Original Research Article

Lugrandoside attenuates spinal cord injury by targeting peli1 and TLR4/NF-κB pathway to exert anti-inflammatory and anti-apoptotic effects

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Abstract

Purpose: To investigate the curative effect and mechanism of lugrandoside (LG) on spinal cord injury (SCI).

Methods: We probed the expression of Pellino1 (peli1) in microglia and spinal cord tissues with different treatments of LG. Lipopolysaccharide (LPS) was used to activate the microglia. Furthermore, rats were used to establish SCI model, and LG, at low and high concentrations, was administered to injured animals to ascertain whether LG exerted a therapeutic effect on SCI.

Results: LG inhibited the activation and recruitment of glial cells by acting as a negative regulator of glial inflammation, and this reverse the targeting of peli1 and TLR4/NF-κB pathway. Furthermore, the in vivo data showed that LG exerted a neuroprotective effect, following SCI, via anti-inflammatory and anti-apoptotic effects. Furthermore, Peli1 and TLR4/NF-κB were suppressed by LG stimuli.

Conclusion: These results suggest that LG protects neural tissue against neuroinflammation and apoptosis by suppressing TLR4/NF-κB pathway and negatively targeting peli1. The findings may provide new insights into the treatment of spinal cord injury.

Keywords: Spinal cord injury (SCI), Lugrandoside, Apoptosis, Neuroinflammation, Peli1

INTRODUCTION

Spinal cord injury (SCI) causes grievous neurologic disorders following trauma, leading to autonomic dysfunction, paralysis and even death [1,2]. Neurotrauma provokes the recruitment and activation of resident glial groups, chemotaxis and migration peripheric leukocytes in response to the primary injury [3]. Neuroinflammatory cascades break out and diffuse to the adjacent area, destroying normal spinal cord tissue by inducing apoptosis of neurons and glia groups. The above events eventually result in the loss of neurostructure and a demyelination procedure on a larger scale on the spinal cord [4,5]. Microglia regulates neurofunction in the normal stages, but there is activation of inflammation caused by trauma or infection in neurological diseases [6,7]. Previous studies have suggested that microglial inflammation is intimately relative to demyelination via the induction of oligodendroglia...
apoptosis [8]. Besides, neurons loss [9] and axonal injury [10] depend intently on the degree and the range of neuroinflammation. It is, therefore, very important to effectively restrain microglial activation that causes inflammation, in order to avoid secondary neural damage.

The peli protein family is a group of proteins with E3 ubiquitin ligase activity, including peli1, peli2 as well as peli3 [11]. Both of peli protein and toll family are involved in non-specific immune process [12]. In vitro studies have demonstrated that peli can regulate signal transduction mediated by TLRs/ IL-1R [13]. Moreover, peli1 also plays an important role in TLRs/TRIF-mediated signaling pathway activation and pro-inflammatory cytokine production decreases after peli1 knockout [14]. Recent studies have shown that peli1 regulates microglia-mediated neuroinflammation and affects the survival of dopaminergic neurons in Parkinson's disease [15]. However, whether peli protein regulates inflammatory function and improves neuronal function recovery post-SCI remains unknown.

Lugrandoside (LG), an emerging phenylpropanoid glycoside, is extracted from the culinary leaves of Digitalis lutea L. and Digitalis grandiflora Miller [16,17]. Earlier studies reported that phenylpropanoid glycosides play a role in antitumor, immune modulation as well as anti-inflammatory [18]. Lee et al. [19] suggested phenylpropanoid glycosides possess anti-asthmatic effect in guinea pigs. Li et al. [20] demonstrated LG ameliorates acute respiratory distress syndrome through inhibiting inflammation and apoptosis in mice. However, LG has not been implicated in neuroinflammatory responses as a promising anti-immune inflammatory agent. In this study, we hypothesized that LG might be a novel selective drug to improve the effect of neuroinflammatory response after SCI, and attempted to explore the therapeutic mechanism of LG in ameliorating inflammatory levels.

**EXPERIMENTAL**

**Animals**

Male Sprague Dawley (SD) rats (180 - 200 g) were obtained and sacrificed for the study. They were housed in groups of three per cage, and provided with accessible food and water, suitable temperature and humidity (22 -26 °C, 50 – 60 %), and 12 h light/dark cycle. All experiments were approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (no. SHTCM-AEC-no.17-32). All procedures were conducted in accordance with the ‘Animal Research: Reporting in vivo experiments guidelines 2.0’.

**Cell culture and drug treatment**

BV2, the microglia cell line, was purchased and cultured in Dulbecco’s modified Eagle’s medium (containing 100 U/mL penicillin and 100 µg/mL streptomycin) with 10 % fetal calf serum at 37 °C and 5 % CO2. When cells grew to 90 % confluence, different concentrations of LG (10, 20, or 30 ng/mL) were treated for 24 h before stimulation with LPS (100 ng/mL, 24 h). Then the cell cultured supernatants were gathered for determination of the inflammatory cytokines, and the cells were extracted into protein for the subsequent study.

**Surgical procedure and sample grouping**

Rat models were anesthetized using 10% chloral hydrate, and the skin was prepared. An incision was then made on their backs, the facial muscles were separated, and a laminectomy was performed to expose the T10-T11 spinal cord. A contusion was done using the NYU impactor with a 10 g rod dropped 5 cm from the cord surface. Grouping was as follows: the Sham group (n = 8) which was designated by a laminectomy without a contusion, the SCI group (n = 8) which conducted a contusion on the spinal cord, and the LG group (n = 8) which administered 30 mg/kg LG one week after the SCI.

**Western blot technique**

Cells and spinal cord tissues were extracted to obtain protein using total protein extraction kit (Keygen, Nanjing, China) following protocol. The extracted protein was measured using bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) and balanced for used in western blot. Protein was dispersed in 10 % sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Following blocking with 5 % skim milk, the membrane was incubated overnight with primary antibodies (anti-iNOS (Abcam, Cambridge, MA, USA, 1:500), anti-TLR4 (Abcam, Cambridge, MA, USA, 1:1000), anti-p-p65 (Abcam, Cambridge, MA, USA, 1:1000), anti-Peli1 (Abcam, Cambridge, MA, USA, 1:1000), anti-IκB (Abcam, Cambridge, MA, USA, 1:1000), anti-Bcl-2 (Abcam, Cambridge, MA, USA, 1:3000), anti-Bax (Abcam, Cambridge, MA, USA, 1:1000), anti-caspase-3/9 (Abcam, Cambridge, MA, USA, 1:1000), and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 1:2000)). It was washed with Tris-buffered saline with Tween-20.
Sheng et al

Trop J Pharm Res, February 2022; 21(2): 247

(TBST), the membrane was combined with secondary antibody (Abcam, Cambridge, MA, USA, 1:2000), then washed, protein bands were imaged using the enhanced chemiluminescence system and analyzed using ImageJ software.

**Enzyme linked immunosorbent assay (ELISA)**

The cell cultured supernatant and spinal cord samples was used to detect inflammatory cytokines using ELISA kit (Keygen, Nanjing, China) complied with instruction. Briefly, standard product and test samples were added into 96-well plate successively and performed lucifuge incubation at room temperature for 30 minutes. After washing, the enzyme standard reagent was added to each well and then the colorant and terminating agent were consecutively added to the wells. The absorbance of each sample was obtained and the standard curve was plotted to calculate sample concentration.

**Immunofluorescence (IF)**

Spinal cord tissue was added to 4 % paraformaldehyde (PFA), 15 and 30 % sucrose solution successively for fixation and dehydration. The spinal cord samples were cut into the paraffin sections (5 μm). Following deparaffinage, hydration and antigen retrieval, the sections were blocked using BSA and integrated with primary antibodies (anti-iNOS, 1:100; anti-IBA-1, 1:400) overnight. After washing with phosphate buffered solution (PBS), the sections were incubated with Cy3-conjugated goat anti-rabbit IgG antibody (Abcam, Cambridge, MA, USA, 1:200) for 2 h at room temperature. After washing, nuclei counterstaining and section mounting were performed using DAPI-Fluoromount-G (Southernbiotech, Birmingham, AL, USA). Then, fluorescence imaging was conducted under a fluorescence microscope (Zeiss, Oberkochen, Germany).

**Basso-Beattie-Bresnahan (BBB) locomotor rating assay**

BBB locomotor rating assay was performed to observe and evaluate the activity of rats’ hindlimb and two blinded researchers scored each sample according to the rating scale. All rats were allowed to move in an open field for 3 minutes at 1, 3, 7, 14, 28 days respectively following SCI.

**Statistical analysis**

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS IBM, Armonk, NY USA) statistical software was used to analyze the data. All data were described as the means ± standard deviations. Differences between two groups were analyzed by Student’s t-test. Comparison between multiple groups was done using one-way ANOVA test followed by post hoc test (least significant difference). \( P < 0.05 \) was deemed statistically significant.

**RESULTS**

Lugrandoside (LG) lowered the activation and inflammation levels of microglial cells *in vitro*

After processing with different concentrations of LG and lipopolysaccharide (LPS), the culture supernatant of the microglial cells was taken to aid the detection of the inflammatory factors using enzyme-linked immunosorbent assay (ELISA), so as to observe the influence of LG on the glial inflammation level. The results showed that after activation by LPS, the levels of interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) in the cell supernatant were elevated, compared with those in the control group. In LG pretreatment group however, it was found that the decrease in these inflammatory factors were inversely related to the gradient increase in LG concentration, and the difference was significant (Figure 1 A).

The inhibitory effect of LG on the release of inflammatory factors was caused by a decline in the inflammatory polarization degree in microglial cells (Figure 1 B). Therefore, inducible nitric oxide synthase (iNOS), a marker of inflammatory microglial cells located in cell proteins, was found to reflect the degree of inflammation in glial cells, and it was revealed that the iNOS content declined with the increase in LG concentration, which was consistent with the change in the trend of the inflammatory factors (Figure 1 C).

In order to determine the role of LG in the Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) pathway, an inflammatory pathway, the protein levels of TLR4, inhibitor of NF-κB (IκB) and phosphorylated-p65 (p-p65) were measured. The results indicated that the high-dose LG enhanced the expression of IκB but repressed the levels of TLR4 and p-p65 (Figure 1 D and E). In addition, it was discovered that the application of LG down-regulated the protein level of pellino E3 ubiquitin protein ligase 1 (Peli1), a regulatory factor of the TLR4/NF-κB pathway (Figure 1 F). All those findings manifest that LG inhibits the inflammation mediated by microglial cells in a concentration-dependent manner, and such a process is realized by down-regulating the
TLR4/NF-κB pathway through negative regulation on Peli1.

Figure 1: Concentration-dependent Lugrandoside (LG) lowered the activation and inflammation levels of microglial cells in vitro. (A) the levels of IL-1β, IL-6 and TNF-α in cultured microglia with 10, 20 and 30 ng/ml LG treatment. (B) the protein bands of iNOS, TLR4, p-p65 and Peli1 in microglia with 10, 20 and 30 ng/ml LG treatment. (C) the relative expression of iNOS protein. (D) the relative expression of TLR4 protein. (E) the relative expression of p-p65 protein. (F) the relative expression of Peli1 protein. *P < 0.05, **p < 0.01

LG attenuated the inflammation in tissues after spinal cord injury (SCI)

In order to understand the therapeutic effect and mechanism of LG on SCI in vivo, we treated SCI rats with LG and observed the inflammatory progression post SCI. At 3 days after SCI, the spinal cord tissues from the center of the injury site were used to detect the inflammatory factors via ELISA and Western blotting. The measurement results manifested that the outbreak of inflammation after SCI evidently increased the expressions of IL-1β, IL-6 and TNF-α in tissues, while the treatment with 30 mg/kg LG in vivo weakened the release of inflammatory factors remarkably (Figure 2 A - C).

Moreover, LG obviously decreased the expressions of TLR4 and p-p65 but increased the protein level of IκB. Meanwhile, it was indicated in the measurement of Peli1 protein that the protein expression of Peli1 declined after the treatment with LG (Figure 2 D). Furthermore, inflammatory polarization and chemotaxis in microglial cells were observed at 3 d after SCI by means of IF co-staining for iNOS and IBA-1(Figure 2 E). It was exhibited that LG could significantly reduce the number of iNOS-positive cells and chemotaxis at the margin of the injury site (Figure 2 F), further illustrating the interference of LG in glial inflammation. These results were all in consistence with the results of in-vitro cell experiments. Hence, it was concluded that LG in vivo represses the TLR4/NF-κB pathway by inhibiting Peli1, thus relieving the activation and recruitment of microglial cells in the acute phase of SCI and slowing down the progression of inflammation.

Figure 2: LG attenuated the inflammation level in tissues after spinal cord injury (SCI) by down-regulating the TLR4/NF-κB pathway. (A) IL-1β level in Sham group, SCI group and SCI+LG group at 3 days following SCI. (B) IL-6 level in Sham group, SCI group and SCI+LG group at 3 days following SCI. (C) TNF-α level in Sham group, SCI group and SCI+LG group at 3 days following SCI. (D) the protein bands of TLR4, p-p65, IκB and Peli1 in Sham group, SCI group and SCI+LG group at 3 days following SCI. (E) the immunofluorescent co-staining of IBA-1(green) and iNOS(red) at 3 days following SCI in SCI group and SCI+LG group. (F) the relative amount of iNOS-positive microglia. *P<0.05, **p < 0.01

Application of LG after SCI ameliorated cell apoptosis in injured spinal cord tissue

Apoptosis is closely associated with the inflammation level, and cells can initiate their apoptosis program under the stimulation of excessive inflammation. It was assumed that LG regulated the cell apoptosis while repressing inflammatory responses. Therefore, at 14 d after SCI, a series of apoptosis-associated indices were found to reflect the adjustment of apoptosis program by LG. The apoptosis-related proteins [B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), caspase-3 and caspase-9] were measured (Figure 3 A). It was shown that the expression of Bcl-2 was decreased after injury, while the expressions of Bax as well as activated caspase-3 and caspase-9 were raised prominently, so the ratio of Bcl-2 to Bax was decreased. Nevertheless, after the treatment with LG, the level of Bcl-2 was elevated notably, while the expressions of Bax as well as activated caspase-3 and caspase-9 were inhibited markedly, and the ratio of Bcl-2 to Bax was increased obviously (Figure 3 B). The
aforementioned findings elaborate that LG decreases the inflammation and regulate the apoptosis degree at the same time, so as to save the survival cells in tissues.

**Figure 3:** Application of LG after SCI ameliorated cell apoptosis in injured spinal cord tissue. (A) the protein bands of Bcl-2, Bax, caspase-3 and caspase-9 at 7 days post-SCI in Sham group, SCI group and SCI+LG group. (B) the relative protein expressions among the three groups. *P < 0.05, **p < 0.01

**Figure 4:** Treatment with LG improved motor function recovery after SCI in rats. The BBB rating scores in Sham group, SCI group and SCI+LG group at 1,3,7,14,28 days following SCI. *P < 0.05, **p < 0.01

**Treatment with LG improved motor function recovery after SCI in rats**

The recovery of motor function in each group of rats was assessed and scored for four weeks using BBB scale. The results indicated that the motor function score in Sham group was lower than normal score within 3 days and returned to the normal level after one week, which may be attributed to postoperative pain. However, the motor function in the other two groups remained at a low level within 3 d after SCI due to contusion of spinal cord, which was improved to some extent one week later. Besides, the recovery of lower limb function in LG-treated rats was superior to that in untreated rats. More noticeably, the LG-treated rats had a distinctly higher motor function score than untreated rats at 1 - 4 weeks after SCI (Figure 4), demonstrating that the treatment with LG improves the motor function recovery after SCI in rats.

**DISCUSSION**

A majority of secondary injuries after SCI are triggered by inflammatory responses and the biological event accompanied, including oxidative stress, edema and cell apoptosis [21], while the secondary injuries can mediate repeated injury at the original site and expand the area of injury. It can be seen that how to effectively and rapidly control the degree of inflammation after trauma is particularly important for the prevention of excess damage to tissues. The activation of responses of in situ microglial cells to trauma is an indispensable link of the initiation and progression of inflammation [22]. Previous studies reported that the microglial cells activated after SCI persistently release a large number of inflammatory factors and reactive oxygen species, resulting in the activation and recruitment of astrocytes [23], which probably has a close correlation with the apoptosis of oligodendrocytes [24]. In spite of various prospective studies on the treatment of SCI at present, there are few specific treatment methods that can evidently reduce the injuries induced by neuroimmune-mediated inflammations. LG is a kind of widely applied phenylpropanoid glycoside and possesses potent anti-inflammatory property. In the aspects of inhibiting tumor growth, increasing blood glucose concentration and regulating immunity, phenylpropanoid glycoside is proven to have very crucial effects, but its effect in regulating neuroinflammation after SCI is still unknown. Hence, the therapeutic mechanism and efficacy of LG after SCI was investigated in this research.

It was discovered that LG could inhibit the inflammatory microglial cells activated by LPS in vitro and efficiently lower the release of multiple inflammatory factors. In addition, the further exploration indicated that the anti-inflammatory effects of LG were realized by modulating the protein expression of Peli1 to repress the TLR4/NF-κB pathway. LG also had protective effects on the occurrence of immune-mediated glial inflammation and apoptosis of impaired nerve cells after SCI. NF-κB, as a classical inflammation-related transcription factor, can produce inflammatory responses, promote the expression of inflammatory factors and induce the initiation of apoptosis program after activation, which is inseparable from the regulatory role of Peli1 protein.
Huang et al [25] discovered that Peli1 induces the activation of microglial cells, inflammatory responses and neurological deficit after neurological diseases. Wang et al [26] demonstrated that Peli1 has dual regulatory effects on necrosis and apoptosis via mediating RIPK1 and c-FLIP. Importantly, more attention should be paid to the fact that Peli1 stimulates the incidence of neuroinflammation after viral infections [27]. Nevertheless, the medicines capable of effectively controlling the Peli1-mediated neuroinflammatory responses are rarely reported. It was revealed that LG repressed the activation of microglial cells and inflammatory responses, in which it negatively regulated Peli1 and the TLR4/NF-κB pathway modulated thereby. Besides, LG significantly alleviated the neuroinflammation and reduced the apoptosis level through the target after SCI, thus exerting neuroprotective effects.

In this study, the ability of LG to improve cell apoptosis was elucidated only from the aspect of the inhibition on inflammation, but the mechanism of LG in weakening the Peli1-mediated apoptosis was not revealed, and this still further investigation. It was argued in this research that the amelioration of motor function after SCI by LG was achieved due to its powerful anti-inflammatory and anti-apoptotic effects. It was also shown that the regulation of LG on Peli1 and TLR4/NF-κB pathway lowered the levels of neuroinflammation and neuronal apoptosis. The treatment of SCI provided the researchers with a brand-new perspective for drug research, and the treatment strategies for SCI can be further supplemented and developed on the basis of this research.

CONCLUSION

LG inhibits microglia inflammatory activation in vitro, attenuates microglia-induced neuroinflammation and neural apoptosis in vivo, and facilitates locomotor functional recovery following SCI. The findings of this study are important for us to understand the exact molecular mechanism, and may provide new insights for the treatment of spinal cord injury.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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