

Resveratrol attenuates inflammatory hyperalgesia by inhibiting glial activation in mice spinal cords

LIN-LIN WANG^{1,2}, DONG-LING SHI^{1,2}, HUI-YAO GU³, MING-ZHI ZHENG⁴,
JUE HU⁴, XING-HUI SONG⁵, YUE-LIANG SHEN³ and YING-YING CHEN³

¹Department of Physiology; ²Center for Stem Cell and Tissue Engineering;

³Department of Pathology and Pathophysiology, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058;

⁴Department of Pharmacology, Zhejiang Medical College, Hangzhou, Zhejiang 310053;

⁵Core Facilities, Department of Basic Medical Sciences, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, P.R. China

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Abstract. The present study aimed to investigate the effect of resveratrol on inflammatory pain. Mice were injected intraperitoneally with lipopolysaccharide (LPS) for 5 consecutive days to induce subacute systemic inflammation. Acetic acid-induced writhing tests and tail-flick tests were performed following the final LPS injection. Glial fibrillary acidic protein (GFAP; an astrocyte-specific activation marker), ionized calcium binding adapter molecule 1 (Iba-1; a microglia-specific activation marker) and sirtuin 1 (SIRT1) protein expression levels were detected using immunohistochemistry analysis or western blotting. Following administration of LPS for 5 days, the number of writhes increased and the tail-flick latency decreased. Resveratrol (10 or 20 mg/kg) partly inhibited LPS-induced hyperalgesia and prevented the increase in tumor necrosis factor- α and interleukin 6 levels induced by LPS. LPS injection reduced the SIRT1 protein expression and increased the number of GFAP-positive and Iba-1-positive cells in the spinal cord. Resveratrol increased the SIRT1 protein expression levels and decreased the number of GFAP-positive and Iba-1-positive cells in LPS-treated mice. The protective effect of resveratrol was partly blocked by a selective SIRT1 inhibitor, EX-257. Results from the present study suggest that subacute treatment with LPS induced the activation of glial cells and hyperalgesia. Resveratrol was demonstrated to inhibit the activation of glial cells and attenuate inflammatory hyperalgesia in a SIRT1-dependent manner.

Introduction

Pain facilitation has been reported to induce numerous nervous system-mediated sickness responses (1). Inflammation is crucial in the development and maintenance of persistent pain and inflammatory mediators are often altered regionally in acute pain (2); however, chronic pain patients have demonstrated a significant increase in pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-2, and IL-6 in plasma, which is associated with increased pain intensity (3,4). Prolonged upregulation of inflammatory mediators in the injury epicenter, and regions above and below the lesion may be involved in chronic neuropathic pain in spinal cord injury (5). The systemic inflammatory response is also associated with pain and other symptoms in a cohort of cancer patients (6). Thus, anti-inflammatory intervention may be a useful therapeutic strategy in the management of pathological pain.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a non-flavonoid polyphenol present in grapes and red wine, which has been demonstrated to elicit a broad spectrum of biological responses *in vitro* and *in vivo*, including anticarcinogenic effects and neuroprotective and cardioprotective activities (7,8). Previous studies have indicated that resveratrol is a potential therapeutic agent to attenuate bone cancer pain (9) and diabetic neuropathic pain (10). Injection of resveratrol into the cerebral ventricles also exerts evident central analgesic effects (11). Resveratrol has been demonstrated to interact with multiple molecular targets, a number of which are associated with inflammation and immunity (12). Thus, the current study hypothesized that resveratrol may also attenuate systemic inflammation-induced pain. The aim of the present study was to investigate the effect of resveratrol on inflammatory pain induced by the bacterial endotoxin, lipopolysaccharide (LPS), and investigate its underlying molecular mechanism.

Correspondence to: Dr Ying-Ying Chen, Department of Pathology and Pathophysiology, Zhejiang University School of Medicine, 866 Yu-Hang-Tang Road, Hangzhou, Zhejiang 310058, P.R. China
E-mail: bchenyy@zju.edu.cn

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Materials and methods

Reagents. LPS (from *Escherichia coli* 055:B5), resveratrol, sodium pentobarbital and EX-257 were purchased from

Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), acetic acid, paraformaldehyde, 0.1 M phosphate buffered saline (PBS; pH 7.4), paraffin, xylene, ethanol, hematoxylin and 3,3-diaminobenzidine were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin and 1% Triton X-100 were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Radioimmunoprecipitation lysis buffer, citrate buffer and BeyoECL Plus kit were purchased from Beyotime Institute of Biotechnology (Haimen, China). Primary antibodies for glial fibrillary acidic protein (GFAP; mouse monoclonal; sc-33673), sirtuin 1 (SIRT1; rabbit monoclonal; sc-15404), and β -actin (mouse monoclonal; sc-47778) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary antibodies for ionized calcium binding adapter molecule 1 (Iba-1; rabbit polyclonal; 019-19741) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Biotin-labeled goat anti-rabbit IgG (A0277) and biotin-labeled goat anti-mouse IgG (A0286) secondary antibodies used in immunohistochemistry were obtained from Beyotime Institute of Biotechnology. Goat anti-rabbit IgG (7074) and horse anti-mouse IgG (7074) used in western blotting were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The enzyme-linked immunosorbent assay (ELISA) kits for TNF- α and IL-6 were obtained from R&D Systems, Inc. (Minneapolis, MN, USA).

Mice. A total of 120 male ICR mice (age, 8-10 weeks; weight, 22-24 g) were obtained from the Experimental Animal Center of Zhejiang University (Hangzhou, China). Five mice per cage were housed in transparent plastic cages in controlled conditions at 20-24°C with 40-60% humidity and a 12 h light/dark cycle. All mice were allowed *ad libitum* access to water and food. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health (Bethesda, MD, USA) (13). All experimental protocols were approved by the Ethics Committee on Animal Experimentation of Zhejiang University.

Mice were divided into the following groups (n=12): i) Control group, which received the same volume of 0.1% DMSO vehicle diluted in saline (0.1 ml/10 g body weight); ii) LPS group, which received an intraperitoneal (i.p.) injection of LPS (0.25, 0.5, or 1 mg/kg) for 5 consecutive days; iii) LPS + resveratrol group, which received an i.p. injection with resveratrol (5, 10 or 20 mg/kg) 30 min prior to administration of LPS (1 mg/kg); iv) resveratrol group, which received an i.p. injection of resveratrol (20 mg/kg) for 5 consecutive days; v) LPS + resveratrol + EX-257 group, which received EX-257 (2 mg/kg) by i.p. injection 30 min prior to resveratrol (20 mg/kg) administration, which was followed by injection of LPS; and vi) EX-257 group, which received an i.p. injection of EX-257 (2 mg/kg). Behavioral assessments, histology and molecular biology analysis were performed 6 h after the final LPS injection of the experimental groups, or after the vehicle injection on the control group at the same time point. Resveratrol and EX-257 were dissolved in DMSO and diluted in saline. The concentration of DMSO was <0.1%, which exerted no marked physiological action or pharmacological anti-inflammatory effect.

Acetic acid-induced writhing test. The mice received an i.p. injection of 0.7% acetic acid (0.1 ml/10 g) and were then placed into separate plastic animal cages. The number of writhes was counted for 20 min after the i.p. injection according to the method described by Zhao *et al* (14). Writhing was defined as the contraction of abdominal muscles, which were accompanied by the stretching of the hind limbs.

Tail-flick test. To examine thermal hyperalgesia, the tail-flick test was employed according to the method described by Sora *et al* (15). Briefly, mice were restrained in conical polypropylene tubes with an opening, and their tails were immersed ~2 cm into a 50±0.2°C water bath. The period from the immersion to the removal of the whole tail from the water was recorded as tail-flick latency.

Measurement of cytokines. Blood samples (1 ml) were collected in test tubes containing ethylenediaminetetraacetic acid (1 mg/ml) and centrifuged immediately at 1,000 x g for 10 min at room temperature. The plasma was stored at -80°C for analysis for cytokines. The plasma TNF- α and IL-6 levels were measured using ELISA kits according to manufacturer's protocols.

Immunohistochemistry. Mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused with PBS via the ascending aorta to remove blood in the tissue prior to the fix-perfusion of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The mice were then sacrificed by exsanguination. Spinal cord segments (lumbar (L)4-L5) were removed, post-fixed in the same fixative for 72 h, and then embedded in paraffin. For immunohistochemistry, each specimen was cut into 5- μ m sections. The sections were deparaffinized in glass centrifuge tubes with two 10 ml changes of xylene each 10 min. Then, sections were rehydrated at room temperature in a sequence of decreasing concentrations of ethanol by 2 min incubations in 10 ml of the following: 100% Ethanol 2 times, 90% ethanol 2 times, 70% ethanol and 50% ethanol. After pretreatment with citrate buffer to enhance immunoreactivity, the sections were blocked in 5% bovine serum albumin for 1 h. The sections were incubated with anti-GFAP antibody (1:500) or anti-Iba-1 (1:500) overnight at 4°C. After washing with PBS 3 times, the sections were incubated with biotin-conjugated secondary antibodies (1:200) for 1 h at room temperature. Sections were counterstained with hematoxylin to label nuclei. The staining was visualized using 3,3-diaminobenzidine, and observed with a BX51 microscope (Olympus Corporation, Tokyo, Japan). To quantify GFAP-positive and Iba-1-positive cells, images were captured of the dorsal horn of the spinal cord (magnification, x40). Immunoreactive astrocytes and microglial cells with clearly identifiable nuclei were counted manually by an investigator who was blinded to the treatment and experienced in the field.

Western blotting. The spinal cords segments (dorsal part of L4-L5) were collected and homogenized in radioimmunoprecipitation lysis buffer containing 1% Triton X-100. Following centrifugation at 10,000 x g at 4°C for 30 min, the supernatant was collected and frozen at -80°C until use. Lysates from the spinal cord were mixed with sodium dodecyl sulfate

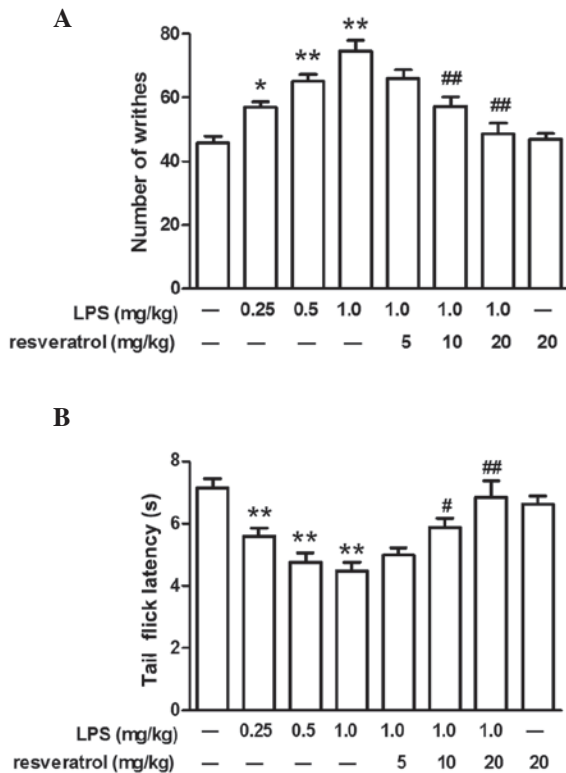


Figure 1. Effect of resveratrol on LPS-induced hyperalgesia in mice. (A) Number of writhes was obtained from the acetic acid-induced writhing test. (B) Latency was recorded in the tail-flick test. Data are presented as the mean \pm standard error of the mean (n=12). *P<0.05, **P<0.01 vs. the control group; #P<0.05, ##P<0.01 vs. the LPS (1.0 mg/kg) group. LPS, lipopolysaccharide.

sample buffer, heated at 100°C for 5 min and separated on 10% SDS-PAGE gels at 200 V for 40 min prior to transfer to a nitrocellulose membrane. Following blocking for 1 h with 5% skim milk, the membrane was incubated with primary antibodies (1:1,000) at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies (1:2,000) for 45 min at room temperature. All reactions were detected by the enhanced chemiluminescence detection method. The enhanced chemiluminescence signal was captured using a G:BOX Chemi XR5 imaging system (Syngene, Cambridge, UK). The band density was analyzed with Quality One software (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to β -actin.

Statistical analysis. Data were expressed as the mean \pm standard error of the mean and analyzed by one-way analysis of variance with a Bonferroni post hoc test as required using Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). P<0.05 was considered to indicate a statistically significant difference.

Results

LPS increased the number of writhes and decreased the latency of tail flicks. LPS-induced hyperalgesia in mice was evaluated by the acetic acid-induced writhing test and tail-flick test. Following the administration of LPS (0.25, 0.5, or 1.0 mg/kg, i.p.) for 5 days, the number of writhes increased

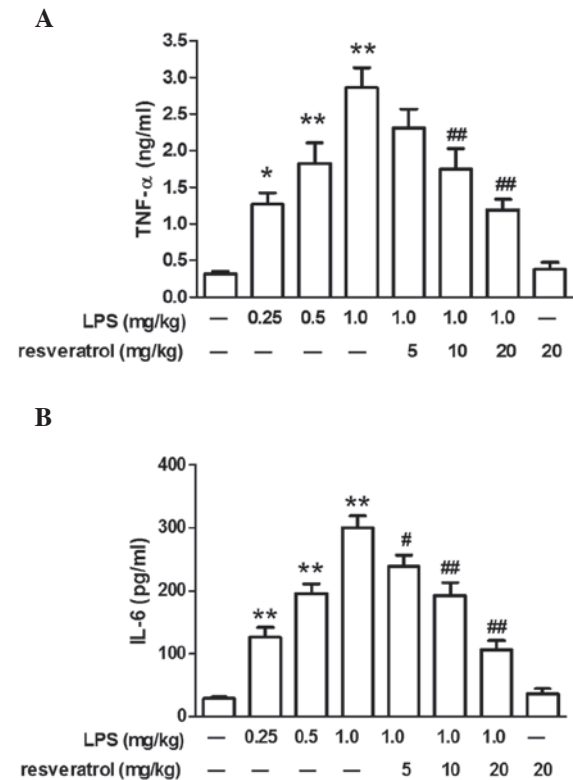


Figure 2. Effect of resveratrol on LPS-induced inflammation in mice. (A) Serum TNF- α level. (B) Serum IL-6 level. Data are presented as the mean \pm standard error of the mean (n=12). *P<0.05, **P<0.01 vs. the control group; #P<0.05, ##P<0.01 vs. the LPS (1.0 mg/kg) group. LPS, lipopolysaccharide; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6.

and the tail-flick latency decreased in a dose-dependent manner (P<0.05; Fig. 1). As mice that were exposed to the highest concentration of LPS (1.0 mg/kg) exhibited marked hyperalgesia, this concentration of LPS was used for subsequent experiments. Administration of LPS once per day for 5 days did not alter body weight and although treatment with LPS induced slight behavioral alterations, one day after the final injection these behaviors, including grip tone, motor activity and swimming speed, were comparable with that of control group (data not shown).

Resveratrol partly inhibited LPS-induced hyperalgesia at 10 or 20 mg/kg. Compared with the mice injected with LPS (1.0 mg/kg), resveratrol (10 or 20 mg/kg) partly inhibited the LPS-induced hyperalgesia in the acetic acid-induced writhing test and tail-flick test, however, a lower dose of resveratrol (5 mg/kg) did not demonstrate any marked inhibitory effect on LPS-induced hyperalgesia. Injection of resveratrol alone did not influence the pain behavioral response in mice (P<0.05; Fig. 1).

Resveratrol blocked LPS-induced inflammation in mice. LPS injection significantly increased plasma TNF- α and IL-6 levels in mice (P<0.05). Resveratrol (10 or 20 mg/kg) treatment reduced changes in IL-6 or TNF- α expression levels induced by LPS (P<0.01; Fig. 2).

Resveratrol reduced the number of GFAP and Iba-1 positive cells in LPS treated mice. The astrocyte-specific activation

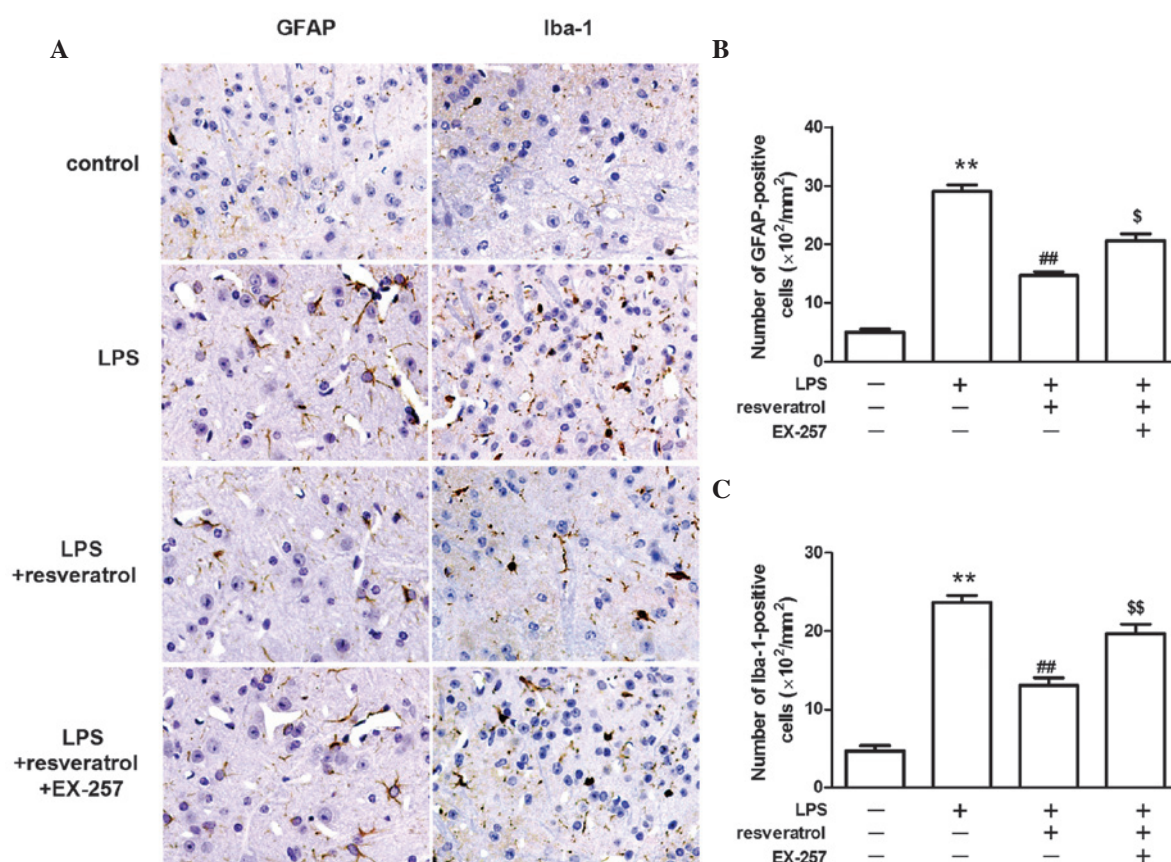


Figure 3. Immunohistochemical staining of GFAP and Iba-1 in the spinal cord of mice. (A) Representative microscopic field images (magnification, x400) of the dorsal horn of the spinal cord immunostained with GFAP and Iba-1. Number of (B) GFAP-positive and (C) Iba-1-positive glial cells. Data are presented as the mean \pm standard error of the mean (n=3). **P<0.01 vs. the control group; ##P<0.01 vs. the LPS group; \$P<0.05, \$\$P<0.01 vs. the LPS + resveratrol group. GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adapter molecule 1; LPS, lipopolysaccharide.

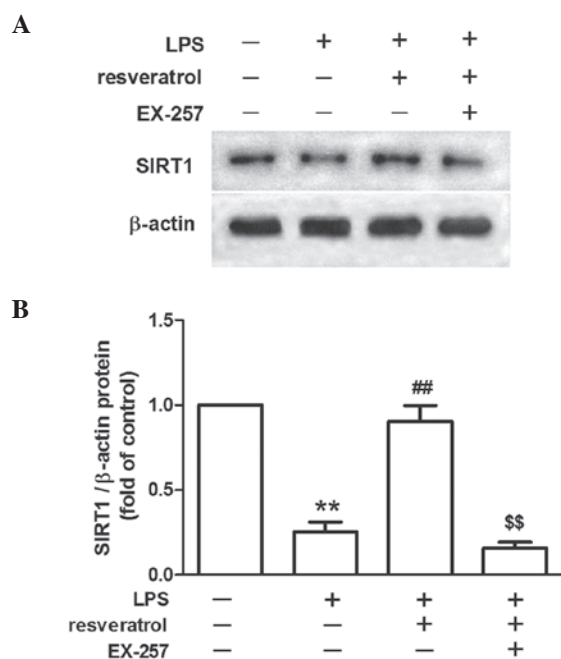


Figure 4. Western blotting analysis of SIRT1 protein expression in the dorsal spinal cord in mice. (A) Representative blots and (B) densitometric quantification of SIRT1 protein expression normalized to β -actin level. Data are presented as the mean \pm standard error of the mean (n=3) and expressed as fold increase relative to the control value. **P<0.01 vs. the control group; ##P<0.01 vs. the LPS group; \$\$P<0.01 vs. the LPS + resveratrol group. LPS, lipopolysaccharide; SIRT1, sirtuin 1.

marker, GFAP and the microglia-specific activation marker, Iba-1 were detected by immunohistochemical staining. GFAP and Iba-1 staining was weak in the control group, however, the staining was markedly increased in the dorsal horns of the spinal cord following the injection of LPS. The number of GFAP-positive and Iba-1-positive cells in the spinal cord was markedly lower in the LPS + resveratrol group, when compared with the LPS treated group (P<0.01; Fig. 3).

The inhibitory effect of resveratrol on LPS-induced hyperalgesia was blocked by a SIRT1 inhibitor. LPS injection reduced SIRT1 protein expression in the spinal cord. Resveratrol increased SIRT1 protein expression levels in the LPS treated mice (P<0.01; Fig. 4). EX-257, a selective SIRT1 inhibitor, blocked the resveratrol-induced analgesic effect (P<0.05; Fig. 5), and EX-257 inhibited the anti-inflammatory effect exerted by resveratrol, and increased astrocyte and microglial activation, when compared with the LPS + resveratrol group (P<0.05; Figs. 3 and 6).

Discussion

In a number of chronic pain conditions, including neuropathic pain, cancer and obesity-associated pain, and other chronic inflammatory conditions, systemic inflammation is important in the pathophysiology of hyperalgesia, which may be mediated by the neural response in the spinal cord

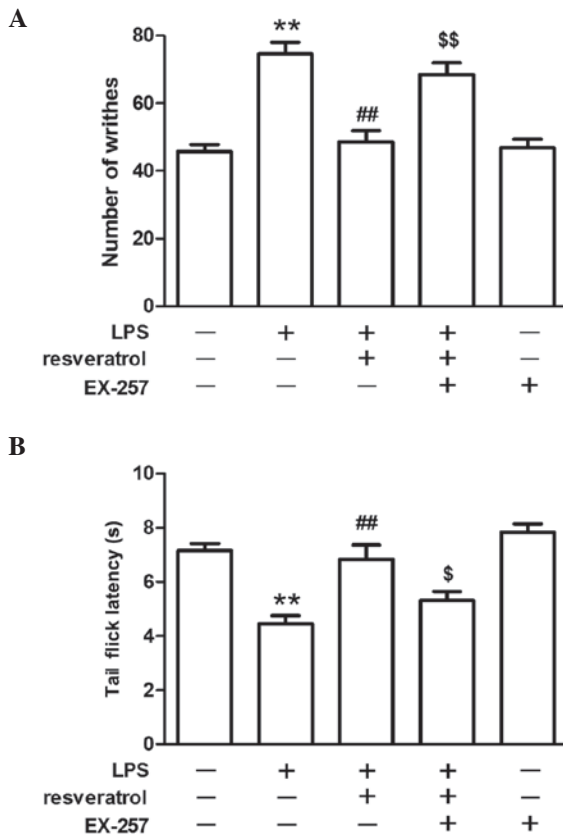


Figure 5. Effect of EX-257 on resveratrol-induced analgesia in mice. (A) Number of writhes observed in the acetic acid-induced writhing test. (B) Latency was recorded in the tail-flick test. Data are presented as the mean \pm standard error of the mean (n=12). **P<0.01 vs. the control group; ##P<0.01 vs. the LPS group; \$P<0.05, \$\$P<0.01 vs. the LPS + resveratrol group. LPS, lipopolysaccharide.

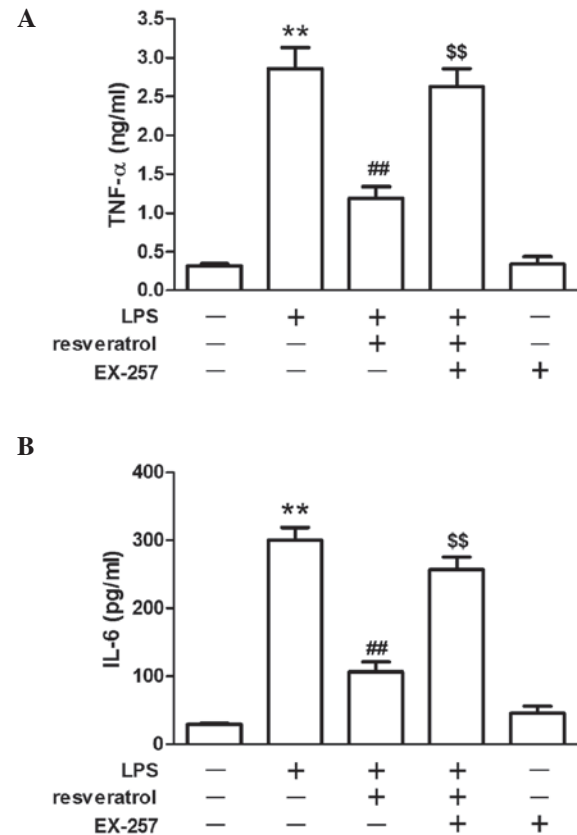


Figure 6. Effect of EX-257 on resveratrol-induced anti-inflammation in mice. (A) TNF- α levels and (B) IL-6 levels measured in the serum. Data are presented as the mean \pm standard error of the mean (n=12). **P<0.01 vs. the control group; ##P<0.01 vs. the LPS group; \$\$P<0.01 vs. the LPS + resveratrol group. LPS, lipopolysaccharide; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6.

or brain (5,6,16). In the present study, subacute i.p. injections of LPS were administered daily for five consecutive days to assess the hyperalgesia. Two different hyperalgesic tests were used, the acetic acid-induced writhing test and the tail flick test. In the acetic acid-induced abdominal writhing test (a visceral pain model), acetic acid nociceptive activity may be the result of a release of cytokines, including TNF- α , IL-1 and IL-8 (17). In the present study, LPS increased the number of writhing events, which was consistent with the increased level of TNF- α and IL-6 in the plasma. However, the tail-flick tests were performed to assess whether resveratrol possesses central analgesic properties. In the tail-flick test, a thermal stimulus is focused on the tail skin of the animal to activate nociceptors in the superficial layers of the skin. The activation of peripheral nociceptors triggers a complex series of processes at the spinal level and results in the tail flick response. The latency of the tail-flick was decreased by LPS administration.

Glial cells, predominantly astrocytes and microglia, are important cell populations in the central nervous system (18). The glial cells are involved in maintaining homeostasis of the brain via regulation of pH and ionic balance, neurotransmitter uptake and degradation, and neuroinflammation modulation in physiological and pathophysiological conditions (19,20). Increasing evidence indicates that the glial cells are crucial in the generation and maintenance of

chronic pain (21,22). Ji *et al* (23) reported that spinal astrocytic activation contributes to mechanical allodynia in a rat chemotherapy-induced neuropathic pain model. Activation of astrocytes in the anterior cingulate cortex has been demonstrated to be an affective component of pain in an inflammatory pain model (24). Astrocytic activation in the spinal cord also contributes to the development and maintenance of pain in rat models of chronic pancreatitis, chronic constriction of the sciatic nerve, and bone cancer (25-27). Furthermore, activated spinal microglia are key in neuropathic pain (28-30). GFAP is a specific astrocytic activation marker and Iba-1 is a specific microglial activation marker in the central nervous system (31). Although LPS may induce satellite glial cell activation in dorsal root ganglia (32), recent evidence suggests the importance of glial activation in the spinal cord (33,34). In the present study, GFAP and Iba-1 expression levels were observed to be increased following administration of LPS for 5 days. The activation of glial cells may a result of the release of inflammatory cytokines in the rat dorsal horn by LPS (35,36). Pharmacological inhibition of glial reactions may be a potential therapeutic strategy to treat inflammatory pain in animal models.

SIRT1 is a nicotinamide adenine dinucleotide-dependent histone deacetylase, which is involved in lifespan extension, age-associated disease delay, metabolism and apoptosis regulation (37-40). Recently, SIRT1 was demonstrated to regulate

algesia. Upregulation of the expression of SIRT1 in the spinal dorsal horn reversed chronic morphine antinociceptive tolerance (41). Increased spinal SIRT1 expression attenuated mechanical allodynia and thermal hyperalgesia in a chronic constriction injury model (42,43), and SIRT1 has also been observed to be crucial in inflammatory diseases, including obesity, type 2 diabetes mellitus, and atherosclerosis (44,45). The present study showed that SIRT1 was downregulated in mice injected with LPS, suggesting that SIRT1 is important in modulating the development and progression of pain in subacute systemic inflammation. Thus, upregulation of SIRT1 appears to be a promising therapeutic strategy for systemic inflammation-induced pain.

SIRT1 has been reported to be one of the key targeted molecule of resveratrol and involved in its multiple biological effects (46-48). Resveratrol has potential anti-oxidative and anti-inflammatory properties and has been indicated to attenuate bone cancer pain (9). Chronic treatment with resveratrol for 4 weeks decreased the serum concentration of TNF- α and attenuated diabetic neuropathic pain (10). Systemic or spinal administration of resveratrol significantly suppresses morphine-induced microglial activation in the spinal cord (49). However, whether resveratrol relieved inflammation-induced pain remains to be elucidated. In the present study, it was observed that resveratrol inhibited the LPS-induced increase in serum inflammatory cytokines levels and attenuated inflammatory hyperalgesia in a dose-dependent manner. Furthermore, resveratrol inhibited the activation of glial cells in the dorsal horns of the spinal cord. The biological effect of resveratrol may be mediated by SIRT1-dependent (50) or SIRT1-independent (51) mechanisms, and phosphatidylinositol 3-kinase is essential for resveratrol-mediated expression of SIRT1 (50). The present study demonstrated that resveratrol increased the expression levels of SIRT1, and inhibition of SIRT1 expression by EX-257 blocked the resveratrol-induced analgesic effect.

A limitation of the present study is that the acute effect of resveratrol following the five treatments with LPS was not evaluated. Furthermore, whether the anti-hyperalgesia effect of resveratrol is more effective by intrathecal injection remains to be elucidated. However, the results of the present study suggest that resveratrol attenuates systemic inflammation-induced pain via upregulation of SIRT1. The protective effect of SIRT1 may occur via promotion of p65 deacetylation, regulation of mitogen activated protein kinase pathway, or inhibition of nuclear factor- κ B activity (52,53).

In conclusion, subacute administration of LPS induced the activation of glial cells and hyperalgesia. Resveratrol inhibited the activation of glial cells and attenuated inflammatory hyperalgesia in a SIRT1-dependent manner. The present study suggests that the beneficial role of resveratrol will help provide a potential therapeutic strategy in the treatment of pathological inflammatory pain.

Acknowledgements

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