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Suppression of interleukin-6 by minocycline in a rat model of neuropathic pain

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Abstract

Inflammatory mediators produced in the injured nerve have been proposed as contributing factors in the development of neuropathic pain. In this regard an important role is assigned to interleukin-6. The present study, evaluated the effect of pretreatment with minocycline, on pain behavior (hyperalgesia and allodynia) and serum level of interleukin-6 in chronic constriction injury (CCI) model of neuropathic pain in rat. Minocycline (5, 10, 20 and 40 mg/kg, i.p.) was injected 1 h before surgery and continued daily to day 14 post-ligation. Behavioral tests were recorded before surgery and on postoperative days 1, 3, 5, 7, 9, 10, 14, and the serum concentration of interleukin-6 was determined at day 14. We observed that minocycline which was reported to have a neuroprotective effect in some neurodegenerative diseases, reversed hyperalgesia and allodynia due to sciatic nerve ligation and inhibited the interleukin-6 production. It seems that minocycline could have an anti-inflammatory and analgesic effect in some chronic pain states.

Keywords: Minocycline; Neuropathic pain; Hyperalgesia; Allodynia; Interleukin-6

1. Introduction

Interest in neuroinflammation and neuroimmune activation has grown rapidly in recent years with the recognition of the role of central nervous system (CNS) inflammation and immune responses in the etiology of neurological disorders such as brain and spinal cord injury which are often associated with persistent pain states (Deleo and Yezierski, 2001). Neuropathic pain is defined as pain initiated or caused by a primary lesion or dysfunction in the nervous system. It may occur following a lesion at nearly any level of the neuraxis that contains parts of the nociceptive system. Nerve injury often result in the development of hyperalgesia characterized by spontaneous pain, increased responsiveness to painful stimuli and allodynia which is pain perceived in response to normally non-noxious stimuli (Cherney et al., 1994; Zimmermann, 2001). Chronic neuropathic pain is a physically and emotionally debilitating condition for which there is no adequate treatment to prevent the development, nor to adequately, predictably and specifically control established neuropathic pain (Koltzenburg, 1998). Pain facilitation (hyperalgesia) has been the focus of intense study over the past decade. Insults to the central and peripheral nervous system can range from traumatic injury, to chemical insult and to immunologic challenge (Sweitzer et al., 1999; Watkins et al., 1995). When tissue is destroyed, pain arises, tissue destruction as well as wound healing are associated with an inflammatory reaction. This leads to activation of nociceptors (pain receptors) which can cross-communicate with the inflammatory infiltrate (Bartfai, 2001; Rittner et al., 2002). The majority of recent pain research related to the arena of neuroimmune function has postulated on the involvement of cytokines and growth factors in the generation and/or maintenance of chronic pain (Clatworthy et al., 1995; Laughlin et al., 2000; Woolf et al., 1997). Immune cells and a variety of additional immune mediators should not be ignored in the context of the dynamic interaction that occurs following

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injury to the nervous system (Deleo and Yezierski, 2001). Evidence for a role of the immune system in hyperalgesic pain states is increasing (Watkins et al., 1995). It is now estimated that half of all clinical cases of neuropathic pain are associated with infection or inflammation of peripheral nerves rather than with nerve trauma (Watkins and Maier, 2002). Both inflammatory pain and neuropathic pain of peripheral origin often present with local cutaneous hypersensitivity in the form of hyperalgesia and allodynia (Levine et al., 1988; Watkins et al., 1995). Among cytokines involved in pathological states, an important role is assigned to interleukin-6, an inflammatory cytokine, in the physiology of nociception and the pathophysiology of pain (Dejongh et al., 2003; Lacroix et al., 2002). To date, research using models in rat has mainly focused on injury to a peripheral nerve, usually the sciatic or spinal nerve, to reliably produce behaviors suggestive of neuropathic pain in humans (Bennett and Xie, 1988; Kim and Chung, 1992; Seltzer et al., 1990; Wall et al., 1979). It has been reported that minocycline, a semisynthetic tetracycline derivate has protective effect against many neurodegenerative diseases (He et al., 2001; Tikka and Koistinaho, 2001; Tikka et al., 2001; Wu et al., 2002; Yrjanheikki et al., 1998). In addition, minocycline have produced analgesic effect in patients with rheumatoid arthritis disease (Kloppenburg et al., 1996). It seems that, inflammatory mediators play an important role in the development of hypersensitivity following nerve injuries.

The aim of the present study was to determine the inflammatory response associated with sciatic nerve injury under the influence of minocycline. Here, we have studied the effect of pretreatment of minocycline on pain behavior and also evaluated the serum level of interleukin-6 in chronic constriction injury (CCI) model of neuropathic pain simulating the clinical condition of chronic nerve compression such as the one that occurs in nerve entrapment neuropathy or spinal root irritation by a lumbar disk herniation (Zimmermann, 2001).

2. Materials and methods

2.1. Animals

Experiments were carried out on male Wistar rats, (weight 150–200 g) that were housed one rat per cage and placed under a 12 h light/dark cycle in a room with controlled temperature (22 ± 1 °C). Animals had free access to food and water. Rats were divided randomly into several experimental groups, each group compromising 6 animals. All experiments followed the IASP guidelines on ethical standard for investigation of experimental pain in animals (Zimmermann, 1983). Animals were allowed to habituate to the housing facilities for one week before the experiments began. Behavioral studies were performed in a quiet room between the hours 9:00 and 11:00 AM. Efforts were made to limit distress and use the minimum number of animals necessary to achieve statistical significance.

2.2. Surgery

We used chronic constriction injury (CCI) model of neuropathic pain (Bennett and Xie, 1988). The surgical procedure was performed under ketamine anesthesia (60 mg/kg) and xylizine (10 mg/kg). The left sciatic nerve was exposed and 4 loose chromic gut ligatures were placed around the nerve proximal to the trifurcation. The distance between two adjacent ligatures was 1 mm. The wound was irrigated with normal saline and closed in two layer with 4-0 silk (fascial plane) and surgical skin staples. In sham-operated group, rats undergo surgical procedure except for the ligation. All surgical procedures were carried out under normal sterile conditions and were performed by the same person.

2.3. Drug preparation

Minocycline hydrochloride (Sigma, U.S.A) was dissolved in 0.9% saline. Ketamine hydrochloride (Sigma, U.S.A) and Xylizine hydrochloride (Sigma, U.S.A) were used for anesthesia. All drugs were injected by the i.p. route.

2.4. Drug administration

Animals were divided randomly into three experimental groups: 1 — CCI, 2 — Sham-operated and 3 — CCI drug-treated. Normal saline was injected i.p. to CCI and sham-operated animals. Minocycline 5, 10, 20 and 40 mg/kg were injected 1 h before surgery and continued daily (15 h before experiments) to day 14 post-ligation. The selection of minocycline doses (10, 20 and 40 mg/kg) and the rationale for the dosing regime is within the range at which it was reported to be neuroprotectant in rodents (Wu et al., 2002; Yrjanheikki et al., 1999). All behavioral tests were recorded on day 0 (control day) before surgery and on days 1, 3, 5, 7, 10 and 14 post-nerve injury. On day 14, rats were euthanized by CO_2 asphyxiation and then were rapidly guillotined and the blood was collected for serum evaluation of interleukin-6 (Raghavendra et al., 2002).

2.5. Behavioral tests and experimental design

The sciatic nerve territory (mid-plantar hind paw) was tested for sensitivity to noxious and innocuous stimuli at several intervals following surgery up to 14 days using standard behavioral assays done sequentially. Animals were acclimated to the testing chambers for 30 min prior to testing. Hyperalgesia (decreased threshold to noxious stimuli) and allodynia (heightened response to normally non-noxious stimuli), were evaluated in animals. The order of behavioral tests was therefore defined as follows: thermal hyperalgesia, mechanical, chemical and cold allodynia. Animals were left for 30 min undisturbed between each assay to habituate to the testing environment.

2.6. Thermal hyperalgesia

We used the paw immersion test (hot bath) to assess the sensitivity to heat stimulus which consist the immersion of the paw in a water bath of 42 °C (Seltzer et al., 1990). We recorded the latency of withdrawal for each paw. Three measurement with an interval of 5 min were made per hind paw, and the mean was calculated. The paw withdrawal latency was obtained by substracting the latency of the controlateral unaffected hind

paw from the ipsilateral experimental paw. A cutoff time of 15 s was considered to avoid tissue damage. The negative difference scores are indicative of thermal hyperalgesia (Attal et al., 1990; Bennett and Xie, 1988; Boivie et al., 1994; Seltzer et al., 1990).

2.7. Mechanical allodynia

Mechanical sensitivity to non-noxious stimuli was measured by applying a set of calibrated nylon monofilaments (Stoelting, USA). The von Frey methodology was used to assess the sensitivity of the skin to tactile stimulation. Von Frey filaments are calibrated to have a characteristic bending force when pressure is applied. Each rat was placed under a transparent plexiglass cage on an elevated metal screen surface with 1 cm mesh openings. Increasing strengths of von Frey filaments were applied sequentially to the plantar surface of the left hind paw of each animal. The minimum paw withdrawal threshold, defined as the minimum gram strength eliciting two sequential responses at 3 min intervals between them (withdrawal from pressure) was recorded for the left paw. The intensity of mechanical stimulation was increased from 2 to 60 g in a graded manner using successively greater diameter filaments until the hind paw was withdrawn, a paw withdrawal threshold decrease indicates that allodynia has developed. For successive tests, the placement of these stimuli was varied slightly from one trial to the next to avoid sensitization of the hind paw (Stuesse et al., 2001).

2.8. Chemical allodynia

Acetone test: A slightly modified method of Choi et al. (1994) was used for the determination of the reactivity to a chemical stimulus. Rats were placed under a transparent plexiglass cage as described previously and an acetone bubble was formed at the end of a piece of small polyethylene tubing that was connected to a syringe, then the bubble was slightly touched to the heel. The acetone was applied 5 times with an interval of 1 min and the number of paw lifting from surface was considered as response. The response was calculated as the percent of paw withdrawal frequency using the following equation: (Number of paw withdrawals / 5 trials) × 100 (Stuesse et al., 2001).

2.9. Cold allodynia

The paw immersion test (cold bath) was used to test cold allodynia. Similar to the heat bath method, the paw was immersed in a 10 °C water bath and the latency of withdrawal was recorded (Attal et al., 1990). Cold allodynia, defined as those rats with a negative paw withdrawal latency difference (injured side minus uninjured side). A negative paw withdrawal latency indicates that the paw ipsilateral to the CCI is more sensitive than the paw controlateral to the CCI.

2.10. Interleukin-6 protein analysis by ELISA

Cytokine level in serum of rats were measured by commercial available ELISA specific for interleukin-6 (Biosource International, England) with a lower detection limit of < 8 pg/ml. Blood sample of different groups was centrifugated at 2500 rpm/20 min and the serum was collected and frozen at −70 °C. The analysis of cytokine protein expression was made according to the manufacturer's instruction. Rat interleukin-6 kit is a solid phase Sandwich Enzyme-Linked-Immuno-Sorbent-Assay (ELISA). An antibody specific for rat interleukin-6 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known rat interleukin-6 content, control specimens and unknowns, are pipetted into these wells. During the first incubation, the rat interleukin-6 antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated antibody specific for rat interleukin-6 is added. During the second incubation, this antibody binds to the immobilized rat interleukin-6 captured during the first incubation. After removal of excess second antibody, Streptavidin–Peroxidase (enzyme) is added. This bind to the biotynilated antibody to complete the four member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of rat interleukin-6 present in the original specimen, then the plates are read by a microplate reader at 450 nm. The cytokine protein concentration was obtained from a standard curve.

2.11. Statistical analysis

Data were analysed for significance using an analysis of variance (ANOVA) followed by a post hoc Tukey's test. In all cases $P<0.05$ was considered significant.

3. Results

All animals experienced normal weight gain over the course of the study. Different stimuli were tested over a 14 day time frame, and included the measurement of thermal, mechanical, chemical and cold stimuli.

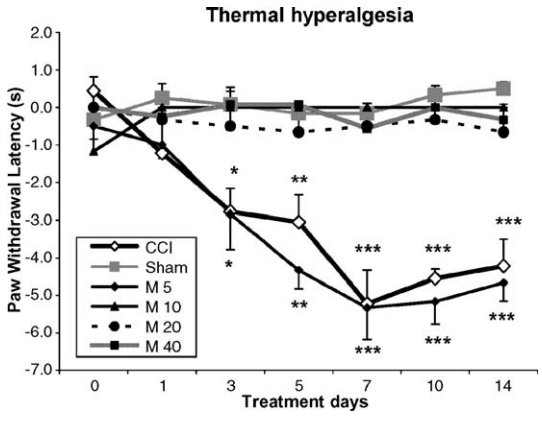

Fig. 1. The latency of paw withdrawal (in seconds) in response to 42 °C hot water bath before and at several time points after surgery in CCI saline-treated, sham-operated and CCI minocycline treated-groups. Minocycline (5, 10, 20 and 40 mg/kg) was injected i.p. Data are presented as means ± S.E.M. of 6 rats in each group. Asterisks (*$P<0.05$; **$P<0.01$; ***$P<0.001$) indicate a statistically significant difference when compared to day 0 paw withdrawal latency value.

3.1. Response to thermal hyperalgesia

In the paw immersion test (hot bath), while sham-operated rats did not exhibit variation in pain behavior during the 14 days of the study compared to pre-ligation day, all CCI saline-treated animals have shown confirmed heat hyperalgesia ($P<0.05$) at the third day, following "loose ligation" of the sciatic nerve, compared to pre-surgery control day, this was sustained throughout the experimental period. The animals treated with minocycline 10, 20 and 40 mg/kg did not develop hyperalgesia during the period of the study when compared to the control day. However, thermal hyperalgesia was seen on day 3 post-surgery in rats treated with minocycline 5 mg/kg ($P<0.05$) compared to day 0 which persisted until the end of the study (Fig. 1).

3.2. Response to mechanical allodynia

In the von Frey test, all CCI saline-treated animals were strongly allodynic at the third day (post-ligation) ($P<0.001$) compared to control day, this effect was sustained until the end of the study. Contrary, sham-operated animals did not produce mechanical allodynia throughout the experimental period as compared to pre-surgery day. Moreover, during the period of the study, tactile sensitivity was not produced in rats treated with minocycline 10, 20 and 40 mg/kg when compared to the control day. However, animals treated with minocycline 5 mg/kg exhibited pain behavior ($P<0.001$) at 3 days post-ligation compared to day 0 which persisted during the study period (Fig. 2).

3.3. Response to chemical allodynia

3.3.1. Acetone test

In CCI saline-treated rats, a significant difference in pain behavior ($P<0.001$) was seen at the third day post-injury compared to day 0. The previously mentioned effect continued until the end of the study. However, chemical allodynia was not observed in sham-operated group during the experimental period. Furthermore, following the nerve ligation, animals treated with minocycline 10, 20 and 40 mg/kg, did not develop pain behavior when compared to day 0, while rats treated with minocycline 5 mg/kg showed pain behavior ($P<0.05$) at third day post-surgery compared to the control day. This effect was sustained throughout the study (Fig. 3).

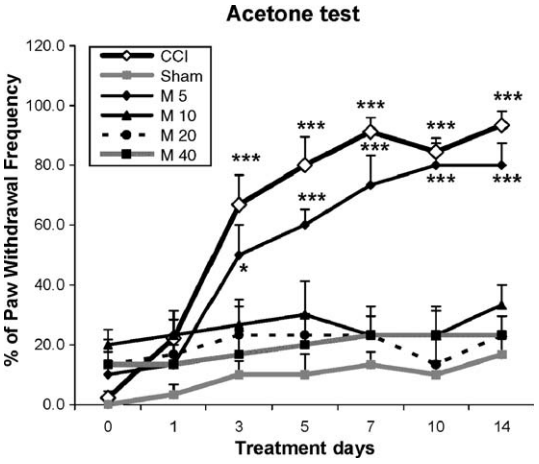

Fig. 3. The frequency of paw withdrawal in response to acetone before and at several time points after surgery in CCI saline-treated, sham-operated and CCI minocycline treated-groups. Minocycline (5, 10, 20 and 40 mg/kg) was injected i.p. Data are presented as means±S.E.M. of 6 rats in each group. Asterisks (*$P<0.05$; ***$P<0.001$) indicate a statistically significant difference when compared to day 0 paw withdrawal frequency value.

3.4. Response to cold allodynia

In the paw immersion test, cold allodynia was developed at 3 days post-ligation in CCI saline-treated group ($P<0.01$) compared to control day which persisted during the period of the study. However, sham-operated animals did not exhibit pain behavior

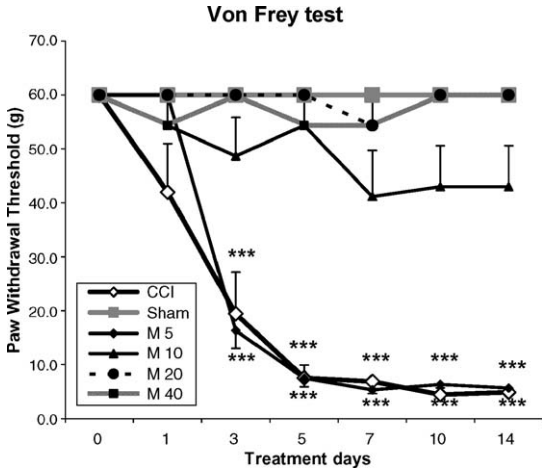

Fig. 2. Paw withdrawal threshold in response to von Frey filaments before and at several time points after surgery in CCI saline-treated, sham-operated and CCI minocycline-treated groups. Minocycline (5, 10, 20 and 40 mg/kg) was injected i.p. Data are presented as means±S.E.M. of 6 rats in each group. Asterisks (***$P<0.001$) indicate a statistically significant difference when compared to day 0 paw withdrawal threshold value.

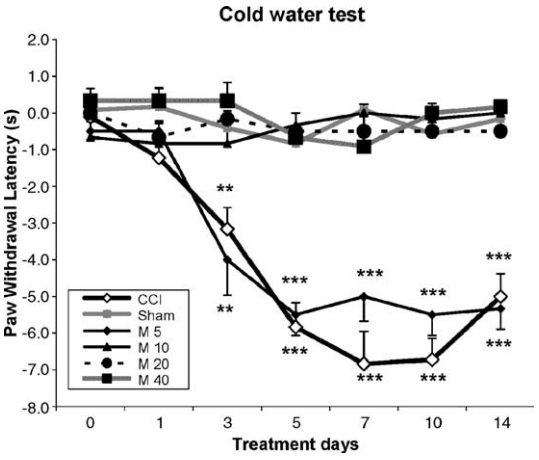

Fig. 4. The latency of paw withdrawal (in seconds) in response to 10 °C cold water bath before and at several time points after surgery in CCI saline-treated, sham-operated and CCI minocycline treated-groups. Minocycline (5, 10, 20 and 40 mg/kg) was injected i.p. Data are presented as means±S.E.M. of 6 rats in each group. Asterisks (**$P<0.01$; ***$P<0.001$) indicate a statistically significant difference when compared to day 0 paw withdrawal latency value.

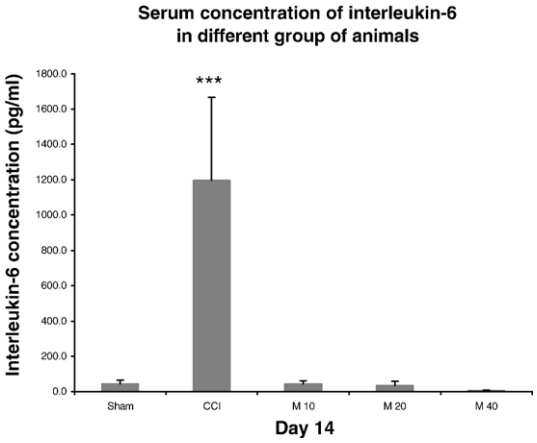

Fig. 5. Serum concentration of interleukin-6 in CCI saline-treated, sham-operated and CCI minocycline-treated rats on day 14 post-ligation. Data are presented as means±S.E.M. of 6 rats in each group. Asterisks (***$P<0.001$) indicate a statistically significant difference when compared to CCI saline-treated rats. M10=minocycline 10 mg/kg, M20=minocycline 20 mg/kg, M40=minocycline 40 mg/kg.

throughout the study. Moreover, during the experimental period, cold allodynia was not seen in animals treated with minocycline 10, 20 and 40 mg/kg compared to control day. However, rats treated with minocycline 5 mg/kg showed pain behavior ($P<0.01$) at day 3 following sciatic nerve ligation compared to day 0 which persisted during the experimental period (Fig. 4).

3.5. Cytokine protein analysis

To understand the involvement of the immune system under the influence of minocycline following sciatic nerve injury, we evaluated the serum concentration of pro-inflammatory cytokine interleukin-6 in different group of animals. Protein analysis (by ELISA) revealed an increase in the serum level of interleukin-6 in CCI saline-treated animals ($P<0.001$) compared to sham-operated and animals pretreated with minocycline 10, 20 and 40 mg/kg (Fig. 5). However, in animals treated with minocycline 5 mg/kg there was no significant difference in serum level of interleukin-6 compared to CCI saline-treated rats (data not shown).

4. Discussion

In this study we evaluated the anti-inflammatory and analgesic effects of minocycline in neuropathic pain in rat. We have chosen the CCI model of nerve injury because it has both an inflammatory and a nerve injury component, and it is reported to mimic types of neuropathic pain found in humans (Bennett and Xie, 1988). Minocycline is a semisynthetic derivate of tetracycline antibiotic drug. It has a superior penetration to the CNS via the brain–blood barrier (Aronson, 1980). It has been shown that minocycline have reduced pain in patients with rheumatoid arthritis disease (Kloppenburg et al., 1996). Recent data have pointed to the anti-inflammatory effect of minocycline that are completely separate and distinct from its antimicrobial action (He et al., 2001; Kloppenburg et al., 1995). Nowadays enormous interests are focused on the role of neuroinflammation and neuroimmune activation in the development of acute and chronic pain (Clatworthy et al., 1995; Deleo and Yezierski, 2001; Laughlin et al., 2000; Woolf et al., 1997). Furthermore, it has been postulated that cytokines induce hyperexcitable sensory states that induce the development of hyperalgesia (Watkins et al., 1995). It is of critical importance to understand the contribution of inflammation and immune responses to the evaluation of chronic pain states. Recent reports have described that systemic minocycline (a specific microglial inhibitor) blocks the development of neuropathic pain states in rats with L5 nerve transection model, but does not reduce pain that is already established (Raghavendra et al., 2003).

In addition, it was shown that intrathecal administration of minocycline produced a potent and consistent antinociception in models of tissue injury and inflammation-evoked pain (Hua et al., 2005). In our experiments we have determined the effect of systemic minocycline on nerve injury induced neuropathic pain with the inflammatory component. We observed that minocycline prevented hyperalgesia and allodynia due to sciatic nerve ligation. Our data are consistent with the above mentioned research since minocycline has prevented hyperalgesia and allodynia in chronic constriction injury of the sciatic nerve. This effect is also consistent with the fact that minocycline provides neuroprotection against some neurodegenerative diseases like brain ischemia, Huntington and Parkinson diseases (Tikka and Koistinaho, 2001; Wu et al., 2002; Yrjanheikki et al., 1998, 1999). Recently, it has been reported that minocycline was not effective in reducing established pain in L5 nerve transection (Raghavendra et al., 2003) and in chronic constriction injury models of neuropathic pain (Ledboer et al., 2005). We have similarly found that minocycline had no analgesic effect on existing hyperalgesia and allodynia (data not shown). There are several lines of evidence implicating spinal cord microglia and astrocytes in creating exaggerated pain states. Microglia, the intrinsic immune effector cells of the CNS are involved in the induction and maintenance of chronic pain following injury (Colburn et al., 1997, 1999; Coyle, 1998; Watkins et al., 2001a,b; Watkins and Maier, 2003). Therefore, microglia might be responsible for the initiation of neuropathic pain states and astrocytes may be involved in their maintenance (Kreutzberg, 1996; Marchand et al., 2005). In a rat model of sciatic nerve inflammation, it was speculated that microglial activation initiates and maintains the astroglial reaction, explaining why prevention of microglial activation (and thereby prevention of astrocytic reaction) is able to block allodynia for a prolonged period (Ledboer et al., 2005). As stated before, tissue destruction is associated with an inflammatory reaction which involves the release of proinflammatory cytokines from immune cells (Cui et al., 2000; Dejongh et al., 2003; Deleo and Yezierski, 2001; Marchand et al., 2005; Watkins et al., 1995). Interleukin-6 is an interesting target in the study of pain, which has an important role in the physiology of nociception and the pathophysiology of pain (Dejongh et al., 2003; Sweitzer et al., 2001). Interleukin-6 is secreted by a wide range of cells including fibroblasts, monocytes, B cells, endothelial cells, T cells, microglial cells, astrocytes and neurons (Benveniste, 1998). This proinflammatory cytokine plays a key role in peripheral nerve-injury-induced

mechanical allodynia and thermal hyperalgesia in both rodents and humans (Deleo et al., 1996; Oka et al., 1995). In addition, it has been shown that neurons of the spinal cord produce interleukin-6 mRNA in response to peripheral nerve injury resulting in neuropathic pain behaviors (Arruda et al., 1998). Furthermore, two weeks following partial sciatic nerve ligation, interleukin-6 release from cultured cells, derived from injured nerves, was increased significantly compared with uninjured nerves (Ma and Quirion, 2005). Interleukin-6 is potentially important in pain etiologies with potential nociceptive actions. The importance of interleukin-6 in nociception has been further demonstrated with interleukin-6 knock-out mice, which do not develop heat and pressure hypersensitivity in response to a chronic constriction injury (Murphy et al., 1999). Relatively minor or moderate insults to tissue integrity induce production of a variety of cytokines that act locally, in a paracrine or autocrine manner. However, a few cytokines (for example, interleukin-6 and the macrophage colony stimulating factor) appear to exert at least part of their tissue function by entering the circulation, where they recruit systemic responses that are important for maintaining the integrity of the injured tissue (Hopkins and Rothwell, 1995). It is well established that interleukin-6 has been implicated in neuropathic pain (Dejongh et al., 2003). Mechanical allodynia has been correlated with levels of interleukin-6 immunoreactivity or mRNA in the sciatic nerve and dorsal root ganglia, respectively, after nerve constriction injury. Moreover it has been reported that in this model of neuropathic pain, interleukin-6 knock-out mice showed less thermal hyperalgesia and mechanical allodynia compared with wild-type mice (Murphy et al., 1999; Ramer et al., 1998). We also found that in the presence of minocycline the serum concentration of interleukin-6 declined compared to CCI saline-treated animals. This finding can be compared to the analgesic effect of minocycline in patients with rheumatoid arthritis disease which have shown a decline in their serum interleukin-6 concentration (Kloppenburg et al., 1996). In conclusion, our results showed that minocycline could have a protective effect in reducing pain behaviors in CCI model of neuropathic pain. It seems that, the antihyperalgesic effect of minocycline may be due in part by preventing interleukin-6 production following sciatic nerve injury. On the other hand, previous study have shown that generation and maintenance of enhanced pain states involve activation of non-neuronal cells such as microglia following injury (Watkins et al., 2001a,b; Watkins and Maier, 2003). According to the fact that cytokines are involved in the generation of pain after nerve injury, further studies are needed to determine the level of interleukin-6 immunoreactivity or mRNA in the sciatic nerve and in microglial cells under the influence of minocycline in chronic constriction injury model of neuropathic pain.

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