Animal Study

The Analgesic Effects of Celecoxib on the Formalin-induced Short- and Long-term Inflammatory Pain

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The non-steroidal anti-inflammatory drug celecoxib has long been used for reducing pain, in spite of moderate gastrointestinal side effects. In previous studies, it has been shown that celecoxib can inhibit formalin-induced spontaneous pain and secondary hyperalgesia. Injecting formalin into a rodent's hind paw not only induces acute pain behaviors, but also produces long-lasting hyperalgesia. Whether celecoxib can also have long-lasting effects is still unknown. Our results show that pretreatment with an intraperitoneal injection of celecoxib at one hour before formalin injection induced inhibition on the spontaneous flinch and licking behaviors in the second phase but not the first phase. Meanwhile, FOS expressions were also reduced with celecoxib pretreatment. Consecutive administration of celecoxib also protects the hind paw from hypoalgesia and relieves formalin-induced, long-lasting hyperalgesia in the ipsilateral hind paw. These analgesic effects may be related to suppression of the activation of neurons and astrocytes indicated by FOS and GFAP expressions. Based on the above findings, celecoxib demonstrated analgesic effects not only on acute spontaneous pain behavior but also on long-lasting hyperalgesia induced by formalin injection. The inhibition of neurons and astrocytes by celecoxib may be possible reasons for its analgesia.

Key words: Formalin test, celecoxib, FOS, GFAP, hyperalgesia

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To pre-clinically evaluate the analgesic effects of analgesics, 2 animal models are commonly used, i.e. an acute pain model and a chronic pain model. In previous studies, it has been shown that injecting formalin into a rodent's hind paw can induce both acute spontaneous pain and long-term hyperalgesia behaviors (1-3). Long-term peripheral inflammation or tissue injury and central sensitization in the spinal cord and brain nucleus are all involved in the mediation or modulation of formalin-induced pain (4-6). There are advantages for precise clarity as to anti-nociceptive properties mediated by different mechanisms, such as peripheral anti-inflammation, and/ or central desensitization. Therefore, the potential role

of any therapeutic analgesic agent through screening its effects in such an animal model can assist basic and clinical scientists as they seek out new treatment options in different pain states (7).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used medications targeting different components of pain. Celecoxib, one of the selective cyclooxygenase (COX)-2 inhibitors, has been extensively used in the treatment of osteoarthritis and rheumatoid arthritis (8,9). This compound and other selective COX-2 inhibitors exhibit antipyretic, anti-inflammatory and analgesic (10)-attributed effects due to their inhibition of prostaglandin biosynthesis (11). Besides other mechanisms such as activating the

endogenous opioid/cannabinoid systems (12), inhibiting protein kinase C epsilon translocation to modulate TRPV1 function and inhibiting substance P synthesis and release (13) were recently suggested to be possible contributors to celecoxib analgesia. Further, celecoxib evaluated as an anti-inflammatory drug can produce analgesia through suppressing immune response in the spinal dorsal horn, which includes the activation of microglia and astrocytes (14,15). Moreover, the releasing of cytokines can also be inhibited in different pain models (16,17).

Our previous study has shown that pretreatment with an intraperitoneal (IP) injection of celecoxib at 1 hour before formalin injection induced dose-dependent inhibition on the second, but not first phase, pain response. What is more, the effect of celecoxib on second phase flinches was calculated and the 50% effective dose (ED_{50}) of celecoxib on second phase flinches was 19.91 mg/kg (18). Actually, there are 2 different spontaneous behaviors following formalin injected into the hind paw, which include the flinch and licking behaviors (3). The effects of celecoxib on formalin-induced licking behaviors are still unclear. On the other hand, it has been widely accepted that injection of formalin can produce long-term hyperalgesia, which may be similar to the real situations that are faced by patients. Therefore, it is urgent and necessary to evaluate the analgesic or anti-inflammatory effects of celecoxib on long-term pain.

METHODS

Animals

Male C57BL/6 mice (about 8 weeks old) were housed in a temperature-controlled environment on a 12 hour light/dark cycle with access to food and water ad libitum. All experimental procedures received prior approval from the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi'an, China), and the ethical guidelines for investigating experimental pain in conscious animals were followed.

Experimental Design

According to our previous studies (3,4,18), the behavioral features of mice receiving a subcutaneous (SC) saline injection were similar to those of naïve mice, thus, in the current study, the data obtained from the naïve mice were not included. Celecoxib (Pifzer Pharmaceuticals LLC, New York City, NY) was purchased and freshly dissolved in sterile saline, filtered before use, and delivered intraperitoneally.

Experiment One aimed to evaluate the analgesic effects of IP administration of celecoxib on formalin-induced spontaneous pain including flinching and licking behaviors, as well as spinal neuronal activation. After a one week acclimation period, the mice were randomly assigned to one of the following groups: group 1 mice received an IP injection of 50 μL saline followed by an SC injection of 25 μL of 5% formalin one hour later (Forma+Sal group); group 2 mice received an IP injection of 50 μL of 20 mg/kg of celecoxib followed by an SC injection of 25 μL of 5% formalin one hour later (Forma+Cel group). All the animals from the 2 groups were video and audio recorded for later offline analysis during the one hour time window.

Experiment 2 aimed to observe the analgesic effects of celecoxib on formalin-induced, long-term mechanical hyperalgesia as well as neuronal and astrocytic activation. After a one week acclimation period, the animals were randomly assigned to two groups. Group 1 mice received an IP injection of 50 μL saline each day with 25 μL of 5% formalin one hour post-injection on day 10 (Forma+Sal group). Group 2 mice received an IP injection of 50 μL of 20 mg/kg of celecoxib each day after pre-injection (SC) with 25 μL of 5% formalin one hour post-injection on day 10 (Forma+Cel group).

Assessment of Spontaneous Pain-Related Behaviors

The formalin test was used to induce flinching or licking of the injected hind paw as described in our previous studies (3,4,18). All the behavioral observations were performed in a low, illuminated, soundproof room. A sound-attenuated clear Perspex® (Lucite International, Hampshire, United Kingdom) testing cage (25x25x40 cm) was fitted with a reverse video camera to record video for offline behavioral analysis. After the mice's acclimation to the testing chamber for about 20 minutes, the formalin solution (dissolved in saline) was subcutaneously injected into the plantar surface of the right hind paw using a microsyringe (Hamilton Co. Reno, NV) with a 30G needle. After formalin administration, the mice were returned to the observing cage and video and audio recordings were made for 60 minutes. A trained observer conducted a behavioral analysis of the video recordings. The pain behaviors were manually recorded with a stopwatch by retrieving spontaneous flinches or lickings of the injected hind paw from the recorded videos.

Measurement of Behavior to Mechanical Stimuli

The mice were habituated to the testing environment for 3 days before baseline testing, and then were placed under inverted plastic boxes (7x7x10 cm) on an elevated mesh floor and allowed to habituate for 30 minutes before the threshold testing. A logarithmic series of 8 calibrated Semmes-Weinstein monofilaments (von-Frey hairs; Stoelting, Kiel, WI) were applied to the lateral edge on the plantar surface of the injected paw, as well as on the plantar surface of the contralateral paw to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. A von-Frey filament was applied 10 times (3 seconds for each stimulus) to each testing area. The bending force of the von Frey filament able to evoke a 50% occurrence of paw withdrawal reflex was expressed as the paw withdrawal threshold (PWT). The stimulus was stopped if the threshold exceeded 10.0 g force (cutoff value). Assessments were made before formalin injection as a baseline value. The behavioral tests were performed on 1 day, 3 days, 7 days, and 10 days post-injection.

The threshold was defined as the minimum pressure required for eliciting a withdrawal reflex. The percent change in PWT was determined for the injected paw relative to the contralateral paw, according to the formula (ipsilateral PWT – contralateral PWT) / contralateral PWT. This index was used as a measure of mechanical hyperalgesia associated with the injected hind paw relative to the contralateral hind paw. Positive values indicated a state of hypoalgesia, whereas negative values indicated a state of hyperalgesia associated with the injected hind paw. All the tests were performed in a double-blind manner.

Immunohistochemistry Staining

After deep anesthesia by using pentobarbital (100 mg/kg IP), and perfusion with 30 mL of 0.9% saline followed by 100 mL of 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde, the fifth lumbar spinal cord segments (L5) were removed and post-fixed in the same agent for 6 hours and then immersed into 30% sucrose in 0.1 M PB for 48 hours at 4°C. Transverse spinal sections (the contralateral side was labeled by piercing on the ventral horn) were cut into 25 μm thickness on a cryostat (Leica CM1800, Heidelberg, Germany) at -20°C and sections were collected serially into dishes containing 0.01 M phosphate-buffered saline (PBS, pH 7.4).

Immunohistochemistry staining for FOS or GFAP was performed with an avidin-biotin-peroxidase complex (ABC) method. The sections were rinsed in 0.01 M PBS three times (10 minutes each), and blocked with 10% donkey serum in 0.01 M PBS containing 0.3% Triton X-100 for 30 minutes at room temperature (RT, 20–25°C), then used for immunohistochemistry staining. The sections were incubated overnight at RT with mouse anti-FOS antiserum (1:500; ab11959; Abcam, Cambridge, MA) or mouse anti-GFAP antiserum (1:4,000; MAB3402; Millipore, Billerica, MA) in 0.01 M PBS containing 5% normal donkey serum, 0.03% Triton X-100, 0.05% NaN3 and 0.25% carrageenan (PBS-NDS, pH 7.4), followed by incubation with biotinylated donkey anti-mouse IgG (1:500; AP192B; Millipore) diluted in PBS-NDS for 5 hours. Finally, sections were incubated with ABC-Elite kit (1:100; Vector Laboratories, Burlingame, CA) at RT for 2 hours, and then sections were further incubated in a solution containing 0.05 M Tris-HCl, 0.13% diaminobenzidine, and 0.005% hydrogen peroxide for 20 to 30 minutes in order to complete the diaminobenzidine reaction. Sections were washed completely with 0.01 M PBS between each step. Finally, all sections were mounted onto gelatin-coated glass slides, air dried, dehydrated in a graded series of diluted ethanol, cleared in xylene, and cover slipped.

Statistical Analysis

The results were expressed as mean value ± standard deviation (SD). The area under the curve (AUC) of individual mice for formalin-induced flinch or licking responses were group pooled and Student's t test was performed using GraphPad Prism version 5.01 for Windows (Graph Pad Software, San Diego, CA). Two-way analysis of variance (ANOVA) with Bonferroni confidence interval adjustment was used for the mechanical hyperalgesia analysis. One-way ANOVA and Bonferroni's multiple comparison test were used for the expressions of FOS and GFAP analyses.

Results

Effects of IP celecoxib on the spontaneous flinch and licking behaviors and neuronal activation induced by SC formalin injection

Injection of 5% formalin SC into the hind paw produced biphasic flinching and licking of the injected paw, consisting of the first transient phase lasting for the first 10 min and the second phase from 15 to 60 min. Pretreatment with IP celecoxib significantly affected the second but not the first phase flinch and licking, calculated based on the AUC. Moreover, there is a right
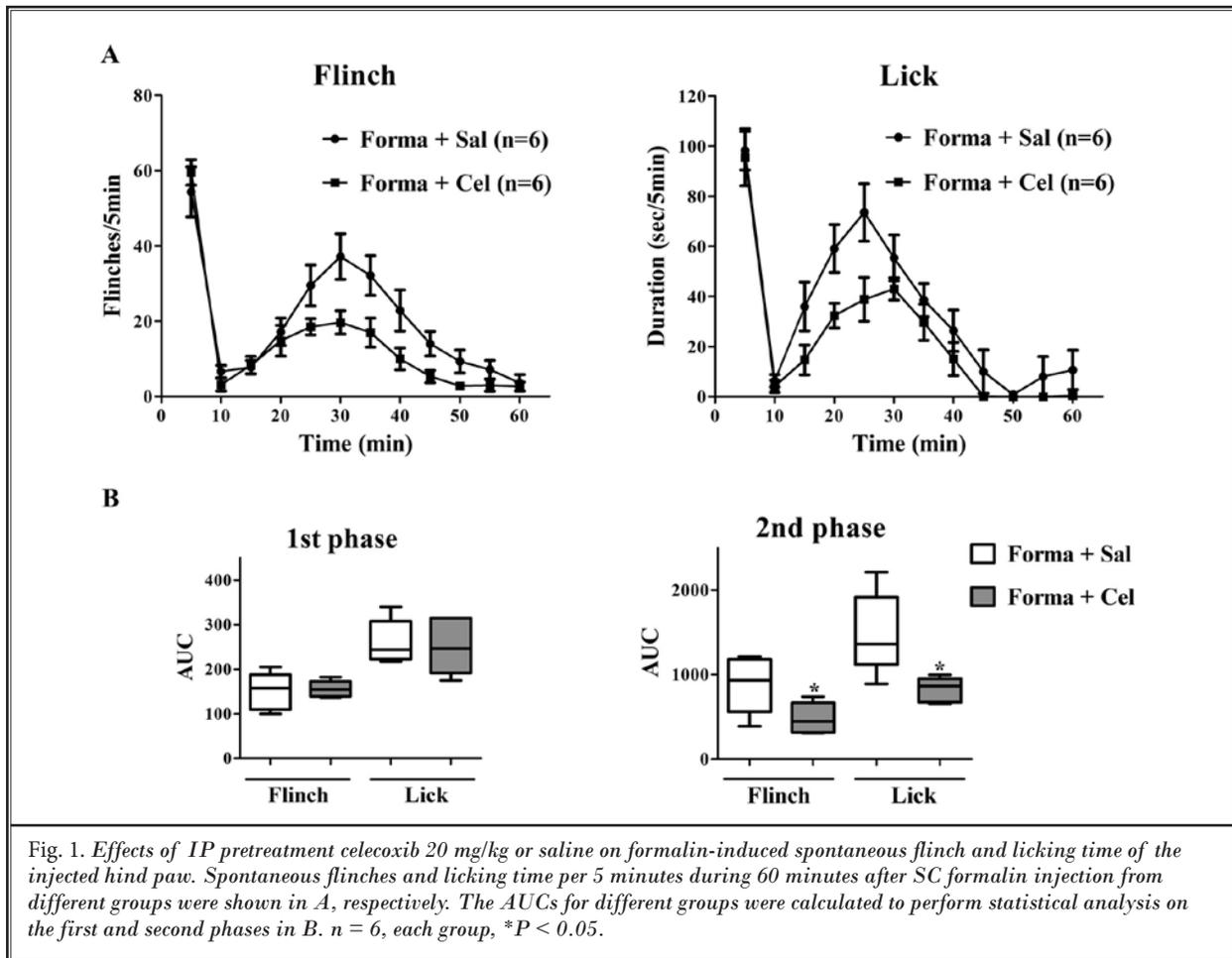
shift of the curve with celecoxib pretreatment. By comparing the AUC of flinch and licking behaviors, there were no significant differences between the Forma+Sal and Forma+Cel group in the first phase (Fig. 1 t test; $P = 0.8243$ and $P = 0.7137$, respectively). The analgesic effects of celecoxib on the second phase presented a significant group difference (Fig. 1 t test; $P = 0.0337$ for flinch and $P = 0.0247$ for licking).

FOS protein has been widely used as a marker for neuronal activity that can be used to map functionally related neural pathways involved in specific behaviors including acute and chronic pain (6,19). The number of FOS- immunoreactive (IR) neurons was counted in the spinal doral horn to evaluate the degree of spinal neuronal activation. Therefore, IP celecoxib pretreatments were investigated to check whether they inhibited neuronal activation by decreasing the expression of FOS, correlating with analgesic effects on spontaneous behaviors. FOS-IR neurons were most densely observed within the medial half of the ipsilateral spinal dorsal horn, including superficial layers (lamina I-II) and deep layers (lamina III-VI) at 2 hours after formalin injection (Fig. 2B). Meanwhile, there were few FOS-IR neurons distributed in the contralateral spinal dorsal horn (Fig. 2E). The total number of FOS positive neurons per section in the Forma+Sal group was 54.4 ± 8.4; in the Forma+Cel group it was 28.9 ± 7.0. Compared with the IP saline injection, the IP celecoxib administration significantly decreased the total number of FOS positive neurons per section (Fig. 2 and Fig. 3A; t test, $P < 0.0001$).

Effects of IP celecoxib on mechanical hyposensitivity, or hypersensitivity and spinal neuronal activation and GFAP-IR expressions

There were no significant differences between the injected hind paw and noninjected hind paw on

Fig. 1. *Effects of IP pretreatment celecoxib 20 mg/kg or saline on formalin-induced spontaneous flinch and licking time of the injected hind paw. Spontaneous flinches and licking time per 5 minutes during 60 minutes after SC formalin injection from different groups were shown in A, respectively. The AUCs for different groups were calculated to perform statistical analysis on the first and second phases in B. n = 6, each group, *P < 0.05.*
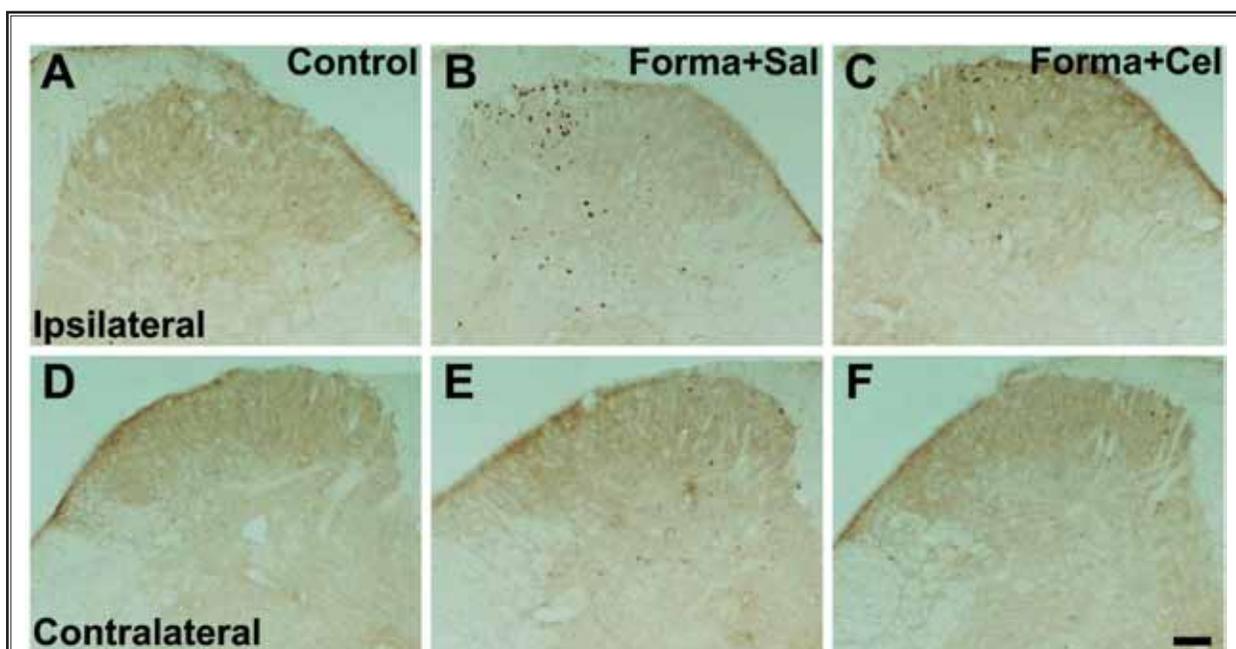
Fig. 2. *Effects of IP pretreatment of celecoxib 20 mg/kg or saline on the SC injection of formalin-induced FOS protein expression in the spinal cord. Representative photomicrographs demonstrate immunohistochemical staining for FOS in the ipsilateral spinal cord of control (A), Forma+Sal (B), Forma+Cel(C) group and the contralateral spinal cord of the control (D), Forma+Sal (E), Forma+Cel(F) group. There was almost no labeling in the lateral dorsal horn. Scale bar = 100 μm.*

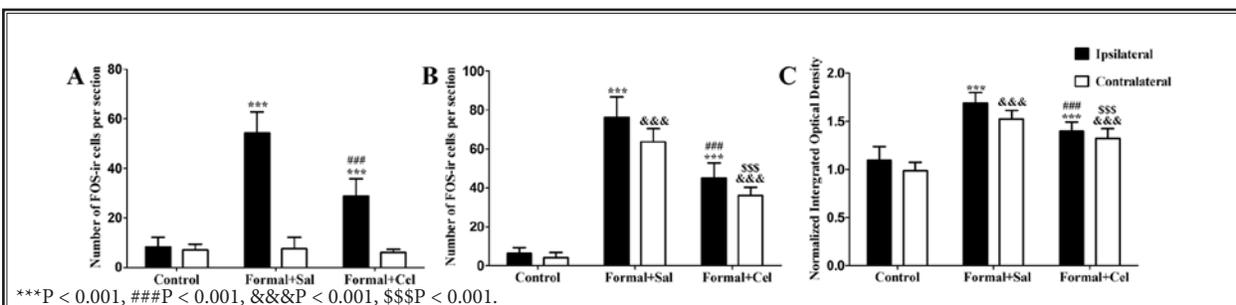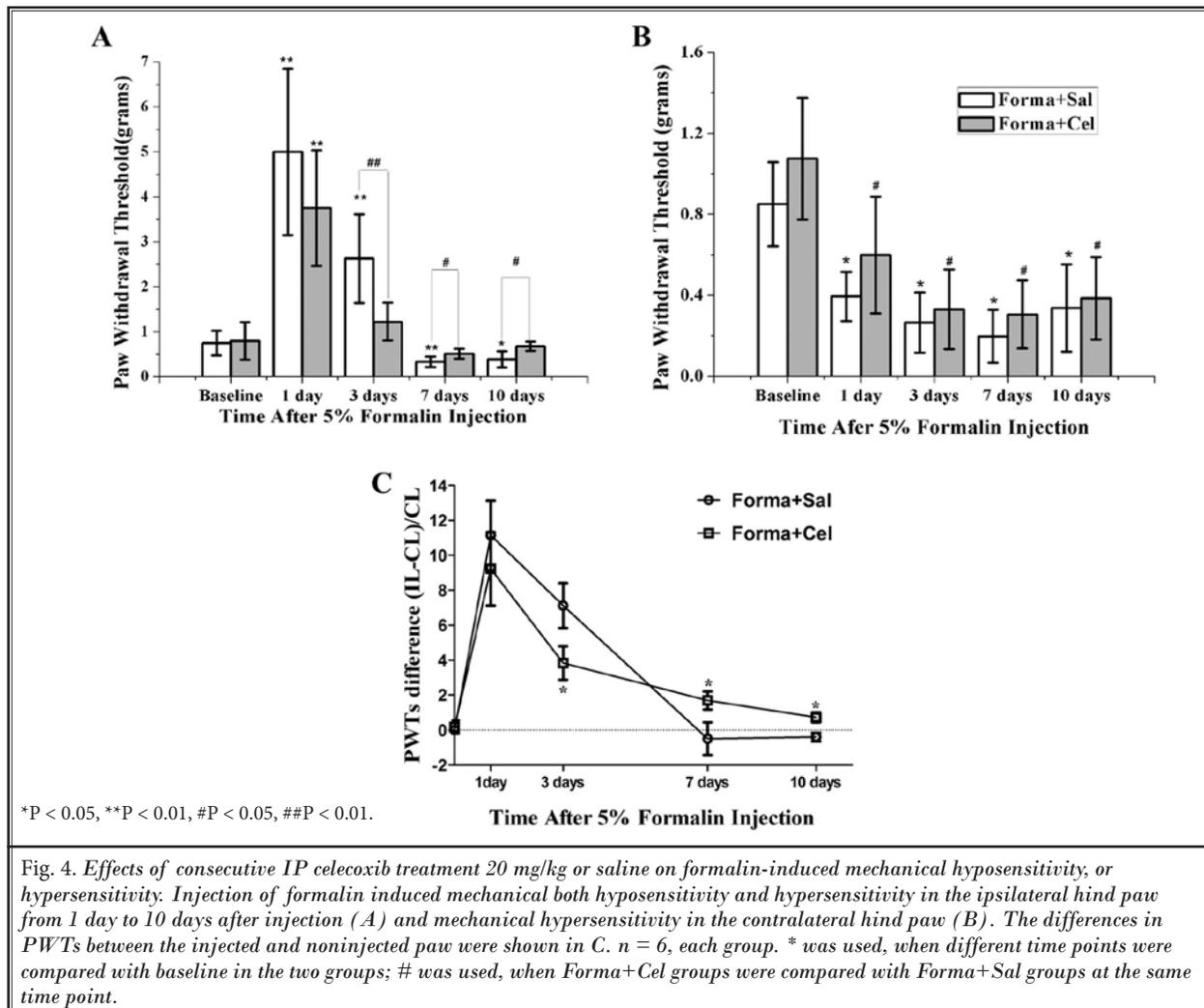***P < 0.001, ###P < 0.001, &&&P < 0.001, $$$P < 0.001.

Fig. 3. *Statistical analysis of FOS expressions after a single IP pretreatment of celecoxib and the expressions of FOS and GFAP after consecutive IP celecoxib treatments in the spinal dorsal horn of the ipsilateral and contralateral hind paws, following formalin injection at 2 hours and 10 days. The numbers of FOS per section in each group following formalin injection at 2 hours were analyzed in (A). The numbers of FOS and optical density of GFAP per section in each group following formalin injection at 10 days analyzed in (B and C). n = 18 sections from 3 animals, each group. *was used, when groups were compared with control group in the ipsilateral spinal cord; & was used, when groups were compared with control group in the contralateral spinal cord; # was used, when Forma+Cel group were compared with Forma+Sal group in the ipsilateral spinal cord; $ was used, when Forma+Cel group were compared with Forma+Sal group in the contralateral spinal cord.*

baseline PWTs to mechanical stimulus on the plantar surface. After formalin was injected into the plantar surface of the right hind paw, PWTs on the plantar surface of the injected paws increased relative to baseline pre-injection values at one day ($P < 0.001$). This hypo-

algesic state of the injected hind paw was also evident at 3 days ($P < 0.01$). Then, PWTs on the plantar surface of the injected paws decreased and presented hyperalgesia after 3 days. So, injection of formalin produced both hypoalgesia and hyperalgesia on the ipsilateral

Fig. 4. *Effects of consecutive IP celecoxib treatment 20 mg/kg or saline on formalin-induced mechanical hyposensitivity, or hypersensitivity. Injection of formalin induced mechanical both hyposensitivity and hypersensitivity in the ipsilateral hind paw from 1 day to 10 days after injection (A) and mechanical hypersensitivity in the contralateral hind paw (B). The differences in PWTs between the injected and noninjected paw were shown in C. n = 6, each group. * was used, when different time points were compared with baseline in the two groups; # was used, when Forma+Cel groups were compared with Forma+Sal groups at the same time point.*

paw. Consecutive IP celecoxib treatment reduced not only hypoalgesia, but also hyperalgesia (Fig. 4A; two-way ANOVA, *P* < 0.05). With regard to differences from the injected paw, hyperalgesic responses to mechanical stimuli were detected on the noninjected paw from one day to 10 days after formalin injection. However, there were no analgesic effects of IP celecoxib on the contralateral paw (Fig. 4B; two-way ANOVA, *P* > 0.05). The hypoalgesic state was also observed at one day (*P* = 0.003) and 3 days (*P* = 0.003) by comparison of the injected paw to the noninjected paw (Fig. 4). There-fore, there was a protective effect of celecoxib on this formalin-induced hypoalgesic state at 3 days (Fig. 4A; t test, *P* < 0.001). However, there were no obvious protec-tive effects at one day (Fig. 4A; t test, *P* > 0.05).

Following the above train of thought in the spon-taneous pain investigation, the number of FOS-IR neu-rons was counted in the spinal dorsal horn to evaluate the degree of the spinal neuronal activation. Therefore, the numbers of FOS-IR neurons were also investigated to ascertain the analgesic effects of IP celecoxib pre-treatments on long-lasting hyperalgesia. There are still many FOS-IR neurons in the spinal dorsal horn at 10 days after formalin injection. The number of FOS-IR cells in the ipsilateral spinal dorsal horn was 76.4 ± 10.4, while the number in the contralateral was 63.5 ± 6.8 (Figs. 3 and 5). Surprisingly, celecoxib reduced the num-bers of FOS-IR neurons not only in the ipsilateral (Figs. 3B and 5; one-way ANOVA and Bonferroni's multiple comparison test, *P* < 0.001) but also in the contralateral (*P* < 0.001) dorsal horn.

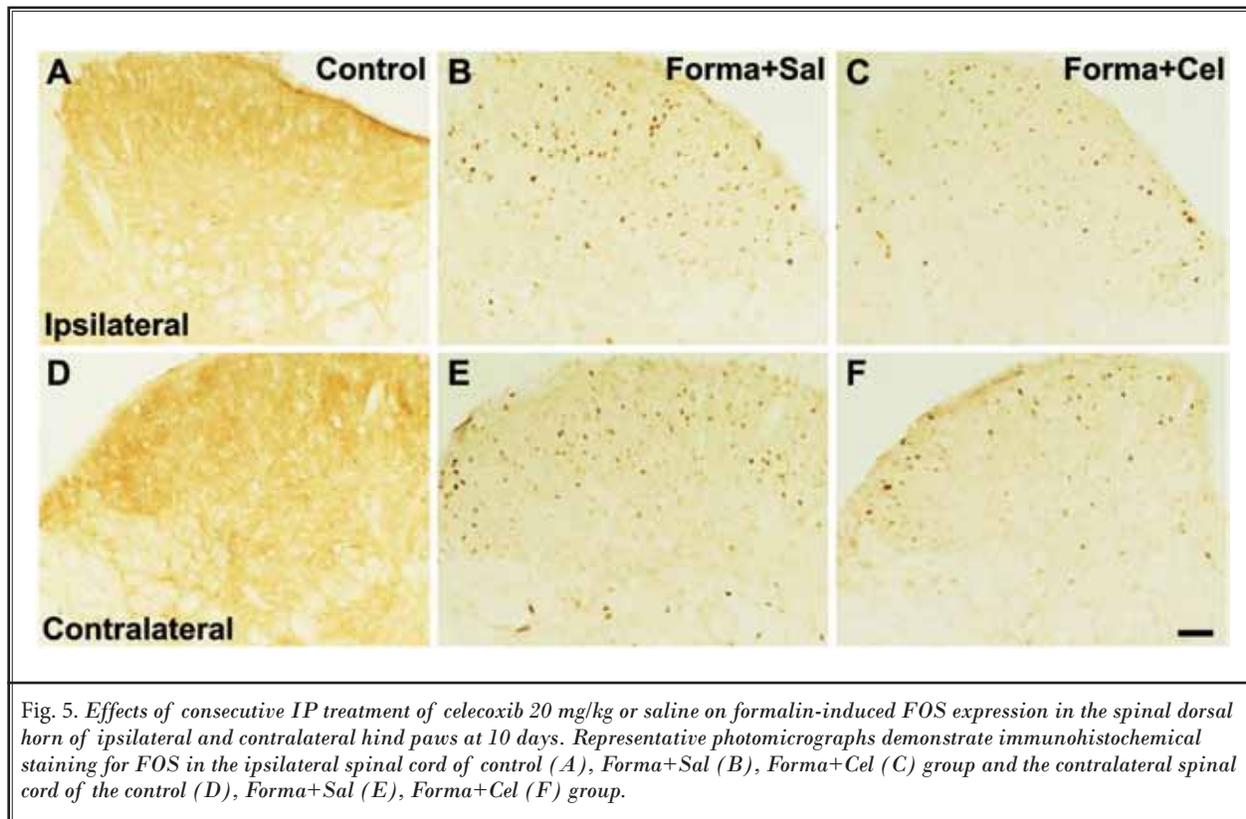Our previous studies suggest that the maintenance

Fig. 5. *Effects of consecutive IP treatment of celecoxib 20 mg/kg or saline on formalin-induced FOS expression in the spinal dorsal horn of ipsilateral and contralateral hind paws at 10 days. Representative photomicrographs demonstrate immunohistochemical staining for FOS in the ipsilateral spinal cord of control (A), Forma+Sal (B), Forma+Cel (C) group and the contralateral spinal cord of the control (D), Forma+Sal (E), Forma+Cel (F) group.*

of chronic pain is correlated with astrocytic activation. Thus, we further wondered whether astrocytic inhibition contributed to analgesia. We observed that formalin induced a significant astrocytic activation indicated by GFAP up-regulation in the formalin injection group compared with the control group. Immunohistochemistry indicated that activated astrocytes presented as hypertrophied cell bodies and a thickened process with enhanced GFAP-IR. Consecutive IP treatment with celecoxib significantly reduced the expressions of GFAP in the ipsilateral (Figs. 3C and 6; one-way ANOVA and Bonferroni's multiple comparison test, $P < 0.001$) and contralateral ($P < 0.001$) spinal dorsal horn.

DISCUSSION

To our knowledge, this is the first report demonstrating analgesia for celecoxib on long-lasting inflammatory pain induced by SC injection of formalin into the hind paw. Although celecoxib has been commonly used in the treatment of pain, especially for acute inflammatory or neuropathic pain, the gastrointestinal side effects of celecoxib limit its clinic application. The long-lasting analgesia observed in the current study may help promote the concept of a new long-lasting analgesic strategy.

The two-phase spontaneous pain responses are the shared features for both orofacial and hind paw formalin tests and are regarded to be associated with 2 at least partially distinct mechanisms for nociception: the first phase is associated with direct stimulation of nociceptors via direct activation of transient receptor potential ankyrin (TRPA)-1 receptors (20), whereas the second phase reflects integration between peripheral (nociceptors) and central (spinal/supraspinal) signaling including neuronal and glial responses (21). Moreover, the spontaneous flinch and licking of the injected hind paw seem to be a reliable parameter for evaluating biphasic pain responses induced by SC formalin injection; this model was used in our previous studies as well (3,4). Celecoxib shows an analgesic effect when administered acutely (22) or chronically (23). Our previous data suggested that acutely administered celecoxib induced analgesia on somatic pain responses but not emotional pain responses. It was also reported that a single administration of celecoxib did not change stress-related behavior as evaluated with both an open field or elevated plus maze (18).

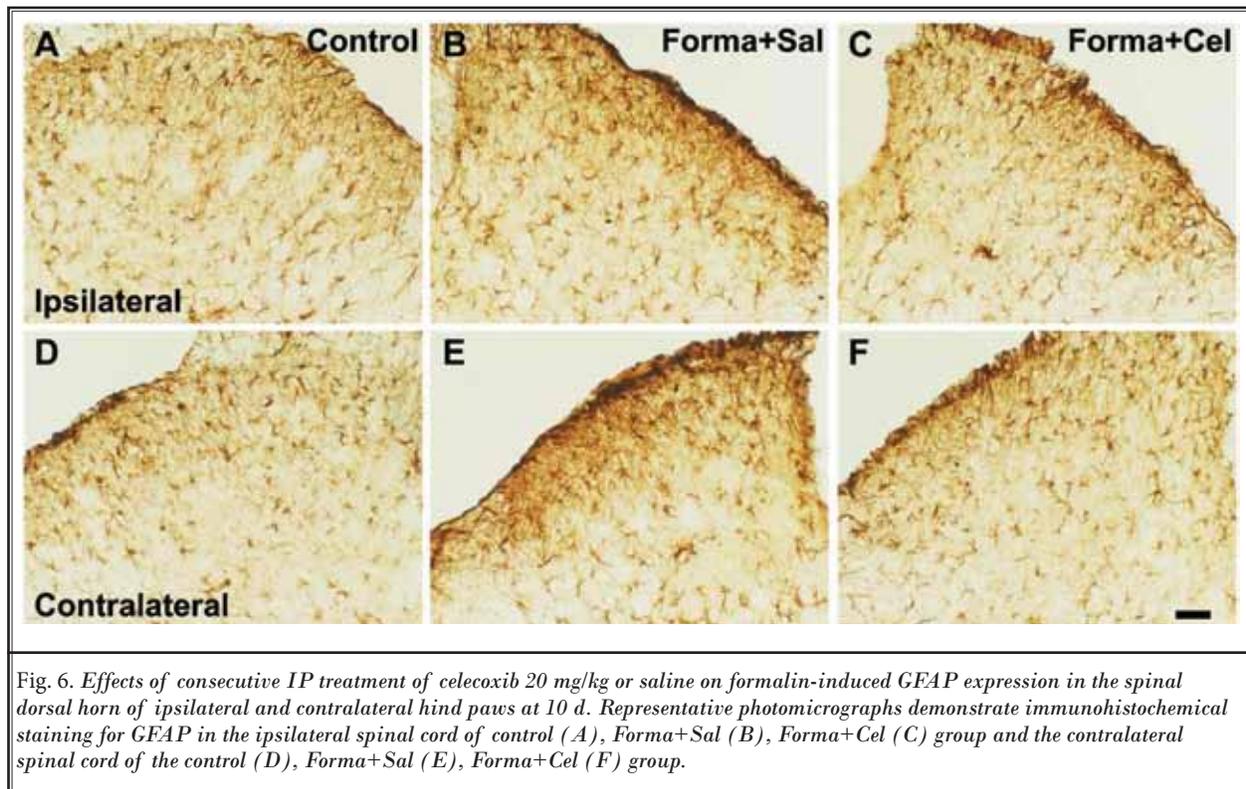Our study suggests that celecoxib pretreatment at-

Fig. 6. *Effects of consecutive IP treatment of celecoxib 20 mg/kg or saline on formalin-induced GFAP expression in the spinal dorsal horn of ipsilateral and contralateral hind paws at 10 d. Representative photomicrographs demonstrate immunohistochemical staining for GFAP in the ipsilateral spinal cord of control (A), Forma+Sal (B), Forma+Cel (C) group and the contralateral spinal cord of the control (D), Forma+Sal (E), Forma+Cel (F) group.*

tenuates the second phase of formalin pain responses more than those of the first phase. This acute analgesic effect of celecoxib not only include the reduction of the AUC of flinches and licking, but also shifted the spontaneous pain behaviors to curve right, which may be preferentially mediated by spinal versus supraspinal mechanisms. Celecoxib inhibits nociceptive responses for the suppression of prostaglandin E generation by inhibiting COX-2. Hind paw formalin injection increased COX-1 expression, beginning at one day after injection and lasting at least 2 weeks. The COX-2 expression changed considerably less, with a significant increase of COX-2 protein level only observed at 2 hours after injection (24). We have also shown that the inhibitory expressions of COX-2 by Danggui-Shaoyao San (3) contribute to suppression of spontaneous pain behaviors and FOS expression after formalin injection. Theoretically, IP-injected celecoxib can reach the higher brain structures and spinal cord to mediate serotonergic, noradrenergic, and prostanoid systems to render analgesia for second phase pain responses. However, acute administration may not induce a sustained increase of drug concentration in higher brain structures, but the involved primary nociceptive integrating system might be mainly involved in analgesia and the relatively rapid increase of 5-HT and NE levels at the spinal level is a feasible mechanism underlying the analgesia effect.

Within minutes following an insult to a peripheral nerve or tissue, glial cells in the spinal dorsal horn respond with a change in morphology and an up-regulation and secretion of immune and growth factors (25,26). Astrocytes, being in intimate contact with neurons, may respond first with an up-regulation of a gap junction protein, connexin-43, suggesting an early change in neuronal-astrocyte interactions (25). Following injury, microglial cells have also been observed to rapidly become less ramified and begin to proliferate. At later times following insult, astrocytes up-regulate the expression of GFAP and become hypertrophied and less ramified in appearance (19,27). Facial nerve injury (27,28), chemically induced neurodegeneration (19), spinal nerve injury (26), and spinal contusion all result in a rapid activation of microglia followed by a delayed astrocytic activation (as determined via up-regulation of GFAP).

The increase of GFAP expressions was not observed, however the up-regulation of interleukin-1β was significant at day 7 following formalin SC injection

(29). A quality and quantitative increase of OX-42-IR microglial cells were observed in the medial portion of the dorsal horn and in the gracile nucleus of the brain stem on the side ipsilateral to the formalin SC injection, starting on days 1-3 and peaking on day 7 postinjection (30,31). Actually, the ligation of a spinal nerve also results in activation of microglia, most prominently in the ipsilateral dorsal horn, starting soon after the lesion (< 1 day) and persisting for > 3 weeks (32-34). Meanwhile, astrocytes were also activated following peripheral inflammation and nerve injury (33,35). Therefore, it was assumed that the activation of astrocytes also played an important role in the maintenance of hyperalgesia. Suppressing the expressions of GFAP might be a good choice to produce analgesia.

Acute treatment with celecoxib did not affect mechanical allodynia or a difference in weight bearing in rats with bone cancer pain, in which significant enhancement of GFAP staining in the corresponding segments of the ipsilateral spinal cord highlights the possible involvement of astrocytes (36). This indicates that a single treatment with a COX-2 inhibitor has no influence on GFAP expressions. The expressions of GFAP in the spinal dorsal horn were reduced following consecutive treatment with celecoxib. Meanwhile, celecoxib attenuated venom-induced hyperalgesia, which can activate dorsal horn astrocytes and microglia (37). The osteomyelitis-induced pain behavior was reversed by celecoxib, in which a significant increase of both microglia and astrocytes were observed in the spinal cord (15). Celecoxib also presented profoundly, analgesic effects and reduced astrocyte activity in a collagen-induced arthritis pain model (14).

In summary, our present study offers experimental support that IP treatment with celecoxib produces short- and long-term analgesic effects after formalin injection. This analgesia is accompanied by suppressions of neuronal and astrocytic activations. However, the side effects for chronic or repeated treatment to provide sustained analgesia needs to be investigated in the future.

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