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Original Article

Baicalin protects against thrombin induced cell injury in SH-SY5Y cells

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Abstract: Baicalin, an extract from the dried root of Scutellaria baicalensis Georgi, was shown to be neuroprotective. However, the precise mechanisms are incompletely known. In this study, we determined the effect of baicalin on thrombin induced cell injury in SH-SY5Y cells, and explored the possible mechanisms. SH-SY5Y cells was treated with thrombin alone or pre-treated with baicalin (5, 10, 20 μM) for 2 h followed by thrombin treatment. Cells without thrombin and baicalin treatment were used as controls. Cell viability was detected by MTT assay. Cell apoptosis was analyzed by flow cytometry. Real-time PCR was performed to determine the mRNA expression of protease-activated receptor-1 (*PAR-1)*. Western blotting was conducted to determine the protein expression of PAR-1, Caspase-3 and NF-κB. Baicalin reduced cell death following thrombin treatment in a dose-dependent manner, with concomitant inhibition of NF-κB activation and suppression of PAR-1 expression. In addition, baicalin reduced Caspase-3 expression. The above findings indicated that baicalin prevents against cell injury after thrombin stimulation possibly through inhibition of PAR-1 expression and NF-κB activation.

Keywords: Baicalin, neuroprotection, protease-activated receptor-1, thrombin

Introduction

Ischemia-reperfusion leads to neuronal damage [1, 2]. One of the key events following cerebral ischemia is thrombin toxicity [3, 4]. In patients with ischemic cerebrovascular disease (ICVD) or those with high risk of ICVD, thrombin expression is elevated [5, 6].

Thrombin inhibitor such as argatroban ameliorated thrombin induced neural injury [7]. Accumulating evidence showed that the action of thrombin is mediated by PAR-1 [8-11]. Protease-activated receptors (PARs) are a family of G-protein coupled receptors comprising four members (PAR-1, 2, 3 and 4) [12, 13]. PAR-1 is predominantly expressed in the central nervous system [14, 15]. In rodent models, early increase in PAR-1 expression has been confirmed in brain tissue after thrombin induction [16, 17]. Conversely, inhibition of PAR-1 activity attenuated thrombin-induced brain injury and neurological deficits in mice [5].

NF-κB pathway plays an essential role in inflammatory processes. The NF-κB expression was showed to be up-regulated upon thrombin stimulation and was suggested to contribute to cell injury in ischemic stroke [9, 18]. As an important transcriptional regulatory factor, NF-κB participates in transcription regulation of multiple target genes. Activation of NF-κB signaling pathway leads to sustained inflammatory responses [19]. Inhibition of NF-κB protects against thrombin induced cell injury.

Baicalin is one of the principal flavonoids extracted from the dried root of Scutellaria baicalensis Georgi (Huang Qin). Baicalin possesses multiple biological activities in peripheral organs or tissues, including anti-inflammation [20]; anti-oxidant [21]; anti-tumor [22] and anti-virus [23]. In our previous study, we showed that baicalin has protective effects on focal cerebral ischemia reperfusion injury through attenuating the thrombin toxicity in mice [5]. In this study, we used SH-SY5Y cell line to deter-

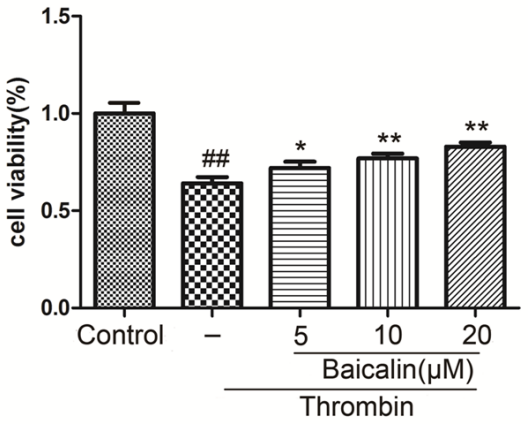

Figure 1. Effects of baicalin against cytotoxicity of thrombin stimulation. In thrombin group, cells were pre-incubated with baicalin (5, 10, 20 μM) for 2 h before exposed to thrombin (40 U/ml) for 6 h. Data are expressed as mean ± SEM of 3 independent experiments. $^{\#\#}P<0.01$ compared with control group; $^{*}P<0.05$, $^{**}P<0.01$ versus thrombin group.

mine whether baicalin has a direct effect on thrombin-mediated cell injury in vitro, and to explore the possible mechanisms.

Materials and methods

Chemicals and reagents

Baicalin, 7-D-glucuronic acid-5, 6-dihydroxy flavone, was provided by Haotian Pharmaceutical Company, China (Chinese Drug Approval Number: H20073931, purity was 95%, Weifang, China). Thrombin (T6884) and dimethyl sulfoxide (DMSO; D5879) were purchased from Sigma-Aldrich (St. Louis, MO, USA); Fetal bovine serum (FBS; 10099-141) was received from Gibco; RPMI-1640 (SH30809.01B) was obtained from Hyclone; Thiazolyl blue tetrazolium bromide (MTT; 0793) was from Amresco; TRIzol (15596-026) Reagent was purchased from Invitrogen (CA, USA). PAR-1(ab183083), NF-κB (ab32536) antibodies were purchased from Abcam. Caspase-3 (9664) antibody was purchased from Cell Signaling Technology; β-actin (sc-47778) antibody was purchased from Santa Cruz Biotechnology.

Cell culture and experimental design

SH-SY5Y cell line was purchased from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). The cells were cultured in RPMI-1640 medium (Hyclone) supplemented with 15% fetal bovine serum (Gibco) at 37°C in an air atmosphere containing 95% air and 5% CO_2 with a saturated humidity. Upon a confluence of 60~70%, the SH-SY5Y cells were divided into: (i) control group, incubated in RPMI-1640 medium; (ii) thrombin group, which was subject to thrombin induction (40 U/L) for 6 h based on our pre-experiment; and (iii) baicalin groups, which were treated by baicalin (5 μM, 10 μM, or 20 μM) for 2 h before induction of thrombin.

Cell viability assay

Cell viability was measured using MTT assay as previous described [24]. Briefly, 15 μl of the MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37°C. After removing the supernatant, 80 μL DMSO were added into each well. The absorbance was measured at 492 nm using a microplate reader (Thermo, Multiskan MK3, USA). All experiments were performed in triplicate.

Apoptosis detection by flow cytometry

Cells were harvested by trypsinization without EDTA, and washed twice in PBS. After staining with AnnexinV/fluorescein isothiocyanate (FITC) and propidium iodide (PI), the cells were immediately analyzed by flow cytometer (FACS Calibur, Becton Dickinson).

Real-time PCR analysis of PAR-1 mRNA

Total RNA was extracted from SH-SY5Y cells by using TriZol RNA extraction reagent (Invitrogen, CA, USA). RNA concentration was determined by UV absorbance at 260 nm. Subsequently, single-strand cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's specifications. Quantitative real-time PCR was performed using SYBR green on an ABI 7000 PCR System (Applied Biosystems, USA) using specific primers listed as followed: β-actin: 5'-TGACGTGGACATCCGCAAAG-3' (forward primer), 5'-CTGGAAGGTGGACAGCGAGG-3' (reverse primer); PAR-1: 5'-CACAAACGTCCTCCTGATTG-3' (forward primer) and 5'-ATGCTGCTGACACAGACACA-3' (reverse primer). Standard curves of the target genes were taken sharp with results of parallel PCR reactions performed on serial dilutions of a standard DNA. Finally, the amplification results were calculated using the $2^{(-\Delta\Delta Ct)}$ method [25].

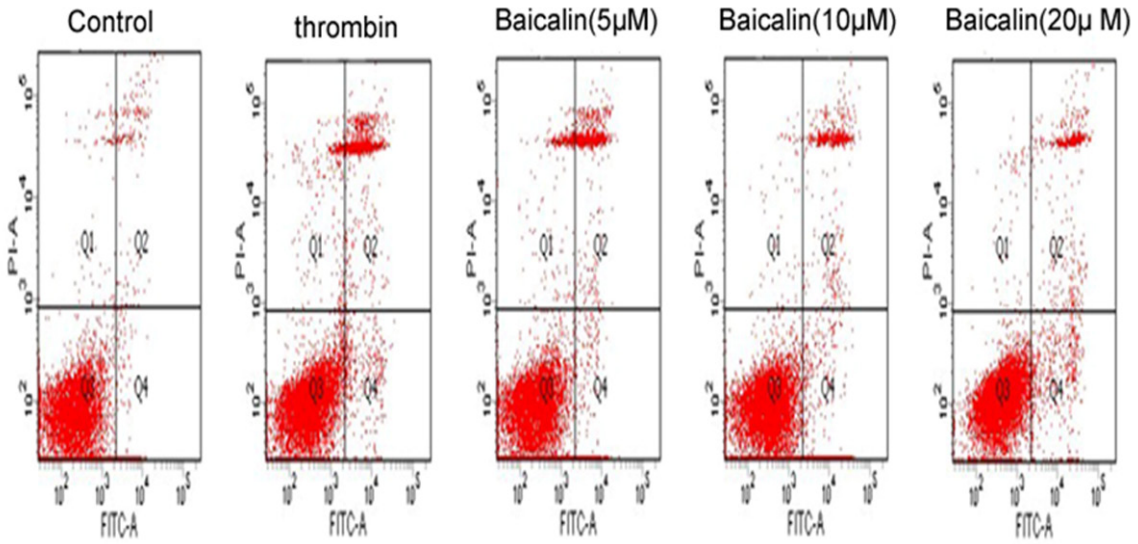

Figure 2. Baicalin inhibited thrombin-induced apoptosis. Four populations was possible to identify: viable cells in the lower-left quadrant (low PI and FITC signals); early apoptotic cells in the lower-right quadrant (low PI and high FITC signals); necrotic cells in the upper-left quadrant (high PI and low FITC signals); and late apoptotic cells in the upper-right quadrant (high PI and high FITC signals). The flow cytometric data in thrombin treated SH-SY5Y cells.

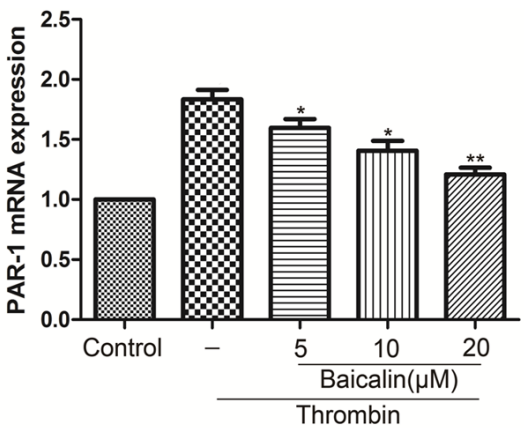

Figure 3. Baicalin suppressed PAR-1 mRNA expression following thrombin-induced injury. *PAR-1* mRNA expression was determined by the quantitative real-time PCR system. Data are expressed as mean ± SEM of 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus thrombin group.

Western blot analysis

Initially, the SH-SY5Y cells were homogenized in RIPA lysis buffer containing protease inhibitor. The concentration of the protein was determined by Coomassie brilliant blue protein assay. Equal amounts of the protein were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Subsequently, the membranes were blocked with 5% non-fat dry milk and incubated with the primary antibodies against PAR-1, NF-κB (1:2,000, Abcam, Cambridge, MA, USA), and Caspase-3 (1:1,000, Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. Afterwards, these membranes were incubated with the peroxidase-conjugated goat anti-rabbit secondary antibodies for 1.5 h at room temperature. The same membrane was probed for β-actin for loading control. After washing with TBST buffer, the immunocomplexes were detected with the enhanced chemiluminescence plus kit (Millipore Corporation, Billerica, MA01821 USA).

Statistical analysis

All data were analyzed by SPSS 15.0 software. The data were expressed as mean ± standard error. Differences between groups were analyzed by t test. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of baicalin on thrombin induced cell injury

Cell viability was determined by MTT assay. Compared with control cells, cell viability was significantly decreased in SH-SY5Y cells treated with thrombin. Pre-treatment with baicalin increased cell viability in a dose-dependent manner compared with cells treated thrombin alone (**Figure 1**).

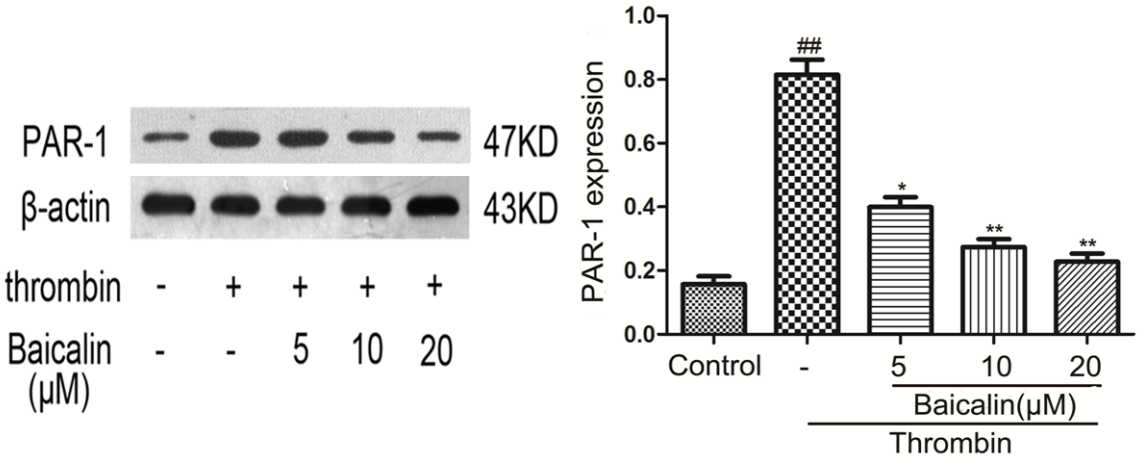

Figure 4. Baicalin suppressed PAR-1 protein expression following thrombin-mediated injury. Anti-β-actin antibody was used for normalization in the Western blotting analysis. The intensity of bands was quantified by densitometric analysis. All values represent mean ± SEM of three independent experiments. $^{\#\#}P<0.01$ compared with control group; $^{*}P<0.05$, $^{**}P<0.01$ versus thrombin group.

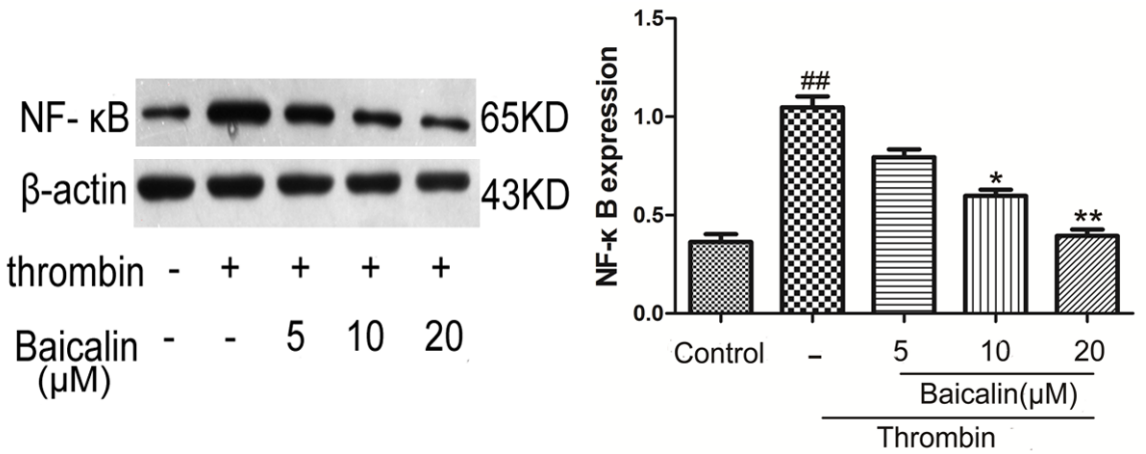

Figure 5. Effects of baicalin on the protein level of NF-κB in thrombin-stimulated SH-SY5Y cells. Histograms represent mean ± SEM of the relative intensity of NF-κB protein bands normalized to β-actin. $^{\#\#}P<0.01$ compared with control group; $^{*}P<0.05$, $^{**}P<0.01$ versus thrombin group.

As MTT assay cannot distinguish between necrosis and apoptosis, flow cytometry was performed. As shown in **Figure 2**, approximately 19.4% of SH-SY5Y cells showed typical features of apoptosis after thrombin induction. In baicalin treated groups, the apoptosis rates in the low, moderate and high dose groups were 15.5%, 11.3%, and 7.4%, respectively.

Baicalin inhibited thrombin induced PAR-1 mRNA and protein expression

As shown in **Figure 3**, thrombin significantly increased *PAR-1* mRNA expression, which was partly attenuated by baicalin pre-treatment. Similarly, baicalin pre-treatment also attenuated thrombin induced PAR-1 protein expression (**Figure 4**).

Baicalin inhibited thrombin induced NF-κB and Caspase-3 protein expression

The effects of baicalin on the NF-κB and Caspase-3 protein expression were determined by western blotting. Compared with the control group, thrombin increased NF-κB protein expression, which was significantly attenuated by medium or high dose of baicalin (10, 20 μM)

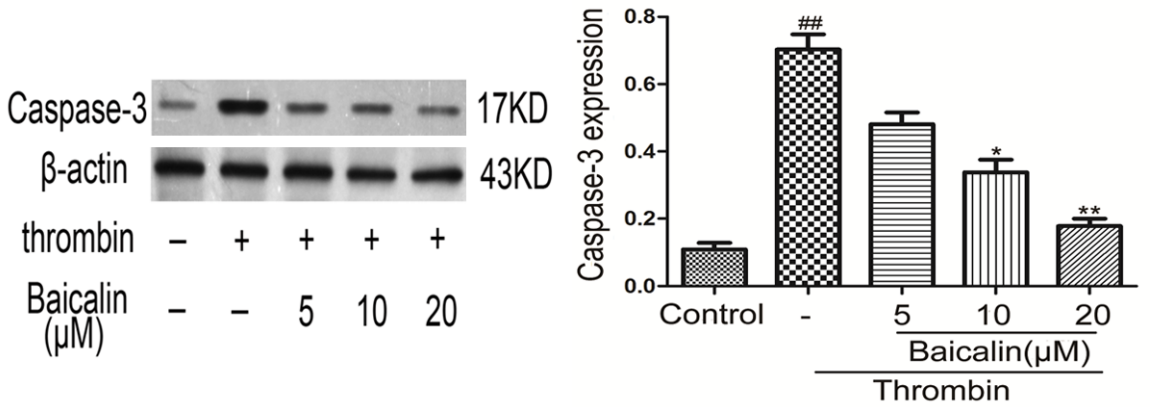

Figure 6. Effects of baicalin on the expression of Caspase-3 protein in thrombin-treated SH-SY5Y cells. The β-actin acts as the internal standard. Data are expressed as mean ± standard deviation. $^{\#\#}P<0.01$ compared with control group; $^{*}P<0.05$, $^{**}P<0.01$ versus thrombin group.

(**Figure 5**). In addition, thrombin also induced Caspase-3 protein expression, which was significantly attenuated by high dose of bacailin (**Figure 6**).

Discussion

In the present study, we demonstrated that baicalin attenuated thrombin induced cell injury in SH-SY5Y cells. This protective effect of baicalin is associated with the inhibition of PAR-1, NF-κB and Caspase-3 expression.

As a serine protease, thrombin is an essential component of the coagulation cascade, which is produced by the cleavage of pro-thrombin. Evidence showed that brain may also be a source of pro-thrombin. Pro-thrombin mRNA is not only expressed in the cells of the nervous system but also up-regulated after cerebral ischemia and spinal cord injury [26-28]. Thrombin is generated in the brain either immediately after cerebral hemorrhage or after the blood brain barrier (BBB) breakdown that induced by many kinds of brain damages [26, 29].

In the present study, we showed that thrombin (40 U/L) caused obvious cell injury in SH-SY5Y cells, which was significantly attenuated by pre-treatment with baicalin in a dose-dependent manner. Our results were consistent with previous studies showing that baicalin was neuroprotective following cerebral ischemia in animal models [30-32].

It was proposed that the extra-vascular effects of thrombin were mediated by a family of PARs [10, 11]. PARs are a family of seven transmembrane G protein-coupled receptors that include PAR-1, PAR-2, PAR-3 and PAR-4. Of these different receptors, PAR-1, PAR-3, and PAR-4 can be activated by thrombin, whereas PAR-2 is activated by trypsin [33]. PAR-1 is predominantly expressed in the brain and has been suggested to mediate the thrombin toxicity in cerebral ischemia-reperfusion damage [5]. To explore the possible mechanism by which baicalin reduces thrombin-induced cell injury, we determined the effect of baicalin on the PAR-1 expression. Our results showed that the PAR-1 expression was significantly increased after thrombin stimulation within 6 h at both mRNA and protein levels, which were attenuated by baicalin in a dose-dependent manner.

NF-κB is a critical regulator of inflammation. It exists in the cytoplasm as a dimer predominantly formed by the p65/p50 complex in an inactive state combined with members of the NF-κB inhibitor (I-κB) family. In an external activation pathway, I-κB is phosphorylated by I-κB kinases (IKKs), which results in its degradation, and thus liberating the active NF-κB complex which migrates into the cell nucleus and initiates the transcription of target genes [34, 35]. According to previous studies, thrombin could induce NF-κB activation and subsequent inflammatory responses [9, 18]. In line with previous studies, our results showed that thrombin treatment significantly up-regulated the NF-κB (p65) expression, and this was partly attenuated by pre-treatment with baicalin.

Previous studies showed that the over-expression of NF-κB or PAR-1 could induce the expres-

sion of pro-apoptotic proteins, which finally led to cell apoptosis [8, 36, 37]. Caspase-3 is a key player in apoptotic signaling pathways, and Caspase-3 expression has been reported to be induced by various herbs [38, 39]. In the present study, we determined the effect of thrombin on Caspase-3 expression in SH-SY5Y cells. Our results showed that Caspase-3 expression was up-regulated by thrombin, which was attenuated by bacailin in a dose-dependent manner.

Taken together, our results demonstrated that baicalin effectively attenuated thrombin induced cell injury in SH-SY5Y cells. The mechanisms may be partly through the inhibition of PAR-1, NF-κB and Caspase-3 expression. These findings raise the possibility of the use of baicalin as a therapeutic agent for ischemic cerebrovascular disease.

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Disclosure of conflict of interest

None.

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