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Research article

Neuroprotective effects of crocin against traumatic brain injury in mice: Involvement of notch signaling pathway

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HIGHLIGHTS

- Pretreatment of crocin (20 mg/kg) conferred neuroprotective effects on the mice against TBI.
- Pretreatment of crocin (20 mg/kg) significantly increased the Notch signaling activation after TBI.
- Inhibition of Notch signaling attenuated the ability of crocin to protect mice against TBI.

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ABSTRACT

This study investigated the protective effects and mechanisms of crocin, an extract of saffron, on brain damage after traumatic brain injury (TBI) in mice. C57BL/6 mice were subjected to controlled cortical impact (CCI)-induced TBI. Pretreatment with crocin (20 mg/kg) had protective effects against TBI, demonstrated by improved neurological severity score (NSS) and brain edema, decreased microglial activation and release of several pro-inflammatory cytokines, and decreased cell apoptosis. TBI activated Notch signaling, as shown by upregulated levels of Notch intracellular domain (NICD) and Hes1 mRNA, and pretreatment with crocin further increased Notch activation. However, pretreatment with DAPT (100 mg/kg), a gamma-secretase inhibitor, significantly suppressed crocin-induced activation of Notch signaling and attenuated the ability of crocin to protect mice against TBI-induced inflammation and apoptosis. Therefore, these results suggest that crocin has neuroprotective effects against TBI in mice, and these effects are at least partially dependent on activation of Notch signaling.

1. Introduction

Traumatic brain injury (TBI) represents physical injury to brain tissue by mechanical forces of shearing, tearing, or stretching, resulting in contusion, hemorrhage, and immediate clinical effects. TBI presents a heavy economic and social burden, and there are currently no effective neuroprotective agents available for TBI patients. Nowadays, it is widely accepted that brain damage following TBI can be divided into initial and secondary injury phases, and prevention of secondary injury is the major target in therapeutic management of TBI. Post-traumatic cerebral inflammation, which is triggered by the initial injury and is characterized by microglia activation, leukocyte recruitment, and a subsequent upregulation of cytokines, plays an important role in the progression of secondary injury [12]. This neuroinflammation has been reported to promote edema formation and neuronal death and to ultimately lead to functional defects [2]. Therefore, it is imperative to search for an effective treatment based on anti-inflammatory strategies.

Crocin, a pharmacologically-active component of *Crocus sativus* L. (saffron), has been studied in many animal disease models. Crocin has a broad spectrum of pharmacological properties, including anti-hyperglycemic [24], anti-oxidant [24], and anti-tumor [10] effects, and it was recently found to exhibit anti-inflammatory activity in several rodent disease models. Specifically, crocin decreased the number of neutrophils in the carrageenan model of local inflammation in rats [4], alleviated inflammation by downregulating pro-inflammatory cytokines in a mouse colitis model when included in the diet [7], and improved spatial cognition

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after cerebral ischemia in rats [6]. However, whether crocin has effects on TBI and its related mechanisms has not been investigated.

The Notch signaling pathway, a well-conserved signaling pathway in animals, is activated by continuous processes: Notch ligand-receptor binding, cleavage of the Notch intracellular domain (NICD), localization of NICD to the nucleus, and transcriptional regulation of genes (e.g., Hes1 and Hes5). Notch signaling is fundamental for neuronal development and specification [14]. Accumulating studies have shown the Notch pathway to be extensively involved in various types of brain injury [8,19]. However, the effect of crocin on Notch signaling is unknown.

Therefore, this study investigated the effects of crocin and the Notch signaling pathway in a well-characterized TBI model, i.e., controlled cortical impact (CCI) in mice.

2. Material and methods

2.1. Animals

Male C57BL/6 mice (12–14 weeks of age, 28–32 g) were purchased from the Fourth Military Medical University Animal Services. Animals were housed under controlled conditions on a 12 h light/dark cycle at 21 ± 2 °C and 60–70% humidity. All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH) guidelines for the use of experimental animals and were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University.

2.2. Drug treatments

Crocin (Sigma–Aldrich Inc., St. Louis, MO, USA) was dissolved in normal saline, and the gamma-secretase inhibitor DAPT (Sigma–Aldrich Inc.) was dissolved in DMSO. Both crocin and DAPT were administered 30 min before TBI. The dose and method of crocin and DAPT infusion was chosen based on previous ischemia studies [13,18,23].

2.3. Animal models

TBI was produced via CCI (Hatteras Instruments, Cary, NC, USA) with some modification of previous methods [1]. Briefly, mice were anesthetized with 4% isoflurane in oxygen and placed in the stereotaxic frame on a thermostatically-controlled heating pad to maintain body temperature at 37 ± 0.5 °C. A portable drill was used to create a 3.5 mm diameter craniotomy over the right parietal cortex between bregma and lambda, 1 mm lateral to the midline. The dura mater was kept intact. To induce injury, a pneumatic piston impactor device (3 mm diameter, rounded tip) was used to impact the brain at a depth of 1 mm (velocity 4.5 m/s). Sham-operated mice underwent identical surgical procedures, but did not receive a CCI.

Testing at 24 h following TBI was completed as follows: (1) evaluation of neurological impairment (n = 5 per group); (2) evaluation of brain edema (n = 5 per group); (3) Western blot analysis (n = 5 per group); (4) real-time PCR (n = 5 per group); (5) terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and immunofluorescence (n = 5 per group); (6) enzyme-linked immunosorbent assay (n = 5 per group).

2.4. Evaluation of neurological impairment

Neurological deficits were evaluated using the Neurological Severity Score (NSS) on a 10-point scale according to Chen's method [21] by a researcher blinded to treatment. On this scale, one point is awarded for failure of each of 10 tasks, such that the maximum score of 10 points represents severe neurological dysfunction, whereas 0 points indicates normal function.

2.5. Evaluation of brain edema

According to Hatashita's wet-dry method [17], mice were killed by cervical dislocation, and their brains were immediately removed and placed onto a frozen plate, then weighed to determine wet weight. Next, brains were dried in a desiccating oven at 110 °C for 24 h and weighed again to determine dry weight. Brain water content was calculated using the following formula: brain water content (%) = (wet weight − dry weight) × 100/wet weight.

2.6. Western blot analysis

Western blot analysis was used to detect expression of cleaved caspase3 and NICD. In brief, injured cerebral cortices were individually homogenized in cold lysis buffer, and protein concentrations were determined using a Bio-Rad Protein Assay kit (Bio-Rad-Los Angeles, CA, USA). Samples (40–50 μg) were separated using 10% SDS-PAGE and blotted onto polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies, and immunoreactivity was detected by HRP-conjugated secondary antibody and visualized using chemiluminescence (Amersham, Piscataway, NJ, USA). The following primary antibodies were used: anti-cleaved caspase3 (Cell signaling Technology, Danvers, MA, USA, 1:1000), anti-caspase3 (cell signaling technology, 1:1000), anti-NICD (Abcam, Cambridge, MA, USA, 1:500), and anti-β-Actin (Santa Cruz Biotechnology, Santa Cruz, USA, 1:1000). The secondary antibody was goat anti-rabbit IgG-B (Santa Cruz Biotechnology, 1:20,000). Western blot band intensity was analyzed using Image-Pro Plus software.

2.7. Real-time PCR

The total RNA of each ipsilateral hemisphere was extracted using RNAiso Plus (TaKaRa, Dalian, China), as described previously [22]. Quantitative PCR was completed with the Bio-Rad iQ5 Gradient Real-Time PCR system (Bio-Rad-Los Angeles, CA, USA). Quantified values of RNA were normalized with those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were used in the current study: Hes1: (Fwd: TGT CAA CAC GAC ACC GGA CA, Rev: GCC TCT TCT CCA TGA TAG GCT TTG); GAPDH: (Fwd: GGC ACA GTC AAG GCT GAG AAT G, Rev: ATG GTG GTG AAG ACG CCA GTA).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Mice were sacrificed 24 h after TBI, their brains were extracted, and the injured cerebral hemisphere was homogenized. The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-1β were assessed using specific ELISA kits (Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer's instructions.

2.9. Immunofluorescence

Mice were anesthetized with isoflurane and intracardially perfused with 4% paraformaldehyde in phosphate buffered saline. Brains were extracted and sectioned coronally on a cryostat. Sections were permeabilized with 3% Triton X-100 for 10 min, blocked with 10% normal donkey serum in PBS for 60 min at room temperature, and incubated overnight with primary antibodies at 4 °C. Activated microglia were detected using rabbit anti-mouse IBA-1 antibody (Wako Pure Chemical Industries Ltd., Osaka, Japan; 1:800), followed by Alexa Fluor 488 donkey anti-rabbit secondary