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Effect of spinal cyclooxygenase inhibitors in rat using the formalin test and in vitro prostaglandin E_2 release

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Abstract

Spinally delivery of the non-specific cyclooxygenase inhibitor, *S*(+)-ibuprofen, reduces the second phase of the formalin test and the evoked release of prostaglandin E_2 (prostaglandin E_2) from rat spinal cord in vitro. Using two selective cyclooxygenase-2 inhibitors, SC58125 (1-[(4-methysufonyl)phenyl]-3-tri-fluoromethyl-5-(4-fluorophenyl)pyrazole) and SC-236 (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide), we observed that neither agent at the highest dose/concentration employed altered the second phase of the formalin test after intrathecal delivery or K^+-evoked prostaglandin E_2 release from spinal cord in vitro, although ibuprofen was effective in both models. These observations suggest that cyclooxygenase-2 may not be associated with spinal prostanoid synthesis acutely or with facilitated nociception which occurs within the limited time frame of the formalin test. © 1997 Elsevier Science B.V.

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1. Introduction

Prostaglandin synthesis by cyclooxygenase and its inhibition by non-steroidal anti-inflammatory drugs (NSAIDs) have long been associated with peripheral inflammation and pain (Vane, 1971). More recently, spinal nociceptive mechanisms of prostanoid action have been proposed based on several observations: (i) spinal delivery of cyclooxygenase inhibitors have been shown to diminish the facilitated component of processing evoked by persistent afferent activity generated by peripheral injection of irritants (Yaksh, 1982; Malmberg and Yaksh, 1992); (ii) spinal delivery of prostanoids will evoke a hyperalgesic state (Uda et al., 1990) and (iii) peripheral injury or inflammation will increase spinal prostanoid release (Malmberg and Yaksh, 1995; Yang et al., 1996). The mechanism of this facilitation is unclear, however, prostanoids have been shown to facilitate transmitter release from sensory afferents (Hingtgen and Vasko, 1994; Hingtgen et al., 1995).

Two isozymes of the cyclooxygenase enzyme have now been shown to exist: cyclooxygenase-1 and cyclooxygenase-2. Investigation of the pharmacology of cyclooxygenase inhibition indicates that NSAIDs, such as ibuprofen, are mixed inhibitors of these two enzymes (Seibert et al., 1994; Gierse et al., 1996). Current developments in the pharmacology of cyclooxygenase inhibitors have led to the synthesis of highly selective cyclooxygenase-2 inhibitors. The importance of this differential activity has arisen from several issues. Originally, it was believed that cyclooxygenase-1 was constitutively expressed while cyclooxygenase-2 was upregulated in response to several cellular stimuli. Thus, cyclooxygenase-2 expression is increased in the hippocampus by seizures or *N*-methyl-D-aspartate (NMDA) injection (Yamagata et al., 1993) and is rapidly induced in several inflammatory states and by growth factors (Raz et al., 1988; Masferrer et al., 1990; Sirois and Richards, 1992). However, recent findings have demonstrated constitutive expression of cyclooxygenase-2 in several tissues such as macula densa, testis and, more importantly, both brain (Breder et al., 1995) and spinal cord (Beiche et al., 1996). Significantly, cyclooxygenase-2 mRNA appears to represent the most common isoform in spinal cord (Beiche et al., 1996). These observations thus

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lead to questions regarding the pharmacology of spinal prostanoid synthetic dependence upon cyclooxygenase-1, cyclooxygenase-2, or both. The development of specific cyclooxygenase-2 inhibitors allows the re-examination of studies employing mixed cyclooxygenase-1/cyclooxygenase-2 inhibitors (i.e., NSAIDs) in order to investigate which isoform(s) of cyclooxygenase are involved in nociception. The current study uses specific cyclooxygenase-2 inhibitors to investigate the role of rat spinal cyclooxygenase isozymes in formalin-associated nociception as well as evoked release of prostanoids from an in vitro spinal cord preparation.

2. Materials and methods

2.1. Animal preparation

Male Sprague Dawley rats (300–325 g; Harlan Industries, Indianapolis, IN, USA) were housed pair-wise in cages and maintained on a 12 h light/dark cycle with access to food and water at all times. Chronic intrathecal (i.t.) catheters were implanted under halothane anesthesia according to a modification of the procedure described by Yaksh and Rudy (1976). A polyethylene catheter (PE-10) was inserted through an incision in the atlanto–occipital membrane and advanced caudally to the rostral edge of the lumbar enlargement. The rostral segment of the catheter was tunneled subcutaneously and externalized on top of the head. After implantation, rats were housed individually as described above and monitored daily for signs of neurological dysfunction. All studies were carried out under protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

2.2. Behavioral testing paradigm

Prior to any testing and at least three days post-implantation, all animals were weighed and checked for i.t. catheter patency with 5 μl of physiological saline. Animals were placed individually in Plexiglas cylinders (20 × 30 cm) on absorbent paper and allowed to acclimate to the apparatus for a minimum of 10 min before testing. A mirror was placed behind the cylinder for unencumbered observation of the animal during the test. All agents were administered intrathecally 10 min prior to formalin injection.

For formalin injection, animals were gently restrained, and 50 μl of formalin (5%) was injected subcutaneously into the dorsal surface of the right hind paw. Time zero of the study was defined as injection of formalin. Flinches were counted for one min intervals from 1–2 min, 5–6 min, and every 5 min for 60 min, after which subjects were immediately euthanized. Criteria for exclusion from the study included > 20% weight loss, catheter occlusion, incomplete formalin injection, or excessive bleeding from injection site.

2.3. In vitro spinal cord preparation

After terminal halothane anesthesia (4%), rats were decapitated, and spinal cords were hydraulically extruded. Spinal cords were placed in ice-cold isotonic buffer and then dissected on a filter paper-covered glass plate placed on crushed ice. A 2 cm segment of the lumbar enlargement was isolated and hemisected longitudinally into lateral halves. These halves were hemisected again, and the dorsal quadrants were retained. These dorsal segments were chopped cross-sectionally into 2 mm prisms. Prisms were dispersed on Millipore filters (13 mm diam, 5 μm pore size) which were placed inside perfusion chambers (Modified Millipore filter units, Bedford, MA, USA).

2.4. In vitro perfusion and stimulation

The prisms of one lumbar enlargement were used in each perfusion study and dispersed at random to 3 or 4 perfusion chambers (5 prisms per chamber). Perfusion chambers maintained at 37°C in a water bath were perfused with artificial cerebral spinal fluid (ACSF) at a rate of 200 μl/min via peristaltic pump. ACSF consisted of $NaHCO_3$, 21.0 mM; $Na_2HPO_4 \cdot 7H_2O$, 2.5 mM; NaCl, 125.0 mM; KCl, 2.6 mM; $MgCl_2 \cdot 6H_2O$, 0.9 mM; $CaCl_2$, 1.3 mM; D-glucose, 3.9 mM. When a high K^+ solution was employed as a stimulus, KCl was increased to 60 mM, and NaCl reduced to 67.6 mM to maintain osmolarity. The ACSF reservoir for each study was placed in the same water bath as the perfusion chambers, and pH was adjusted by bubbling with 5% CO_2/95% O_2 for 30 min prior to and throughout the study. After an initial washout period of 45 min to allow tissue stabilization, a ten min baseline and a ten min stimulation (60 mM K^+) sample were collected. Chamber temperature was monitored continuously using a thermocouple (36Ga, Type T, Omega Instruments) with digital readout permanently implanted in one perfusion chamber.

2.5. Prostaglandin E_2 radioimmunoassay

In vitro samples were collected on ice, frozen at −70°C, lyophilized, and stored at −70°C until reconstitution for competitive radioimmunoassay (RIA) using a polyclonal rabbit anti-prostaglandin E_2 antibody in conjunction with a magnetic particle-coupled, goat anti-rabbit antibody (PerSeptive Biosystems, Framington, MA, USA). Specifics of this methodology have been previously published (Jobke et al., 1973; Granstrom and Kindahl, 1978). Briefly, lyophilized perfusate samples or prostaglandin E_2 (as standard) were reconstituted in assay buffer (0.01 M phosphate, 0.1% bovine gamma globulin and 0.1% sodium azide, pH 7.0) and incubated with the rabbit anti-prostaglandin E_2 for 2 h at 4°C. ^{125}I-labeled prostaglandin E_2 was then added, and the mixture was incubated overnight at 4°C. After precipitation of the bound complex using

goat anti-rabbit serum and centrifugation (15 min, 4°C, $1000 \times g$), the pellet was counted in a γ-counter. Assays were carried out with non-specific binding and blanks, and minimum assay sensitivity was 4 pg/assay tube.

2.6. Drug delivery

Drugs were delivered intrathecally in a total volume of 10 μl using a gear-driven syringe pump. This drug delivery was followed immediately by a 10 μl volume of vehicle to flush the catheter. The following drugs were used in this study: *S*(+)-ibuprofen, SC58125 (1-[(4-methysufonyl)phenyl]-3-tri-fluoromethyl-5-(4-fluorophenyl)pyrazole), and SC-236 (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide). SC58125 and SC-236 were obtained from G.D. Searle (St. Louis, MO, USA). Drugs were dissolved in dimethyl sulfoxide (DMSO) and then diluted 1:1 with 50% β-hydroxy-propyl cyclodextrin in sterile water. Vehicle controls used the same combination of DMSO and cyclodextrin. For in vitro experiments, drugs were dissolved in 100% DMSO to a concentration of 5 mg/ml, and then diluted to final concentration with ACSF or 60 mM K^+ in ACSF. The final DMSO concentration did not exceed 0.75%. All drugs were reconstituted on the morning of testing and used only on that day.

2.7. Data analysis and statistics

Flinch counts were separated into Phase 1 (0–9 min) and Phase 2 (10–60 min) responses and expressed as mean total flinches/phase of five animals per treatment group ± S.E.M. Additionally, Phase 2 was separated into 2A (20–40 min) and 2B (40–60 min) as described by Malmberg and Yaksh (1992). Each drug cohort was analyzed with respect to changes in flinching behavior relative to the vehicle control group. Any significant difference indicated by analysis of variance (ANOVA) was examined further using Student Neuman Keuls post-hoc multiple comparison test. Resting in vitro release of prostaglandin E_2 was compared across multiple trials using ANOVA with Scheffe's post-hoc correction for different values of *n* and multiple comparisons. No difference was observed in basal release, which was then pooled and compared to K^+-evoked release in the presence or absence of cyclooxygenase inhibitors, using Bonferroni-Dunn comparison of all means to control. In all tests, the minimum criteria for statistical significance was $P < 0.05$.

3. Results

3.1. Formalin responses

Besides the mild activation evoked by vehicle, no changes in motor tone or other behavioral effects were observed after administration of any drug. Vehicle-injected animals were agitated and vocalized immediately after intrathecal injection, but this subsided within one min of injection. These effects were transient, and animals displayed a classic biphasic formalin response (Wheeler-Aceto et al., 1990) which was not different from that observed after i.t. saline (Dirig and Yaksh, 1995).

As shown in Fig. 1, pre-treatment ($t = -10$ min) with *S*(+)-ibuprofen (80 nmol, i.t.) did not affect Phase 1 or

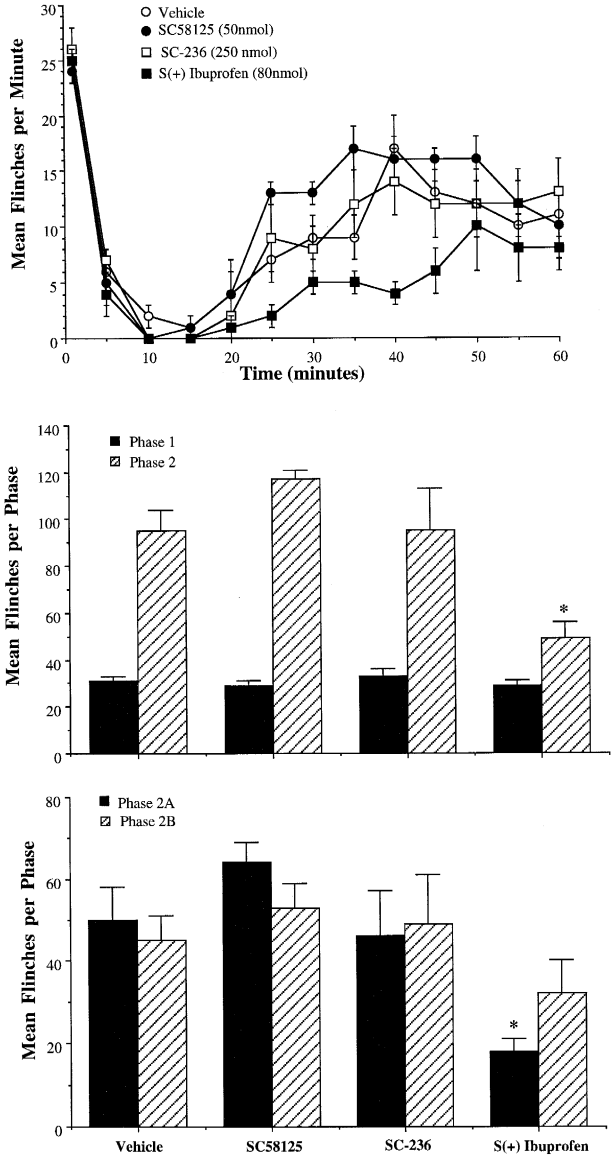

Fig. 1. Top panel describes the time course of formalin responses of rats after intrathecal injection with Vehicle (open circles), SC58125 (50 nmol, closed circles), SC-236 (250 nmol, open squares), and *S*(+)-ibuprofen (80 nmol, closed squares). Time course for each drug group represents the mean of five animals ± S.E.M. The middle panel represents the same time course data as mean flinches per phase. Note that *S*(+)-ibuprofen significantly suppressed flinching during Phase 2 only. When Phase 2 responses are split into Phase 2A and 2B in the bottom panel, the statistically significant suppression ($P < 0.05$) occurs during Phase 2A only.

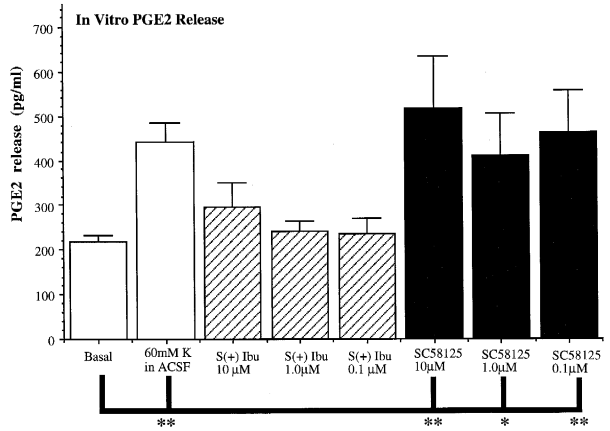

Fig. 2. ACSF containing 60 mM K^+ evoked a significant increase from basal prostaglandin E_2 in vitro, as shown by the open bars. Drug treatment groups are presented as K^+-evoked prostaglandin E_2 release across drug treatment groups (5 perfusion chambers per group ± S.E.M.). *S*(+)-ibuprofen blocked this increase (hatched bars), but the specific cyclooxygenase-2 inhibitor, SC58125, did not block the increase in prostaglandin E_2 release evoked by 60 mM K^+ (filled bars). Significant increases from baseline were determined by ANOVA with Bonferroni/Dunn comparison of all means to control indicated by $^{*}P < 0.05$ and $^{**}P < 0.0001$.

Phase 2B relative to vehicle controls, but significantly suppressed Phase 2A ($P < 0.05$). The formalin response after pre-treatment with either SC58125 (50 nmol, i.t.) or SC-236 (250 nmol, i.t.) did not differ from control animals. These doses were selected on the basis of solubility and for their maximum efficacy in other behavioral studies (Hammond and Gregory, 1996).

3.2. In vitro release

As shown in Fig. 2, K^+ (60 mM) evoked a significant 2-fold increase in extracellular PGE2 levels ($P = 0.0001$). Inclusion of *S*(+)-ibuprofen (1–100 μM) in K^+-containing ACSF blocked the K^+-evoked increase in prostaglandin E_2, but did not affect basal levels. Consistent with the formalin data described above, SC58125 (1–100 μM) did not affect K^+-evoked release of prostaglandin E_2 ($P < 0.05$).

4. Discussion

4.1. Pharmacology of the formalin model

The first phase of the formalin test is representative of a short-lasting burst of small afferent activity. During the second phase, there is a low, but non-zero level of peripheral afferent activity (Puig and Sorkin, 1996). Examination of the firing of dorsal horn neurons reveals a prominent biphasic response pattern with the first phase corresponding to the initial elevation in afferent input, while the second phase is believed to reflect a state of facilitated processing driven by the moderate ongoing peripheral input (Dickenson and Sullivan, 1987). Afferent input evokes release of excitatory amino acids and peptides such as neurokinins that lead to the initiation of a state of facilitation, mediated in part by activation of *N*-methyl-D-aspartate (NMDA) subtype glutamate receptors and tachykinin NK_1 receptors. This activation is thought to lead to the release of nitric oxide and several prostanoids that jointly serve to initiate a state of facilitated processing (for review, see Yaksh, 1993, 1997). One possible mechanism of the prostanoid-mediated spinal sensitization may be represented by cultured sensory neuron studies demonstrating that prostanoids (prostaglandin E_2 and prostaglandin I_2) potentiate the evoked release of Substance P, an endogenous tachykinin NK_1 receptor ligand. NMDA and NK_1 receptor antagonists are only effective intrathecally if given before formalin injection; if administered after Phase 1, these agents have little or no effect (Yamamoto and Yaksh, 1991, 1992), whereas i.t. administration of mixed-function cyclooxygenase inhibitors (NSAIDs) are effective in blocking the early part of Phase 2 (Phase 2A) whether given before or after formalin. Malmberg and Yaksh (1992) showed that pre- or post-treatment with various NSAIDs suppressed Phase 2A flinching in a stereospecific manner that mirrored the known structure-activity relationships for the various compounds. Such findings suggest that the pharmacology of Phase 1 and 2 responses differ substantially, with early activation of NMDA and NK_1 receptors and later production of prostanoids during Phase 2 being critical for the development of protracted nociception during the formalin test.

4.2. Cyclooxygenase isozymes and nociception

In agreement with Malmberg and Yaksh (1992), the current study showed that intrathecal administration of *S*(+)-ibuprofen, an NSAID which inhibits both cyclooxygenase-1 and cyclooxygenase-2, suppresses Phase 2A of the formalin test. The inactive enantiomer, *R*(−)-ibuprofen, was not employed, but the stereoselectivity of cyclooxygenase inhibition by *S*(+), but not *R*(−)-ibuprofen, is well documented (Adams et al., 1976; Buttinoni et al., 1983; Malmberg and Yaksh, 1994; Gierse et al., 1995). Given the constitutive expression of cyclooxygenase-1 and cyclooxygenase-2 message within the rat spinal cord (Beiche et al., 1996), one possible outcome of this study was that NSAIDs were acting to inhibit both cyclooxygenase isozymes to produce the anti-hyperalgesic effects. Alternatively, induction of cyclooxygenase-2 expression may be increased by the peripheral injury, leading to the development of cyclooxygenase-2 inhibitor sensitivity that may or may not be observed within the 1 h window of the

formalin test. Surprisingly, intrathecal administration of two different cyclooxygenase-2 inhibitors did not affect Phase 1 or Phase 2 of the formalin test. There are several possible explanations for this outcome.

While cyclooxygenase-2 mRNA has been shown to be present in the rat spinal cord (Beiche et al., 1996), post-transcriptional degradation of cyclooxygenase-2 message cannot be ruled out. Studies demonstrating presence of cyclooxygenase-2 protein within the rat spinal cord have yet to be published. Beiche and colleagues demonstrated an increase in spinal cyclooxygenase-2 mRNA with 6 h of inflammation, but 20–40 min of tissue injury after formalin injection would not appear a sufficient time interval to allow upregulation of cyclooxygenase-2 mRNA and translation of new enzyme. Thus, the presence of constitutive cyclooxygenase-2 message or its induction may not play a role in the spinal processing of nociception associated with the formalin test.

SC58125 and SC-236 are specific inhibitors of cyclooxygenase-2 and do not inhibit cyclooxygenase-1 (Seibert et al., 1994; Gierse et al., 1996; Penning et al., 1997). While these novel compounds are useful, they are insoluble in aqueous solvents. Due to the adverse effects of 100% DMSO as a vehicle, we attempted to find an alternative by dissolving the drug in DMSO and then diluting (1:1) with a cyclodextrin solution. Unfortunately, below 25% β-hydroxy-propyl cyclodextrin (final concentration), neither drug remained in solution. The negative data generated with respect to these cyclooxygenase-2 inhibitors could also be the result of precipitation of the compounds from the vehicle or lack of drug dissolution from the viscous cyclodextrin solution in vivo. To address these concerns, we repeated the formalin testing of all three compounds using the same doses in 100% DMSO and generated similar results (data not shown). Our results and those of Hammond and Gregory (1996) bear out this method, in that both of these agents were effective in the same vehicle and concentration intrathecally using the more protracted paw carrageenan model of thermal hyperalgesia. Additionally, the utility of using cyclodextrin as an intrathecal vehicle for hydrophobic compounds has been previously described, such that highly lipophilic compounds may be administered intrathecally using β-hydroxy-propyl cyclodextrin without changes in drug availability or ED_{50} (Yaksh et al., 1991). Thus, it is unlikely that the lack of suppression observed with the cyclooxygenase-2 inhibitors employed results from a lack of drug availability at a putative spinal site of action, but rather suggests an insensitivity of the formalin model to spinal cyclooxygenase-2 inhibition.

The current study suggests that cyclooxygenase-1, but not cyclooxygenase-2, is relevant for the development of phase 2 hyperalgesic formalin responses. The dichotomy presented by the constitutive expression of cyclooxygenase-2 message within the cord, but no suppression of behavioral responses after pre-treatment with intrathecal cyclooxygenase-2 inhibitors, is curious. Smith and DeWitt (1996) have proposed a separate role for cyclooxygenase isozymes based on intracellular localization, whereby cyclooxygenase-1 is responsible for the extracellular physiological responses associated with prostaglandins, and cyclooxygenase-2 prostaglandin synthesis is associated with intra-nuclear events. Cyclooxygenase-2 is more concentrated on the nuclear envelope (Morita et al., 1995), and its synthesis of prostanoids may be associated with nuclear events such as gene regulation or induction. Alternatively, we suggest that the downstream nuclear events of cyclooxygenase-2 activation (i.e. gene activation) may be important for the development of hyperalgesia over an interval of persistent afferent activation. The formalin test reflects upon an early component of the post-inflammatory process and reveals that cyclooxygenase-2 may not be relevant to spinal prostaglandins synthesis within this shortened time frame.

4.3. *In vitro* spinal prostaglandin E_2 release

The lack of acute sensitivity to cyclooxygenase-2 inhibition within the spinal cord is also supported by the in vitro data presented above. In acutely harvested spinal cords, cyclooxygenase-2 inhibitors were ineffective in inhibiting K^+-evoked prostaglandin E_2 release whereas the mixed-inhibitor, *S*(+)-ibuprofen, blocked evoked release of prostaglandin E_2. It is interesting to note that resting release was not changed in the presence of ibuprofen. This may be due to the presence of a previously-synthesized pool of prostaglandin E_2 before inhibitor and K^+ were added. Bishai and Coceani (1980) demonstrated prostaglandin E_2 catabolism by 15-hydroxyprostaglandin dehydrogenase in cat spinal roots, but not spinal cord tissue itself, and the current preparation strips off the spinal roots before spinal cord dissection. Accordingly, in an in vitro preparation without access to catabolic enzymes, it is reasonable to assume that prostanoid levels may be artificially maintained even in the presence of synthesis inhibitors.

In conclusion, the present study demonstrates in both acutely harvested in vitro studies and the in vivo formalin test, that a mixed cyclooxygenase inhibitor was effective in blocking prostaglandin E_2 release and hyperalgesic formalin responses, whereas specific cyclooxygenase-2 inhibitors were not. Thus, it appears likely that in acute situations, spinal cyclooxygenase-1, but not cyclooxygenase-2, is involved in the synthesis of prostaglandin E_2 and protracted nociception. The question of whether the time frame employed biases these studies against the development of a longer term cyclooxygenase-2 sensitivity will require intrathecal studies using protracted nociceptive models and in vitro release from spinal cords of animals after a prolonged inflammatory state (e.g., paw carrageenan).

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