessary to identify the signaling mechanisms by which KHG21834 attenuates glutamate-induced toxicity in neuronal cells because the protective effects of KHG21834 may involve other pathways. For example, KHG21834 showed antioxidative effects against amyloid β-induced oxidative cell death via the NF-κB, GSK-3β, and β-catenin signaling pathways in cultured cortical neurons[20], pathways that may be involved in glutamate-induced toxicity.[51),52)]

To our knowledge, this is the first report showing that KHG21834 protected SH-SY5Y cells against glutamate-induced toxicity. Additional studies, however, are required to determine the *in vivo* effects and pharmacokinetics of KHG21834.

Conclusions

We exhibited that KHG21834 protected SH-SY5Y cells against glutamate-induced toxicity. KHG21834 didn't disturb cell viability but had effect on reducing glutamate induced high intracellular calcium ion concentration and oxidative stress. It also maintained mitochondrial function and homeostasis against ER stress and its downstream apoptosis signaling induced by glutamate. Persistent glutamate excitotoxicity induces neurodegenerative diseases, so KHG21834 may have protective effect on neurodegenerative diseases.

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Korean abstracts

Glutamate에 유도되는 독성에 대해 길항작용을 가지는 새로운 물질들이 많이 개발되고 있다. 본 연구는 새로운 thiazole 유도체인 KHG21834가 인간 신경모세포 SH-SY5Y에서 glutamate에 의해 유도된 독성에 대해 길항 효과가 있는지 확인하였다. SH-SY5Y 세포에 KHG21834를 처리하였을 때 약물 농도에 따라 glutamate 유도 산화 스트레스를 50 μM 농도에서 50% 까지 줄이는 효과가 있음을 확인하였다. KHG21834는 또한 glutamate에 의해 높아진 세포내 칼슘 이온 농도 및 미토콘드리아 세포막전위, ATP를 줄이는 효과가 있었으며, 소포체 스트레스 및 NLRP3 inflammasome activation을 각각 59%, 65% 까지 줄임을 확인하였다. Glutamate에 의해 유도되는 소포체 스트레스, 미토콘드리아 기능 장애 등으로 인해 결국 세포자연사가 일어나게 되는데, 이때 관여하는 물질들 Bax, Bcl-2, caspase-3, caspase-4, 인산화된 p38 및 JNK 가 glutamate 자극에 의해 증가하였다가 KHG21834를 처리하였을 때 감소함을 확인하였다. 본 연구를 통해 처음으로 KHG21834가 SH-SY5Y 세포에서 gluamate에 의도된 독성에 대해

길항작용을 함을 밝혔고 따라서 glutamate 독성에 대한 후보물질로서의 가능성을 제시하였다.