Increased Gene Expression and Production of Spinal Cyclooxygenase 1 and 2 during Experimental Osteoarthritis Pain

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Summary
Knowledge on the involvement of spinal COX-1 and COX-2 in pain due to osteoarthritis could be useful for better understanding of its pathogenesis and therapy. In this study we have investigated a long-term pattern of expression and production of spinal COX-1 and COX-2 in the model of osteoarthritis induced in rats by injection of monoiodoacetate (MIA) into the knee joint. MIA injection produced thermal hyperalgesia (assessed by the plantar test) and tactile allodynia (measured with von Frey hairs). The pain measures reached maximum on the 5th day, then remained relatively stable. The expression of spinal COX-2 mRNA reached maximum on day 5 (5.2 times; P<0.001) and remained increased until day 31 (4.9 times; P<0.001). Expression of spinal COX-1 mRNA increased gradually reaching maximum on the day 31 (4.5 times; P<0.001) when the relative expression of both genes was almost equal. The production of both proteins was almost similar at the beginning of the experiment. The highest production of COX-2 protein was observed on day 5 after the induction of osteoarthritis (increased 3.9 times). The levels of COX-1 protein increased gradually with maximum on day 31 (3.4 times). The present findings indicate that not only expression of COX-2 mRNA but also that of COX-1 mRNA is significantly increased in the spine during osteoarthritis pain. Thus, in contrast to inflammatory pain, the upregulation of spinal COX-1 may be important in osteoarthritis pain.

Key words
Osteoarthritis • Cyclooxygenase (COX) • Pain • Spinal cord

Introduction
Cyclooxygenases are the enzymes that catalyze the conversion of arachidonic acid to prostaglandins which play an important role in inflammation and pain. There are two identified cyclooxygenase (COX) isoenzymes: COX-1 and COX-2. COX-1 is the „house-keeping“ form, expressed by a wide variety of cells. COX-2 is highly inducible in response to inflammatory and noxious stimuli. Both cyclooxygenases are constitutively expressed in the spinal cord (Kaufmann et al. 1997).

The involvement of spinal COX-1 and COX-2 in various pain states is not fully understood. COX-2 gene expression and production in the spinal cord was significantly increased in rats with peripheral inflammation induced by intraplantar injection of Freund’s complete adjuvant (Beiche et al. 1996, Hay et al. 1997, Beiche et al. 1998). No increase of COX-1 gene expression and production in the spinal cord was found in this model which was associated with swelling, hyperalgesia and allodynia (Beiche et al. 1996, Hay et al. 1997, Beiche et al. 1998). In agreement with these findings, spinal COX-2 mRNA were markedly increased and spinal levels of COX-1 mRNA were not significantly altered in peripheral inflammation induced by intraplantar injection of carrageenan (Procházková et al. 2006).

Rats with streptozotocin-induced diabetes exhibited significantly increased levels of spinal COX-2 protein and activity along with hyperalgesia (Ramos et al. 2007). Intrathecal administration of COX-2 inhibitors has an anti-hyperalgesic effect on streptozotocin-induced

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mechanical hyperalgesia (Matsunaga et al. 2007). A sharp upregulation of spinal COX-2 was reported in the mouse model of amyotrophic lateral sclerosis (McGeer and McGeer 2002).

On the other hand, the expression of COX-2 mRNA in the spine was less dominant in postoperative pain model than in inflammatory pain, while expression of spinal COX-1 mRNA was significantly increased in postoperative pain (Procházková et al. 2006). The important role of COX-1 in the model of postoperative pain was also shown by Zhu et al. (2003).

Spinal COX-1 also appears to play a role in the model of neuropathic pain. Spinal COX-1 expression was increased after partial peripheral nerve transection (Zhu and Eisenach 2003). The inhibition of COX-1 prevented the development of allodynia and hyperalgesia after peripheral nerve ligation (Hefferan et al. 2003).

Induction of osteoarthritis

For induction of osteoarthritis, rats were anesthetized with halothane (Narcotan, Zentiva). Eight rats per group received single injection of monoiodoacetate (2 mg) (Sigma–Aldrich) into the right knee joint in a total volume of 25 µl. Control animals (n=8) were injected 25 µl of vehicle into the right knee joint under the same conditions.

Paw withdrawal testing

The response to noxious thermal stimulus was determined using thermal plantar device (Ugo Basile, Italy) according to the procedure described by Hargreaves et al. (1988) before and in defined times during 31 days after the injection of monoiodoacetate. Animals were placed to opaque plastic chambers (22 cm in width x 17 cm in length x 14 cm in height) for 10 min prior to the each experiment. Movable infrared radiant heat source was placed directly under the plantar surface of the hind paw and the time taken for hind paw withdrawal was monitored. A cut-off time of 20 s was used in all experiments. Three tests were carried out at 10 min intervals and then the mean value was taken as the nociceptive threshold.

Following three baseline measurements, rats received intraarticular injection of monoiodoacetate or saline. In the defined times after injection of monoiodoacetate or saline, paw withdrawal latencies were recorded.

von Frey hairs

Tactile allodynia was measured with von Frey hairs (Ugo Basile, Italy). Animals were placed into wire mesh bottom cages and allowed to acclimatize prior the start of the experiment. Tactile allodynia was tested by
touching the plantar surface of the animal's hind paw with von Frey hairs in ascending order of force until a paw withdrawal response was elicited. Each von Frey hair was applied to the paw for 5 s or until a response occurred. Once a withdrawal response was established, the paw was retested. The lowest amount of force required to elicit a response was recorded as withdrawal threshold in grams.

Following three baseline measurements, rats received intraarticular injection of monoiodoacetate or saline. Paw withdrawal thresholds were measured in defined times after the injection of monoiodoacetate or saline.

**Tissue preparation**

In four different times after monoiodoacetate or saline injection, animals (eight per group) were euthanized in halothane anesthesia. Lumbar section of the spinal cord was removed and given in RNAlater solution (Qiagen).

**RNA isolation**

Disruption and homogenization of small parts of the lumbar section of spinal cord weighing approximately 100 mg stabilized with the RNAlater (Qiagen) was performed using Ultra-Turrax (Ika). Total RNA was isolated with the RNeasy lipid tissue isolation kit (Qiagen) according to the manufacturer’s instruction. RNA integrity was determined by gel electrophoresis in 2 % agarose gel stained with ethidium bromide. The purity of the RNA was assessed by the ratio of absorbance at 260 nm and 280 nm. RNA was stored in aliquots at −70 °C until used for reverse transcription.

**Reverse transcription**

RNA samples were reverse transcribed using RT buffer, 25 mM MgCl₂, 10 mM dNTPs (2.5 mM of each), 50 µM random hexamers, RNase inhibitor (20 U/µl) and reverse transcriptase (50 U/µl), all from Applied Biosystems. The mix was aliquoted into individual tubes and RNA was added. Samples were incubated for 10 min at 25 ºC, 30 min at 48 ºC and then for 5 min at 95 ºC.

**Real-time PCR**

A reaction mix for real-time PCR was made with TaqMan Universal PCR master mix, water and Assays on Demand gene expression products (all Applied Biosystems).

Reaction mix was aliquoted to the wells on a real-time PCR plate. Each sample was made in duplicate. A volume of 5 µl of cDNA was added to each well. A no-template control contained water instead of cDNA. PCR reaction was run on ABI PRISM 7300 (ABI PRISM 7300 SDS analytical cycler, Applied Biosystems) using standard conditions.

Expression of COX-1 and COX-2 was normalized to RNA loading for each sample using the β2-microglobulin as an internal standard. The quantity of mRNA was given as $2^{-\Delta\Delta ct}$. $\Delta\Delta ct$ was calculated as follows: $\Delta ct = \Delta ct (\text{gene of interest}) – \Delta ct (\text{endogenous control})$.

**ELISA**

For detection of antigens, sandwich enzyme-linked immunosorbent assay (ELISA) was used. Microtiter NUNC plates (Schoeller) were coated with monoclonal antibody specific for cyclooxygenase 1 (Alpha Diagnostic) and for cyclooxygenase 2 (Kamiya Biomedical Company) diluted in coating buffer and incubated overnight.

After 24 h the plates were washed two times with washing buffer PBS (phosphate buffered saline) and two times with PBST (PBS containing 0.05 % Tween 20, Sigma-Aldrich). Each well was then filled with PBST and incubated for one hour at room temperature to prevent non-specific adsorption of protein to the well surfaces. During this time, samples from spinal cord of monoiodoacetate-injected or control animals were homogenized in 5 % FBS (fetal bovine serum, Sigma-Aldrich) and then added to the wells. Two hours after this incubation at room temperature, the plates were washed two times with PBS and two times with PBST (PBS containing 0.05 % Tween 20, Sigma-Aldrich).

The secondary biotinylated antibodies (Acris Antibodies) were added to the wells and incubated for next two hours. The plates were then washed, followed by incubation of 1:1000 dilution of streptavidin (Beckman Coulter) for 20 min. After washing the coated well, TMB (tetramethylbenzidine, Sigma-Aldrich) and citric buffer with peroxide were added.

For development of the color reaction the plates were incubated in the dark and the reaction was stopped by the addition of H₂SO₄ to each well. The color intensity was determined at 450 nm on Multiscan RC reader. Results are expressed as stimulation index (OD of monoiodoacetate treated vs. OD of control animals).

**Drugs**

Monoiodoacetate was purchased from Sigma-Aldrich and halothane (Narcotan) was obtained from Zentiva.
Statistical analysis

All results are expressed as mean values ± S.E.M. Statistical analysis was carried out using two-way repeated measures ANOVA with a post-hoc Student-Newman-Keuls test in the case of repetitive testing of paw withdrawal. P<0.05 was accepted as significant. The evaluation of real-time PCR data was done by one-way ANOVA with a post-hoc Turkey’s test using 2^ΔΔct values of each samples. P<0.05 value was considered significant. Data from ELISA method are presented as stimulation index and were analyzed using unpaired t-test. The results were considered significant if P value was less than 0.05.

Results

Effect of monoiodoacetate on paw withdrawal latency

Paw withdrawal latencies were measured before and in defined times after application of monoiodoacetate or saline. Intraarticular injection of monoiodoacetate into the right knee joint produced marked and significant reduction of paw withdrawal latencies to noxious radiant heat stimuli. Decreased paw withdrawal latencies were evident from the first day following injection of monoiodoacetate, with the maximum on day 5 after induction of osteoarthritis (statistically significant at all observed times compared to baseline and to control animals; P<0.001). The paw withdrawal latencies remained decreased until day 31 (Fig. 1).

Effect of monoiodoacetate on tactile allodynia

Tactile allodynia was measured with von Frey hairs before and following intraarticular injection of monoiodoacetate or saline. The injection of monoiodoacetate into the right knee joint induced marked allodynia. The onset of allodynia was evident from the first day following injection of monoiodoacetate. Figure 2 shows that tactile allodynia in the monoiodoacetate-injected knee joints reaches the maximum on day 5 and was observed throughout the experiment (statistically significant at all observed times compared to baseline and to control animals, P<0.001).

Expression of COX-1 and COX-2 mRNA in the spinal cord after induction of osteoarthritis

Expression of COX isoenzymes was measured at four different times. First day after monoiodoacetate injection, spinal levels of COX-1 mRNA and COX-2 mRNA were moderately increased (2.3 and 2.6 times, respectively; P<0.05). The expression of spinal COX-2 mRNA was much higher on day 5 (5.2 times; P<0.001) and remained increased at this level until the day 31 (4.9 times; P<0.001). On the other hand, expression of spinal COX-1 mRNA increased gradually during the whole testing period reaching maximum on the day 31 (4.5 times; P<0.001) when the relative expression of both genes was almost equal. All results are expressed in comparison with control animals (Fig. 3).
Production of COX-1 and COX-2 proteins after induction of osteoarthritis

The production of spinal COX proteins was comparable to the expression results. The production of COX-1 and COX-2 proteins was almost similar at the beginning of the experiment (1.9 and 2.0 times, respectively; \( P<0.001 \)). The highest production of COX-2 protein was observed on day 5 after the induction of osteoarthritis (increased 3.9 times; \( P<0.001 \)). The levels of COX-1 protein increased gradually from the 5th day after induction of osteoarthritis (1.7 times; \( P<0.001 \)) with maximum on day 31 (3.4 times; \( P<0.001 \)). Results are expressed as stimulation index (OD of monoiodoacetate-treated vs. OD of control animals) (Fig. 4).

Discussion

Monoiodoacetate injection produced pain as measured by von Frey thresholds and paw withdrawal latencies in the plantar test. The pain measures reached maximum on the 5th day, then remained relatively stable. Expression and production of spinal COX-2 was rapidly increased in parallel to pain measures reaching maximum on the 5th day and then remained relatively stable. On the other hand, the expression and production of spinal COX-1 mRNA increased at a slower pace but 31 days after the induction of osteoarthritis there was almost no difference between relative amount of COX-1 and COX-2 mRNA.

The present results indicate that osteoarthritis pain has different patterns of expression of spinal mRNA for COX-1 and COX-2 compared with chronic inflammatory or postoperative pain. Expression and production of spinal COX-2 were significantly increased 22 days after the induction of arthritis by CFA (Complete Freund's Adjuvant) while spinal COX-1 mRNA and protein levels remained unchanged at this time (Beiche et al. 1996, Beiche et al. 1998). In contrast, COX-1 might play a more important role in postoperative pain. Expression of spinal COX-1 mRNA raised gradually after rat paw incision and 6 hours after the surgery there was no difference between relative amount of COX-1 and COX-2 mRNA in the spinal cord (Procházková et al. 2006).

The increased expression and production of spinal COX-1 in some types of pain may have therapeutic implications. The lack of analgesic effect of intrathecally administered selective inhibitor of COX-2 (NS-398) in the model of postoperative pain induced by skin incision suggest that spinal COX-2 might play a less important role in this type of pain (Yamamoto and Sakashita 1999). Intrathecal administration of COX-1 inhibitors, but not of COX-2 inhibitor, dose-dependently reduced pain in the model of postoperative pain (Zhu et al. 2003). Perioperative intrathecal administration of COX-1 inhibitors (COX-1 preferring inhibitor ketorolac and COX-1 selective inhibitor SC-560), but not of selective COX-2 inhibitor (NS-398), reduced paw incision induced hypersensitivity in the model of postoperative pain in rats (Zhu et al. 2005). Intrathecal administration of selective COX-1 inhibitor (SC-560), but not selective inhibitor of COX-2 (NS-398), restores normal exploratory activity (rearing behavior) after laparotomy (Martin et al. 2006).

The increased expression of spinal COX-1 mRNA found in the present study corroborates other
findings suggesting important role of this enzyme in osteoarthritis. Knorth et al. (2004) demonstrated the important role of synovial COX-1 in patients with primary osteoarthritis.

The expression of COX-1 and COX-2 mRNA from synovial tissue from patients with osteoarthritis or from patients with inflammatory arthritis (rheumatoid arthritis) was compared by Siegle et al. (1998). The expression of COX-2 was found to be elevated in both groups of the patients, but the difference in expression of both cyclooxygenases was more evident in the group of patients with rheumatoid arthritis. The amount of COX-2 mRNA was significantly higher in patients with rheumatoid arthritis compared with patients with osteoarthritis.

The expression of both, COX-1 and COX-2 mRNA, was detected in cells from synovial fluid of patients with acute and chronic arthritis. COX-1 was the most abundant isoform with the strong immunostaining observed in a special fraction of mononuclear cells (Iniguez et al. 1998).

In summary, the present findings indicate that not only expression and production of COX-2 but also that of COX-1 is significantly increased in the spine during osteoarthritis pain. In contrast to inflammatory pain, the increased expression and production of spinal COX-1 might play an important role in osteoarthritis pain.

Conflict of Interest
There is no conflict of interest.

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References


YAMAMOTO T, SAKASHITA Y: The role of the spinal opioid receptor like1 receptor, the NK-1 receptor, and cyclooxygenase-2 in maintaining postoperative pain in the rat. *Anesth Analg* **89**: 1203-1208, 1999.


