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Systemic minocycline differentially influences changes in spinal microglial markers following formalin-induced nociception

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ABSTRACT

In the present study, intraperitoneal administration of minocycline attenuated enhancing nociceptive behaviors in those rats receiving dual formalin injections (5% formalin followed at 7 days later by 1% formalin). The minocycline treatment did not prevent the increase in OX-42 and MHC class I labeling and morphological changes, but significantly attenuated upregulation of phospho-p38 in activated microglia. These results suggest that the later days of microglial activation with upregulated immune markers in the spinal cord contributes to enhancing long-term pain response by a pathway of p38 activation in microglia.

1. Introduction

There is increasing evidence that activated glia (microglia and astrocytes) in the spinal cord contribute to the induction and maintenance of pathological pain. Microglial activation has been described in several ways including changes in morphology, an increase in the expression of microglial markers, or the number of microglia. Changes in microglial morphology from ramified to amoeboid were evident at 2–3 days after peripheral tissue injury (Coyle, 1998; Fu et al., 1999; Graeber et al., 1988; Hashizume et al., 2000; Honore et al., 2000; Lin et al., 2007b; Molander et al., 1997). In our previous study, peripheral formalin injection upregulated the expression of CD45 and MHC class I antigen in activated microglia, beginning 3 days after the formalin injection and up to at least day 7 (Fu et al., 2009). The time course of these immune marker changes corresponded with that of changes in microglial morphology detected by OX-42 immunohistochemistry (Fu et al., 1999). Moreover, while microglia undergo an early and rapid activation without observable morphological changes (Hua et al., 2005; Svensson et al., 2003), changes in both morphology and immune markers are observed only at a late stage (days to weeks) after tissue injury (Fu et al., 2009). Thus, microglia may undergo two distinct stages of changes after tissue injury (Fu et al., 2009): (1) Early-activated microglia indicated by phospho-p38MAPK still have a "resting" ramified morphology with a relatively small cell body and weakly express molecules normally present in other haematopoietic lineages, such as CD45, MHC class I antigen, and other immunomolecules. (2) Late-activated microglia show upregulation of CD45 and MHC class I as well as morphology characterized by a hypertrophic cell body and the shortening of cellular processes.

Glial cells, upon activation, are the source of a number of neuromodulators such as pro-inflammatory cytokines, chemokines, nitric oxide, prostaglandins and ATP, contributing to the mechanisms of central sensitization (De Leo et al., 2006; Marchand et al., 2009; McMahon et al., 2005; Moalem and Tracey, 2006; Owolabi and Saab, 2006; Watkins et al., 2007). The morphological differences may relate to the function of microglia at different times following peripheral injury. For example, each stage of activated microglia may produce and release different neuromodulators and differentially contribute to nociceptive behaviors. If so, it would be possible for microglial activation with or without changes in morphology and immune markers to contribute differently to nociceptive behavior induced by the formalin injection into a rat's hindpaw.

Minocycline, a tetracycline-like anti-biotic, has been used as a microglial activation inhibitor and shown to ameliorate several neurodegenerative conditions (Hua and Walz, 2006; Ravina et al., 2003; Tikka and Koistinaho, 2001; Yong et al., 2004). Systemic or spinal injections of minocycline have been shown to attenuate neuropathic

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and inflammatory pain behavior and inhibit microglial activation as demonstrated by decreased OX-42 expression in the spinal cord (Cho et al., 2006; Hains and Waxman, 2006; Lin et al., 2007a; Piao et al., 2006). In the present study, we utilized a rat model of dual formalin injections to examine the functional implication of differential microglial changes in formalin-induced nociceptive behavior. The first formalin injection (5%, 100 µl) was used to replicate two stages of microglial changes, i.e., activation of microglia without (at day 1) and with morphological changes (at day 7) after the injection, respectively. A second formalin (1%, 50 µl) injection was given to examine the influence of the microglial changes on nociceptive behavior with or without systemic minocycline treatment.

2. Materials and methods

2.1. Animals and treatments

The experimental protocol was approved by our Institutional Animal Care and Use Committee. A total of 48 adult male Sprague–Dawley rats weighing 200–225 g (Charles River Laboratories, Wilmington, MA) were used. The animal room was artificially lighted from 7:00 AM until 7:00 PM with a free access to food and water.

2.1.1. Experiment 1

Eighteen rats were randomly assigned to three groups: Formalin 7d Group ($n=6$), Formalin 1d Group ($n=6$), and a Control Group ($n=6$). For the Formalin 7d Group, each rat was given a first (conditioning) injection of 100 µl of 5% formalin subcutaneously into the *plantar surface* of the right hind paw. On day 7 after this first formalin injection, when microglial morphological changes were clearly demonstrated (Fu et al., 1999), a second injection with 50 µl of 1% formalin was given to the *dorsal surface* of the same hind paw. For the Formalin 1d Group, 100 µl of 5% formalin was injected into the plantar surface of the right hind paw. Twenty four hours later, when no significant microglial morphological changes were observed (Fu et al., 1999), a second injection of 50 µl of 1% formalin was given into the dorsal surface of the same hindpaw. For the Control Group, 50 µl 1% formalin was injected into the dorsal surface of the right hind paw without the first 5% formalin injection as in the other groups. For each group, an investigator blinded to the treatment condition recorded the number of formalin-induced paw flinches every 5 min for 1 h following the 1% formalin injection. Our previous reports indicated that 5% formalin injection damaged nociceptive receptors at the injection site (Fu et al., 2001; Lin et al., 2007b). In the present study the second injection of 1% formalin was given into the undamaged dorsal surface of the same hindpaw, instead of the plantar surface for the first formalin injection, to avoid this confounding condition.

2.1.2. Experiment 2

Fifteen rats were randomly assigned into three groups: a Minocycline + formalin Group ($n=5$), a Saline + formalin Group ($n=5$), and a Saline + saline Group ($n=5$). For the Minocycline + formalin Group, rats were injected intraperitoneally (I.P.) with 40 mg/kg minocycline (Sigma-Aldrich, USA) each day for 7 days (from day 0 to day 6). This dose significantly decreased nociceptive behavior after formalin injection or peripheral nerve injury and inhibited spinal cord microglial activation (Cho et al., 2006; Raghavendra et al., 2003). One hour after the first minocycline injections, 100 µl of 5% formalin was injected subcutaneously into the plantar surface of the right hind paw.

The Saline + formalin Group received I.P. saline daily for 7 days (a same total volume at each injection as minocycline) instead of minocycline and received the same 5% formalin injection as the Minocycline + formalin Group.

The Saline + saline Group, received saline instead of minocycline daily for 7 days, as in the Saline + formalin Group, and also received 100 µl saline instead of 5% formalin injected into the rat hind paw.

On day 7 after the initial injection of 5% formalin or saline, all three groups of rats received a second injection of formalin (1%, 50 µl) into the dorsal surface of the right hind paw. Immediately after this injection, the number of paw flinches was recorded in a 5-minute interval for 1 h. Rats were then sacrificed for the OX-42 and MHC class I immunostaining.

2.1.3. Experiment 3

The mitogen-activated protein kinases (MAPKs) are a family of intracellular signaling molecules that respond to a wide variety of extracellular stimuli. The p38 MAPK is activated by phosphorylation in spinal cord microglia following peripheral inflammation and nerve injury, and plays a role in spinal nociceptive processing (Hua et al., 2005; Jin et al., 2003; Kim et al., 2002; Svensson et al., 2003; Svensson et al., 2005; Tsuda et al., 2004). We previously observed that phosphorylated p38 (phospho-p38) was reliably increased on day 3 after the injection with 5% formalin injection (unpublished data). In this experiment, we investigated whether minocycline might produce antinociceptive effects by blocking the p-p38 expression as determined with immunohistochemistry and Western blots. The same experimental design as for Experiment 2 was used including a Minocycline + formalin Group ($n=5$), Saline + formalin Group ($n=5$), and Saline + saline Group ($n=5$), except that minocycline or saline was given I.P. once per day for three days beginning at 1 h before the first formalin injection. On day 3 after the 5% formalin injection all rats were sacrificed to obtain spinal cord samples for immunohistochemical staining or Western blots.

2.2. Assessment of formalin-induced nociceptive behavior

Subcutaneous formalin injection into a hindpaw produces two phases of reproducible nociceptive behaviors including licking, biting, or flinching/shaking of the injected paw. To observe these behaviors, animals were acclimated individually to a clear Plexiglas cage for at least 1 h prior to testing. In this study, we recorded the number of paw flinches per 5-minute unit for a total of 1 h. The behavioral response consisted of an early, short-lasting (3–5 min) period of activity (Phase I) that occurred immediately after the injection. This phase was followed 10–15 min later by a prolonged phase of behavioral activity lasting 20–40 min (Phase II). To quantitatively evaluate the formalin-induced pain behavior, we compared the mean number of paw flinches during the 0–5 min and 20–40 min time block after the formalin injection.

2.3. Immunohistochemistry

Animals (4 rats for each group) were anesthetized with an overdose of pentobarbital sodium and euthanized by trans-cardiac perfusion (250 ml body temperature 0.1 M PBS pH 7.4 followed by 200–300 ml ice-cold 4% paraformaldehyde/4% sucrose in 0.1 M PB pH 7.4). After perfusion, the lumbar spinal cords (L4-5) were removed, postfixed in the same 4% paraformaldehyde fixative for 4 h and then placed in 30% sucrose solution (in 0.1 M PB) overnight at 4 °C. Thirty micron thick tissue sections were cut transversely on a cryostat for free-floating immunohistochemical staining for OX-42 (monoclonal mouse anti-rat CD11b, 1:200, Serotec, U.K.), MHC class I antigen (mouse anti-rat MHC class I RT1A, MRC OX-18, 1:2000, Serotec), and p-p38 (rabbit polyclonal anti-phospho-p38, 1:200, Cell Signaling). All of the sections were blocked with 5% normal goat serum in 0.3% Triton X-100 for 1 h at room temperature (RT) and incubated for 48 h at 4 °C with primary antibody. The sections were then incubated for 90 min at RT with a corresponding FITC-conjugated secondary antibody. For double immunofluorescence,

tissues were incubated with a mixture of primary antibody p-p38 with OX-42. The single- or double-stained images were captured with a CCD spot camera and processed using Adobe Photoshop.

To analyze OX-42 and MHC class I immunoreactivity, the immunofluorescence intensity was measured. The medial portion of the spinal cord dorsal horn was outlined as an area of interest, as described in our previous reports (Fu et al., 1999; Lin et al., 2007b). The average percentage of area with OX-42-IR (immunoreactivity) or MHC class I-IR relative to the total outlined area of interest was calculated from three selected tissue sections for each animal. The immunoreactivity level was expressed as the fold increase compared to controls.

2.4. Western blotting

Rats (3 rats for each group) were deeply anesthetized and decapitated. The spinal cord segments (L4–L5) ipsilateral to the formalin injection were removed rapidly and homogenized in a lysis buffer (20 mM Tris buffer, pH 7.6, containing 150 mM NaCl, 1% NP-40, 5% sodium deoxycholate, 1 mM EDTA, 2 mM sodium orthovanadate, 1 mM PMSF, phosphatase and protease inhibitor cocktail; Sigma). The homogenate was centrifuged at 15,000 *g* for 45 min at 4 °C. The protein concentration of tissue lysates was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Twenty microgram aliquots were subjected to 12% SDS-PAGE, and proteins were transferred electrophoretically to PVDF filters (Millipore, Bedford, MA, USA). After being blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h in room temperature, membranes were incubated with an anti-phospho-p38 antibody (1:1000, in 5% BSA; Cell Signaling), p38 (1:1000, Cell Signaling) and β-actin (1:1000, Santa Cruz) antibody overnight at 4 °C. After washing, the antibody–protein complexes were probed with HRP-conjugated secondary antibody (1:10,000, Jackson ImmunoResearch), developed in ECL solution for 3 min, and exposed onto Kodak hyperfilms. The density of immunoreactive bands was quantified using NIH ImageJ 1.38 software (National Institutes of Health, Bethesda, MD, USA), normalized to the density of internal control (phospho-p38/β-actin, total p38/β-actin) and expressed as the fold change as compared to Control Group.

2.5. Statistical analysis

Paw flinches, OX-42 and MHC class I immunoreactivity, and phospho-p38 protein level were statistically analyzed. All data were reported as the mean ± SEM. Differences between groups were compared by one-way ANOVA followed by Fisher's PLSD post-hoc analysis (multiple groups). The criterion for statistical significance was $P<0.05$.

3. Results

3.1. Enhanced pain behavior following second formalin injection

In our previous study, injection of 5% formalin into a rat hind paw induced spinal microglial activation as indicated by morphological changes and OX-42 labeling at 3–7 days after the injection (Fu et al., 1999, 2009). In this experiment, animals were tested with a second injection of 1% formalin in order to examine differences in nociceptive behavior at 1 and 7 days after the first injection of 5% formalin. This design allowed us to examine whether the initial 5% formalin injection-induced microglial changes contributed to nociceptive behavior evoked by 1% formalin injection. Our results showed that nociceptive behavior from both Phase I and Phase II after the 1% formalin test was enhanced in those rats receiving the first formalin (5%) injection 7 days before, as compared to the Control Group (Fig 1A), such that the mean number of paw flinches from the time

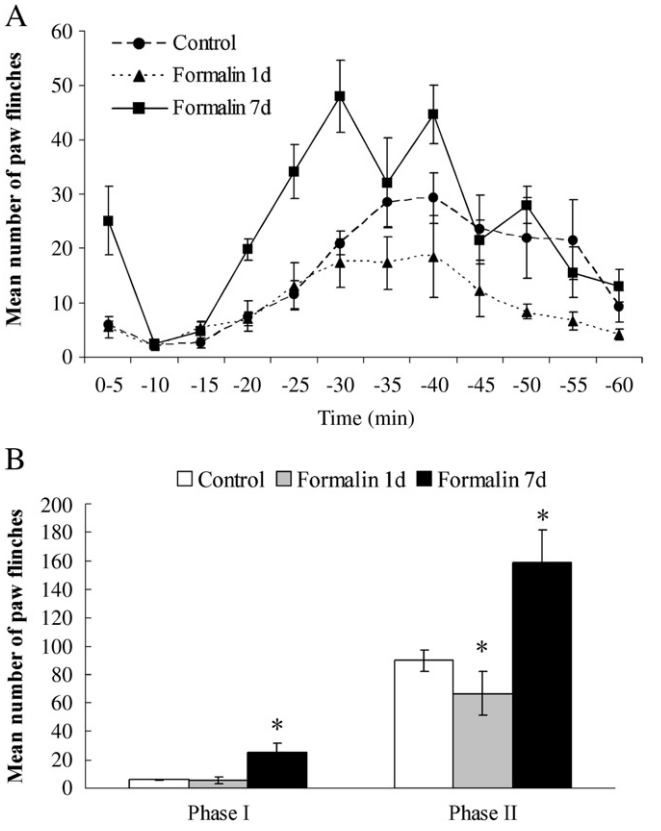

Fig. 1. Time course of nociceptive behavior indicated by paw flinchings following second formalin (1%) injection into the dorsal side of a hindpaw (A). The number of paw flinches induced by 1% formalin injection in Phase I (0–5 min) and Phase II (20–40 min) was quantitatively compared among three groups (B). Formalin 7d: rats received 5% formalin injection at 7 days before (into plantar surface of paw); Formalin 1d: rats received 5% formalin injection at 1 day before (into plantar surface of paw); Control: without prior 5% formalin injection. *$P<0.05$, as compared with the Control Group.

block 0–5 min (Phase I) and 20–40 min (Phase II) was significantly higher in the Formalin 7d Group than the Control Group (Fig 1B). This enhancement of nociceptive behavior was not observed in the group of rats tested with 1% formalin one day after the 5% formalin injection (Fig. 1A, B). Instead, there was a decrease in Phase II nociceptive behavior in these rats (Fig. 1A, B).

3.2. Effect of minocycline on nociceptive behavior

Rats in the Minocycline + formalin Group received the minocycline treatment once per day for 7 consecutive days, beginning 1 h before the first formalin (5%) injection. This treatment significantly attenuated nociceptive behavior induced by the second formalin injection on day 7 (Fig. 2). Because pain behavior was also reduced as measured with the 1% formalin test at one day after the 5% formalin injection (see Fig. 1A, B), we could not test whether minocycline also reduced nociceptive behavior at this time point. In contrast, rats in the Saline + formalin Group received no minocycline treatment and showed significantly increased biphasic spontaneous pain behaviors induced by the formalin (1%) test at 7 days after the first formalin (5%) injection, as compared to the Saline + saline Group (Fig. 2).

3.3. Lack of immune marker changes after minocycline

As reported previously (Fu et al., 1999, 2009), 5% formalin injection produced spinal microglial changes characterized by the morphological and immune marker changes (e.g., OX-42 and MHC class I antigen immunohistochemistry), which began on day 3 and

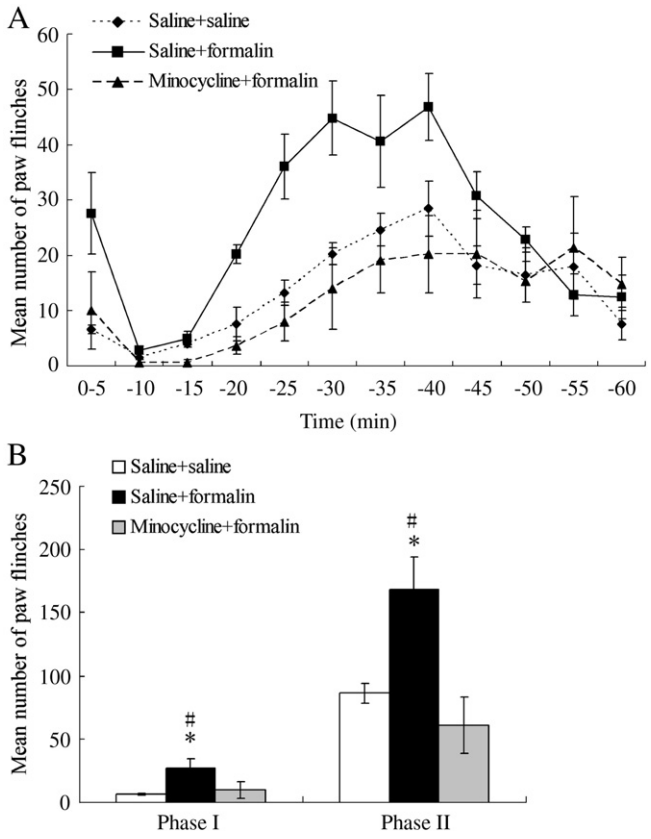

Fig. 2. Effect of minocycline treatment on the second (1%) formalin-induced nociceptive behavior on day 7 after the first formalin (5%) injection. All animals were tested after 1% formalin injection into the dorsal surface of the hindpaw. A: Time courses of nociceptive behavior following the second formalin injection. B: Quantitative comparison for the mean number of paw flinches in Phase I and Phase II among different treatment groups. *Minocycline + formalin*, the plantar hind paw was injected 5% formalin 1 h after the first of 7 daily I.P. minocycline injections; *Saline + formalin*, the plantar hind paw was injected 5% formalin 1 h after the first of 7 daily I.P. saline injections; *Saline + saline*, the plantar surface was injected with saline (instead of 5% formalin) after the first of 7 daily I.P. saline injections. $^{*}P<0.05$, as compared with the Saline + saline Group; #$P<0.05$, as compared with the Minocycline + formalin Group.

continued through at least day 7 after the injection. In this experiment, we specifically examined whether these microglial changes could be blocked by the minocycline treatment. The results showed that the minocycline treatment failed to alter the significant upregulation of OX-42 and MHC class I antigen expression and the associated morphological changes observed at 7 days after formalin injection (Figs. 3 and 4). This observation was further supported by semi-quantitative measurement of immunofluorescence intensity of each immune marker measured (Figs. 3 and 4). Thus, systemic minocycline treatment at the current dose did not alter microglia morphology and immune marker changes including an increase in MHC class I antigen in microglia.

3.4. Inhibition of microglial p38 activation after minocycline

As reported previously (Svensson et al., 2003), formalin injection rapidly increased (within minutes) p38 phosphorylation (phospho-p38) in spinal cord microglia before any morphological changes were observed. Because the results from our pilot experiments showed that the p38 activation began within 1 h after the formalin injection and peaked on day 3, we selected day 3 as the time point to examine whether the minocycline treatment could inhibit the microglial p38 activation. A significant increase of p38 activation as determined by immunohistochemistry was detected in the ipsilateral spinal cord dorsal horn at 3 days after the 5% formalin injection (Fig. 5A, B). The phospho-p38 positive cells were identified as microglia by double-labeling with OX-42 (Fig. 5D). The increased phospho-p38 expression in microglia was significantly reduced by the minocycline treatment (Fig. 5C), which was also shown quantitatively by the Western blot analysis (Fig. 5E, F), whereas the total p38 protein level was not changed after the minocycline treatment (data not shown).

4. Discussion

4.1. Methodological considerations

Subcutaneous formalin injection is extensively used to study the mechanisms of persistent nociception and to evaluate the antinociceptive effect of various endogenous and exogenous substances. Injection of formalin into the rat's hind paw produces two phases of

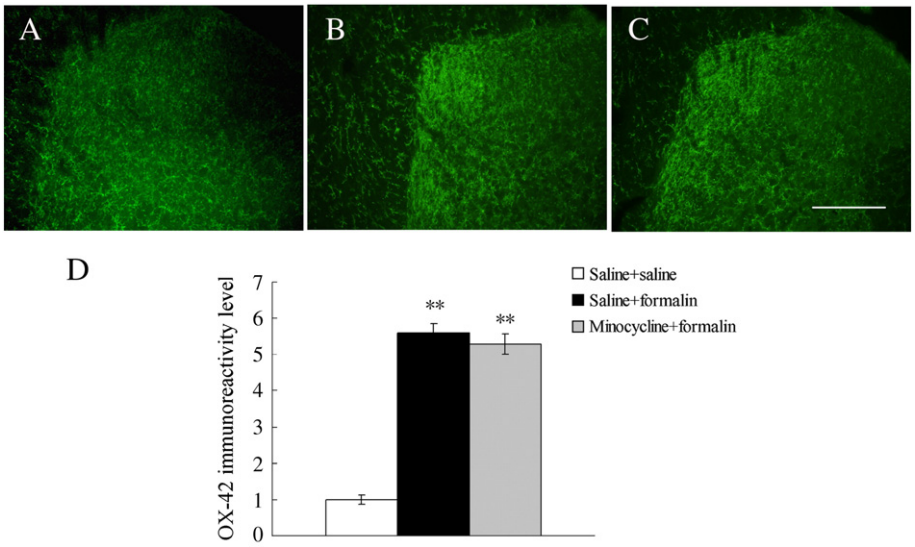

Fig. 3. Effect of the minocycline treatment on formalin-induced OX-42 immunoreactivity in spinal microglia on day 7 after 5% formalin injection. OX-42 immunoreactivity was shown on the ipsilateral side of the spinal cord dorsal horn in the Saline + saline Group (A), Saline + formalin Group (B), and Minocycline + formalin Group (C). Scale bar, 200 μm. The intensity of OX-42 immunoreactivity was compared among three groups (D). $^{**}P<0.01$, as compared with the Saline + saline Group.

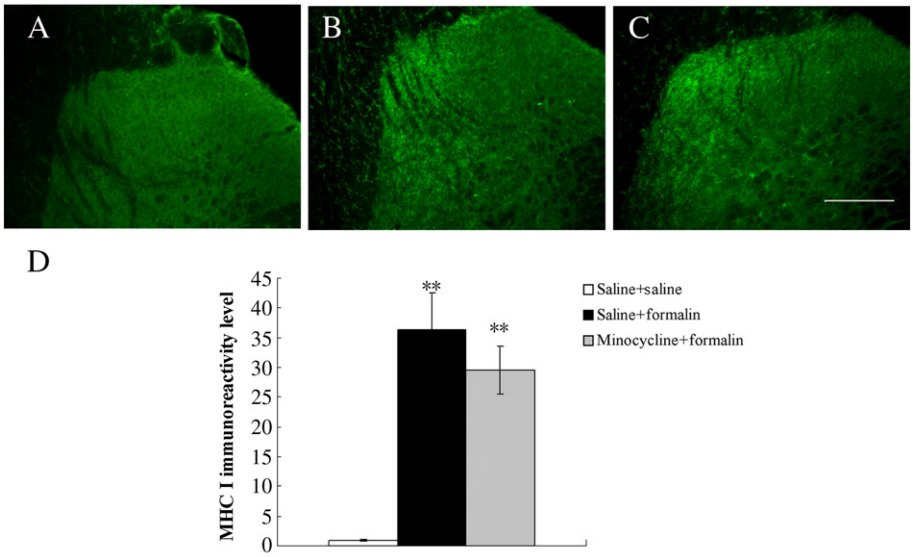

Fig. 4. Effect of the minocycline treatment on formalin-induced MHC class I immunoreactivity in spinal microglia on day 7 after 5% formalin injection. MHC class I was not expressed in the Saline + saline Group (A), but highly expressed in the medial portion of the ipsilateral side of the spinal cord dorsal horn in the Saline + formalin Group (B) and Minocycline + formalin Group (C). Scale bar, 200 μm. The intensity of MHC class I immunoreactivity was compared among three groups (D). $^{**}P<0.01$, as compared with the Saline + saline Group.

nociceptive behaviors. The first phase lasts about 5 min and results from direct chemical activation of myelinated and unmyelinated nociceptive afferent fibers. After a short quiescent period, the second phase, characterized by persistent shaking, flinching, or licking of the injected paw, begins and lasts approximately 40 min (Dubuisson and Dennis, 1977). It has been suggested that central sensitization in the spinal cord plays an important role in the late phase of formalin-induced hyperalgesia (Bianchi and Panerai, 1997; Cadet et al., 1995; Coderre et al., 1990; Wiertelak et al., 1994). In our previous study, we demonstrated that 5% formalin injection resulted in prolonged secondary hyperalgesia and allodynia on the hind paw surface (e.g., dorsal surface) opposite to the surface (e.g., plantar) of formalin

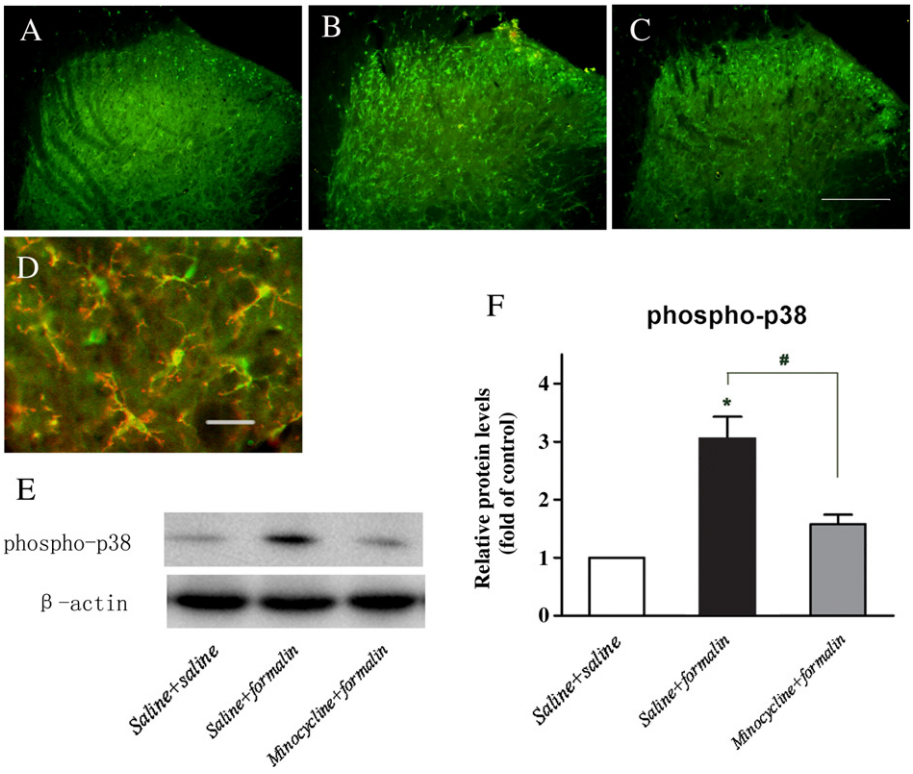

Fig. 5. Effect of the minocycline treatment on formalin-induced microglial p38 activation on the ipsilateral side of the spinal cord dorsal horn on day 3 after 5% formalin injection. Less phospho-p38 expression was shown in the Saline + saline Group (A). A significant increase of p38 activation was present following formalin injection without minocycline (B), which was reduced in the presence of the minocycline treatment (C). Scale bar, 200 μm. Phospho-p38 positive cells were colocalized with OX-42 (D). Scale bar, 40 μm. (E) Representative bands of Western blot showing levels of phospho-p38 from three groups. (F) Percentage change of the spinal phospho-p38 with and without minocycline treatment was compared to the Control Group. Data were normalized against the beta-actin expression in each sample. $^{*}P<0.05$ as compared with the Saline + saline Group; $\#P<0.05$, comparison between the Saline + formalin Group and Minocycline + formalin Group.

injection for 2 weeks (Fu et al., 2001). Cadet et al (1995) injected 10% formalin into the left upper lip and evaluated nociceptive behavior triggered by another 5% formalin injection in the contralateral right upper lip. Their results showed that an enhanced nociceptive response to the formalin test was present for at least 7 to 14 days after the first formalin injection. These findings indicate that after a single high concentration (5% or 10%) formalin injection, central sensitization may be developed and maintained for at least 1–2 weeks, contributing to the enhanced response to a subsequent formalin injection (Cadet et al., 1995). Interestingly, investigators have indicated that "double hits" of mild nociceptive stimuli may induce very long-lasting and more wide-spread hypersensitivity in muscles. For example, two injections of acid saline into muscles 2 days or 5 days apart produced up to a month of muscle wide-spread pain enhancement (Sharma et al., 2009; Skyba et al., 2002). It is possible that some of the changes in phospho-p38 in the spinal cord we observed here may be involved in this long-term enhancement of pain.

In the present study, we demonstrated that the initial 5% formalin injection enhanced the spontaneous nociceptive responses caused by a second injection of 1% formalin given 7 days but not 1 day later, indicating that the later days (2–7 days) of microglial activation with upregulated immune markers in the spinal cord contributed to the enhanced nociceptive response. Of interest is that spontaneous nociceptive behaviors were actually decreased in those rats receiving the 1% formalin injection 1 day after the 5% formalin injection relative to controls receiving no prior injection, only the one injection of 1% formalin. This observation was consistent with our previous report that nociceptive behavior evoked by heat or mechanical stimulation was reduced at 24 h after 5% formalin injection but was increased again at 2 days after the 5% formalin injection and this increase lasted for at least 2 weeks (Fu et al., 2001). Perhaps a short-term, central inhibitory mechanism lasting 24–36 h is evoked by 5% formalin injection.

4.2. Effect of minocycline on nociceptive behavior and microglial p38 activation

Minocycline is a putative inhibitor of microglial activation and proliferation without a direct action on astrocytes or neurons (Amin et al., 1996; Tikka and Koistinaho, 2001). Several groups have recently demonstrated that administration of minocycline either systemically or intrathecally attenuated mechanical hyperalgesia and/or allodynia in rat models of neuropathy, traumatic spinal cord injury, and peripheral inflammation (Cho et al., 2006; Hains and Waxman, 2006; Hua et al., 2005; Ledeboer et al., 2005; Piao et al., 2006; Raghavendra et al., 2003). This effect was associated with the inhibition of spinal microglial activation as determined by OX-42 labeling, inhibition of expression of pro-inflammatory cytokines as determined by quantitative mRNA expression, and cytokine protein measurements. On the other hand, several other studies have shown the lack of effect by the minocycline treatment on microglial activation and morphological changes in activated microglia (Fendrick et al., 2005; Fox et al., 2005; Hua and Walz, 2006). In the present study, systemic minocycline treatment attenuated the enhanced spontaneous nociceptive behaviors in those rats receiving 5% formalin injections when tested with 1% formalin injections 7 days later. While, importantly, we identified differential changes in spinal microglial markers. The minocycline treatment did not prevent the increase in OX-42 and MHC class I labeling and morphological changes, but significantly attenuated upregulation of phospho-p38 in activated microglia induced by the formalin injection. Minocycline is a tetracycline anti-biotic and as such has profound anti-inflammatory properties. Since we injected the minocycline IP, it could have a peripheral effect in our experiments. Minocycline's anti-biotic activity could alter peripheral inflammation and immune response at the formalin injection site, thus influencing the signaling to the spinal cord and microglial marker staining. However, our previous reports showed that peripheral inflammation alone did not induce microglial activation with morphological changes and phenotypic changes such as CD11b and MHC class I (Fu et al., 2009; Lin et al., 2007b). Also peripheral inflammation alone did not increase p38 phosphorylation in the spinal dorsal horn (Ji et al., 2002). Thus, the potential anti-inflammatory effects of minocycline at the formalin injection site could not account for either the morphological activation of the microglia (these effects would have been seen whether or not the minocycline was an effective anti-inflammatory agent), or the decrease in phospho-p38 observed (inflammation does not increase p38 phosphorylation), therefore blocking peripheral inflammation should not have caused a decrease in phospho-p38. These observations suggest that spinal cord, not systemic effects of minocycline caused the effects we observed here. Still, we cannot be certain that minocycline also had an effect at the formalin injection site. In our another study (unpublished data), intrathecal treatment of the p38 inhibitor SB203580 (10 μg in 10 μl) once per day for 7 days significantly reversed 5% formalin-induced mechanical hyperalgesia. Collectively, systemic minocycline differentially influences changes in spinal microglial markers following formalin-induced nociception. There appears to be a strong correlation between p38 activation in microglia and the enhanced nociceptive behavior, which is consistent with previous studies demonstrating that inhibition of spinal p38 activation reduced nociceptive behaviors in animal models of inflammation and peripheral neuropathy (Ji and Suter, 2007; Jin et al., 2003; Svensson et al., 2003; Svensson et al., 2005; Tsuda et al., 2004).

In summary, our results demonstrate that nociceptive behavior evoked by a second formalin (1%) injection was enhanced at 7 days, but not 1 day, after the initial 5% formalin injection. This enhanced pain behavior was prevented by 7 consecutive days of systemic minocycline administration. Minocycline also reduced spinal microglial activation as determined by the attenuation of phospho-p38 expression without altering later morphological and immune marker changes such as OX-42 and MHC class I immunohistochemical staining. These results suggest that the later days of microglial activation with upregulated immune markers in the spinal cord contribute to enhancing long-term pain response by a pathway of p38 activation in microglia.

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