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Quercetin ameliorates glutamate toxicity-induced neuronal cell death by controlling calcium-binding protein parvalbumin

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ABSTRACT

Background: Glutamate is the main excitatory neurotransmitter. Excessive glutamate causes excitatory toxicity and increases intracellular calcium, leading to neuronal death. Parvalbumin is a calcium-binding protein that regulates calcium homeostasis. Quercetin is a polyphenol found in plant and has neuroprotective effects against neurodegenerative diseases.
Objectives: We investigated whether quercetin regulates apoptosis by modulating parvalbumin expression in glutamate induced neuronal damage.
Methods: Glutamate was treated in hippocampal-derived cell line, and quercetin or vehicle was treated 1 h before glutamate exposure. Cells were collected for experimental procedure 24 h after glutamate treatment and intracellular calcium concentration and parvalbumin expression were examined. Parvalbumin small interfering RNA (siRNA) transfection was performed to detect the relation between parvalbumin and apoptosis.
Results: Glutamate reduced cell viability and increased intracellular calcium concentration, while quercetin preserved calcium concentration and neuronal damage. Moreover, glutamate reduced parvalbumin expression and quercetin alleviated this reduction. Glutamate increased caspase-3 expression, and quercetin attenuated this increase in both parvalbumin siRNA transfected and non-transfected cells. The alleviative effect of quercetin was statistically significant in non-transfected cells. Moreover, glutamate decreased bcl-2 and increased bax expressions, while quercetin alleviated these changes. The alleviative effect of quercetin in bcl-2 family protein expression was more remarkable in non-transfected cells.
Conclusions: These results demonstrate that parvalbumin contributes to the maintainace of intracellular calcium concentration and the prevention of apoptosis, and quercetin modulates parvalbumin expression in glutamate-exposed cells. Thus, these findings suggest that quercetin performs neuroprotective function against glutamate toxicity by regulating parvalbumin expression.

Keywords: Neuroprotection; parvalbumin; quercetin

INTRODUCTION

Glutamate is a major excitatory neurotransmitter that abundantly presents in the central nervous system [1]. It is involved in neuroplasticity, learning, and memory function in the

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Conflict of Interest
The authors declare no conflicts of interest.

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brain [2,3]. However, excessive glutamate causes excitability toxicity and leads to nerve damage. Therefore, the regulation of proper glutamate concentration is required to perform normal brain function [4]. Glutamate excitatory toxicity is associated with N-methyl-D-aspartate receptors, which induce neuronal calcium influx, reactive oxygen species (ROS) generation, and apoptosis [5-7]. It induces various neurodegenerative diseases such as stroke, Alzheimer's disease, and Parkinson's disease [8,9]. It also increases the intracellular calcium concentration, which finally causes cell damage [10,11].

Excessive intracellular calcium triggers oxidative stress, lipid peroxidation, mitochondrial depolarization, and neurodegeneration [12]. Moreover, it activates the caspase cascade signaling pathway and causes apoptotic cell death [13]. Parvalbumin is a calcium-binding protein that widely distributed in various areas of the brain. It plays an important role in maintaining intracellular calcium concentration [14]. Parvalbumin protects the mitochondria from calcium overload by inhibiting a depolarization-induced calcium increase and buffering calcium in an intracellular environment [15]. It modulates synaptic plasticity and regulates neuronal excitability by controlling the electrical properties of neurons [16]. Down-regulation of parvalbumin expression induces ROS formation and nerve damage [17]. Based on previous studies, the down-regulation of calcium-regulating proteins is closely linked to neuronal damage.

Quercetin is a polyphenolic substance that can be easily found in various fruits and vegetables [18]. It acts as a potent antioxidant and exerts beneficial effects on cancer, diabetes, and cardiovascular disease [19-21]. It has neuroprotective effects in various neurodegenerative diseases, including traumatic brain injury and stroke [22,23]. It also protects neurons against glutamate toxicity [24]. Moreover, quercetin exerts neuroprotective effects on styrene oxide-induced neuronal cell death by reducing ROS and controlling calcium concentrations [25]. We have previously shown that quercetin exerts neuroprotective effects by regulating the calcium sensor protein hippocalcin in stroke animal model [26]. Quercetin also alleviates the increase in calcium concentration in neuronal damage caused by glutamate toxicity [26]. We have previously reported that quercetin attenuates the reduction of pavalbumin in stroke animal models [27]. However, the exact molecular biological mechanism for the neuroprotective effect of quercetin against glutamate toxicity has not been fully elucidated. We propose that quercetin regulates calcium-binding protein and protects neurons against glutamate toxicity. Thus, we investigated whether quercetin regulates intracellular calcium concentration and apoptosis-related protein expression through the modulation of parvalbumin expression in glutamate-induced neuronal damage.

MATERIALS AND METHODS

Hippocampal cell line culture

Hippocampal cell line (HT22) was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) that contains heat-inactivated 10% fetal bovine serum (FBS) and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin). HT22 cells were cultured in a humid incubator at 37°C in 5% CO_2 and culture media was replaced to maintain proper nutritional environment. Quercetin (Sigma-Aldrich, USA) was dissolved in phosphate buffer saline (PBS) and PBS was used for vehicle solution. Quercetin (1, 3, and 5 μM) or PBS was supplemented into culture media 1 h before glutamate (5 mM, Sigma-Aldrich) treatment [26]. Cells were collected 24 h after glutamate treatment for further experimental procedure.

Cell viability test

3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out to measure cell viability. HT22 cells were seeded at 5×10^3 cells/well density in 96-well culture plate and incubated for 24 h. Glutamate and/or quercetin were treated as previous described dose. MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37°C in light blocked place. MTT formazan crystals were dissolved into dimethyl sulfoxide and absorbance of 570 nm was measured. Cell viability was measured by the ratio of absorbance value (%) and determined as a percentage of the absorbance of each group to that of PBS group.

Intracellular calcium concentration measurement

Fluo-3-acetoxymethyl (Fluo-3 AM, Thermo Fisher Scientific, USA) was applied to measure the concentration of intracellular calcium as a previously described [28]. HT22 cells were incubated in DMEM containing Fluo-3 AM (3 μM) for 1 h at 37°C and trypsinized by 0.05% trypsin solution. Detached cells were centrifuged at 1,000 g for 10 min and re-suspended in Locke's solution (pH 7.3, 130 mM NaCl, 15 mM glucose, 10 mM HEPES, 5 mM KCl, 2.2 mM $CaCl_2$, and 2 mM $MgCl_2$) at a proper cell density (approximately 1×10^6 cells/mL). Cells were subjected to fluorescence-activated cell sorting (FACS) and intensity of Fluo-3 AM fluorescence was measured by FACSVerse flow cytometer (BD Bioscience, USA). Fluo-3 AM in Locke's solution was excited at 488 nm and measured at 530 ± 20 nm. Measured fluorescence intensity was analyzed by FACSuite software (BD Bioscience). Intracellular calcium concentration was calculated accoding to a previously described formula $[Ca^{2+}]_i = K_d \times (F - F_{min})/(F_{max} - F)$ [29]. Ca^{2+} dissociation constant of Fluo-3 AM is expressed as K_d and is approximately 400 nM at vertebrate ionic strength. F indicates the mean fluorescence intensity of total cell sample at 530 ± 20 nm. F_{max} means the maximal fluorescence intensity of Fluo-3 AM saturated with Ca^{2+} by ionomycin. F_{min} means the minimal fluorescence intensity of Ca^{2+}-free environment made by ethylene glycol tetra acetic acid.

Parvalbumin small interfering RNA (siRNA) transfection

Parvalbumin siRNA transfection was performed according to the manufacturer's instructions. HT22 cells were cultured in DMEM medium and culture medium was changed to DMEM medium without FBS and antibiotics 24 h before transfection. Liposome solution (DMEM containing Lipofectamine 3000, Thermo Fisher Scientific) was mixed with parvalbumin siRNA (Thermo Fisher Scientific) and incubated for 20 min. Mixture was added to culture medium and medium was changed 48 h after transfection. Quercetin (5 μM) or PBS was treated to transfected cells 1 h before glutamate (5 mM) treatment, and transfected cells were collected 24 h after glutamate treatment.

Western blot analysis

Collected cells were lysed in lysis buffer (1% Triton X-100, 1 mM EDTA in PBS [pH 7.4]) contained phenylmethanesulfonyl fluoride (Sigma-Aldrich) and sonicated. Sonicated lysates were centrifuge at 15,000 g for 1 h at 4°C and supernatants were collected. Concentration of proteins was analyzed using bicinchoninic acid protein assay kit (Pierce, USA). Proteins (30 μg) were electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gels at 10 mA for 30 min and consecutively run at 20 mA for 90 min. Loaded gels were transferred to polyvinyl difluoride membrane (Sigma-Aldrich) at 120 V for 2 h. Transferred membranes were incubated with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h to block non-specific reaction and washed with TBST for 10 min. Washed membranes were reacted with anti-parvalbumin (diluted 1:1,000, Thermo Fisher Scientific), anti-caspase-3, anti-cleaved caspase-3, anti-bcl-2, anti-bax (diluted 1:1,000, Cell Signaling Technology,

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USA), and anti-β-actin (diluted 1:1,000, Santa Cruz Biotechnology, USA) for overnight at 4°C. They were washed with TBST and incubated with horseradish peroxide-conjugated anti-rabbit IgG or anti-mouse IgG (diluted 1:5,000, Cell Signaling Technology) for 2 h at room temperature. Membranes were washed with TBST for 10 min and reacted with enhanced chemiluminescence reagents (GE Healthcare, UK). They were exposed on X-ray film (FujiFilm Co., Japan) and developed with developer solution until protein bands were visualized. They were continuously washed with water, fixed with fixation solution, washed with water, and dried. The intensity of band was statically analyzed by Image J software. They were expressed as a ratio of specific protein band intensity to β-actin band intensity

Immunocytochemistry

Cells were cultured on gelatin-coated glass slide, fixed with 4% natural buffered paraformaldehyde solution (pH 7.4), and washed with PBS for 10 min. They were reacted with normal goat serum for 1 h at room temperature to block non-specific binding and incubated with anti- parvalbumin polyclonal antibody (1:100, Thermo Fisher Scientific) for overnight at 4°C. Cells were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody for 2 h at room temperature. They were stained with 4,6-diamino-2-phenylindole dihydrochloride (DAPI) as a counter staining, mounted with fluorescent mounting medium (Dako, North America Inc., USA), and observed with confocal fluorescence microscope (FV-1000, Olympus, Japan)

Statistical analysis

Data were presented as means ± SD. Results of each groups were compared by two-way analysis of variance (ANOVA) followed by post-hoc Scheffe's test and the effect of quercetin by dose-dependent manner were compared by two-way ANOVA followed by Dunnett's multiple comparison. $p < 0.05$ was considered to be statistically significant.

RESULTS

We confirmed the neuroprotective effect of quercetin in glutamate-exposed neurons. **Figure 1A** presented representative photos of neurons in glutamate and/or quercetin-treated groups. Glutamate toxicity significantly reduced cell viability, whereas quercetin administration prevented this reduction. The protective effects of quercetin were dose-dependent. Cell viability values were 17.7 ± 5.3 in the glutamate-only treated cells and 28.0 ± 8.6, 64.3 ± 4.3, and 82.0 ± 3.2 in the quercetin (1, 3, and 5 μM) and glutamate co-treated cells, respectively (**Fig. 1B**). Intracellular calcium levels were significantly elevated in the glutamate-only treated cells, while quercetin administration attenuated this rise (**Fig. 1C**). Quercetin regulated intracellular calcium levels in a dose-dependent manner. The intracellular calcium concentration was 740 nM in the glutamate-only treated cells and 433, 231, and 167 in the the quercetin (1, 3, and 5 μM) and glutamate co-treated cells, respectively (**Fig. 1D**).

Parvalbumin expression was decreased in the glutamate-only treated cells, while quercetin prevented this decrease (**Fig. 2**). Immunocytochemical staining revealed that the number of parvalbumin-positive cells was remarkably reduced in the glutamate-only treated cells, but quercetin pretreatment alleviated this decrease in a dose-dependent manner (**Fig. 2A and B**). Parvalbumin was strongly expressed in the cytoplasm of neurons. In results of Western blot analysis, parvalbumin protein levels were 0.18 ± 0.03 in the glutamate-only treated cells and

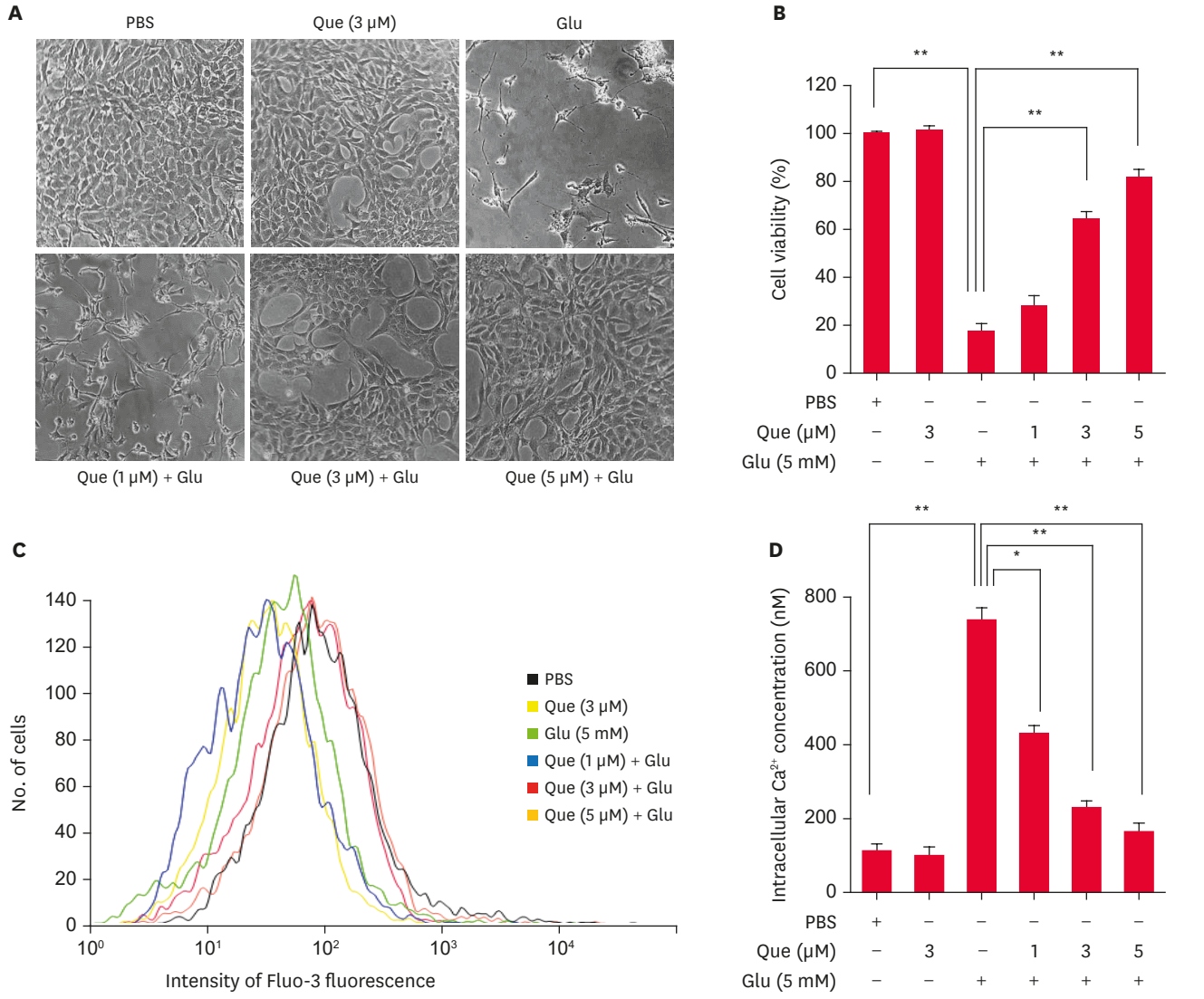

Fig. 1. Representative photos of cells (A), cell viability (B), and intracellular calcium concentration (C and D) in glutamate (Glu)- and/or quercetin (Que)-treated hippocampal neuronal cells (HT22). HT22 cells were treated with glutamate (5 mM) for 24 h and quercetin (1, 3, and 5 μM) was treated 1 h before glutamate exposure. Cell viability was assessed with the MTT assay (B). Cell survival was expressed as a percentage of PBS-treated cells. Neurons were labeled with Fluo-3 AM were measured using a luminescence spectrophotometer (D). Data (n = 5) are presented as means ± SEM.
$^{*}p < 0.05$, $^{**}p < 0.001$.

0.22 ± 0.03, 0.75 ± 0.04, and 0.80 ± 0.03 in the the quercetin (1, 3, and 5 μM) and glutamate co-treated cells, respectively (**Fig. 2C and D**).

Fig. 3 showed parvalbumin and caspase-3 expression levels in glutamate- and/or querectin-treated cells with parvalbumin siRNA transfection. We confirmed that the expression level of parvalbumin was lower in parvalbumin siRNA-transfected cells than in non-transfected neurons. Glutamate exposure reduced the expression of parvalbumin in both siRNA-transfected and non-transfected cells, and quercetin alleviated this reduction (**Fig. 3A**). Parvalbumin expression levels in non-transfected cells were 0.10 ± 0.03 and 0.77 ± 0.05 in the glutamate- and in the quercetin and glutamate co-treated cells, respectively (**Fig. 3C**). Parvalbumin levels in siRNA-transfected cells were 0.04 ± 0.01 in the glutamate-only treated cells and 0.27 ± 0.03 in the quercetin and glutamate co-treated cells (**Fig. 3C**). Glutamate

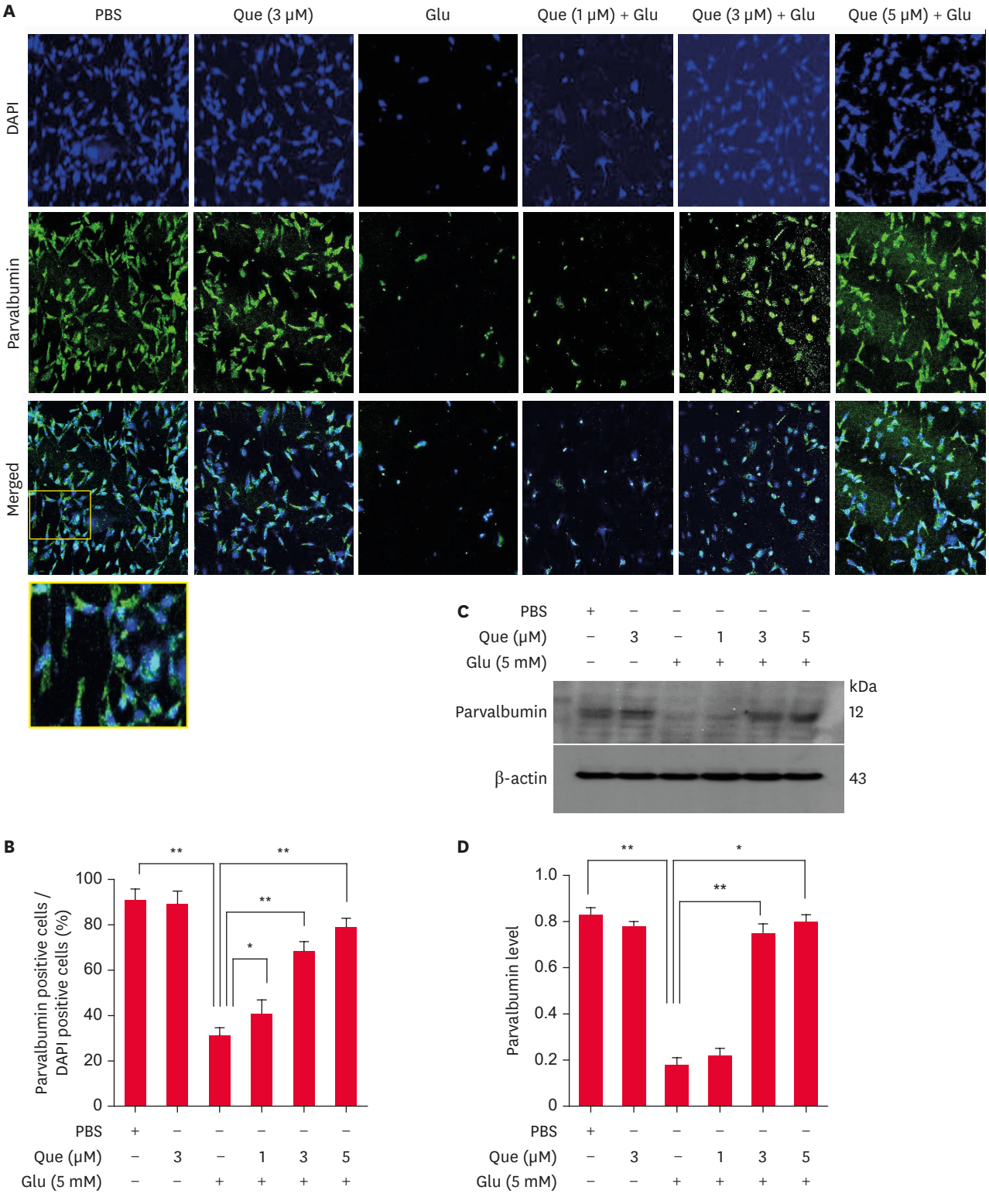

Fig. 2. Representative images of double immunofluorescence labeling (A and B) with parvalbumin (green color) and DAPI (nuclei marker, blue) taken at a magnification of 200x. Western blot analysis (C and D) of parvalbumin in glutamate (Glu)- and/or quercetin (Que)-treated hippocampal neuronal cells. Glutamate (5 mM) was administrated for 24 h and quercetin (1, 3, and 5 μM) was treated 1 h before glutamate exposure. Magnified photo is an enlarged image of square area (400×). Parvalbumin-positive cells expression was calculated the percentage of the number of parvalbumin-positive cells to the number of DAPI-positive cells (B). Densitometric analysis results are presented as the ratio of parvalbumin intensity to β-actin intensity (D). Data (n = 5) are presented as means ± SEM.
$^{*}p < 0.05$, $^{**}p < 0.001$.

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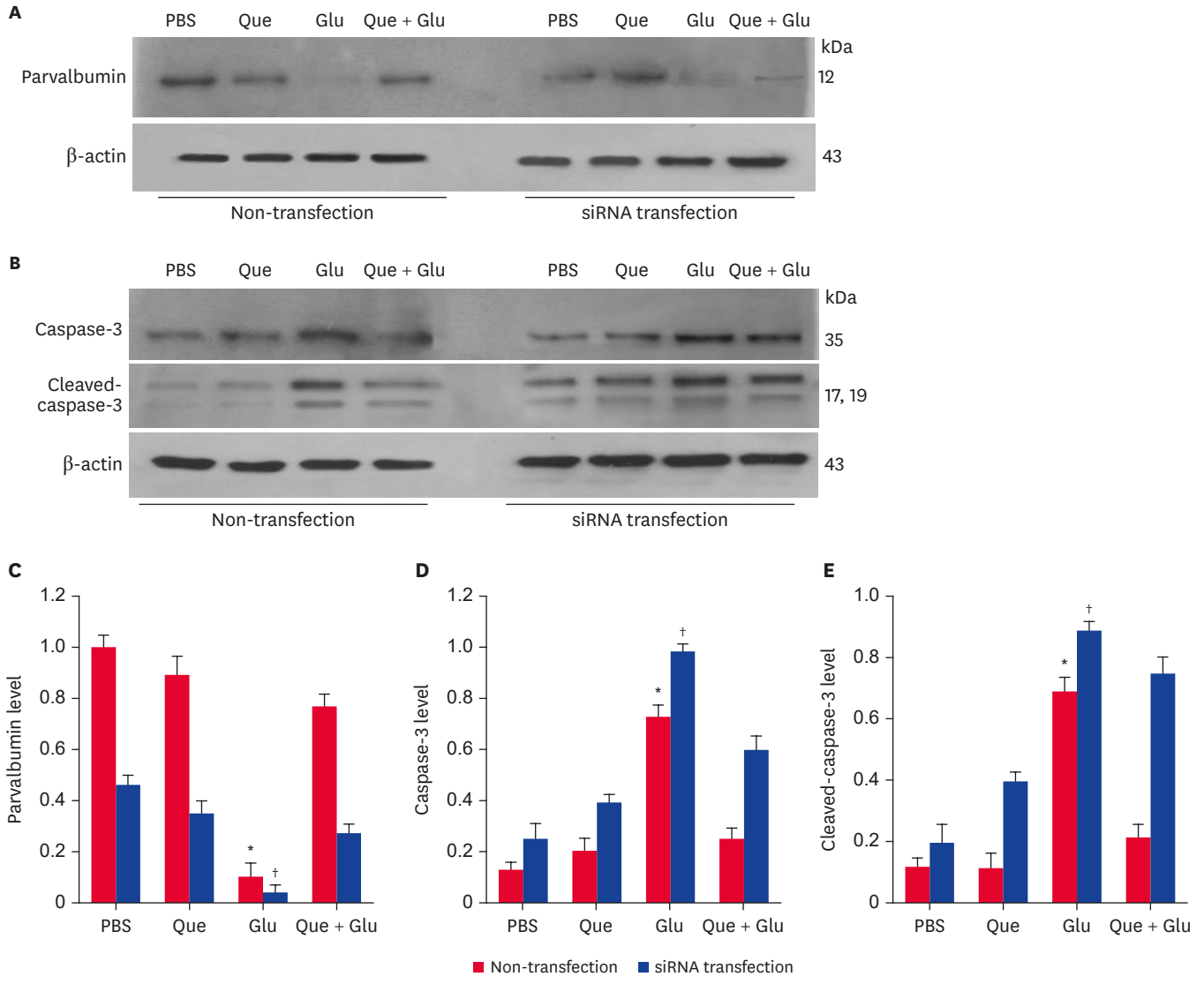

Fig. 3. Western blot analysis of parvalbumin (A and C), caspase-3 (B and D), and cleaved caspase-3 (B and E) in glutamate (Glu)- and/or quercetin (Que)-treated hippocampal neuronal cells with or without parvalbumin siRNA transfection. Glutamate (5 mM) was exposed for 24 h and quercetin (3 μM) was treated 1 h before glutamate exposure. Data are presented as the ratio of detected protein intensity to β-actin intensity. Data (n = 5) are presented as means ± SEM.
$^{*}p < 0.05$ in non-transfected cells, $^{\dagger}p < 0.05$ in siRNA transfected cells.

treatment increased caspase-3 and cleaved caspase-3 expressions regardless of the siRNA transfection, while quercetin attenuated the glutamate-induced these increases. Caspase-3 and cleaved caspase-3 expressions were greater in parvalbumin siRNA-transfected cells than in non-transfected cells (**Fig. 3B**). Caspase-3 levels in parvalbumin siRNA-transfected cells were 0.98 ± 0.03 in exposed to glutamate and 0.60 ± 0.05 in subjected to quercetin and glutamate (**Fig. 3D**). Cleaved caspase-3 levels in siRNA-transfected cells were 0.89 ± 0.03 in the glutamate-treated cells and 0.75 ± 0.03 in the quercetin and glutamate co-treated cells (**Fig. 3E**).

We evaluated the expression levels of bcl-2 and bax proteins in glutamate-treated cells with parvalbumin siRNA transfection. Glutamate treatment significantly reduced the expression of bcl-2, but quercetin treatment alleviated this reduction. Bcl-2 expression levels in parvalbumin siRNA-transfected cells were lower than in non-transfected cells (**Fig. 4A**). Bcl-2 expression levels in siRNA-transfected cells were 0.06 ± 0.01 in the glutamate-only treated

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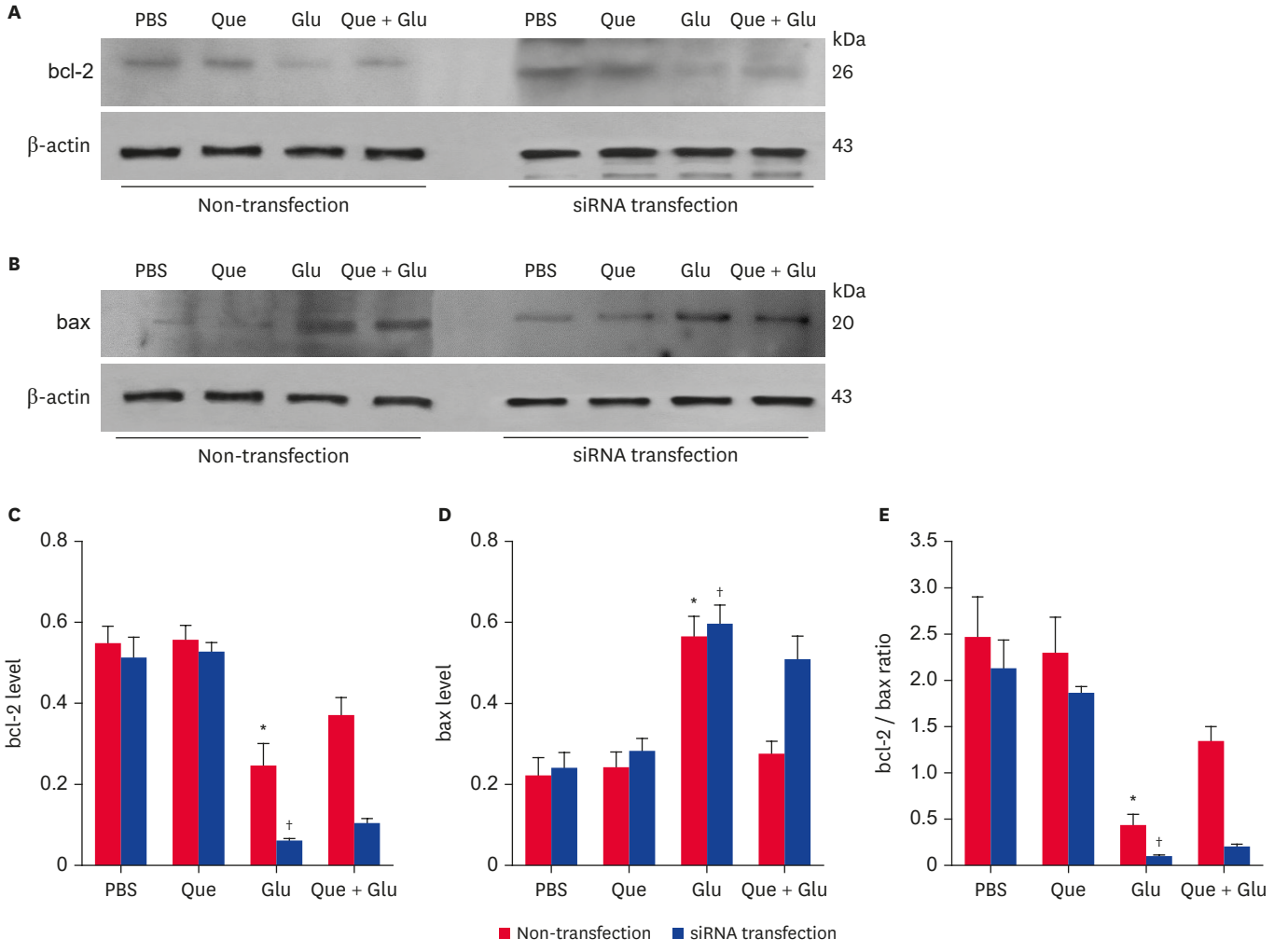

Fig. 4. Western blot analysis of bcl-2 (A and C), bax (B and D), and bcl to bax ratio (E) in glutamate (Glu)- and/or quercetin (Que)-treated HT22 cells with or without parvalbumin siRNA transfection. Glutamate (5 mM) was exposed for 24 h and quercetin (3 μM) was treated 1 h before glutamate exposure. Data are presented as the ratio of bcl-2 or bax intensity to β-actin intensity. Data (n = 5) are presented as means ± SEM.
$^{*}p < 0.05$ in non-transfected cells, $^{\dagger}p < 0.05$ in siRNA transfected cells.

cells and 0.10 ± 0.01 in the quercetin and glutamate co-treated cells (**Fig. 4C**). In contrast with bcl-2 expression, bax expression was increased in the glutamate-only treated cells, but quercetin co-treatment attenuated this increase. Bax expression levels in the parvalbumin siRNA-transfected cells were higher than in the non-transfected cells (**Fig. 4B**). They were 0.60 ± 0.03 in the glutamate-only treated cells and 0.51 ± 0.06 in the quercetin and glutamate co-treated cells (**Fig. 4D**). Moreover, the ratio of bcl-2 to bax decreases in glutamate-only treated cells, quercetin treatment prevented this decrease. These levels were lower in siRNA-transfected cells than in non-transfected cells. They were 0.10 ± 0.01 in the glutamate-only treated cells and 0.20 ± 0.02 in the quercetin and glutamate co-treated cells with siRNA transfection (**Fig. 4E**).

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DISCUSSION

Glutamate toxicity causes morphological changes such as dendritic atrophy and neuronal body contraction, induces neuronal cell death, and eventually reduces cell viability [30]. We confirmed that quercetin exerts a neuroprotective effect against glutamate toxicity in a dose-dependent manner. Moreover, glutamate exposure dramatically raises intracellular calcium concentration in neurons, while quercetin attenuates this rise. Quercetin treatment also alleviates the glutamate-induced decline in parvalbumin expression. The hippocampus plays an important role in learning and memory. It is considered an area vulnerable to glutamate toxicity. HT22 cells have been accepted as an immortalized mouse hippocampal cell line widely used for studies of nerve damage or recovery. In particular, excitatory glutamate toxicity induces apoptosis in HT22 cells through the release of the mitochondrial apoptosis-inducing factor [31]. In this study, HT22 cells were used to investigate the effects of quercetin on glutamate toxicity in the hippocampus. We clearly showed that glutamate toxicity induces neuronal damage and quercetin has a protective effect on glutamate in HT22 cells.

Parvalbumin is a calcium-buffering protein that regulates the intracellular calcium concentration [32]. It is an important protein that constantly maintains the intracellular calcium concentration in nerve cells [33]. Overload of intracellular calcium concentration induces cell degradation and damage. An excessively high intracellular calcium level initiates the activation of protease and lipase, induces ROS generation and cell structure damage, and eventually leads to cell death [34]. In addition, excessive calcium influx increases mitochondrial permeability, releases cytochrome *c* from mitochondria to cytosol, and activates caspase cascade [35,36]. Imbalance of intracellular calcium concentration causes various neurological disorders. Thus, it is accepted that the maintenance of intracellular calcium concentration is important for the survival of neurons [37]. We have previously shown that glutamate exposure reduces the expression of parvalbumin in neurons [38]. This study additionally showed that quercetin attenuates the glutamate-induced decrease in parvalbumin and preserves neurons against glutamate exposure. Quercetin restores nerve damage caused by glutamate toxicity and exerts protective effects. It also regulates intracellular calcium concentration and parvalbumin expression in glutamate-exposed neurons.

Quercetin attenuates neurobehavioral disorder and alleviates the reduction of pavalbumin expression in stroke animal models [27]. We additionally investigated whether quercetin prevents apoptotic cell death through parvalbumin regulation using an siRNA transfection manual. Our results demonstrated a decrease in parvalbumin expression and an increase in caspase-3 expression in siRNA-transfected cells. This finding demonstrated that parvalbumin regulates caspase-3 activation and contributes to apoptosis inhibition. Moreover, glutamate exposure induces increases in caspase-3 expression regardless of parvalbumin siRNA transfection, and these increases are higher in siRNA-transfected cells than in non-transfected cells. This study showed that quercetin alleviates an increase in caspase-3 in glutamate-exposed neurons. The alleviation effect of quercetin was more pronounced in non-transfected cells than in transfected cells. Caspase-3 is accepted as a representative protein that related to apoptosis. Bcl-2 family members are key regulators of cell death [39]. Bcl-2 is an inhibitor that prevents apoptosis and bax is an inducer that promotes apoptosis [40,41]. Moreover, glutamate exposure causes the down-regulation of bcl-2 and the up-regulation of bax. The degree of change in bcl-2 and bax in the parvalbumin siRNA-transfected condition was higher than in the non-transfected condition. Quercetin prevents glutamate-induced changes regardless of siRNA transfection, but the effect of quercetin on bcl-2 and bax

regulation was weakened in parvalbumin siRNA-transfected cells. Our results also revealed a decrease in the ratio of bcl-2 to bax in glutamate-treated neurons. The ratio of bcl-2 to bax acts as a critical factor in determining cell death and cell survival. The decrease in the bcl-2 to bax ratio is more remarkable in siRNA-transfected neurons than in non-transfected neurons. Quercetin attenuates this decrease by glutamate exposure, and the recovery ability of quercetin on these protein changes was reduced in parvalbumin siRNA-transfected neurons. These results showed that parvalbumin contributes to bcl-2 and bax expression, and caspase-3 expression in glutamate-exposed neurons. Quercetin modulates both the down- and up-regulation of these proteins caused by glutamate toxicity. Our results indicate that quercetin regulates glutamate-induced apoptosis through the modulation of parvalbumin. We clearly elucidated the fact that quercetin modulates parvalbumin expression in glutamate-exposed cells, regulates intracellular calcium concentration, and results in the prevention of apoptosis by regulating bcl-2 family proteins and attenuating caspase-3 expression. In conclusion, these findings suggest that quercetin exerts a neuroprotective effect through the modulation of parvalbumin in glutamate-exposed neurons.

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