Brief Communication

Downregulation of Cytosolic Prostaglandin E_2 Synthase Results in Decreased Nociceptive Behavior in Rats

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Nociception-evoked prostaglandin E_2 (PGE_2) release in the spinal cord contributes considerably to the development of hyperalgesia and allodynia. Biosynthesis of PGE_2 involves the conversion of arachidonic acid to PGH_2 by cyclooxygenases (COXs), followed by an isomerization of PGH_2 to PGE_2 by PGE_2 synthases (PGESs). The roles of COX-1, COX-2, and the inducible microsomal PGES-1 have been studied in models of pain and inflammation. In contrast, in nociceptive processes, very little is known about the role of cytosolic PGES (cPGES), which has been described as being functionally coupled to COX-1. Here we show by *in situ* hybridization and immunohistological analysis that COX-1 and cPGES are constitutively expressed in neuronal and non-neuronal cells of the dorsal and ventral horns in the spinal cord of adult rats. The protein levels of both enzymes were not regulated by nociceptive stimuli; however, reduction of cPGES in rat spinal cord with intrathecal application of cPGES antisense oligonucleotides reduced the nociceptive behavior in zymosan-evoked thermal hyperalgesia and in the formalin assay. The data indicate that cPGES plays an important role in mediating early responses during spinal nociceptive processing.

Key words: **pain; spinal cord; nociception; prostaglandin; prostaglandin synthase; hyperalgesia**

Introduction

Prostaglandin E_2 (PGE_2) is an important mediator of spinal nociceptive processing, inflammation, and fever (Ballou et al., 2000; Baba et al., 2001; Samad et al., 2001; Vanegas and Schaible, 2001; Bar et al., 2004). The biosynthesis of PGE_2 comprises several well studied steps. After its release by phospholipase A_2 from the membrane, arachidonic acid is converted by the constitutively expressed cyclooxygenase-1 (COX-1) or the inducible COX-2 to PGH_2 (Maihofner et al., 2000; Smith et al., 2000; Murakami and Kudo, 2004). PGH_2 is then converted to PGE_2 by PGE_2 synthases (PGESs) (Murakami and Kudo, 2004). According to biochemical and histological studies, the two COX isoforms seem to be functionally coupled to specific PGE_2 synthases. COX-1 has been shown to be coupled to cytosolic PGES (cPGES) (Tanioka et al., 2000) and COX-2 to microsomal PGES-1 (mPGES-1) (Jakobsson et al., 1999; Murakami et al., 2000; Ek et al., 2001). Thus, as for COX-2, we would expect mPGES-1 to play an important role in pain, inflammation, and fever. Indeed, it was shown recently that mPGES-1-deficient mice have a decreased PGE_2 synthesis (Boulet et al., 2004) and a reduced response in fever, inflammation, and some models of nociception (Engblom et al., 2003; Trebino et al., 2003; Kamei et al., 2004).

The role of COX-1 and cPGES in spinal nociceptive processing has been less well studied; however, there is mounting evidence that COX-1 is involved in nociceptive processing. Ballou et al. (2000) showed that COX-1 was the primary COX isoform that is involved in spontaneous pain (hotplate assay) and slowly developing, diffuse pain (writhing). Additionally, in acute inflammatory nociception after the injection of formalin, rapid PGE_2 release is mediated in part by COX-1 (Tanioka et al., 2000; Tegeder et al., 2001). In contrast, several groups found that COX-1-selective inhibitors did not influence nociceptive behavior in carrageenan-evoked hyperalgesia (Zhang et al., 1997; Smith et al., 1998; Guay et al., 2004). Because no selective PGES inhibitors and no cPGES-deficient mice have been available so far, the role of cPGES in spinal nociceptive processing has not been investigated; therefore, we investigated the functional effects of reduced cPGES expression in the spinal cord by using antisense oligonucleotides (ODNs) in models of nociception.

Materials and Methods

Animals. Male Sprague Dawley rats were purchased from Charles River Wiga (Sulzfeld, Germany). Animals were maintained in climate- and light-controlled rooms and had *ad libitum* access to food and water. In all experiments, the ethics guidelines for investigations in conscious animals were followed, and the procedures were approved by the local Ethics Committee.

Section preparation. Adult rats were fixed by perfusion with 4% paraformaldehyde in 0.1 M PBS, pH 7.2. The fixed tissues were cryoprotected with 10–30% sucrose in 0.1 M PBS. Tissues were cryostat-sectioned in a horizontal plane at a thickness of 10–12 μm. Sections were mounted on Superfrost Plus Slides (Fisher Scientific, Pittsburgh, PA) and stored at −80°C until use.

In situ *hybridization.* To generate riboprobes, the cDNA clone of cPGES was generated by reverse transcription (RT)-PCR with spinal

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cord RNA from adult rats. The following primers were used: 5′-ATGCAGCCTGCTTCTGCAA-3′ (forward) and 5′-TTACTCCA-GATCTGGCAT-3′ (reverse). Full-length cPGES cDNA was cloned in pCR4bluntTOPO (Invitrogen, Carlsbad, CA), excised with *Eco*RI, and subcloned in the *Eco*RI site of pBluescript II KS (+/−) (Stratagene, La Jolla, CA). Antisense and sense riboprobes were obtained with T7 and T3 polymerases (Promega, Madison, WI) after the plasmid was linearized with *Hin*dIII or *Bam*HI. *In vitro* transcription was performed in the presence of UTP-α[^{35}S] (MP Biomedicals, Irvine, CA), linearized cDNA, and NTP at 37°C for 1 h. Transcripts were purified with the RNA Probe Purification Kit (Peqlab, Erlangen, Germany). *In situ* hybridization was performed as described previously (Ehnert et al., 2004).

Immunohistochemical analyses. Immunohistochemistry was performed as described previously (Ehnert et al., 2004). Rat spinal cord slices were incubated for 1 h with anti-cPGES or anti-COX-1 (Cayman Chemical, Ann Arbor, MI) and anti-neuronal-specific nuclear protein (NeuN) antibody (Chemicon, Temecula, CA), followed by an incubation with FITC-labeled goat anti-rabbit and cyanine 3-labeled goat anti-mouse antibody (Sigma, St. Louis, MO).

Tissue preparation. Lumbar spinal cords were homogenized in 1 ml of lysis buffer (10 mM Tris–HCl, pH 7.4, 20 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Homogenates were centrifuged at 18,000 × *g* for 10 min at 4°C, and the Bradford assay was used to measure the protein content of the supernatant.

Western blot. Western blots were performed with 10–17% SDS-PAGE. COX-1, COX-2, and cPGES were detected by using antibodies obtained from Cayman Chemical. Anti-heat shock protein 70 (Hsp70; BD Biosciences, Heidelberg, Germany) or anti-extracellular signal-regulated kinase 1/2 (Erk1/2; Promega) antibodies were used as controls for equal loading. Signal strength was determined densitometrically, and the data were analyzed with Student's *t* test; $p < 0.05$ was considered statistically significant.

RT-PCR. Total RNA (2 μg) from rat spinal cord was annealed with oligo-dT primers (0.6 μM each) and reverse transcribed with reverse transcriptases (Promega) for 30 min at 37°C. Oligonucleotide primers for glyceraldehyde-3-phosphate dehydrogenase amplification were used as described previously (Trajkovic et al., 2000). Primers for cPGES were 5′-ATGCAGCCTGCTTCTGCAAAGTGG-3′ (forward) and 5′-TTACTCCAGATCTGGCAT-3′ (reverse). The sequences of primers for COX-1 (Abassi et al., 2001) and COX-2 (Hirst et al., 1999) were the same as described previously. Quantitative PCR was performed with the TaqMan system and TaqMan reagents (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions and previous descriptions (Ehnert et al., 2004).

Spinal delivery of cPGES antisense and sense oligonucleotides: drug delivery system. ALZET mini-osmotic pumps (model 2001; Charles River Wiga) were filled with antisense or sense ODN solution (5 mg/ml in artificial CSF) and connected to 100-mm-long polyethylene catheters (inner diameter, 0.28 mm; outer diameter, 0.61 mm) according to the manufacturer's instructions. The pumps were incubated in 0.9% NaCl for 15–18 h at 37°C and implanted in the animals.

Implantation of lumbar intrathecal catheters. Rats were anesthetized with ketamine (60 mg/kg, i.p.) and midazolam (0.5–1 mg/kg, i.p.). The skin was incised above the vertebral column from vertebrae Th13 to L3. Muscle tissue around L2–L3 was cleared away. The processus spinosus of L3 was removed, and a laminectomy was done at L2. The catheter, connected to the osmotic pump and shortened according to the dimensions of the animal, was inserted into the peridural space so that the tip reached Th12–Th13 and was fixed with cyanacrylate glue. A small "pocket" was made by blunt dissection under the skin of the midsacral region, and the osmotic pump was inserted into it. The skin was sutured, and the animal was returned in its home cage. Only rats without disturbances of neurological functions were used for behavioral experiments. After 7 d, we performed a formalin or a Hargreaves assay. Behavioral testing was performed by investigators unaware of the respective treatment. Rats were killed by cardiac puncture under deep isoflurane anesthesia immediately after the test. Lumbar spinal cords were excised, frozen in liquid nitrogen, and stored at −80°C until further analysis.

Formalin assay. A 5% formaldehyde solution (50 μl) was injected subcutaneously into the dorsal surface of one hindpaw. Flinches were counted in 1 min intervals for up to 60 min starting just after the formalin injection. Flinches of 5 min intervals were summarized as mean flinches per minute. To compare the nociceptive behavior between groups, the sum of flinches during the 1 h observation period was submitted to the Student's *t* test.

Zymosan-evoked inflammation. To induce inflammation, we subcutaneously injected 6 mg of zymosan A (Sigma) suspended in 100 μl of PBS, pH 7.5, into the midplantar region of the right hindpaw. A Hargreaves plantar test (Hargreaves et al., 1988) was used to assess thermal hyperalgesia. After zymosan A injection into the plantar side of the right hindpaw, we determined paw withdrawal latencies (PWLs) on exposure of the paw to a defined thermal stimulus with a commercially available device (Hargreaves Test; Ugo Basile Biological Research Apparatus, Comerio, Italy). Rats were kept in the test cages for 1 d. During the day, the rats were tested several times to gain the baseline PWL. On day 2, thermal hyperalgesia was assessed for 6 h starting 15 min after the injection of zymosan (100 μl of a 10 mg/ml solution). Left and right hindpaws were measured alternately in intervals of 5–10 min. At 1 h intervals, the PWLs were averaged.

Statistical analysis. The measurement of PWLs is reported in seconds, and data are presented as mean ± SEM. For statistical evaluation, PWLs are calculated as a percentage difference between the right and the left hindpaws according to the following formula, as described previously (Meller and Gebhart, 1997): (right − left)/left × 100.

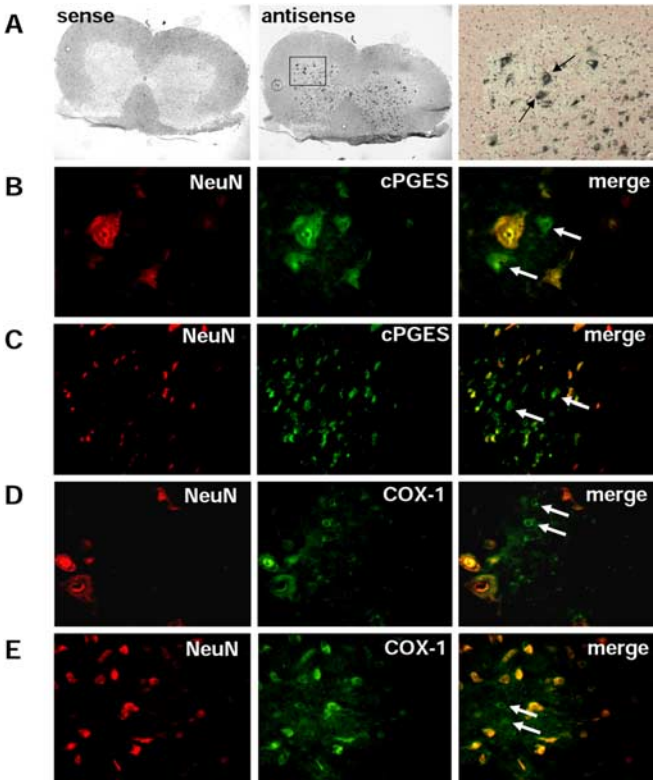

Figure 1. cPGES is expressed in neurons of the adult rat spinal cord. ***A***, *In situ* hybridization of rat spinal cord sections. *In situ* hybridization was performed with horizontal sections of spinal cords from adult rats. The left and middle panels (magnification, 5×) depict the entire spinal cord section with sense and antisense probes. The outlined area in the middle panel (the ventral horn) is shown in the right panel at higher magnification (40×). Black arrows mark the locations of motoneurons in the ventral horn. ***B–E***, Immunohistochemical analysis of spinal cord sections from ventral (***B***, ***D***) and dorsal (***C***, ***E***) horns with antibodies against cPGES (***B***, ***C***) or COX-1 (***D***, ***E***) and NeuN (magnification, 40×). Arrows indicate cPGES- or COX-1-expressing non-neuronal cells.

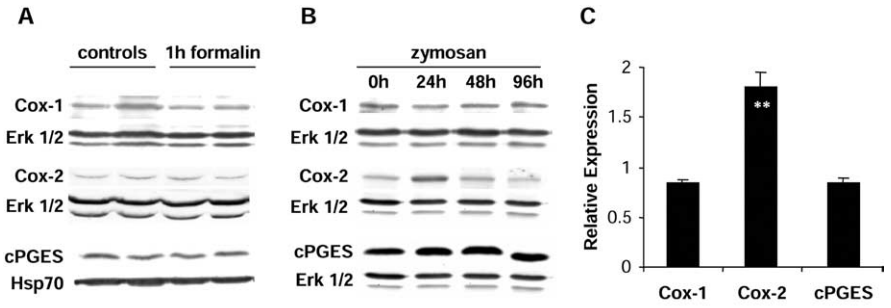

Figure 2. cPGES expression is not altered after treatment with zymosan or formalin. ***A***, Western blot analyses with rat spinal cord lysates (30 μg) of control animals or animals treated with formalin for 1 h with antibodies against COX-1, COX-2, and cPGES. Equal loading was controlled by reprobing with anti-Erk1/2 or anti-Hsp70 antibodies. Three similar experiments are represented. ***B***, Western blot analysis with rat spinal cord lysates (30 μg) of control animals or animals treated with zymosan for 24, 48, and 96 h. Equal loading was controlled by reprobing with anti-Erk1/2 antibody. Four similar experiments are represented. ***C***, Quantitative RT-PCR with RNA from spinal cords of animals 24 h after zymosan injection compared with spinal cord from untreated rats. Error bars indicate the mean ± SEM of two to four experiments, each done in triplicate (Student's *t* test; $^{**}p \leq 0.05$).

Table 1. cPGES sense and antisense oligonucleotides

ODN	Sequence	Base pairs
AS1	5′-TGCAGAAGCAGGCTGCAT-3′	52–69
AS2	5′-GTACCACTTTGCAGAAGC-3′	61–78
AS3	5′-CCTTCGGTCGTACCACTT-3′	70–87
S1	5′-ATGCAGCCTGCTTCTGCA-3′	52–69

cPGES sense and antisense oligonucleotides were tested with intrathecal applications for downregulation of cPGES. The base pairs indicate the position of the oligonucleotides in the rat cPGES cDNA sequence.

Results

cPGES is constitutively expressed in various cells and tissues (Tanioka et al., 2000), but little is known about its function in spinal nociceptive processing. To determine cPGES expression in the spinal cord of adult rats, we first used *in situ* hybridization. cPGES mRNA was detected throughout the gray matter, but not in the white matter, of the spinal cord of adult rats (Fig. 1*A*). In the ventral horn, cPGES mRNA could be found in the motoneurons. To verify this finding, we performed immunohistochemical analyses of the localization of cPGES and COX-1 in the spinal cord of adult rats. Costaining for cPGES and NeuN showed that cPGES was expressed in neuronal and non-neuronal cells of the ventral (Fig. 1*B*) and dorsal (Fig. 1*C*) horns. Because cPGES has been described as being functionally coupled to COX-1 (Tanioka et al., 2000), in the next step we determined the localization of COX-1 in the spinal cord. Indeed, as seen for cPGES, immunohistochemical staining with anti-COX-1 antibody showed immunoreactivity throughout the gray matter in neuronal and non-neuronal cells in the ventral (Fig. 1*D*) and dorsal (Fig. 1*E*) horns.

Next we examined the expression of cPGES and COX-1 in the spinal cord to determine whether it is regulated after peripheral nociceptive stimulation. One hour after the injection of formalin, the expression of cPGES and COX-1 in the spinal cord was not altered compared with untreated control rats (Fig. 2*A*). We then induced a peripheral inflammation in the rat hindpaw by zymosan injection, and the expression of the cyclooxygenases and cPGESs in the spinal cord was determined after 24, 48, or 96 h. No changes were seen in the expression of COX-1 and cPGES (Fig. 2*B*) by Western blot analysis. COX-2 is known to be induced by proinflammatory stimuli, and its expression was monitored for control purposes (Murakami et al., 2000). As expected, we found an upregulation of COX-2 expression 24 h after the zymosan injection (Fig. 2*B*). To confirm these results, quantitative PCR was performed. Again, 24 h after zymosan treatment we found an upregulation of COX-2 compared with untreated rats, whereas the amounts of COX-1 and cPGES mRNA were not altered (Fig. 2*C*). In addition, *in situ* hybridization with spinal cord slices of rats 24, 48, and 96 h after zymosan injection did not reveal any change in the amount or distribution of cPGES mRNA (data not shown).

To determine the role of cPGES in spinal nociceptive processing, rats were infused intrathecally with cPGES antisense ODNs to decrease the endogenous cPGES expression. One of the tested cPGES antisense ODNs (named AS1) (Table 1) decreased the expression of cPGES in the spinal cord by ~40%, as shown by Western blot (Fig. 3*A*). To examine the nociceptive behavior of rats treated with cPGES antisense (AS1) or sense (S1) ODNs, we performed formalin assays. Animals treated with cPGES antisense ODN showed a significant decrease in nociceptive behavior >1 h after formalin injection compared with cPGES sense treatment ($p = 0.034$) (Fig. 3*B*). Next we analyzed the nociceptive behavior of cPGES antisense and sense ODN-treated rats during thermal hyperalgesia by using the Hargreaves assay after treatment with zymosan. Rats treated with cPGES antisense ODN showed a significant decrease in nociceptive behavior 1 and 2 h after zymosan injection (Fig. 3*C*). At later time points, the antisense ODN-treated animals tended to show decreased nociceptive behavior, although the differences between sense and antisense ODN-treated animals were not significant.

Discussion

After inflammation, allodynia and hyperalgesia can occur because of an increase in PGE_2 in the inflamed tissue and in the spinal cord that is associated with an induction and activation of COX-2 (Ebersberger et al., 1999; Smith et al., 2000; Vanegas and Schaible, 2001; Guay et al., 2004; Murakami and Kudo, 2004). COX-2 has been demonstrated to be the major source of PGE_2 in many pain models, and COX-2-selective inhibitors are potent antihyperalgesic substances. In animal models of inflammatory pain, such as carrageenan- or zymosan-evoked thermal hyperalgesia, COX-2-selective inhibitors reduced markedly the nociceptive response (Zhang et al., 1997; Smith et al., 1998; Niederberger et al., 2001; Vetter et al., 2001; Niederberger et al., 2003). In contrast, PGE_2 production by COX-1 and cPGES is thought to be involved mainly in the immediate release of PGE_2 after inflammation (Tanioka et al., 2000). Indeed, the selective COX-1 inhibitor SC560 significantly reduced the nociceptive response in the formalin assay and completely abolished the formalin-evoked increase in PGE_2, whereas the COX-2-selective inhibitor celecoxib did not alter nociceptive behavior or PGE_2 release in the same assay (Tanioka et al., 2000; Tegeder et al., 2001). Moreover, comparison of nociceptive behavior in COX-1 and COX-2 knock-out mice revealed that immediately transmitted pain (hotplate assay) and slowly developing, diffuse pain (writhing) are decreased more markedly in COX-$1^{-/-}$ mice than in COX-$2^{-/-}$ mice (Ballou et al., 2000). Thus it seems that COX-1-dependent PGE_2 synthesis is responsible for early nociceptive responses, whereas COX-2-dependent PGE_2 synthesis plays a major role in the delayed responses as seen in zymosan- or

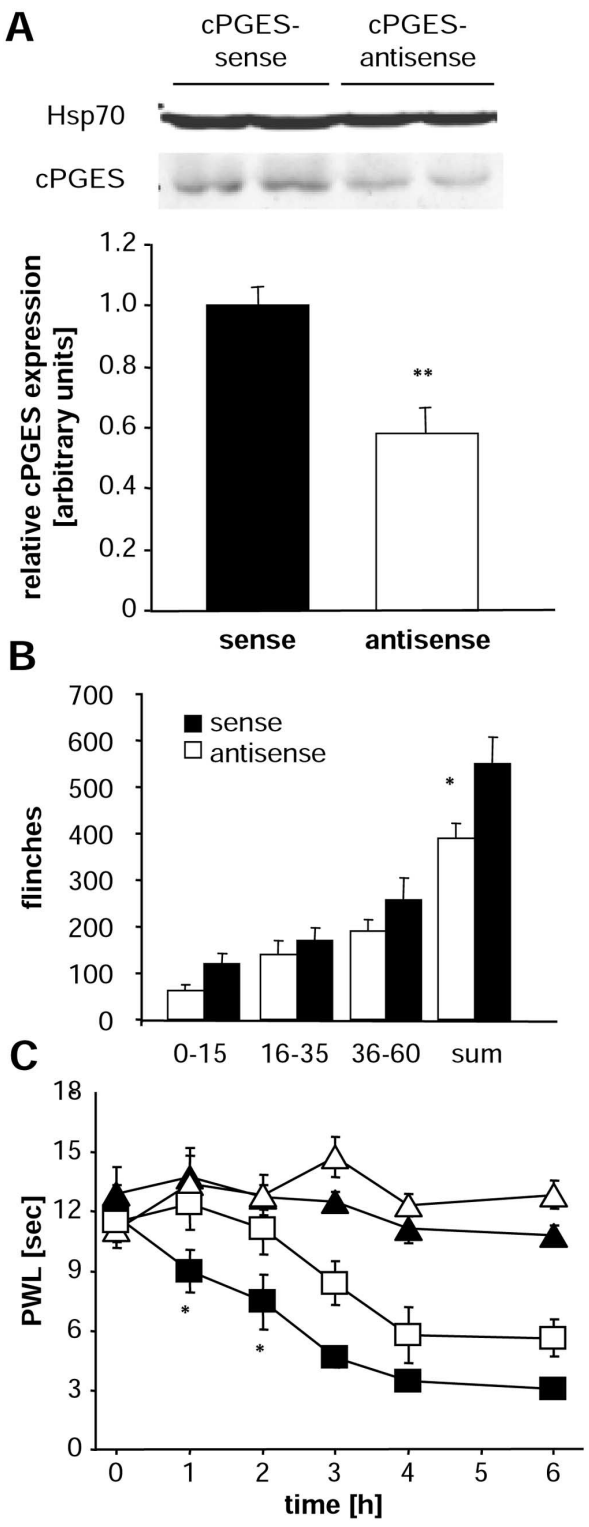

Figure 3. Intrathecal application of antisense ODN against cPGES decreases nociceptive behavior. ***A***, Adult rats were given intrathecal cPGES sense and antisense ODN. After treatment with formalin, the spinal cord was removed and analyzed by Western blot. For the relative cPGES expression, the mean ± SEM of seven to nine determinations is shown (Student's *t* test; $^{**}p \leq 0.002$). ***B***, Formalin assay of animals treated with cPGES sense or antisense ODN. The number of flinches from 0 to 15, 16 to 35, and 36 to 60 min and the total number of flinches during 1 h are shown. Error bars indicate the mean ± SEM of seven to nine determinations (Student's *t* test; $^{*}p \leq 0.034$). ***C***, Thermal hyperalgesia of animals treated with cPGES sense (black squares and triangles) or antisense (white squares and triangles) ODN. Squares represent ipsilateral behavioral responses; triangles represent contralateral behavioral responses. Error bars indicate the mean ± SEM of at least six determinations.

carrageenan-evoked thermal hyperalgesia. Accordingly, no effect of the selective COX-1 inhibitor SC560 was found on carrageenan-evoked thermal hyperalgesia (Smith et al., 1998; Yaksh et al., 2001).

Because of the lack of selective inhibitors for the PGE_2 synthases, only the generation of PGES-deficient mice or the use of antisense technology to reduce tissue specifically the PGES expression can be used to study the role of the different enzymes in spinal nociceptive processing. Although mPGES-1-deficient mice have been described recently, to date, the role of mPGES-1 in nociceptive processing has been investigated only with the writhing test (Trebino et al., 2003; Kamei et al., 2004). Here, as described for COX-2-deficient mice, the mPGES-1-deficient mice exhibited markedly decreased nociceptive behavior compared with wild-type animals.

We found that in rats with reduced cPGES expression in the spinal cord, the nociceptive behavior was significantly decreased in the formalin assay as well as in zymosan-evoked thermal hyperalgesia. Thus we propose the hypothesis that in the formalin-evoked pain response, the immediate release of PGE_2 in the spinal cord depends mainly on the COX-1–cPGES pathway, and therefore a change in nociceptive behavior after downregulation of cPGES would be expected (Tanioka et al., 2000; Tegeder et al., 2001). Interestingly, we found that a reduction in endogenous spinal cPGES expression also results in decreased nociceptive behavior in the first hours of zymosan-evoked thermal hyperalgesia. In addition, cPGES downregulation in the spinal cord did not affect thermal withdrawal response thresholds in the uninjected contralateral hindpaw of zymosan-treated rats. This finding suggests that spinal cPGES downregulation has an antihyperalgesic rather than an analgesic effect. This may be explained as a result of decreased spinal PGE_2 production; similar results have been seen previously after administration of the nonselective COX-inhibitor indomethacin (Turnbach and Randich, 2001) in the carrageenan-induced thermal hyperalgesia model.

In conclusion, our data show that COX-1–cPGES-mediated PGE_2 synthesis plays a role in spinal nociceptive processing during the early phases of processing. Thus it seems that PGE_2 synthesized by the constitutively expressed COX-1 and cPGES is especially important under conditions during which COX-2 and mPGES-1 are not yet upregulated and have not yet become the major source of PGE_2 synthesis.

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