Chinese Journal of Physiology 55(3): 219-226, 2012
DOI: 10.4077/CJP.2012.BAA050

Effects of Baicalin on Protease-Activated Receptor-1 Expression and Brain Injury in a Rat Model of Intracerebral Hemorrhage

Qing-Bo Zhou[1], Qing Jia[2], Yuan Zhang[1], Lu-Yang Li[3], Zhao-Fu Chi[3], and Ping Liu[4]

[1]*Department of Neurology, The Second Hospital, Shandong University, Jinan 250033*
[2]*Department of Experimental Pathology, Institute of Basic Medicine*
Shangdong Academy of Medical Sciences, Jinan 250012
[3]*Department of Neurology, Qilu Hospital, Shandong University, Jinan 250012*
and
[4]*Institute of Pharmacology, College of Medicine, Shandong University, Jinan 250012*
Shandong, People's Republic of China

Abstract

Baicalin, a major flavonoid compound isolated from the dry roots of *Scutellaria baicalensis* Georgi, has been shown to be neuroprotective after ischemic brain injury. However, little is known about its effects on brain injury following intracerebral hemorrhage (ICH). In this study, we evaluated the effects of baicalin on ICH-induced brain injury in an ICH rat model. Male Wistar rats were injected intracerebrally with 0.5 U collagenaseVII to induce ICH, while control rats were injected with an equal volume of saline. After ICH induction, the rats were randomly divided into four groups and treated with baicalin at different doses (0, 25, 50 or 100 mg/kg) through peritoneal injection. The control rats were injected with an equal volume of vehicle. Brain tissues around the hemorrhage areas were collected on day 1, 3, 5 and 10 after treatment. Brain water content was analyzed by desiccation method; mRNA and protein levels of brain protease-activated receptor-1 (PAR-1) were determined by RT-PCR and Western blot, respectively; cell apoptosis was evaluated by terminal transferase dUTP nick end labeling staining. The results showed that baicalin effectively attenuated brain edema and inhibited cell apoptosis following ICH in a dose- and time-dependent manner, with concomitant suppression of PAR-1 expression at both the mRNA and protein levels. These findings indicate that baicalin has protective effects on ICH-induced brain injury. The effects of baicalin may involve a mechanism of inhibition of PAR-1 expression.

Key Words: baicalin, intracerebral hemorrhage, protease-activated receptor, neuroprotection

Introduction

Intracerebral hemorrhage (ICH) is a devastating neurological disorder accounting for 15-20% of all strokes. Patients with ICH have a high mortality rate and poor prognosis. It has been reported that as high as 50% ICH patients hardly survive the condition beyond 30 days. Of those survivors after the acute phase, only 20% can achieve full recovery within 6 months and 80% may suffer prolonged neurological deficits and brain atrophies (17). To date, no curative therapy is available for the treatment of ICH, and supportive therapy is the major option for most cases (33).

ICH-associated brain damage involves numerous mediators. Thrombin, produced in the brain either immediately after ICH (primary or secondary to brain hematoma) or after breakdown of blood brain barrier may play an essential role in this process. The thrombin-induced neurotoxicity has been demonstrated both

Corresponding author: Dr. Qing-Bo Zhou, The Second Hospital, Shandong University, #247 Beiyuan Street, Jinan 250033, Shandong, People's Republic of China. Tel: +86-531-85875207, E-mail: sdeyzqb@yahoo.cn
Received: June 24, 2011; Revised (Final Version): September 3, 2011; Accepted: September 20, 2011.
 ISSN : 0304-4920. http://www.cps.org.tw

in vitro and *in vivo* (11, 25, 27, 28, 31). Given that thrombin-induced extravascular neurotoxicity has been shown to be mediated by protease activated receptors (PARs), and that persistent overexpression of PAR-1 is commonly associated with brain damage following ICH (1, 34), targeting PAR-1 might offer a potential therapeutic option for patients with ICH.

Baicalin (7-glucuronic acid, 5, 6-dihydroxy-flavone) (Fig. 1) is one of the major flavonoid compounds isolated from the dry roots of *Scutellaria baicalensis* Georgi. *Scutellaria baicalensis* Georgi, as an important medical herb, has been widely used as an anti-allergic and anti-inflammatory agent in traditional Chinese medicine. Numerous studies have shown that baicalin has multiple biological activities such as anti-oxidant (4), anti-inflammatory (21), anti-tumor agent (8), anti-viral (28) and anti-apoptosis (24). In a model of cerebral ischemia reperfusion injury, baicalin showed neuroprotective effect through inhibition of nuclear factor κB p65 activation and caspase-3 overexpression (13, 32). A recent study has suggested that baicalin may have neuroprotective effects on ischemic cerebral injury raising the possibility of using baicalin as a potential agent for stroke therapy (2). However, little is known about the effects of baicalin on hemorrhagic brain injury. A previous report has shown that complex prescriptions containing *Scutellaria baicalensis* Georgi were able to inhibit PAR-1 expression in rat brain after ICH (36). The aim of this study was to evaluate the effects of baicalin on ICH-induced brain injury using an ICH rat model. In addition, the mechanisms underlying were also investigated.

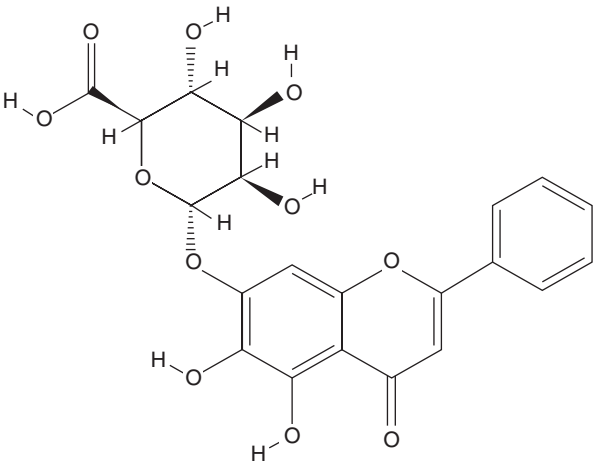


Fig. 1. The chemical structure of baicalin MW = 446.36.

Materials and Methods

Animal experiments were conducted in accordance to the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985) and the Regulations of the Committee on the Use and Care of Animals of Fudan University in Shanghai, China, and approved by the Ethics Committee of the Second Hospital of Shandong University in Jinan, China.

Chemicals and Reagents

Baicalin was purchased from Sichuan Xieli pharmaceutical Co. Ltd (Chinese Drug Approval Number: H20053191, Purity was 98.1%, Chengdu, China). CollagenaseVII was purchased from Sigma-Aldrich (St. Louis, MO, USA). 10% chloral hydrate was purchased from Qilu pharmaceutical Co. Ltd (Jinan, PRC). Goat-anti-rat *PAR-1* antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hybond-P PVDF membrane blot was purchased from GE Healthcare Life Sciences (Beijing, PRC). TRIZOL Reagent was purchased from Invitrogen (CA, USA). AMV one-step RT-PCR amplification kit was purchased from Sangon Biotechnology Company (Shanghai, PRC). *In Situ* Cell Death Detection Kit was purchased from Roche Diagnostics (Shanghai, PRC).

Animals and Experimental Proceeding

Male Wistar rats, aged 12 weeks and weighing 320-350 g, were provided by the Laboratory Animal Center of Shandong University (Jinan, Shandong, China). Prior to the experiments, the animals were allowed to adapt to the new environment for one week. During the adaptation and the whole experimental periods, the animals were housed in separate cages under diurnal lightning conditions with free access to food and water. ICH was induced by intracerebral administration of 0.5 U collagenaseVII in 1 μl saline into right the caudate nucleus of rats at a rate of 0.2 μl/min as previously described (20); sham operation rats were administered with an equal volume of saline without collagenaseVII. ICH was confirmed by the appearance of hematoma in the caudate nucleus. Rats with ICH were randomly divided into four groups: Group B (vehicle-treated); Group C (baicalin, 25 mg/kg); Group D (baicalin, 50 mg/kg); Group E (baicalin, 100 mg/kg). Rats with sham operation were used as controls (Group A). Two hours after the ICH induction, the animals were injected either with saline (Groups A and B) or baicalin (Groups C, D and E) at the indicated doses through intraperitoneal injection, and the same treatments were conducted once a day thereafter. On day 1, day 3, day 5 and day 10, six rats from each group were sacrificed by decapitation under anesthesia with 10% chloral hydrate. Brain tissues were collected from the perihematoma areas and stored at -70°C before assays.

Neurobehavioral Evaluation

Neurobehavioral function was evaluated by a score system as described previously (15). The score system consisted of 3 individual tests, each with a score range of 0-4 (0 = best; 4 = worst). The maximum total score value was 12. The tests included: [1] spontaneous ipsilateral circling behavior; [2] contralateral forelimb and hindlimb retraction capability; and [3] ability to walk a 70-cm-long 32.4-cm-wide wood beam. The evaluation was conducted by a masked observer at day 1, day 3, day 5 and day 10 after the induction of ICH.

Measurement of Brain Water Content

After the rats were sacrificed, brain tissues were immediately removed. Coronal slices of 4 mm each from the frontal poles of the right brain were made. Wet weight (WW) of each slice was determined by an electronic analytic balance. Subsequently, the slices were dried in an oven at 110°C for 24 h and weighed again to get the data of dry weights (DW). The water content (%) of the brain tissues was calculated as [(WW- DW)/WW] × 100%.

Western Blotting Analysis for PAR-1 Expression

Western blotting analysis was performed as previously described (26). Briefly, the brain tissues were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Proteins were separated by electrophoresis on a 10% SDS-PAGE gel and transferred to a Hybond-P PVDF membrane. Subsequently, the membrane was blocked in 5% non-fat milk and incubated with an *PAR-1* primary antibody (1:1000 dilution) overnight at 4°C, followed by incubation with the peroxidase-conjugated rabbit anti-goat secondary antibody (1:1000 dilution) for 1 h at room temperature. After washing with PBS, the bound primary antibody was visualized with the Enhanced Chemiluminescence System from Amersham (Piscataway, NJ, USA) and exposed to film. The same membrane was probed for β-actin for loading control. The relative density of PAR to β-actin was analyzed with the TotalLab TL120 Image software (TotalLab Life Science Analysis Essentials, Greensboro, NC, USA).

RT-PCR Analysis for PAR-1 *mRNA*

RNA was extracted using TRIZOL Reagent (3) and subject to measurement of *PAR-1* mRNA abundance using AnAMV one-step RT-PCR amplification kit according to manufacturer's instructions. Briefly, the first-strand cDNA was synthesized by RT using a random primer. The RT product was subsequently amplified by PCR on a Perkin-Elmer 9600 thermal cycler. The sequences of the sense and antisense primers used in the PCR reaction were: 5-ACT ATT TCT CCG CC TTC TCC GCC AT-3' and 5-TCA CGC AGA CGC AGA GGA GGA GGT AAG C-3', corresponding to nucleotides 900-924 and 1148-1172 of the rat *PAR-1* gene, respectively (35). β-actin was amplified under the same reaction conditions to serve as an internal control. The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. The predicted sizes for the *PAR-1* and β-actin products were 273 bp and 224 bp, respectively. Band intensities were analyzed using a Gel Documentation System and semi-quantitative analysis of *PAR-1* mRNA abundance was performed with the TotalLab TL120 Image software.

Measurement of Apoptotic Cells

Terminal Transferase dUTP Nick End Labeling (TUNEL) assay was performed with the *In Situ* Cell Death Detection Kit according to the manufacturer's protocol. Briefly, paraffin sections (two slides for each of the animals) were made from the brain tissue specimens collected at the time of animal sacrifice. Following deparaffinization, dehydration, pretreatment with proteinase K and peroxidase activity blocking, the sections were incubated with TdT enzyme at 37°C for 1 h. After washing with PBS, the sections were incubated with streptavidin-horseradish peroxidase for 15 min. After washing with PBS, diaminobenzidine tetrahydrochlorine (DAB) was added to visualize the TUNEL-positive signal. The number of TUNEL-positive cells in 10 non-overlapping fields around the hematoma on each of the slides was counted under an Olympus CH1 microscope by a research staff blinded to the slide identities using the Leica QWin V3 image analysis system (Leica, Wetzlar, Germany).

Statistical Analyses

All data were presented as means ± SD and analyzed with one-way analysis of variance (ANOVA) and Student's *t*-test. P value < 0.05 was considered statistically significant.

Results

Effect of Baicalin on Neurobehavioral Deficits Following ICH

Neurobehavioral deficits was evaluated by a score system. After the induction of ICH, all ICH

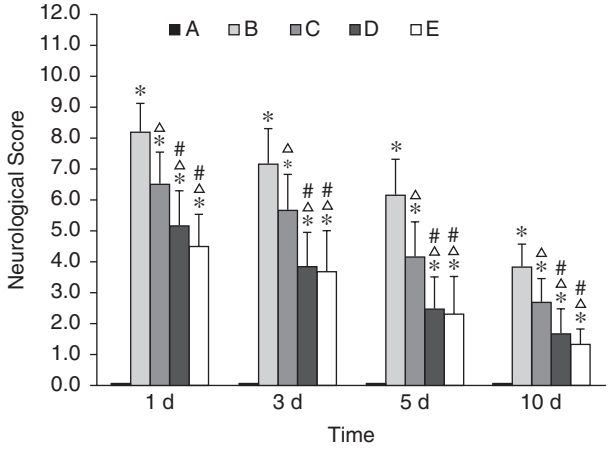


Fig. 2. Baicalin improved neurobehavioral deficits following ICH. At each time point, neurobehavioral deficits was evaluated by a score system as described in "Materials and Methods". The aggregate score represents the average neurobehavioral function per animal (n = 6 for each group). $^{*}P < 0.01$ *vs.* group A, $^{\Delta}P < 0.05$ *vs.* group B, $^{\#}P < 0.05$ *vs.* group C. Group A: control; Group B: vehicle treated ICH; Group C: baicalin (25 mg/kg) treated ICH; Group D: baicalin (50 mg/kg) treated ICH; Group E: baicalin (100 mg/kg) treated ICH.

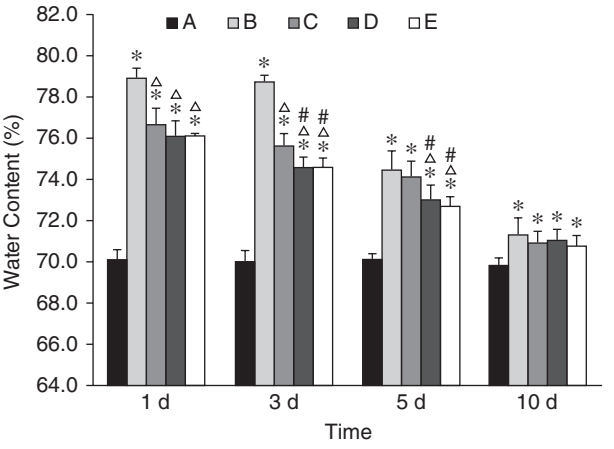


Fig. 3. Baicalin reduced brain water content following ICH. Brain water content was measured as described in "Materials and Methods". The quantitation represents the average value of the brain water content per animal (n = 6 for each group). $^{*}P < 0.01$ *vs.* group A, $^{\Delta}P < 0.01$ *vs.* group B, $^{\#}P < 0.05$ *vs.* group C. Group A: control; Group B: vehicle treated ICH; Group C: baicalin (25 mg/kg) treated ICH; Group D: baicalin (50 mg/kg) treated ICH; Group E: baicalin (100 mg/kg) treated ICH.

groups showed neurobehavioral deficits as compared with the sham operation group (Group A) (Fig. 2). The neurobehavioral deficits were alleviated over time in each group suggestive of self-recovery ability of the rats. At each time point, baicalin significantly improved the neurobehavioral function in a dose-dependent manner.

Effect of Baicalin on Brain Edema Following ICH

Brain edema was evaluated by measurement of the brain water content (Fig. 3). After the induction of ICH, brain water content in vehicle treated ICH group (Group B) was increased significantly on day 1 as compared with that of the sham operation group (Group A) and peaked on day 3. At each time point, the brain water content in Group B was significantly higher than those of the control group ($P < 0.01$, respectively). On day 3 and day 5 after ICH, baicalin significantly reduced the brain water content in a dose-dependent manner. On day 10, the brain water contents in all ICH groups were still higher than that of the control group, whereas the brain water content of the vehicle-treated ICH group was not significantly different from those of the baicalin-treated groups.

Effects of Baicalin on PAR-1 Protein Expression

PAR-1 protein in the control Group (group A) was maintained at a constant level throughout the whole observation period (Fig. 4). Compared with the control group, the PAR-1 protein level in the vehicle-treated ICH group (Group B) was increased significantly on day 1 and peaked on day 3. At each time point, the PAR-1 protein level in the vehicle-treated ICH group (Group B) was significantly higher than that of the control group ($P < 0.01$, respectively). At each time point, baicalin at all doses significantly reduced the PAR-1 protein levels as compared with those of the vehicle-treated ICH group ($P < 0.01$, respectively). On day 3 and 5, baicalin suppressed the PAR-1 protein expression dose-dependently.

Effects of Baicalin on PAR-1 *mRNA Expression*

To investigate if changes in the PAR-1 protein levels were related to changes in the mRNA levels, *PAR-1* mRNA levels were next examined. *PAR-1* mRNA was found to be maintained at a low and constant level in the control group (Fig. 5). After the induction of ICH, *PAR-1* mRNA level in the vehicle-treated ICH group (Group B) increased significantly on day 1 and peaked on day 3. At each time point, the *PAR-1* mRNA level in the vehicle-treated ICH group was significantly higher than that of the control group ($P < 0.01$, respectively). Baicalin at all doses significantly reduced the *PAR-1* mRNA expression as compared with that of the vehicle-treated ICH group ($P < 0.01$, respectively). On day 3 and 5, baicalin was

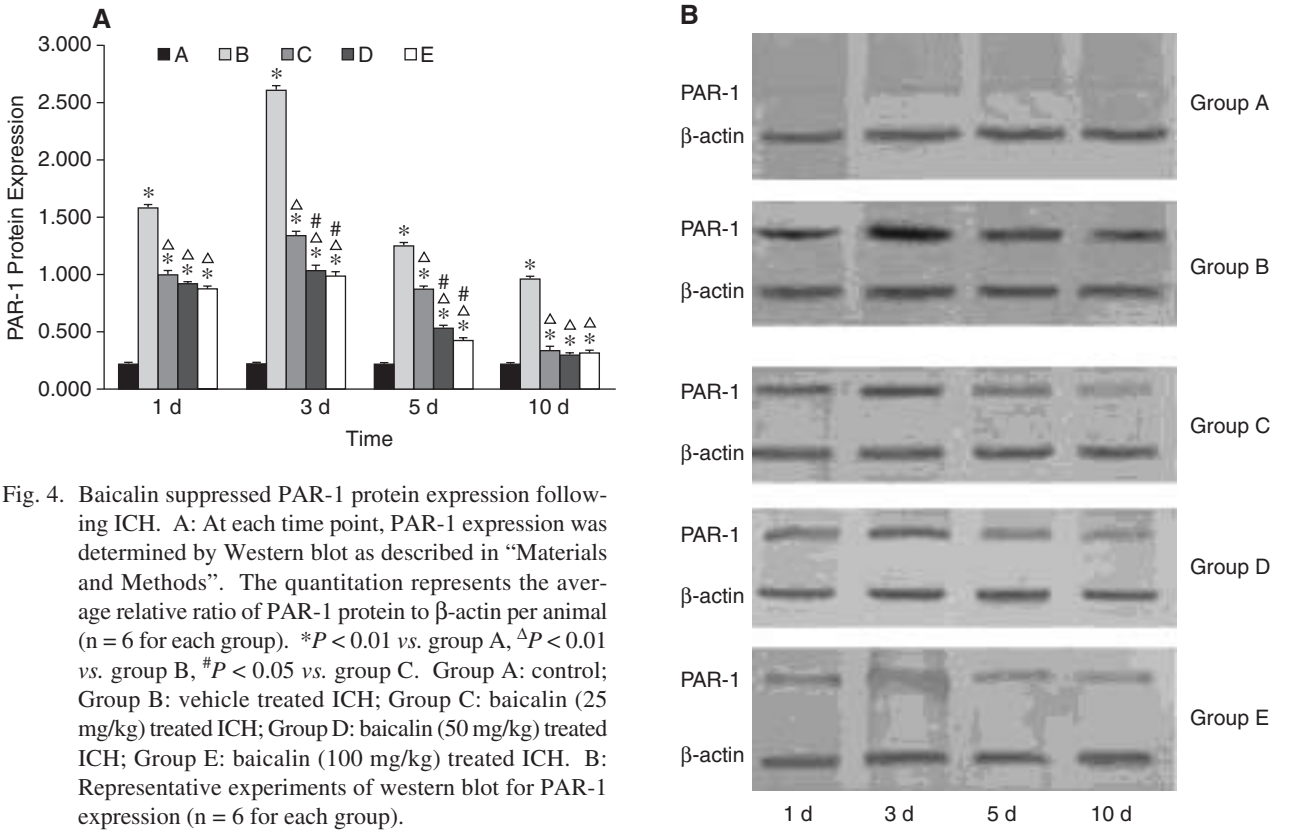


Fig. 4. Baicalin suppressed PAR-1 protein expression following ICH. A: At each time point, PAR-1 expression was determined by Western blot as described in "Materials and Methods". The quantitation represents the average relative ratio of PAR-1 protein to β-actin per animal (n = 6 for each group). $^{*}P < 0.01$ *vs.* group A, $^{\Delta}P < 0.01$ *vs.* group B, $^{\#}P < 0.05$ *vs.* group C. Group A: control; Group B: vehicle treated ICH; Group C: baicalin (25 mg/kg) treated ICH; Group D: baicalin (50 mg/kg) treated ICH; Group E: baicalin (100 mg/kg) treated ICH. B: Representative experiments of western blot for PAR-1 expression (n = 6 for each group).

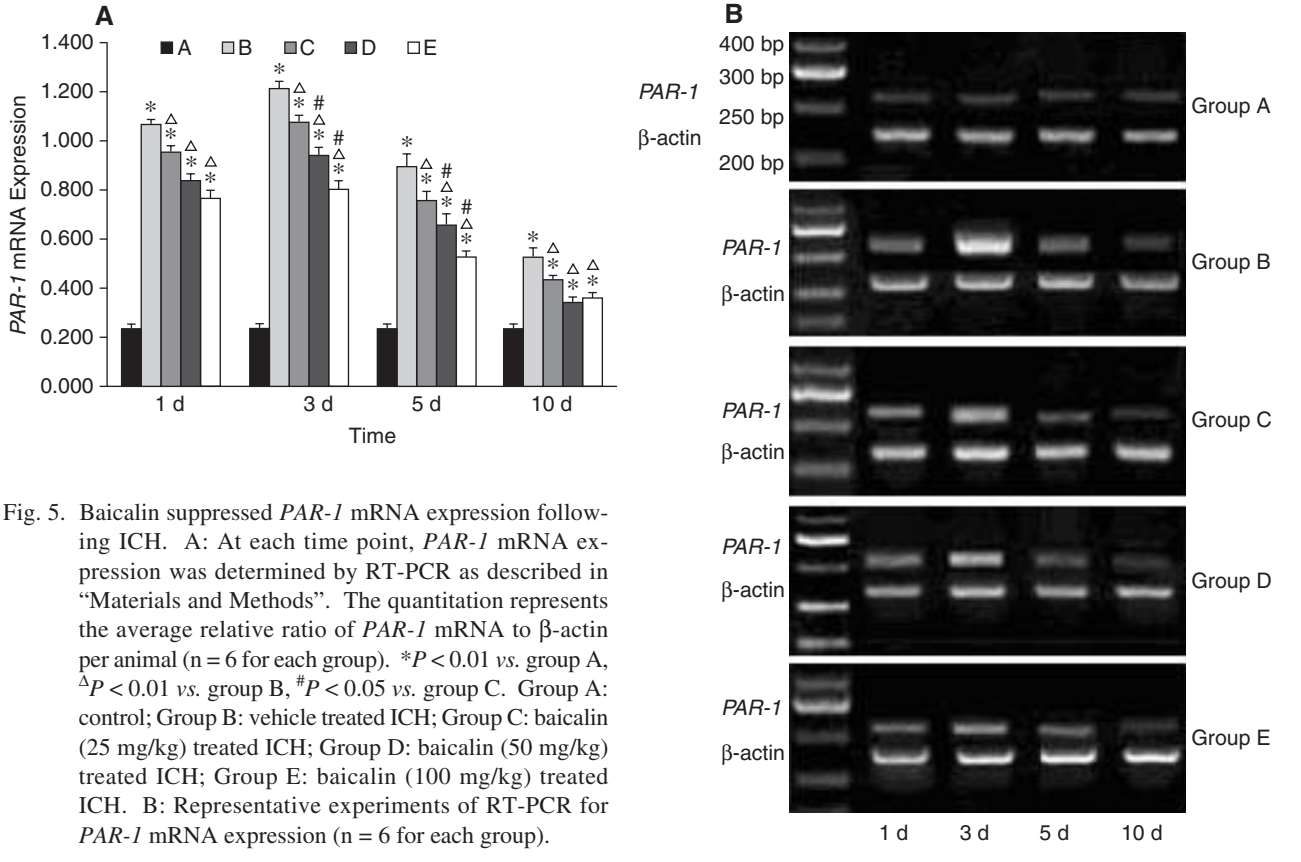
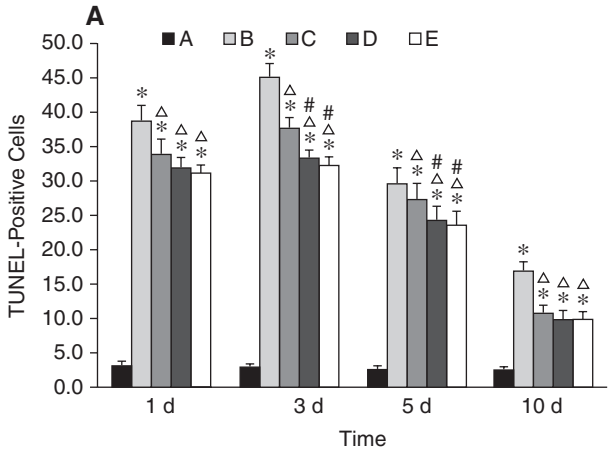


Fig. 5. Baicalin suppressed *PAR-1* mRNA expression following ICH. A: At each time point, *PAR-1* mRNA expression was determined by RT-PCR as described in "Materials and Methods". The quantitation represents the average relative ratio of *PAR-1* mRNA to β-actin per animal (n = 6 for each group). $^{*}P < 0.01$ *vs.* group A, $^{\Delta}P < 0.01$ *vs.* group B, $^{\#}P < 0.05$ *vs.* group C. Group A: control; Group B: vehicle treated ICH; Group C: baicalin (25 mg/kg) treated ICH; Group D: baicalin (50 mg/kg) treated ICH; Group E: baicalin (100 mg/kg) treated ICH. B: Representative experiments of RT-PCR for *PAR-1* mRNA expression (n = 6 for each group).



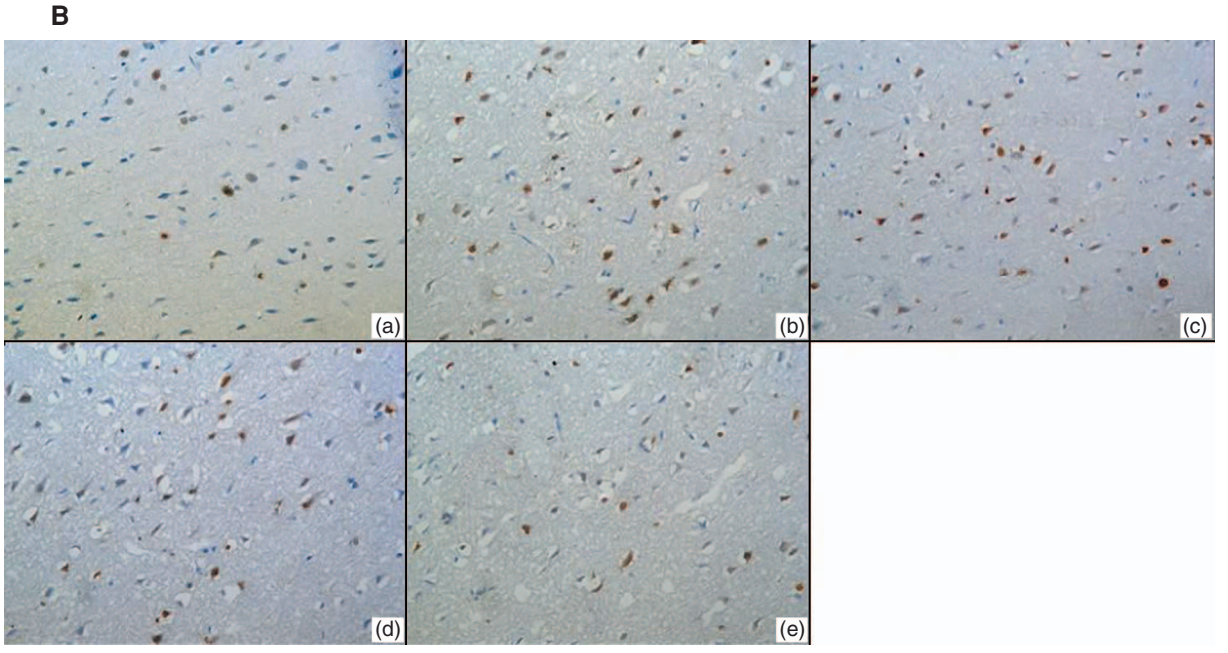


Fig. 6. Baicalin inhibits ICH induced cell apoptosis following ICH. A: At each time point, cell apoptosis was determined by TUNEL as described in "Materials and Methods". The quantitation represents the average number of TUNEL-positive cellsfield of 6 fields per animal (n = 6 for each group). $^{*}P < 0.01$ *vs.* group A, $^{\Delta}P < 0.01$ *vs.* group B, $^{\#}P < 0.05$ *vs.* group C. Group A: control; Group B: vehicle treated ICH; Group C: baicalin (25 mg/kg) treated ICH; Group D: baicalin (50 mg/kg) treated ICH; Group E: baicalin (100 mg/kg) treated ICH. B: Representative experiments of TUNEL staining at 1 day after ICH (n = 6 for each group).

shown to inhibit the *PAR-1* mRNA expression in a dose-dependent manner.

Effects of Baicalin on Cell Apoptosis

Following ICH induction, cell apoptosis around the hemorrhage areas in the vehicle-treated ICH group (Group B) increased significantly on day 1 as compared with the control group ($P < 0.01$), and peaked on day 3. Baicalin at all doses significantly reduced cell apoptosis at each time point ($P < 0.01$, respectively). At earlier phase following ICH (day 1 to day 5), baicalin inhibited cell apoptosis in a dose-dependent manner (Fig. 6).

Discussion

ICH is a severe neurological disorder with a poor prognosis. At present, curative therapy for ICH is still lacking. In this study, we have taken the advantage of an ICH animal model to investigate the effects of baicalin on ICH-induced brain injury. Several approaches are currently available for the establishment of animal models of ICH among which the injection of autologous blood or collagenase is most commonly used. It has been reported that disruption of basal lamina of cerebral capillaries and bleeding in the brain parenchyma start 30 min after intrastriatal injection of collagenase and hematoma appears in about 4 h (23). In the present study, we successfully induced ICH in rats by intrastriatal injection of collagenaseVII as confirmed by the appearance of hematoma in the caudate nucleus. Neurobehavioral deficits were apparent after ICH. Baicalin significantly improved the neurobehavioral functioning in a dose-dependent manner.

Brain damage from acute hemorrhagic stroke is a complex process involving multiple pathways. In-

jury can be raised from tissue reactions secondary to hematoma resulting in ischemia, edema, intense inflammation, apoptosis and ultimately cell death (6). Thrombin is a serine protease generated by proteolysis of prothrombin and is an essential component in the coagulation cascade. Generated upon the clotting of hematoma, thrombin is believed to play a key role in the processes of ICH (7). The biological activities of thrombin are primarily mediated by its receptors, PARs. PAR-1 is the major isoform of PAR receptors that are expressed in brain tissues (*e.g.* hippocampus, thalamus, hypothalamus and striatum). Previous studies have shown that the thrombin expression was markedly increased at both mRNA and protein levels in the brain of rats after ICH induction (26, 29, 34). In the present study, we demonstrated that expression of the thrombin receptor PAR-1 was dramatically induced as early as 24 h after ICH and peaked on day 3, an observation similar to that reported previously (34). Additionally, we demonstrated that, in parallel with the increase in PAR-1 protein level, the *PAR-1* mRNA level was also elevated suggesting that the increased expression of PAR-1 protein might be due to increased gene transcription or mRNA stabilization. These results strongly suggest the aberrant thrombin-PAR-1 signaling is involved in ICH-induced brain injury.

Edema is one of major pathological features in the brain tissue after ICH, and is believed to play a role in subsequent neural cell death (25). Edema and cell death are commonly seen in the region surrounding brain hematoma after hemorrhage stroke (19). In line with these ICH-associated pathologic changes, our results showed that collagenase challenge robustly increased brain water content and cell apoptosis in the perihematoma regions. The present study extended our previous study (36) by demonstrating that baicalin had neuroprotective effects on ICH-induced brain injury. Various molecular mechanisms have been proposed on the neuroprotective activities of baicalin including inhibition of protein kinase C-alpha translocation (12), inactivation of N-methyl-D-aspartate receptors (5), reduction of calcium overload (14) or inhibition of matrix metallopeptidase-9 (31). In addition, it has been shown that the formation of edema induced by injection of blood into the striatum of rats could be prevented by various thrombin inhibitors (9, 10). In the present study, we demonstrated that baicalin treatment significantly reduced brain edema and cell apoptosis after the induction of ICH with commitment suppression of thrombin receptor PAR-1 expression. The results suggested that the protective effects of baicalin may be partially through the disruption of thrombin-PAR-1 signaling pathway.

At present, the mechanism underlying the neurotoxicity of thrombin-PAR-1 signaling remains unclear. Xue *et al*. demonstrated that thrombin-induced neurotoxicity in ICH was only partially mediated by PAR-1 activation but largely mediated by its direct activation of matrix metalloproteinases (30). However, we could not exclude the possibility that the effects of baicalin observed in this study may involve other mechanism(s). As mentioned previously, *Scutellaria baicalensis* Georgi is a widely used traditional Chinese herb that exhibits anti-inflammatory property (18). Thrombin has been shown to stimulate the production of the proinflammatory cytokines, including interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF), in several types of cells (16, 22). It is not clear whether baicalin has a direct effect on inflammatory processes independently of thrombin signaling. As one limit of the present study, we did not check the effects of baicalin on the ICH-induced inflammatory process. Further studies are needed to address this issue.

Acknowledgments

This work was supported by Shandong Natural Science Fund Program of China (No.Q2008C15), Shandong Traditional Chinese Medicine Administration Fund Program (2007-143) and Free Exploration Program of Shandong University (2009TS009).

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