Up-regulation of cyclooxygenase-2 mRNA in the rat spinal cord following peripheral inflammation

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Abstract Prostaglandins (PG) have been described as mediators in spinal nociceptive processing after peripheral inflammation. Enzymes essential for PG biosynthesis, cyclooxygenase isozymes COX-1 and COX-2, have not yet been investigated in the spinal cord. In two studies on rats with adjuvant-induced peripheral inflammation levels of mRNA expression of both COX isoforms were analyzed in the lumbar section of the spinal cord using reverse transcription-polymerase chain reaction (RT-PCR) technique. We could show that mRNA of both COX isoforms is expressed constitutively in the spinal cord with COX-2 as the predominant isoform. Six hours after induction of peripheral inflammation, levels of COX-2 mRNA expression were raised significantly in respect to untreated control rats and returned to baseline within 3 days after induction of inflammation. COX-2 might therefore be regarded as the COX isozyme responsible for spinal PG release in nociceptive processing under a peripheral inflammatory stimulus.

Key words: Cyclooxygenase-2; Inflammation; Spinal cord; RT-PCR

1. Introduction

Prostaglandins (PG) have been known as important pro-inflammatory mediators for a long time and cyclooxygenases as the enzymes responsible for PG formation have been well characterized over the last years. Two isoforms of cyclooxygenase have been identified. Beside the well-studied 'classical' isoform of cyclooxygenase, COX-1, now often referred to as the constitutive isoform that is expressed in most tissues and thought to mediate physiological responses, a second inducible isoform of cyclooxygenase, COX-2, is known today [1]. COX-2 is barely detectable in cultured cells but its expression is highly regulated in response to growth factors or cytokines. Induction of this isozyme could be shown in a variety of cell types by a number of stimuli [2,3]. In vivo experiments revealed expression of COX-2 to be up-regulated by inflammatory processes [4–7]. In some tissues, however, COX-2 is present without obvious stimulatory processes as in the macula densa of the kidney [8] the testis or the brain, where COX-2 is not only expressed constitutively but also considered to be the dominating COX isoform [9–11]. In the central nervous system (CNS) prostaglandins maintain important functions as neuroregulators [12]. It has been reported that PGs are released from the spinal cord by various processes as stimulation of afferent nerves [13], noxious thermal stimulation [14] and by increased potassium levels [15]. Prostaglandins are also known to be involved in transmission of nociceptive information in the spinal cord after peripheral inflammation [16]. Functional evidence thus indicates a role for cyclooxygenases in the spinal cord, expression of the enzyme itself, however, has not yet been investigated. We conducted two studies to determine the spinal presence and distribution of COX isozymes using the rat model of adjuvant-induced inflammation. Tissue samples of hindpaws and the lumbar section of the spinal cord were taken before and after induction of inflammation and examined for levels of COX mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Animals and tissue preparation

Experiments were performed on two groups of male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 280–320 g and 160–200 g, respectively. Arthritis was induced by plantar injection of complete Freund's type adjuvant (CFA) in one hind footpad [17]. Animals were treated with a dosis of 100 μl of CFA (*Mycobacterium sp.* suspended in mineral oil at 10 mg/ml; DIFCO Laboratories, Detroit, MI) or physiological saline and killed under CO_2 at 6 h, 24 h, 3 days and 7 days after inoculation in the first study and 3 h, 6 h, 12 h, 24 h and 72 h in the second study (groups of 3). The study protocol was approved by the local Ethics Committee. The spinal cord was ejected from the lumbar section of the spine by a water-filled syringe and shock-frozen in liquid nitrogen. For the first study the lumbar section of the spinal cord was divided laterally into an ipsi- and contralateral half and frozen separately. Tissue from both inflamed and untreated hindpaws was removed in total without bones.

2.2. RNA isolation

Tissue samples from spinal cord and hindpaws of individual animals were homogenized and total RNAs isolated by a slightly modified protocol of Chomczynski and Sacchi [18]. RNA (40 μg) was treated with 10 U of RNase-free DNase I (Boehringer Mannheim) for 1–2 h at 25°C to remove any contaminating DNA. RNA was then phenol-extracted, ethanol-precipitated and stored in aliquots at −20°C. RNA integrity and yields were assessed by UV spectrophotometry and ethidium bromide staining. To exactly determine the amount of RNA taken into the RT-PCR experiments, 2 μg aliquots of RNA were run out on 1.2% formaldehyde agarose gels (1×MOPS as running buffer) every time RNA was used in reverse transcription experiments and the amount of 18S-rRNA evaluated densitometrically after ethidium bromide staining. RNA concentrations were then corrected for 18S-rRNA values.

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Abbreviations: bp, basepair; CFA, complete Freund's adjuvant; CNS, central nervous system; COX, cyclooxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase; MOPS, 4-morpholinepropanesulfonic acid; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; RT-PCR, reverse transcription-polymerase chain reaction; SSC, saline sodium citrate

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2.3. Northern blotting

RNA was separated on 1.2% formaldehyde agarose gels and transferred to nylon membrane by capillary blotting using 20×SSC. Northern hybridizations and washes were conducted as described previously [19]. For detection of hindpaw mRNAs the following specific cDNA probes from the coding regions of the genes were used: GAPDH probe as described by Stroebel and Goppelt-Struebe [19], 2.767 kb and 1.156 kb *Eco*RI fragments from the 5'-end of the *COX-1* and *COX-2* genes, respectively [21].

2.4. Reverse transcription-polymerase chain reaction

DNase-treated RNA (0.05–0.2 μg) was reverse-transcribed with random hexamer or oligo-dT primers (Eurogentec, Belgium) using 5 mM $MgCl_2$, 1 U of RNase inhibitor (Promega, WI), 4 mM dNTPs, 50 U of Moloney murine leukemia virus reverse transcriptase (M-MLV-RT, Promega) and 1× buffer supplied by the manufacturer in a total volume of 20 μl. A parallel reaction without reverse transcriptase was run for each sample. The RT reaction was incubated at 37°C and 42°C for 20 min each and then stopped by heating to 99°C for 5 min. Aliquots (5 μl) of the reaction were diluted to 25 μl with 1× PCR-buffer (10 mM Tris-HCl, 3.5 mM $MgCl_2$, 75 mM KCl, pH 8.3) and used for PCR amplification with COX- or GAPDH-specific primers (0.15 μM) and 0.2 U of Taq polymerase (Boehringer Mannheim). Samples were placed in a trioblock thermal cycler (Biometra, Göttingen) and initially denatured at 95°C for 2 min. They were then denatured at 95°C for 1 min, annealed and extended at 64°C (GAPDH primers) or 72°C (COX-1 and COX-2 primers) for 5 min in repetitive cycles. Final extension time was 7 min at 72°C. Ten microliters of the PCR reaction were resolved by electrophoresis through a 2% agarose gel. Amplified cDNA bands were detected by ethidium bromide staining and the volumes evaluated by densitometry (Vilber-Lourmat, Bio 1D). The yield of the amplified product was tested to be linear for amount of input RNA and PCR cycle number. For RT-PCR of spinal cord RNA the following optimized conditions were chosen: 0.2 μg of total RNA in 30–34 cycles for assessment of COX-1 expression and 0.05 μg RNA in 28–32 PCR cycles for COX-2 mRNA expression (data not shown). Experiments were therefore conducted at 0.2 μg RNA/32 cycles for COX-1 and 0.05 μg RNA/30 cycles for COX-2 mRNA amplification. RNAs from hindpaws were investigated at 0.2 μg/28 cycles for COX-1 and COX-2 and 0.05 μg/22 cycles for GAPDH expression.

2.5. PCR primers and probes

PCR primers were selected from the published cDNA sequences [6,20] and commercially synthesized (Eurogentec, Belgium). The following primers were used for the genes of interest: COX-1 5'-forward primer CATGGATCCGGATTGGTGGGGGTAG and 3'-reverse primer ATCTCGAGGGGCAGGTCTTGGTGTTG, COX-2 5'-forward CTGTATCCCGCCCTGCTGGTG and 3'-reverse primer ACTTGCGTTGATGGTGGCTGTCTT, GAPDH 5'-forward primer CCTTCATTGACCTCAACTAC and 3'-reverse primer GGAAGGCCATGCCAGTGAGC. These primer pairs were found to yield amplified products of the expected sizes of 447 bp for the COX-1 cDNA, 279 bp for COX-2-cDNA and 574 bp for GAPDH. Southern blot analysis using specific cDNA-probes for COX-1 and COX-2 (see below) confirmed the identity of the amplified products.

Table 1
Increase in paw volumes of adjuvant- and saline-injected animals

Time	increase in paw volume [% over control]			
	CFA-treated animals		saline-treated animals	
(h)	ipsilateral	contralateral	ipsilateral	contralateral
First study				
6	80 ± 15	1.2 ± 0.2	nd	nd
24	101 ± 17	1.2 ± 0.9	nd	nd
72	95 ± 22	7.3 ± 0.6	12 ± 5	18 ± 416
8	162 ± 51	1 ± 0.2	11 ± 9	1 ± 2
Second study				
3	73 ± 21	0 ± 1	0 ± 1	0 ± 1
6	97 ± 14	30 ± 15	3.2 ± 5	2.3 ± 1.2
12	83 ± 12	18 ± 10	1.1 ± 2	2.6 ± 2.3
24	85 ± 13	3 ± 4	8.2 ± 6	16 ± 10
72	89 ± 6	8 ± 7	5 ± 4	20 ± 17

Development of inflammation was assessed by increase in paw volumes in adjuvant (CFA)- and saline-treated animals (% over control). Paw volumes were determined by plethysmographic measurement of both inflamed (ipsilateral) and uninflamed (contralateral) hindpaws (nd, not done).

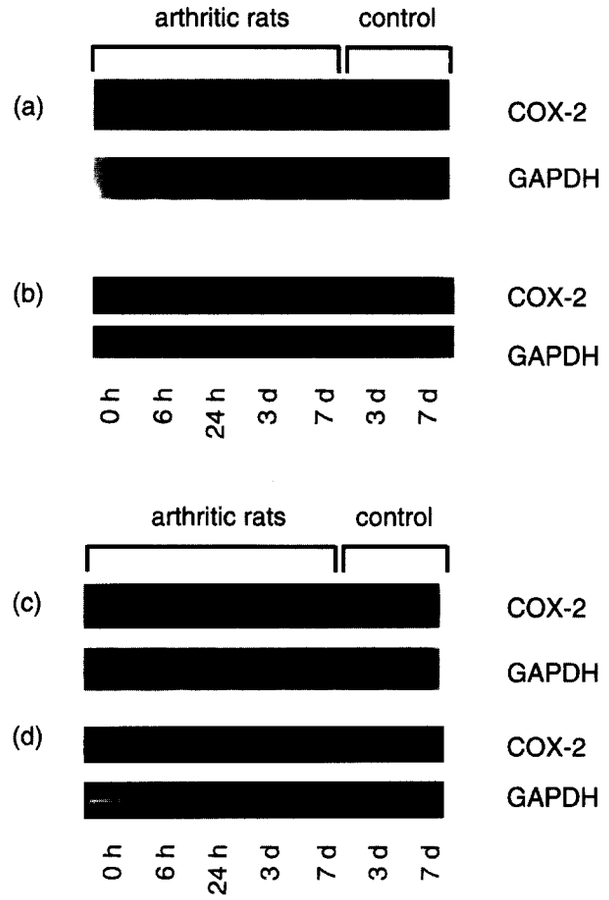

Fig. 1. Expression of COX-2 mRNA in hindpaw tissue. COX-2 mRNA expression was analyzed in hindfoot tissue after adjuvant-induced peripheral inflammation in ipsilateral inflamed paws (a+b) and contralateral uninflamed paws (c+d). RNA levels were monitored over a timecourse of 7 days by Northern blot analysis (a and c) and RT-PCR (b and d). GAPDH expression was used as internal control for RNA loading.

2.6. Statistics

Data were statistically assessed by analysis of variance (ANOVA). The significance level was set at $P < 0.05$.

3. Results

3.1. Expression of cyclooxygenase mRNA in inflamed peripheral tissue

Peripheral inflammation was induced in the rat hindfoot by injections of CFA [17]. Development of arthritis was monitored by increase in paw volume of the animals (Table 1). In both studies CFA-inoculated paws were swollen distinctly 6 h after induction of the inflammation. Hindfeet had about twice the normal size throughout the first 3 days in both experiments and reached a maximum swelling at the seventh day (162% over control). Over a time course of 7 days tissues from inflamed hindpaws as well as from contralateral untreated paws of individual animals were screened for COX-2

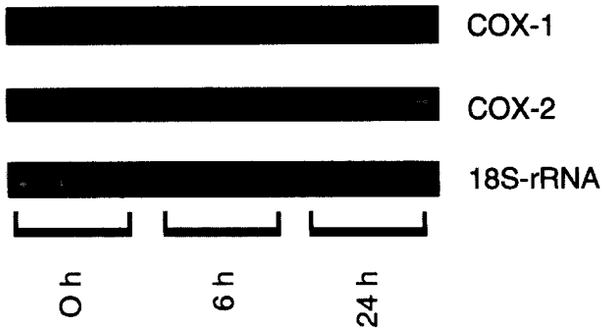

Fig. 2. Expression of COX mRNA in the lumbar spinal cord after peripheral inflammation. Total RNA was isolated from the ipsilaterally located sections of the lumbar spinal cord of animals killed at 0 h, 6 h and 24 h after induction of peripheral inflammation and screened for expression of COX-1 and COX-2 mRNA by RT-PCR. 0.2 μg RNA/32 cycles were used to detect COX-1, 0.05 μg RNA/30 cycles for COX-2. 18S-rRNA was used as control for RNA load. (Data shown are from 6 animals killed at times indicated.)

mRNA expression by Northern analysis and RT-PCR. The profile of COX-2 mRNA expression in hindfoot tissue over the first days of adjuvant-induced inflammation is shown in Fig. 1. Tissue from untreated (0 h) or saline-inoculated (control) paws did not show distinctable levels of COX-2 mRNA expression. The level of COX-2 mRNA expression was significantly elevated ($P < 0.05$) 6 h after inoculation of the adjuvant. High levels of COX-2 mRNA were maintained in the inflamed hindpaws throughout the monitored time course.

Data from the Northern analysis of COX-2 mRNA expression (Fig. 1a,c) were in agreement with data obtained from the RT-PCR (Fig. 1b,d). This technique was further applied when analyzing levels of COX mRNA expression in tissue samples from the spinal cord, as RNA preparations from spinal cord tissue of individual animals did not yield sufficient amounts of specific COX mRNA to be detected by Northern analysis.

3.2. Investigation of cyclooxygenase isoform expression in the spinal cord

Cyclooxygenase expression in the spinal cord was investigated in two separate studies on male Sprague-Dawley rats. Animals were killed at the mentioned timepoints after induction of inflammation and the lumbar sections of the spinal cords removed as described. Total RNA was isolated and levels of COX-1 and COX-2 mRNA expression analyzed by RT-PCR. For validation of RT-PCR see Section 2.

3.3. Results from the first study

The first study was conducted on 21 animals in groups of three which were inoculated with CFA or physiological saline and killed after 6 h, 24 h, 3 days and 7 days, saline controls being taken after 3 and 7 days only. Untreated animals were used as controls for baseline mRNA expression. Lumbar spinal cords were cut longitudinally and both halves were analyzed separately as sections of the cord correlating to the ipsilateral inflamed and the contralateral uninflamed hindfoot. Both sections of the spinal cord tissue from untreated control rats showed a basic expression of COX-1 and COX-2 isozyme mRNA (Fig. 2). COX-2 was found to be the predominant isoform in the spinal cord. A 4-fold amount of RNA and additional cycles had to be used for COX-1 mRNA detection. Cyclooxygenase mRNA levels were compared in each group of animals as well as between groups of different timepoints. A marked difference in COX-2 mRNA levels could be detected at 6 h and 24 h after induction of peripheral inflammation in both ipsi- and contralateral sections of the lumbar spinal cord, whereas COX-1 mRNA expression seemed to be unchanged in this setting. In spinal tissue COX-2 mRNA levels were significantly elevated at 6 h in all screens and had returned to baseline after 3 days (Fig. 5).

3.4. Results from the second study

A second study was performed to analyze the early phase of inflammation in more detail. This study included 33 animals in groups of three which received plantar injections of adjuvant and normal saline, respectively, and were killed at 3 h, 6 h, 12 h, 24 h and 72 h after injection. Spinal cord tissue was not split longitudinally in this case as the first study could not reveal significant differences between COX mRNA expression in the ipsi- and contralateral halves of the lumbar cord. Spinal COX mRNA levels were compared in the first three groups of animals, killed at 0 h, 3 h and 6 h after injection of CFA (Fig. 3). Densitometric quantitation revealed that levels of COX-1 mRNA were comparable in all animals ($n = 9$). In contrast, COX-2 mRNA expression was enhanced over time, being significantly increased at 6 h after induction of the peripheral inflammation. As observed in the first study, COX-2 mRNA levels returned to baseline within 3 days. These findings were confirmed in screens comparing animals from all

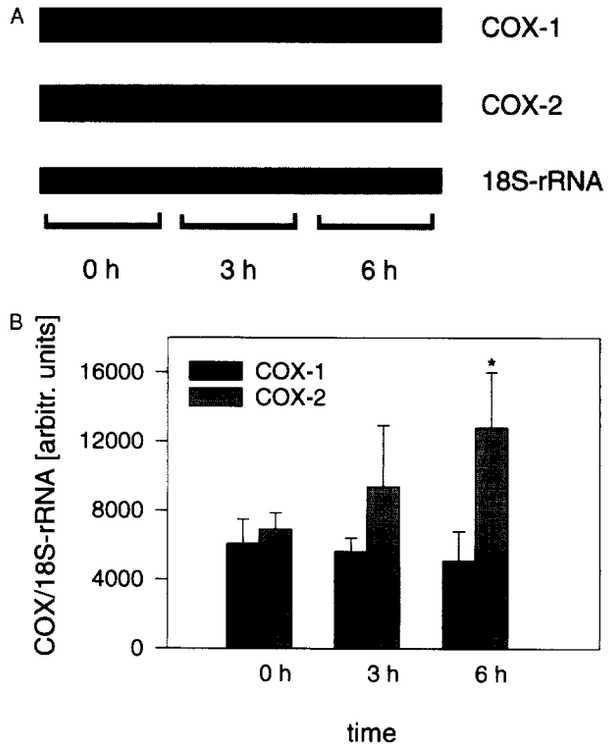

Fig. 3. Spinal COX mRNA expression within 6 h of induction of inflammation. RNA was isolated from total lumbar spinal cord of animals killed at the given timepoints ($n = 3$) and analyzed for COX mRNA expression by RT-PCR (a). A significant increase in COX-2 mRNA levels could be detected at 6 h after induction of peripheral inflammation, whereas COX-1 mRNA expression remained unchanged in the spinal inflammatory response (b). (Data obtained from second study.) $^*P < 0.05$.

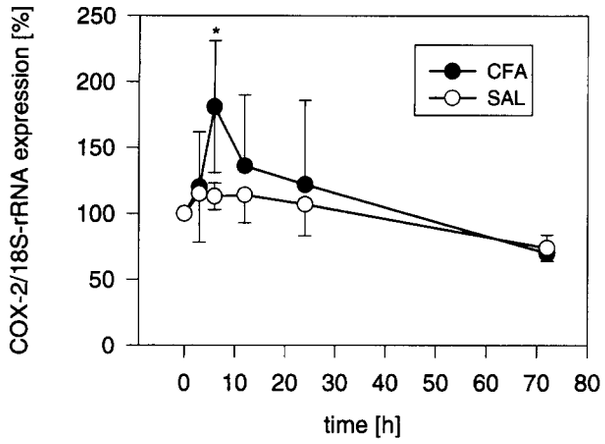

Fig. 4. Levels of lumbar COX-2 mRNA expression. Levels of spinal COX-2 expression were compared in adjuvant (CFA)- and saline (SAL)-treated animals over a timecourse of 72 h. (Data obtained from the second study.) COX-2 mRNA levels were transitionally increased after 6 h and had returned to baseline at 24 h latest ($n = 3$ per timepoint, duplicate values). COX-2 mRNA expression is corrected for 18S-rRNA levels, expression in untreated animals is set to 100% in each screen. $^*P < 0.05$.

different time points (Fig. 4). Animals injected with saline and killed at the same time as adjuvant-treated animals showed no significant elevation in the spinal expression of COX-2 mRNA over the monitored time course (Fig. 4). Fig. 5 gives a summary on COX-2 mRNA levels in the two studies. Comparable time courses for COX-2 mRNA expression were found in both studies with rapid increases after 6 h and declines to baseline after 3 days at latest.

4. Discussion

A number of investigations has been conducted in recent years to show that prostaglandins are involved in nociceptive processing in the CNS [22]. A functional role for prostaglandins in the spinal cord has been confirmed by intrathecal administration of COX products such as PGE_2, PGD_2 and $PGF_{2\alpha}$ [23,24]. Evidence has also been presented for spinal actions of NSAIDs, which are known to inhibit COX function [25]. In vivo studies showed direct influence of intrathecally administered NSAIDs [16,26,27] on spinal nociceptive processing, hereby diminishing nociceptive behaviour. Malmberg and Yaksh [28] could also demonstrate that NSAIDs are 100–500 times as active when administered spinally in comparison to systemic administration. In this context, several studies have investigated the role of prostaglandins in the spinal modulation of thermal and mechanical hyperalgesia [27–29]. Although opinions on whether PGs are involved in both these hyperalgesic processes are still contradictory, it has already become evident that there is a definite role for PGs in the modulation of the nociceptive responses from the periphery.

It has been discussed for some time that the analgesic action of NSAIDs should be dissociated from their anti-inflammatory actions [16,30,31]. PG synthesis inhibition in the inflamed tissue is obviously related to the anti-inflammatory effect of NSAIDs, whereas the analgesic action seems to be related to the antinociceptive effect on a possible spinal site of action [30,32,33].

The cerebral presence of the prostanoid-forming cyclooxygenases has been documented in several studies [9–11]. In our studies presented here we could now show direct evidence for the presence of both COX isoforms in the rat spinal cord. COX-2 was found to be the predominantly expressed isoform in the lumbar section of the cord. Predominance of COX isozyme expression thus seems to be similar as described for sections of the brain [9].

In this model of peripheral inflammation the functional role of spinal cyclooxygenases was investigated. Injection of Freund's adjuvant led to paw swelling accompanied by a rise in COX-2 mRNA expression in the inflamed paw 6 h after induction of inflammation. At that timepoint we could also detect a rise in the level of COX-2 mRNA expression in the lumbar section of the spinal cord, while COX-1 mRNA expression remained steady at baseline. In both studies a maximum increase in COX-2 mRNA was observed after 6 h. The decline was slightly slower in the first study but also reached background levels after 3 days. This timecourse covers most of the acute phase of inflammation. Whether the slow rise in spinal COX-2 mRNA levels after 7 days of peripheral inflammation leads to a second induction of expression towards the chronical phase of inflammation needs further investigation. In contrast to the transient rise in the spinal cord, COX-2 expression remained elevated at the peripheral site of inflammation from 6 h after induction of inflammation over the whole timecourse of 7 days, correlating with increased volumes of the inflamed hindfeet.

A 6 h lag phase for induction of spinal mRNAs was observed by Ji et al. [34] in the same model of adjuvant-induced arthritis. They could demonstrate a rapid increase in neuropeptide Y and neuropeptide Y receptor mRNA expression in the ipsilateral dorsal horn neurons 6 h after inoculation of unilateral inflammation. In a lateral separation of the lumbar spinal cord in sections relating to the inflamed or uninflamed hindfoot we could not distinguish significant differences in COX-2 mRNA expression between the ipsi- and contralateral sections.

With the methods used in our studies it was not yet possible to locate the exact site of COX expression and action. It will be interesting to investigate the distribution of cyclooxy-

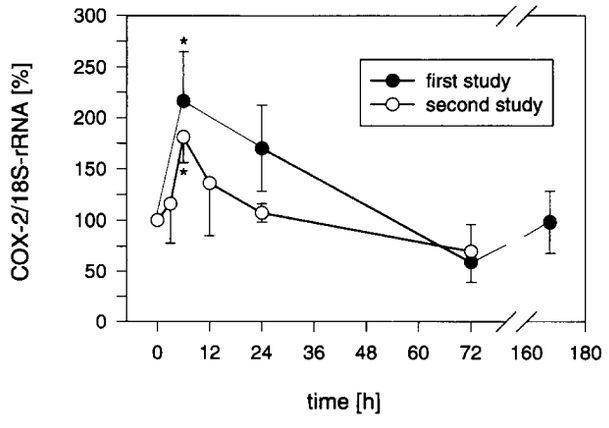

Fig. 5. Comparison of the studies presented. COX-2 mRNA levels are significantly elevated in animals killed 6 h after the peripheral inflammatory stimulus in both studies. This transient elevation has returned to baseline after 3 days in the first and 24 h in the second study. At 7 days another small increase in COX-2 mRNA is detectable. $^*P < 0.05$.

genases in the spinal cord to be able to determine the sites of spinal PG synthesis and actions. In the mapping of COX-2-like immunostaining in the brain, for instance, high levels of expression have been found in dendrites and neuronal cell bodies, i.e. typical postsynaptic structures [9]. Exact mapping of the isozymes will give further insight into the functional role of cyclooxygenases in mediating nociception.

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