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Cyclooxygenase inhibitors suppress the expression of $P2X_3$ receptors in the DRG and attenuate hyperalgesia following chronic constriction injury in rats

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ABSTRACT

Recent evidence suggests that $P2X_3$ receptors express abundantly in nociceptive sensory neurons and play an important role in neuropathic pain. Upregulation of prostaglandin E2 (PGE2) after nerve injure is involved in the pathogenesis of neuropathic pain. An increase of $P2X_3$ receptors after chronic constriction injury (CCI) to the sciatic nerve has also been reported, the mechanisms are not known clearly. In this study, we examined the effects of systemic administration of cyclooxygenase (COX) inhibitors on analgesia and the expression of $P2X_3$ receptors in the dorsal root ganglia (DRG) in CCI rats. Rats received 0.9% saline, the nonselective COX inhibitor ibuprofen (40 mg kg^{-1} day^{-1}) or the selective COX-2 inhibitor celecoxib (30 mg kg^{-1} day^{-1}) by gavage twice daily from 3 to 14 days after surgery. Mechanical allodynia and thermal hyperalgesia induced by CCI were markedly attenuated by celecoxib from 5 to 14 days after surgery, and relieved by ibuprofen treatment from 7 to 10 days after surgery. The increase of $P2X_3$ receptors in the DRG in CCI rats on day 14 after surgery was also significantly inhibited; the effect of ibuprofen was stronger than that of celecoxib. These results demonstrate that up-regulated COX/PGE2 after nerve damage may play an important role in neuropathic pain. They are highly involved in the expression of $P2X_3$ receptors in the DRG in CCI rats.

Neuropathic pain is caused by damage or inflammation of the nervous system, and is chronic and characterized by hyperalgesia, allodynia and spontaneous pain [18,20]. In 1995 [4,14], $P2X_3$ receptors were found selectively expressed in small and medium diameter nociceptive sensory neurons. Since then, application of $P2X_3$ receptors anti-sense oligonucleotides [1], RNA interference [8], gene knockout technology [5] or selective antagonists [11,13] have shown that ATP and $P2X_3$ receptors play an important role in the development and maintenance of neuropathic pain. Nowadays, the inhibition of $P2X_3$ receptors is regarded as useful in relieving neuropathic pain [3,12,14,22]. Recent evidence suggests that PGE2 is up-regulated after nerve injury and involved in the pathogenesis of neuropathic pain [16–19]. Although an increase of $P2X_3$ receptors in CCI rats have been reported [23], the mechanisms for this increase remain unknown. Wang [28] observed that PGE2 could enhance ATP currents and was relevant to hyperalgesia and allodynia mediated by $P2X_3$ receptors. Whether PGE2 can increase the expression of $P2X_3$ receptors is still unclear. Cyclooxygenase (COX), existing in the form of COX-1 and COX-2, is a major rate-limiting enzyme for synthesis of PGE2. In this experiment, CCI was used to explore analgesic effects of the nonselective COX inhibitor ibuprofen and the COX-2 selective inhibitor celecoxib on neuropathic pain and their effects on the expression of $P2X_3$ receptors in the DRG.

This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal Care Committee of Central South University in China approved all the procedures. 36 male adult rats of Sprague–Dawley (SD), provided by animal experiment center of Xiangya Medicine School, Central South University, weighing 250–280 g, were randomly divided into sham operation group ($n = 9$) and three CCI groups (vehicle-treated, ibuprofen-treated and celecoxib-treated groups, each $n = 9$). Under deep anesthesia induced by intraperitoneal injection of 10% chloral hydrate (300–350 mg/kg), a modified CCI to the sciatic nerve was performed on the left side according to Bennett and Xie [2], and a sham operation was just exposing the nerve but not ligating it. CCI rats received either vehicle (0.9% saline), the nonselective COX inhibitor ibuprofen (GlaxoSmithKline, CA, UK, 40 mg kg^{-1} day^{-1}) or the selective COX-2 inhibitor celecoxib (Pfizer, CA, USA, 30 mg kg^{-1} day^{-1}) by gavage twice daily from 3 days to 14 days after surgery. Mechanical allodynia and thermal hyperalgesia of all rats were monitored. Recent studies show that the electronic algometer provides a sensitive, objective, quantitative and convenient method to assess mechanical allodynia [21,27]. So, mechanical allodynia was tested with 2390 Electronic von-Frey Anesthesiometer (IITC Life Science, Woodland Hills, CA, USA) as previously reported [6] with adaption of a 0.8 mm rigid tip. Thermal hyperalgesia was assessed with Hargreaves Tes7370

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(Ugo Basile, Comerio, Italy). The end-point was characterized by the removal of the paw followed by clear flinching movements. After paw withdrawal, the intensity was recorded automatically. Each rat was measured five times, with an exposure interval of 5 min. Mechanical withdrawal threshold (MWT) and Thermal withdrawal latency (TWL) were averaged by removing the maximum and minimum values. MWT and TWL of left rear foot were measured before, 3, 5, 7, 10 and 14 days after surgery. All the behavioral testing was done by observers unaware of animal treatments.

On day 14 after surgery, 3 rats from each group were chosen randomly to observe $P2X_3$ receptors expression using immunohistochemistry approach. Under deep anesthesia induced by intraperitoneal injection of 10% chloral hydrate (300–350 mg/kg), 200 ml normal saline, followed with 400 ml 4% paraformaldehyde were perfused into rats through their ascending aorta, then, their left lateral L4-6DRG were dissected. The remaining 6 rats in each group were used to observe $P2X_3$ mRNA expression with reverse transcription PCR (RT-PCR) approach, and $P2X_3$ receptors expression with Western blot analysis. On day 14 after surgery, the 6 remaining rats in each group were killed under deep anesthesia, and their ipsilateral L4-6DRG were dissected freshly.

For immunocytochemistry, tissues were fixed with 4% paraformaldehyde for 6 h and then were soaked in 30% sucrose solution until sinked to the bottom. Frozen tissues were sectioned at 25 μm thickness using a cryostat, the sections were collected in 0.01 mol/l phosphate buffered saline (PBS). During immunestaining, the sections were treated with 3% H_2O_2 solution for 30 min; permeabilized and blocked with blocking solution (10% bovine serum albumin and 0.2% TritonX-100) at room temperature for 2 h, then, incubated with rabbit polyclonal anti-$P2X_3$ antibody (1:150) (ABCAM, CA, USA) at room temperature for 2 h and then at 4 °C overnight. On the second day, sections were incubated with biotinylated goat anti-rabbit IgG (1:200) (Vector, CA, USA) at room temperature for 2 h and ABC solution (1:200) (Vector, CA, USA) at room temperature for 2 h. Finally, sections were incubated with 0.05% DAB (Vector, CA, USA) and 0.01% H_2O_2 solution for 1–2 min for coloration. Among each incubation, sections were washed with 0.01 mol/l PBS 3 times, each time for 5 min. PBS replaced the primary antibody as a negative control. Cells that had brown-yellow granules in cytoplasm and membrane were positive ones.For RT-PCR analysis, primers were designed according to relevant gene sequences, produced by Shanghai Bio-Engineering Company in China. $P2X_3$ primer: upstream: 5-TCACCGACAAGGACATAAA-3, downstream: 5-TGCCCACTCCCACAGAAG-3; length: 432 bp. β-Actin primers: upstream: 5-GAGAGGGAAATCGTGCGTGAC-3, downstream: 5-CATCTGCTGGAAGGTGGACA-3, length: 452 bp. Total RNA was extracted from all the freshly dissected tissues using Trizol Reagent (Invitrogen, CA, USA). Then their concentration and purity were detected. cDNA templates were synthesized by reverse transcription and saved at −20 °C. PCR was carried out in 20 μl volumes containing 1 μl cDNA templates, 12 μl double-distilled water, 2 μl 10 × PCR buffer, 1.5 μl 25 mmol/l Mg^{2+}, 2.0 μl 2 mmol/l dNTP, 0.5 μl 100 μmol/l primer and anti-sense primer and 0.5 μl 500U Tap enzyme. PCR conditions used were 1 cycle of pre-denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72°C for 30 s; 1 cycle of extension at 72 °C for 5 min, and termination at 4 °C. 10 μl of each PCR product was size-fractionated by 1.5% (w/v) agarose gel electrophoresis, the gel stained with ethidium bromide (EB) and photographed under UV light. Positive bands were analyzed with Quantity One software (Bio-Rad, CA, USA) to assay the value of their optical density. Each $P2X_3$ expression level was normalized by β-actin as previously reported [25].For Western blot analysis, total proteins were extracted from tissues and their concentrations were assayed. Proteins were heated for 3 min to denaturation. 15 μl of each protein sample was separated by a 12% polyacrylamide gel and electrotransferred to a nitrocellulose membrane in the Mini Teans-Blot lecreophoresis Transfer Cell (Bio-Rad, CA, USA). Membranes were blocked in 5% skimmed milk powder (get from PBS) at 37 °C for 2 h. Membranes were incubated with anti-rabbit $P2X_3$ antibody (1:1000) (ABCAM, CA, USA), (molecular weight 47.6 kD) and anti-mouse GAPDH antibody (1:4000) (ProMab, CA, USA), (molecular weight 37 kD) at room temperature in the shaker for 2 h and then 4 °C overnight, followed by incubated with anti-Goat anti-rabbit IgG/HRP (1:40,000) and anti-goat anti-mouse IgG/HRP (1:40,000) at room temperature in the shaker for 1 h. The mmunocomplexes were visualized by chemiluminescence using the ECL kit (Pierce, CA, USA). Positive bands were analyzed with Quantity One software (Bio-Rad, CA, USA) to assay the value of their optical density. Each $P2X_3$ expression level was normalized by GAPDH like previously reported [25].

Data were given as mean ± S.E.M. For comparison of change of hyperalgesia, a two-way ANOVA for repeated measures data was used and then SNK-q test and LSD test were used to compare groups with each other. For comparison of $P2X_3$ receptors

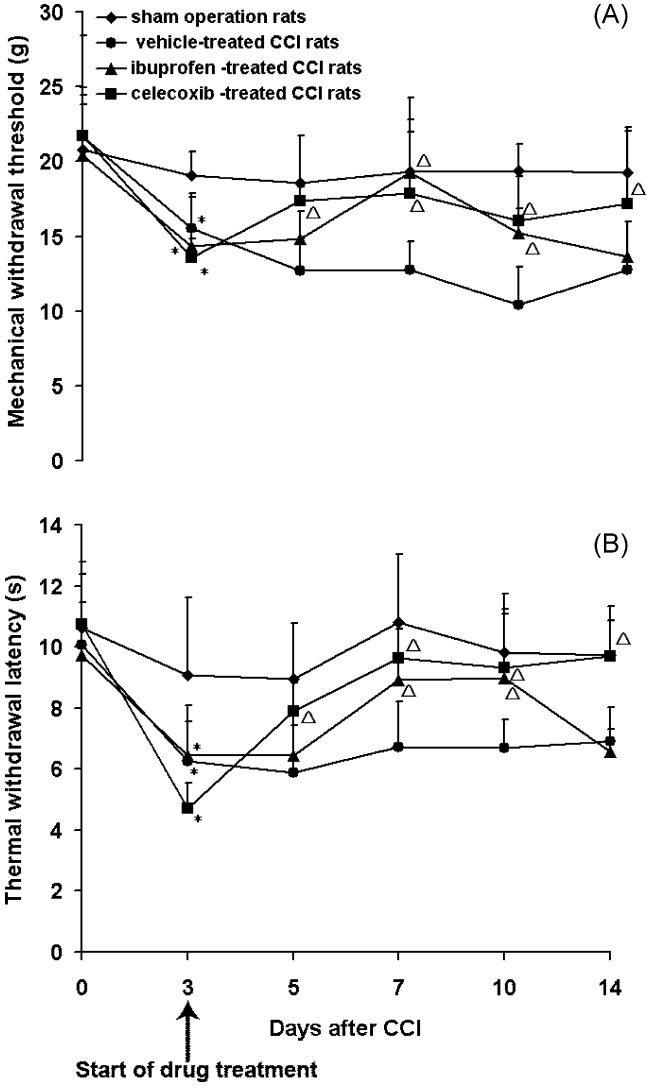

Fig. 1. The mean (±S.E.M.) data of mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in all rats. (A) Change of mechanical allodynia in all rats from before to 14 days after surgery. (B) Change of thermal hyperalgesia in all rats from before to 14 days after surgery. Comparison of MWT and TWL in rats with different treatments before, 3, 5, 7, 10 and 14 days after surgery: * vs before operation, $P < 0.05$; △ vs vehicle-treated CCI rats, $P < 0.05$.