Carrageenan-induced Paw Edema in Rat Elicits a Predominant Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) Response in the Central Nervous System Associated with the Induction of Microsomal PGE\textsubscript{2} Synthase-1*  

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Peripheral inflammation involves an increase in cyclooxygenase-2 (COX-2)-mediated prostaglandin (PG) synthesis in the central nervous system (CNS), which contributes to allostynia and hyperalgesia. In the present study we have determined the changes in prostanooid tissue levels and in expression of terminal prostanooid synthases in both the CNS and inflamed peripheral tissue during carrageenan-induced paw inflammation in the rat. Prostanoid levels were measured by liquid chromatography-mass spectrometry and enzyme expression at the RNA level by quantitative PCR analysis during both the early (1–6 h) and late (12 and 24 h) phases of the inflammatory response. In the paw, the early phase was associated with increases in PGE\textsubscript{2} and thromboxane (TX)B\textsubscript{2} levels and with a peak of COX-2 expression that preceded that of microsomal prostaglandin-E\textsubscript{2} synthase-1 (mPGES-1). COX-2 and mPGES-1 remained elevated during the late phase, and PGE\textsubscript{2} continued to further increase through 24 h. The cytosolic PGE\textsubscript{2} synthase (cPGES) showed a small transient increase during the early phase, whereas mPGES-2 expression was not affected by inflammation. In the cerebrospinal fluid, elevated levels of PGE\textsubscript{2}, 6-keto-PGF\textsubscript{1α}, TXB\textsubscript{2}, and TXA\textsubscript{2} were detected during the early phase. PGE\textsubscript{2} levels also increased in the spinal cord and, to a lesser extent, in the brain and remained elevated in both the cerebrospinal fluid and the spinal cord during the late phase. The expression of mPGES-1 was strongly up-regulated in the brain and spinal cord during inflammation, whereas no change was detected for the expression of cPGES, mPGES-2, COX-1, and terminal PGD, TX, or PGI synthases. The results show that the carrageenan-induced edema in the paw elicits an early phase of COX-2 induction in the CNS leading to an increase synthesis in PGD\textsubscript{2}, 6-keto-PGF\textsubscript{1α}, and TXB\textsubscript{2} in addition to the major PGE\textsubscript{2} response. The data also indicate that the up-regulation of mPGES-1 contributes to COX-2-mediated PGE\textsubscript{2} production in the CNS during peripheral inflammation.

Carrageenan-induced inflammation in the rat paw represents a classical model of edema formation and hyperalgesia, which has been extensively used in the development of nonsteroidal anti-inflammatory drugs and selective COX\textsuperscript{2}-inhibitors. Several lines of evidence indicate that the COX-2-mediated increase in prostaglandin (PG) E\textsubscript{2} production in the central nervous system (CNS) contributes to the severity of the inflammatory and pain responses in this model. COX-2 is rapidly induced in the spinal cord and other regions of the CNS following carrageenan injection in the paw (1). The administration of selective COX-2 inhibitors, but not COX-1 inhibitors, reduces the levels of PGE\textsubscript{2} in the cerebrospinal fluid (CSF) and hyperalgesia (2–5). In addition, it has been shown that the intrathecal administration of PGE\textsubscript{2} potentiates carrageenan-induced inflammation (6) and that the direct microinjection of PGE\textsubscript{2} in the brain causes hyperalgesia (7). Selective COX-2 inhibitors can also inhibit peripheral pain responses when given intrathecally (3, 8, 9), whereas a selective COX-1 inhibitor has no effect (10). The central effects of PGE\textsubscript{2} appear to be mediated via the EP\textsubscript{3} receptor based on observations that the microinjection of an agonist of the EP\textsubscript{3} receptor in the brain directly causes hyperalgesia (7), and the inflammatory responses are strongly reduced in the mice deficient in the EP\textsubscript{3} receptor (11).

Altogether these studies indicate that the central production of PGE\textsubscript{2} mediated by COX-2 during inflammation contributes to nociception and hyperalgesia at the site of peripheral inflammation. However, the role of the different prostanooid synthases in inflammation and the identity of the critical prostanooids involved in the inflammatory processes have not been well defined. In addition to PGE\textsubscript{2}, PGI\textsubscript{2} also has been proposed to represent an important mediator of inflammation based on the reduced edema and pain response in the prostacyclin receptor (IP) knock-out mice (12, 13). Clinical studies have indicated that selective COX-2 inhibitors inhibit the production of urinary PGI\textsubscript{2} metabolites in addition to those of the PGE\textsubscript{2} pathway (14) indicating that COX-2 plays a role in the synthesis of both prostanooid mediators in humans.

PGE\textsubscript{2} is synthesized by the sequential reactions catalyzed by COX, which converts arachidonic acid to PGH\textsubscript{2}, and by PGE synthase, which converts PGH\textsubscript{2} to PGE\textsubscript{2}. Several proteins that possess variable levels of PGE synthase activity have been identified, microsomal PGES-1 (mPGES-1) (15), glutathione transferases (16), cytosolic PGES (cPGES)/p23 (17) and mPGES-2 (18, 19). The mPGES-1 is considered to be the major form implicated in COX-2-mediated PGE\textsubscript{2} production based on co-transfection experiments in cultured cells indicating a better coupling with COX-2 than with COX-1 for the production of PGE\textsubscript{2} (20) and based on the inducibility of the enzyme in thase; mPGES, microsomal prostaglandin-E\textsubscript{2} synthase; PG, prostaglandin; PGDS, lipooxygenase-type prostaglandin D synthase; PGI\textsubscript{2}, prostacyclin synthase; TX, thromboxane; TXS, thromboxane synthase; IL, interleukin; LC-MS, liquid chromatography-mass spectrometry; IP, prostacyclin receptor.
response to pro-inflammatory mediators (15, 21, 22), and in various models of inflammation and fever in vivo (23–26). Furthermore, the mPGES-1 has been shown to co-localize with COX-2 in endothelial cells of the brain vasculature after induction with interleukin (IL)-1β (23, 27). The profile of the mPGES-1 knock-out mice is strongly supportive for a role of this enzyme in the production of inflammatory PGE₂ production. Indeed, a reduction of lipopolysaccharide-stimulated PGE₂ synthesis in peritoneal macrophages (28) and a reduction in inflammation in the collagen-induced arthritis model (29).

Most of the studies on the regulation of mPGES-1 in vivo have been performed using models of IL-1- or lipopolysaccharide-induced pyrexia in which the expression of the enzyme has been shown to increase in the CNS and in certain peripheral tissues (23–25, 27, 28, 30, 31). Recently, mPGES-1 has also been shown to be inducible during a chronic model of adjuvant-induced arthritis (26, 30). In the present study we report on the up-regulation of mPGES-1 in the CNS during the acute model of carrageenan-induced inflammation of the paw in the rat, providing further evidence for the role of mPGES-1 in the increased synthesis of PGE₂ associated with inflammatory responses.

MATERIALS AND METHODS

Model of Carrageenan-induced Edema in Rat—All procedures for in vivo experiments were approved by the Animal Care Committee at the Merck Frosst Centre for Therapeutic Research (Kirkland, Quebec, Canada) and were performed according to guidelines established by the Canadian Council on Animal Care. Paw edema was induced in Sprague-Dawley rats (150–200 g) (Charles River Laboratories, St-Constant, Quebec, Canada) by injection of 100 μl of 1% carrageenan (λ-carrageenan, type IV, Sigma) diluted in saline in the left hind foot pad (31). Paw volumes were determined using a water plethysmometer (Ugo Basile, type IV, Sigma) diluted in saline in the left hind foot pad (31). The tissues were flash frozen in liquid nitrogen and kept at −80 °C until processing. The CSF was collected and kept at −80 °C for prostanoid analyses.

Preparation of Tissue Extracts—Frozen tissues were pulverized in liquid nitrogen using a mortar and pestle, homogenized in 5 volumes of 0.5 M dithiothreitol and 1 mM dNTP, 2.5 M random hexamers, 0.4 units/ΜL Multiscribe reverse transcriptase. Real time quantitative PCR for mPGES-1, COX-2, COX-1, cPGES, mPGES-2, and prostacyclin synthase (PGIS) was performed using probes and primers as fully described by Claveau et al. (26). The oligonucleotide sequences used for lipidic-type prostaglandin-D synthase (PDGS) and thromboxane synthase (TXS) probes and primers were as follows: PDGS probe, 5'-ACGCTACTCTAGTGGTGTTTACACA-3', PDGS forward primer, 5'-CCCGGACATCAGTACCTACACAGC-3'; TXS reverse primer, 5'-TGGTTTGCTTGAAAC-3'; TXS probe, 5'-TGGATGGCCAAAGGCCACACTTG-3'; TXS forward primer, 5'-CCAGAGTTGTTACTGCGTTT-3'; TXS reverse primer, 5'-GGAGCATCTGAGGATGTTAC-3'.

Analyses of mRNA by Quantitative PCR—The mRNA isolation and quantification were performed as described previously (26) with some modifications. Briefly, frozen tissues were pulverized in liquid nitrogen using mortar and pestle and then homogenized in TRIzol reagent (Cayman Chemical). Chemiluminescence detection was performed using Fuji Film LAS-1000 charge-couple device and Image Gauge software for quantitative analysis or by film exposure (Kodak BioMax MR, Eastman Kodak Co.) using a Bio-Rad GS-800 densitometer and Quantity One software for analysis.

Analyses of MS conditions for the analyses of prostaglandins and TXB₂

| Prostaglandin     | Q1 mass | Q3 mass | DP² voltage | Collision energy |
|-------------------|---------|---------|-------------|------------------|
| PGE₁ and PGD₁     | 351.3   | 315.1   | −70         | −16              |
| d₁-PGE₂          | 355.3   | 319.1   | −70         | −25              |
| PGG₂             | 353.3   | 309.0   | 85          | −36              |
| d₁-PGG₂          | 357.3   | 313.0   | 85          | −29              |
| 6-keto-PGF₂       | 389.1   | 163.2   | −75         | −36              |
| d₂-6-keto-PGF₂₁   | 373.1   | 167.2   | −75         | −36              |
| TXB₂             | 369.1   | 168.8   | −65         | −28              |
| d₂-TXB₂          | 373.1   | 168.8   | −65         | −28              |

* Q, quadrupole.
† DP, declustering potential.

Western Blot Analyses—Aliquots containing 25 or 50 μg of protein were loaded on SDS-PAGE using 4–20% gradient gels (Novex, Invitrogen) and transferred electrophoretically to polyvinylidene difluoride membranes using a Novex immoblot apparatus according to the manufacturer’s instructions. Immunodetection was performed using COX-2 (1/500 dilution) and mPGES-1 (1/300) purified antibodies (Cayman Chemical). Chemiluminescence detection was performed using Fuji Film LAS-1000 charge-couple device and Image Gauge software for quantitative analysis or by film exposure (Kodak BioMax MR, Eastman Kodak Co.) using a Bio-Rad GS-800 densitometer and Quantity One software for analysis.

Time Course of Edema Formation—Carrageenan-induced edema in the hind foot pad was used as a model to determine the temporal relationships between edema formation, prostanoid synthesis, and expression of selected terminal prostanoid synthases both at the site of peripheral inflammation and in the CNS. Under the conditions used for carrageenan-induced inflammation, swelling of the paw occurred rapidly after the injection of carrageenan with an increase in volume of 1.5-fold at 1 h, which reached a maximum at 6 h (2.7-fold) and remained elevated until the last measurement at 24 h (Fig. 1).

**Increased PGE₂ and TXB₂ Levels in Inflamed Paw**—The soft
A 2-fold increase in TXB2 levels in the paw at time 0 (and using 18 S rRNA to normalize). Both COX-2 and mPGES-1 mRNAs were elevated in inflamed paws during the early phase of inflammation (Fig. 3A). The induction of COX-2 in the tissues from the paw caused a marked increase of PGE2 in the spinal cord with a maximum at 6 h (6-fold), and a time course (Fig. 5B) that was similar to that observed for the CSF. In the brain, the increase in PGE2 levels (2-fold increase at 3–6 h) was less pronounced than in the spinal cord (Fig. 5B).

Elevation in Prostaglandin and TXB2 Levels in the CNS during the Early Phase of Inflammation—The tissues from the CNS were also analyzed for their contents of the other prostanoids. In the CSF, 6-keto-PGF1α, PGD2, and TXB2 levels were all at the limit of detection of the LC-MS analysis for samples from untreated animals or from the control animals collected at different times after saline injection. After carrageenan injection, 6-keto-PGF1α was detected at elevated levels in the CSF at 3, 5, and 6 h, while PGD2 and TXB2 showed increases at 6 h (Fig. 6A). PGF2α could not be detected at any time point in the CSF. Only small changes for these prostanoids were detected in the brain and spinal cord extracts. In the spinal cord, small transient increases (~2-fold) were observed at 6 h for 6-keto-PGF1α, PGD2, TXB2, and PGF2α (data not shown). In the brain, PGD2, PGF2α, TXB2, and 6-keto-PGF1α all showed minor increases at 3–6 h (1.5–2-fold), which reached...
mPGES-1 Induction in the CNS during Peripheral Inflammation

Both PGE_2 and PGL_2 have been implicated as mediators of the inflammatory and pain responses. In the present study, we seek to obtain more information on the role of these and other prostanoids by examining the changes in tissue levels of the major prostanoids and the expression of terminal prostanoid synthases in the CNS as compared with the site of peripheral footpad edema.

**Induction of COX-2 and mPGES-1 during Sustained PGE_2 Production in Inflamed Paws**—Previous studies (33–35) have shown that COX-2 is detected at elevated levels in paw tissues and in the CNS following carrageenan-induced inflammation. In the current model, swelling of the paw progressively increased over the first 6 h, and the edema persisted for at least 24 h. A very early induction of COX-2 occurred in the paw, with maximal mRNA expression at 1 h, together with an increased expression of mPGES-1 that peaked at 6 h and remained elevated over controls until the last 24-h time point. The sequential induction of COX-2 and mPGES-1 observed in vivo paralleled the in vitro data obtained with cell lines during stimulation of PGE_2 production (21, 36). Both enzymes accumulate at the protein level in paws at later time points during the maintenance of inflammation as PGE_2 levels continued to increase. These data indicate the early phase of the modest increase in PGE_2 might be primarily COX-2-dependent and that both elevated COX-2 and mPGES-1 contribute to a more sustained production and accumulation of PGE_2 at the inflammation site. Of the three PGE_2 synthases evaluated (cPGES, mPGES-1, and mPGES-2), only mPGES-1 mRNA was strongly up-regulated (a transient doubling of expression was detected for cPGES) as observed in the adjuvant-induced arthritis model in rat (26) and after IL-1β-induced inflammation in mouse brain (37), although a small increase in cPGES protein could also be detected in these models. Although no induction was seen in the current model, mPGES-2 (designated as GBF-1) has been reported recently to be inducible in response to interferon-γ (38). It should be noted that the mPGES-1 protein was detected in extracts of normal paws and thus the constitutive expression of the enzyme might contribute to basal PGE_2 levels as well as to the early phase of PGE_2 production. Constitutive expression of mPGES-1 has also been reported previously in the mouse hypothalamus (39).

Carrageenan-induced Edema of the Paw Causes an Up-regulation of COX-2 and a General Increase in Prostanoids in the CNS during the Early Phase of Inflammation—The up-regulation of expression of COX-2 mRNA in the CNS was rapid, detectable at the first 1-h time point, and was more pronounced in the spinal cord than in whole brain tissue extracts. In both tissues the elevation of COX-2 was accompanied by an increase in PGE_2 (2–5-fold) and a more modest increase in PGD_2, PGF_2α, TXB_2, and 6-keto-PGF_1α, during the early phase. PGF_2α and PGD_2 have also been implicated in centrally mediated pain responses. It has been shown that the intrathecal administration of PGE_2 and PGF_2α at low doses produced hyperalgesia to mechanical stimuli in rats (40). These two prostanoids have also been reported to cause touch-evoked allodynia in mice (41, 42) but have much weaker effects in rats (40), which may reflect species differences in prostanoid responses. In mice, there is strong evidence that PGD_2 plays a role in nociception (43) and is essential for PGE_2-induced allodynia (44). In the present study with rats, PGD_2 was the most abundant prostanoid in brain extracts, consistent with previous reports (45), and the increase in PGD_2 in the brain during the early phase of inflammation was statistically significant compared with both time 0 and saline controls. Although the level of stimulation was very low, the maximal levels reached in the spinal cord...
during the early phase were 1.3 ± 0.3 ng/mg protein for PGD$_2$ and 0.28 ± 0.04 ng/mg protein for PGE$_2$, as compared with 0.6 ± 0.1 ng/mg protein for PGE$_2$. Thus it is possible that both PGD$_2$ and PGE$_2$ contribute to pain responses in carrageenan-induced inflammation. In the CSF where the amounts of prostanoids are extremely low in untreated animals, marked increases in PGD$_2$, TXB$_2$, and 6-keto-PGF$_{1\alpha}$ were detected during the early phase. These prostanoids were also found to increase in the CNS after kainate-induced COX-2 expression (46) and neuronal COX-2 overexpression (47). All these observations are consistent with the present data suggesting that COX-2 plays a role in the activation of prostanoid synthetic pathways other than the PGE$_2$ pathway in the CNS. Although 6-keto-PGF$_{1\alpha}$, TXB$_2$, and PGD$_2$ were all found to increase in the CSF, no increase in the corresponding terminal synthases could be detected. These data are consistent with the up-regulation of COX-2 resulting in an increased synthesis of the PGH$_2$ substrate available for each of the prostacyclin, TX, and PGD synthetic pathways during the acute phase of inflammation.

Peripheral Inflammation Causes a Pronounced Elevation of PGE$_2$ and the Selective Induction of mPGES-1 in the CNS—As compared with the other prostanoids measured, PGE$_2$ showed the largest induction in the spinal cord and reached the highest levels in the CSF among the prostanoids tested (by ~3-fold) during the early phase. In contrast to the large accumulation of PGE$_2$ observed for the paw, PGE$_2$ levels in the CNS decreased after the peak at 6 h (but were still slightly higher in the spinal cord and in the CSF than in the corresponding saline controls at 24 h). The present data indicate that this elevation in PGE$_2$ correlated with the marked and selective induction of mPGES-1 in the spinal cord. In whole brain extracts, a large increase of mPGES-1 also occurred, but the increases in PGE$_2$ and COX-2 were less pronounced.

COX-2 induction in the CNS has been proposed to result from the afferent neuronal input and from an increase in circulating pro-inflammatory cytokines. Strong evidence that IL-1$\beta$, whose levels are highly up-regulated in the paw and in the CSF following injection of the paw with Freund adjuvant, represents a major mediator of COX-2 induction in the CNS (8). The inducibility of mPGES-1 has been extensively characterized in rat brain following treatment with IL-1$\beta$ or lipopolysaccharide (23, 24, 27, 48, 49) and during adjuvant-induced arthritis (30). In these studies, the peak of expression of COX-2 preceded that of mPGES-1 following the induction of fever with lipopolysaccharide or the intravenous administration of IL-1$\beta$ (8, 24, 27). The characteristics of the present model are thus consistent with an IL-1 mediated process where both COX-2 and mPGES-1 are rapidly induced in sequence to contribute the maximal PGE$_2$ production (at 6 h) and elevated levels...
throughout the inflammation response. PGE$_2$ in the spinal cord can contribute to potentiation of peripheral edema (6), to the enhanced neuron hyperexcitability (50), and to hyperalgesia (7, 33).

Prostacyclin Levels Are Elevated in the CNS during the Early Phase Carrageenan-induced Paw Edema without Significant Up-regulation of PGIS—Studies with the IP receptor knock-out mice have suggested a role for prostacyclin in this model (12). We have not detected any significant increase in the stable prostacyclin metabolite 6-keto-PGF$_{1 \alpha}$ in inflamed paws. In another model of inflammation in the rat (adjuvant-induced arthritis), 6-keto-PGF$_{1 \alpha}$ was found to show a transient increase in the paw (26) and to be elevated in the spinal cord during chronic inflammation (51). In the early phase of carrageenan-induced edema we observed small increases in 6-keto-PGF$_{1 \alpha}$ in the brain and the spinal cord comparable with those observed for the other prostanoids. Interestingly, the increase in 6-keto-PGF$_{1 \alpha}$ in the CSF appears to occur earlier than that of PGD$_2$ and TXB$_2$. In whole extracts of brain, spinal cord, or paw tissues, no major change of PGIS expression (2-fold) was detected in contrast to mPGES-1. The increase in 6-keto-PGF$_{1 \alpha}$ detected in the CNS thus appears to reflect the increase in COX-2 activity leading to an increase in PGH$_2$ substrate avail-

ability for the different prostaglandins, prostacyclin, and TX pathways. It has been shown recently (52) that the expression of the IP receptor increases in the spinal cord and that the IP receptor agonist cicaprost induces mechanical hyperalgesia in the inflamed paw, suggesting of a role for PGI$_2$ in acute inflammation.

In summary, we have found that the induction of COX-2 that occurs in the CNS during carrageenan-induced paw inflammation leads to an increase in PGs, prostacyclin, and TX in the early phase and to a large increase in PGE$_2$ production associated with selective up-regulation of mPGES-1. The results of this study provide further evidence for the implication of mPGES-1 as an important terminal enzyme for COX-2-mediated synthesis of inflammatory PGE$_2$ not only at the site of inflammation but also in the CNS. The data from this study, along with other observations on the induction of the mPGES-1 during fever and adjuvant-induced arthritis, and on the profile of the mPGES-1 knock-out mice showing reduced sensitivity to collagen-induced arthritis are all consistent with the proposals that mPGES-1 plays a role in inflammatory responses and represents a potential therapeutic target for novel anti-inflammatory agents.

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