Activation of p38 Mitogen-activated Protein Kinase in Spinal Microglia Contributes to Incision-induced Mechanical Allodynia

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Background: Recent studies have implicated the activation of stress-activated mitogen-activated protein kinase (MAPK) p38 in spinal microglial cells for development of neuropathic and inflammatory pain. The aim of the present study was to investigate whether phosphorylation of p38 (p-p38) also mediates mechanical allodynia and thermal hyperalgesia induced by plantar incision.

Methods: After rats received a plantar incision surgery, mechanical allodynia and thermal hyperalgesia were determined by von Frey filaments and radiant heat, respectively, and the number of p-p38 immunoreactive cells in the dorsal horn was quantified to determine p38 activation at different time points after incision. The p38 inhibitor FR167653 was administered intrathecally 30 min before hind paw plantar incision to determine the role of p38 in postoperative pain.

Results: A significant increase in number of p-p38 immunoreactive cells was observed in the ipsilateral L4-5 spinal dorsal horn from 1 h to 3 days after the incision. p-p38 was found predominantly in microglia. However, microglial activation (assessed by OX-42 upregulation) was not evident until 3 days after plantar incision. Intrathecal pretreatment of FR167653 attenuated incision-induced mechanical allodynia from 1 h to day 2 and significantly reduced activation of p38 in the dorsal horn 1 day after plantar incision. However, FR167653 only inhibited heat hyperalgesia at an early time point.

Conclusions: Plantar incision-induced mechanical allodynia can be prevented by the p38 inhibitor. Our results suggest that p38 activation in spinal microglia play a role in incision-induced mechanical allodynia in rats. Therefore, p38 inhibition may be useful in treating postsurgical pain.

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POSTOPERATIVE pain results from surgical injuries in skin, fascia, muscle, and small nerves innervating these tissues and is a common form of acute pain in clinics. Despite increased basic and clinical research and improved understanding of pathologic mechanisms, optimal postsurgical pain therapy remains a challenge for physicians. To investigate the underlying mechanisms of postsurgical pain, Brennan *et al.*[1] have developed a rat model of incisional pain that is characterized by increased mechanical sensitivity after a surgical incision made in the plantar aspect of rat hind paw. This model has been shown to possess a battery of nociceptive responses that parallel the time course of postoperative pain in humans.[1,2]

Several studies based on this surgical pain model have suggested discrepancies between surgical incision and other pathologic pain models.[3–5] For example, surgical incision-induced central sensitization is more likely mediated by activation of non-*N*-methyl-D-aspartate, metabotropic glutamate receptor, and neurokinin-1 receptors in spinal dorsal horn,[4,6,7] but not by *N*-methyl-D-aspartate receptors,[5,8] which play an important role in inflammatory hyperalgesia.[9] Therefore, our knowledge of inflammatory or neuropathic pain may not apply to postsurgical pain conditions.

Mitogen-activated protein kinases (MAPKs) are activated through various upstream protein kinases and transduce diverse extracellular stimuli into intracellular posttranslational and transcriptional responses.[10] The members of the MAPK family, including extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38, have been shown to be critical for the development and maintenance of inflammation-induced and nerve injury-induced pain hypersensitivity.[11,12] p38 is a stress-activated protein kinase, and growing evidence suggests that activation of p38 MAPK in the spinal cord and dorsal root ganglion contributes to the development and maintenance of inflammatory[13–16] and neuropathic pain.[14,17–20] However, the role of p38 MAPK in incision-induced pain hypersensitivity remains unclear.

The present study was designed to investigate whether activation (phosphorylation) of p38 MAPK in the spinal cord is involved in pain hypersensitivity after a plantar incision in the rat hind paw. Using immunohistochemical and behavioral approaches, we examined the temporal correlation between the incision-induced nocifensive behaviors and p38 activation in the spinal cord dorsal horn. We also investigated the preventive effects of intrathecal injection of p38 inhibitor, FR167653, before

plantar incision on postsurgical mechanical allodynia and thermal hyperalgesia.

Materials and Methods

Animals

Male Sprague-Dawley rats (250–300 g, CD® [SD] IGS; National Laboratory Animal Center, Taiwan) were used. The animals were housed in groups of three to four per cage and acclimatized to the laboratory conditions (12-h light/dark cycle; 22 ± 1°C room temperature) 1 week before experiments. Animals had free access to food and water. All experiments were carried out in accordance with the guidelines established by the Institutional Animal Care and Utilization Committee, Fu Jen Catholic University, Taipei County, Taiwan. Efforts were made to minimize animal suffering and the number of animals.

Surgery

Plantar Incision. The plantar surgery was performed as previously described.[1] All animals were anesthetized with isoflurane (4% to induce; 1.5–2% to maintain) in air delivered *via* a nose cone. The plantar aspect of the left hind paw was sterilized with a 10% povidone-iodine solution before and after surgeries and was placed through a hole in a sterile drape. A 1-cm longitudinal incision was made through skin and fascia of the plantar aspect of the foot, starting 0.5 cm from the proximal edge of the heel and extending toward the toes. The plantaris muscle was elevated and longitudinally incised through, leaving muscle origin and insertion intact. After hemostasis with gentle pressure, the skin was apposed with 2 mattress sutures of 5-0 nylon. The animals were allowed to recover. The incision was checked daily, and any sign of wound infection or dehiscence excluded the animal from the study.

Intrathecal Cannulation. Animals were anesthetized with chloral hydrate (intraperitoneal injection, 400 mg/kg; Kanto Chemical Co. Inc., Tokyo, Japan), and a thoracic laminectomy was performed. A midline incision was made at the lower thoracic spines. The dura was cut, a PE5 polyethylene tubing (OD = 0.355 mm; ID = 0.2 mm) adhering to PE50 tubing (OD = 0.956 mm; ID = 0.58 mm) was inserted into the subarachnoid space, and the tip of the catheter was located near the lumbar enlargement of the spinal cord. The catheter was secured to the paravertebral muscles with suture, and the external end of the catheter was sealed by heat. The wound was then closed in layers, and animals were returned to their home cages for recovery at least 3 days before plantar incision. The patency of catheter was confirmed by paralysis of hindlimbs after a 10-μl 2% lidocaine injection the next day after catheterization. Rats with any neurologic deficits were excluded from the experiment.

Spinal Drug Delivery

The p38 inhibitor FR167653 (a gift from Fujisawa Pharmaceutical Company, Osaka, Japan) was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to obtain a concentration of 50 mg/ml as a stocking solution and then diluted to 10 μg/μl (20% DMSO), 5 μg/μl (10% DMSO), and 2 μg/μl (4% DMSO) in sterile saline on the injection day. FR167653 (100 μg, 50 μg, and 20 μg) or diluted vehicle (20%, 10%, and 4% DMSO) was injected intrathecally (10 μl) followed by flush with 20 μl of saline 30 min before plantar incision.

Behavioral Experiments

All animals were habituated to the testing environment from 2 days before baseline testing. For mechanical stimulation, animals were individually placed in a chamber (10 × 10 × 20 cm) of a Plexiglas cage on an elevated iron mesh floor and allowed to acclimate for 20 min before testing. The von Frey filaments were applied from underneath the mesh openings to stimulate the plantar surface at an area adjacent to the wound near the medial heel. A series of von Frey filaments with incremental stiffness (0.4, 0.6, 1, 2, 4, 6, 8, and 15 g; Stoelting, Wood Dale, IL), always started from the 2.0-g filament, were presented perpendicular to the plantar surface for 5–6 s for each filament. Stimulation was conducted in a consecutive fashion, whether ascending or descending using up-down method as previously described.[21] In the absence of paw withdrawal response to the initial selected filament, a stronger stimulus was presented; in the event of paw withdrawal, the next weaker stimulus was chosen. In cases in which threshold fell outside the range of detection, 0.4 and 15.0 g were assigned for the minimal and maximal value, respectively. Threshold value was an average of two test measurements at each time point and was used for calculation of the 50% withdrawal threshold.[22] The animals were tested daily from 2 days before surgery, and then 1, 3, and 5 h and 1, 2, 3, 5, and 7 days after surgical incisions.

For thermal hyperalgesia, rats were placed individually in the Plexiglas cage on a glass floor that had been preheated at constant 30°C (Plantar Test Apparatus; IITC, Woodland Hills, CA). After animal accommodation, a focused radiant heat source underneath the glass floor was applied at the incision midpoint of the left paw. The intensity of the emitting heat was adjusted to produce withdrawal latency at about 20–25 s in normal rats. Cut-off latency was predetermined at 30 s to avoid paw injury. Each rat was tested three times at an interval of 5 min, and the average of the three measurements was recorded as withdrawal latency at the time point. The data were obtained before surgery, then 1, 3, and 5 h and 1, 2, 3, 5, and 7 days after surgery. Preoperative baselines of both mechanical and thermal tests were tested daily for 2 days and averaged.

DMSO was reported to have antiinflammatory[23] and analgesic properties; therefore,[24] its use as a solvent for FR167653 may be of concern. As a result, two control groups, saline and DMSO, are included in the studies of p38 inhibitor administration. Specifically, 10% and 20% DMSO were given as vehicle controls of 50 μg and 100 μg FR167653, respectively. At least 8 rats were included for each group in the behavioral experiment with FR167653 and vehicle treatment. The same experimenter who did the mechanical and thermal stimulation was blinded to the group allocation of the rats.

Immunohistochemistry

At 1 h, 3 h, 1 day, 3 days, and 7 days postincisional time points, animals were sacrificed with an overdose of intraperitoneal injection of chloral hydrate (650 mg/kg) and perfused transcardially with saline (room temperature) followed by 4°C 4% paraformaldehyde in 0.1 M phosphate buffer. The L4-5 spinal segments were carefully removed, postfixed in the same fixative for overnight, and then cryoprotected in cold 30% sucrose/phosphate buffer overnight. The tissues were frozen at –20°C in a cryostat and transverse free-floating spinal sections (30 μm) were cut and collected in 0.1 M phosphate buffer. After blocking with 2% normal goat serum containing 0.3% Triton X-100 for 1 h at room temperature, all the sections were incubated with either a rabbit polyclonal antiphospho-p38 MAPK primary antibody (1:500; Cell Signaling Technology, Beverly, MA) or a mouse monoclonal anti-OX-42 (CD11b, microglia marker, 1:2000; Serotec, Indianapolis, IN) at 4°C for two nights. Then, sections were incubated with either goat biotinylated anti-rabbit or goat biotinylated anti-mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 2 h and subsequently with ExtraAvidin peroxidase (1:1000, Sigma-Aldrich) for 1 h at room temperature. After rinsing in 0.1 M phosphate buffer for 20 min, sections were reacted with 0.25% 3,3′-diaminobenzidine tetrahydrochloride solution in phosphate buffer containing 0.0025% hydrogen peroxide (Sigma-Aldrich) for 6 min. Finally, sections were mounted onto gelatin-coated glass slides, air-dried, dehydrated, cleared with xylene, and coverslipped with Entellan mounting medium (Merck, Darmstadt, Germany).

For double immunofluorescence, sections were incubated with a mixture of primary antibodies rabbit polyclonal antibody against phosphorylated p38 (p-p38) (1:500; Cell Signaling Technology) and mouse monoclonal antibody against neuronal specific nuclear protein (NeuN, a neuronal marker) (1:4000; Chemicon, Temecula, CA), glial fibrillary acidic protein (GFAP; an astrocyte marker) (1:5000; Chemicon), or OX-42 (1:4000, Serotec) for 36–48 h at 4°C, followed by a mixture of goat anti-mouse fluorescein isothiocyanate– conjugated and goat anti-rabbit indocarbocyanine–conjugated secondary antibodies (1:200; Jackson ImmunoResearch Inc., West Grove, PA) for 1 h at room temperature. Then, the double-stained sections were observed with a Nikon fluorescence microscopy (Tokyo, Japan), and images were captured

with a CCD camera (SPOT; Diagnostic Instruments, Sterling Heights, MI).

Quantification and Statistical Analyses

All the results were expressed as mean ± SEM. To assess the incision-induced mechanical and thermal thresholds before and after plantar incision, the main effects of different time points and treatments as well as interaction of treatment × control effect were analyzed by two-way analysis of variance (ANOVA). Then, repeated-measures of ANOVA were applied to assess the incision-induced mechanical and thermal threshold before and after plantar incision by using the preincisional baselines as control, and one-way ANOVA was applied to compare group differences after FR167653 treatment at each time point. All tests were followed by Bonferroni test as the multiple comparison analysis. Analysis was performed with SPSS software (14.0; SPSS Inc., Chicago, IL).

For immunohistochemistry, phospho-p38-immunoreactive (p-p38-ir) cells in L4-5 spinal dorsal horns (laminae I-V) were counted on six randomly chosen sections from each animal and averaged for each animal. In all cases, the number of p-p38-ir cells was quantified at a magnification of ×100. The density of OX-42 labeled microglia ipsilateral to the incision paw was measured microscopically under ×100 magnification in 8 to 10 randomly selected sections from the L4 and L5 spinal cord. Images were digitally captured with a computer-assisted image analysis system (Image J; NIH Image, National Institutes of Health, Bethesda, MD). The images with 2 predefined rectangles located at the medial one half of laminae I–II and 3 rectangles at the white matter ipsilateral to the operated paw were digitally adjusted with gray scales ranging from 0 to 255, and the pixel numbers of each area was counted, according to the method described by Lin et al.[25] The pixel ratio of the average of the medial superficial dorsal horn to the average of the white matter was calculated. Group differences in the number of p-p38-ir cells and the pixel ratio of OX-42 staining were compared with either one-way ANOVA followed by post hoc Bonferroni test, or Student t test for two-group comparison when appropriate. $P < 0.05$ was considered statistically significant.

Results

Activation of p38 in the Spinal Cord after Plantar Incision

To investigate whether p38 MAPK is activated after surgery, we used immunohistochemistry to examine changes of phosphorylated p38 immunoreactive (p-p38-ir) cells in the spinal dorsal horn at different postoperative time points. A very low constitutive expression of p-p38 was found in the L4-5 spinal dorsal horn of naïve rats (n = 6; fig. 1A). Incisions induced an increase in the number of p-p38-ir cells evident in the ipsilateral L4-5
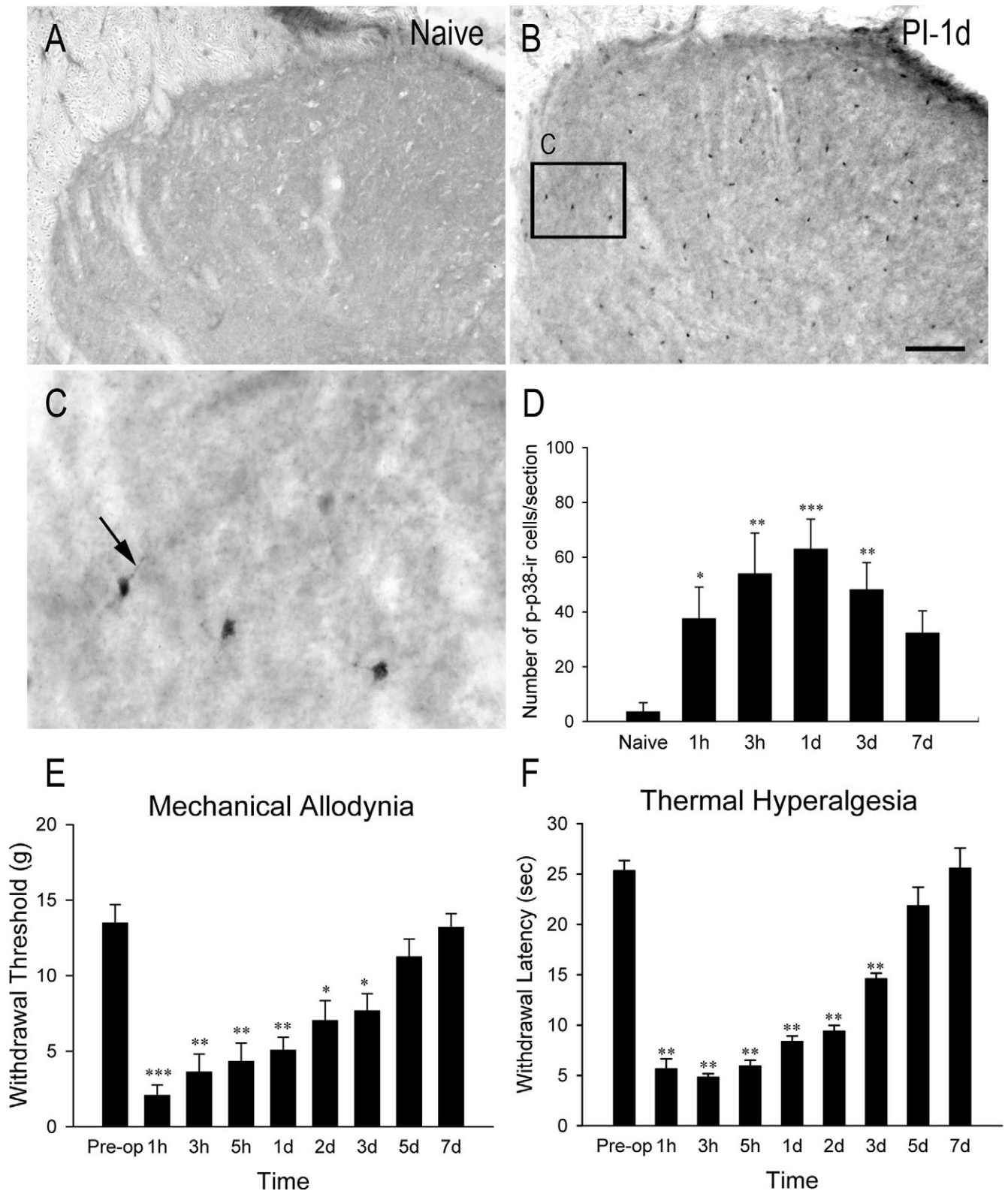
Fig. 1. Plantar incision (PI) induces p38 activation in the ipsilateral L4-5 spinal dorsal horns from (*A*) naïve rats and (*B*) rats at 1 day postoperatively. Higher magnification of p-p38–immunoreactive (ir) cells with long cytoplasmic processes (*arrowhead*) shown in panel *C* from the square marked in panel *B*. (*D*) Quantification of the number of the p-p38 immunoreactive cells per section in the spinal dorsal horn after plantar incision. A significant increase is observed at 1 h, 3 h, day 1, and day 3 postoperative time. Plantar incision reduces paw withdrawal threshold to (*E*) von Frey filaments (n = 6) and to (*F*) radiant heat (n = 8). Data represent the mean ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (repeated measurement ANOVA; n = 6) *vs.* before incision (Preop). Scale bar = 100 μm for *A* and *B*, 20 μm for *C*.

spinal segments (fig. 1, B and C). Quantification of p-p38-ir cells in the L4-5 dorsal horn revealed an increase in p38 activation at 1 h (n = 5), 3 h (n = 7), 1 day (n = 7), 3 days (n = 6), and 7 days (n = 7) after plantar incisions. p-p38 level began to increase at 1 h, reached the peak at day 1, and then maintained for 3 days. Analysis of data revealed a significant difference in the number of p-p38-ir cells in the dorsal horn of the ipsilateral lumbar spinal cord [$F(5,32)$ = 3.914; one-way ANOVA; $P < 0.01$)], and significant increases between the naïve and the incision group were found at 1 h ($P < 0.05$), 3 h ($P < 0.01$), 1 day ($P < 0.001$), and 3 days ($P < 0.01$), postoperatively (fig. 1D). Then, p38 activation decreased at day 7 and did not show statistical significance ($P = 0.062$) compared to the naïve group.

Postoperative Nociceptive Behavior

As reported previously,[1] plantar incision produced a rapid mechanical allodynia beginning at 1 h postoperatively. Analysis revealed that plantar incision resulted in a significant reduction of mechanical withdrawal threshold to von Frey stimulation at 1 h (2.08 ± 0.68 g; $P < 0.001$), 3 h (3.62 ± 1.19 g; $P < 0.01$), 5 h (4.34 ± 1.19 g; $P < 0.01$), 1 day (5.08 ± 0.84 g; $P < 0.01$), 2 days (7.04 ± 1.31 g; $P < 0.05$), and 3 days (7.69 ± 1.12 g; $P < 0.05$) when compared with the baseline value (13.5 ± 1.19 g; n = 6; fig. 1E). This reduction in thresholds recovered at day 5 (11.26 ± 1.16 g; $P > 0.05$).

In addition, plantar incision caused a rapid decrease in the paw withdrawal latency to noxious thermal stimuli (fig. 1F). The mean withdrawal latency to radiant heat was 25.33 ± 1.01 s (n = 8) before incision. After incision, a significant decrease in withdrawal latency was shown at 1 h (5.68 ± 0.98 s; $P < 0.01$), 3 h (4.83 ± 0.34 s; $P < 0.01$), 5 h (5.94 ± 0.58 s; $P < 0.01$), 1 day (8.36 ± 0.55 s; $P < 0.01$), 2 days (9.4 ± 0.58 s; $P < 0.01$), and 3 days (14.59 ± 0.57 s; $P < 0.01$) postoperatively. On postoperative day 5, the withdrawal latency returned to preoperative baseline level (21.85 ± 1.93 s; $P > 0.05$).

Plantar Incision-induced p-p38 Is Localized Primarily in Microglia and in Some Neurons

Previous reports have demonstrated colocalization of p-p38 with OX-42, a marker for microglial cells, in spinal cord in several pain models.[17,26] Here, to identify whether p-p38-ir was expressed in the microglial cells in the plantar incision model, we performed double immunofluorescence staining of p-p38 with several cell-specific markers: NeuN (neurons), GFAP (astrocytes), and OX-42 (microglia) at 1 h, 3 h, and 1 day postoperatively (fig. 2; n = 2 for each time point). p-p38 staining was found in a limited number of the spinal dorsal horn neurons at 1 h (fig. 2, A and B) and 3 h (fig. 2, G and H). Neurons had close contacts with p-p38 immunoreactive cells at the time point of 1 day, but no colocalization was observed between p-p38 and NeuN (fig. 2, M and N).

The majority of p-p38 was localized in OX-42-positive microglia at 1 h (fig. 2, D, E, and F), 3 h (fig. 2, J, K, and L) and 1 day (fig. 2, P, Q, and R). The morphology of p-p38-ir cells, as shown in figure 2, shows small cell bodies with ramified processes. However, no p-p38 was localized in GFAP-positive astrocytes at all the time points examined (fig. 2, C, I, and O).

Plantar Incision–induced Spinal Microglial Activation

The OX-42 immunoreactivity in the L4-5 spinal dorsal horn at different time points after plantar incision was shown in figure 3. In tissue from rats in naïve group and 3 h postoperative time, microglia showed small soma with thin branched processes (fig. 3A). The topographic expression of OX-42 developed gradually from the superficial dorsal horn (at 1 day; fig. 3B) to the whole dorsal horns (at 3 days; fig. 3C) after surgery. Activated microglia were markedly found in the laminae I-II of spinal dorsal horn 3 days after plantar incision, especially at the medial half of the dorsal horn, with marked morphological changes such as increased cell size and thickened branches (fig. 3C). Thereafter, the microglial activation was decreased to basal level at 7 days after surgery (fig. 3D). Statistical analysis showed that there is a significant increase in the pixel ratio of OX-42 immunoreactivity of the ipsilateral superficial dorsal horn to the white matter in sections at 3 days (1.41 ± 0.05; $P < 0.001$, post hoc comparison; fig. 3F) postoperatively, but not at 3 h (1.17 ± 0.05), 1 day (1.24 ± 0.06), and 7 days (1.21 ± 0.02) when compared with those from naïve rats (1.12 ± 0.04; fig. 3E). These results indicate that the most remarkable microglial activation was observed on day 3 after plantar incision.

Inhibition of p38 Prevented Mechanical Hypersensitivity

To investigate the functional roles of activation of p38 in incision-induced mechanical hyperalgesia, we tested whether intrathecal administration of FR167653, a specific p38 inhibitor,[27-29] attenuated mechanical allodynia. First, to examine whether blocking p38 activation in the spinal cord has an effect on baseline withdrawal threshold, we injected saline, vehicle (10% DMSO in saline), or 20 or 50 μg FR167653 intrathecally 30 min before plantar incision. Basal withdrawal thresholds to von Frey filaments were not different after FR167653 injection (fig. 4A). The baseline values of paw withdrawal thresholds for rats in saline, vehicle, and 20 μg and 50 μg FR167653-treatment groups were 14.19 ± 0.45 g (n = 10), 13.93 ± 0.75 g (n = 9), 13.95 ± 0.38 g (n = 8), and 12.54 ± 0.72 g (n = 8), respectively ($P > 0.05$).

Then we tested the effects of pretreatment of FR167653 (20 μg or 50 μg) and the vehicle on mechanical hypersensitivity after plantar incision. An initial analysis of two-way ANOVA showed that the treatment effect, the
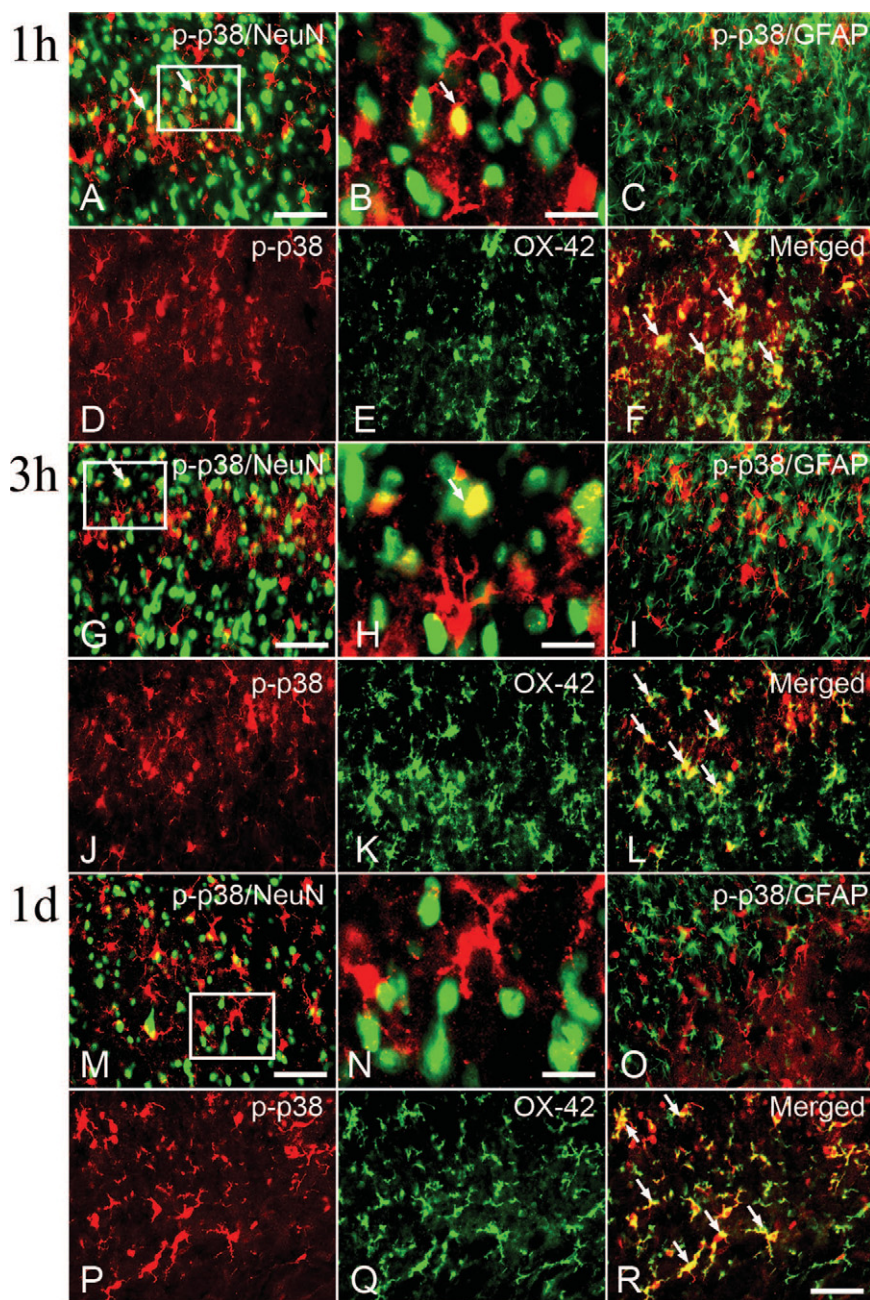
Fig. 2. Double-labeled immunofluorescence of p-p38, neuronal specific nuclear protein (NeuN, a neuronal marker), glial fibrillary acidic protein (GFAP, an astrocyte marker), and OX-42 (a microglia marker) in the spinal dorsal horn ipsilateral to paw incision at 1 h (*A–F*), 3 h (*G–L*), and 1 day (*M–R*) postoperatively. (*A, G,* and *H*) Double-staining demonstrates colocalization (*arrows*) of p-p38 (*red*) and NeuN (*green*) in neurons from spinal cord of the 1-h and 3-h groups, but not in the 1-day group. (*B, H,* and *N*) Higher magnification of colocalization from panels *A, G,* and *M*, respectively. (*C, I,* and *O*) Double-labeling of p-p38 (*red*) with GFAP (*green*) showed no colocalization in the spinal dorsal horn at every time point. (*D–F, J–L,* and *P–R*) Double-staining for p-p38 (*red*) and OX-42 (*green*) reveals a heavy colocalization. Scale bar for *A, C–G, I–M,* and *O–R* = 200 μm; scale bar for *B, H,* and *N* = 50 μm.

time effect, and the interaction of time × treatment were all significantly different (all *P* < 0.001), indicating different time trends in the four groups. Then, analysis of one-way ANOVA revealed that inhibition of spinal p38 activation by 50 μg of FR167653 significantly prevented incision-induced mechanical allodynia at 1 h (*P* < 0.001), 3 h (*P* < 0.001), 5 h (*P* < 0.001), 1 day (*P* < 0.001) and 2 days (*P* < 0.001) (fig. 4A) when compared with animals intrathecally treated with saline. Intrathecal injection of 10% DMSO seemingly produced a mild antiallodynic effect lasting for about 1 day; however, the effects were not statistically significant at any time point. The group receiving 50 μg of FR167653 still showed significant differences compared to the vehicle group at 1 h (*P* < 0.001), 3 h (*P* < 0.01), 5 h

(*P* < 0.01), 1 day (*P* < 0.05), and 2 days (*P* < 0.05). Moreover, treatment with 50 μg of FR167653 results in significant increase of withdrawal threshold in a dose-dependent manner when compared with 20-μg FR167653 treatment at 3 h (*P* < 0.05), 1 day (*P* < 0.01), and 2 days (*P* < 0.05). Intrathecal 20-μg FR167653 treatment could only inhibit postoperative allodynia for 1 h (*P* < 0.01 *versus* saline and vehicle), and it soon lost its antiallodynic effect.

Inhibition of p38 Only Inhibited Thermal Hyperalgesia at an Early Time Point

To elucidate whether inhibition of p38 activation altered withdrawal responses to thermal stimulation,
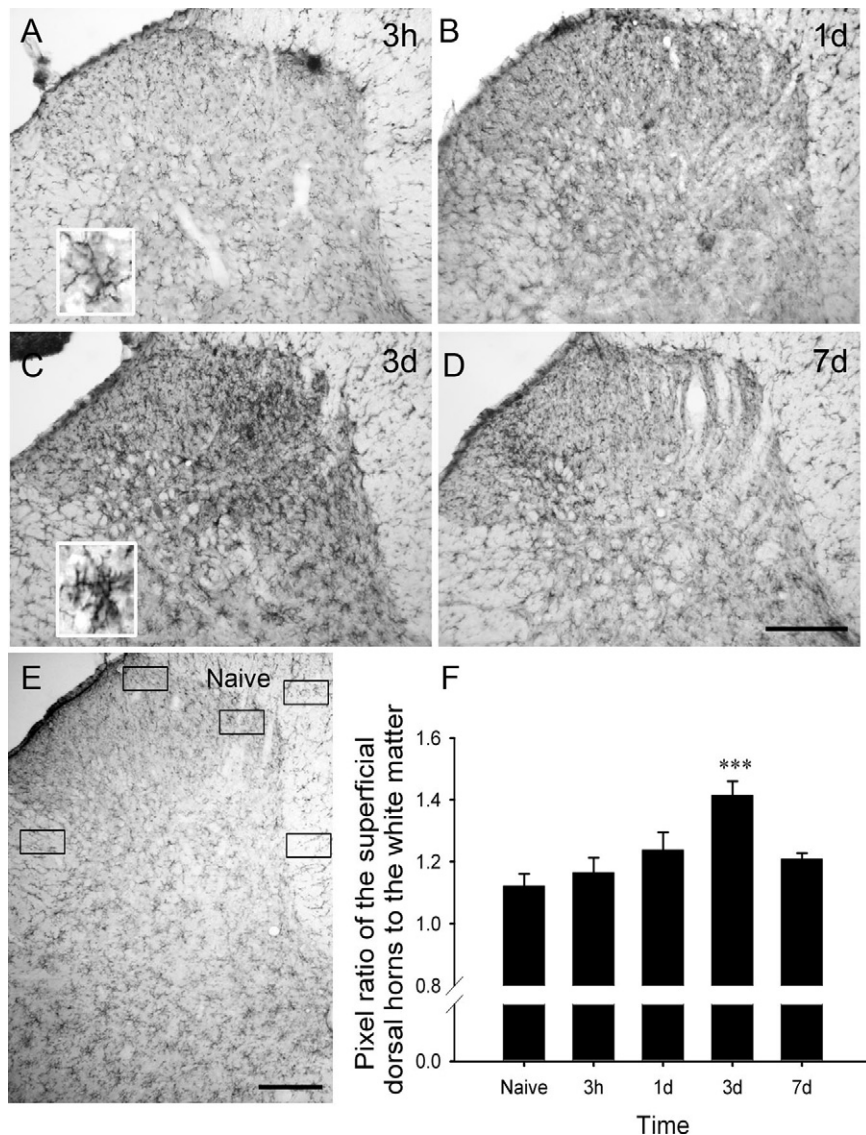
Fig. 3. Representative photomicrographs illustrating OX-42 immunoreactivity in ipsilateral spinal dorsal horn (*A*) 3 h, (*B*) 1 day, (*C*) 3 days, and (*D*) 7 days after hind paw incision. (*E*) Predefined rectangles at the superficial dorsal horn of a naïve rat were shown. The images were digitally captured to calculate the pixel numbers. Insets in *A* and *C* show the morphological changes of the activated microglia after incision. (*F*) Spinal microglial activation after a unilateral hind paw incision was indicated by the pixel ratio of the L4-5 superficial dorsal horn to the white matter. The pixel ratio was increased and reached the peak 3 days after incision. Scale bar = 200 μm for *A–D* and *E*. *** *P* < 0.001 *versus* naive, ANOVA with Bonferroni *post hoc* test; n = 4–5 for each time point.

FR167653 was injected intrathecally 30 min before plantar incision. Pretreatment of saline, vehicle (20% DMSO), and 100 μg of FR167653 (intrathecal injection) had no effect on the baseline values of withdrawal latency (fig. 4B). The baseline values of paw withdrawal latencies for rats in the saline, vehicle, and 100-μg FR167653 treatment groups were 23.99 ± 1.03 s (n = 12), 25.35 ± 1.21 s (n = 8), and 24.69 ± 1.27 s (n = 9), respectively (*P* > 0.05; two-way ANOVA). Moreover, it is surprising that a very high dose of FR167653 (100 μg) could only produce a transient reduction of thermal hyperalgesia at 1 h after surgery (*P* < 0.05; *post hoc* comparison; fig. 4B) when compared with saline or vehicle (20% DMSO in saline) group, and it had no effect at any other time points. Lower dose of FR167653 (50 μg) was tested in a small number of rats and showed no effect (data not shown). Unlike von Frey test, postincision thermal hyperalgesia was not affected by intrathecal DMSO, though a high concentration of 20% was used.

FR167653 Reduced Incision-induced Number of p-p38-ir Cells

Because there was a maximal increase of p-p38-ir cells at 1 day after plantar incision, we examined whether intrathecal pretreatment of p38 MAPK inhibitor reduced incision-induced activation of p38 in the spinal dorsal horn at this time point. Results from this study showed that pretreatment with 20 and 50 μg of FR167653 produced a significant reduction in the number of p-p38-ir cell in lumbar dorsal horn (37.5 ± 16.8 at the 50-μg group and 47.0 ± 4.7 at the 20-μg group *vs.* 157.0 ± 9.7 at the vehicle group, *P* < 0.001; F(2,11) = 40.551; one-way ANOVA) (fig. 5).

Discussion

This study shows that, in the L4-5 spinal dorsal horn, the plantar incision induced an increase in phosphorylation of p38 starting immediately after surgery, and the
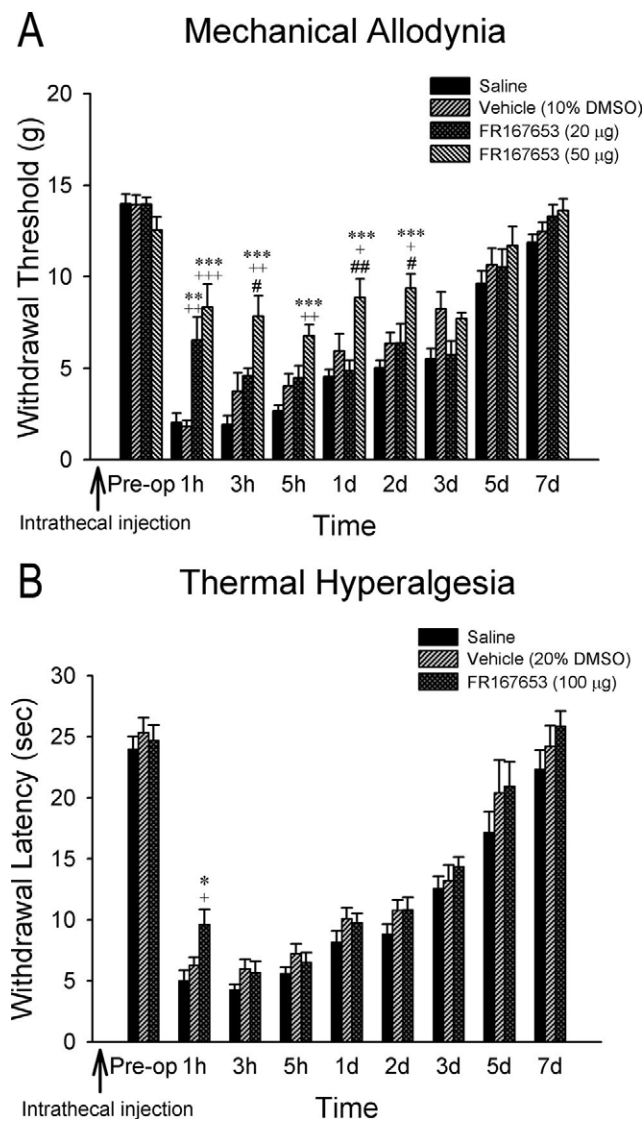
Fig. 4. Effects of p38 inhibitor FR167653 pretreatment on withdrawal threshold to von Frey stimulation and withdrawal threshold to thermal stimulation before and after plantar incision. (*A*) FR167653 attenuates plantar incision-induced mechanical hypersensitivity. Rats were pretreated with intrathecal bolus of saline (n = 10), vehicle (10% DMSO in saline; n = 9), 20 μg (n = 8), or 50 μg (n = 8) FR167653. (*B*) FR167653 only prevents incision-induced thermal hyperalgesia at 1 h. Rat from saline (n = 12), vehicle (20% DMSO in saline; n = 8), or 100 μg (n = 9) FR167653. Data are represented as the mean ± SEM. * P < 0.05, ** P < 0.01, and *** P < 0.001 *versus* saline; + P < 0.05, ++ P < 0.01, and +++ P < 0.001 *versus* vehicle; # P < 0.05 and ## P < 0.01 *versus* 20 μg FR167653; one-way ANOVA with Bonferroni *post hoc* test.

activation of p38 is coincident with the development of incisional pain. Activation of p38 was found mainly in microglia and in a limited number of spinal dorsal horn neurons at 1 and 3 h, but exclusively in microglia at 1 day after incision. Plantar incision produced a delayed microglial activation (OX-42 upregulation) at day 3. Pretreatment of p38 inhibitor FR167653 significantly prevented incision-induced mechanical allodynia and reduced p38 activation in the lumbar spinal dorsal horn,

but it only attenuated thermal hyperalgesia at an early time point. These results suggest that p38 activation in spinal microglial cells plays a role in the development of postoperative mechanical hypersensitivity.

p38 Activation and Pain Hypersensitivity

The present study disclosed that activation of p38 MAPK plays a role in incision-induced mechanical hypersensitivity because blocking its activation with pretreatment of p38 inhibitor FR167653 effectively prevented postoperative tactile allodynia. These results are in agreement with previous observations in various experimental pain models, including models of nerve injury,[14,17,19] inflammation,[14,15] diabetes[30] and capsaicin.[31] All these studies identified the colocalization of p-p38 in microglia after injury. However, the appearance of p-p38 in different spinal cells is also observed in a adjuvant immunized rats,[26] venom-induced inflammation,[32] and neuropathic pain model.[33] Our study supports these studies, indicating that involvement of p38 MAPK cascade in neurons/microglia follows a time-sequence and is not exclusively in one cell type in incisional pain.

This study investigated the temporal relationship between the p38 activation and incision-induced mechanical hypersensitivity. The immunoreactivity of p-p38 reaches the maximum at 1 day after paw incision; however, the mechanical allodynia is most obvious at 1 h. Pretreatment of FR167653 at two different doses dose-dependently prevented postincisional allodynia, although they showed similar inhibition on p-p38. Because we treated the animals with single dose of p38 inhibitor before plantar incision, the p-p38-ir existing on day 1 represents a direct result of an extinct effect of FR167653. In contrast, the differential antiallodynic effects of two inhibitor doses reflect an integrated consequence of inhibition on nociceptive development or broadly on complicated neuron-glial interactions. These results indicate that the activation of spinal p38 is required for incision-induced mechanical allodynia, but the quantitative p-p38 expression is only partially correlated with the nociceptive responses. The appearance of p38 activation later than that of the maximal allodynia suggests that p-p38 contributes to the nociceptive development rather than initiation. In addition, previous reports have demonstrated that the other members of MAPK, including extracellular signal-regulated kinase 1 and 2, and c-Jun N-terminal kinase, play distinct roles in the development and maintenance of inflammation-induced and nerve injury-induced pain hypersensitivity.[30,34,35] However, their functional roles in incisional pain remain to be elucidated.

Surprisingly, with one single intrathecal injection, 50 μg of FR167653 produced a long inhibition on tactile allodynia lasting for 2 days after incision, whereas a higher dose of FR167653 only resulted in a very brief inhibition on heat hyperalgesia. It is suggested that spi-
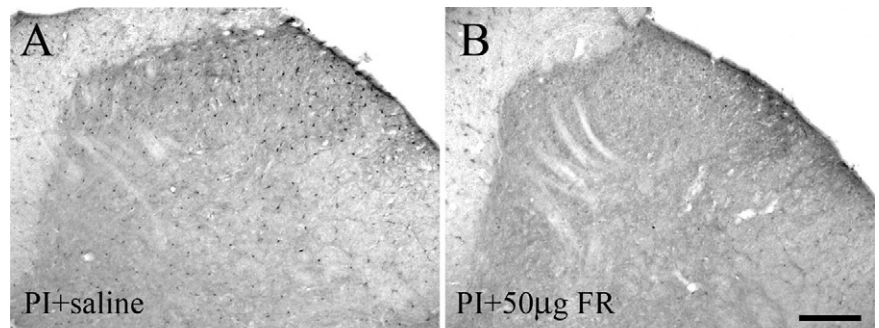
Fig. 5. Intrathecal injection of p38 inhibitor FR167653 attenuates plantar incision-induced p38 activation in the ipsilateral spinal dorsal horn. Rats were pretreated with either (*A*) saline or (*B*) FR167653 (50 μg). (*C*) A significant decrease of p-p38-immunoreactive cells was observed both at dosage of 20 μg and 50 μg. Data are represented as the mean ± SEM. *** $P < 0.001$ *versus* saline-treated rats; ANOVA (n = 5 for each group). Scale bar = 200 μm for *A* and *B*.
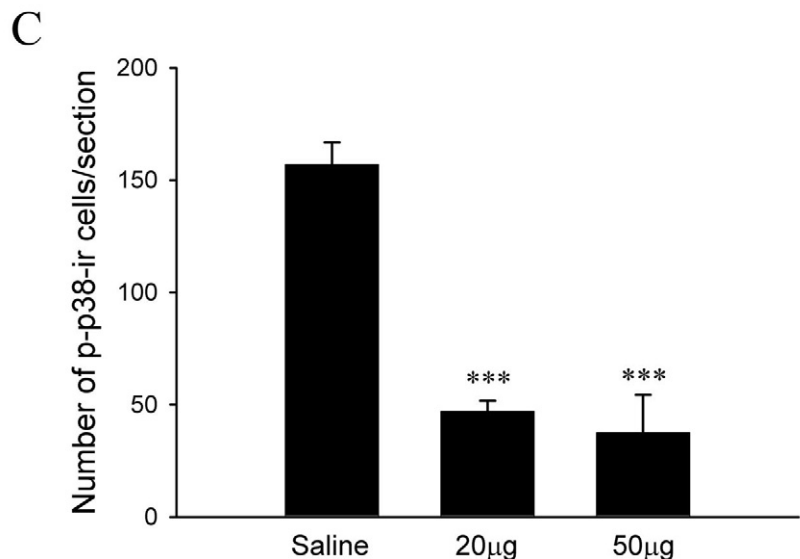
nal p38 activation is involved more in the pathophysiology of mechanical allodynia than thermal hyperalgesia in surgical pain. In clinics, the most common pain complaints after surgery are mechanically evoked such as dressing change, coughing, and ambulation.[36] Furthermore, different pain modalities may have discrepant drug responses. Single injection of 50 μg of FR167653 prevented incision-induced mechanical allodynia for 2 days, whereas in the spared nerve injury, after the pain related behavior was established, it could reverse mechanical allodynia for only 6 h.[37] This supports the idea that different pain models have no identically underlying mechanisms and may require different specific treatments at a given time point.

In experimental studies, drugs such as FR167653, SB203580, and CNI1493 were used to test the functional roles of p38 MAPK in inflammation-induced and nerve injury-induced hyperalgesia and allodynia. They all diminish p38 activity to reduce inflammation-induced and nerve injury-induced pain hypersensitivity.[15,18,37,38] Among them, FR167653 has a potent antiinflammatory action by inhibiting interleukin-1β and tumor necrosis factor-α production,[39,40] and these pro-inflammatory cytokines can modulate the synaptic transmission activity and promote pain.[41] *In vivo* studies showed that FR167653 can effectively prevent and attenuate isch-

emia/reperfusion injury through inhibiting p38 MAPK phosphorylation,[27–29] an action different from SB203580, which binds to the adenosine triphosphate pocket in the enzyme to block p38 activity.[42] Our results provide further evidence that FR167653 has a role in blocking surgical pain through inhibition of p38 phosphorylation.

In contrast, our results did not show blocking of heating pain hypersensitivity by FR167653, except a brief inhibition at the first postsurgical hour. Although thermal hyperalgesia is less noteworthy in the postoperative patients, there is growing evidence that the excitation of dorsal root ganglion (DRG) neurons plays an important role in heat hypersensitivity.[43–45] Tissue injury induced thermal hyperalgesia by sensitization in DRG neurons and activation of a candidate nociceptor, the cation-selective ion-channel transient receptor potential vanilloid 1.[43] Behavioral study has suggested that transient receptor potential vanilloid 1 is important in generating heat hyperalgesia by incision in a transient receptor potential vanilloid 1 knockout model.[46] Furthermore, Mizushima *et al.*[47] proved that capsaicin injection into the hind paw induced phosphorylation of p38 MAPK in transient receptor potential vanilloid 1-positive neurons in the DRG immediately after capsaicin injection, and capsaicin-induced thermal hyperalgesia was reversed by intrathecal

FR167653 administration when the intrathecal catheter tip is placed close to the L4-5 DRG level.

A site-dependent expression of p-p38 MAPK should be considered. Peripheral inflammatory pain by complete Freund's adjuvant caused p-p38 expression in DRG neurons of C fibers for several days, but it did not activate p38 phosphorylation in the spinal dorsal horn.[13] Because the dural membrane in rats extends over the capsule of the DRG[17,48,49] and intrathecal FR167653 might diffuse to affect cells in the DRG, we suggest that, in this study, FR167653 could inhibit the p38 activation in the DRG[47] at the immediate postsurgical period. This hypothesis provides a logical explanation that the immediate blocking of mechanical allodynia is mediated *via* p-p38 inhibition in the DRG but few in the spinal cord.

Importantly, blocking of p38 activation with FR167653 did not result in change of baseline nociceptive responses to tactile and thermal stimuli before plantar incision, indicating that this agent does not alter the physiologic responses to phasic pain. These results are consistent with previous pharmacological studies demonstrating that spinal p38 inactivation does not modulate baseline nociception.[15,17,19,31,47] This pharmacological character adds to the merit of this agent for the potential use in clinic.

Microglia Activation and Postoperative Pain

Compared with an early activation of p38, the increased microglial activation was observed much delayed, *i.e.*, at 3 days, but not at 3 h and 1 day. This finding is not surprising because previous studies indicated that there is a dissociation between microglial activation and tactile allodynia in plantar incision[50] and nerve injury models.[51] Our results correspond to current concepts regarding the emergence of "activated" microglia, by evidence of upregulation of CD11b expression, as a finding of proliferation of "reactive" microglia and invading monocytes from vessels in the vicinity, rather than an initiating sign of pathologic cascades leading to pain.[52,53]

The relationship between p38 phosphorylation and microglial activation in the nociceptive hypersensitization has not been well established or understood. Most pain modalities, including formalin,[15,54] complete Freund's adjuvant,[26] capsaicin injection,[13] nerve injury,[17,19,37] spinal cord injury,[55] and incision (this study) proved to activate the spinal p38 MAPK phosphorylation in the microglia. However, the detection of microglia activation depending on immunohistological upregulation of the surface marker CD11b (OX-42) would reflect rather a morphological change than a functional alteration. Furthermore, pain states may not always be associated with the upregulation of this surface marker,[50,51,56] and other characteristics of activation should be sought in the future. Another recent study supported our finding by using the expression of ionized calcium-binding adapter molecule 1 (Iba-1), another microglia marker. When measuring the whole spinal dorsal horn, Iba-1 expression 1 day after incision is significantly increased when compared with that of the contralateral side and the naïve rats; however, when the measuring area is limited to laminae I-II, the increase of Iba-1 expression is insignificant.[57] Therefore, p38 MAPK phosphorylation may be a better marker than OX-42 expression in incision-induced pain model.

Conclusions

In conclusion, activation (phosphorylation) of p38 MAPK in spinal microglial cells is partially coincident with the development of the tactile allodynia induced by surgical incision. Most importantly, intrathecal administration of p38 MAPK inhibitor FR167653 before surgery can effectively prevent incision-induced mechanical pain hypersensitivity, a common problem in clinics. Accordingly, we suggest that p38 inhibitor could be used as an effective therapeutic agent to relieve postoperative pain and that microglia inhibition may be a new strategy for postoperative pain treatment.

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