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Intrathecal administration of clonidine attenuates spinal neuroimmune activation in a rat model of neuropathic pain with existing hyperalgesia

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ABSTRACT

Central neuroimmune activation contributes to the initiation and maintenance of neuropathic pain after nerve injury. The current study was aimed to examine the modulation of neuroimmune activation in the spinal cord by the α_2 adrenoceptor agonist, clonidine, in a rat model of neuropathic pain induced by partial sciatic nerve ligation (PSNL). Animals were randomly assigned into 6 groups: sham-operation with 20 μg clonidine or saline; and PSNL with clonidine (5, 10, and 20 μg) or saline. Fourteen days post-operation, various doses of clonidine or saline were injected intrathecally. The paw pressure threshold and paw withdrawal latencies were measured before and at 30, 60, 90, and 120 min after the injection of clonidine. Glial activation markers such as macrophage antigen complex-1 (mac-1) and glial fibrillary acidic protein (GFAP), interleukin (IL)-1β and IL-6, nuclear factor-kappa B (NF-κB) activation, and p-p38 mitogen-activated protein kinase (MAPK) activation in the lumbar spinal cord were determined as well. Administration of clonidine resulted in a dose-dependent attenuation in PSNL-induced mechanical and thermal hyperalgesia. Furthermore, clonidine could markedly inhibit neuroimmune activation characterized by glial activation, production of cytokines, NF-κB activation as well as p38 activation. The antihyperalgesic effect of intrathecal clonidine in rats receiving PSNL might partly attribute to the inhibition of neuroimmune activation associated with the maintenance of neuropathic pain.

1. Introduction

Neuropathic pain is a prevalent condition characterized by spontaneous pain, hyperalgesia and allodynia (Zimmermann, 2001). Currently, it is generally acknowledged that central neuroimmune activation is implicated in the generation and maintenance of hyperalgesia and allodynia following both peripheral and central insults (DeLeo and Yezierski, 2001).

Although the recruitment of neutrophils, macrophages and lymphocytes from blood might participate in the central neuroimmune response, the activation of glia, especially microglia and astrocytes, undoubtedly plays a pivotal role in subsequent production of cytokines and chemokines. There is mounting evidence that proinflammatory cytokines induce the release or expression of cyclooxygenase (COX)-2 and substance P (Inoue et al., 1999). Activation of glia is manifested by the elevated expression of surface markers, macrophage antigen complex-1 (mac-1) for microglia and glial fibrillary acidic protein (GFAP) for astrocytes. The expression of proinflammatory mediators and surface markers further enhance the central immune cascade. Together, neuroimmune activation and neuroinflammation after nerve injury may generate positive feedback loops between multiple inflammatory mediators and glial activation.

Mitogen-activated protein kinases (MAPKs) consist of three major members: extracellular signal regulated kinases (ERK), p38 and c-Jun N-terminal kinase (JNK) (Krishna and Narang, 2008). After nerve injury, MAPKs are differentially activated (phosphorylated) in the glial cells (in microglia mainly p38 and in astrocytes mainly ERK1/2), resulting in the synthesis of proinflammatory and pronociceptive mediators through both posttranslational and transcriptional mechanisms. Translocation of p-p38 MAPK to the nucleus leads to the activation of several transcription factors. Among them, NF-κB has been implicated in neuropathic pain by regulating the gene expression of inflammatory mediators (Ji et al., 2009).

Clonidine, an α_2-adrenoceptor agonist, has been shown to be an effective adjunct to clinical anesthesia and pain management in addition to an antihypertensive agent (Sierralta et al., 1996). There are findings which have highlighted the antinociceptive effects of intrathecal administration of clonidine in animal models of pain (Roh et al., 2008). In addition, perineural and systemic administration of clonidine showed anti-inflammatory effect related to immune modulation (Romero-Sandoval and Eisenach, 2006; Lavand'homme and Eisenach,

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2003). Therefore, we hypothesized that central administration of the drug might produce similar effect. Using a rat model of neuropathic pain following partial sciatic nerve ligation (PSNL), the present study was undertaken to investigate the role of clonidine administered intrathecally in pain behaviors, pro-inflammatory cytokines, glial activation as well as p38 and NF-κB activation.

2. Experimental procedures

2.1. Animals and surgeries

Male Sprague–Dawley rats weighing 200–240 g were housed on a 12-hour light–dark cycle with food and water available ad libitum. Rats were divided randomly into six groups (8 rats/group): sham-operation with 20 μg clonidine or saline; and PSNL with intrathecal injection of clonidine (5, 10, and 20 μg, respectively) or saline. All animal experiments were performed in accordance with "The Principles of Laboratory Animal Care" formulated by the National Society of Medical Research and "The guidelines of the Committee for Research and Ethical Issues of IASP" published in PAIN®, 16 (1983) 109–110. The experiments were approved by the local ethical committee.

Under deep anesthesia with 3% isoflurane in an O_2 carrier, a partial ligation of one sciatic nerve was performed. The left sciatic nerve was exposed at mid-thigh level and liberated from surrounding tissues. One third to one half of the nerve was then tightly ligated at the nerve trunk above its trifurcation of the peroneal, sural, and tibial branches. Muscle and skin layers were closed and animals were allowed to recover from anesthesia. With regard to the sham-operated animals, left sciatic nerve was exposed without ligation.

Fourteen days after the operation, rats received drug injection under anesthesia, awoke immediately thereafter and were freely moving within 1 min after injection. 5, 10, and 20 μg clonidine (Sigma-Aldrich, St Louis, MO, USA) dissolved in 10 μl saline was injected intrathecally in PSNL-induced neuropathic rats. In sham rats, a maximal dose of clonidine (20 μg) was also injected. Control animals were injected with 10 μl saline. The doses of clonidine for intrathecal administration were selected based on the previous studies (Roh et al., 2008). Intrathecal injections were made using the method described by Fairbanks (Fairbanks, 2003). Briefly, a 26-gauge needle connected to a 50 μl microinjection syringe was inserted into the skin and then through the L5–6 intervertebral space directly into the subarachnoid space. A flick of the rat's tail provided a reliable indicator that the needle had penetrated the dura and at this point 10 μl of the drug was subsequently injected into the subarachnoid space. The behavior testing was carried out before and at 30, 60, 90, and 120 min after drug injection. The investigators involved were blinded to the drug given to the animals throughout the experiments.

2.2. Behavioral testing: mechanical hyperalgesia and thermal hypersensitivity

Behavioral studies were conducted in a quiet temperature-controlled (23 ± 1 °C) room and all the behavior data were recorded at each time point investigated. The paw pressure threshold in response to normal innocuous mechanical stimuli and the paw withdrawal latencies in response to heat stimulus were respectively measured by using an Electro VonFrey anesthesiometer (Model 2390CE, IITC Life Science, Inc.) and radiant heat (Model 390, IITC Life Science, Inc.) as previously described (Liu et al., 2007).Briefly, after the rats were acclimated in the inverted ventilated Plexiglas cage, gentle incremental pressure (maximum 200 g) by a rigid von Frey hair was applied to the dorsal surface of the ipsilateral hind paw until the paw was withdrawn. In addition, light intensity was preset to obtain a baseline latency of approximately 10 s and the cutoff time was set at 20 s to avoid the tissue damage. The force (g) applied and *the time period taken* were recorded.

2.3. Tissue harvest

In a separate experiment, rats were immediately sacrificed 60 min after each drug injection ($n = 6$, respectively) that showed the maximal antihyperalgesic effect of clonidine. All rats were deeply anesthetized and perfused intracardially with 250 ml cold heparinized (1 ml/l) saline and the ipsilateral L4–5 spinal cord tissue rostral to the injury site was removed, dissected while on dry ice, collected in polypropylene tubes (RNAase, DNAase, and pyrogen free), and removed quickly to liquid nitrogen for subsequent assays.

2.4. Enzyme-linked immunosorbent assay (ELISA) for cytokine measurement

Spinal production of IL-1β and IL-6 was quantified by ELISA kits for rats according to the manufacturers' instructions (Biosource USA for IL-1β and IL-6).

2.5. Real time reverse-transcription polymerase chain reaction (RT-PCR) for glial activation markers

GFAP and mac-1 were markers for astrocytic and microglial activation, respectively. Total cellular RNA was isolated from spinal cord tissue by using Trizol Reagents (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. The detailed procedure of RT-PCR for GFAP and mac-1 mRNA expression was performed as previously described (Liu et al., 2008). The sense primer for GFAP was 5′-GCTGGAGCAAGACAAACATTC-3′, and the antisense primer was 5′-CCCTACCCACTCCTACATCGT-3′. The sense primer for mac-1 was 5′-TGGCTGCTACTACCGTCCCT-3′, and the antisense primer was 5′-CAGGTTGGAGCCGAATAGGT-3′. The sense primer for GAPDH was 5′-CCCCCAATGTATCCGTTGTG-3′, and the antisense primer was 5′-TAGCCCAGGATGCCCTTTAGT-3′.

2.6. Nuclear protein extract and electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (Feng et al., 2006). 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 ^{32}P-labelled oligonucleotide probe. At last, 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 h. The intensity of the NF-κB was assessed by the densitometry.

2.7. Western blotting analysis for p-p38

To examine changes in p-p38 expression following peripheral nerve injury, all collected tissue samples were homogenized in an SDS sample buffer that contained a mixture of proteinase and phosphatase inhibitors. Protein concentrations of tissue lysates were determined using the BCA Protein Assay Kit (KangChen, Shanghai, China). Protein samples (30 μg per sample) were dissolved, and denatured, then the equivalent amounts of proteins were separated by using SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were incubated overnight at 4 °C with anti-p-p38 antibody (1:1000) or anti-total p38 antibody (1:2000, anti-rabbit, in 5% BSA; Cell Signaling). The blots were incubated for 1 h at RT with HRP-conjugated secondary antibody (Amersham Biosciences; 1:5000), and washed three times in TBS for 30 min. The immune complexes were detected by an enhanced chemiluminescence substrate reaction using a KC™ assay kit (KangChen, Shanghai, China). The scanned images were imported into LabWorks software (UVP, Upland, CA, USA). Scanning densitometry was used for semiquantitative analysis of the data.

2.8. Statistical analysis

Values are expressed as means ± S.E.M for behavioral changes and means ± S.D. for other data. Comparisons between groups were performed using two-way ANOVA followed by Bonferroni or Tamhane's T2 test based on equal variances assumed or not. Data were analyzed using a commercially available statistics software package (SPSS 13.0, Chicago, IL, USA). $P<0.05$ was considered significant.

3. Results

3.1. Results of behavioral testing

In the PSNL surgery group, prominent mechanical and thermal hyperalgesic behaviors were established by 14 days after surgery (Fig. 1A, B). Intrathecal injection of clonidine dose-dependently suppressed paw pressure threshold and paw withdrawal latencies as compared to the values obtained from the saline-treated group (Fig. 1A and B; $*P<0.05$ and $**P<0.01$). After an injection of 20 μg clonidine, a remarkable antihyperalgesic effect was observed for up to 120 min on PSNL-induced mechanical allodynia (Fig. 1A) and a significant antihyperalgesic effect was observed on PSNL-induced thermal hyperalgesia from 30 min to 90 min after injection (Fig. 1B). An intermediate dose of clonidine (10 μg) also produced an antihyperalgesic effect, but over a shorter timeframe (30–60 min). In addition, the minimum dose of clonidine (5 μg) inhibited the behavior changes only at 30 min.

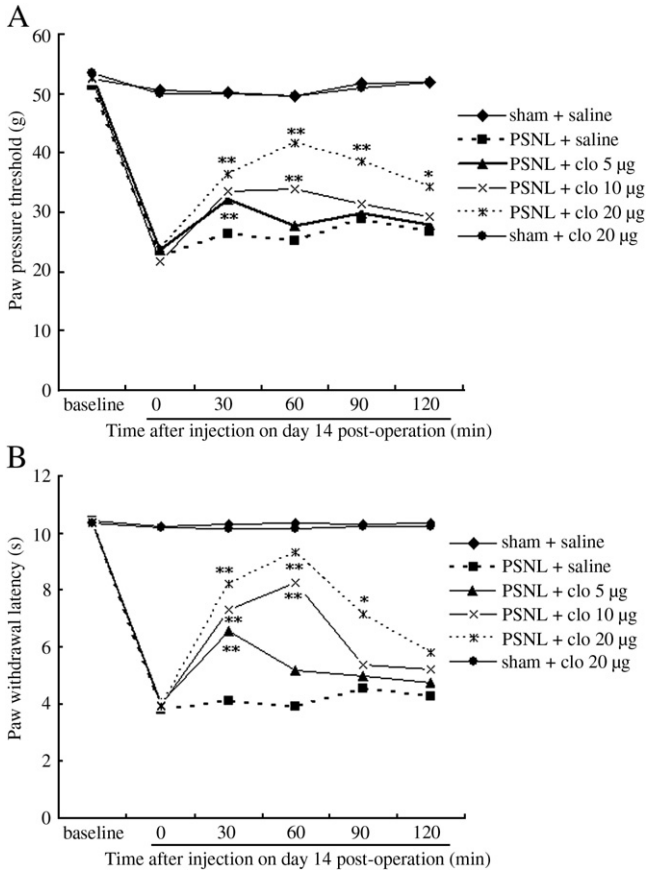

Fig. 1. Changes in rat behavioral hyperalgesia, as evidenced by (A) paw pressure threshold and (B) paw withdrawal latencies, before and at 30, 60, 90, and 120 min after the drug administration. Prominent mechanical and thermal hyperalgesia were established by 14 days after PSNL. 20 μg clonidine exhibited remarkable antiallodynic effect for up to 120 min on PSNL-induced mechanical allodynia (A) and significant antihyperalgesic effect from 30 min to 90 min after injection (B). Clonidine (10 μg) also produced an antihyperalgesic effect, but over a shorter timeframe (30–60 min). Clonidine (5 μg) inhibited the behavior changes only at 30 min. ($n=8$ per group) $*P<0.05$ and $**P<0.01$ vs. saline-treated operation group.

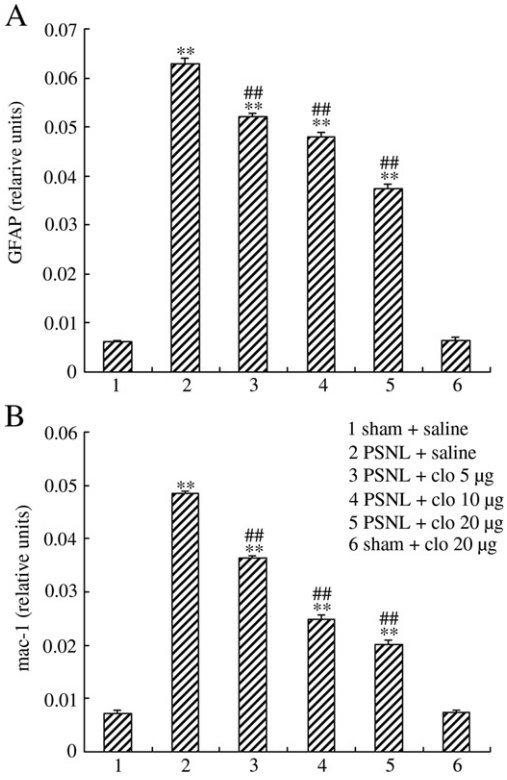

Fig. 2. Expression of glial activation markers, glial fibrillary acidic protein (GFAP) and macrophage antigen complex-1 (mac-1) in the lumbar spinal cord of rats on day 14 after the operation. PSNL increased GFAP and mac-1, while clonidine significantly down-regulated the markers mentioned above. ($n=8$ per group, data are mean ± S.D.) $**P<0.01$ vs. sham-operated group; $##P<0.01$ vs. saline-treated operation group.

3.2. Effects of clonidine on glial activation markers

Clonidine attenuation of behavioral hyperalgesia after nerve injury might be associated with its inhibitory effect on microglia and astrocytes. To test this hypothesis, we studied the expression of glial activation markers in lumbar spinal cord. GFAP (Fig. 2A) and mac-1 (Fig. 2B) expression were markedly increased in nerve-injured rats on day 14 compared with the sham-operated rats ($P<0.01$). Administration of various doses of clonidine could significantly decrease spinal glial activation markers in rats challenged with PSNL compared with rats administered with saline after PSNL dose-dependently ($P<0.01$). Clonidine, especially in the dose of 20 μg, seems to exert higher inhibitive effects on microglia than astrocytes. No significant differences were noted between the saline and clonidine with sham-operation groups.

3.3. Effects of clonidine on inflammatory cytokines

The inhibitory effect of clonidine on glia could result in subsequent decreased production of proinflammatory cytokines. IL-1β (Fig. 3A) and IL-6 (Fig. 3B) from spinal cord were almost the same between sham-operated groups. PSNL induced neuropathic pain was associated with marked increases in the protein levels of IL-1β and IL-6 ($P<0.01$). Clonidine could significantly suppress the elevations of IL-1β and IL-6 dose-dependently ($P<0.05$ for the dose of 5 μg, $P<0.01$ for other doses).

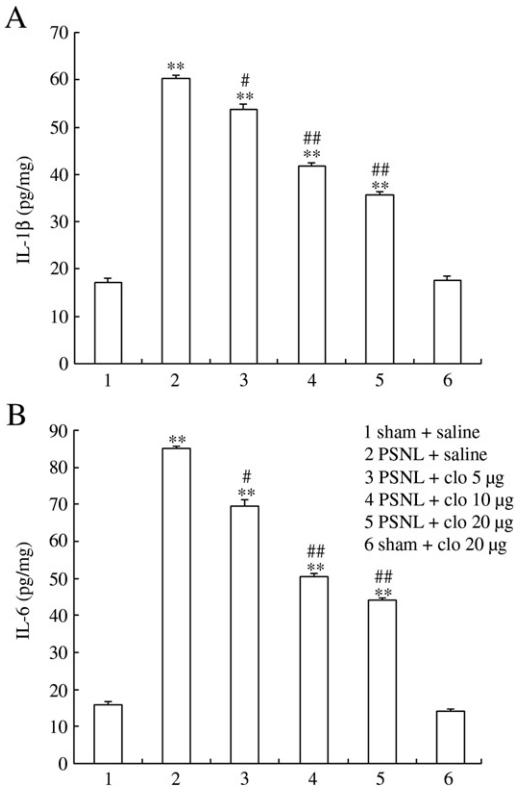

Fig. 3. Production of cytokines in the rat spinal cord on day 14 after the operation. PSNL elevated the expression of interleukin (IL)-1β and IL-6, while clonidine markedly decreased these markers induced by PSNL. ($n=8$ per group, data are mean ± S.D.) $^{**}P<0.01$ vs. sham-operated group; $^{\#\#}P<0.01$ and $^{\#}P<0.05$ vs. saline-treated operation group.

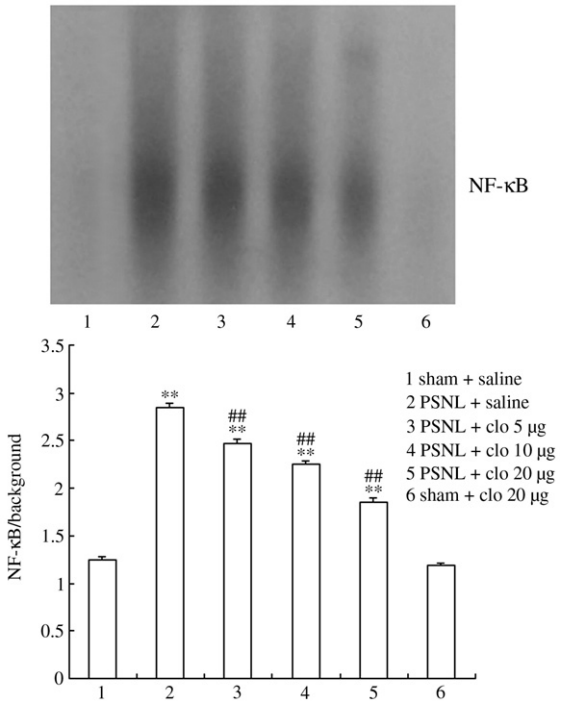

Fig. 4. Nuclear factor-kappa B activation in the spinal cord of rats. The top section shows representative EMSA picture and the bottom section shows quantitative data determined by the mean band intensity of EMSA. PSNL increased NF-κB activation, while clonidine significantly inhibited this response in a dose-dependent way. ($n=8$ per group, data are mean ± S.D.) $^{**}P<0.01$ vs. sham-operated group; $^{\#\#}P<0.01$ vs. saline-treated operation group.

3.4. Effects of clonidine on spinal NF-κB

When compared with the sham-operated group, activation of spinal NF-κB after PSNL was enhanced prominently ($P<0.01$). After injection of clonidine, NF-κB activation decreased markedly in a dose dependent manner ($P<0.01$), and the maximal inhibitory effect was observed at 20 μg (Fig. 4). NF-κB activation remained at baseline level in sham-operated rats.

3.5. Effects of clonidine on p-p38 protein expression

Western blot analysis of rats insulted with PSNL illustrated an increase in p-p38 activation ($P<0.01$). After treated with clonidine, p-p38 protein was reduced markedly ($P<0.01$), especially in 10 μg clonidine group. Protein level of p-p38 was similar between sham-operated groups administered with clonidine or saline (Fig. 5).

4. Discussion

In the present study, we explored the effect of clonidine on the existing mechanical and thermal hypersensitivity using a rat model of neuropathic pain induced by PSNL. Our data showed that a single intrathecal administration of clonidine could dose-dependently attenuate the existing behavioral hyperalgesia. As a previous study has suggested that perineural clonidine injection after PSNL injury could delay the development of hypersensitivity (Romero-Sandoval and Eisenach, 2006), our results might further indicate the therapeutic effects of clonidine on neuropathic pain.

Astrocytes and microglia are key neuromodulatory, neurotrophic and neuroimmune elements in the CNS. Activated spinal glia have been strongly implicated in the development and maintenance of persistent pain states following various kinds of stimuli including traumatic nerve injury (DeLeo and Yezierski, 2001). In addition, it is noteworthy that drugs that suppress glial activation can prevent and reverse neuropathic pain (Watkins and Maier, 2003). It has been demonstrated previously that GFAP and mac-1 mRNA were significantly increased during neuropathic pain induced by complete transection or loose ligature of L5 dorsal roots (Winkelstein et al., 2001). Despite the discrepancy in the animal models, we also observed the increase of GFAP and mac-1 mRNA in the current study. After injection of clonidine, the increase in GFAP and mac-1 were suppressed. Furthermore, effect of clonidine on behavioral pain

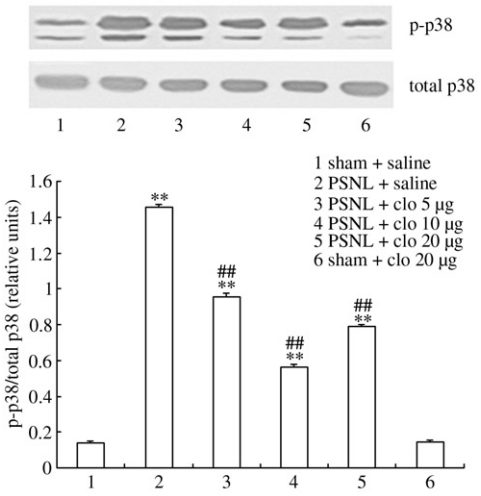

Fig. 5. Activation of p-p38 in the rat spinal cord. The top section shows representative western blot picture and the bottom section shows quantitative data of p-p38 normalized by GADPH. PSNL increased p-p38 activation, while clonidine significantly inhibited this response in a dose-related manner. ($n=8$ per group, data are mean ± S.D.) $^{**}P<0.01$ vs. sham-operated group; $^{\#\#}P<0.01$ vs. saline-treated operation group.

responses appeared to parallel its impact on glial activation, implying that the attenuation of neuropathic pain behavior by clonidine may partly result from its role on glial activation.

Products *mainly* released by activated glia (including cytokines) contribute directly to the pathophysiology of neuropathic pain. The significance of spinal IL-1β and IL-6 in the development and maintenance of peripheral nerve injury-induced allodynia has been demonstrated (Arruda et al., 2000). A previous study showed that IL-1β and IL-6 were increased in ipsilateral tissues 2 weeks after PSNL (Romero-Sandoval et al., 2005), which was exactly in agreement with our current results. Moreover, our data demonstrated that clonidine could inhibit production of cytokines, which contributed to its role on neuroimmune activation associated with neuropathic pain. However, a previous in vitro study found that clonidine did not affect the release of proinflammatory mediators from endotoxin stimulated microglia (Mori et al., 2002), which seemed to be disagreement with our results. This disparity might be attributed to the difference between multiple possible stimulators (chemokines, ATP, heat shock protein, et al.) for microglia in vivo and simple single activator (endotoxin) for cultured microglia. Moreover, in addition to microglia, astrocytes are well known to express adrenergic receptors (ARs), including β_1, α_1, and α_2 (Mantyh et al., 1995; Milner et al., 1998). Therefore, even if clonidine did not directly affect the release of proinflammatory mediators from activated microglia, we cannot rule out the possibilities that clonidine could act on astrocytes and neurons which have complicated feedback with microglia in vivo.

As induction of certain inflammatory genes including the cytokines IL-1β and IL-6 is directly regulated by NF-κB, we focused on NF-κB activation to dissect out the underlying mechanism. Activation of NF-κB has been detected in animal models of neuropathic pain by our previous studies and others (Liu et al., 2007; Xie et al., 2006). Additionally, hyperalgesia in neuropathic pain can be reduced by inhibition of IκB kinase (Tegeder et al., 2004). Our data highlighted that the elevation of NF-κB activation during neuropathic pain induced by PSNL could be suppressed by clonidine, which *might underlie* the mechanism of action of clonidine on pain responses and the associated neuroimmune activation as revealed by glial activation and production of cytokines. Conversely, a previous study has reported that clonidine may enhance NF-κB activation in endotoxin-stimulated C6 glioma cells (Su et al., 2008). However, Nemeth et al. reported that clonidine significantly inhibited rather than enhanced NF-κB activation in LPS-stimulated human umbilical endothelial vein cells (Németh et al., 2002). The precise mechanisms responsible for the effects of clonidine on NF-κB activation remain to be elucidated. Nevertheless, our data did indicate that astrocytes and microglia may react differently from macrophages during neuropathic pain in contrast to exposing to LPS.

One of the most important findings was that clonidine could suppress p38 phosphorylation in the lumbar spinal cord of rats receiving PSNL. In the present study, only p38 was chosen for the following reasons: First, all MAPKs can induce gene transcription by activating transcription factors such as NF-κB. Second, p-p38 is constitutively present in spinal glia. Activation of MAPKs in microglia and astroglia results in production of multiple inflammatory mediators, including the proinflammatory cytokines IL-1β and IL-6 (DeLeo et al., 2004). A previous study has shown that PSNL increased p38-MAPK activation in small and medium sensory afferents (Liu and Eisenach, 2006). Furthermore, perineural clonidine has been reported to reduce activation of p38 MARK in sensory neurons after nerve injury. These results strongly supported our findings. The suppression of p38 activation by clonidine further added to its role on neuropathic pain and the associated neuroimmune activation.

The results presented herein added relatively little to our understanding regarding the invlovement of α_2-adrenoceptors in the mechanism of action of clonidine on glial activation. It has been reported that α_2-adrenergic agonists could produce antinociception (Khasar et al., 1996), and both μ-opioid and A_1-adenosine agonists have been shown to produce a potent antinociception (Aley et al., 1995). In addition, it is reported that clonidine antinociception was blocked not only by yohimbine (α_2-antagonist) but also by PACPX (A_1-antagonist) and by naloxone (μ-antagonist) (Aley and Levine, 1997). Future studies are warranted to clarify the underlying mechanism of clonidine on glial activation by using several kinds of antagonists, such as yohimbine, naloxone, and so on.

Central activation of glial cells often results in contralateral hypersensitivity in animals (Milligan et al., 2003; Twining et al., 2004) and occasionally in humans (Oaklander et al., 1998). Some researchers have observed changes in cytokine mRNA expression in neural structures contralateral to peripheral nerve transection. In addition, Romero-Sandoval et al. also found that PSNL was accompanied by increased proinflammatory cytokines and hypersensitivity both ipsilaterally and contralaterally (Romero-Sandoval and Eisenach, 2006). In the current study, we did focus on ipsilateral side, more future studies are needed to observe the effects of clonidine administration on the response of the contralateral paw.

5. Conclusions

Our present study demonstrated that clonidine may be antiallodynic in a rat model of neuropathic pain by inhibiting glial activation and activation of NF-κB and p38, thus, directly inhibiting production of proinflammatory cytokines. The effects of clonidine on neuroimmune activation may contribute to its clinical application on neuropathic pain.

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