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

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Abstract

**Background:** Prion diseases are a group of unvaryingly 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.

**Results:** In the present study, we investigated whether baicalein attenuates prion peptide-mediated neurotoxicity and reduces calcineurin. 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.

**Conclusions:** 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 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], Aβ-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 prion peptide-induced neuronal apoptosis by inhibiting JNK activation. 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 observed the neuroprotective effects of baicalein against PrP (106-126)-mediated apoptosis, calcineurin and autophagy activation.

**Results**

**Baicalein inhibits prion peptide-induced neuronal apoptosis.**

We investigated whether baicalein affected prion peptide-induced neurotoxicity. The results showed that PrP (106-126) induced neuronal cell death, and baicalein reduced prion protein-mediated neurotoxicity (Figures 1A and B). Using TUNEL assay, we demonstrated that baicalein attenuated DNA strand breakage caused by prion peptide (Figure 1C). These results suggest that baicalein attenuates PrP (106-126)–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. Ca$^{2+}$ content was directly evaluated by prion peptide, 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 ($\sim$), 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 DNA strand breakage (Figure 3C). 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 induction of SQSTM1/p62 protein and the reduction of LC3-II protein (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 (arrows) were reduced by treatment of 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 DNA strand breakage caused by prion protein (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].

Although prion peptide 106-126 was used more than 20 years as a model of prion disorders, the relevance of pathogenic mechanism is still controversial. The prion peptide PrP106-126 has been widely used as a suitable model peptide because this fragment is soluble in water and exhibits several physicochemical and biological properties similar to those of PrP<sub>Sc</sub>, such as aggregation in solution, anti-proteinase K digestion, and neurotoxicity [42, 43]. However, an important argument about PrP peptide 106-126 is the facts that it is not actually a fragment found in the brains of humans or animals with prion diseases[44]. Even we investigated baicalein effect in this study, we will further study the issue using longer fragments or full-length PrP mutant models to examine therapeutic potential of baicalein in prion disease.

**Conclusions**

In the future, we will further study the neuroprotective effects of baicalein, autophagy and the calcineurin pathway in mouse models to examine baicalein's potential therapeutic role in prion disease.

**Methods**

**Cell culture**

SK-N-SH cell lines were obtained from the ATCC (Rockville, MD, USA) and cell culture method has been previously described [33]. Cells were incubated at 37°C with 5% CO2.

**PrP (106-126) treatment**

Synthetic PrP (106-126) was synthesized as previously described [45]. Sequences of PrP (106-126) used in this study were Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly. PrP (106-126) were synthesized by Peptron (Seoul, Korea).

**Annexin V/Propidium iodide (PI) test**

Apoptosis in detached 5,000 cells was assessed with an annexin V Assay kit to determine apoptosis according to the manufacturer's protocol. The staining procedure for PI and annexin V and incubated for 30 min in the dark. Flow cytometry was used to Guava EasyCyte HT System as described previously [46].

**Measurement of [Ca<sup>2+</sup>]i**

Measurement of Ca<sup>2+</sup> contents was implemented as previously reported [47]. Briefly, cells were plated on collagen-coated confocal dish. The SK-N-SH cells were loaded with 5 μM Fluo-4 AM (Invitrogen) for 40 min at 37°C. The image was processed using a confocal microscope (Zeiss). Fluorescence intensity was measured using Image J program (NIH).
Calcineurin activity assay

SK-N-SH cells were used to determine calcineurin phosphatase activity. Calcineurin activity was measured with calcineurin cellular activity assay kit (#BML-AK816-0001; Enzo Life Sciences, Inc., Farmingdale, NY, USA) and assay was performed following manufacturer's instructions. Calcineurin activity was measured as previously reported [48].

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

Cellular apoptosis was measured using TUNEL assay (BioVision, Mountain View, CA, USA) and described previously [49].

Western blot analysis

Western blot analysis was measured as described previously [50]. The 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).

Fluorescence microscopy

After treatment, coverslips were mounted with 70% ethanol in DW for 10 min at RT. Cells were stained with AO (0.1 µg/mL) for 20 min in a cell culture incubator. Finally, these cells were visualized under a fluorescence microscope (#451203, Nikon ECLIPSE 80i; Nikon Corporation, Tokyo, Japan; magnification, x200 and fluorescence intensity was measured using Image J program (NIH).

Transmission electron microscopy (TEM)

TEM was performed as described previously [51]. Briefly, SK-N-SH cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.2. Cells were post-fixed with 1% osmium tetroxide for further fixation. Specimens were dehydrated in graded ethanol and changed to propylene oxide, and then embedded in Epoxy resin. 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 statistical analysis was carried out using GraphPad Prism software (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). All experiments were expressed as mean ± standard error. And all experiments were compared using the one-way ANOVA followed by the Tukey test. 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 analyzed 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 information

JH and JM analyzed and interpreted all the data. JH performed the experiments and was a major contributor in writing the manuscript. SP contributed to study design and the writing of the manuscript. All authors read and approved the final manuscript.

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