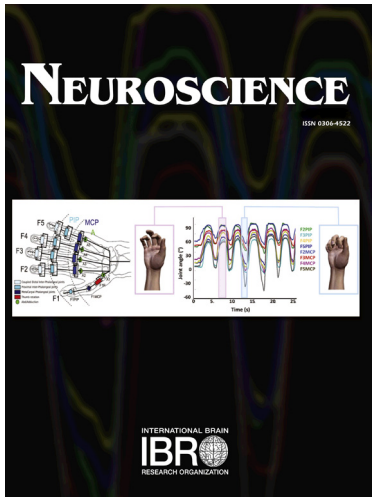

Research Article

β-elemene enhances GAP-43 expression and neurite outgrowth by inhibiting RhoA kinase activation in rats with spinal cord injury

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PII: S0306-4522(18)30319-1
DOI: https://doi.org/10.1016/j.neuroscience.2018.04.045
Reference: NSC 18434

To appear in: *Neuroscience*

Received Date: 9 October 2017
Accepted Date: 28 April 2018

Please cite this article as: J. Wang, H. Li, Y. Yao, Y. Ren, J. Lin, J. Hu, M. Zheng, X. Song, T. Zhao, Y-Y. Chen, Y. Shen, Y-J. Zhu, L-L. Wang, β-elemene enhances GAP-43 expression and neurite outgrowth by inhibiting RhoA kinase activation in rats with spinal cord injury, *Neuroscience* (2018), doi: https://doi.org/10.1016/j.neuroscience.2018.04.045

Title page

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Short Title: β-elemene enhances GAP-43 expression and neurite outgrowth

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Abstract

RhoA signalling pathway inhibitors such as Y27632 (a ROCK inhibitor) have recently been applied as treatments for spinal cord injury (SCI) because they promote neurite outgrowth and axonal regeneration in neurons. β-elemene, a compound that is extracted from a natural plant (Curcuma zedoary), influences the expression level of RhoA protein. Whether it can promote neurite outgrowth in motor neurons or enhance locomotor recovery in SCI remains unclear. Here, we initially demonstrated that β-elemene promotes neurite outgrowth of ventral spinal cord 4.1 (VSC4.1) motoneuronal cells and primary cortical neurons. Pull-down assays showed that β-elemene significantly inhibits the activation of RhoA kinase. Western blotting assays suggested β-elemene markedly inhibits the phosphorylation of limk and confilin and significantly increases the expression level of GAP-43. Then, in a rat model of SCI, haematoxylin-eosin and myelin staining showed that β-elemene reduces the area of lesion cavity and spares the white matter. BBB scores showed β-elemene significantly promotes locomotor behavioural recovery. In addition, western blotting assays and immunofluorescence staining demonstrated that the expression level of GAP-43 is upregulated by β-elemene treatment *in vivo*. Thus, our study provided an encouraging novel strategy for the potential treatment of SCI patients with β-elemene.

Key words

β-elemene; Neurite outgrowth; Spinal cord injury; RhoA; GAP-43

Abbreviations used in this paper

GAP-43	Growth-associated Protein-43
SCI	Spinal cord injury

H&E	Haematoxylin and Eosin
NF-200	Neurofilament protein 200

β-elemene enhances GAP-43 expression and neurite outgrowth by inhibiting RhoA kinase activation in rats with spinal cord injury

Introduction

Spinal cord injury (SCI) has become a severe global problem that leads to permanent motor and sensory deficits (Jackson, 2016; Peng et al., 2017). It has been estimated that approximately 3 million people in the world are suffering from SCI (Anna et al., 2017). Persistent functional deficits following SCI are due to the failure of spontaneous neurite regeneration and neurological connectivity restoration, which are caused by both the intrinsic characteristics of neurons in the central nervous system (CNS) and the proteome profile of the CNS (Chen et al., 2017; Jianli Hu and Selzer, 2017; Kaplan et al., 2017). Two classes of inhibitors, including inhibitory molecules of the extracellular matrix and inhibitory proteins associated with adult myelin, accumulate at the lesion site after SCI (Snow et al., 1990). In addition, following injury, microtubule destabilization limits neurite outgrowth (Knoferle et al., 2010). Thus, inhibiting the inhibitory environmental molecular cues and stimulating neurite outgrowth are effective strategies for repairing the damaged connections.

Ras homologue gene family member A (RhoA) is a small GTPase that is activated via guanine nucleotide exchange factors (GEFs) that catalyse the transition from an inactive GDP-bound state to an active GTP-bound state (Burridge and Wennerberg, 2004). Activated RhoA stimulates effector proteins, including Rho-associated kinase (ROCK) and p21-activated kinase (PAK), to participate in neuronal differentiation processes (neuronal migration, axonal growth, neurite outgrowth) (Kim et al., 2017). Meanwhile, ROCK promotes the phosphorylation of downstream effector protein limk, and then, p-limk promotes the phosphorylation of confilin protein, which eventually stabilizes actin and inhibits neurite outgrowth (Sit and Manser, 2011).

Several studies have certified that the increase in RhoA expression and activation that occurs after SCI restricts recovery (Lehmann et al., 1999; Dergham et al., 2002). Application of Y27632 (a ROCK inhibitor) or specific knockdown of RhoA significantly reduced neuronal apoptosis and enhanced axonal regeneration (Koch et al., 2014; Devaux et al., 2017; Gwak et al., 2017; J. Hu et al., 2017).

β-elemene extracted from a natural plant (Curcuma zedoary) is well known as an antitumoural drug (Zhu et al., 2015; Wu et al., 2017). However, its antioxidation activity in human umbilical vein endothelial cells revealed its potential for cytoprotective effects (Chen et al., 2014). Intriguingly, β-elemene can inhibit the expression of RhoA in hepatic stellate cells. Therefore, we next explored whether β-elemene promotes neurite outgrowth after SCI via influencing the RhoA signalling pathway. The relevant molecular mechanisms were also evaluated.

Materials and Methods

Cell culture and treatment

The ventral spinal cord 4.1 (VSC4.1) motoneuronal cell line was cultured as previously described (Gu et al., 2017). VSC4.1 motor neurons were grown in RPMI1640 medium containing foetal bovine serum (10%, v/v; Gibco, Invitrogen) and 1% penicillin and streptomycin at 37 °C with 5% CO2 in a fully humidified incubator, as recommended by the suppliers. First, we explored the effect of β-elemene (Sigma, USA) on cell viability. VSC4.1 motor neurons were disseminated in 96-well plates at a concentration of 5×10^3 per well, and then, five doses of β-elemene (2.5, 5, 10, 20, 40 μg/ml) were added to the medium (without foetal bovine serum) for 24 h. A CCK-8 assay was conducted to detect cell viability. Next, we explored the effect of β-elemene on neurite outgrowth, VSC4.1 motor neurons were disseminated in 24-well

plates at a concentration of 2×10^4 per well and grown in RPMI1640 medium without foetal bovine serum. Then, VSC4.1 motor neurons were exposed to β-elemene (0, 2.5, 5 μg/ml) or Y27632 (Selleckchem, USA, 10 μM) for 24 h. Then, immunofluorescence staining with beta-Ⅲ-tubulin was performed. For western blotting and RhoA kinase activation assays (pull-down assay), 5×10^5 VSC4.1 motor neurons were disseminated in 6 cm dishes and exposed to β-elemene (5 μg/ml) or Y27632 (10 μM) for 24 h.

Primary cortical neuron cultures

Primary cortical neuron culture was performed as described previously (Kaplan et al., 2017). Three E18 Sprague Dawley rats with 21 embryos were used to generate the culture. Briefly, cortices from the embryos were dissected from E18 Sprague Dawley rats in cold D-hanks medium (Jinuo Biology, China) under a microscope and incubated in 0.25% trypsin-EDTA at 37 °C for 15 min. Then, neurons were dissociated via gentle trituration and seeded onto culture dishes coated with 100 μg/mL poly-D-lysine (PDL, Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) and grown in Neurobasal medium (Gibco, Invitrogen) with 2% B27 (Gibco, Invitrogen), 1% penicillin/streptomycin and 2 mM L-glutamine. For neurite outgrowth assays, neurons were seeded in 12-well plates at a concentration of 2×10^5 per well. They were cultured with β-elemene (0, 0.5, 1 μg/ml) or Y27632 (10 μM) for 24 h to observe neurite outgrowth.

Neurite outgrowth assays

Images of the VSC 4.1 motor neurons and primary cortical neurons were obtained using a microscope (BX61, Olympus, Japan). A blinded quantitative analysis of VSC 4.1 motor neurons was performed by counting the percentage of neurons with neurites and the average length of neurites in four microscopic fields from each well using

Image-Pro Plus 6.0. A blinded quantitative analysis of primary cortical neurons was performed by counting the average length of neurites and the average amount of neurites per neuron. The numbers and lengths of neurites per neuron were analysed in four randomized fields of 3 wells per group and were displayed as the mean quantity and length of neurites per neuron in each group. All quantifications were conducted by two independent examiners blinded to the experimental conditions.

CCK-8 assay

The viability of VSC 4.1 motor neurons was measured using a CCK-8 Kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. After the designated experiment, cells were cultured with 100 μl of the CCK-8 mixture containing 90 μl medium and 10 μl CCK-8 at 37 °C in the incubator. After 2 h, cell viability was determined by the absorbance at a wavelength of 450 nm using a TECAN-infinite M5 (Molecular Devices, USA).

Animals and surgery

All experiments were performed in accordance with approved Institutional Animal Care and Use Committee protocols of the Second Affiliated Hospital of Medical School, Zhejiang University. Forty-five female Sprague-Dawley rats (250-275 g) at age 6-8 weeks were purchased from the Animal Center of Zhejiang University (Zhejiang, China). The rats were housed under a 12 h light/dark cycle with ad libitum access to water and food. β-elemene has been widely used in clinical settings in China, and the chosen doses were calculated based on the conversion of body surface area from humans to rats.

The spinal contusion injury model was established via Allen's method as previously

described (Lemmon et al., 2014; Gu et al., 2017). Briefly, rats were deeply anaesthetized with 10% chloral hydrate. Then, the skin over the midline was incised, and the dura was exposed at the T9/T10 vertebral level by vertebral laminectomy. The rats were subjected to a contusive injury by rapidly dropping an impactor (10 g) with a diameter of 2 mm onto the exposed spinal cord from a height of 50 mm using a New York University (NYC) Ⅱ struck instrument. If the rat died from bleeding during the surgery, it was directly excluded. No rats died after the surgery. Thus, after surgery, the rats (n=6/group) were randomly divided into five experimental groups as follows: Sham; Sham with 320 μg/kg β-elemene treated; SCI with vehicle; SCI with 80 μg/kg β-elemene treated and SCI with 320 μg/kg β-elemene treated. The same procedure without the contusive injury was performed in the sham group. Meanwhile, the contusive area of the treatment groups was covered by absorbable gelatin sponges (3 mm ×3 mm ×3 mm; Xiang En Medical Technology, Jiang Xi, China) containing different concentrations of β-elemene. In the SCI with vehicle group, an equal volume of normal saline on gelatin sponges was administered by the same procedure. Eventually, the incision was sutured, and the bladder was massaged two times daily to aid urination until bladder function was recovered.

Locomotion recovery assessment

The hind limb motor function of all rats (n=(6+3)/group) was assessed by the Basso, Beattie, and Bresnahan (BBB) scores composed of 21 different criteria for movement of the hind limb in an open-field at 0, 7, 14, 21 and 28 days after SCI, as previous reported(Basso et al., 1995). The scale is based on the accurate observation of coordination, joint movements, and hind limb stepping for 4 minutes via two trained observers blinded to the experimental conditions.

Luxol fast blue (LFB) staining

A supplementary experiment involving LFB staining was also performed. Fifteen rats (n=3/group) were deeply anaesthetized by 2% isoflurane fixed with 100% O_2, 600 ml/min. The spinal contusion injury model was established and treated with the same procedure as described above. The new method of anaesthesia showed no influence on the BBB score, which means the treatment effect of β-elemene was replicated. This part of the BBB score data was combined with the previous BBB score data and were pooled for statistical analysis. Four weeks after β-elemene treatment, transverse sections (n=4/rat) of spinal cord were stained with Luxol Fast Blue Stain (Myelin Stain) (Abcam, China) for myelin sheath staining.

Haematoxylin-eosin (H&E)

Four weeks after β-elemene treatment, fifteen rats (n=3/group) were chosen randomly, anaesthetized, and intracardially perfused with 50 ml 0.9% NaCl, followed by 150 ml of 4% paraformaldehyde solution. The T9-T12 spinal cord segments centred on and enclosing the injured site were collected and post-fixed in 4% paraformaldehyde at 4 °C overnight. Then, the spinal cords were embedded in paraffin for longitudinal sectioning. Subsequently, all spinal cords were cut into 4-μm-thick sections that were mounted onto poly-L-lysine-coated glass slides for histopathological examination and immunofluorescence staining. Longitudinal sections (n=4/rat) were stained with haematoxylin and eosin according to the manufacturer's instructions using an H&E staining kit (Beyotime Institute of Biotechnology, Jiangsu, China). Some longitudinal sections (n=4/rat) were used for immunofluorescence staining to detect the expression of NF-200 and GAP-43.

Immunofluorescence staining

The slides containing VSC 4.1 motor neurons and primary cortical neurons used for immunofluorescence staining were fixed with a 4% paraformaldehyde solution for 15 min at room temperature, followed by blocking with a solution containing 10% goat serum, 3% BSA, and 0.1% Triton X-100 for 30 min. Then, the slides (n=4 per rat) were incubated with beta-III-tubulin antibody (1:200, CST) at 4 °C overnight. Then, the slides were incubated with FITC-labelled goat anti-mouse IgG for 1 h at room temperature.

As for longitudinal sections of the spinal cord, sections (n=4 per rat) were deparaffinized via xylene and graded ethanol solutions, followed by antigen retrieval. Then, the tissue sections were co-incubated with primary antibodies, including NF-200 (1:200, CST) and GAP-43 (1:200, CST) at 4 °C overnight, followed by co-incubation with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG and FITC-labelled goat anti-rabbit IgG for 1 h at room temperature.

Date quantification

For H&E, LFB, and immunofluorescence staining, all images were captured with an Olympus BX61 microscope (Olympus, Japan) with a mounted camera. The spared areas and myelin sheath areas were determined automatically using Image-Pro Plus software (IPP, version 5.0) and normalized against the total sectional area. Four sections (the interval between each slice is approximately 50 μm, in the central region of the injury in each group) of the spinal cord in one rat were analysed and were displayed as the mean area of the spared region and myelin sheath areas per rat in each group. The mean density of GAP-43 protein in the cavity determined using IPP software was analysed in three randomized fields per section and was displayed as the

mean density of expression of GAP-43 in each section. All quantifications were conducted by two independent examiners blinded to the experimental conditions.

Western blotting analysis

Four weeks after β-elemene treatment, fifteen rats (n=3/group) were euthanized via an overdose of pentobarbital sodium, followed by rapid isolation of the spinal cords at the lesion site and flash-freezing at -80 °C. The spinal cords were triturated by Tissue Prep (Gering Scientific Instruments, Beijing, China) and after the designated treatment, cells were dissolved in WB and IP lysis buffer containing phenylmethanesulfonylfluoride (PMSF) and phosphatase inhibitor and centrifuged at 1.3×10^4 g for 10 min at 4 °C. Then, the protein concentration (3 mg/ml) was quantified using a BCA kit (Beyotime Institute of Biotechnology, Jiangsu, China). All samples (20 μl) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes that were incubated in primary antibodies at 4 °C overnight after blocking in 5% skimmed milk powder dissolved in TBS-T for 1 h. The primary antibodies included the following: rabbit anti-RhoA (2117s, 1:1000), rabbit anti-GAP43 (8945s, 1:1000), rabbit anti-p-limk1 (3841s, 1:1000), rabbit anti-p-confilin (3313s, 1:1000) were purchased from Cell Signalling Technology, Inc. (Danvers, MA, USA); rabbit anti-limk1 (sc-48346, 1:1000), rabbit anti-confilin (sc-32158, 1:1000) was purchased from Santa Cruz (USA); rabbit anti-GAPDH (1:5000) was purchased from Earth Ox (USA). After several washes with TBS-T, membranes were incubated with infrared-labelled secondary antibodies (Li-COR Biosciences). An Odyssey infrared imaging system (LI-COR® Biosciences, NE, USA) was used for visualizing the immunoblot bands. Band intensity was analysed with an Image Studio Ver 5.2 system and compared with the GAPDH internal standard.

RhoA kinase activation assay

The activation of RhoA kinase was investigated using the RhoA activation assay kit (New East, Malvern, PA) as previously described(Kim et al., 2017). Briefly, cell samples were washed with PBS and then lysed in the dish in Assay/Lysis buffer solution. Then, the lysates were centrifuged at 12000 × g for 10 min at 4 ℃. The supernatant was incubated with active RhoA antibody and a protein A/G gel column to detect RhoA-GTP. The bound proteins were washed 3 times with Assay/Lysis buffer and eluted with 2 × SDS-PAGE buffer by boiling, followed by electrophoresis and analysis via western blotting with the anti-RhoA antibody.

Statistical analysis

All data are presented as the mean ± S.E.M. of at least three independent experiments. A one-way ANOVA with a Newman-Keuls post hoc test or a two-way repeated measures ANOVA with a Bonferroni post hoc test was used to compare groups using the GraphPad Prism 6.0 programme. Differences were considered significant at $P < 0.05$.

Results

β-elemene promotes neurite outgrowth *in vitro*

To assess the role of β-elemene in neurite outgrowth, we treated VSC 4.1 motor neurons with 2.5, 5 μg/ml β-elemene or 10 μM Y27632 for 24 h. We found that β-elemene increased the percentage of neurite-bearing VSC 4.1 motor neurons in a dose-dependent manner (Fig. 1A and 1B, 70.93% ± 0.96% *vs* 12.42% ± 1.25%, $F_{1.636, 18} = 162.8$, $P < 0.0001$) and increased the average neurite length per neuron compared to the control group (Fig. 1C, 171.2 ± 7.22 *vs* 87.50 ± 4.49, $F_{2.347, 25.82} = 48.41$, $P < 0.0001$). Y27632 showed similar results, but with shorter average neurite lengths per

neuron compared to the β-elemene treatment group (Fig. 1B and 1C, 131.9 ± 1.68 *vs* 171.2 ± 7.22, $F_{2.347,\ 25.82} = 48.41$, $P < 0.01$). This was not accompanied by any significant reduction in cell survival within the concentration range we used (Fig. 1D).

In addition, we treated primary cultured rat cortical neurons with β-elemene and Y27632. Similar to Y27632, β-elemene (at a lower concentration range, 0, 0.5, 1 μg/ml) markedly increased neurite growth in a dose-dependent manner. Cortical neurons in the β-elemene (1 μg/ml) group had the longest processes, with an increase in the average neurite length (Figure 1E and 1F, 186.90 ± 5.40 *vs* 73.06 ± 5.48, $F_{1.708,\ 18.79} = 102.8$, $P < 0.0001$) and in the average amount of neurites (Figure 1G, 4.84 ± 0.32 *vs* 2.94 ± 0.20, $F_{2.197,\ 24.17} = 10.64$, $P < 0.001$).

β-elemene inhibits RhoA kinase activation and RhoA-mediated signalling.

We have confirmed that β-elemene promotes neurite outgrowth in neurons, which is similar to the effect of Y27632, an inhibitor of ROCK. Thus, we speculated that β-elemene may affect RhoA-mediated signalling. First, to explore whether β-elemene affects RhoA kinase activity, a pull-down assay was performed in rat VSC 4.1 motoneuronal cell lines. The results showed that the level of RhoA-GTP in the β-elemene-treated group was significantly lower than that of the control group (Fig. 2A and 2B, 0.36 ± 0.03 *vs* 0.57 ± 0.05, $F_{2,\ 6} = 14.14$, $P < 0.01$), while the total level of RhoA did not change, revealing that β-elemene negatively regulates RhoA kinase activity in neurons.

Next, we further investigated whether β-elemene regulates the downstream signalling pathway of RhoA, such as RhoA-ROCK-LIMK-confilin, which has been verified to play an important role in neurite outgrowth. The results showed that β-elemene, as

well as Y27632, significantly inhibits the phosphorylation of limk1 and confilin, while the expression levels of total limk1 and confilin were not significantly changed (Fig. 2C, 2E, and 2F, 0.31 ± 0.05 *vs* 0.46 ± 0.05, $F_{2, 4} = 12.48$, $P < 0.05$, 0.09 ± 0.02 *vs* 0.22 ± 0.03, $F_{2, 4} = 175.6$, $P < 0.0001$).

The growth-associated protein-43 (GAP-43) is a crucial protein associated with neurite growth. VSC4.1 motor neurons were exposed to β-elemene or Y27632 for 24 h. The expression level of GAP-43 was measured using western blotting assays. The results showed that the expression level of GAP-43 was significantly increased with β-elemene or Y27632 treatment (Figure 2C and 2D, 0.51 ± 0.07 *vs* 0.14 ± 0.03,0.49 ± 0.05 *vs* 0.14 ± 0.04, $F_{1.328, 2.657} = 61.00$, $P < 0.05$).

β-elemene reduces the area of lesion cavity and improves locomotor functional recovery in rats after SCI.

To investigate whether β-elemene promotes recovery in rats after SCI, H&E and myelin sheath staining were performed on the lesion of rats after SCI. As shown in Fig. 3A-3C, the lesion cavity area in the high dose of β-elemene-treated group was markedly reduced compared to the SCI group (0.68 ± 0.03-fold of SCI group, $F_{1.412, 15.53} = 37.69$, $P < 0.0001$), and the area of LFB staining was markedly increased compared to the SCI group (0.75 ± 0.02 *vs* 0.63 ± 0.02, $F_{1.401, 14.01} = 50.56$, $P < 0.05$). Moreover, the recovery of locomotor function was evaluated using BBB scores. All adult rats had a normal score of 21 before SCI, but the score dropped to zero at day 1 after SCI. The BBB score in the 320 μg/kg β-elemene-treated group showed that they had significantly improved locomotor functional recovery by day 14 after treatment compared with the SCI group (Fig. 3D, 8.56 ± 0.84 *vs* 4.67 ± 0.63, 9.50 ± 0.60 *vs* 5.67 ± 0.51, 9.89 ± 0.54 *vs* 6.17 ± 0.43, $F_{16, 128} = 29.27$, $P < 0.0001$).

β-elemene promotes neurite outgrowth *in vivo*

To evaluate the mechanism of β-elemene on functional recovery and neurite outgrowth in SCI rats, the expression of GAP-43, a crucial marker of neurite growth, was measured using immunofluorescence and western blotting (Fig. 4). Meanwhile, the expression of NF-200 was used to label neurons in the injured spinal cord. The results of GAP-43 immunofluorescence demonstrated that the expression of GAP-43 was significantly decreased in the SCI+vehicle, SCI+80 μg/kg β-elemene and SCI+ SCI+320 μg/kg β-elemene group compared with the sham+vehicle group after 28 days treatment. However, the SCI+320 μg/kg β-elemene group showed significantly higher expression of GAP-43 when compared with the SCI+vehicle group (Fig. 4A and 4D, 0.72 ± 0.03 *vs* 0.43 ± 0.02, $F_{2.384, 23.84} = 91.25$, $P < 0.0001$). The results of the western blotting assay of GAP-43 demonstrated the same trends as immunofluorescence. Western blotting assays suggested that the expression level of GAP-43 in the 320 μg/kg β-elemene treatment group was significantly increased compared to the SCI group (Fig. 4C and 4E, 1.39 ± 0.04 vs 0.95 ± 0.04, $F_{1.056, 2.113} = 38.48$, $P < 0.01$).

Discussion and Conclusion

Permanent neurite damage and failure of neurological connectivity restoration following SCI eventually results in paraplegia or quadriplegia (Garcia-Alias et al., 2004), and there is currently little effective therapy for SCI. It has been reported that the activation of RhoA kinase plays an important role in the failure of neurite regeneration after SCI (McGee and Strittmatter, 2003; Inoue et al., 2004). In this study, we first found that β-elemene and Y27632 (a ROCK inhibitor) increased the percentage of neurite-bearing cells, promoted neurite outgrowth of VSC 4.1 motoneuronal cell lines and primary cultured cortical neurons. In VSC 4.1

motoneuronal cell lines, the β-elemene treated group showed even longer neurite growth than that of the Y27632 treated group.

On the basis of the results stated above, β-elemene showed similar and occasionally stronger effects compared to Y27632. Thus, we speculate that β-elemene may affect the upstream signalling of ROCK, such as the activation of RhoA kinase. RhoA kinase is best known for its function in increasing actin–myosin contractility through direct activation of myosin light chain and inhibition of myosin light chain phosphatase, which eventually inhibits neurite initiation, retracts axons, and collapses the growth cone (Nusser et al., 2002; Ahnert-Hilger et al., 2004; Govek et al., 2005). Interestingly, pull-down assays confirmed that β-elemene could inhibit the activation of RhoA kinase. Recently, many studies have demonstrated that RhoA-GTP activates LIMKs, which phosphorylate and inactivate the actin depolymerizing factor, cofilin, thus inhibiting neurite outgrowth after neuronal injury (Chesarone and Goode, 2009). Our study showed that β-elemene reduces the phosphorylation of limk and confilin without changing the total levels of limk and cofilin. This result was similar to the increased neurite extension and neurite length seen with application of the ROCK inhibitor Y27632, suggesting that β-elemene exerts its influence on neurite outgrowth by inhibiting the activation of the RhoA-ROCK-LIMK-CONFILIN pathway. However, one drawback of our study is that we have not tested any agonists of the RhoA signalling pathway to see whether they block the effects of β-elemene.

Importantly, we further demonstrated that β-elemene improves locomotor functional recovery in rats at 28 days after SCI. Because the BBB score did not reached the platform at 28 days after SCI, we believe that locomotor functional recovery will continue to rise in the later stage of SCI, for example, at 3 or 6 months. Next, we tried to confirm whether the improvement in function results from neuronal structural

repair. Histological analysis showed the area of cavity induced by SCI was significantly reduced with β-elemene treatment. It is interesting that, although the sponge containing β-elemene was put on the surface of the spinal dura meninges where the contusion had been performed, the recovery in cavity size was found not only dorsally and ventrally but also rostrally and caudally. This finding may be attributed to the ability of β-elemene to penetrate into the spinal cord and distribute evenly in the damaged area quickly.

On one hand, we speculated that β-elemene may alleviate secondary injuries in the spinal cord after SCI. Thus, the effects of β-elemene on the immune-inflammatory response and on apoptosis of neurons in the early stage of SCI will be further explored in our future studies. On the other hand, we speculated that β-elemene may promote the regeneration of neural tissue, thus reducing the lesion cavity, and promote locomotor function recovery in the late stage of SCI. LFB staining showed a greater proportion of myelin after β-elemene treatment, which may be due to the preservation of the myelin sheath. Moreover, increased GAP-43 expression raised the possibility that newborn neurites may also be involved in the protective effect of β-elemene. GAP-43 is an intrinsic determinant of neuronal development and plasticity. It plays key roles in neurite outgrowth and growth cone navigation (Stokowska et al., 2017). Western blotting assays confirmed that β-elemene promotes the expression of GAP-43 in motor neurons *in vitro*. Moreover, Western blotting and immunofluorescence staining demonstrated that the expression level of GAP-43 was significantly upregulated by β-elemene *in vivo*.

In conclusion, β-elemene promotes neurite outgrowth *in vivo* and *in vitro* via regulating the RhoA signalling pathway and promotes locomotor functional recovery in SCI rats. All these results suggest that β-elemene can be used as a new treatment

strategy for SCI patient.

Author contributions

Jingyu Wang[#], Heyangzi Li[#] and Ying Yao[#] contributed equally to conduct the experiments and drafted the article; Yucheng Ren, Jiangtao Lin and Tengfei Zhao conducted the experiments; Jue Hu, Mingzhi Zheng and Yueliang Shen performed data acquisition and assembly of data; Xinghui Song, Ying-Ying Chen: data analysis and interpretation; Yong-Jian Zhu and Lin-Lin Wang: conception and design, financial support, manuscript revising and final approval of manuscript.

Additional information

The authors declare that they have no conflict of interest.

The work has not been published previously.

Acknowledgements

This study was supported by National natural science foundation of China (No. 81572229, 81673777, 81501943 and 81401011) and the Natural Science Foundation of Zhejiang, China (No. LY15H060004).

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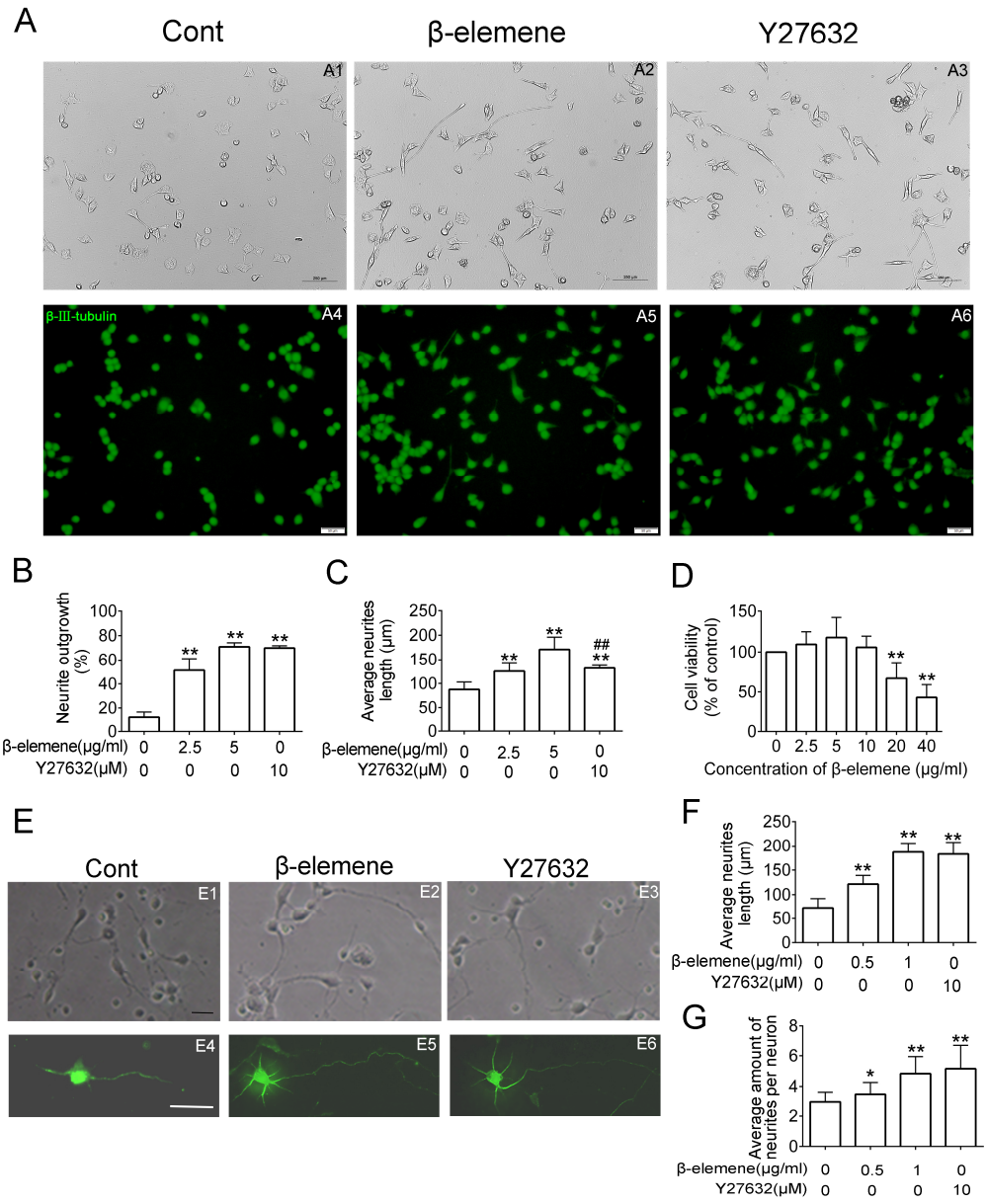
Legends

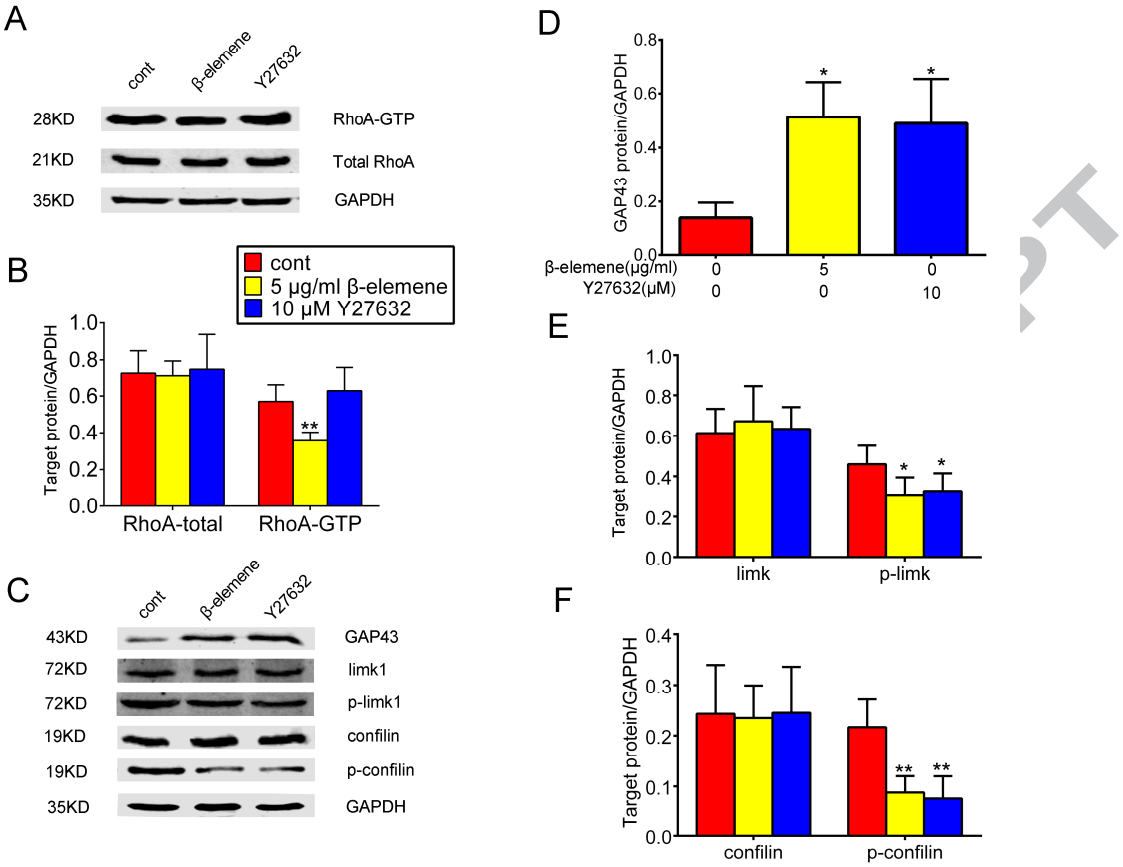
Figure 1. β-elemene promotes neurite outgrowth *in vitro*. VSC4.1 motoneuronal cell lines were cultured with β-elemene or Y27632 for 24 h in serum free RPMI1640 medium (A1-A3: Light microscopy, scale bar=500 μm, A4-A5: immunofluorescence staining of ß-III-tubulin, scale bar=300 μm). The percentage of cells with neurites (B) and the average length of neurites (C) were analysed. Cell viability was examined by CCK-8 assay (D). Light microscopy (E1-E3) and immunofluorescence staining of ß-III-tubulin (E4-E6) of rat cortical neurons to show the neurite outgrowth in β-elemene group or Y27632 group (scale bar = 25 μm). The average numbers and the average length of neurites per neuron were analysed (F, G). The given values are mean ± SEM (n=4, $^{**}p < 0.01$ *vs* control, $^{\#\#}p < 0.01$ *vs* 5 μg/ml β-elemene-treated group). Experiments were performed in triplicate.

Figure 2. β-elemene inhibits RhoA kinase activation and RhoA-mediated signalling pathway. (A and B) Detection of RhoA-GTP by GST-Rhotekin-RBD pull-down assay in VSC4.1 motoneuronal cell lines with or without β-elemene or Y27632 treatment. The level of RhoA-GTP was determined by western blotting with anti-RhoA antibody following a pull-down assay. (C-F) Phosphorylation of limk and confilin and the expression level of GAP-43 were analysed by western blotting in VSC4.1 motor neurons with or without β-elemene or Y27632 treatment. Experiments were performed in triplicate. The corresponding densitometric analyses of the protein bands were measured in the immunoblots and normalized to the signal of GAPDH. The given values are mean ± SEM (n=3, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* control).

Figure 3. β-elemene reduces lesion cavity area and improves locomotor functional recovery in rats after SCI. (A) Spinal cord sections in the injured region were stained with H&E staining (A1-A5, scale bar = 500 μm) and Luxol Fast Blue staining (A6-A10, scale bar = 100 μm). The lesion cavity area (B) and the area of LFB staining (C) were analysed by IPP software. The given values are mean ± SEM (n=3, $^{**}p < 0.01$ *vs* SCI group, ## $p < 0.01$ *vs* sham group). (D) Basso Beattie Bresnahan (BBB) open field score at days 1, 7, 14, 21 and 28 after SCI in rats with or without β-elemene. The given values are mean ± SEM (n=9, $^{**}p < 0.01$ *vs* SCI group)

Figure 4. β-elemene promotes neurite outgrowth in rats after SCI. (A) Spinal cord sections in the injured region were stained with immunofluorescence staining of GAP-43, NF-200, and DAPI (scale bar = 50 μm). (C) The rectangular insert shows the region of interest. (D) The mean density of GAP-43 was analysed by IPP software. (B and E) The expression level of GAP-43 after SCI with or without β-elemene treatment was analysed by western blotting. The corresponding densitometric analyses of the protein bands measured in the immunoblots and normalized to the signal of GAPDH are shown. Quantification of the optical densities of the protein bands to determine the levels of protein expressed as percentages of control values. The given values are mean ± SEM (n=3, $^{**}p < 0.01$ *vs* sham group, ##$p < 0.01$ *vs* SCI group).





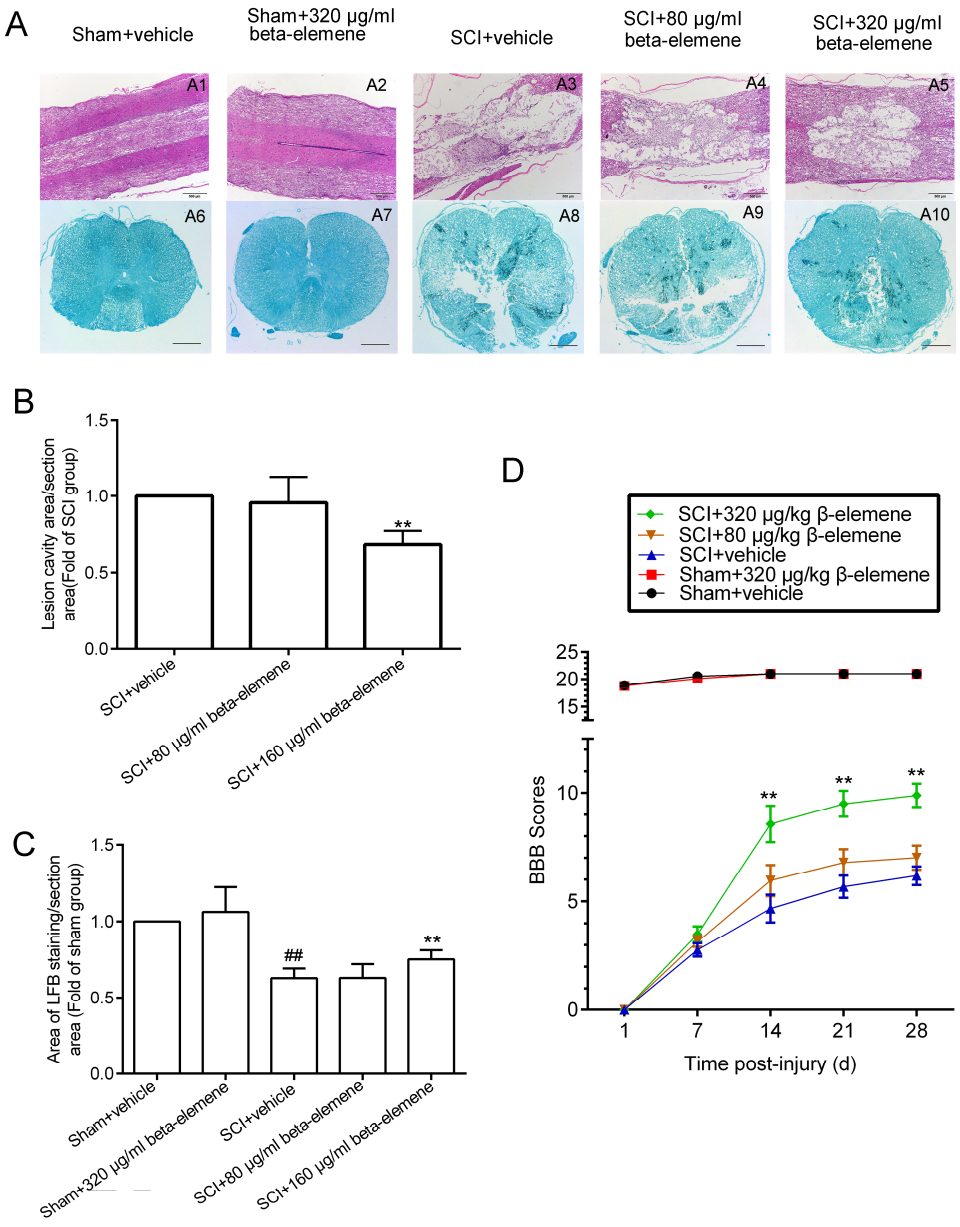
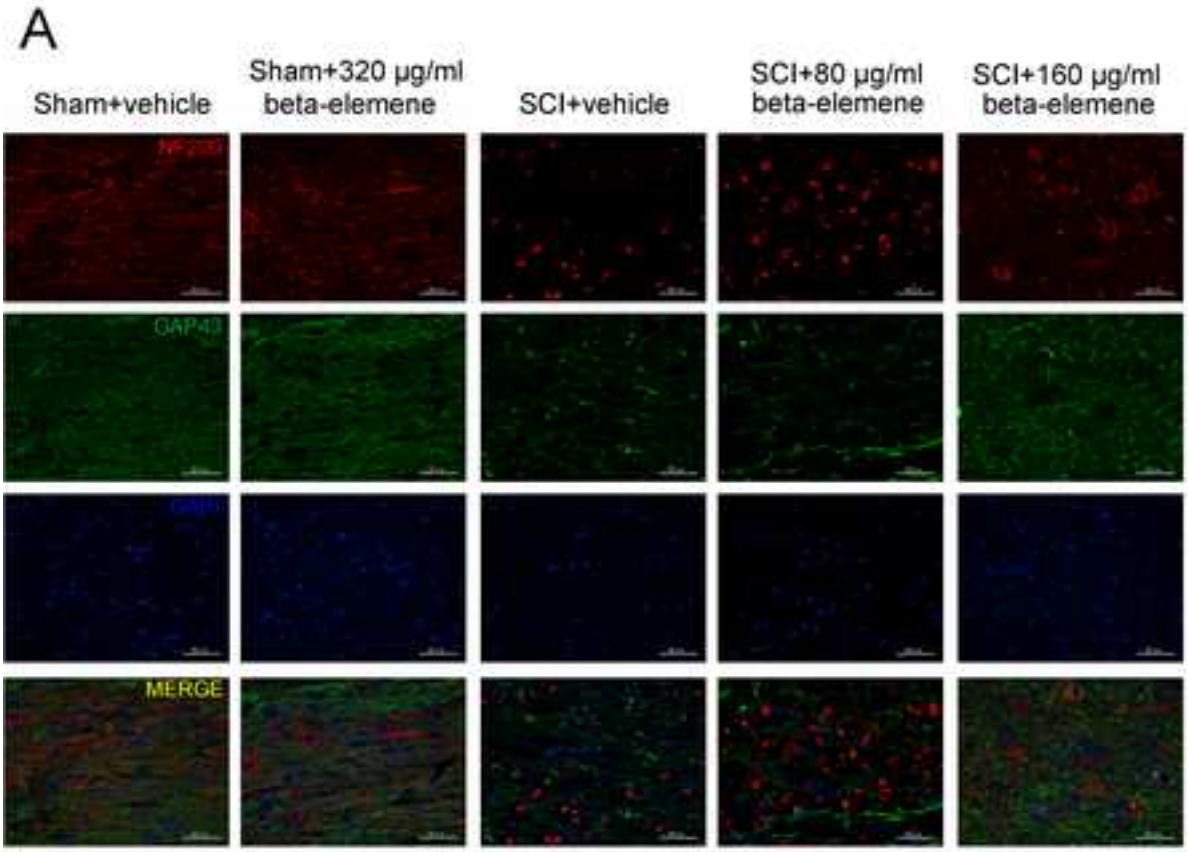
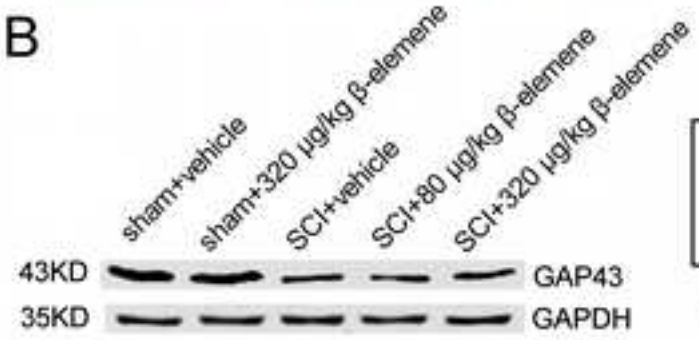
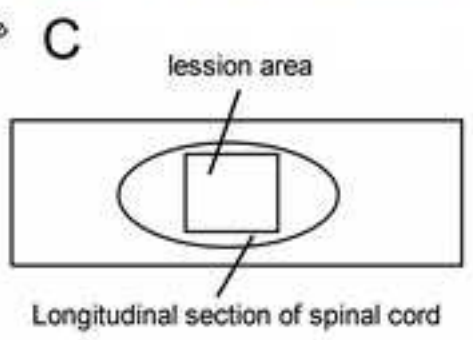
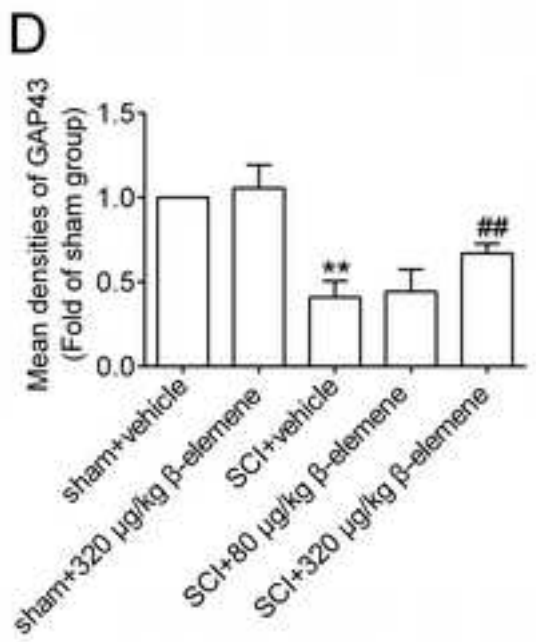


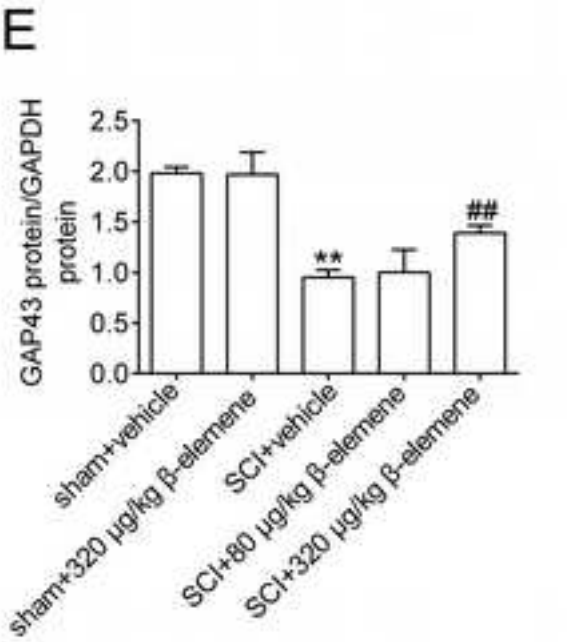
Figure4

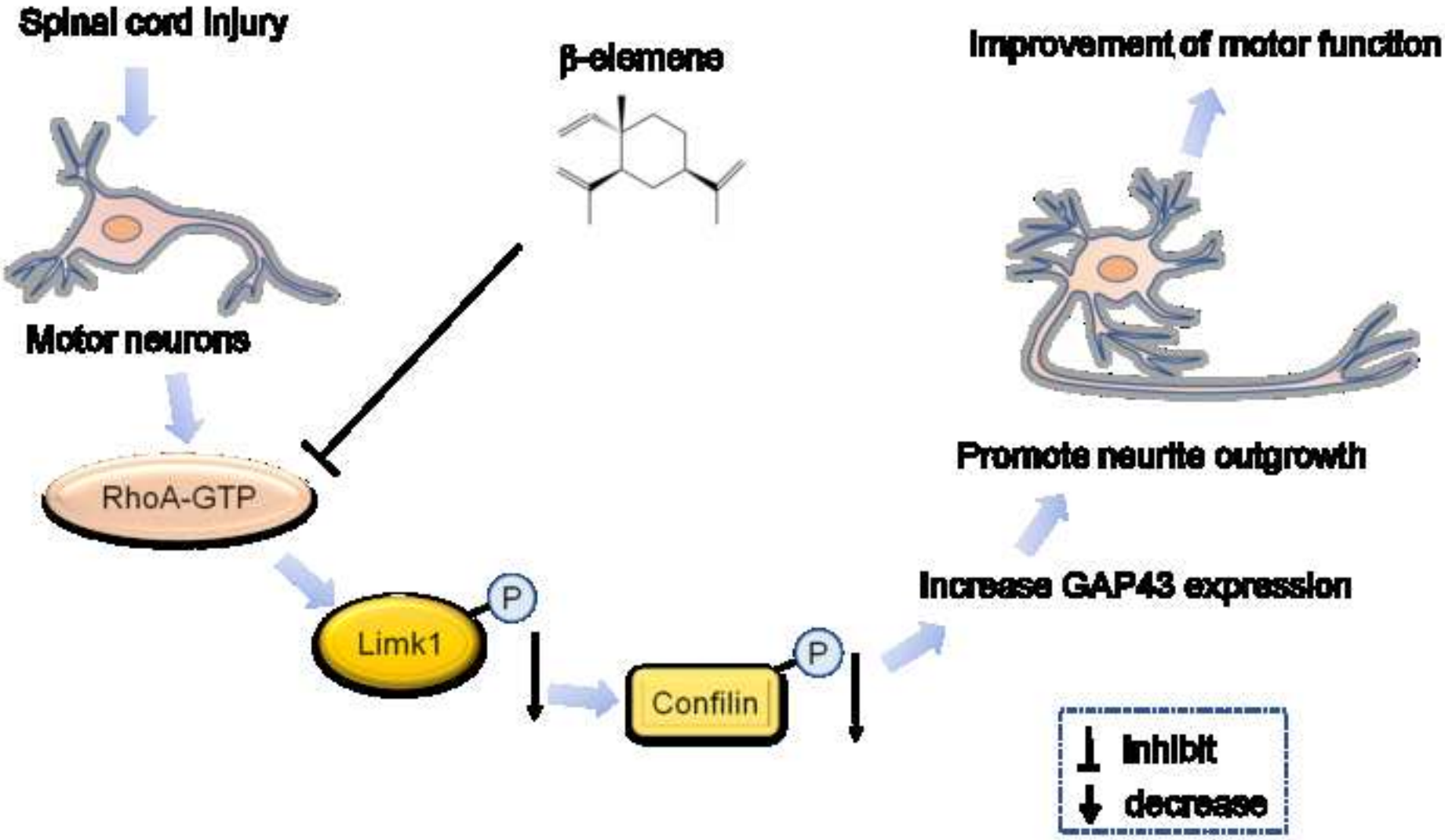











Spinal cord injury
β-elemene
Improvement of motor function
Motor neurons
RhoA-GTP
Promote neurite outgrowth
Limk1
P
Increase GAP43 expression
Confilin
P
Inhibit
decrease

Highlights

- β-elemene enhances GAP-43 expression and neurite outgrowth in neurons
- β-elemene inhibits RhoA kinase activation and RhoA-mediated signaling in neurons
- β-elemene improved behavioral outcome in SCI rats
- β-elemene enhances GAP-43 expression and neurite outgrowth in SCI rats