Genetics and Molecular Research

Alleviation of spinal cord injury by Ginkgolide B via the inhibition of STAT1 expression

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Genet. Mol. Res. 15 (2): gmr.15027673
Received September 18, 2015
Accepted December 8, 2015
Published June 17, 2016
DOI http://dx.doi.org/10.4238/gmr.15027673

ABSTRACT. Ginkgolide B has been known to inhibit cell apoptosis by modulating multiple cytokines and plays an important role in neuroprotection. Signal transducer and activator of transcription 1 (STAT1) has been studied in a spinal cord injury (SCI) model. However, the role of Ginkgolide B in SCI treatment remains unclear. This study investigated the potential mechanism of Ginkgolide B using an SCI rat model. SD rats were used to generate an SCI model followed by Ginkgolide B injection (4 mg/kg) for 14 days. Spinal cord tissue samples were examined using hematoxylin and eosin (H&E) staining. The expression of STAT1 was determined by western blot. Using a dyskinesia scale, intervention with Ginkgolide B significantly decreased the severity of SCI. H&E staining revealed less nuclear condensation and cell necrosis in SCI rats after treatment with Ginkgolide B. STAT1 expression was significantly increased in SCI model rats, but was lower after Ginkgolide B treatment. Therefore, Ginkgolide B can effectively inhibit STAT1 expression and alleviate SCI.

Key words: Ginkgolide B; Signal transducer and activator of transcription 1; Spinal cord injury

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INTRODUCTION

Spinal cord injury (SCI) can be caused by multiple factors including trauma and degenerative disease. Although significant progress has been made regarding its diagnosis, treatment and rehabilitation, a cure for SCI is still a major hurdle in neurosurgery (Dasari et al., 2008; Zhao et al., 2012). Primary and secondary injuries occur sequentially in SCI. Primary injury occurs within a short-time window and causes irreversible physical damage to neural tissues. On the other hand, secondary injury leads to a series of pathological changes such as focal ischemia, tissue edema, and inflammation (Park et al., 2008).

During the SCI process, signal transduction plays an important role as a complex-regulatory network is involved including mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K)/Akt, glycogen synthase kinase 3β (GSK-3β), and Janus kinase (JAK)/signal transducer and activator of transcription 1 (STAT1). Among these well-studied pathways, STAT1 plays a critical role in signal transduction as a transcription factor by binding specifically onto surface receptors, resulting in its phosphorylation and nuclear translocation (Calapai et al., 2000; Wang et al., 2006; Dominguez et al., 2010).

Extracts of ginkgo biloba have significant pharmaceutical value and have been used in both basic and clinical studies, particularly with respect to the nervous system. Animal models have shown the alleviation of ischemia-induced neural injuries by ginkgo biloba extracts by their inhibition of nitric oxide synthase. Clinical studies also revealed the remarkable role of extracts in treating Alzheimer's disease and other brain disorders (Tchantchou et al., 2007). A recent *in vivo* study showed the protection of spinal cord neurons by the inhibition of cytosolic phospholipase A2 (cPLA2) and caspase-3 expression, thus depressing cell apoptosis (Zhao et al., 2011). As the major component of ginkgo biloba extracts, Ginkgolide B is a potent platelet activating factor and can penetrate the blood-brain barrier (Zhao et al., 2011). The purpose of this study was to investigate the mechanism of Ginkgolide B in alleviating SCI.

MATERIAL AND METHODS

SCI animal model

Rats were provided by the Shandong University Animal Experiment Center (license key: SYXK-2013-1239). A total of 30 SD rats were anesthetized and an area of 9 mm^2 was exposed around the T10 spine process as previously described (Vawda and Fehlings, 2013). The spinal cord was clipped for 3 s to generate SCI. A spastic tail movement and retraction of hind limbs followed by paralysis indicated the successful SCI model. The sham group received anesthesia, but did not underwent any surgery. Rats were used for all experiments and all procedures were approved by the Animal Ethics Committee of Jinan Military General Hospital.

The motor function of rats was evaluated 1, 3, 7, and 14 days after surgery. Using the Basso, Beattie and Bresnahan (BBB) scale, each animal was observed walking for 15-30 min. A score higher than 20 was considered normal walking; a score less than 8 was identified as motor dysfunction; and a score of 0 represented complete loss of motor function. Each animal also underwent IPT, the ramp test which is another method of motion detection.

Hematoxylin and eosin (H&E) staining

Spinal cord segment tissues were fixed in formalin overnight, followed by serial dehydration in 70% ethanol (3 h), 80% ethanol (3 h), 95% ethanol (2 h), 100% ethanol (1.5 h, twice) and xylene (0.5 h, twice). After immersion in paraffin (1 h at 55°C and 2 h at 60°C), tissues were embedded and sectioned in to 3-μm slices. Using xylene and gradient ethanol for dewaxing, slices were sequentially stained in hematoxylin (1 min) and eosin (10 s), and were mounted on glass slides. The pathological changes were evaluated by three independent observers in a blinded manner.

Western blotting

Total proteins were extracted from spinal cord tissues using protein extraction kit (Beyotime Biotechnology, Shanghai, China) and equal amounts of protein from each sample were loaded onto SDS-PAGE for separation. The electrophoresis was carried out at a voltage of 80 V for 1 h and 120 V until the die-front reached the bottom of the gel. Proteins were then transferred to PVDF membrane under a 250-mA electrical field for 90 min. After gentle rinsing in TBS-T, the membrane was blocked in 5% skim milk powder for 1 h. Rabbit anti-rat STAT1 primary antibody in 1:100 dilution (Santa Cruz, CA, USA) was applied for overnight incubation. The next day, the membrane was rinsed in TBS-T three times, followed by incubation with goat anti-rabbit secondary antibody (1:100 dilution, Santa Cruz). ECL chromogenic substrate was then used to develop the membrane.

Statistical analysis

The SPSS 11.0 package software (Chicago, IL, USA) was used to process all collected data. The Student *t*-test was used to compare means between groups. Pearson analysis revealed correlation between parameters. A statistical significance was defined when $P < 0.05$.

RESULTS

Ginkgolide B facilitates motor function recovery after SCI

Significant motor dysfunction can be observed by the naked eye after SCI model generation. The sham group had no significant change in motor function. IPT score revealed elevated motor function in SCI rats with Ginkgolide B intervention compared to the SCI-untreated group ($P < 0.05$; Figure 1A). BBB score showed consistent results as Ginkgolide B improved motor score significantly with longer treatment times ($P < 0.05$; Figure 1B).

Cell morphology changes in SCI

In sham rats, we found clear cell shape with normal nuclear morphology. In SCI model animals, nuclear condensation was observed at day 3 with the occurrence of necrotic cells at day 14. At the same time points, we found less nuclear condensation and cell necrosis in rats with Ginkgolide B treatment (Figure 2). These results support the hypothesis that cellular integrity is maintained with Ginkgolide B treatment after induction of SCI.

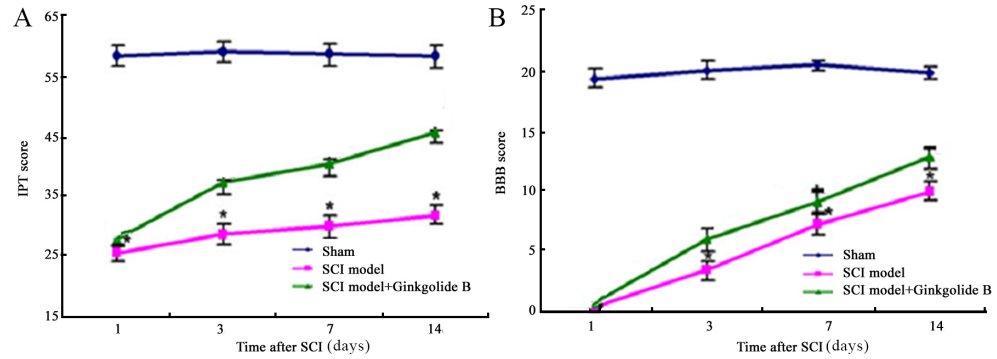

Figure 1. Motor function of rats after SCI. **A.** IPT score was lower in model rats but was higher after Ginkgolide B application. **B.** BBB score showed similar patterns. In both scenarios, Ginkgolide B resulted in significant improvements with longer treatment. *P < 0.05 compared to sham group.

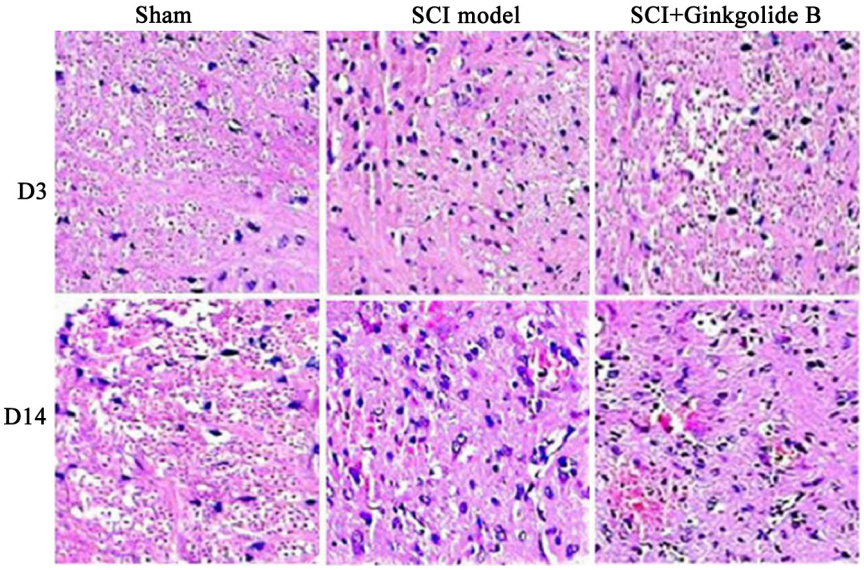

Figure 2. Spinal cord tissue morphology as shown by H&E staining. Sham animals (left panels) had clear nuclei without necrotic cells. SCI model (middle panels) had significantly increased nuclear condensation and cell necrosis. The application of Ginkgolide B (right panel) alleviated tissue injury compared to SCI model group. Cells are shown on day 3 (D3) and day 14 (D14).

Ginkgolide B inhibits STAT1 expression

As STAT1 is a major transcription factor expressed during signal transduction, we tested STAT1 expression in all animals. Specifically, the nuclear fraction of proteins was quantified, as phosphorylated STAT1 migrates into the nucleus to initiate transcription. Western blotting results showed that STAT1 activity was potentiated in SCI model group. Treatment with Ginkgolide B reduced STAT1 activity to levels similar to the sham group ($P < 0.05$). These results are shown in Figure 3.

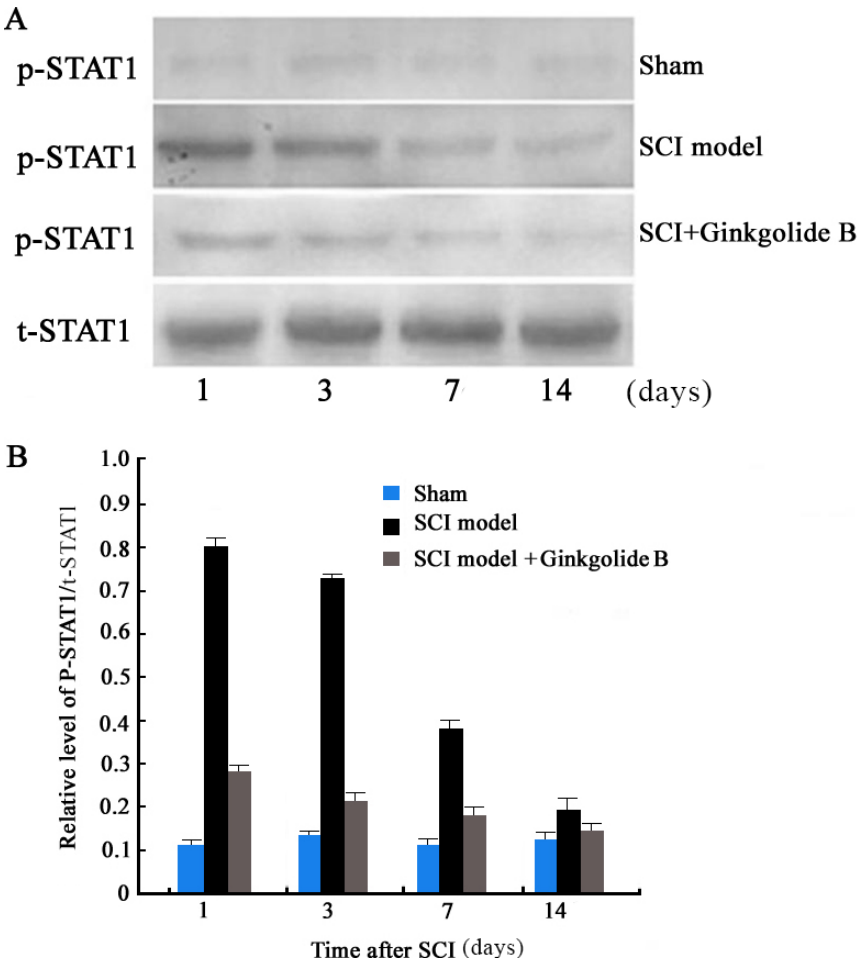

Figure 3. Expression of phosphorylated STAT1 (p-STAT1) and total STAT1 (t-STAT1) in rat spinal cords. **A.** Western blot and **B.** quantitation of p-STAT1/t-STAT1 at different time points.

DISCUSSION

SCI is mainly caused by traffic accidents, sports injuries and other traumas, and can cause severe neuropathological damage and neural dysfunction (Massieu et al., 2004). Secondary injury during SCI can cause apoptosis of neurons and glial cells, increasing the permeability of the spinal cord barrier and increasing neural reaction response time (Lin et al., 2008; Yoshimura et al., 2011). The secondary inflammation reaction in the spinal cord is even more severe than in brain tissue. Some studies have tried to manipulate the composition of inflammatory cells in mouse models of SCI but did not obtain satisfactory results (Shi et al., 2010; Lukáš et al., 2011), suggesting the necessity for the development of alternative treatments for secondary SCI.

The JAK/STAT signaling pathway is activated immediately after SCI and is accompanied with morphological changes and cell apoptosis (Cayli et al., 2004). Other studies described the activation of STAT1 in cerebral focal ischemia and brain damage and many potent antioxidants exert their protective functions via inhibition of STAT1 phosphorylation and nuclear translocation (Choi et al., 2011; Xu et al., 2011; Rong et al., 2012). The protective role of Ginkgolide B in SCI has not been reported; therefore, we hypothesized that Ginkgolide B might exert its protection of the spinal cord through the STAT1 pathway. This is based on evidence showing the alteration of important transcription factors, such as STAT1 and tumor growth factor β (TGF-β), in other injured tissues such as bone (Han et al., 2012; Ishii et al., 2013). Thus, we hypothesized that Ginkgolide B might exert similar effects through STAT1 in SCI.

Our study showed the important role of STAT1 in spinal cord tissues, where STAT1 is dephosphorylated under normal physiological conditions. The phosphorylation of STAT1 was initiated immediately after SCI induction and reached a peak 1 day after injury, following which it gradually decreased. These results suggested the activation of STAT1 after SCI, leading to nuclear condensation and cell necrosis and resulting in a lower motor score. Intervention of SCI with Ginkgolide B significantly decreased STAT1 phosphorylation levels, decreased nuclear condensation and cell necrosis, and initiated partial recovery of motor function.

In summary, this study demonstrated the facilitation of spinal cord function occurs through Ginkgolide B, possibly via STAT1 activation. Our results provide more insights regarding the clinical treatment of SCI.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

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