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European Journal of Pharmacology 554 (2007) 12–17
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# Intrathecally administered COX-2 but not COX-1 or COX-3 inhibitors attenuate streptozotocin-induced mechanical hyperalgesia in rats

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Received 27 March 2006; received in revised form 14 September 2006; accepted 22 September 2006
Available online 20 October 2006

**Abstract**

Members of the cyclooxygenase (COX) family are known to catalyze the rate-limiting steps of prostaglandins synthesis and reported to be involved in neuropathic pain. Diabetic neuropathy is a type of neuropathic pain, though it is not clear if COX is relevant to the condition. Recently, spinal COX-2 protein was found to be increasing in streptozotocin-induced rats as compared to the constitutive expression. We attempted to determine which cyclooxygenase isoforms are involved in streptozotocin-induced mechanical hyperalgesia, which was induced by a single intraperitoneal injection of 75 mg/kg of streptozotocin. Intrathecal administrations of the COX-2 inhibitors SC-58125 (7–100 μg) and NS-398 (7–60 μg), as well as a high dose (100 μg) of the COX-1 inhibitor SC-560 attenuated hyperalgesia, whereas intrathecal administrations of a low dose (10 μg) of SC-560 and the COX-3 inhibitor acetaminophen (1–7 mg) did not. Further, intrathecal administration of SC-58125 (100 μg) did not produce an analgesic effect in normal rats. These results indicate that intrathecal administration of COX-2 inhibitors has an anti-hyperalgesic effect on streptozotocin-induced mechanical hyperalgesia and we concluded that spinal COX-2 is pivotal in streptozotocin-induced hyperalgesia.


*Keywords:* COX-2; Diabetes; Hyperalgesia

## 1. Introduction

Diabetic neuropathy is one of the most common complications of diabetes and occurs predominantly in the distal extremities (Vinik et al., 1992; Clark and Lee, 1995; Morley et al., 1984). Patients with this condition can suffer spontaneously as a result of exposure to light but painful stimuli (i.e., hyperalgesia) as well as stimuli not normally perceived as painful (i.e., allodynia) (Brown and Asbury, 1984). Diabetic neuropathy is difficult to treat, because it is relatively resistant to standard analgesics such as nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids (Clark and Lee, 1995), which are common agents used for treating acute pain.

NSAIDs are used as analgesic drugs, as they are able to inhibit prostaglandin synthesis in peripheral tissues for a long period (McCormack, 1994). Two cyclooxygenase (COX) isozymes, COX-1 and COX-2, known to catalyze the rate-limiting steps of prostaglandins synthesis, are targets of NSAIDs (Kujubu et al., 1991). In normal cells, COX-1 is thought to be constitutive, whereas COX-2 is up-regulated in response to inflammation or injury (Seibert et al., 1994; Beiche et al., 1996; Willingale et al., 1997). However, in the spinal cord, both COX-1 and COX-2 are present in a constitutive manner (Willingale et al., 1997; Ebersberger et al., 1999). In 2002, a new isoform of COX-1 that is sensitive to acetaminophen was identified in canines and designated as COX-3 (Chandrasekharan et al., 2002), while COX-3 mRNA expression was more recently shown in rat spinal cord tissues (Kis et al., 2004).

Allodynia in nerve ligation rats, a model of neuropathic pain, has been shown to be suppressed by intrathecal administrations of a nonselective COX inhibitor as well as a COX-2 selective inhibitor (Zhao et al., 2000; Takeda et al., 2005), while the levels of COX-2 protein in the spine were shown to be increasing in the same rat model (Zhao et al., 2000). On the other hand, hyperalgesia in nerve injury and TNF-induced models was not suppressed by intrathecal administrations of COX-2 selective

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doi:10.1016/j.ejphar.2006.09.072

inhibitors (Padi and Kulkarni, 2004; Schafers et al., 2004). There are conflicting reports regarding the roles of COX-2 in the spinal cord after nerve injury, and though diabetic neuropathy is a type of neuropathic pain, its mechanism is unclear. It has also been reported that systemic or intrathecal administrations of nonselective COX inhibitors did not have an effect on streptozotocin-induced mechanical hyperalgesia (Ahlgren and Levine, 1993; Calcutt and Chaplan, 1997). Further, in a recent study of streptozotocin-induced hyperalgesia rats, spinal COX-2 protein levels were shown to be increasing at 4 weeks after streptozotocin induction, as compared to the constitutive expression (Freshwater et al., 2002). Nevertheless, it remains controversial whether the COX family is involved in diabetic neuropathic pain.

In the present study, we used selective COX-1, COX-2, and COX-3 inhibitors to investigate which isoforms of cyclooxygenase are involved in streptozotocin-induced mechanical hyperalgesia.

## 2. Materials and methods

### 2.1. Animals

The experimental protocols used were approved by the Institutional Animal Care Committee of Hiroshima University and consistent with the guidelines of the Ethical Committee of the International Association for the Study of Pain (Zimmermann, 1983). One hundred seventy-four adult male Sprague–Dawley rats, initially weighing 200–230 g, were used. They were housed individually at a constant room temperature of 23 ± 2 °C under a 12-hour light–dark cycle (lights on at 8:00 am) throughout the study.

### 2.2. Induction of diabetes

Diabetes was induced by a single intraperitoneal injection of 75 mg/kg of streptozotocin (Sigma, St. Louis, MO) freshly dissolved in 0.5 ml of sterile saline. Intraperitoneal streptozotocin is known to ablate pancreatic β cells and induce insulin deficient diabetes (Type I) (Rakieten et al., 1963). One week later, diabetes was confirmed by measuring plasma glucose concentration enzymatically (FreeStyle™, Nipro, Osaka, Japan) from tail vein samples and animals showing hyperglycemia (concentrations over 350 mg/dl) were considered diabetic. We measured plasma glucose concentrations once each week thereafter and if the concentration had decreased to 350 mg/dl or less, the rat was excluded from the experiment. Results of other previous studies (Fox et al., 1999; Hefferan et al., 2003b) as well as our own (Kajiyama et al., 2005) have demonstrated that streptozotocin-induced rats display reproducible hyperalgesia within 3 weeks after injection, which remains for at least 6 weeks.

### 2.3. Behavioral assessment of streptozotocin-induced mechanical hyperalgesia

To assess the development of mechanical hyperalgesia in the streptozotocin-induced rats, hind paw-withdrawal thresholds were measured weekly by using an electronic von Frey anesthesimeter (model 2290, Life Science Instruments, Woodland Hills, CA). The animals were placed in individual plastic chambers on a metal mesh floor for 15–30 min before testing to allow for adaptation to the environment. Next, the rigid tip of the anesthesimeter with a contact area approximately 1 mm in diameter was applied perpendicular to the plantar surface of the hind paw with sufficient force to cause brisk withdrawal or paw flinching. When the animal showed limb withdrawal, the paw-withdrawal threshold was digitally recorded in grams. Rats were selected when the mechanical withdrawal threshold became lower than 50% as compared to the controls (Kajiyama et al., 2005).

### 2.4. Implantation of intrathecal catheter

Three weeks after streptozotocin induction, 147 streptozotocin-induced and 27 normal rats were anesthetized with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital prior to surgical implantation of an intrathecal catheter. A polyethylene catheter (Intramedic PE-10, Becton Dickinson and Co., Franklin Lakes, NJ) was implanted with the tip positioned at the level of lumbar enlargement (Yaksh and Rudy, 1976). The catheter was then externalized to the back of the neck and sealed with a steel wire, and the musculature and skin were ligated with 3-0 silk sutures (Kajiyama et al., 2005). After a 1-week recovery period following catheterization, the rats were subjected to behavioral testing. Animals showing neurological deficits or a fatigued condition were excluded from further investigation.

### 2.5. Experimental protocols

COX inhibitors or acetaminophen were administered 4 weeks after streptozotocin induction. All pharmacological experiments were performed by using the same time schedule in order to avoid circadian rhythm variations. With nearly all of the dosages, the agents were given by intrathecal administration after being dissolved in 80% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) and 20% saline, or SC-58125 at 100 μg and acetaminophen at 7 mg dissolved in 100% DMSO. All reagents were dissolved in a total volume of 10 μl. After the agents were injected, 10 μl saline was injected to flush the catheter.

### 2.6. Agents

The agents used in this study were the COX-1 selective inhibitor, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560), the COX-2 selective inhibitors, 5-(4-fluorophenyl)-1-[4-(methylsulfonyl)phenyl]-3-(trifluoromethyl)-1H-pyrazole (SC-58125) and *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) were obtained from Cayman Chemicals (Ann Arbor, MI). 4-acetamidophenol (acetaminophen) was obtained from Sigma (St. Louis, MO). Each was dissolved in 100% DMSO and diluted with saline before administration, with the final concentration of DMSO ranging from 80% to 100%.

### 2.7. Mechanical nociceptive effects of intrathecally injected COX-1, -2, -3 inhibitors

SC-58125 at doses of 7, 20, 60, and 100 μg, NS-398 at doses of 7, 20, and 60 μg, SC-560 at doses of 10 and 100 μg, acetaminophen at doses of 1 and 7 mg, or the vehicle (DMSO) were intrathecally administered to streptozotocin-induced rats and age-matched normal rats, after the baseline threshold was determined. Thereafter, mechanical nociceptive thresholds were measured at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 8.0, and 24 h after intrathecal administration.

### 2.8. Data analysis

To determine whether an intraperitoneal injection of streptozotocin-induced significant mechanical hyperalgesia, we compared the pre-injection thresholds with thresholds obtained 3 to 4 weeks after treatment by using a paired *t* test.

To analyze the dose–response effects of the COX inhibitors, values were converted to the percent maximum possible effect (% MPE) by using the following formula; %MPE = {[post-treatment threshold − pretreatment baseline threshold]/[control threshold (60 g) − pretreatment baseline threshold]} • 100. The effect of each agent was determined by analysis of variance, followed by a post-hoc Tukey–Kramer test. A $P$ value of less than 0.05 was considered statistically significant. All values are expressed as mean ± S.E.M.

## 3. Results

At 1 week after streptozotocin induction, 68% of the rats developed hyperglycemia. The plasma glucose concentrations of the diabetic and age-matched normal rats were 419 ± 14 and 106 ± 4 mg/dl ($P < 0.05$), respectively. First, we confirmed mechanical hyperalgesia. Paw-withdrawal threshold before streptozotocin induction was 59.8 ± 1.6 g in all rats, while mechanical threshold was decreased slightly (53.9 ± 1.9 g; $P < 0.05$) at 1 week and markedly (41.3 ± 2.9 g; $P < 0.01$) at 2 weeks after streptozotocin induction. Sustained diabetic hyperalgesia at 4 weeks after streptozotocin induction was 21.2 ± 2.1 g, which was significantly lower than the value observed prior to streptozotocin induction (58 ± 1.3 g) ($P < 0.05$). At 4 weeks after streptozotocin induction, 60% of the streptozotocin-induced rats showed hyperalgesia. Thus, mechanical hyperalgesia started 1 week after streptozotocin induction and persisted for more than 5 weeks in 60% of the streptozotocin-injected rats showing hyperalgesia (Fig. 1).

SC-58125, a COX-2 inhibitor, significantly increased paw-withdrawal threshold in a dose-dependent manner and its anti-mechanical hyperalgesic effect at doses of 20, 60, and 100 μg was evident from 0.5 h after treatment. As the dose of SC-58125 increased, the peak effect tended to appear earlier. At doses of 20 and 60 μg, the effect was gradually reduced to the baseline level within 4 h, while the return to baseline occurred within 3 h with 100 μg (Figs. 2(a), 3(a)).

We investigated another COX-2 inhibitor, NS-398, in regards to its attenuation of streptozotocin-induced hyperalgesia to confirm the involvement of spinal COX-2. We found that the anti-mechanical hyperalgesic effect of NS-398 on paw-withdrawal threshold started from 2 h after treatment and returned to baseline within 24 h (Figs. 2(b), 3(b)).

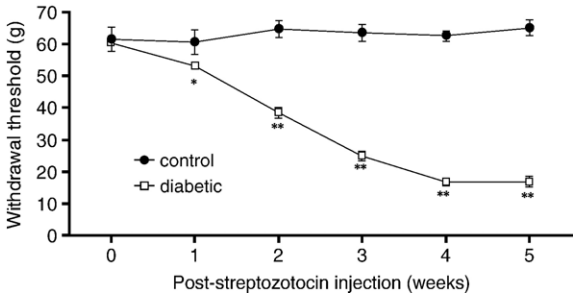


Fig. 1. Changes in mechanical nociceptive thresholds in diabetic and control animals. The time course of mechanical hyperalgesia was assessed by using paw withdrawal threshold to mechanical stimuli after intraperitoneal injection of streptozotocin or saline. "Diabetic" represents streptozotocin-induced mechanical hyperalgesia. Values are shown as the mean ± S.E.M. Control: $n$=5, diabetic: $n$=10, *$P$<0.05, **$P$<0.01.

We also investigated whether an inhibitor of COX-1 and COX-3 attenuated streptozotocin-induced hyperalgesia. Intrathecal administration of SC-560 at 100 μg increased the paw-withdrawal threshold, whereas that at 10 μg, which was thought to inhibit COX-1 completely, did not increase paw-withdrawal threshold. Evidence of the anti-mechanical hyperalgesic effect of SC-560 at 100 μg on paw-withdrawal threshold in streptozotocin-induced hyperalgesia rats was observed at 1 h after treatment and then returned to the baseline within 2 h. In addition, an intrathecal administration of acetaminophen did not increase paw-withdrawal threshold at a dose of 1 or 7 mg (data not shown). Although we attempted to determine whether the COX-2 inhibitor SC-58125 had an analgesic effect on age-matched normal rats, it was shown that a dose of 100 μg did not increase the paw-withdrawal threshold in response to applied mechanical stimuli (data not shown).

## 4. Discussion

We demonstrated that intrathecal administration of COX-2 inhibitors reduced streptozotocin-induced hyperalgesia in a dose-dependent manner, in contrast to COX-1 and COX-3 inhibitors, which did not show any effect. These findings suggest that spinal COX-2, but not COX-1 or COX-3, plays an important role in streptozotocin-induced hyperalgesia.

It was previously reported that intradermal administration of indomethacin, a nonselective COX inhibitor, at doses of 1 to 10000 ng did not significantly affect streptozotocin-induced hyperalgesia (Ahlgren and Levine, 1993), while intrathecal administration of the nonselective COX inhibitor ketorolac at 100 μg did not have an effect on tactile allodynia in streptozotocin-induced rats (Calcutt and Chaplan, 1997). Those results suggest that neither intradermal nor intrathecal administration of nonselective COX inhibitors produce an anti-hyperalgesic effect toward streptozotocin-induced hyperalgesia. In the present study, 2 different COX-2 selective inhibitors reduced hyperalgesia in streptozotocin-induced rats. Indomethacin (molecular weight 357.8), ketorolac (molecular weight 376.4), SC-

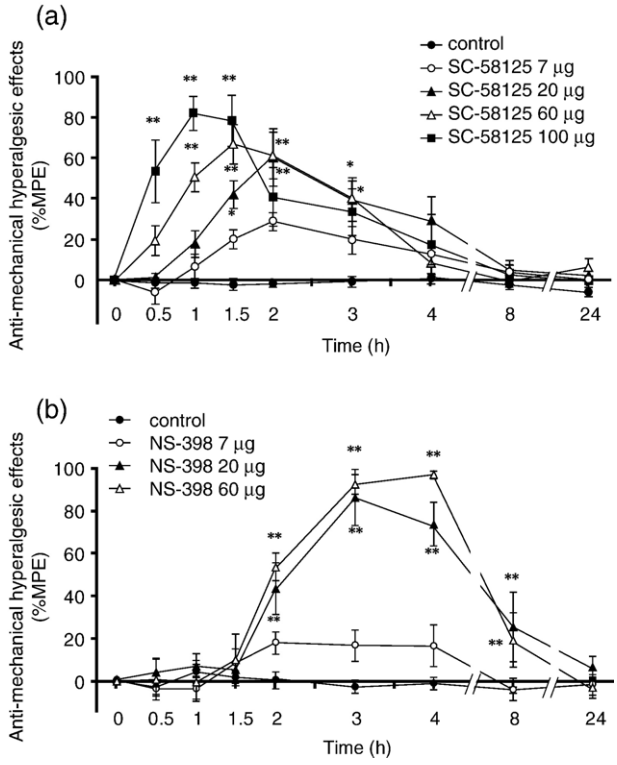


Fig. 2. Time course of dose-dependent anti-mechanical hyperalgesic effects of (a) 7, 20, 60, and 100 μg of SC-58125, and (b) 7, 20, and 60 μg of NS-398, as well as the control (DMSO), which were intrathecally administered to streptozotocin-induced mechanical hyperalgesia model rats. Six and 4 rats received SC-58125 and NS-398, respectively. Paw-withdrawal threshold was determined by withdrawal response of the hind paw to mechanical stimuli by using an electronic von Frey anesthesimeter. Values are shown as the mean threshold (%MPE) ± S.E.M., where %MPE = {[post-treatment threshold - pretreatment baseline threshold] / [control threshold (60g) - pretreatment baseline threshold]} ● 100. *$P$<0.05, **$P$<0.01, as compared to the respective baselines of the control at the corresponding time points.

58125 (molecular weight 384.3), and NS-398 (molecular weight 314.4) inhibited COX-2 with $IC_{50}$ values of 24.6 μM, 60.5 μM, 70 nM, and 1.77 μM, respectively, and the COX-2 inhibitor was shown to be more potent than the nonselective inhibitor in its efficacy to block COX-2. There is a possibility that doses of indomethacin and ketorolac used in previous experiments were insufficient to block COX-2. Generally, spinal administration of an agent is approximately 100 to 1000 times more potent than systemic administration. (Taiwo and Levine, 1988; Malmberg and Yaksh, 1992; Bjorkman, 1995; Jurna et al., 1992).

In another study, a continuous intrathecal administration of meloxicam, a selective COX-2 inhibitor, was started immediately after spinal nerve ligation and found to prevent the development of mechanical allodynia. Further, the involvement of spinal COX-2 was suggested in the initiation of mechanical allodynia, because the treatment did not suppress allodynia even when the rats were treated after the condition had become established (Takeda et al., 2005). In a study of streptozotocin-induced hyperalgesia rats, there was a 3-fold increase in the amount of spinal COX-2 protein as compared to the constitutive expression, which remained for up to 4 weeks after streptozotocin-induction (Freshwater et al., 2002). Consistent with those findings, intrathecal administration of the COX-2 selective inhibitors in the present experiments had anti-hyperalgesic effects on streptozotocin-induced hyperalgesia. In addition, intrathecal administration of SC-58125, a COX-2 selective inhibitor, did not produce an analgesic effect in normal rats. Thus, constitutive COX-2 protein is considered to not have a relationship with mechanical nociceptive thresholds in normal rats.

There is a conflicting evidence about the roles of COX-2 and prostaglandin $E_2$ ($PGE_2$) in the spinal cord after a nerve injury (Broom et al., 2004; Schafers et al., 2004; Ma et al., 2002; Marchand et al., 2005). Of those studies, Broom et al. reported that nerve injury seemed to be independent of COX-2 and $PGE_2$ in the spinal cord, whereas Ma et al. found that a COX-2 inhibitor reversed tactile allodynia after nerve injury. $PGE_2$ in the spinal cord may modulate pain processing in several ways, as it acts on E-series prostaglandin (EP) receptors expressed in the dorsal horn and primary sensory neurons (Yaksh et al., 2001). More recently, it was reported that $PGE_2$ may inhibit a glycine receptor subtype (GlyR alpha3) by phosphorylation, leading to disinhibition and, thereby, increased spinal pain processing (Harvey et al., 2004). Freshwater et al. found that diabetic rats had elevated COX-2 protein levels in the spinal cord and that hyperalgesia after noxious stimulus was accompanied by protracted spinal $PGE_2$ release (Freshwater et al., 2002). However, it was not clear

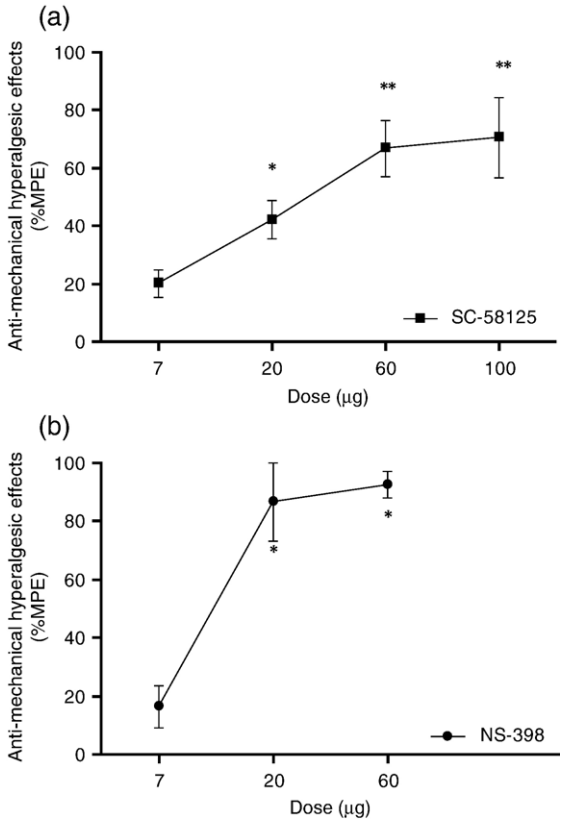


Fig. 3. Dose–response curves showing the anti-mechanical hyperalgesic effects of intrathecally administered (a) SC-58125 and (b) NS-398 in streptozotocin-induced mechanical hyperalgesia model rats. The ordinate shows the peak anti-mechanical hyperalgesic effect induced by SC-58125 or NS-398. Each point represents the mean±S.E.M. SC-58125 was administered to 6 rats and NS-398 to 4. *$P$<0.05, **$P$<0.01, as compared with the lowest dose (7 μg).

whether mechanical hyperalgesia had developed in those diabetic rats. The present study rats were confirmed to have mechanical hyperalgesia, which was inhibited by intrathecal administration of the COX-2 inhibitors. We speculated that $PGE_2$ was released as long as hyperalgesia continued.

The COX-1 inhibitor SC-560, given intrathecally to rats with streptozotocin-induced hyperalgesia, showed an anti-hyperalgesic effect at the high dose of 100 μg, but not the low dose of 10 μg. Further, though an intrathecal administration of 10 μg of SC-560 was thought to fully inhibit COX-1, no anti-hyperalgesic effect appeared at this dose. In addition, administration of SC-560 at 100 μg is considered to fully inhibit COX-1 and slightly inhibit COX-2. Therefore, we speculated that the administration of 100 μg of SC-560 attenuated streptozotocin-induced hyperalgesia by inhibiting COX-2. Spinal COX-1 is known to be induced in nerve ligation models and intrathecal administrations of COX-1 inhibitors have been shown to have anti-hyperalgesic effects (Zhu and Eisenach, 2003; Hefferan et al., 2003a).

A new isoform of COX-1 that is sensitive to acetaminophen was recently identified in canines and designated as COX-3 (Chandrasekharan et al., 2002), after which COX-3 mRNA expression was found in rat spinal cords (Kis et al., 2004). However, retention of the 98-bp intron-1 in rats causes a frame shift and COX-3 mRNA does not encode the acetaminophen-sensitive COX-3 protein in rats (Hersh et al., 2005; Snipes et al., 2005), thus the existence of COX-3 protein in rodents is controversial. Acetaminophen induced a significant antinociceptive effect following intravenous and intrathecal administrations in carrageenin-treated rats (Alloui et al., 2002). In other reports, oral and intrathecal administrations of acetaminophen inhibited nociceptive behavior in both phases of formalin test and chemotherapy-induced neuropathic pain (Choi et al., 2001; Bonnefont et al., 2003; Lynch et al., 2004), as well as in phase 2 of formalin test and carrageenin treatment (Malmberg and Yaksh, 1992; Alloui et al., 2002). Further, the analgesic effects of acetaminophen have been reported in some neuropathic pain models. But, we found that an intrathecal administration of acetaminophen had no effect on streptozotocin-induced hyperalgesia in the present study.

It remains controversial which cells in the spinal cord express COX-2, though it has been reported that spinal COX-2 mediates allodynia and hyperalgesia in various pain models (Zhao et al., 2000; Takeda et al., 2005). Further, one study found that astrocytes were activated when spinal COX-2 protein was increased in nerve ligation model rats (Takeda et al., 2005), while another showed that COX-2 protein in the dorsal horn was increased in a spared nerve injury model (Broom et al., 2004). In the present study, we did not examine the localization of COX-2 protein in the spinal cord. It will be necessary for a future examination to determine which cells in the spinal cord take part in the appearance of COX-2 in streptozotocin-induced hyperalgesia rats.

Diabetic mechanical hyperalgesia becomes evident within 8 days following streptozotocin induction and lasts for at least 4 weeks (Fox et al., 1999; Ahlgren and Levine, 1993; Chen and Pan, 2002; Malcangio and Tomlinson, 1998; Courteix et al., 1993; Lynch et al., 1999). It has also been reported that changes in thermal nociceptive thresholds are highly variable. Streptozotocin-induced thermal hyperalgesia has also been shown (Courteix et al., 1993) and other reports have found that loss of thermal sensation occurred in the early stage, but did not continue in experiments that lasted for at least 4 weeks (Fox et al., 1999; Malcangio and Tomlinson, 1998). Together, these results suggest that thermal sensation in streptozotocin-induced rats is variable, which is why we did not investigate thermal nociceptive thresholds in the present study.

In conclusion, intrathecal administration of COX-2 inhibitors showed anti-hyperalgesic effects toward streptozotocin-induced hyperalgesia. We concluded that spinal COX-2 is pivotal in streptozotocin-induced hyperalgesia.

## Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C), No. 17791032, from Japan Society for the Promotion of Science, and a Grant-in-Aid for Scientific Research from Tsuchiya Foundation. We express our appreciation to the staff of the animal experiment facilities of Hiroshima University for their kind assistance.

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