Research Report

Effects of dihydroxyphenyl lactic acid on inflammatory responses in spinal cord injury

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ABSTRACT

The initial mechanical tissue disruption of spinal cord injury (SCI) is followed by a period of secondary injury that increases the size of the lesion. Secondary injuries are associated with edema, inflammation, excessive cytokine release, excitotoxicity and cell apoptosis. 3,4-dihydroxyphenyl lactic acid (DLA) is one of the major water-soluble components of chemical constituents from Salvia miltiorrhiza (SM). To investigate the inhibition effects of DLA on secondary injury of SCI, focusing especially on suppression of inflammatory responses and the mechanism of this effect, the following studies were performed: Basso, Beattie, and Bresnahan (BBB) scores to assess motor functions till 10 days after SCI; Nissl and Fast Blue histological staining and immunohistochemistry of inhibitory-kappa B-alpha (IκB-α) and nuclear factor-kappa B (NF-κB) p65 subunit protein; levels of myeloperoxidase (MPO) activity analysis as an indicator of polymorphonuclear infiltration; IL-6 production in plasma 10 days after SCI; Western blot analysis to determine cytoplasm levels of IκB-α and NF-κB p65 subunit proteins in the nuclear fractions 10 days after SCI. DLA significantly attenuated the motor function and tissue damage following SCI in rats, significant reduced polymorphonuclear cell infiltration and IL-6 production, as well as reduced cytoplasm IκB-α degradation and the nuclear translocation of NF-κB p65 subunit protein after SCI. In conclusion, the results clearly demonstrate that DLA inhibit the inflammation responses induced by SCI via inhibiting effect of production of IL-6 and nuclear translocation of NF-κB.

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1. Introduction

Spinal cord injury (SCI) affects people of all ages and can lead to loss of sensory and motor functions below the level of the lesion. Traumatic injury of the spinal cord initiates a series of cellular and molecular events that include both primary and secondary injury cascades. Inflammatory responses are a major component of secondary injury and play a central role in regulating the

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pathogenesis of spinal SCI (Blight, 1992; Dusart and Schwab, 1994). Progressive vascular events, especially activated neutrophil-induced endothelial cell damage, have been shown to be implicated. The accumulation of neutrophils at the site of compression, as evaluated by measuring tissue myeloperoxidase (MPO) activity, significantly increased with time following the compression, peaking 3 h post-compression (Taoka et al., 1997; Taoka et al., 1998). Many inflammatory responses are mediated by enhanced and/or induced gene expression. A principal player in the regulation of inflammatory gene expression is the nuclear factor-kappa B (NF-κB) family of transcription factors (Baueuerle, 1991; Baueuerle and Henkel, 1994; Han et al., 2009). NF-κB is a critical transcription factor that regulates the expression of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) in central nervous system (CNS) inflammatory responses (Barnes and Karin, 1997; O’Neill et al., 1997; Bethea et al., 1998; Li and Verma, 2002). These pro-inflammatory cytokines were already known to exhibit cytotoxicity, including induction of apoptosis of neurons and oligodendrocytes. Reports have identified apoptosis as a critical event in the secondary damages following SCI (Crowe et al., 1997) being detected from a couple of hours up to 3 weeks following SCI (Liu et al., 1997) and apoptosis subsequent to necrosis is a delayed process that contributes to progressive secondary degeneration and, ultimately, results in spinal cord dysfunction below the injury site (Spriger et al., 1999; Lewen et al., 2000; yune et al., 2003). Because SCI involves a complex pathophysiology, an effective therapy should employ either multiple agents or a multipartite agent.

For several decades, Salvia miltiorrhiza (SM) has been widely used in clinics in many Asian countries for the treatment of various microcirculatory disturbance-related diseases, such as cardiovascular disease, cerebrovascular disease, liver dysfunction, renal deficiency and diabetic vascular complication (Han et al., 2008). 3,4-dihydroxyphenyl lactic acid (DLA, also called danshensu) is one of the major water-soluble components of chemical constituents from SM. Water-soluble fraction from SM was reported to inhibit the expression of intercellular adhesion molecules (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) on human umbilical vein endothelial cells (HUVECs) induced by TNF-α (Oskouian et al., 2002) and TNF-α induced translocation of NF-κB from the cytoplasm to the nucleus (Ding et al., 2005). Our previous study have demonstrated that DLA is able to ameliorate the expression of adhesion molecules CD11b and CD18 and the production of peroxides in leukocyte, and to inhibit leukocyte adhesion to mesenteric venular wall in rat challenged by lipopolysaccharide (Guo et al., 2008). Furthermore, thrombosis elicited by photochemical reaction was depressed by administration of DLA, through either scavenging the peroxides produced or depressing the expression of adhesion molecules in neutrophil (Wang et al., 2009). DLA was also reported to protect against endothelial cell damage caused by homocysteine (Chan et al., 2004) and to abolish oxysterol-induced endothelial cell apoptosis in vitro and in vivo (Nakazawa et al., 2005). However, little is known about the effect of DLA on secondary damage in SCI.

To determine whether DLA could prevent spinal cord injury and improve neurologic outcome in SCI rat model and to analyze whether this drug could prevent inflammatory responses in spinal cord and the mechanism of these effects, we investigated the effects of DLA on recovery of motor functions and tissue injury, neutrophil infiltration, production of IL-6, inhibitory-kappa B-alpha (IκB-α) degradation, and NF-κB p65 subunit protein expression in the nuclear fractions in experimental SCI model.

2. Results

2.1. Effects of DLA on functional recovery

In all the rats used in this study, hindlimb movement was abolished immediately after SCI. Thereafter, spontaneous recovery of function was observed over the next 10 days in all groups. Control rats progressively improved from day 5, and DLA group improved more rapidly, and showed statistically significant better scores from day 3 to day 10. 10 days after injury, the BBB average score of DLA-treated rats was 7.0, reflecting their capacity to perform extensive movement of the three joints in the hindlimbs, whereas saline-treated rats presented only an average score of 2.7 at the same study period, revealing their capacity for only extensive movement of one joint and slight movement of the other. (p<0.05, Fig. 1).

2.2. Histological Nissl staining

Light microscopic examination of sham group cord samples revealed normal findings, as expected (Figs. 2A1 and A2). In SCI group, significant damage to the spinal cord was observed in the spinal cord tissue 10 days after SCI. The motor neurons in the gray matter exhibited cell lost (Figs. 2B1 and B2). In both white and gray matter zones there were hemorrhagic, widespread edema, and vascular thrombosis (Figs. 2B1 and B2). Notably, a significant protection was observed in the tissues collected from the DLA treated rats 10 days after SCI (Figs. 2C1 and C2).

2.3. Immunohistochemical staining of IκB-α and NF-κB p65

The immunohistochemical reaction of IκB-α was expressed strongly by motor neurons in Sham spinal cord (Fig. 2A3), faint
in SCI (Fig. 2B3), and a little more staining in DLA treated rats 10 days after SCI (Fig. 2C3). However, the immunohistochemical expression of NF-κB p65 was nearly negative in Sham spinal cord (Fig. 2A4), positive in the gray matter of SCI (even though motor neurons were lost) (Fig. 2B4), and a little depigment in DLA treated rats (Fig. 2C4).

2.4. Fast blue staining and quantification of myelin

In sham animals, myelin structure was clearly stained by fast blue in both horizontal and vertical sections of the dorsal funiculi of the spinal cord (Figs. 3A1, A2, D1, and D2). 10 days after the injury, the quantified analysis showed (Fig. 3G) a significant loss of myelin in dorsal funiculi was observed in SCI-operated rats (Figs. 3B1, B2, E1, and E2). In contrast, the myelin degradation was attenuated in the dorsal funiculi from DLA treated rats 10 days after SCI (Figs. 3C1, C2, F1, and F2).

2.5. Effects of DLA on MPO activity

As shown in Fig. 4A, the accumulation of neutrophils in SCI segments of the spinal cord as reflected by tissue MPO activity was enhanced dramatically at 4 h after SCI in SCI-operated rats compared with those in the sham group. In DLA-treated rats 4 h after SCI, MPO activity was attenuated markedly compared with the animals in the SCI group.

2.6. Effects of DLA on production of IL-6 in plasma 4 h after SCI

The concentrations of the cytokines IL-6 in plasma are presented in Fig. 4B. IL-6 concentration was significantly elevated at 4 h after SCI in SCI-operated rats compared with those in the Sham group. In DLA-treated rats, the production of IL-6 was significantly attenuated in comparison with the animals in the SCI group.
Fig. 3 – Effect of DLA on the myelin structure of the spinal cord tissue after SCI. Normal myelin structure was observed by Fast Blue staining in sham operated rats at different magnification. A1 and A2 in horizontal and B1 and B2 in vertical. 10 days after the injury in SCI-operated rats, a significant loss of myelin was observed (B1, B2, E1, and E2). In contrast, in SCI-operated rats treated with DLA, myelin degradation was attenuated (C1, C2, F1, and F2). Scale bar: A1–C1=200 μm; and A2–F2=50 μm. G. The quantified analysis showed a significant loss of myelin in the dorsal funiculi was observed in SCI-operated rats. In contrast, the myelin degradation was attenuated in the dorsal funiculi from DLA treated rats 10 days after SCI. *p<0.05 versus Sham group, and #p<0.05 versus SCI group. Arrows shown myelin structure in Sham, demyelin in SCI and remyelin in DLA.
2.7. Effects of DLA on cytosolic IκB-α and NF-κB p65 subunit proteins in the nuclear fractions

The presence of IκB-α in the cytosolic fraction of the spinal cord tissues was detected by western blot analysis 10 days after SCI. A basal level of IκB-α was detectable in the cytosolic fraction of the spinal cord tissues from animals in the Sham group, indicating the presence of the inactive NF-κB in the cytoplasm. Cytosolic IκB-α of lesion segment samples from the SCI group showed a significant decrease as compared to that from sham-treated animals and this decrease was attenuated significantly by treatment with DLA (Fig. 4C). The NF-κB p65 subunit protein in the nuclear fractions of the spinal cord tissue showed a weakly detectable band in extracts from uninjured spinal cord, and it was significantly increased 10 days after SCI. DLA treatment significantly reduced NF-κB p65 subunit protein in the nuclear fractions (Fig. 4D).

3. Discussion

In this study on experimental spinal cord injury in the rat, we showed that DLA promoted recovery of hind limb function, attenuated tissue damage, inhibited IκB degradation and the nuclear translocation of NF-κB, inhibited the production of IL-6 in plasma, as well as inhibited inflammatory cell infiltration at the site of injury in the spinal cords.

In most human SCI, the mechanism of the primary injury is acute compression or laceration of the SC due to displacement of bone or disk into the SC during fracture dislocation or burst fracture of the spine. To mimic most of the mechanical events that lead to various forms of human SCI, several experimental models have been developed. The most commonly used model is that of the contusion model. This model induces instantaneous mechanical deformation of the SC by dropping a weight, an impactor rod, or an impounder with computer-guided...
assistance. Another model used in SCI research is the compression model; in this model, injury is induced by applying either a weight or an aneurysm clip to the SC. This model aims to add to that of the contusion model by replicating the persistence of cord compression that is commonly observed in human SCI (Genovese et al., 2007).

As previous study (Nakamura et al., 2003), the expression of IL-6 is sharply increased 4 h after SCI, which plays a critical role as a pro-inflammatory cytokine and to be associated with the secondary tissue damage in SCI. After spinal cord trauma, the ruptured blood vessels disturb the blood-brain barrier and the injury site is rapidly infiltrated by blood-borne neutrophils. This process may contribute to the secondary damage that follows the initial primary injury. Blood-brain barrier restriction to large molecules was restored by 4–5 h after injury but smaller molecules were still able to enter the brain as long as 4 days post-injury (Habgood et al., 2007). IL-6 receptor monoclonal antibody treatment suppressed the astrocytic differentiation, decreased the number of inflammatory cells and the severity of connective tissue scar formation (Okada et al., 2004). It has been suggest that blocking of the IL-6 signals can suppress inflammatory reactions and secondary injury, thus attenuating injury of intact tissue (Nakamura et al., 2005). Forced expression of IL-6 signals after spinal cord injury resulted in an approximately sixfold increase of neutrophil infiltration, a twofold increase of macrophage infiltration and activated microglia and a fourfold decrease in axonal growth at the lesion site (Lacroix et al., 2002).

The inhibitory effect of DLA on IL-6 was observed 10 days after SCI further sustaining a key role of infiltrated neutrophils in the production of cytokines observed after traumatic SCI. The expression of IL-6 was regulated by NF-κB (Barnes and Karin, 1997; O’Neill et al., 1997; Bethea et al., 1998; Li and Verma, 2002). NF-κB is a generic term for a dimeric transcription factor formed by the hetero- or homodimerization of proteins from the family of genes named Rel. There are 5 rel proteins: RelA (p65), RelB and cRel, which contain transactivation domains, and p50 and p52, which are expressed as the precursor proteins p105 and p100, respectively that require post-transcriptional processing. NF-κB is usually kept inactive in the cytoplasm associated with an endogenous inhibitor protein called IκB. Once IκB-α has been degraded, NF-κB dimerizes, translocates to the nucleus, and binds to the DNA for modulation of expression of its target genes (Kucharczak et al., 2003; Schaecher et al., 2004). It is activated by various signals, including cytokines, neurotrophic factors, neurotransmitters, free radicals, stimulation of glutamate receptors, and elevation of intracellular calcium levels (Clemens et al., 1997; Blondeau et al., 2001). Activation of NF-κB and its involvement in inflammation has been demonstrated in the lesion of spinal cord after injury. NF-κB activation occurred as early as 0.5 h post-injury and persisted for at least 72 h (Bethea et al., 1998). Dusart and Schwab (1994) demonstrate that neutrophils and macrophages enter the spinal cord after SCI in an orchestrated temporal sequence. Neutrophils begin to accumulate within 1 h, are most abundant at 24 h, and begin to decline at 48 h. Neutrophils are able to release reactive oxygen and nitrosyl radicals as well as cytokines, chemokines, and a variety of enzymes and they have been proposed to participate in enlargement of the lesion and promote tissue destruction (Dusart and Schwab, 1994). Therefore, reduction of secondary tissue damage is a major goal of treatment in cases of spinal cord injury (Dumont et al., 2002). Our results are in agreement with studies that show a persistent increase in active NF-κB protein after traumatic injury to spinal cord (Bethea et al., 1998; Zhang et al., 1998). Our results show that after SCI, cytosolic levels of IκB-α decreased and levels of nuclear NF-κB increased. This indicates a translocation of NF-κB from the cytosol to the nucleus. Our results also show that in the SCI models, treatment with DLA significantly increased the levels of cytosolic IκB-α and significantly decreased the levels of nuclear NF-κB, as compared to that in SCI group.

In conclusion, DLA can inhabit SCI-induced inflammatory responses by reducing the activation of NF-κB p65, IL-6 production and polymorphonuclear cell infiltration. Therefore, DLA administration may represent a novel approach in the treatment of SCI.

4. Experimental procedures

4.1. Animal groups and administration

Male adult Wistar rats weighing 200 to 250 g were obtained from the Animal Center of Peking University Health Science Center. The rats were housed in a controlled environment and provided with standard rodent chow and water. All studies were approved by and all animals were handled according to the guidelines of the Peking University Animal Research Committee.

Rats were randomly divided into three groups, each with 18 animals. Sham group: laminectomy is performed but clip was not applied plus intravenous administration of 1 ml saline; 2. SCI group: rats were subjected to SCI plus intravenous administration of 1 ml saline; 3. SCI + DLA group.

Rats were subjected to SCI plus intravenous administration of DLA (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) at the dose of 5 mg/kg body weight (in 1 ml saline) 1 h after SCI. In the experiments investigating the motor score, the animals were treated with DLA or saline 1 h after SCI and daily until day 10. Six rats from each group were killed 4 h and 10 days, respectively after SCI to collect samples for the evaluation of the parameters as described below. The doses of DLA used here were based on previous in vivo study (Guo et al., 2008).

4.2. Spinal cord injury

Rats were anesthetized with urethane (1.25 mg/kg body weight) by intraperitoneal (i.p.) injection. A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected away exposing T9–T10 vertebrae. The spinous processes of T9 and T10 were removed with rongeurs and a laminectomy performed using a dental drill and rongeurs to expose the dorsal spinal cord. The clip (Yasargil aneurism clip, force of closure 50 g) was applied extradural to the spinal cord and remained compressing the cord for 60 s in all injured groups. Sham animals were only subjected to laminectomy. After surgery, 1.0 ml of saline was administered subcutaneously to replace the blood volume lost during the surgery. Animals were allowed to recover on a heating pad maintained at

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36.5 °C. The rats were returned to their cages with free access to water and food for a survival period of 4 h or 10 days. Manual bladder expression was performed twice per day until reflex bladder emptying was re-established.

4.3. Functional recovery

Functional tests were performed once a day for 10 days after SCI. Behavioral recovery was scored according to the Basso, Beattie, and Bresnahan (BBB) scale, which is composed of 21 different criteria for the movement of the hindlimb from complete paralysis to complete mobility. BBB scale is a valid and predictive measure of locomotor recovery able to distinguish behavioral outcomes due to different injuries and to predict anatomical alterations at the lesion center. Interrater reliability tests indicate that examiners with widely varying behavioral testing experience can apply the scale consistently and obtain similar scores. The overall locomotor behaviour was assessed using the BBB locomotor recovery scale, which consists of a 21 points scale. This scale is based on the precise observation of hind limb movements, stepping and coordination in an open field. Spinal cord injured animals were observed for 4 min in an open field by an observer blinded to the treatments and scored from 0 (no observable hind limb movements) to 21 (normal locomotion with consistent plantar stepping and coordinated gait, toe clearance, parallel paw position, trunk stability and tail consistently in an upper position).

4.4. Histological staining of Nissl and fast blue

Spinal cord specimens were taken 10 days after SCI or sham operations. Tissue segments tissue segments (10 mm long) of the spinal cord containing 4.5 mm caudal and 4.5 mm rostral to the apparent lesion epicenter of injury (1.0 mm long), were dissected and were fixed in paraformaldehyde 4% in 0.1 M phosphate buffer and embedded in paraffin. Coronal sections (5 μm thick) from these regions were cut and deparaffinized with Nissl and Fast Blue staining (used to assess demyelination). The sections of spinal cord were treated with 0.3% hydrogen peroxide (H2O2) in PBS for 30 min and 10% normal horse serum in 0.05 M PBS for 30 min. The sections were next incubated with diluted rabbit anti-L-B-a Ab or rabbit anti-p65 Ab (Santa Cruz Biotechnology, Santa Cruz, Calif) diluted 1:200 overnight at room temperature. Thereafter, the sections were sequentially exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (diluted 1:200, Vector, USA). The sections were visualized with 3,3’-diaminobenzidine tetrachloride in 0.1 M Tris buffer and mounted on the gelatin-coated slides.

4.5. The quantification of myelin

The quantitative analysis for demyelination in LFB staining section was performed as described previously (Patani et al., 2007). To measure the extent of demyelination, images of the entire lesions in the dorsal funiculus were captured from LFB stained sections. A magnification of ×20 provided sufficient resolution to allow individual fiber to be visualized, so that demyelination could be identified accurately. The demyelination in section were traced and measured using an image analysis system [Imaging-Pro-Plus (OLYMPUS)] and a OLYMPUS BX51 microscope with digital camera (DP70, OLYMPUS) allowed the automated capture of adjacent frames and then reconstruction of an image of the entire selected area at high resolution (4080×3072).

4.6. Immunohistochemistry staining

The methods for immunohistochemical staining have been described previously (Guo et al., 2008). The sections of spinal cord were treated with 0.3% hydrogen peroxide (H2O2) in PBS for 30 min and 10% normal horse serum in 0.05 M PBS for 30 min. The sections were next incubated with diluted rabbit anti-L-B-a Ab or rabbit anti-p65 Ab (Santa Cruz Biotechnology, Santa Cruz, Calif) diluted 1:200 overnight at room temperature. Thereafter, the sections were sequentially exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (diluted 1:200, Vector, USA). The sections were visualized with 3,3’-diaminobenzidine tetrachloride in 0.1 M Tris buffer and mounted on the gelatin-coated slides.

4.7. MPO activity

MPO activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described (Genovese et al., 2006). 4 h after SCI or sham operations, tissue segments (10 mm long) of the spinal cord containing 4.5 mm caudal and 4.5 mm rostral to the apparent lesion epicenter of injury (1.0 mm long), were dissected and weighed, and each piece was homogenized in a solution containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000×g at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mmol/L tetramethylbenzidine and 0.1 mmol/L H2O2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37 °C and was expressed in milliunits per gram of wet tissue.

4.8. Measurement of plasma cytokines IL-6

The animals were deeply anesthetized with urethane (1.25 mg/kg body weight, i.p.), and blood was collected from the abdominal aorta of each animal and anticoagulated with heparin (20 U/ml blood) 4 h after SCI or sham operations. The plasma was isolated by centrifugation (AllegraTM 64R Centrifuge, Beckman Coulter, German). The concentrations of IL-6 were measured by flow cytometry with a BD cytometric bead array kit (BD Biosciences Pharmingen, USA). Fifty microliters of plasma or standard was incubated with 50 μl capture beads for 1 h at room temperature, and then mixed with 50 μl phycoerythrin (PE)-labeled IL-6 (BD Biosciences Pharmingen, USA) detection antibodies and incubated for 2 h at room temperature to form a sandwich complex. Following incubations, 1 ml of washing buffer (BD Biosciences Pharmingen, USA) was added to each tube, and the mean fluorescence intensity was detected using flow cytometry (FACS Calibur, B.D. Co., USA). The data were analyzed with BD Cytometric Bead Array analysis software (Chan et al., 2004; Wong et al., 2004; Sun et al., 2007).

4.9. Western blot

Animals were deeply anesthetized with urethane (1.25 mg/kg body weight, i.p.) 10 days after SCI or sham operations, followed by intracardiac perfusion with 0.9% ice-cold saline to remove the
blood and to chill the spinal cord tissue rapidly. After exsanguinations, tissue segment (as described in the method of MPO analysis) was quickly dissected, immediately stored at −80 °C until homogenization. Cytoplasmic and nuclear protein extracts were prepared according to methods previously reported, with some modifications (Qiu et al., 2001). Briefly, dissected tissues were homogenized with a homogenizer on ice in an ice-cold buffer (3.5 μg/mg tissue) containing 20 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mg/ml each of the protease inhibitors antipain, chymotatin, pepstatin, and leupeptin. The lysate was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant, containing soluble cytoplasmic proteins, was removed and saved in aliquots. The pellet was reconstituted in ice-cold buffer (1.5 μg/mg tissue) containing 20 mM HEPES, pH 7.8, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 2 mg/ml each of antipain, chymotatin, pepstatin, and leupeptin, followed by vigorous shaking for 20 min at 4 °C. The lysate was centrifuged at 14,000 rpm for 20 min. The supernatant, containing soluble nuclear proteins, was removed and saved in aliquots. Proteins were quantitated by using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Both nuclear and cytoplasmic protein extracts were separated on SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were placed in 10% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature, then cytoplasmic protein extracts were incubated with rabbit anti-iB-α Ab, and nuclear protein extracts were incubated with rabbit anti-p65 Ab (Santa Cruz Biotechnology, Santa Cruz, Calif) diluted 1:1000 in TBST overnight at 4 °C. The membranes were washed three times for 10 min in TBST and incubated for 1 h with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary Ab (RBD Systems, Minneapolis) diluted in 1:1000 blocking solution. After three washes in TBST, the blots were visualized with an enhanced chemiluminescence reagent kit (ECL; Amersham, Arlington Heights, IL).

4.10. Statistical evaluation

All data were reported as mean±SEM. All statistical analyses were performed using statistical analysis software (Statistical Package for Social Sciences Program, SPSS, Version 11.0). Statistical analysis was primarily conducted using a one-way analysis of variance with post hoc multiple comparison test. P values of less than 0.05 were considered statistically significant.

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