Regular Article

Triggering of Autophagy by Baicalein in Response to Apoptosis after Spinal Cord Injury: Possible Involvement of the PI3K Activation

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High level apoptosis induced by spinal cord injury (SCI) evokes serious damage because of the loss and dysfunction of motor neurons. Our previous studies showed that inhibition of autophagy evokes the activation of apoptosis. Interestingly, Baicalein, a medicine with anti-apoptosis activity that is derived from the roots of herb Scutellaria baicalensis, largely induces autophagy by activating phosphatidylinositol 3-kinase (PI3K). In this study, we investigated the effects of intraperitoneal injection of Baicalein on autophagy and apoptosis in SCI mice and evaluated the relationship between autophagy and apoptosis. We demonstrated that Baicalein promoted the functional recovery of motor neurons at 7d after SCI. In addition, Baicalein enhanced neuronal autophagy and the autophagy-related factor PI3K, while inhibiting the p62 protein. Baicalein treatment decreased neuronal apoptosis at 7d after SCI. Moreover, when inhibiting autophagy, apoptosis was upgraded by Baicalein treatment after injury. Thus, Baicalein attenuated SCI by inducing autophagy to reduce apoptosis in neurons potentially via activating PI3K.

Key words apoptosis; autophagy; Baicalein; spinal cord injury; phosphatidylinositol 3-kinase (PI3K)

Spinal cord injury (SCI) is one of the most critical diseases of the central nervous system (CNS). Recent clinical data showed that, in China, the incidence of SCI is 23.7–60.6/1 million individuals, and the two main reasons are injury due to falls (37.8%) and traffic accidents (40.0%). According to a report by Oyinbo, the pathophysiological phase of SCI included primary SCI and secondary SCI. Secondary SCI includes a series of pathophysiological changes that occur after primary SCI. These changes involve multiple mechanisms, such as autophagy, inflammation, apoptosis, necrosis, and edema in spinal cord tissues, which appropriately affected neurological function, glial activation, and the recovery of white matter. Although considerable efforts have been made to treat the condition, SCI can induce several serious dysfunctions and a limited recovery time. Moreover, the irreversible phenomenon of neuronal degeneration and local imbalance of the microenvironment caused by secondary SCI are irreversible. Thus, it is of utmost importance to restore neuron dysfunction and local pathophysiological changes to increase recovery after SCI.

Autophagy is a common way for cells to degrade proteins and organelles in the cytoplasm. Interestingly, autophagy plays an important role in the repair of nerve function in the CNS. Moreover, we found that levels of autophagy are significantly increased after SCI, and that neuronal autophagy is associated with neurological function recovery as well as inhibition of apoptosis in the spinal cord. Apoptosis plays an essential role during animal recovery after injury. Zhang et al. reported that secondary SCI led to neuronal apoptosis in spinal cord tissues that seriously affected the survival and function of neurons. Moreover, in recent study, it was demonstrated that the level of autophagy and apoptosis were closely related to phosphatidylinositol 3-kinase (PI3K). Thus, to regulate apoptosis, we intervened PI3K.

We established an effective way to enhance autophagy in spinal cord tissues for recovery after SCI. Baicalein (5,6,7-trihydroxyflavone, a flavone subclass of flavonoids), is one of the oldest Chinese medicines that is still in use and readily available. It is a major constituent of the roots of the herb Scutellaria baicalensis. Due to its anti-inflammatory, anti-apoptosis, anti-oxidative, and neurodegeneration effects, Baicalein has successfully been used for the treatment of cerebral injury, Parkinson’s disease (PD), lung injury and liver injury. Recent studies have shown that, Baicalein has neuroprotective activities against liver ischemia/reperfusion (I/R) and liver injury by inducing autophagy. In addition, Baicalein can be transported across the blood–brain barrier (BBB) and benefit the integrity of the BBB. Only few reports are available that focus on the effects of Baicalein on autophagy levels in spinal cord tissues after injury.

In our study, we used a SCI mouse model to investigate the mechanism of neuroprotection after Baicalein treatment. The results demonstrated that Baicalein treatment increased the level of autophagy via the PI3K signaling at 7d after SCI, indicating that Baicalein inhibited apoptosis byactivating autophagy. Thus, our results suggested that Baicalein is a potential therapeutic drug that benefits SCI recovery by affecting autophagy possibly via activating PI3K.

MATERIALS AND METHODS

Spinal Cord Injury Model and Baicalein Treatment

Experimental animals (C57BL/6, adult male, 25–30g, SCXK (Liao) 2014–0004) were purchased form the animal Experimental Center of Jinzhou Medical University (Liaoning, China). Mice were randomly divided into three groups, including a sham group, vehicle group, and Baicalein group and surgeries were as follows: (1) sham group, the vertebral plate (T9–T10) was opened; (2) vehicle group, the spinal cords were completely exposed, and after a 2mm diameter impactor weight (10g) device was dropped from a height of 20mm to hit the T9–T10 spinal cord (Allen’s method), the vertebral plate was opened as in the Sham group; (3) Baicalein group, mice were injured as in the Vehicle group, and treated...
with Baicalein solution (100mg/kg, Sigma, St. Louis, MO, U.S.A., ab120372). Treatment was started immediately after SCI and continued once daily from 1 week to 4 weeks after SCI through intraperitoneal (i.p.) injection. Antibiotics were injected intramuscularly for 3 d and the bladder was massaged 3 times daily until bladder function turned to normal. All experimental procedures were in complied with the Experimental Animal Ethics Committee of Jinzhou Medical University (Liaoning, China).

**3-Methyladenine Inhibition**  At 30 min after SCI, mice were given an i.p. injection with 3-methyladenine (3-MA, PI3K inhibitor, Selleck, Houston, TX, U.S.A., S2767) (30 mg/kg) and were randomly divided into the following four groups: SCI, SCI+3-MA, SCI+Baicalein and SCI+3-MA+Baicalein.

**Assessment of Locomotion Behavior**  Basso Mouse Scale (BMS scores) were used to assess the recovery of locomotion function at 1, 3, 7, 14, and 28 d after SCI. BMS scores ranged from 0 (complete paralysis of hind limb) to 9 (normal locomotion) and were based on the changes of weight support, hind limb joint movement, paw position, and tail control. Moreover, the inclined plane test was used to evaluate the balance ability of the limb by placing the mice on a testing apparatus (a board covered with a rubber mat). All behavioral tasks were performed by 3 experienced individuals and performed in a double blinded manner.

**Tissue Preparation**  Mice were perfused with 0.9 saline and 4% paraformaldehyde (PFA, pH=7.4) as follows: after the limbs completely stiff, a section of the spinal cord was removed (T9–T10, 4 mm, including the site of injury). Then, spinal cords were soaked in 4% PFA for 1 d, then transferred to 30% sucrose (in 4% PFA) for 3 d and frozen in liquid nitrogen. Subsequently, 5 µm sections (2 mm from the site of injury) were cut using a cryostat Microtome (Leica, Heidelberg, Germany).

**Nissl Staining**  The morphology of spinal cord neurons was evaluated by Nissl staining. Briefly, sections were placed overnight in chloroform ethanol solution (1:1), soaked twice in 95% ethanol, absolute ethanol, anhydrous ethanol, xylene solution, and mounted using neutral balsam. Sections were observed using a microscope (Leica) and five sections were randomly selected for calculation of the proportion and sizes of lesions and average quantities of spinal cord anterior horn motor neurons using ImageJ2x software (National Institutes of Health, Bethesda, U.S.A.).

**Immunofluorescence Analysis and Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Staining**  For immunofluorescence analysis, sections were soaked with 0.3% Triton X-100 (in phosphate buffered saline (PBS)) and incubated with 10% normal goat serum. Then, sections were incubated overnight at 4°C with a primary rabbit anti-NeuN monoclonal antibody (1:1000, Abcam, Cambridge, MA, U.S.A., ab177487). The next day, the sections were incubated with goat anti-mouse immunoglobulin G (IgG) H&L Alexa Fluor® 488 (1:500, Abcam, ab150117), then incubated with cytoskeletal marker anti-vimentin antibody Alexa Fluor® 568 (1:500, Abcam, ab202504). Subsequently, sections were incubated with TUNEL staining mixture (TUNEL Apo-Green Detection Kit, Biotool, U.S.A., B31118). Sections were mounted using Fluoroshield mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI) (Abcam, ab104139) for visualization of spinal cord nuclei. Next, the sections were observed under a fluorescence microscope (Olympus, Tokyo, Japan), and the percentage of TUNEL positive cells was determined using ImageJ2x software.

**Transmission Electron Microscopy (TEM)**  After transcardial perfusion at day 7 post injury, tissue was fixed in 1% osmium acid for 2h, then washed by PBS, and dehydrated in a series of acetone washes. Next, tissue was blocked in 2.5% (w/v) glutaraldehyde overnight, and placed in a heating polymerizer at 70°C for one night. Finally, tissue was sliced and observed using TEM (Hitachi, Tokyo, Japan, HT770).

**Western Blot Analysis**  The membranes were incubated using the following primary antibodies: anti-p-PI3K antibody (1:1000, Cell Signaling Technology, Danvers, MA, U.S.A., 4228S), anti-LC3B antibody (1:1000, Abcam, Cambridge, ab48394), anti-Beclin-1 antibody (1:1000, Abcam, U.S.A., ab62557), anti-p62 antibody (1:1000, Abcam, ab56416), anti-Caspase3 antibody (1:1000, Abcam, ab13585), anti-Caspase9 antibody (1:1000, Abcam, ab32539), anti-Bcl-2 antibody (1:1000, Abcam, ab32124), anti-Bax antibody (1:1000, Abcam, ab32503) and anti-β-actin antibody (1:1000, Abcam, ab8226). Next, antibodies were washed and incubated with the following secondary antibodies, AffinPure horseradish peroxidase conjugate (Aimerbio, Beijing, China) and incubated with 3-mercaptopropionic acid (3-MA) or 3-MA + Baicalein for 1 h. The membranes were washed with Tris-buffered saline solution (TBS) and incubated with H2O2 (3%) and 4-chloronaphthol (1%). The bands were visualized under the ImageJ2 software (National Institutes of Health, Bethesda, U.S.A.).

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**Fig. 1. Improved Motor Function Recovery in the Baicalein Group after Spinal Cord Injury.**

(A) We rigorously adopted Basso Mouse Scale for Locomotion (BMS) scores to determine the motor function at days 1, 3, 7, 14, and 28 after spinal cord injury (SCI). BMS scores the Sham group were stable at day 21. Compared to the Vehicle group, the BMS scores of the Baicalein group were significantly increased at days 7, 14, and 28 after SCI (n=5 in each group; *p<0.05; **p<0.01). (B) Similarly, the inclined plane test score was used to determine motor function, which showed that the scores of the Baicalein group were higher compared to the scores of the than Vehicle at the days of 7, 14, and 28. (Data were compared by two-way repeated measures ANOVA post hoc Bonferroni test and expressed as the mean±S.E.M.; n=5 per group; *p<0.05; **p<0.01).
peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000, Haoranbio, Shanghai, China, HSA0003) or AffinPure HRP-conjugated goat anti-mouse IgG (1:10000, Haoranbio, HSA0001). Membranes were washed and proteins were visualized using ECL solution, imaged by ChemiDoc-ItTMTS2 Imager (Shilianboyan, Beijing, China), and quantified using ImageJ2x software.

**Statistical Analysis** Data were presented as the mean±standard error of mean (S.E.M.) from three groups and analyzed by SPSS software (17.0). Two-way repeated measures ANOVA post hoc Bonferroni test was used among the BBB scores. One-way ANOVA post hoc Dunnett’s test

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Fig. 2. Enhanced Number of Neurons in the Baicalein Group at 7 Days after Spinal Cord Injury

(A) Nissl staining was used to determine the number of spinal anterior horn motor neurons in each group. (B) Nissl staining indicated that compared to the Vehicle group, the number of spinal anterior horn motor neurons was higher in the Baicalein group and significantly higher compared to the Sham group (data were compared by one-way ANOVA post hoc Dunnett’s test and expressed as the mean±S.E.M.; n=5 sections per group; **p<0.01, ***p<0.001; bar=100 µm).

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Fig. 3. Expression of Autophagy-Related Proteins in the Baicalein Group by Western Blot Analysis at 7 Days after Spinal Cord Injury

(A) Western blot analysis was used to evaluate the protein expression of p-PI3K, LC3B-I, LC3B-II, p62 and Beclin-1. (B–E) Western blot results showing that, compared to the Baicalein group, protein level of p62 were higher in the Vehicle group, and significantly higher in the Sham group. However, compared to the Baicalein group, the protein expression levels of p-PI3K, LC3B-II/ LC3B-I, and Beclin-1 were lower in the Vehicle group and significantly lower in the Sham group (data were compared by one-way ANOVA post hoc Dunnett’s test and expressed as the mean±S.E.M.; n=5 per group; *p<0.05, **p<0.01).
was used to compare two groups. One-way ANOVA post hoc Tukey–Kramer test were used when more than three groups were compared. Values of $p<0.05$ were considered statistically significant.

RESULTS

**Baicalein Protects Motor Function and Motor Neurons of Spinal Cord after Acute Spinal Cord Injury**

Because of the reduction of neuronal function and microenvironment disorders caused by acute SCI, the motor function of the post-surgery mice was estimated by performing animal behavioral experiments using BMS scores and inclined plane tests. The BMS scores of vehicle-treated and Baicalein-treated groups were lower compared to those in the sham-treated group. Moreover, the curve exhibited that the ascending trend of Baicalein group was significantly higher compared to that of the vehicle group, and significant differences were found between vehicle and Baicalein groups at days 7 ($p<0.05$), 14 ($p<0.01$) and 28 ($p<0.01$, Fig. 1A). Similarly, the inclined plane test scores of the Baicalein group were significantly higher compared to the vehicle group at days 7 ($p<0.05$), 14 ($p<0.05$), 28 ($p<0.01$, Fig. 1B).

The behavior text results unexpectedly indicated that Ba-

![Fig. 4. Analysis of Baicalein-Induced Autophagy Using Spinal Cord Sections at 7 Days after Spinal Cord Injury](image-url)

(A, B) Analysis of Baicalein treatment at autophagy morphology using immunofluorescence analysis and transmission electron microscopy (TEM). (C) Immunofluorescence analysis showing that, compared to the Vehicle group, LC3B-NeuN positive cells/total cells was higher in the Baicalein-treated group ($n=5$ sections per group, *$p<0.05$; bar=$100\mu m$). (D) Similarly, using TEM, the number of autophagosomes in the Baicalein-treated group after SCI was increased compared to that in the Vehicle group. (Data were compared by one-way ANOVA post hoc Dunnett’s test and expressed as the mean±S.E.M.; $n=5$ sections per groups; *$p<0.05$; **$p<0.01$; bar=$500nm$).
icalein treatment improved motor function recovery of the damage caused by acute SCI. Therefore, next we estimated the quantities of motor neurons in tissue sections of each group by Nissl staining. Our results indicated that compared with the sham-treated group, the vehicle group showed a reduction in size of Nissl bodies reduced ($p<0.001$). In addition, a greater number of Nissl bodies survived in the Baicalein-treated group compared with the vehicle-treated group ($p<0.01$, Figs. 2A, B).

**Baicalein Improves Autophagy via Activating PI3K after Spinal Cord Injury at 7 Days**

Next, we investigated whether Baicalein treatment influenced autophagy after SCI at day 7. To evaluate the biochemical effects of Baicalein treatment after SCI, autophagy-related protein, including p-PI3K, LC3B, Beclin-1, and p62 were quantified in the mouse spinal cord. Compared with mice in the sham group, Baicalein-treated mice showed a reduction in expression level of p62 ($p<0.05$), which was significantly reduced compared with the vehicle group ($p<0.01$). However, the expression of p-PI3K in Baicalein was higher compared with the sham and vehicle group ($p<0.05$). Compared with the vehicle, the Baicalein-treated group showed that expression levels of LC3-II/LC3-I and Beclin-1 after SCI were higher at 7 d ($p<0.01$ and $p<0.05$, respectively), and significantly higher compared with the sham group ($p<0.01$, Fig. 3).

Representative images of NeuN and LC3B positive neurons in spinal cord at 7 d are shown in Fig. 4A. In spinal cord sections at 7 d after injury the Baicalein-treated group showed a higher number of LC3B-positive neurons compared to the vehicle ($p<0.05$). Moreover, to evaluate autophagy at 7 d, we compared the differences at the organelle level of spinal cord by using TEM (Fig. 4B). Our findings indicated that the number of autophagosomes in the Baicalein group was higher compared to that in the vehicle group ($p<0.05$). It is noteworthy that Baicalein treatment of mice with SCI at 7 d after surgery significantly upregulated autophagy in the spinal cord, possibly via PI3K signaling.

**Baicalein Induces Autophagy to Inhibit Apoptosis after Spinal Cord Injury**

In the SCI model, the level of apoptosis may be significantly upregulated followed by inhibition of autophagy. Thus, we tested whether apoptosis was inhibited by Baicalein treatment after SCI. The data indicated that, compared with the vehicle, the Baicalein group showed a reduced expression of Caspase-3, Caspase-9, and Bax/Bcl-2 ($p<0.05$). Moreover, compared with the sham group, the Baicalein-treated group showed that the expression of Caspase-3 and Bax/Bcl-2 was higher after acute SCI at 7 d ($p<0.05$, Fig. 5A). Similarly, the proportion of TUNEL-positive neurons was lower in the Baicalein-treated group compared with the vehicle group ($p<0.05$, Fig. 5B). Combined, the autophagy and apoptosis-related data show that in the SCI mouse model, Baicalein treatment inhibited apoptosis, followed by the activation of autophagy. Thus, our observations suggested that these two phenomena may be interconnected.

To further evaluate the relationship between autophagy and apoptosis after Baicalein treatment, we used 3-MA (an autophagy inhibitor that specifically inhibits PI3K) to inhibit Baicalein-induced autophagy and evaluated changes in apo-

![Fig. 5. Analysis of Apoptosis Inhibition by Baicalein Treatment at 7 Days in a Spinal Cord Injury Model](image-url)

(A) Apoptosis-related proteins, such as Caspase 3, Caspase 9, Bax, and Bcl-2 were evaluated by Western blot analysis. (B) TUNEL staining and NeuN were used to estimate the level of apoptosis in the anterior horn of the injured area. (C–F) Statistical analysis of the indicators of apoptosis showing that, compared to the Baicalein group, expression levels of Caspase 3 and Caspase 9 were higher in the Vehicle-treated group, whereas the expression of Caspase 3 was lower in the Sham group. Similarly, in the Baicalein-treated group, Bax/Bcl-2 levels were lower compared to that in the Vehicle-treated group, but higher compared to that in the Sham-treated group. Moreover, compared to Baicalein group, the proportion of TUNEL positive-neurons was higher in the Vehicle group. (data were compared by one-way ANOVA post hoc Dunnett’s test and expressed as the mean±S.E.M.; n=5 sections per group; *p<0.05; **p<0.01; bar=100 µm).
ptosis. Figures 6, 7 and 8 show examples of autophagy and apoptosis-related protein expression of Beclin-1, Bax, Bcl-2, and TUNEL. We found that, in the SCI mouse model, administration of 3-MA decreased Beclin-1, followed by upregulation of apoptosis. Our data suggested that, Baiaclein induced autophagy to inhibit apoptosis after acute SCI at 7 d, possibly via PI3K signaling.

DISCUSSION

To confirm the neuroprotective effects of Biaeclein, we performed the functional morphological recovery with Baiaclein.
treatment after acute SCI in mice. We found that, Baicalein enhanced the recovery of motor neurons after acute SCI. In short, our findings indicated that, Baicalein plays a beneficial role during the recovery of spinal cord tissue after acute SCI.

Several previous reports have shown that Baicalein largely increased the level of autophagy in cancer and liver, however only few studies have focused on the effects in SCI. Autophagy plays a critical role on nerve function repair after CNS injury. However, the dysfunction of autophagy can lead to programmed cell death (PCD). Interestingly, even though autophagy can cause PCD, it protects the cells from several injuries, including SCI, Parkinson's disease, and Alzheimer's disease. The regulation of autophagy is very complex and includes many signaling pathways and players, such as P53, p62, Bcl-2, UV radiation resistance-associated gene protein (UVRAG), and Beclin-1. Thapalia et al. showed that PI3K was a key player in the regulation of autophagy, which can be inhibited in presence of 3-MA. Our results showed that activated protein (p-PI3K) was induced by Baicalein after SCI. In addition, LC3, a mammalian homolog of Atg8 in yeast, was found a critical factor of autophagy. LC3-I is a ubiquitin-like molecule, which was covalently bound to the membrane of phagocytes to form LC3-II (a marker of the autophagosome). Several studies showed that, LC3-II expression levels were regulated by the reaction of autophagic induction, as well as Beclin-1 (part of the Class III phosphatidylinositol-3-kinase). p62 is a ubiquitin-binding protein that decreases proteins via the lysosome, and autophagy can inhibit the level of p62. Moreover, Baicalein increased the levels of LC3-II and Beclin-1, whereas it decreased the levels of LC3-I and p62. Similar findings showed that Baicalein increased the number of LC3B-NeuN positive cells. Indeed, the number of autophagosomes was significantly increased in the Baicalein-treated group. Based on these findings, we verified that Baicalein treatment enhanced autophagy likely via activating PI3K after SCI.

Baicalein treatment decreased apoptosis in the CNS, liver, and lung after injury. Moreover, recent studies indicated that apoptosis-positive cells (pre-death cells) were found in damaged areas, which led to demyelination of the white matter after SCI, and significantly limited the recovery of neurological function. We evaluated the pro-apoptotic proteins Caspase 3, Caspase 9 and Bax as well as the anti-apoptotic protein Bcl-2, and showed that Caspase 3, Caspase 9, and Bax levels were up-regulated after Baicalein treatment, whereas Bcl-2 levels were down-regulated. Simultaneously, the proportion of TUNEL-positive neurons decreased with Baicalein treatment. Based on these results, Baicalein likely enhanced neuroprotective effects by inhibiting apoptosis at 7 d after SCI.

In this study, we showed an opposite trend between apoptosis-related and autophagy-related proteins after Baicalein treatment in SCI. Recent study reported that autophagy can reduce neuronal damage via inhibition of apoptosis after SCI. However, the mechanism of autophagy to reduce apoptosis is complex, and one of the principal factors involved is autophagic flux blockade. The disorder of autophagic flux aggravates endoplasmic reticulum (ER) stress, resulting in ER stress-induced apoptosis after SCI. When autophagic flux is blocked, the level of apoptosis increases after injury. Moreover, a high level of autophagic flux can inhibit apoptosis in SCI models. Thus, we infer that, Baicalein treatment inhibited apoptosis by inducing autophagy to promote the recovery of nerve cells after SCI. To test this hypothesis, we used 3-MA to inhibit autophagy after Baicalein treatment. The results showed that, the expression of apoptosis-related proteins was increased, while autophagy-related proteins significantly decreased after treatment with Baicalein and 3-MA. Thus, we suggested that Baicalein-induced autophagy in SCI mice inhibited apoptosis.

In this study, we only investigated 3-MA to mediate PI3K
and autophagy after SCI. However, the mechanism of the PI3K signaling pathway in SCI recovery involves other pathways in addition to autophagy, as reported in our present study. For example, PI3K inhibitors (3-MA) can affect SCI by inflammatory effect in vitro. Moreover, Baicalein may activate other pathway to inhibit apoptosis, for example, as present study reported, Baicalein can block tumor necrosis factor alpha (TNF-α) to inhibit apoptosis. Therefore, additional studies will be required to further test the relationship between Baicalein and nerve function recovery after SCI.

In summary, we investigated the role of Baicalein treatment on SCI mice. The data showed that Baicalein has several benefits for the recovery of liver cells after SCI, such as activation of autophagy, through apoptosis via the PI3K signaling.

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Conflict of Interest The authors declare no conflict of interest.

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