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Minocycline blocks lipopolysaccharide induced hyperalgesia by suppression of microglia but not astrocytes

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Abstract

Systemic injection of lipopolysaccharide (LPS) induces a robust immune response as well as thermal and mechanical hyperalgesia. Spinal and peripheral glial cells have been implicated as important mediators in this hyperalgesia but the specific contributions of microglia versus astrocytes are not entirely clear. To better define these mechanisms, this study examined the febrile response, nociceptive sensitivity, glial cell reactivity and cytokine production in the dorsal root ganglion (DRG) and spinal cord in rats following systemic treatment with LPS and the effects of minocycline in countering these responses. Intraperitoneal LPS injection resulted in an increase in core body temperature and produced hyperalgesia to heat and mechanical stimuli. Western blot studies revealed increased expression of microgial cell, macrophage and satellite cell markers in DRG and microglial and astrocyte markers in spinal cord following LPS treatment. Real-time RT-PCR indicated that LPS treatment increased cytokine mRNA expression levels in both the DRG and the spinal cord. Minocycline suppressed all LPS-induced behavioral effects but not the febrile response. Moreover, minocycline prevented LPS induced microglia/macrophage activation and cytokine responses in spinal cord and DRG, but did not affect the activation of astrocytes/satellite cells. These data demonstrate that LPS-induced changes in nociceptive sensitivity are likely mediated by activation of microglial cells and/or macrophages in the spinal cord and DRG.

Keywords

Lipopolysaccharide; Minocycline; Hyperalgesia; Dorsal root ganglion; Spinal cord; Inflammation; Microglia; Astrocyte; Satellite cell

Introduction

Lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria induces most clinical aspects of acute bacterial infection, including inflammation

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and changes in immune functions when introduced systemically that lead to systemic illness response such as anorexia, fever and lethargy (Dantzer et al.,1998). These effects of LPS are all dependent on the induction of pro-inflammatory cytokines. Moreover, intraperitoneal injection of LPS or specific cytokines enhances nociceptive sensitivity to various somatic stimuli in rodents suggesting hyperalgesia (Maier et al.,1993). Because nociceptive sensitivity is mediated by neural circuitry in the spinal cord or brain, this hyperalgesia appears to be part of the CNS response to peripherally injected LPS.

Ascending nociceptive information passes through the peripheral dorsal root ganglion (DRG) to the spinal cord and then hence to the brain (Woolf,2004). Various inflammatory mediators such as cytokines, eicosanoids, and nitric oxide increase the sensitivity of the DRG and spinal cord to nociceptive signals, thereby reducing thresholds for nociceptive stimuli in acute and chronic pain models (Woolf,2004). Glial cells, as the main source of cytokines in the CNS, are active participants in the neuroimmune response to a variety of stressors (Wieseler-Frank et al.,2004). However, the relative contribution of glial subtypes, microglia versus astrocytes, appears to vary in hyperalgesia with differing etiologies. Microglial cells appear to play a key role in hyperalgesia produced by direct nerve injuries (Milligan et al.,2003; Raghavendra et al.,2003), whereas astrocytes appear to play the key role in neuropathic pain produced by chemotherapeutics (Zhang et al.,2011). The relative contribution of microglial cells versus astrocytes in LPS-induced hyperalgesia is not defined (Guo and Schluesener,2006; Lee et al.,2010).

Minocycline, a second-generation semisynthetic tetracycline antibiotic, also has inhibitory effects on immune and glial cells. Minocycline is a lipid soluble drug that can readily cross the blood-brain barrier (BBB) into the CNS parenchyma (Saivin and Houin,1988). Several studies demonstrated that minocycline possesses anti-inflammatory properties independent of its antimicrobial effects (Stirling et al.,2005). Animal studies indicate minocycline inhibits the production of immune activators by macrophages, microglia, and neurons (Nikodemova et al.,2007a; Zhao et al.,2007; Zink et al.,2005). In vitro, minocycline has an inhibitory effect on the activation, proliferation, and viral replication of microglia, astrocytes macrophages, and lymphocytes (Nikodemova et al.,2007a; Si et al.,2004; Zink et al.,2005). For example, minocycline decreased the activity of microglia and the CNS release of cytokines in experimental allergic encephalitis (Nikodemova et al.,2007a) and blocked the deleterious effects of neuroinflammation on neurogenesis, long-term potentiation, and neuronal survival (Kim et al.,2009; Wang et al.,2004). Recently, interest has arisen on its possible use in the management of chronic pain (Hains and Waxman,2006).

Although several studies have shown that peripheral injection of LPS produces behavioral changes in response to sensory stimuli (Maier et al.,1993), in addition to systemic illness responses, as noted above the glial cell response that drives this phenotype is not established nor is the potential for differential effects of minocycline in abrogating this response. The goal here was to address these gaps in knowledge.

2. Materials and Methods

2.1. Animals

Experiments were performed on male Sprague-Dawley rats (330-380 g; Harlan Laboratories, Indianapolis, IN, USA) housed with free access to food and water and maintained in temperature- and light-controlled rooms ($23 \pm 2°C$, 12/12-hour light/dark cycle with lights on at 07:00) for at least 1 week prior to the study. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and conformed to National Institutes of Health guidelines (NIH publication No. 86-23, revised 1985).

2.2. Drug administration and behavioral tests

2.2.1. Drug administration—To examine body temperature and pain behavior, we randomly assigned 26 rats to 1 of 4 treatment group as follows: minocycline/LPS (n = 6), minocycline/vehicle (n = 6), vehicle/LPS (n = 6) and vehicle/vehicle (n = 8). Minocycline (Sigma, CA, USA) was diluted in saline to a concentration of 12.5 mg/ml and buffered by 5 M sodium hydroxide. Rats received an intraperitoneal injection of either the vehicle (saline) or minocycline (25 mg/kg) for 3 consecutive days (day 0, 1 and 2). A repeated injection regimen was selected for minocycline because this was shown necessary to attenuate neuroinflammation in numerous experimental models (Ekdahl et al.,2003; Griffin et al., 2006; Henry et al.,2008a; Nikodemova et al.,2007b; Tomas-Camardiel et al.,2004). LPS (E. coli 055:B5; Sigma) was diluted in sterile normal sterile, and on the third day (at day 2) of the minocycline (or vehicle) injections, unanesthetized rats were injected intraperitoneally with 1 mg/kg LPS or the vehicle alone 30 minutes after minocycline treatment.

2.2.2. Core temperature evaluation—Food was withheld for 2 hours before LPS injection to avoid the thermic effects of digestion. Rectal temperature was measured in conscious, unrestrained rats by gently inserting a small thermistor probe (Vicks V901h-24; Procter & Gamble, Cincinnati, OH, USA) 3 cm into the rectum. Measurements were taken immediately prior to the LPS or vehicle and then at 2, 4, 6, 8, and 24 hours afterward. For all shared time points body temperature was always measured first to minimize potential influences of stress.

2.2.3. Behavioral assessments—Behavioral experiments were conducted in a quiet testing room by an investigator blinded to drug treatment.

2.2.4. Response to thermal stimuli—The hot plate test was used to evaluate thermal nociceptive sensitivity (IITC Life science, Woodland Hills, CA,). Animals were gently placed into an acrylic box with a metal floor that was preheated to 52°C. Paw withdrawal latency was calculated using a timer that was started when the animal was released onto the preheated plate and stopped at the moment of withdrawal, shaking, or licking of either hind paw. Animals were tested at 3, 6, 9, and 24 hours after LPS injections, with 3 trials for each animal at each of these times. Withdrawal threshold for each rat was calculated at the mean of the three trials.

2.2.5. Response to mechanical stimuli—Mechanical withdrawal threshold was determined using an ascending series of von Frey filaments that delivered approximately logarithmic incremental forces (1, 4, 10, 8, 15, 26, and 60 g). Each rat was placed in a clear Plexiglas compartment (25.4 × 25.4 × 10.16 cm; 10 × 10 × 4 in.) on a metal mesh grid. Before each test session, animals were acclimated to the new environment for 10–20 minutes. Each monofilament, starting with the lowest force (1 g), was applied 6 times to the mid-plantar region of the left hind paw of each rat. The monofilament that produced a response of paw withdrawal, flinching, or licking in 3 of the 6 applications was defined as the 50% paw withdrawal threshold (50% PWT). Each animal's 50% PWT was assessed at 3, 6, 9, and 24 hours after LPS injections.

2.3. Tissue collection

Spinal cord and DRG samples were collected to ascertain cytokine mRNA expression and glial cell activity at 6 hours after LPS injection. In separate groups (*n* = 6 per group), following deep anesthesia with intraperitoneal injection of pentobarbitone (90 mg/kg body weight; Lundbeck, Deerfield, IL, USA), lumbar spinal cord segments 3-6 and their DRGs were dissected and quickly frozen in liquid nitrogen and stored at −80°C until further

processing for real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting.

2.4. Western blotting

Spinal cord and DRG tissues were homogenized using a Polytron homogenizer (PRO scientific, CT, USA) for 1 minute in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na$_2$ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM - glycerophosphate, 1 mM Na$_3$VO$_4$, 1 g/ml leupeptin, and protease and phosphatase inhibitor cocktails (Sigma). Each homogenate was centrifuged for 15 minutes at 13,000 rotations per minute at 4°C to yield supernatant. The protein concentration of the resulting supernatant was determined by Lowry protein assay (Bio-Rad, Hercules, CA, USA). Protein samples (30 g) were heated at 95°C for 5 minutes, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane using a Transblot SD apparatus (Bio-Rad). After blots had been washed with twice-buffered saline and Tween-20 (TBST; 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20), the membranes were blocked with 5% skim milk/TBST for 30 minutes and incubated overnight with the primary antibody, followed by a horseradish peroxidase-conjugated appropriate secondary antibody. Primary antibodies included those to the microglial marker ionized calcium-binding adaptor molecule 1 (Iba-1,1:1000; Wako, Osaka, Japan); the astrocyte/ satellite cell marker glial fibrillary acidic protein (GFAP, 1:5000, Cell Signaling Technology, Beverly, MA, USA); and macrophage marker, ED-1 (1:1000, AbD Serotec, Raleigh, NC, USA). β-actin was used as the loading control (Sigma, CA, USA). The membranes were then washed, and primary antibodies were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

2.5. Quantitative real-time PCR

Total RNA was isolated from spinal cord and DRGs using TRIzole Reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. RNA was then air-dried and re-suspended in RNAse-free pure water and the concentration was measured at 260 nm in a NanoDrop spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, USA). First-strand cDNA synthesis was performed with random hexamers using a reverse transcription kit (Invitrogen). Quantitative real-time PCR amplification was performed in a final volume of 25 l containing SYBR Green PCR Master Mix (Ambion, Austin, TX, USA) using the Applied Biosystems 7000 sequence detection system (Foster City, CA, USA). The following messages were probed: interleukin-1 beta (IL-1β); interleukin-6 (IL-6); tumor necrosis factor alpha (TNF-α); monocyte chemotactic protein-1 (MCP-1); and RANTES (regulated on activation, normal T expressed and secreted). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was used as a control. Primer sequences were obtained from GenBank (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov; Table 1). Amplification steps consisted of 50°C for 2 minutes; 95°C for 10 minutes once; then 95°C for 15 seconds, 55°C for 25 seconds, and 72°C for 1 minute for 40 cycles. The threshold cycle (Ct; the number of cycles needed to reach the threshold of detection) was determined for each gene and the relative expression level of each gene was computed using the following formula: relative expression of mRNA = $2^{-(\Delta Ct_{sample} - Ct_{control})}$, where Ct_{sample} is the Ct for the target gene and $Ct_{control}$ is the Ct for GAPDH (for details on the formula, see Applied Biosystems User Bulletin #2).

2.6. Statistical analysis

Results are expressed as means ± standard errors. Data analysis and statistical comparisons were performed using GraphPad Prism version 5.0 software (GraphPad Software, San

Diego, CA, USA). Differences between two groups were assessed using Student's *t*-test. For multiple comparisons to analyze the RT-PCR and western blotting or weight loss, analysis of variance (ANOVA) followed by post hoc Dunnett test was performed. Data on pain sensitivity or fever response at the different time points under different treatment conditions were analyzed using 2-way ANOVA followed by post hoc Bonferroni analysis. Differences with $p < 0.05$ were considered significant.

3. Results

3.1. LPS-induced systemic illness response

3.1.1. Fever—The effects of LPS on body temperature, with and without minocycline treatment, are shown in Figure 1. Rats treated with LPS (vehicle/LPS) showed a significant increase in mean body temperature at 4, 6, 8, and 24 hours after treatment ($p < 0.01$ at 6 hours; p<0.001 8 hours; $p < 0.05$ at 24 hours) compared with rats in the vehicle/vehicle control group at these times (treatment group factor: $F_{(3,110)} = 9.179$, $p < 0.001$; time factor: $F_{(5,110)} = 5.779$, $p < 0.001$).

Minocycline did not block LPS-induced fever as these also developed a significant febrile response (Figure 1). Rats in the minocycline/LPS treatment group showed a significant increase in body temperature at 6, 8, and 24 hours after LPS treatment compared with rats in the vehicle/vehicle control group ($p < 0.01$ at 6 hours; p<0.001 8 hours; $p < 0.05$ at 24 hours) and this effect was not different from that shown by the LPS treated animals (vehicle/LPS treatment group).

3.2. LPS-induced pain behavior

3.2.1. Thermal stimulus response—The effects of LPS treatment on the responses to thermal stimuli (paw withdrawal latency), with and without minocycline treatment are shown in Figure 2. In the non-minocycline-treated group, rats with LPS treatment group (vehicle/LPS) showed decreased mean paw withdrawal latency at 6 hours ($p < 0.05$) and 9 hours ($p < 0.05$) but not at 24 hours after treatment compared with rats in the vehicle/vehicle control group (treatment group factor: $F_{(3,88)} = 1.898$; time factor: $F_{(4,88)} = 6.584$, $p < 0.001$).

Minocycline appeared to block the thermal hyperalgesia effect induced by LPS; rats in the minocycline/LPS treatment group did not show a significant decrease in mean paw withdrawal latency compared with rats in the vehicle/vehicle control group.

3.2.2. Mechanical stimulus response—The effects of LPS treatment on response to mechanical stimuli (50% PWT), with and without minocycline treatment, are shown in Figure 3. Though there was some degree of variability, the baseline 50% PWT values were not statistically different between groups (p=0.23). Compared with rats in the vehicle/vehicle control group, rats in the vehicle/LPS treatment group showed reduced mean 50% PWTs at 6, 9, and 24 hours after treatment ($p < 0.05$ at 6 and 9 hours, p<0.01 at 24hours in LPS; treatment group factor: $F_{(3,88)} = 4.417$, $p < 0.05$; time factor: $F_{(4,88)} = 5.733$, $p < 0.001$). Maximal reduction in mean 50% PWTs was shown at 24 hours after treatment. Minocycline appeared to block the LPS-induced mechanical hyperalgesia; rats in the minocycline/LPS treatment group did not show significantly different mean 50% PWTs from rats in vehicle/vehicle control group at any measurement time.

3.3. Iba-1, GFAP and ED-1 expression in spinal cord and DRG

Because the maximal change in behavioral responses occurred at 6 hours after LPS and then gradually decreased from this point, we choose this time point to test glial reactivity and

pro-inflammatory responses in the spinal cord and DRG and its modification by minocycline. Western blot data examining glial cell activation by systemic LPS in DRG and spinal cord is shown in Figures 4 and 5. The mean relative Iba-1 expression was significantly different in DRG among each treatment group (Fig.4A, $F(3.24) = 12.04$, $p < 0.001$). Post-hoc analysis showed that DRG samples from the LPS treatment group (vehicle/LPS) showed significantly higher mean relative Iba-1 expression than samples from the vehicle/vehicle control group ($p < 0.001$). Minocycline treatment blocked the effects of LPS treatment as the mean relative expression levels of Iba-1 in samples from the minocycline/LPS treatment group was not statistically different from the mean relative expression levels in samples from the respective minocycline/vehicle treatment group.

Mean relative GFAP expression was also higher in samples from vehicle/LPS treatment group compared with relative GFAP expression levels in samples from the vehicle/vehicle control group (Fig.4B, $F(3.24) = 5.756$, $p < 0.01$; $p < 0.05$ for the comparison of vehicle/ LPS vs. vehicle/vehicle). In contrast, DRG samples from the minocycline/LPS treated group did not show any significant difference in mean GFAP expression levels compared with samples from the vehicle/LPS group but higher GFAP expression level compared to the vehicle/vehicle and minocycline/vehicle group ($p < 0.01$), suggesting that minocycline treatment did not affect satellite cell activation.

The mean relative ED-1 expression was higher in DRG samples from the vehicle/LPS treatment group compared with samples from the vehicle/vehicle control group (Fig.4C, $F(3.24) = 10.49$, $p < 0.001$; $p < 0.01$ for the comparison of vehicle/LPS vs. vehicle/vehicle). Minocycline treatment blocked the LPS-induced increase in ED-1 expression levels, suggesting that minocycline also blocked DRG macrophage activation.

When spinal cord samples were analyzed, the Iba-1 expression pattern among the treatment groups was almost the same as in the DRG samples (Fig.5). The vehicle/LPS treatment groups showed higher mean relative spinal cord Iba-1 expression than samples from the vehicle/vehicle control group (Fig.5A, $F(3.24) = 12.04$, $p < 0.001$; $p < 0.001$ for the comparison of vehicle/LPS vs. vehicle/vehicle). Moreover, samples from the minocycline/ LPS treated group showed lower mean relative Iba-1 expression levels than samples from the respective vehicle/LPS treatment group ($p < 0.001$), suggesting that minocycline blocked the effect of LPS on microglial activation in the spinal cord.

Spinal cord samples from the LPS treatment group (vehicle/LPS) showed higher mean relative GFAP expression levels than samples from the vehicle/vehicle control group (Fig. 5B, $F(3.24) = 5.756$, $p < 0.01$; $p < 0.05$ for the comparison of vehicle/LPS vs. vehicle/ vehicle), and, as in the DRG samples, mean relative GFAP expression levels in the spinal cord samples from minocycline/LPS treated group was not significantly different from those in samples from the vehicle/LPS group. Minocycline/LPS group still showed higher GFAP expression level compared than that of minocycline/vehicle group ($p < 0.05$), suggesting that minocycline treatment did not affect the activation of spinal astrocytes.

Finally, the mean relative ED-1 expression levels in spinal cord were very weak across all treatment groups indicating very low levels of tissue resident macrophages. This expression levels in spinal cord samples from both minocycline-treated and non-minocycline-treated group with LPS were not significantly different from mean relative expression levels in samples from the vehicle/vehicle control group (Fig.5C, $F(3.24) = 0.894$), suggesting that LPS failed to provoked macrophage activation in the spinal cord.

3.4. Cytokine mRNA expression

Analysis of cytokine mRNA expression levels in the DRG (Fig. 6A, C, E, G, and I) revealed a significant effect of LPS treatment in increasing the expression of IL-1β, IL-6, TNF-α, MCP-1, and RANTES compared to vehicle/vehicle ($F_{(3,20)}$ = 8.401, $p < 0.001$; $F_{(3,20)}$ = 8.960, $p < 0.001$; $F_{(3,20)}$ = 9.095, $p < 0.001$; $F_{(3,20)}$ = 5.847, $p < 0.01$; and $F_{(3,20)}$ = 6.948, $p < 0.01$ respectively). Minocycline treatment prevented this increase as DRG from LPS plus minocycline-treated rats showed significantly lower mean mRNA expression levels for all of tested cytokines compared to the vehicle/LPS group ($p < 0.01$ for IL-1β, IL-6 and TNF-α; $p < 0.001$ for MCP-1; $p < 0.05$ for RANTES). DRG from the minocycline/vehicle group did not show any significant differences in mean relative cytokine mRNA expression levels from those of rats in the vehicle/vehicle control group.

In the spinal cord (Fig. 6B, D, F, H, and J), LPS similarly increased mRNA expression levels for all cytokines except IL-6 ($F_{(3,20)}$ = 8.401, $p < 0.001$ for IL-1β; $F_{(3,20)}$ = 8.960, $p < 0.001$ for TNF-α; $F_{(3,20)}$ = 9.095, $p < 0.001$ MCP-1; $F_{(3,20)}$ = 5.847, $p < 0.01$ for RANTES respectively). Spinal cord samples from minocycline-treated rats (minocycline/LPS group) showed noticeably lower mean mRNA expression levels compared to the vehicle/LPS group across all cytokines measured ($p < 0.001$ for IL-1β; $p < 0.01$ for TNF-α and RANTES; $p < 0.05$ for MCP-1). Spinal cords from the minocycline/vehicle group did not show any significant differences in mean relative cytokine mRNA expression levels from those of rats in the vehicle/vehicle control group.

4. Discussion

The results have shown that LPS treatment produced fever and changes in nociceptive sensitivity that were paralleled by increases in expression markers of glial cells and macrophages in the DRG, glial cells only in the spinal cord, and increased mRNA expression levels for several pro-inflammatory cytokines in both compartments. Minocycline suppressed LPS-induced hyperalgesia and increases in microglial/macrophage cell markers and cytokine expression but did not affect increases in astrocyte markers or LPS-induced increases in core temperature. These results suggest that microglial cells in the spinal cord and DRG are key mediators in sickness, and possibly localized infection, related hyperalgesia through production of proinflammatory cytokines that in turn alter the signaling of primary and spinal neurons in response to cutaneous stimuli.

LPS treatment markedly increased expression of several pro-inflammatory cytokines including IL-1β, IL-6, TNF-α, MCP-1, and RANTES in the DRG and spinal cord. Systemic or perineural administration of numerous pro-inflammatory cytokines, including TNFα, IL-1β and IL-6, induces mechanical hypersensitivity and thermal hyperalgesia (Cunha et al., 1992; Cunha et al.,2000; Cunha et al.,2005; Jin and Gereau,2006; Perkins and Kelly,1994; Safieh-Garabedian et al.,1995; Schäfers et al.,2003a; Schäfers et al.,2003b; Wacnik et al., 2005; Woolf et al.,1997). Increase in the expression of many of these pro-inflammatory cytokines following nerve injury is observed in and around peripheral nerves and in the dorsal root ganglia (Cui et al.,2000; Murphy et al.,1995; Okamoto et al.,2001; Shubayev and Myers,2000; Shubayev and Myers,2001). Peripheral blockade of pro-inflammatory cytokines prevents the development of both inflammatory and neuropathic pain (Cunha et al.,1992; Cunha et al.,2000; Cunha et al.,2005; Safieh-Garabedian et al.,1995; Sommer et al.,1998a; Sommer et al.,1998b; Sommer et al.,2001a; Sommer et al.,2001b; Sorkin and Doom,2000; Woolf et al.,1997). The likely mechanism by which cytokines increase behavioral pain phenotypes is by directly influencing the function of primary afferent neurons. For example, TNFα not only induces transcriptional regulation of down-stream inflammatory mediators, such as induction of IL-1β, IL-6 and Nuclear Factor-B in neurons and Schwann cells (Cunha et al.,2005; Lee et al.,2009; Watkins et al.,1995), but also has a

rapid sensitizing effect on primary afferent neurons. TNFα promotes heat-induced CGRP release from nociceptor terminals in skin (Opree and Kress,2000), lowers activation threshold in Aβ- and C-fibers (Junger and Sorkin,2000; Liu et al.,2002; Schäfers et al., 2003a; Zhang et al.,2002), sensitizes transient receptor potential vanilloid receptor 1 (TRPV1) (Nicol et al.,1997) and enhances TTX-resistant sodium currents in primary sensory neurons (Jin and Gereau,2006). In addition to inducing the synthesis of several sensitizers of nociceptors, IL-1β also directly activates peripheral nociceptors. IL-1β acts in a p38 mitogen-activated protein kinase (p38 MAP kinase)-dependent manner, to increase the excitability of primary sensory neurons and increase action potential generation by relieving resting slow inactivation of tetrodotoxin-resistant voltage-gated sodium channels and enhancing persistent TTX-resistant currents (Binshtok et al.,2008). The chemokine MCP-1, also called CCL2, also increases the excitability of primary sensory neurons perhaps acting in an autocrine fashion to increase pain signaling following nerve injury (Jung et al.,2008; Sun et al.,2006; White et al.,2005).

The central nervous system effects of pro-inflammatory cytokines/chemokines have been explored in a more limited fashion. TNFα, IL-1β, IL-6 and MCP-1 are all increased in spinal cord after peripheral nerve injury or inflammation (DeLeo et al.,1997; Holguin et al., 2004; Milligan et al.,2001; Pineau and Lacroix,2007; Sweitzer et al.,2001). Intrathecal application of TNFα, IL-6 or MCP-1 all induce mechanical allodynia and thermal hyperalgesia (Arruda et al.,1998; DeLeo et al.,1996; Gao et al.,2009); whereas in contrast, spinal administration of neutralizing antibodies to TNFα, IL-1β or IL-6 prevents the development of inflammatory and neuropathic pain (Arruda et al.,2000; Choi et al.,2010; Schafers et al.,2001; Schäfers et al.,2003c; Schoeniger-Skinner et al.,2007; Sweitzer et al., 2001). The mechanisms underlying the pronociceptive effects of pro-inflammatory cytokines and chemokines in the spinal cord are largely unknown. TNFα, IL-1β, IL-6 and MCP-1 modulate excitatory and inhibitory synaptic transmission in unidentified dorsal horn neurons (Gao et al.,2009; Kawasaki et al.,2008). Yet, the identity of the specific functional subtypes of neurons that are affected, the receptors and their location, and the second messenger systems involved are not known. Enhancement of excitatory spinal transmission by TNFα has only very recently been shown as dependent upon suppression of on-going inhibitory synaptic transmission (Zhang et al.,2010; Zhang and Dougherty,2011). TNFα is also involved in recruiting Ca^{2+} permeable AMPA receptors into the plasma membrane of dorsal horn neurons during carrageenan-induced inflammation (Choi et al.,2010). Finally, IL-1β increases the expression of cyclooxygenase-2 in spinal cord dorsal horn during complete Freund's adjuvant induced paw inflammation (Narita et al.,2008; Samad et al., 2001)

Rats treated with minocycline prior to LPS injections did not demonstrate the LPS-induced hyperalgesia in response to thermal and mechanical stimulation and this appears to have been due to the fact that the normal cytokine response induced by LPS was abrogated. The surprising and important issue that arises based on this observation concerns the relative role of microglia and related cells versus astrocytes in the expression of pain phenotypes. Minocycline is a second-generation semisynthetic tetracycline antibiotic that has unusual anti-inflammatory properties (Dheen et al.,2007). Cell culture and animal experiments have demonstrated that minocycline readily crosses the blood-brain barrier and attenuates microglial cell activation and limits production of inflammatory mediators in a number of conditions (Chen-Roetling et al.,2009; Keilhoff et al.,2011). For instance, minocycline pretreatment of BV-2 (microglia) cultures decreased LPS-stimulated cytokine production (Horvath et al.,2008). Moreover, minocycline blocks impaired working memory that accompanied LPS-induced neuroinflammation (Henry et al.,2008b). In this study, microglia activation was inferred by measurement of increases in Iba-1 protein which is restricted to microglia but not neurons, astrocytes or oligodendrocytes both in vitro and in vivo (Ito et al.,

1998). There is increasing evidence that uncontrolled activation of microglia under several pain conditions induces the release of pro-inflammatory cytokines, complement components and other substances leading to the facilitation of pain transmission (Mika,2008). Therefore the results shown here imply that minocycline inhibits spinal microglia release of cytokines that subsequently blocks the LPS-induced pain hyperalgesia.

The results concerning the existence of Iba-1 positive microglia-like immune cells in DRG remain uncertain given that microglia are considered specific CNS resident immune cells. Recently, the presence of Iba-1 positive microglial-like cells in DRG was observed in a model of sciatic nerve transection in rats (Patro et al.,2010). In that study, Iba-1 positive cells were activated following peripheral nerve transection consistent with spinal microglia, which were not co-localized with GFAP (the marker for satellite cells) indicating that Iba-1 positive microglial-like cells are different from satellite cells in DRG. Moreover, all of the Iba-1 positive cells were co-localized with MHC II markers (such as OX-6), characteristic of the microglial phenotype. Although the migration of the peripheral macrophages in DRGs following injury cannot be excluded, Iba-1 positive cells even in the absence of injury supports the presence of resident microglia-like cells in the DRG similar to CNS. Together with previous finding, the data here further suggest that microglia-like immune cells might have a similar role in LPS-induced cytokine release in DRG as spinal cord inducing an increase in neuronal activity by local pro-inflammatory cytokine release.

The increased expression of ED-1 expression in DRG that was significantly increased after LPS injection compared than vehicle group suggests that macrophage infiltration into the DRG is provoked by LPS treatment (Jeon et al.,2011). However, in the spinal cord, ED-1 signal was very weak and was not different from vehicle group. Macrophages enter the DRG after a signal from a peripheral conditioning lesion, and contribute to the pathology of many inflammatory diseases (Schaible et al.,2010). Our study indicates that macrophages infiltration to the DRG might play a key role in production of inflammatory factors that influence nociceptive sensitivity.

Minocycline did not affect the up-regulation of GFAP expression in either the DRG or spinal dorsal horn. GFAP is expressed in satellite cells in the DRG and in astrocytes in the dorsal horn. Because minocycline blocked pain sensitivity caused by LPS, it appears that neither satellite cells in the DRG nor astrocytes in the spinal cord were involved in the LPS-induced changes in nociceptive sensitivity (Cui et al.,2008). Thus, inflammatory, direct nerve injury and infection induced hyperalgesias all appear to include a key microglial but a less important astrocyte component. In contrast, neuropathic pain produced by chemotherapeutics show the opposite, wherein astrocytes play a key role but microglia do not (Zhang et al.,2011; Zheng et al.,2011). Interestingly, minocycline blocks the behavioral phenotype equally well in models of chemotherapy related hyperalgesia as in the nerve injury and LPS models (Boyette-Davis et al.,2011; Boyette-Davis and Dougherty,2011; Zhang et al.,2011). The basis for selectivity of the drug under the differing hyperalgesia conditions is not clear and will likely be of keen interest for further investigation. Moreover, the potential importance of defining differential roles of glial cell subtypes in various specific pain conditions will no doubt also be of interest for further investigation.

Interestingly, minocycline did not affect the LPS-induced fever response. The generation of fever is closely related to the induction of circulating cytokines in blood (Netea et al.,2000). Although not measured here, another study has focused on the effects of minocycline on plasma versus brain levels of interleukins 1 and 6 (Henry et al.,2008a). While, minocycline lowered LPS-induced cytokine levels in hippocampus and cortex there was no effect on the plasma levels. Presumably, the same effect explains the current results with the fever

mediated by circulating cytokines that reached the critical regions in the hypothalamus that mediate the pyrogenic response.

In conclusion, systemic LPS induced changes in nociceptive sensitivity, increased glial cell activity and cytokine production. Minocycline blocks the behavioral phenotype by apparently preventing microglial and resident macrophage activation in the dorsal horn and DRG respectively by LPS that result in reduced pro-inflammatory cytokine expression. A lack of effect of astrocytes in the LPS model may suggest differential glial cell involvement in particular models of hyperalgesia.

Acknowledgments

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Research highlights

LPS hyperalgesia involves activation of microglial/macrophage activity in DRG and spinal cord.

LPS induced hyperalgesia is inhibited by minocycline.

Minocycline suppresses activation of microglia/macrophages in DRG and spinal cord but does not affect astrocytes.
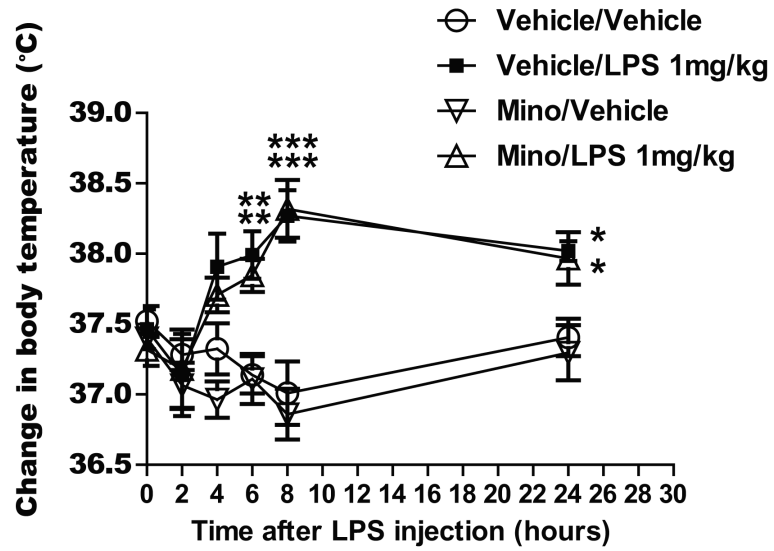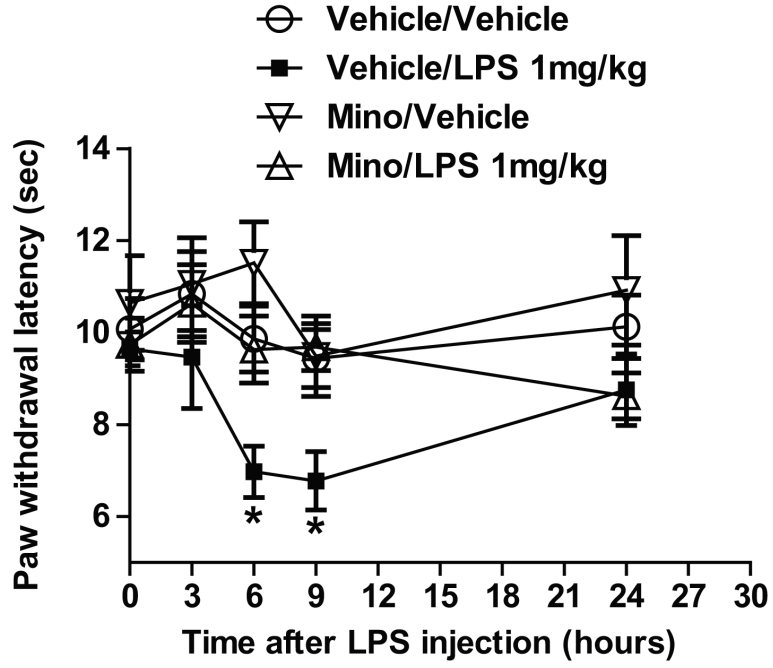
Fig. 1.
Graphs illustrating the effect of lipopolysaccharide (LPS) injection following 3 days of treatment with minocycline or vehicle (saline) on change in body temperature following LPS injection. ***p<0.001, **p<0.01 or *$p < 0.05$ versus the value of the vehicle/vehicle control group at the same time point.

Fig. 2.
Graphs illustrating the effect of lipopolysaccharide (LPS) injection following 3 days of treatment with minocycline or vehicle (saline) on the paw withdrawal latency (sec) response to thermal stimulation (hot-plate test). $*p < 0.05$ versus the vehicle/vehicle control group at the same time point.
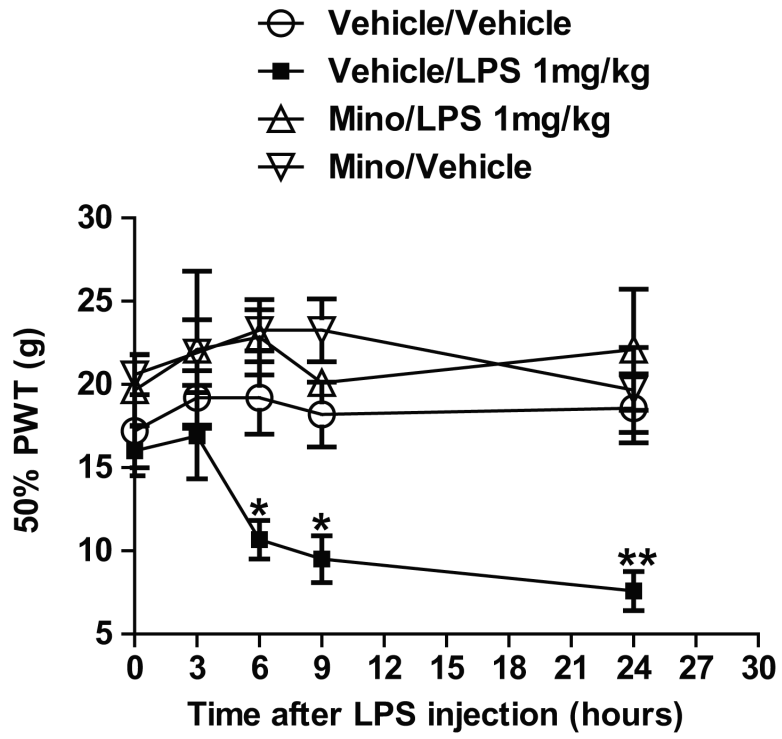
Fig. 3.
Graphs illustrating the effect of lipopolysaccharide (LPS) injection following 3 days of treatment with minocycline or vehicle (saline) on the 50% paw withdrawal threshold (50% PWT; g) response to mechanical stimulation. $**p < 0.01$ or $*p < 0.05$ versus the vehicle/vehicle control group at the same time point.
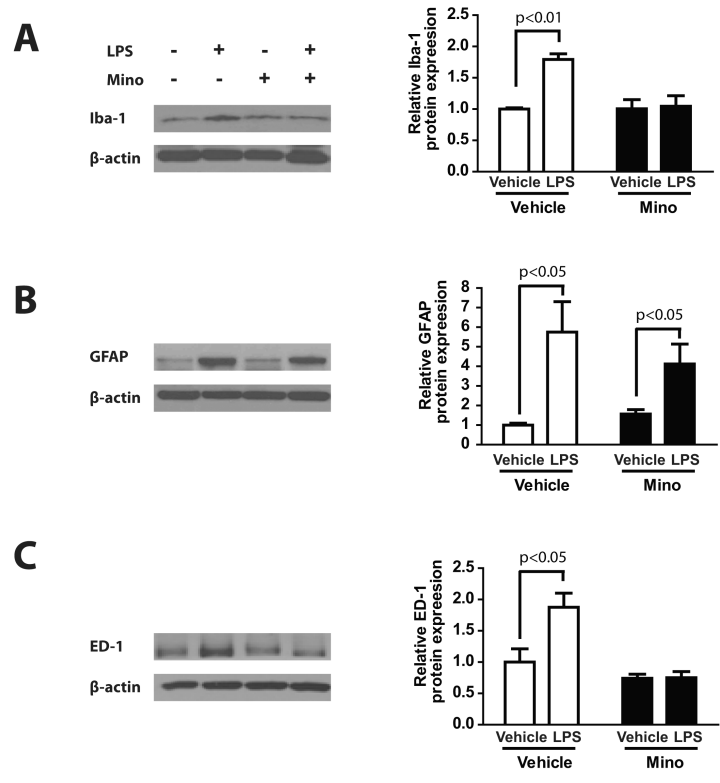
Fig. 4.
Western blots and graphs illustrating the effect of lipopolysaccharide (LPS) injection
following 3 days of treatment with minocycline or vehicle (saline) on relative expression
levels of (A) ionized calcium-binding adaptor molecule 1 (Iba-1; microglia-like cell
marker), (B) glial fibrillary acidic protein (GFAP; satellite cell marker), and (C) ED-1
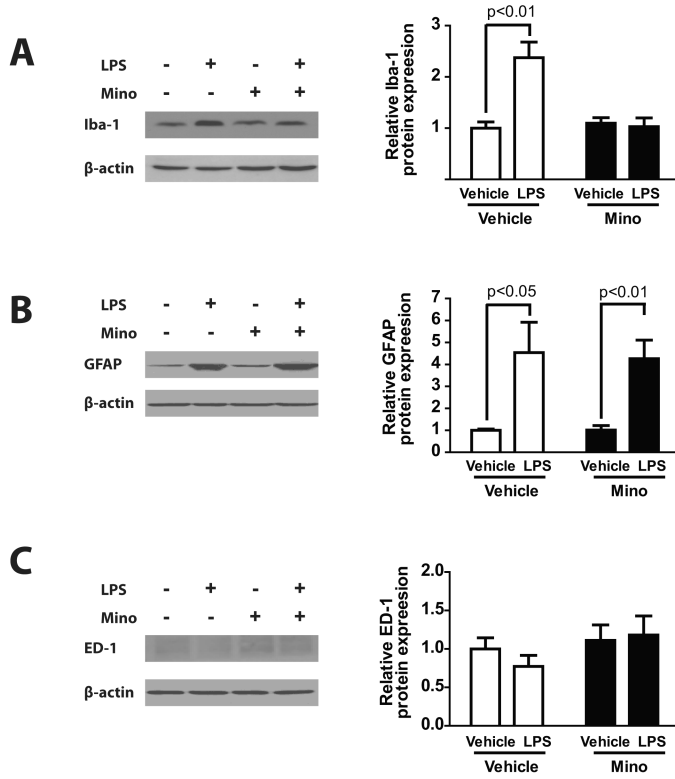(macrophage marker) proteins in the dorsal root ganglion (DRG).

Fig. 5.
Western blots and graphs illustrating the effect of lipopolysaccharide (LPS) injection following 3 days of treatment with minocycline (25 mg/kg; Mino + LPS)or vehicle (saline) on relative expression levels of (A) ionized calcium-binding adaptor molecule 1 (Iba-1; microglia-like cell marker), (B) glial fibrillary acidic protein (GFAP; astrocyte cell marker), and (C) ED-1 (macrophage marker) proteins in the spinal cord.
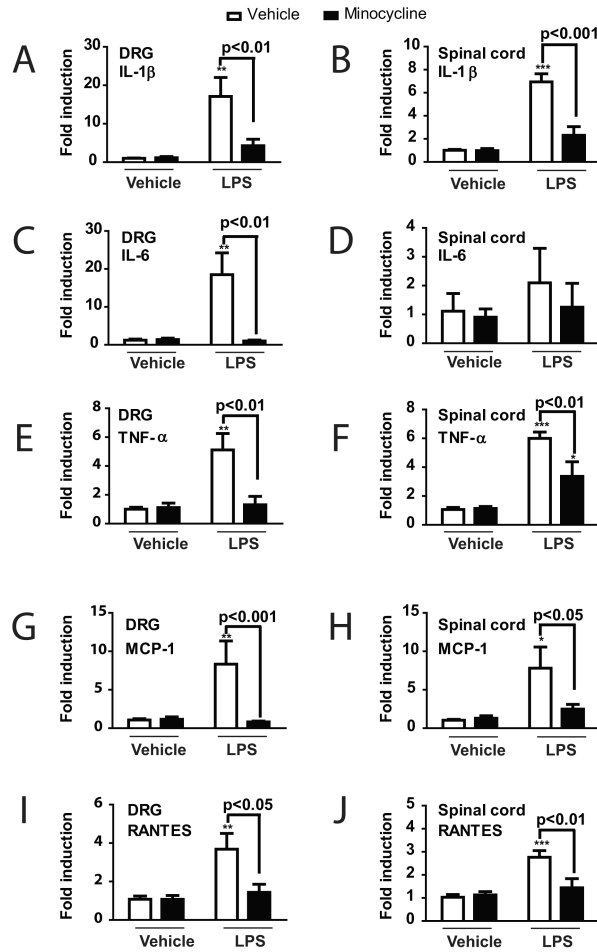
Fig. 6.
Graphs illustrating the effect of lipopolysaccharide (LPS) injection following 3 days of treatment with minocycline or vehicle (saline) on relative mRNA expression levels, expressed as a fold change compared with the control group values for (A, B) interleukin-1 beta (IL-1β), (C, D) interleukin-6 (IL-6), (E, F) tumor necrosis factor alpha (TNF-α), (G, H) monocyte chemotactic protein-1 (MCP-1), and (I, J) RANTES (regulated on activation, normal T expressed and secreted in (A, C, E, G, I) the dorsal root ganglion (DRG) and (B, D, F, H, J) the spinal cord. ***$p < 0.001$, ** $p < 0.01$ or *$p < 0.05$ versus the vehicle/vehicle control group.

Table 1

Primer sequences used for quantitative real-time polymerase chain reaction.

Gene[a]	Primer sequence (5′–3′)	GenBank accession no.
IL-1β	GAAGTCAAGACCAAAGTGG (forward) TGAAGTCAACTATGTCCCG (reverse)	**M98820**
IL-6	AAGTTTCTCTCCGCAAGAGACTTCCAG (forward) AGGCAAATTTCCTGGTTATATCCAGTT (reverse)	**NC005013**
TNF-α	CTTCAAGGGACAAGGCTG (forward) GAGGCTGACTTTCTCCTG (reverse)	**D00475**
MCP-1	TAGCATCCACGTGCTGTCTC (forward) CCGACTCATTGGGATCATCT (reverse)	**NM031530**
RANTES	GCCAACCCAGAGAAGAAGTG (forward) ATCCCCAGCTGGTTAGGACT (reverse)	**NM031116**
GAPDH	TGCCAAGTATGATGACATCAAGAAG (forward) AGCCCAGGATGCCCTTTAGT (reverse)	**NM01008**

[a]TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemotactic protein-1; IL-1β, interleukin-1 beta; IL-6, interleukin-6; RANTES, regulated on activation, normal T expressed and secreted; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2

The effect of minocycline alone on core temperature, thermal and mechanical withdrawal threshold.

Measure	Day 0 (prior to i.p. minocycline)		Day 2 (after i.p. minocycline)	
	Vehicle	Minocycline	Vehicle	Minocycline
Core temperature (°C)	37.56 ± 0.08	37.58 ± 0.06	37.50 ± 0.08	37.46 ± 0.07
Thermal threshold (sec)	9.13 ± 0.50	10.11 ± 0.71	9.53 ± 0.52	10.19 ± 0.58
Mechanical threshold (g)	17.64 ± 1.59	19.17 ± 1.17	17.82 ± 1.42	20.13 ± 1.17

Either the vehicle (saline, N=14) or minocycline (25 mg/kg, N=12) was treated for 3 consecutive days (Day 0, 1 and 2, once a day). On Day 0 (before minocycline injection) and Day 2 (after minocycline injection), core temperature and withdrawal thresholds were measured to examine the effect of minocycline itself prior to LPS injection. Data were expressed by means ± SEM.