Recombinant Human Erythropoietin Attenuates Spinal Neuroimmune Activation of Neuropathic Pain in Rats

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Abstract. Neuropathic pain is a complex syndrome resulting from damage to the peripheral nervous system. Central neuroimmune activation contributes to the generation and maintenance of chronic pain after nerve injury. The current study determined the effects of recombinant human erythropoietin (rhEPO) on behavioral hyperalgesia and neuroimmune activation in a rat model of neuropathic pain induced by L5 spinal nerve transection. Animals were randomly assigned into 3 groups: sham-operation with saline; L5 spinal nerve transection with rhEPO (5000 units/kg); or L5 transection with saline. The rhEPO or saline was given ip on the day before surgery and continued daily to day 7 post-transection. The paw pressure threshold and paw withdrawal latencies were measured before surgery and on days 1, 3, and 7 post-operation. Glial activation markers such as macrophage antigen complex-1 (Mac-1, OX-42) and glial fibrillary acidic protein (GFAP), production of tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-10, as well as nuclear factor-kappa B (NF-κB) activation were determined in the lumbar spinal cord. Administration of rhEPO resulted in attenuation of mechanical and thermal hyperalgesia. Furthermore, rhEPO markedly inhibited neuroimmune activation characterized by glial activation, production of proinflammatory cytokines like TNF-α, IL-1β, and NF-κB activation, but rhEPO enhanced the level of IL-10. These results support the significance of neuroinflammation and neuroimmune activation in the initiation and persistence of behavioral pain responses. The data indicate that rhEPO attenuates behavioral hyperalgesia and neuroimmune activation in neuropathic pain induced by L5 nerve transection.

Keywords: rhEPO, neuropathic pain, astrocytes, microglia, inflammatory cytokines, NF-κB, TNF-α

Introduction

Neuropathic pain results from aberrant sensory processing in either the peripheral nervous system or the central nervous system (CNS); it is characterized by spontaneous pain, increased responsiveness to painful stimuli (hyperalgesia), and pain perceived in response to normally non-noxious stimuli (alldynia). The mechanisms include unusual distributions of Na+ channels as well as abnormal responses to endogenous pain-producing substances such as prostaglandins (PG), substance P, and nitric oxide (NO), and cytokines such as tumor necrosis factor α (TNF-α) [1]. Various animal models have been used to explore the pathophysiology of neuropathic pain. A growing body of literature indicates that neuroimmune activation (which involves glial activation and inflammatory cytokine expression following nerve injury) is associated with the generation and maintenance of behavioral changes during neuropathic pain [2,3].
A neuroimmune response is mounted in the CNS, where neurons and glia produce a variety of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-10. Proinflammatory cytokines like TNF-α, IL-1β, and IL-6 in turn cause the deleterious escalation of a pathological neural activation from microglia to astrocytes and induce expression of cyclooxygenase (COX)-2, inducible nitric oxide (NO) synthase, and substance P, leading to increased nociceptive activity and thus severe and persistent neuropathic pain [4-6]. IL-10, which is a cytokine synthesis inhibiting factor, has also been implicated in the pain [7]. A recent study showed that intrathecal administration of a novel adeno-associated viral (AAV) 2-IL-10 vector encoding IL-10 was successful in transiently preventing and reversing neuropathic pain [8]. Astrocytes upregulate the expression of glial fibrillary acidic protein (GFAP) in the CNS upon activation, while microglia express macrophage antigen complex-1 (Mac-1, OX-42) [3].

A transcription factor, NF-κB, is a crucial signal for glial and neuronal cell function during inflammation, injury, and neuropathic pain [9]. It is present in all neural and glial cell types, including microglia and astrocytes [10]. In addition, it can be activated by some neurotrophic factors and cytokines, and then translocated into the nucleus, where it specifically binds to κB consensus sequences, leading to the increased expression of target genes [11].

Neuropathic pain caused by nerve injury is often treated with nonsteroidal anti-inflammatory drugs (NSAIDs), local anesthetics, or opioids; however, these agents are not always effective and are frequently limited by side effects. Thus, there has been a continuing search for novel drugs to alleviate neuropathic pain. Erythropoietin (EPO), a pleiotropic cytokine originally identified for its role in erythropoiesis, possesses potent neuroprotective activity following cerebral ischemia [12,13], traumatic brain injury [14], and spinal cord injury [15]. Apart from its neuroprotective role, we have found that a single bolus injection of 5000 units/kg of rhEPO 24 hr before insult remarkably decreased TNF-α, IL-6, and ICAM-1 production, which may be due in part to the attenuation of NF-κB activation in a myocardial ischemia-reperfusion injury model [16]. However, studies are limited regarding the underlying mechanism of rhEPO inhibition of neuroimmune activation associated with neuropathic pain.

The present study used a rat model of neuropathic pain following L5 nerve transection to investigate the role of rhEPO administered ip on pain behaviors, inflammatory cytokine expression, glial activation, and NF-κB activation that are involved in neuroimmune activation.

Methods and Materials

Animals. Male Sprague-Dawley rats weighing 200-240 g at the start of surgery were used in these experiments. All animal experiments were performed in accordance with the ethical guidelines for investigations of experimental pain in conscious animals [17]. Efforts were made to limit distress and to use the minimum number of animals necessary to achieve statistical significance. This study was approved by the Institutional Animal Care and Use Committee of Jinling Hospital.

Surgical procedure. Under deep anesthesia with halothane in an O₂ carrier (induction, 4%, and maintenance, 2%), L5 spinal nerve ligation and transection was performed as previously described [18], with slight modification. Briefly, a small incision of the skin overlaying L5-S1 was made, followed by retraction of the paravertebral musculature from the vertebral transverse processes. The L5 spinal nerve was identified, lifted slightly, ligated tightly with 3-0 silk thread, and transected. The wound was closed in two layers. The saline vehicle or the rhEPO (Sigma-Aldrich, St Louis, MO, USA) was injected ip 1 day before surgery and continued daily to day 7 post-transection. With regard to the sham-operated animals, the L5 spinal nerve was exposed without ligation, and the rats were treated with saline vehicle. All injections were given ≥16 hr prior to behavioral testing.

Study group. We established 3 groups: (a) the sham operation group (sham; n = 10); (b) the L5 nerve transection with rhEPO treatment group (rhEPO; n = 10); and (c) the L5 nerve transection with saline group (saline; n = 10). Rats of rhEPO group received injections of rhEPO (5000 units/kg, ip, diluted in 4 ml/kg saline solution) on 1 day before surgery and continued daily to day 7 post-transection. The doses were selected based on the previously established effective dose of rhEpo in a model of neuropathic pain [19]. Rats in the sham and saline groups received equal volumes of 0.9% saline solution at same time (4 ml/kg, ip).

Behavioral tests. Behavioral studies were conducted in a quiet, temperature-controlled (23 ± 1°C) room between the hours of 8 and 10 a.m. The behavioral data were recorded before the surgery, and on days 1, 3, and 7 post-operation. The paw pressure threshold (PPT) in response to normal innocuous mechanical stimuli was measured by using an
Electro VonFrey anesthesiometer (Model 2390CE, IITC Life Science, Inc.). Rats were placed individually beneath an inverted ventilated Plexiglas cage with a metal-mesh floor allowing access to the plantar surface of hind paw. Animals were allowed to acclimatize to the environment and experimenter. Then, gentle incremental pressure (maximum 200 g) by a rigid von Frey hair was applied perpendicularly to the dorsal surface of the ipsilateral hind paw until the paw was withdrawn. Five trials were conducted at intervals of several min and the force (g) applied was recorded. The paw withdrawal latencies (PWL) to noxious heat stimuli were determined using a paw stimulator analgesia meter (Model 390, IITC Life Science, Inc.). Animals were placed on a 6-mm thick glass floor under an inverted clear Plexiglas cage and were allowed to acclimatize to the environment and experimenter. The radiant heat source beneath the glass floor was focused on the plantar surface of the ipsilateral hind paw when in contact with the floor. Light intensity was preset to obtain a baseline latency of about 10 sec and the cutoff time was set at 20 sec to avoid tissue damage. Five trials were carried out with intervals of 8 min.

**Tissue harvesting.** After the behavioral testing on day 7 post-surgery, all rats were deeply anesthetized with sodium pentobarbital and perfused intracardially with 250 ml cold heparinized (1 ml/L) saline (0.9%). The dorsal aspect of the L5 lumbar spinal cord was removed and quickly stored in liquid nitrogen for subsequent assays.

**Western blotting of GFAP.** Tissue homogenates of L5 lumbar spinal cord and standard protein markers were subjected to SDS polyacrylamide gel electrophoresis. Separated proteins were transferred to nitrocellulose filters. Nonspecific binding was blocked by incubation with 1% bovine serum albumin. Primary antibody rabbit anti-rat GFAP (Dako, Carpinteria, CA) was diluted in a buffer containing 0.05% Tween 20. Blots were visualized using 3,5-diaminobenzidine and peroxidase-conjugated goat anti-rabbit immunoglobin. Protein was determined using BCA protein assay kit (Pierce Chemical, Rockford, IL). Intensity of the GFAP was analyzed with densitometric image analysis software.

**Immunohistochemistry for OX-42.** Immunoreactivity for the microglial activation marker OX-42 (antibody against CR3/CD11b) was assessed. Sections were treated with 0.3% H2O2 in Tris-buffered saline (TBS) for 20 min at room temperature to suppress endogenous peroxidase activity. Sections were then incubated overnight at 4°C in monoclonal mouse anti-rat OX-42 (1:1,000; Pharmingen, San Diego, CA) in TBS with 3% normal goat serum and 0.5% Triton-X-100. Subsequently, sections were incubated with the appropriate secondary biotinylated antibodies (1:400; Jackson ImmunoResearch, West Grove, PA) for 2 hr at room temperature, incubated in avidin-biotin complex solution (ABC; 1:400; Vector Laboratories, Burlingame, CA) for 1 hr at room temperature, followed by reaction with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma). Finally, sections were mounted on gelatin-coated slides, dried, dehydrated, and coverslipped with Permount. Staining was evaluated by light microscopy.

**Cytokines by ELISA.** Spinal production of TNF-α, IL-1β, IL-6, and IL-10 was quantified by enzyme-linked immunosorbent assay (ELISA) kits for rats according to the manufacturers’ instructions (Diaclone Research, France; for TNF-α; Biosource International, USA for IL-1β, IL-6, and IL-10). Values were expressed as pg/mg protein. Protein concentrations in the supernatant samples were assayed as described above.

**EMSA for NF-κB.** Electrophoretic mobility shift assay (EMSA) for NF-κB was performed using a kit (Gel Shift Assay System, Promega, Madison, WI, USA) as previously described.

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Fig. 1. Changes in rat mechanical hyperalgesia as evidenced by paw pressure threshold before surgery and days 1, 3, and 7 after the operation. Nerve injury resulted in a significant (p <0.01) decrease in mechanical threshold compared to animals in the sham-operated group. RhEPO treatment attenuated the development of mechanical hyperalgesia in L5 nerve-transected rats compared to the saline treated nerve-transected rats (p <0.01). (n = 10/group, data are means ± SE), **p <0.01 vs saline group, ##p <0.01 vs sham group.

Fig. 2. Changes in rat thermal hyperalgesia as evidenced by paw withdrawal latency before surgery and days 1, 3, and 7 after the operation. Nerve injury resulted in a significant (p <0.01) decrease in thermal threshold compared to animals in the sham-operated group. RhEPO treatment attenuated the development of thermal hyperalgesia in L5 nerve-transected rats compared to the saline treated nerve-transected rats (p <0.01) (n = 10/group, data are means ± SE), **p <0.01 vs saline group, ##p <0.01 vs sham group.
described [20]. Briefly, equal amounts of nuclear extract (10 μg) were added to 9 μl of gel shift binding buffer for 15 min at room temperature. Then the mixture was incubated for 30 min with 1 μl of 32P-labelled oligonucleotide probe. Then 1 μl of loading buffer was added and the sample was electrophoresed in a 4% polyacrylamide gel. The gel was vacuum-dried and exposed to X-ray film (Fuji Hyperfilm) at -70°C for 48 hr. The intensity of the NF-κB was assessed by densitometry.

Statistics. Values were expressed as means ± SE. For behavioral data, comparisons between groups were performed using repeated measures analysis of variance (ANOVA). The effects of surgery and drug injections on other markers were determined by two-way ANOVA followed by Bonferroni or Tamhane’s T2 test based on equal variances assumed or not. Data were analyzed using a statistics software package (SPSS for Windows v. 13.0); p <0.05 was considered significant.

Results

Behavioral tests. As shown in Figs. 1 and 2, baseline responsiveness to mechanical or thermal stimuli was comparable among groups as confirmed by testing sessions before the surgery. Mechanical allodynia and thermal hyperalgesia were evidenced by PPT (Fig. 1) and PWL (Fig. 2). There were no significant differences in the determined behavioral variables (PPT and PWL) in the sham group over time during the experimental process. However, there was a sharp initial decrease in PPT or PWL on day 1 following L5 nerve transection in the ipsilateral hind paw (p <0.01 compared with sham group). Then PPT or PWL gradually decreased and reached its lowest point on day 3 or day 7 (p <0.01 compared to sham group). Administration of rhEPO reduced the development of behavioral symptoms induced by L5 nerve transection compared to vehicle treatment on days 3 and 7 post-operation (p <0.01).

Expression of glial activation markers. We studied GFAP protein expression of astrocytes using Western blot analysis. As shown in Fig. 3, expression of spinal GFAP was markedly increased in nerve-transected rats on day 7 compared to the sham-operated rats (p <0.01). Administration of rhEPO significantly decreased spinal GFAP expression in rats challenged with L5 nerve transection compared to the saline group (p <0.01). Likewise, we assayed the activation marker OX-42 in microglial cells using immunohistochemistry. As shown in Fig. 4A, OX-42 immunoreactive microglia uniformly displayed a ramified morphology in the sham group. After L5 nerve transection, the intensity of OX-42 immunoreactivity was increased, with morphological transformation from bushy type to the hyperramified or hypertrophic type (Fig. 4B). The rhEPO-treated animals showed significant attenuation of OX-42 immunoreactivity (Fig. 4C) compared to the saline-treated group. Thus, we conclude that rhEPO inhibits glial activation in the lumbar spinal cord after L5 nerve transaction.
Production of inflammatory cytokines. As shown in Fig. 5, the expression levels of TNF-α, IL-1β, IL-6, and IL-10 in spinal cord were low in the sham group. L5 nerve transection-induced neuropathic pain was associated with marked increases in the protein levels of TNF-α, IL-1β, and IL-6, but significantly enhanced the expression of IL-10 (p <0.01). Administration of rhEPO significantly suppressed the elevations of TNF-α, IL-1β, and IL-6 (p <0.01 for TNF-α, IL-1β; p <0.05 for IL-6). However, administration of rhEPO significantly increased the expression of the anti-inflammatory cytokine, IL-10, compared to the saline-treated group (p <0.01).

NF-κB activation. EMSA experiments were undertaken to examine the effect of rhEPO on activation of NF-κB in rats undergoing neuropathic pain. As shown in Fig. 6, activation of spinal NF-κB was markedly increased after spinal nerve injury compared to the sham-operated group (p <0.01); rhEPO significantly inhibited NF-κB activation compared to the saline group (p <0.01).

Discussion

This study examined the potential preventive value of rhEPO in the treatment of neuropathic pain using a L5 spinal nerve-transection rat model of neuropathic pain. Our data show that when treatment began 1 day before the surgery, ip administration of rhEPO to rats (a) reduced the development of mechanical and thermal hyperalgesia, (b) prevented astrocytic and microglial activation of GFAP and OX-42, respectively, (c) inhibited the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, while increasing IL-10 production, and (d) suppressed the activation of NF-κB.

Neuropathic pain is a complex syndrome that results from damage to the peripheral nervous system. The presence of neuropathic pain is often characterized by stimulus-independent persistent pain or abnormal sensory perception of pain [21, 22]. Mounting evidence including our previous studies showed that neuropathic pain induced by peripheral nerve injury is manifested by tactile allodynia and thermal hyperalgesia [8,23,24]. Consistent with these observations is our present finding that behavioral hyperalgesia determined by PPT and PWL is initiated after L5 nerve transection. We further showed that rhEPO administration...
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attenuates the development of mechanical and thermal hyperalgesia. Several lines of evidence suggest that systemically administered rhEPO facilitates recovery from behavioral changes during neuropathic pain associated with CCI, L5 spinal nerve crush, or nerve root injury [19,23,25]. These findings support the antinoceptive role of rhEPO in case of neuropathic pain.

Central neuroimmune activation, characterized by glial activation and inflammatory cytokine expression, is integral to the generation and maintenance of hyperalgesia and allodynia following both peripheral and central insults [2]. Astrocytes and microglia are key neuromodulatory, neurotrophic, and neuroimmune elements in the CNS. It is noteworthy that drugs that suppress glial activation can prevent and reverse neuropathic pain [3,26,27]. As demonstrated previously [28], the astrocyte marker, GFAP, and the microglial marker, mac-1, were significantly increased in the nerve injury model of partial sciatic nerve ligation, a well-developed model for neuropathic pain. After injection of rhEPO, the increases in GFAP and mac-1 were remarkably suppressed. The effect of rhEPO on behavioral pain responses appears to parallel its impact on glial activation, implying that the attenuation of neuropathic pain behavior by rhEPO may result from its role on glial activation. Products released by activated glia have been implicated in central sensitization and hence the development and maintenance of persistent pain states [29-31]. Pro-inflammatory cytokines are normally expressed in low concentrations in the spinal cord; however, peripheral nerve injury results in increases of their expression. The significance of spinal TNF-α, IL-1β, and IL-6 in the development and maintenance of peripheral nerve injury-induced allodynia has been demonstrated [32-34]. The activated glia synthesize and release multiple inflammatory mediators, such as TNF-α, IL-1β, IL-6, NO, and PGE2, which can further act either directly on dorsal horn neurons that transmit pain (nociceptive neurons) or indirectly on primary afferents, both leading to increased sensitivity of the nociceptive neurons (central sensitization). Moreover, these inflammatory mediators diffuse to and act on surrounding glia, causing their further activation. Positive feedback loops between inflammatory mediators and glial activation and between glia and neurons lead to the enhancement and maintenance of neuropathic pain.

We found that nerve injury greatly increases production of TNF-α, IL-1β, and IL-6 in the spinal cord. Moreover, our data demonstrated that rhEPO inhibits the production of cytokines, which is consistent with the antinoceptive effect of rhEPO. The results also indicate that systemic administration of rhEPO after spinal nerve transection inhibits the glial activation in the spinal cord, which is the primary source of inflammatory cytokines. The anti-inflammatory cytokine levels also increased, tending to maintain balance and hemostasis. IL-10, a cytokine synthesis-inhibiting factor, has also been involved in pain production [26]. The level of IL-10 was increased progressively and its administration reduced the hyperalgesia induced by CCI injury [30]. It is noteworthy that rhEPO further enhanced IL-10 production in the present study, which may suppress the production of inflammatory cytokines and negatively regulate the positive feedback loop between inflammatory mediators and glial activation.

Having confirmed the antinociceptive activity and anti-inflammatory effects of rhEPO, we moved to another experimental approach in order to gain insight into the underlying molecular mechanisms of rhEPO. Several mechanisms might mediate the neuroprotective actions of rhEPO, including diminished inflammation [35], activation of survival kinases pathways [36], activation of NF-κB [37], and activation of antiapoptotic genes [38]. Among them, we focused on NF-κB. NF-κB is present in microglia and astrocytes and can be activated by some cytokines and in turn regulate the expression of inflammatory cytokines [37]. In cortical neurons, rhEPO counteracts TNF-α-mediated signaling to NF-κB [20], which supports the data presented here that rhEPO inhibits NF-κB activation during neuropathic pain. The tight connections among NF-κB activation, inflammatory mediators, and glial activation suggest that the beneficial effects of rhEPO on NF-κB activation partly contribute to evidence of decreased inflammatory mediators and suppressed glial activation within the spinal cord, and thus alleviate behavioral changes. The role of various signaling pathways
besides NF-κB signaling in the injured peripheral nerve warrants further investigation.

Because gender differences in neuropathic pain sensitivity are well recognized [39], male rats were used in the study due to the reported sensitivity [40,41] and the substantial increase in endogenously produced TNF-α of female rats [42].

The currently available therapies for neuropathic pain are generally not very effective [21]. Patients do not respond well to NSAIDs and the usage of opioids and local anesthetic-type Na+ channel blockers has been limited by serious side effects. Gabapentin and clinically available NMDA receptor antagonists are only effective in a fraction of patients. Thus, there is a continuing search for novel drug molecules to alleviate neuropathic pain. Our findings of protective effects of rhEPO on hyperalgesia, glial activation, and inflammatory responses may offer a novel therapeutic strategy in neuropathic pain.

In conclusion, our present study demonstrates that rhEPO may be antiallodynic in a rat model of neuropathic pain by inhibiting glial activation and activation of NF-κB, thus directly inhibiting production of proinflammatory cytokines and increasing production of an anti-inflammatory cytokine. The effects of rhEPO on neuroimmune activation may lead to its clinical application in neuropathic pain.

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References

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