Journal of Pharmacological Sciences 131 (2016) 275–278

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Short communication

Chondroitin sulfate attenuates formalin-induced persistent tactile allodynia

Wataru Nemoto [a, 1], Kotaro Yamada [b, 1], Yoshiki Ogata [a], Osamu Nakagawasai [a], Katsuhito Onodera [a], Hidetomo Sakurai [b], Koichi Tan-No [a, *]

[a] Department of Pharmacology, Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan

[b] Consumer Healthcare Products Development, Zeria Pharmaceutical Co., Ltd., 10-11 Nihonbashi, Kobuna-cho, Chuo-ku, Tokyo 103-8351, Japan

ARTICLE INFO

Article history:
Received 8 April 2016
Received in revised form
29 June 2016
Accepted 11 July 2016
Available online 5 August 2016

Keywords:
Chondroitin sulfate
Tactile allodynia
Spinal cord

ABSTRACT

We examined the effect of chondroitin sulfate (CS), a compound used in the treatment of osteoarthritis and joint pain, on the formalin-induced tactile allodynia in mice. A repeated oral administration of CS (300 mg/kg, b.i.d.) significantly ameliorated the formalin-induced tactile allodynia from day 10 after formalin injection. On day 14, the phosphorylation of spinal p38 MAPK and subsequent increase in c-Fos-immunoreactive dorsal lumbar neurons were attenuated by the repeated administration of CS. These findings suggest that CS attenuates formalin-induced tactile allodynia through the inhibition of p38 MAPK phosphorylation and subsequent up-regulation of c-Fos expression in the dorsal lumbar spinal cord.

Chondroitin sulfate (CS) is a glycosaminoglycan with polysaccharide chains composed of an alternate sequence of D-glucuronic acid and D-*N*-acetyl galactosamine linked by β(1 → 3) and β(1 → 4) bonds, and is widely distributed in various connective tissues such as cartilage, skin, corpus vitreum and blood vessels. CS contributes to the viscoelastic property of the cartilage, and a reduced level of CS in the cartilage has been reported to be a risk factor for arthritic diseases in elderly people (1). CS is a compound categorized as a symptomatic slow-acting drug for the treatment of osteoarthritis (SYSADOA) because of its anti-inflammatory and chondroprotective properties (2). CS is also popular in North America as a dietary supplement intended to alleviate joint pain (3). In Japan, CS is available as an over-the-counter drug used against knee joint pain. Yet, the pain relief properties and mechanism of CS remain unclear, though it has been suggested that CS may play a role in pain relief through B_2 bradykinin receptor desensitization (4).

Thus, in the present study, we examined the effect of CS through oral administration on the formalin-induced persistent tactile allodynia in mice.

Male ddY mice weighing 22–24 g were used throughout the experiments. Mice were housed in cages with free access to food and water under conditions of constant temperature (22 ± 2 °C) and humidity (55 ± 5%), on a 12 h light–dark cycle (lights on: 7:00 to 19:00). Group of 12 mice for behavioral experiments, 7 mice for Western blotting, and 6–13 mice for immunohistochemical experiments were used in single experiments. All experiments were performed following the approval from the Ethics Committee of Animal Experiment at Tohoku Medical and Pharmaceutical University.

Formalin (Wako Pure Chemical Industries, Osaka, Japan) was prepared as a 2% solution (0.74% formaldehyde in saline) then 20 μl were injected in the right hindpaw under the skin of the plantar surface using a 26-gauge microsyringe. CS (sodium chondroitin sulfate from swine cartilage supplied from Zeria Pharmaceutical Co., Tokyo, Japan) was dissolved in distilled water and orally administered twice daily starting 24 h after the formalin injection.

Tactile allodynia was determined by assessing paw withdrawal using the von Frey filament test. Mice were placed on a mesh floor inside a clear plastic cubicle for an acclimatization period of a least 30 min before the test. After adaptation to the testing environment, the von Frey filament was pressed perpendicularly against the plantar surface of the right hindpaw from beneath the mesh floor and held for 3 s with the filament slightly buckled. A positive response was noted if the paw was sharply withdrawn. Whenever

* Corresponding author. Fax: +81 22 727 0123.
E-mail address: koichi@tohoku-mpu.ac.jp (K. Tan-No).
Peer review under responsibility of Japanese Pharmacological Society.
[1] Contributed equally to this study.

http://dx.doi.org/10.1016/j.jphs.2016.07.009

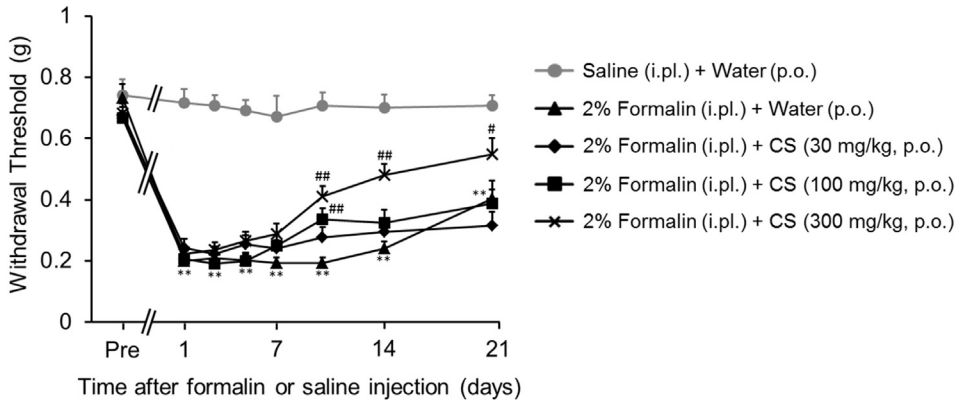

Fig. 1. Effect of CS on formalin-induced tactile allodynia in mice. The threshold for paw withdrawal to von Frey filaments was monitored during 21 days after the injection of formalin or saline. CS or water was orally administered twice daily starting 24 h after the formalin or saline injection. The threshold was measured before the first daily administration of CS or water. Values represent the means ± S.E.M. for groups of 12 mice. Two-way ANOVA: group ($F_{4,55} = 98.11$, $p < 0.01$), time ($F_{7,385} = 73.40$, $p < 0.01$), group × time ($F_{28,385} = 5.42$, $p < 0.01$). One-way ANOVA: pre, $F_{4,55} = 0.68$, $p = 0.61$; 1 day, $F_{4,55} = 54.81$, $p < 0.01$; 3 days, $F_{4,55} = 76.65$, $p < 0.01$; 5 days, $F_{4,55} = 52.44$, $p < 0.01$; 7 days, $F_{4,55} = 23.86$, $p < 0.01$; 10 days, $F_{4,55} = 33.79$, $p < 0.01$; 14 days, $F_{4,55} = 23.75$, $p < 0.01$; 21 days, $F_{4,55} = 10.91$, $p < 0.01$. $^{**}p < 0.01$ compared with the saline-water group and $\#\#p < 0.01$, $\#p < 0.05$ compared with the formalin-water group.

a positive response to a stimulus occurred, the next smaller von Frey hair was applied whereas the pressure was increased one level in the absence of a withdrawal response. Ambulation was considered an ambiguous response, and in such cases the stimulus was repeated.

Confocal microscopy observation and Western blotting were carried out as previously described (5). For immunofluorescence double labeling, 40 μm-thick slices were incubated with mouse anti-neuronal nuclei (NeuN) antibody (Millipore Co., MA, USA) and rabbit anti-c-Fos antibody (Cell Signaling Technology, MA, USA) overnight at 4 °C. These primary antibodies were diluted 1:200 and 1:100, respectively, with PBS containing 0.3% Triton-X and 1% normal goat serum (NGS; Invitrogen, CA, USA). Then, Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, OR, USA) and Alexa Fluor 647-conjugated Fab fragment goat anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, PA, USA) (diluted 1:200 with PBS containing 0.3% Triton-X and 1% NGS) were allowed to react in the dark at room temperature for 2 h. Fluorescence was visualized on a D-Eclipse C1 microscope (Nikon, Tokyo, Japan). For Western blotting, we used rabbit antibodies against p38 MAPK (Cell Signaling Technology) or phospho-p38 MAPK (Cell Signaling Technology), followed by HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technology) as primary and secondary antibodies, respectively. The density of the corresponding bands was analyzed using Image-J 1.43u (National institute of Health).

Data are expressed as means ± SEM. Significant differences were analyzed by a one-way or two-way analysis of variance (ANOVA), followed by Fisher's PLSD test for multiple-comparisons. Student's t-test was used for comparisons between two groups. In all comparisons, $p < 0.05$ was considered statistically significant.

Clinical trials have reported a beneficial effect of CS, either alone (6) or in combination with glucosamine (7) on pain in patient with osteoarthritis. Although it is not a rapidly acting agent such as nonsteroidal anti-inflammatory drugs (NSAIDs), as a SYSADOA, CS contributes to reducing NSAIDs consumption in osteoarthritis patients. In this study, the repeated administration of CS (300 mg/kg, b.i.d.) significantly prevented the decrease in the ipsilateral paw withdrawal threshold from day 10 after formalin injection (Fig. 1). Thus, it took 9 days for CS to exhibit its anti-allodynia properties. One reason that may explain the delayed action is the fact that CS is poorly absorbed through the gastrointestinal tract because of its high molecular weight (5–50 kDa) and highly negative surface charge (8).

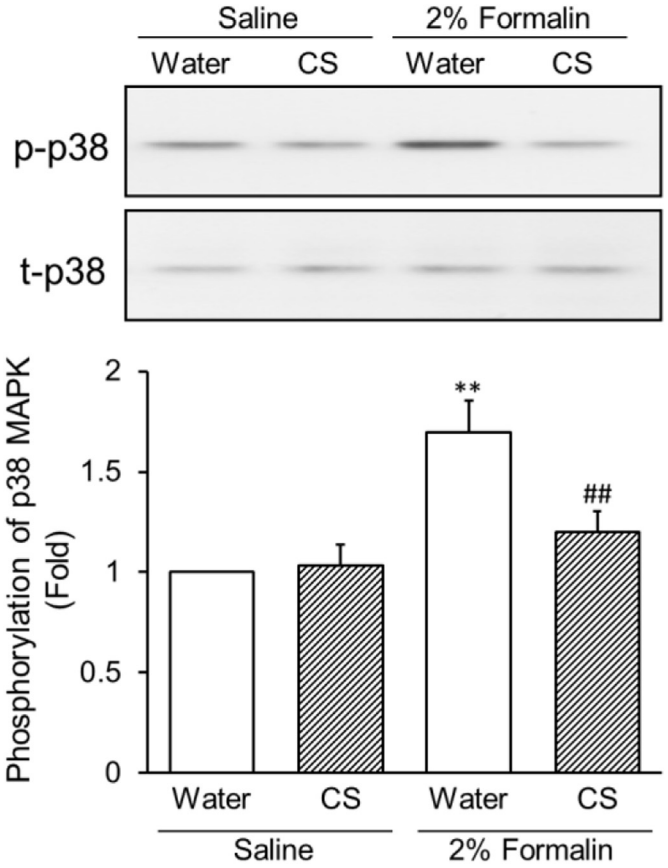

Fig. 2. Change in phospho-p38 MAPK levels in the lumbar spinal cord induced by formalin and the effect of CS treatment. CS (300 mg/kg) or water was orally administered twice daily starting 24 h after the formalin or saline injection, and spinal cord samples were collected on day 14. Top: representative Western blot showing phospho- and total-p38 MAPK. Bottom: relative quantification of phospho-p38 MAPK to total-p38 MAPK set as 1.0 in the saline/water group. Each value represents the means ± S.E.M. of 7 mice in each group. Two-way ANOVA: group ($F_{1,12} = 12.23$, $p < 0.01$), treatment ($F_{1,12} = 6.75$, $p < 0.01$), group × treatment ($F_{1,12} = 8.93$, $p < 0.05$). One-way ANOVA: $F_{3,24} = 8.94$, $p < 0.01$. $^{**}p < 0.01$ compared with the saline/water group, $\#\#p < 0.01$ compared with the formalin/water group.

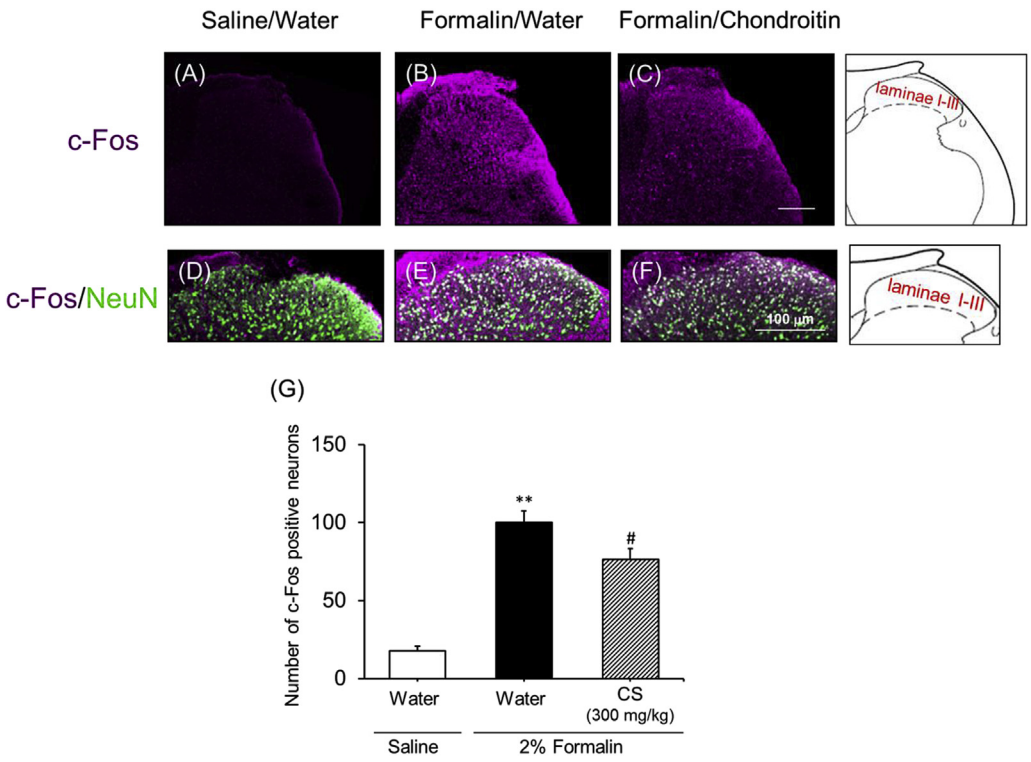

Fig. 3. Change in c-Fos expression in lumbar superficial dorsal horn neurons induced by formalin and the effect of CS treatment. CS (300 mg/kg) or water was orally administered twice daily starting 24 h after the formalin or saline injection, and spinal cord samples were collected on day 14. A–C: photomicrographs showing c-Fos-immunoreactive cells in the ipsilateral dorsal spinal cord (L5) of saline/water (A), formalin/water (B) and formalin/CS (C) groups. D–F: double immunofluorescence staining for c-Fos (magenta) and NeuN (green) in the ipsilateral superficial dorsal horn of saline/water (D), formalin/water (E) and formalin/CS (F) groups. Scale bar = 100 μm. (G) Number of c-Fos/NeuN double positive cells in the superficial dorsal horn (laminae I–III). Values represent the means ± S.E.M. for groups of 6–13 mice. One-way ANOVA: $F_{2,27} = 27.58$, $p < 0.01$. $^{**}p < 0.01$ compared with the saline/water group, $\#p < 0.05$ compared with the formalin/water group.

It has been shown that the inhibitory effect of CS on p38 MAPK phosphorylation is closely related to its anti-inflammatory effects. In chondrocytes, the phosphorylation of p38 MAPK induced by interleukin-1β (9) or lipopolysaccharide (10) was inhibited by CS. On the other hand, in the spinal cord, p38 MAPK activation is involved in formalin-induced tactile allodynia since the latter was ameliorated by the intrathecal injection of the p38 MAPK inhibitor SB203580 (11). In addition, while p38 MAPK is activated by the upstream kinase MKK3/6, the initiation of formalin-induced tactile allodynia was shown to be delayed in MKK3−/− mice (12). Therefore, to investigate whether p38 MAPK phosphorylation can be attenuated by CS, we examined its effect on the phosphorylation of p38 MAPK in the dorsal spinal cord on day 14 after formalin injection, a time-point that showed a marked anti-allodynic effect of CS. As shown in Fig. 2, formalin induced the phosphorylation of p38 MAPK in the dorsal lumbar spinal cord whereas this increase was significantly attenuated by the repeated administration of CS (300 mg/kg, b.i.d. for 13 days). This result suggests that the anti-allodynic effect of CS is due to the inhibition of formalin-induced spinal p38 MAPK phosphorylation.

The immediate early gene protein product c-Fos has been shown to be expressed in the dorsal horn neurons following a nociceptive stimulus (13). The expression of c-Fos has been widely accepted as an endogenous marker for neuronal reactivity and an increase in expression in the superficial dorsal horn was previously observed after the injection of formalin (14). On day 14, the immunofluorescence intensity for c-Fos increased in the superficial dorsal horn (laminae I–III) of the formalin/water group but the increase was attenuated in the CS-treated group (300 mg/kg, b.i.d. for 13 days) (Fig. 3A–C). The number of cells positive for NeuN (a specific marker for mature neurons) that were also positive for c-Fos increased approximately 5.5-fold in the formalin/water group compared with the saline/water group, and the increase in double-positive cells was partially but significantly lessened by CS treatment (Fig. 3D–G). Svensson et al. have reported that the formalin-induced spinal c-Fos expression was attenuated by the intrathecal administration of the p38 MAPK inhibitor SD-282 (14), suggesting that an increase in c-Fos expression after the injection of formalin is preceded by the activation of spinal p38 MAPK. Although ERK1/2 was also phosphorylated in the dorsal spinal cord of the formalin/water group, the increase in phospho-ERK1/2 was not affected by CS treatment (data not shown). Since spinal ERK1/2 activation is also involved in c-Fos up-regulation (15), it seems that CS only partially prevented the increase in the number of c-Fos-immunoreactive neurons by formalin, despite the fact that it almost completely attenuated the formalin-induced phosphorylation of p38 MAPK.

In conclusion, our data show that the repeated oral administration of CS attenuates the formalin-induced persistent tactile allodynia. The mechanism underlying the CS-induced anti-allodynia may be mediated through the inhibition of spinal p38 MAPK activation and subsequent up-regulation of c-Fos expression in the lumbar superficial dorsal horn neurons.

Conflicts of interest statement

Kotaro Yamada and Hidetomo Sakurai are employees of Zeria Pharmaceutical Co., Ltd.

Acknowledgments

This study was supported in part by JSPS KAKENHI Grant Number 26460101 to KT, WN and ON, KAKENHI 16K21311 to WN, and Matching Fund Subsidy for Private University from the Ministry of Education, Culture, Sports, Science and Technology of Japan S1511001L.

References

(1) Mathews MB, Glagov S. Acid mucopolysaccharide patterns in aging human cartilage. J Clin Invest. 1966;45:1103–1111.
(2) du Souich P, Garcia AG, Verges J, Montell E. Immunomodulatory and anti-inflammatory effects of chondroitin sulphate. J Cell Mol Med. 2009;13:1451–1463.
(3) Lamari FN. The potential of chondroitin sulfate as a therapeutic agent. Connect Tissue Res. 2008;49:289–292.
(4) Shimazaki A, Nakagawa T, Mitoma J, Higashi H. Gangliosides and chondroitin sulfate desensitize and internalize B2 bradykinin receptors. Biochem Biophys Res Commun. 2012;420:193–198.
(5) Nemoto W, Ogata Y, Nakagawasai O, Yaoita F, Tadano T, Tan-No K. Involvement of p38 MAPK activation mediated through AT1 receptors on spinal astrocytes and neurons in angiotensin II- and III-induced nociceptive behavior in mice. Neuropharmacology. 2015;99:221–231.
(6) Mazieres B, Hucher M, Zaim M, Garnero P. Effect of chondroitin sulphate in symptomatic knee osteoarthritis: a multicentre, randomised, double-blind, placebo-controlled study. Ann Rheum Dis. 2007;66:639–645.
(7) Hochberg MC, Martel-Pelletier J, Monfort J, Moller I, Castillo JR, Arden N, et al. Combined chondroitin sulfate and glucosamine for painful knee osteoarthritis: a multicentre, randomised, double-blind, non-inferiority trial versus celecoxib. Ann Rheum Dis. 2016;75:37–44.
(8) Kubo M, Ando K, Mimura T, Matsusue Y, Mori K. Chondroitin sulfate for the treatment of hip and knee osteoarthritis: current status and future trends. Life Sci. 2009;85:477–483.
(9) Jomphe C, Gabriac M, Hale TM, Heroux L, Trudeau LE, Deblois D, et al. Chondroitin sulfate inhibits the nuclear translocation of nuclear factor-kappaB in interleukin-1beta-stimulated chondrocytes. Basic Clin Pharmacol Toxicol. 2008;102:59–65.
(10) Holzmann J, Brandl N, Zemann A, Schabus R, Marlovits S, Cowburn R, et al. Assorted effects of TGFbeta and chondroitinsulfate on p38 and ERK1/2 activation levels in human articular chondrocytes stimulated with LPS. Osteoarthr Cartil. 2006;14:519–525.
(11) Li K, Lin T, Cao Y, Light AR, Fu KY. Peripheral formalin injury induces 2 stages of microglial activation in the spinal cord. J Pain. 2010;11:1056–1065.
(12) Sorkin LS, Boyle DL, Hammaker D, Herman DS, Vail E, Firestein GS. MKK3, an upstream activator of p38, contributes to formalin phase 2 and late allodynia in mice. Neuroscience. 2009;162:462–471.
(13) Hunt SP, Pini A, Evan G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature. 1987;328:632–634.
(14) Svensson CI, Marsala M, Westerlund A, Calcutt NA, Campana WM, Freshwater JD, et al. Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. J Neurochem. 2003;86:1534–1544.
(15) Kominato Y, Tachibana T, Dai Y, Tsujino H, Maruo S, Noguchi K. Changes in phosphorylation of ERK and Fos expression in dorsal horn neurons following noxious stimulation in a rat model of neuritis of the nerve root. Brain Res. 2003;967:89–97.