Effects of recombinant human erythropoietin on neuropathic pain and cerebral expressions of cytokines and nuclear factor-kappa B

Effets de l'érythropoïétine humaine recombinante sur la douleur neuropathique et l'expression cérébrale de cytokines et du facteur nucléaire kappa B

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Abstract

Purpose The effect of recombinant human erythropoietin (rhEPO) on neuropathic pain remains unclear. This study aimed to determine the effects of preemptive administration of rhEPO on the behavioural changes and neuroinflammatory responses in a rat model of neuropathic pain.

Methods Fifty rats were randomly allocated into five groups, sham-operation treated with saline and L5 spinal nerve transection treated with different doses of rhEPO (0 [saline], 1000, 3000, or 5000 U · kg⁻¹, respectively). The rats were intraperitoneally treated from 1 day before surgery to post-surgery day 7. The mechanical (paw pressure thresholds, PPT) and thermal thresholds (paw withdrawal latencies, PWL) were measured on post-surgery days 1, 3, and 7. The contralateral brain was obtained on post-surgery day 7 to determine the expressions of tumour necrosis factor (TNF-α), interleukin (IL)-1β, IL-6, IL-10, and nuclear factor-kappa B (NF-κB) activity.

Results There were significant decreases in PPT and PWL after L5 spinal nerve transection ($P < 0.001$). Compared with the saline group, the rhEPO 3000 and 5000 U · kg⁻¹ groups resulted in significant increases in PPT and PWL ($P < 0.001$) and reduced the cerebral expressions of TNF-α, IL-1β, IL-6, and NF-κB activity associated with the increase in IL-10 (rhEPO3000 group, $P < 0.05$, and rhEPO5000 group, $P < 0.001$, respectively).

Administration of rhEPO 1000 U · kg⁻¹ had no significant effects on these variables.

Conclusions Preemptive rhEPO dose-dependently attenuated the mechanical and thermal hyperalgesia in L5 spinal nerve transection rats, which correlated with the decreased cerebral expressions of TNF-α, IL-1β, and IL-6 via downregulating NF-κB activity and the increased expression of IL-10.

Résumé

Objectif L'effet de l'érythropoïétine humaine recombinante (rhEPO) sur la douleur neuropathique est incertain. Cette étude a eu pour objectif de déterminer les effets d'une administration préventive de rhEPO sur les changements comportementaux et les réactions neuroinflammatoires dans un modèle de douleur neuropathique chez le rat.

Méthode Cinquante rats ont été randomisés en 5 groupes: opération fictive traitée avec une solution salée; et coupe transversale du nerf rachidien L5 traitée avec différentes doses de rhEPO (0 [solution saline], 1000, 3000, ou 5000 unités/kg, respectivement). Les rats ont été traités avec une injection intrapéritonéale à partir du jour précédant la chirurgie jusqu'au jour 7 après l'opération. Les seuils mécanique (seuils de pression sur les pattes, PPT) et thermique (temps de latence du retrait des pattes, PWL) ont été mesurés aux jours 1, 3 et 7 après la chirurgie. Le cerveau controlatéral a été obtenu le 7^e jour après la chirurgie afin de déterminer les expressions du facteur onconécrosant (TNF-α), de l'interleukine (IL)-1β, IL-6, IL-10, et l'activité du facteur nucléaire kappa B (NF-κB).

Résultats Les seuils PPT et PWL ont significativement diminué après la coupe transversale du nerf rachidien L5 ($P < 0,001$). Par rapport au groupe solution salée, les

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solutions rhEPO 3000 et 5000 unités/kg ont eu pour résultat des augmentations significatives des seuils PPT et PWL ($P < 0,001$), ainsi qu'une réduction des expressions cérébrales de TNF- α , IL-1 β , IL-6, et de l'activité du NF- κ B associée à une augmentation de IL-10 (pour le groupe rhEPO3000, $P < 0,05$; et pour le groupe rhEPO5000, $P < 0,001$; respectivement). L'administration de rhEPO 1000 unités/kg n'a pas eu d'effets significatifs sur ces variables.

Conclusion Le rhEPO administré de façon préventive a atténué l'hyperalgésie mécanique et thermique de manière dose dépendante chez des rats subissant une coupe transversale du nerf rachidien L5, ce qui a correspondu à des expressions cérébrales réduites de TNF- α , IL-1 β , IL-6 en régulant à la baisse l'activité NF- κ B et une expression accrue de IL-10.

Neuropathic pain results from aberrant sensory processing in either the peripheral nervous system or the central nervous system and is characterized by spontaneous pain, increased responsiveness to painful stimuli (hyperalgesia), and the pain perceived in response to normally non-noxious stimuli (allodynia).¹ A growing body of literature indicates that central neuroimmune and neuroinflammatory activation produces and maintains neuropathic pain after nerve injury.^{2,3}

The typical pro-inflammatory cytokines tumour necrosis factor (TNF- α), interleukin (IL)-1 β , and IL-6 are strongly implicated in the initiation and development of the neuropathic pain after nerve injury.^{3–5} In contrast, as an anti-inflammatory cytokine, IL-10 attenuates the neuropathic pain after nerve injury.^{2,5,6}

Nuclear factor-kappa B (NF- κ B) is one of the most important transcription factors regulating gene expression of the pro-inflammatory cytokines.² Activated NF- κ B is thus involved in the initiation and development of neuropathic pain via a neuron-mediated way of central sensitization and glia-mediated expressions of pro-inflammatory cytokines and pain mediators.^{2,7,8}

Several animal studies^{9–11} have demonstrated that erythropoietin (EPO) or recombinant human erythropoietin (rhEPO) play a potent neuroprotective effect via anti-inflammation following cerebral ischemia or traumatic brain injury. EPO may also alleviate the behavioural changes associated with neuropathic pain^{12–14}; however, the underlying mechanisms remain unclear. We hypothesized that rhEPO could relieve neuropathic pain via altering the cerebral expressions of NF- κ B and cytokines in an animal model.

The objective of this study was to determine whether preemptive administration of rhEPO could attenuate the

pain behaviour in a rat model of neuropathic pain induced by L5 spinal nerve transection, and whether this anti-hyperalgesia effect of rhEPO was associated with the alterations in the cerebral expressions of TNF- α , IL-1 β , IL-6, IL-10, and NF- κ B.

Methods

Animals

The experimental protocol was reviewed and approved by the Institutional Animal Care Committee of Jinling Hospital. Fifty healthy male Sprague-Dawley rats, weighing 200–250 g, were housed in an approved facility and maintained on a 12-hr light-12-hr dark cycle with rodent food and water available ad libitum.

Surgical procedure

Under deep anesthesia with halothane in an O₂ carrier (induction 4%; maintenance 2%), L5 spinal nerve ligation and transection was performed based on the previously described method with slight modification.¹⁵ Briefly, a small incision was made to the skin overlaying L5-S1, followed by retraction of the paravertebral musculature from the vertebral transverse processes. The L5 spinal nerve was identified, lifted slightly, ligated tightly with a 3–0 silk thread, and then transected. In the sham-operated rats, the L5 spinal nerve was exposed without ligation or transection. Finally, the wound was closed in two layers.

Study groups

Using a randomized numeric table, the rats were divided into five groups of ten rats per group: (1) the sham operation treated with saline group (sham); (2) the L5 spinal nerve transection treated with saline group (saline); (3) the L5 spinal nerve transection treated with rhEPO 1000 U · kg⁻¹ group (rhEPO1000) (Sigma-Aldrich, St. Louis, MO, USA); (4) the L5 spinal nerve transection treated with rhEPO 3000 U · kg⁻¹ group (rhEPO3000); (5) the L5 spinal nerve transection treated with rhEPO 5000 U · kg⁻¹ group (rhEPO5000). All rats were intraperitoneally injected with saline 4 mL · kg⁻¹ containing different doses of rhEPO (0, 0, 1000, 3000, or 5000 U · kg⁻¹, respectively) 1 day prior to surgery and daily thereafter to post-surgery day 7. All injections were completed 16 hr before the behavioural tests. The doses of rhEPO were chosen based on the established effective doses of rhEPO in inhibiting pain in a rat model.¹⁶

Behavioural tests

Behavioural tests were conducted in a quiet and temperature-controlled ($23 \pm 1^\circ\text{C}$) room from 8:00 a.m. to 10:00 a.m. All the behavioural data were recorded before surgery and on post-surgery days 1, 3, and 7. The paw pressure thresholds (PPT) were measured by using an Electro Von Frey anesthesia meter (Model 2390CE, IITC Life Science, INC., Woodland Hills, CA, USA). The rats were placed individually beneath an inverted ventilated Plexiglas[®] cage with a metal-mesh floor allowing access to the plantar surface of the hind paw. The animals were allowed to acclimatize to the environment and to the 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 minutes and the pressure (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., Woodland Hills, CA, USA). The 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 to the 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 approximately 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 harvest

After the behavioural tests on post-surgery day 7, all rats were deeply anesthetized with sodium pentobarbital $50 \text{ mg} \cdot \text{kg}^{-1}$ and intracardially perfused with 250 mL cold heparinized saline ($5 \text{ U} \cdot \text{mL}^{-1}$), and the brain was removed quickly to liquid nitrogen for subsequent assays.

Cytokine by ELISA

The anterior half-brain tissue (including mainly prefrontal cortex, weighing 150–200 mg), excluding the olfactory bulbs, which were contralateral to the operation side, was dissected¹⁷ and homogenized in a buffer consisting of a protease inhibitor (Roche Diagnostics, Mannheim, Germany) using Power Gen 124 tissue tearer (Fisher Scientific Co., Suwanee, GA, USA). Samples were spun at 20,000g for 30 min at 4°C . Supernatant was aliquoted and stored at -80°C for future protein quantification. TNF- α (Diaclone Research, Besanc, France), IL-1 β , IL-6, and IL-10

(Biosource International, Camarillo, CA, USA) were determined using the quantitative sandwich enzyme immunoassay according to the manufacturer's protocol. Values were expressed as pictogram per milligram protein.

NF- κ B by EMSA

Electrophoretic mobility shift assay (EMSA) for NF- κ B was performed using a commercial kit (Gel Shift Assay System, Promega, Madison, WI, USA) as previously described.¹⁸ 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. Finally, 1 μL of loading buffer was added, and the sample was electrophoresed in a 4% polyacrylamide gel. The gel was vacuum-dried exposed to *x-ray* film (Fuji Hyperfilm) at -70°C for 48 hr. The intensity of the NF- κ B was assessed by densitometry.

Statistical analysis

Statistical analysis was performed using the Statistical Product for the Social Sciences version 13.0 (SPSS Inc., Chicago, IL, USA). All the figures were drawn as box plots via SPSS 13.0. The primary data are expressed as mean $\pm$ SD (95% confidence interval for mean). Sequential measurements of mechanical thresholds (PPT) and thermal thresholds (PWL) were analyzed using analysis of variance (ANOVA) for repeated measurements, followed by the least significant difference tests for multiple comparisons. Inter-group comparisons of PPT or PWL at the same day were tested using analysis of covariance (dependent variable: PPT or PWL; fixed factor: group; covariate: baseline PPT or PWL). Expressions of TNF- α , IL-1 β , IL-6, IL-10, and NF- κ B were compared using one-way ANOVA, followed by the least significant difference tests for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Behavioural tests

Compared with the sham group, there were significant decreases in PPT and PWL after L5 spinal nerve transection in the ipsilateral hind paw ($P < 0.001$, respectively). Compared with the saline group, rhEPO $3000 \text{ U} \cdot \text{kg}^{-1}$ and rhEPO $5000 \text{ U} \cdot \text{kg}^{-1}$ resulted in significant increases in PPT and PWL ($P < 0.001$, respectively), whereas rhEPO $1000 \text{ U} \cdot \text{kg}^{-1}$ did not (PPT, $P = 0.32$; PWL, $P = 0.33$) (Fig. 1).

Fig. 1 Changes in mechanical thresholds (PPT) and thermal thresholds (PWL) before surgery and on post-surgery days 1, 3, and 7. Compared with sham group, there were significant decreases in PPT and PWL after L5 spinal nerve transection ($P < 0.001$). Compared with saline group, PPT and PWL increased in rhEPO3000 and rhEPO5000 groups ($P < 0.001$), whereas there was no significant increase in rhEPO1000 group (PPT, $P = 0.32$; and PWL, $P = 0.33$). PPT and PWL as final values on post-surgery day 7, increased in rhEPO3000 group (31.5 ± 2.8 [29.5, 33.5] g and 7.5 ± 0.5 [7.1, 7.8] s) and rhEPO5000 group (35.6 ± 2.1 [34.1, 37.1] g and 8.0 ± 0.3 [7.8, 8.2] s) compared with saline group (28.3 ± 2.4 [26.6, 30.0] g and 6.9 ± 0.3 [6.7, 7.1] s) ($P < 0.001$), whereas there was no significant increase in rhEPO1000 group (28.8 ± 2.2 [27.3, 30.3] g and 6.9 ± 0.4 [6.6, 7.1] s) ($P = 0.62$ and 0.977 , respectively)

Since PPT and PWL gradually increased after rhEPO treatment, we only compared PPT and PWL as final values on post-surgery day 7. The results showed that PPT and

PWL increased in the rhEPO3000 group (31.5 ± 2.8 [29.5, 33.5] g and 7.5 ± 0.5 [7.1, 7.8] s) and the rhEPO5000 group (35.6 ± 2.1 [34.1, 37.1] g and 8.0 ± 0.3 [7.8, 8.2] s) compared with the saline group (28.3 ± 2.4 [26.6, 30.0] g and 6.9 ± 0.3 [6.7, 7.1] s) ($P < 0.001$, respectively), whereas the rhEPO1000 group did not (28.8 ± 2.2 [27.3, 30.3] g and 6.9 ± 0.4 [6.6, 7.1] s) ($P = 0.62$, 0.977 , respectively) (Fig. 1).

Expressions of inflammatory cytokines

Compared with the sham-operated rats, the cerebral expressions of TNF- α , IL-1 β , and IL-6 were significantly higher in rats after L5 spinal nerve transection ($P < 0.001$, respectively). rhEPO 3000 U $\cdot$ kg $^{-1}$ and rhEPO 5000 U $\cdot$ kg $^{-1}$ significantly reduced the expressions of the TNF- α , IL-1 β , and IL-6: rhEPO3000 group (TNF- α : 59.9 ± 7.2 [54.7, 65.1] pg $\cdot$ mg $^{-1}$; IL-1 β : 136.5 ± 18.8 [123.1, 149.9] pg $\cdot$ mg $^{-1}$; and IL-6: 376.6 ± 15.2 [365.7, 387.5] pg $\cdot$ mg $^{-1}$) and rhEPO5000 group (TNF- α : 43.2 ± 5.8 [39.1, 47.3] pg $\cdot$ mg $^{-1}$; IL-1 β : 86.9 ± 12.0 [78.3, 95.5] pg $\cdot$ mg $^{-1}$; and IL-6: 254.9 ± 28.1 [234.8, 275.0] pg $\cdot$ mg $^{-1}$) compared with saline group (TNF- α : 68.5 ± 9.8 [61.5, 75.5] pg $\cdot$ mg $^{-1}$; IL-1 β : 154.8 ± 22.6 [138.6, 171.0] pg $\cdot$ mg $^{-1}$; and IL-6: 405.5 ± 20.7 [385.7, 415.3] pg $\cdot$ mg $^{-1}$) (rhEPO3000 group, $P = 0.011$, 0.022 , and 0.017 , respectively; and rhEPO5000 group, $P < 0.001$, respectively). rhEPO 1000 U $\cdot$ kg $^{-1}$ had no significant effects on the expressions of TNF- α , IL-1 β , and IL-6 compared with the saline group ($P = 0.118$, 0.761 , and 0.934 , respectively) (Fig. 2).

Compared with the sham-operated rats, the cerebral expression of IL-10 also increased in rats after L5 spinal nerve transection ($P < 0.001$). Moreover, it increased more significantly in rhEPO3000 group (212.6 ± 26.8 [193.4, 251.8] pg $\cdot$ mg $^{-1}$) and rhEPO5000 group (310.4 ± 19.0 [296.8, 324.0] pg $\cdot$ mg $^{-1}$) compared with saline group (193.7 ± 15.3 [182.7, 204.7] pg $\cdot$ mg $^{-1}$) (rhEPO3000 group, $P = 0.046$; and rhEPO5000 group, $P < 0.001$). There was no significant difference between the rhEPO1000 group and the saline group ($P = 0.779$) (Fig. 2).

NF- κ B activity

Compared with the sham-operated rats, NF- κ B activity increased in rats after L5 spinal nerve transection ($P < 0.001$). NF- κ B activity significantly decreased in rhEPO3000 group (1.97 ± 0.13 [1.87, 2.07]) and rhEPO5000 group (1.82 ± 0.15 [1.71, 1.93]) compared with saline group (2.34 ± 0.25 [2.16, 2.52]) ($P < 0.001$, respectively). There was no significant difference between rhEPO1000 group and saline group ($P = 0.203$) (Fig. 3).

Fig. 2 Cerebral expressions of TNF- α (a), IL-1 β (b), IL-6 (c), and IL-10 (d) on post-surgery day 7. TNF- α , IL-1 β , IL-6, and IL-10 increased in rats after L5 spinal nerve transection compared with the sham-operated rats ($P < 0.001$). Compared with saline group, the cerebral expressions of TNF- α , IL-1 β , and IL-6 decreased and IL-10

increased in rhEPO3000 group ($P = 0.011, 0.022, 0.017$, and 0.046 , respectively) and rhEPO5000 group ($P < 0.001$). There was no significant difference with respect to the expressions of TNF- α , IL-1 β , IL-6, and IL-10 between rhEPO1000 group and saline group ($P = 0.118, 0.761, 0.934$, and 0.779 , respectively)

Discussion

In this study, the behavioural hyperalgesia determined by PPT and PWL initiated, and the cerebral expressions of NF- κ B, TNF- α , IL-1 β , IL-6, and IL-10 increased after L5 nerve transection. Preemptive rhEPO administration dose-dependently attenuated mechanical and thermal hyperalgesia, reduced the cerebral levels of NF- κ B, TNF- α , IL-1 β , and IL-6, and enhanced the cerebral level of IL-10. Taken together, the above results suggested that NF- κ B and these cytokines should be the important mediators in the development of neuropathic pain, and that rhEPO could alleviate neuropathic pain via downregulating the NF- κ B activity and altering the cerebral expressions of cytokines.

Central neuroimmune activation and neuroinflammation mediate and/or modulate the pathogenesis of neuropathic pain.^{2,3,19} In both the peripheral and central models of neuropathic pain, the elevated levels of TNF- α , IL-1 β , and IL-6 mRNA are associated with allodynia and hyperalgesia in response to nerve injury.^{2,3,19} Intrathecal injections of

IL-6 neutralizing antibody significantly reduce nerve injury-induced mechanical allodynia.²⁰ Inhibition of pro-inflammatory cytokines, TNF- α and IL-1 β , attenuates pain-associated behaviour in a dose-dependent manner in rats with neuropathic pain.²¹

NF- κ B is an important transcription factor upregulating the expressions of pro-inflammatory cytokines. Activation of NF- κ B has been detected in dorsal root ganglia in animal models of neuropathic pain.⁸ NF- κ B “decoy” (a new anti-gene approach) alleviates the hyperalgesia induced by spinal nerve ligation injury.⁹ Furthermore, inhibition of I κ B kinase (which phosphorylates I κ B to activate NF- κ B) reduces hyperalgesia in inflammatory and neuropathic pain models of rats.²²

Anti-inflammatory cytokine, IL-10, may inhibit a positive feed-forward loop between pro-inflammatory cytokines and NF- κ B in both inflammation and neuropathic pain states. IL-10 suppresses the production and activity of pro-inflammatory cytokines at several levels, including transcription, translation, and release.²³ In our study, the

Fig. 3 Cerebral NF- κ B activities on post-surgery day 7. Compared with the sham-operated rats, NF- κ B activity increased in rats after L5 spinal nerve transection ($P < 0.001$). NF- κ B activity significantly decreased in the rhEPO3000 group (1.97 ± 0.13 [$1.87, 2.07$]) and the rhEPO5000 group (1.82 ± 0.15 [$1.71, 1.93$]) compared with the saline group (2.34 ± 0.25 [$2.16, 2.52$]) ($P < 0.001$). There was no significant difference between the rhEPO1000 group and the saline group ($P = 0.203$)

sustained increase in the expression of IL-10 suggested a compensatory response to the increases in the potential pro-inflammatory cytokines. Okamoto *et al.* have shown that expression of IL-10 increases gradually in a rat chronic constriction injury (CCI) model.⁵ Wagner *et al.* have demonstrated that the administration of IL-10 can attenuate the CCI induced hyperalgesia.⁶

rhEPO has neuroprotective effects in experimental models of central nervous system injury.^{9–11} The possible mechanisms include anti-inflammation effects,^{9–11} alterations in NF- κ B activity,²¹ and activation of glial cells⁹ and antiapoptotic genes.^{10,12} Few reports have suggested that systemically administered rhEPO can facilitate the recovery from behavioural changes during neuropathic pain associated with CCI, L5 spinal nerve crush, or nerve root injury.^{12,13} These findings seemed to support the antinociceptive role of rhEPO in cases of neuropathic pain.

In our previous study,¹⁴ we found that rhEPO can inhibit glial activation and production of TNF- α , IL-1 β , and IL-6 of spinal nerves, which were consistent with pain behaviour in

rats. Compared with the spinal cord, it seemed that peripheral nerve injury was too anatomically remote to send any direct signals to activate glial cells in the brain. But the sensitization and activation of ascending spinal dorsal neurons would release more excitatory transmitters in the contralateral supraspinal brain region, such as substance P and excitatory amino acids, that might directly activate the supraspinal glial cells or induce neuronal depolarization combined with ion changes that may be major stimuli for glial cell activation.²⁴ NF- κ B is activated during peripheral nerve injury.²² NF- κ B activation during nerve injury can upregulate some downstream antiapoptotic and neuroprotective genes.^{25,26} Digicaylioglu and Lipton²⁵ have reported that neuronal EPO-receptors activated the neuroprotective signalling pathway via Janus Kinase-2 (JAK2), and NF- κ B. Liu *et al.*²⁷ have observed a modest activation of NF- κ B in rhEPO group before ischemia-reperfusion in myocardium and contributed this to the protective effects of rhEPO pretreatment. Although we did not investigate whether this activation was the same in peripheral nerve injury, these findings support our hypothesis that NF- κ B may serve crucial and complex roles in EPO protection. The role of various signalling pathways, besides NF- κ B signalling in the injured peripheral nerve, warrants further investigation.

A recognized limitation of this study is the fact that the studied doses of erythropoietin were much higher than those used clinically. Accordingly, the potential therapeutic effects of rhEPO for treating patients with neuropathic pain will require well-designed dose-response clinical studies.

In conclusion, the systemic effects of preemptive administration of rhEPO could dose-dependently attenuate the mechanical and thermal hyperalgesia in L5 spinal nerve transection rats. The antinociceptive effect of rhEPO might be correlated with the increased cerebral expression of IL-10 and with the decreased cerebral expression of TNF- α , IL-1 β , and IL-6 through inhibition of NF- κ B.

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Conflicts of interest None declared.

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