Calcium-dependent serine-threonine phosphatase, calcineurin inactivation mediated by baicalein attenuates prion protein-mediated neuronal cell damage

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Abstract

Prion diseases are a group of fatal neurodegenerative disorders characterized by neuronal cell death. Calcineurin and autophagy mediate prion-induced neurodegeneration, suggesting that inhibition of calcineurin and autophagy could be a target for therapy. Baicalein has been reported to exert neuroprotective effects against calcium-dependent neuronal cell death. In this study, we investigated the effects of baicalein on the development of prion diseases. We found that baicalein treatment inhibits prion protein-induced apoptosis. Baicalein inhibited calcium up-regulation and protected the cells against prion peptide-induced neuron cell death by calcineurin inactivation. Furthermore, baicalein increased p62 protein levels and decrease LC3-II protein levels indicating autophagic flux inhibition and baicalein inhibited prion protein-induced neurotoxicity through autophagy flux inhibition. Taken together, this study demonstrated that baicalein attenuated prion peptide-induced neurotoxicity via calcineurin inactivation and autophagic flux reduction, and also suggest that baicalein may be an effective therapeutic drug against neurodegenerative diseases, including prion diseases.

Background

Prion diseases, known as transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders. They include kuru and sporadic Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathies in cattle, and scrapie in sheep and goats [1, 2]. Prion diseases are characterized by misfolding of normal cellular prion protein (PrP^{C}) into scrapie prion protein (PrP^{Sc}), which is an abnormal protease-resistant misfolded isoform [3, 4]. PrP^{C} is soluble, with a predominant alpha-helical conformation, but a disease-causing, infectious form (PrP^{Sc}) is insoluble, β -sheet-rich, and protease-resistant [5]. Because of β -sheet-rich conformation, PrP^{Sc} is highly pathogenic and neurotoxic when compared with the largely α -sheet-rich structure of PrP^{C} [6, 7]. PrP^{Sc} is a misfolded protein aggregate accumulating within endosomes or on the neuronal cell surface. It leads to neurodegeneration, suggesting cytotoxicity and cell death [8–10].

Baicalein is a major flavonoid originally extracted from the roots of a traditional Chinese medicinal herb *Scutellaria baicalensis* Georgi. Baicalein exhibits several pharmacological activities, such as anti-inflammatory and anti-oxidative properties [11, 12], and exerts protective effects against ischemic brain injury [13], Parkinson's disease [14, 15], $\text{A}\beta$ -induced toxicity [16], and experimental traumatic brain injury [17]. Baicalein prevents elevation of intracellular calcium in Parkinson's disease [18] and attenuates the expression of calcineurin protein in cardiac remodeling [19].

Calcineurin or protein phosphatase 2B (PP2B) is highly abundant in the brain and regulates synaptic plasticity and neuronal death [20]. Calcineurin immunoreactivity occurs exclusively in the neurons throughout brain [21, 22]. Overactivation of calcineurin by amyloid-beta and prion proteins induces neurotoxicity [3, 23]. Tacrolimus or FK506 is a well-known immunosuppressive drug, which binds to the FK506-binding protein (FKBP). The FK506-FKBP12 complex binds to calcineurin and inhibits its activity [24].

Autophagy is a highly conservative cellular process in which cells degrade and recycle bulk cytosolic proteins and damaged organelles via lysosomal degradation [25, 26]. Importantly, autophagy plays an important role in the survival of neural cells [27, 28]. Autophagy plays a key role in inflammatory diseases, and protein misfolding diseases [29]. However, the role of autophagy in baicalein-mediated neuroprotective effects is not fully understood.

Our previous study showed that baicalein inhibits JNK-reduced neuronal apoptosis caused by the prion peptide. However, the effect of baicalein on prion protein-induced neuronal cell damage via calcineurin and autophagy has yet to be reported. In this study, we investigated the protective effects of baicalein against PrP (106–126)-mediated apoptosis, calcineurin and autophagy activation.

Results

Baicalein inhibits PrP (106-126)-induced neuronal apoptosis.

We investigated whether baicalein affected prion peptide-induced neurotoxicity. The results showed that prion protein induced neuronal cell death, and baicalein reduced prion protein-mediated neurotoxicity (Figures 1A and B). Using TUNEL assay, we demonstrated that the apoptosis in the PrP (106-126)-treated cells led to the emission of green fluorescence, indicating DNA strand breakage (Figure 1C). These results indicate that baicalein is effective in preventing prion peptide-induced neuronal apoptosis.

Baicalein inhibits PrP (106-126)-induced apoptosis by calcineurin inactivation

To determine the effect of baicalein on prion peptide expression in neuronal cells, we investigated the role of prion peptide in altering Ca^{2+} levels in neuronal cells. PrP (106-126) treatment resulted in a rapid increase in Ca^{2+} , and baicalein reduced PrP-induced Ca^{2+} (Figures 2A and B). Fluorescence imaging also indicated that baicalein inhibited calcium increase (Figure 2C). The activity of calcineurin was evaluated by measuring the formation of free phosphate (), using a specific kit assay. The results showed that prion protein elevated calcineurin activity and baicalein reduced prion peptide-induced calcineurin activity (Figure 2D). Next, to investigate whether the inhibition of calcineurin exerts neuroprotective effects against PrP (106-126)-induced neurotoxicity, we analyzed the levels of calcineurin and cell viability using a calcineurin inhibitor (FK506). We also found that baicalein and calcineurin inhibitor blocked the PrP (106-126)-induced neuronal apoptosis (Figures 3A and B). TUNEL assay demonstrated that baicalein and calcineurin inhibitor reduced apoptosis in PrP (106-126)-treated cells resulting in the emission of green fluorescence, indicating DNA strand breakage (Figure 3C). The activity of calcineurin was evaluated by measuring the formation of free phosphate (), using a specific kit assay. The results showed that baicalein and calcineurin inhibitor reduced prion protein-induced calcineurin activity (Figure 3D).

Baicalein treatment inhibits PrP (106-126)-induced apoptosis via autophagy

We investigated whether baicalein affects prion peptide-induced autophagy. We found that baicalein increased SQSTM1/p62 and decreased LC3-II (Figure 4A). Orange fluorescence was recovered by

treatment with calcineurin inhibitor (Figure 4B). Prion peptide-induced autophagic flux was abolished by baicalein and confirmed by the up-regulation of SQSTM1/p62 protein and the down-regulation of LC3-II (Figure 4C). We also conducted TEM to establish the effect of lysosomal inhibition of autophagy by baicalein. As shown in Figure 4D, multiple vesicles including double-membraned autophagosomes (arrowheads) were induced by treatment of cells with baicalein, which indicated inhibition of autophagic flux and lysosomal degradation (Figure 4D). Next, to investigate whether the inhibition of autophagy exerted neuroprotective effects against PrP (106-126)-induced neurotoxicity, we analyzed the levels of autophagy and cell viability using an autophagy inhibitor (chloroquine). We also found that baicalein and autophagy inhibitor blocked PrP (106-126)-induced neuronal apoptosis (Figures 5A and B). TUNEL assay demonstrated that exposure to baicalein and autophagy inhibitor reduced apoptotic process in PrP (106-126)-treated cells that led to the emission of green fluorescence, indicating DNA strand breakage (Figure 5C). Prion peptide-induced autophagic flux was abolished by baicalein and was confirmed by the upregulation of SQSTM1/p62 protein and the downregulation of LC3-II, and inhibition of prion peptide-induced autophagy was confirmed by the upregulation of SQSTM1/p62 protein and LC3-II (Figure 5D).

Discussion

The study was to investigate the role of baicalein in calcineurin inactivation and autophagy, and the regulation of PrP (106–126)-induced apoptosis by baicalein in neuronal cells. The results suggest that the decrease in calcineurin and autophagy by baicalein and the consequent reduction in prion protein-induced neurotoxicity may be the key mechanisms underlying the neuroprotective effects of baicalein.

It has been reported that baicalein inhibits intracellular Ca^{2+} concentrations by reducing phospholipase C activity in C6 rat glioma cells [30]. Wang et al suggested that the protective effect of baicalein is mediated via calcineurin pathway in angiotensin II-treated mice. Baicalein inhibits cardiac remodeling by attenuating calcineurin signaling pathways in mice [19]. Consistent with these findings, our results also demonstrated that baicalein attenuates calcineurin activity. Together, these findings suggest that baicalein effectively inhibits prion protein-induced calcium and calcineurin activity.

Because baicalein has neuroprotective and antioxidant properties, It has been reported to exert a neuroprotective effect against β -amyloid peptide [31, 32], 6-OHDA-induced PD [15, 18] and human prion protein fragment 106–126 (PrP) [33]. A medicinal herb *Scutellaria lateriflora* is known to inhibit PrP replication *in vitro* and delay the onset of prion disease in Mice [34]. The neuroprotection of baicalein has been linked to its anti-apoptotic, anti-inflammatory and pro-differentiation mechanisms. This study explored the neuroprotective role of baicalein against prion protein-induced neurotoxicity.

Baicalein significantly increased the level of autophagy in cancer [35] and liver disease [36]. However, other studies report that baicalein attenuated autophagy [37]. In the present study, we showed that baicalein inhibited autophagy in neuronal cells. Autophagic defects are well documented in neurodegenerative diseases [38, 39], and include accumulation of autophagosomes and undegraded autophagic vacuoles in neuronal cells [39–41].

Conclusions

In conclusion, our current study revealed that baicalein protected neuronal cells against PrP (106–126)-induced neurotoxicity via calcineurin inactivation. In addition, baicalein reduced prion protein-induced autophagy. Information provided in this report is invaluable in designing future studies for possible therapeutic application of baicalein in various neurodegenerative diseases.

Methods

Cell culture

The human neuroblastoma cell line SK-N-SH was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Minimum Essential Medium (MEM, Hyclone Laboratories, Logan, UT, USA) containing 10% fetal bovine serum (Invitrogen-GIBCO, Grand Island, NY, USA) and gentamycin (0.1 mg/mL) in a humidified incubator maintained at 37°C and 5% CO₂.

PrP (106-126) treatment

Synthetic PrP (106-126) peptides (sequence, Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly) were synthesized by Peptron (Seoul, Korea). The peptides were dissolved in sterile dimethyl sulfoxide at a stock concentration of 10 mM and stored at -20°C.

Annexin V/Propidium iodide (PI) test

Apoptosis in detached cells was assessed using an annexin V Assay kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's protocol. Annexin V levels were determined by measuring fluorescence at 488 nm excitation and 525/30 emission using a Guava EasyCyte HT System (Millipore, Bedford, MA, USA).

Measurement of $[Ca^{2+}]_i$

Neuronal cells on collagen-coated confocal dish were incubated with 5 μ M Fluo-4 AM (Invitrogen) in media containing 1% FBS at 37°C for 40 min. The cells were washed three times with Hank's Balanced Salt Solution (HBSS). Changes of $[Ca^{2+}]_i$ were determined at 488 nm excitation/530 nm emission using an air-cooled argon laser system. The fluorescence emitted at 530 nm was collected using a photomultiplier. The image was scanned using a confocal microscope (Zeiss). For the calculation of $[Ca^{2+}]_i$, the method of Tsien *et al.* [42] was used based on the following equation: $[Ca^{2+}]_i = K_d(F - F_{min}) / (F_{max} - F)$, where K_d is 345 nM for fluo-4, and F is the observed fluorescence level. Each tracing was calibrated for maximal fluorescence (F_{max}) via addition of ionomycin (2 μ M) and for minimal fluorescence intensity (F_{min}) via addition of EGTA 5 mM at the end of each measurement.

Calcineurin activity assay

The calcineurin cellular activity assay kit (#BML-AK816-0001; Enzo Life Sciences, Inc., Farmingdale, NY, USA) was used to determine the phosphatase activity of calcineurin in neuronal cells, using the manufacturer's instructions. In brief, the cells were lysed on ice in a lysis buffer containing protease inhibitors. Phosphatase activity was quantified by detection of free phosphate released from the reaction based on the absorbance of malachite green (OD 620 nm).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

TUNEL assay was carried out to measure the stage of cellular apoptosis using a TUNEL-based assay kit (BioVision, Mountain View, CA, USA). TUNEL analysis was performed according to the manufacturer's instructions. PI was used to show the cell nuclei.

Western blot analysis

SK-N-SH cells were lysed in a lysis buffer [25 mM HEPES at pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.1 mM DTT (dithiothreitol), and a protease inhibitor mixture]. Whole cell proteins were electrophoretically resolved on a 10%–15% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. Immunoreactivity was detected via sequential incubation with primary antibodies, horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence reagents (West Save Gold detection kit; AbFrontier, Seoul, Rep. of Korea). The primary antibodies used for immunoblotting were anti-P62 (#5114; Cell Signaling Technology), LC3 (Novus Biologicals, Littleton, CO, USA), and anti- β -actin (A5441; Sigma-Aldrich, St. Louis, MO, USA). Images were analyzed using a Fusion FX7 imaging system (Vilber Lourmat, Torcy Z.I. Sud, France). Densitometry was used to analyze the signal bands with the Bio-1D software package (Vilber Lourmat, Marne La Vallee, France).

Fluorescence microscopy

After treatment, coverslips were mounted with 70% ethanol in DW for 10 min at RT. Cells were stained with AO (0.1 μ g/mL) for 20 min in a cell culture incubator. Excitation wavelengths were 543 nm and 633 nm. Bandpass filters were set at 560–615 nm (Cy3, AlexaFluor568) and 650–750 nm (AlexaFluor647).

Transmission electron microscopy (TEM)

Following fixation of the cells in 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and 2% paraformaldehyde (Electron Microscopy Sciences) in 0.05 M sodium cacodylate (pH 7.2; Electron Microscopy Sciences) for 2 h at 4°C, specimens were fixed in 1% osmium tetroxide (Electron Microscopy Sciences) for 1 h at 4°C, dehydrated with increasing ethanol (25, 50, 70, 90 and 100%) for 5 min each and embedded in epoxy resin (Embed 812; Electron Microscopy Sciences) for 48 h at 60°C according to the manufacturers' instructions. Ultrathin sections (60 nm) were prepared using an LKB-III ultratome (Leica Microsystems GmbH, Wetzlar, Germany) and were stained with 0.5% uranyl acetate (Electron Microscopy Sciences) for 20 min and 0.1% lead citrate (Electron Microscopy Sciences) for 7 min at room temperature.

Images were recorded on a Hitachi H7650 electron microscope (Hitachi, Ltd., Tokyo, Japan; magnification, x10,000) installed at the Center for University-Wide Research Facilities (CURF) at Jeonbuk National University.

Statistical analysis

All data were expressed as mean $\pm$ standard error, and compared using the one-way ANOVA followed by the Tukey test. All statistical analysis was performed using GraphPad Prism software. Results were considered significant at * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$, as appropriate.

Abbreviations

BAI: baicalein; CNS: central nervous system; PrPc: cellular prion protein; PrPsc: scrapie-associated prion protein; LC3-I/II: microtubule-associated protein 1A/1B-*light chain* 3-I/II; CQ: chloroquine

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Author contributions

JH and JM analyzed and interpreted all the data. JH performed the experiments and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Not applicable.

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Figures

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Figure 1

Prion protein increased calcineurin activity. (A) SK-N-SH cells were treated with baicalein for 1 h and then exposed to 100 μM of PrP (106-126) for 12 h. Cell viability was measured by annexin V assay. Cells were treated with FITC-annexin V and PI, which bind to phosphatidylserine in the plasma membrane and nuclei during apoptosis. (B) Bar graph indicating the average number of annexin V-negative cells. (C) Representative immunofluorescence images of TUNEL-positive (green) SK-N-SH cells 12 h after exposure to 100 μM PrP (106 126) in the absence or presence of baicalein (1 h). The cells were counterstained with PI (red) to show all cell nuclei. The bar graph shows the mean $\pm$ standard error of the mean (n = 3). * p < 0.001, significant differences when comparing the control group with each treatment group. PrP, Prion peptide (106-126)

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Figure 2

Baicalein treatment inhibits PrP (106-126)-induced calcineurin. (A) SK-N-SH cells were loaded with fluo-4 AM and the changes in Ca^{2+} levels were measured using confocal microscope. The time point of 40 μ M of baicalein addition is indicated by the $\boxtimes$ arrow and 100 μ M of PrP (106-126) addition is indicated by the $\boxdot$ arrow. Data are mean $\pm$ SEM of $[Ca^{2+}]_i$ at 60 s from three independent experiments. (B) Bar graph indicating the average peak value of intracellular calcium levels. (C) The green fluorescence image indicates peak value of calcium measurement using confocal microscopy. (D) SK-N-SH cells were treated with baicalein for 1 h and then exposed to 100 μ M of PrP (106-126) for 12 h. The treated cells were assessed for calcineurin activity. The bar graph shows the mean $\pm$ standard error of the mean (n = 3). *** p < 0.001, # p < 0.05; significant differences when compared with the control group and each treatment group. PrP, Prion peptide (106-126)

Fig.3 Hong et al.

Figure 3

Baicalein inhibits PrP (106-126)-induced neurotoxicity via calcineurin inhibition. (A) SK-N-SH cells were treated with baicalein and calcineurin inhibitor for 1 h and then exposed to 100 μM of PrP (106-126) for 12 h. Cell viability was measured by annexin V assay. Cells were treated with FITC-annexin V and PI, which binds to phosphatidylserine in the plasma membrane and nuclei during apoptosis. (B) Bar graph indicating the average number of annexin V-negative cells. (C) Representative immunofluorescence images of TUNEL-positive (green) SK-N-SH cells 12 h following exposure to 100 μM PrP (106-126) in the absence or presence of baicalein and calcineurin inhibitor (FK506) (1 h). The cells were counterstained with PI (red) to visualize all cell nuclei. (D) SK-N-SH cells were treated with baicalein and calcineurin inhibitor for 1 h and then exposed to 100 μM of PrP (106-126) for 12 h. The treated cells were assessed for calcineurin activity. The bar graph shows the mean $\pm$ standard error of the mean (n = 3). *** p < 0.001, ## p < 0.01, ### p < 0.001; significant differences when comparing the control group with each treatment group. PrP, Prion peptide (106-126)

Fig.4 Hong et al.

Figure 4

Baicalein inhibited autophagy. (A) SK-N-SH cells were treated with 10, 20, 40 and 80 μ M of baicalein for 12 h. The treated cells were assessed for P62, LC3 by Western blot analysis. (B) Orange fluorescence was recovered with baicalein and autophagy inhibitor. (C) SK-N-SH cells were treated with baicalein for 1 h and then exposed to 100 μ M of PrP (106-126) for 12h. The treated cells were assessed for P62, LC3 by Western blot analysis. (D) SK-N-SH cells were treated with baicalein for 1 h and then exposed to 100 μ M of PrP (106-126) for 12h and analyzed by TEM. Arrowheads indicate autophagosomes and arrows indicate lysosomes and mitochondria.

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Figure 5

Baicalein inhibited PrP(106-126)-induced neurotoxicity via autophagic flux. (A) SK-N-SH cells were treated with baicalein and autophagy inhibitor for 1 h and then exposed to 100 μ M of PrP (106-126) for 12h. Cell viability was measured by annexin V assay. Cells were treated with FITC-annexin V and PI, which binds to phosphatidylserine in the plasma membrane and nuclei during apoptosis. (B) Bar graph indicating the average number of annexin V- negative cells. (C) Representative immunofluorescence images of TUNEL- positive (green) SK-N-SH cells 12 h following exposure to 100 μ M PrP (106 126) in the absence or presence of baicalein and autophagy inhibitor (1 h). The cells were counterstained with PI (red) to show all cell nuclei. (D) SK-N-SH cells were treated with baicalein and autophagy inhibitor for 1 h and then exposed to 100 μ M of PrP (106-126) for 12h. The treated cells were assessed for P62 and LC3 by Western blot analysis.