Prostacyclin Antagonism Reduces Pain and Inflammation in Rodent Models of Hyperalgesia and Chronic Arthritis

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ABSTRACT
The inhibition of prostaglandin (PG) synthesis is at the center of current anti-inflammatory therapies. Because cyclooxygenase-2 (COX-2) inhibitors and nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the formation of multiple PGs, there is currently a strong focus on characterizing the role of the different PGs in the inflammation process and development of arthritis. Evidence to date suggests that both PGE2 and PGI2 act as mediators of pain and inflammation. Most of the data indicating a role for PGI2 in this context have been generated in animal models of acute pain. Herein, we describe the role of PGI2 in models of osteoarthritis (OA) and rheumatoid arthritis using a highly selective PGI2 receptor (IP, Ptgir) antagonist and IP receptor-deficient mice. In the rat OA model using monoiodoacetate injection into the knee joint, the IP antagonist reduced pain with an efficacy approaching that of the NSAID diclofenac. In a chronic model of inflammatory arthritis, collagen-antibody-induced arthritis model in mice, IP receptor-deficient mice displayed a 91% reduction in arthritis score. Interestingly, pretreatment with the IP [N-[4-((imidazolidin-2-ylideneamino)-benzyl]-4-methoxy-benzamide] antagonist in this model also caused a significant reduction of the symptoms, whereas administration of the compound after the initiation of arthritis had no detectable effect. Our data indicate that, in addition to its role in acute inflammation, PGI2 is involved in the development of chronic inflammation. The results also suggest that the inhibition of PGI2 synthesis by NSAIDs and COX-2 inhibitors, in addition to that of PGE2, contributes to their efficacy in treating the signs of arthritis.

Nonsteroidal anti-inflammatory drugs (NSAIDs) block the production of prostaglandins (PGs) by inhibiting the conversion of arachidonic acid by cyclooxygenases (COX-1 and COX-2) to the unstable endoperoxide PGH2. PGH2 is the common substrate for a number of different synthases that produces a spectrum of lipid mediators including PGD2, PGI2, thromboxane A2, PGE2, and PGI2 (prostacyclin) (Funk, 2001). The modulation of PG production with NSAIDs and specific COX-2 inhibitors is at the center of current anti-inflammatory therapies for the treatment of acute pain, as well as the signs and symptoms of osteoarthritis (OA) and rheumatoid arthritis (RA) (Flower, 2003). RA is an inflammatory disease characterized by inflammation of the joints connective tissue (Emery, 2006). In contrast, OA, the most common form of arthritis, is a degenerative joint disease presenting joint pain and progressive loss of articular cartilage. OA is strongly associated with aging and injuries, whereas RA is considered an autoimmune disease (Hunter and Felson, 2006). Synovial fluids from patients with RA and OA have elevated levels of multiple PGs, including PGE2, and PGL2 (Egg, 1984). Based on the contrasting etiologies of these two diseases, we posit that PGL2 would contribute differently to the development and maintenance of inflammation in RA and OA.

Because COX-2 inhibitors and NSAIDs inhibit the formation of multiple PGs, efforts are now focused on identifying which of the individual PGs are directly involved in inflammation and arthritis. Several lines of evidence support the notion that at least some of the beneficial actions of NSAIDs and COX-2 inhibitors are a consequence of their inhibitory effects on PGE2 production. First, PGE2 levels are elevated in inflamed human synovia (Trang et al., 1977; Egg, 1984). In addition, selective neutralization of PGE2 with anti-PGE2
antibody substantially reduced inflammation and hyperalgesia in rats (Portanova et al., 1996). Moreover, genetic ablation of EP4 receptors has revealed that it might be the primary PGE2 receptor accountable for the development of chronic arthritis (McOey et al., 2002), with a possible contribution of EP2 (Honda et al., 2006). Additional evidence has been obtained from studies in which mice deficient for the inducible PGE2 synthase (mPGES-1) are protected from experimental arthritis (Trebin et al., 2003).

Although PGI2 has been better characterized as an anti-thrombotic agent and a vasodilator, it is also an important mediator of inflammation and pain. Specifically, there is an abundance of data supporting the role of PGI2 in acute pain (Bley et al., 1998). For example, PGL2 receptor (IP, Ptgir)-deficient mice display an impaired acute inflammatory response in various models, including the carrageenan-induced paw edema and acetic acid induced-writhing models (Murata et al., 1997). However, there is a paucity of data demonstrating the involvement of IP receptors in chronic inflammation. The evidence implicating PGL2 in chronic inflammation is mostly derived from the presence of PGL2 in synovial fluids isolated from patients with either RA or OA (Brodie et al., 1980).

To delineate the direct contribution of PGL2-mediated effects, we characterized a specific IP receptor antagonist (Keitz et al., 2004) in animal models of human OA and RA-like diseases and further compared its effects to the phenotype of IP receptor-deficient mice (Cheng et al., 2002). First, we established the efficacy of the IP antagonist in vitro in a rat whole-blood assay and in vivo using acute models of inflammation and hyperalgesia in rats. The antagonist was then tested in distinct chronic models of inflammation, i.e., the mono-iodoacetate (MIA) rat model of OA-like weight-bearing pain and the collagen antibody-induced arthritis mouse model (CAIA). In both models, we detected elevated PGL2 levels in inflamed tissue and observed a marked anti-inflammatory effect of the IP antagonist, indicating a major contribution of PGL2 in the development of chronic arthritis.

Materials and Methods

Experimental Animals. All procedures used for the in vivo experiments were approved by the Animal Care Committee at Merck Frosst Centre for Therapeutic Research (Kirkland, QC, Canada) and were performed according to guidelines established by the Canadian Council on Animal Care. IP−/− (Ptgir−/−) were generated as described previously and have been backcrossed extensively onto a C57BL/6 background (n >10) (Cheng et al., 2002). Breeding was performed at Taconic (Germantown, NY). Male Sprague-Dawley rats and Balb/c mice were obtained from Charles River Laboratories (St-Constant, QC, Canada).

Pharmacological Inhibitors. The IP antagonist N-[4-(imidazol-2-yl)deneamino]-benzyl]-4-methoxy-benzamide was synthesized as described previously (Keitz et al., 2004). For pharmacokinetics experiments, rats were fasted overnight and orally dosed in the morning with the IP antagonist. Blood was collected by tail bleeding at different time points in heparinized tubes. Deter-
logical analyses, all four paws were collected, and the skin was removed. Tissues were fixed in 10% neutral buffered formalin, decalcified, and embedded in paraffin. Sections (5 μm) were stained with Safranin O. Histopathological analysis was performed by Bolder BioPATH, Inc (Boulder, CO).

Measurements of PGs by Liquid Chromatography-Mass Spectrometry. Frozen mouse paws from CAIA experiments were pulverized in liquid nitrogen using a mortar and pestle to obtain a fine powder. This powder was homogenized at 4°C in PBS supplemented with 10 μM indomethacin and 1 × Complete protease inhibitor mixture (Roche Applied Science, Laval, QC, Canada) using a tissue tearer (Polytron PRO 200; PRO Scientific Inc., Oxford, CT). The homogenates were subsequently sonicated on ice for 10 to 30 s (Cole-Parmer ultrasonic homogenizer, 50% output; Cole-Parmer Instrument Co., Vernon Hills, IL) and centrifuged at 1000 g for 10 min (4°C). Supernatants were isolated and stored at −80°C until further analysis. Synovial fluids were collected by lavaging the synovial cavity with 100 μl of PBS and also stored at −80°C until further analysis. The levels of 6-keto-PGF1α (stable breakdown product of PGL2) and PGF2α were quantified by LC-MS (Guay et al., 2004). In brief, samples (100 μl) were protein-precipitated by the addition of 120 μl of acetonitrile containing 2 ng/ml deuterated prostanoids that served as internal standards for quantification. Samples were mixed thoroughly by pipetting and centrifuged at 1200 g for 10 min (4°C), and supernatants were transferred to a new 96-well plate for analysis by LC-MS. The detection limit for LC-MS analysis is 16 pg/ml or 0.002 ng/mg protein for each PG. Paw samples were normalized relative to protein concentration determined by standard Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis. Each n value corresponds to a different animal, and all measurements and scorings were performed by a single-blinded observer. Group comparisons for two or more groups were performed using one-way or two-way ANOVA with Bonferroni’s post-test. Student’s t test was performed if only two groups were compared. All data are represented as mean ± S.E.M.

Results

In Vitro Potency and Selectivity of the IP Antagonist. The IP antagonist (Fig. 1) was tested in a panel of binding assays to determine its potency and specificity across eight cloned human prostanoid receptors. The compound showed a high affinity for the IP receptor with a Ki of 67 nM and a high level of selectivity when tested against the other prostanoid receptors (Ki > 5 μM for EP1, EP2, EP3, EP4, PGD2 receptor subtypes 1 and 2, CRTH2 (chemoattractant receptor-homologous molecule expressed on TH2 cells), PGF2α receptor, and thromboxane A2 receptor). Consistent with its pharmacological profile against human prostanoid receptors, this molecule also displayed a relatively high potency (Ki = 7.6 nM) in a rodent cell-based cAMP inhibition assay (Keitz et al., 2004).

The IP antagonist was further characterized for its ability to block the PGL2-evoked effects in a functional whole-blood assay. Previous studies have shown that the PGL2 agonists iloprost and cicaprost inhibited TNFα production by LPS-stimulated mononuclear cells (Czeslick et al., 2003; Eisenhut et al., 1993). Likewise, we observed that the agonist beraprost markedly inhibited the production of TNFα in LPS-stimulated rat whole blood in a dose-dependent manner (data not shown). Subsequently, we tested whether the IP antagonist could reverse the inhibitory effects of beraprost on TNFα production. The compound reversed the effect of the IP agonist in a dose-dependent fashion with an IC50 of 1.7 μM (Fig. 2, A and B).

To evaluate the suitability of this antagonist for in vivo studies, initial pharmacokinetic studies were performed in rats using a single oral dose of 100 mg/kg. At this dose, the compound reached a maximal blood concentration of 2.1 μM at 1 h. After 6 h, the blood compound levels were at 0.5 μM, a concentration lower than that required for antagonistic activity in the whole-blood assay. In rat MIA experiments, ex vivo whole-blood pharmacodynamic assays indicated that the best coverage was obtained with a double dosing regime at time 0 and 3 h (data not shown). This method allows an average blood concentration of 3 μM at 100 mg/kg (two times the whole blood IC50).

We used a similar approach for determining optimal drug exposure in mice. Due to the chronic nature of CAIA and species difference between mice and rats, we performed oral dosing at 300 mg/kg twice day (8 and 16 h) with compound mixed in chow (0.36% w/w) to minimize trough periods during the nighttime. This dosing regime gave a maximal blood concentration of 1.7 μM (1 h after morning dose) and a trough level of 0.9 μM (before morning dose). No overt behavioral abnormalities or weight loss were observed during the course of the experiments.
Efficacy of the IP Antagonist in Acute Models of Inflammation. The effect of the IP antagonist was further characterized in acute models of hyperalgesia and inflammation in rat. Intraplantar injection of beraprost induced thermal hyperalgesia in a dose-dependent manner (data not shown). An effective but submaximal dose of beraprost was used in combination with a dose range of IP antagonist. The IP antagonist blocked the hyperalgesia induced by an injection of 100 ng of beraprost in a dose-dependent manner with 65% reversal at 100 mg/kg (Fig. 3A). The compound was also tested in the acute model of carrageenan-induced inflammation. Administration of the IP antagonist (100 mg/kg) 1 h before carrageenan injection in the rat hindpaw inhibited edema formation to a similar extent than the COX-2 inhibitor MF-tricyclic (10 mg/kg) (Fig. 3B).

PGI₂ Levels Are Elevated in Chronic Models of Arthritis. Injection of MIA into the left synovial cavity (1 mg) resulted in an increase in joint discomfort that can be measured by change in hindpaw weight distribution. Weight distribution between MIA-injected left paw and control right paw was measured on days 0, 3, and 7. We obtained the maximal change in weight redistribution on day 3 postinjection. By day 7, the discomfort was reduced but still significant (data not shown). We determined the levels of the various PGs in the synovial fluid at those two time points. On day 3, the levels of PGE₂ and 6-keto-PGF₁α were found to be elevated by 10- and 3-fold, respectively, compared with saline controls. By day 7, the levels of both PGs returned to basal (saline controls) levels (Fig. 4A). In contrast, the levels of PGD₂, PGF₂α, and TXB₂ did not change significantly at either these two time points (data not shown).

We also assessed PG levels in the hindpaw homogenates isolated from CAIA mice on two different genetic backgrounds. IP receptor-deficient mice are on a C57BL/6 inbred genetic background, and pharmacological characterization of the IP antagonist was performed in Balb/c inbred mice. On day 14 postantibody injection, PGE₂ levels were significantly increased in both mouse strains (Fig. 4B). In contrast, 6-keto-PGF₁α levels were found to be up-regulated only in arthritic Balb/c mice (2-fold increase above control). We could not detect a significant increase in 6-keto-PGF₁α levels in arthritic C57BL/6 mice, which incidentally also develop less severe disease.

Reversal of OA-Like Weight-Bearing Pain with an IP Antagonist. Because 6-keto-PGF₁α levels were elevated in the MIA model, we tested the efficacy of the IP antagonist for reducing joint discomfort in this animal model. The IP antagonist reduced pain associated with MIA injection in a dose-dependent manner both on days 3 and 7 postinjection (Fig. 5, A and B). The relative area under the curves (AUCs) of the paw weight ratio (left/right) graphs was compared for the vehicle-treated animals versus those of the IP antagonist diclofenac and the MF-tricyclic COX-2 inhibitor-treated groups (Fig. 5C). The efficacy of all test compounds was comparable, suggesting that COX-2-mediated PGI₂ synthesis contributes to the development of joint discomfort in this model of OA-like weight-bearing pain.

Reduced Arthritis-Like Disease in IP⁻/⁻ Mice. Collagen-induced arthritis is a well established model of RA in mice. However, robust disease incidence and severity in this model is mostly limited to mice on DBA1 or B10RIII genetic backgrounds. The IP⁻/⁻ mice have been backcrossed to the C57BL/6 background, rendering the model suboptimal for phenotyping and pharmacological characterization. CAIA is an alternate way to induce arthritis in mice and involves the direct i.p. injection of collagen antibodies followed by LPS. The compatibility of CAIA with diverse genetic backgrounds makes it a suitable model to be used in IP⁻/⁻ mice and to compare with the effects of the IP antagonist.

The IP⁻/⁻ mice were injected with 8 mg of a collagen antibody cocktail on day 0 followed by administration of 50 µg of LPS on day 3. In this model, arthritis begins to develop on days 6 to 7. Visual scores of the animals were recorded every 1 to 2 days. There was a significant reduction in both severity and incidence of the disease in IP⁻/⁻ mice compared with their wild-type littermates (Fig. 6A). In the control group, the incidence of arthritis was 100%, whereas in the IP⁻/⁻ mice, only 60% animals were affected and with significantly lower symptoms scores. These differences were detected throughout the time course of the experiment. To further assess disease severity, we analyzed the histopatho-
logical changes associated with experimental arthritis. Mean scores for all parameters (inflammation, bone resorption, cartilage damage, and pannus formation) were reduced by 91% in the IP^{-/-} mice (Fig. 6B and data not shown).

**IP Antagonist Reduces the Severity of Arthritis in a Prophylactic but Not Therapeutic Treatment Paradigm.** We tested the ability of the IP antagonist to reduce the signs of arthritis in the CAIA model. Initial experiments were performed in the Balb/c genetic background because of its higher susceptibility to CAIA. Balb/c mice were injected with 4 mg of collagen antibody cocktail and 50 μg of LPS, respectively, on days 0 and 3. Animals were given the IP antagonist in prophylaxis 1 day before antibody injection. Although the effect was not as pronounced as in the IP^{-/-} mice, the IP antagonist caused a significant decrease in the clinical scores. The incidence was reduced from 100% (6/6) in the control group to 83% (5/6) in the treated group, with an average reduction of 52% of the symptoms scores (Fig. 7A).

Guided by the phenotype of IP^{-/-} mice (Fig. 6), we hypothesized that the pharmacological effects of the IP antagonist could result from a partial occupancy of the IP receptor by the antagonist (Fig. 7A). Therefore, to achieve a more complete reduction of IP-mediated signaling, we used IP^{-/-} mice, which express half of the amount of receptors compared with the wild-type controls. We observed that IP^{+/+} mice had lower arthritis scores compared with their wild-type controls but the difference in score did not attain statistical significance (Fig. 7B). We performed an experiment in IP^{-/-} mice to determine the effect of the IP antagonist in the prophylactic and therapeutic paradigms. In IP^{+/+} mice, treatment with a prophylactic dose (from 1 day before antibody injection) of the IP antagonist caused an almost complete reduction of the arthritis symptoms (Fig. 7B), similar to that obtained with the IP^{-/-} mice (Fig. 6A).

Based on the indication that genetic ablation of IP receptors and prophylactic compound treatment were protective, we were then interested in dissecting whether IP receptors were involved in the maintenance phase of chronic inflammation. To address this question, animals were dosed starting on day 6 and monitored over the remainder of the experiment. In contrast to prophylactic dosages, therapeutic dosages had no effect on the clinical score of arthritis (Fig. 7B).

We also compared the reduction of arthritis severity (AUC of the clinical scores) between IP^{-/-} mice, IP antagonist-treated, and COX-2 inhibitor-treated animals. COX-2-treated animals displayed a 98% reduction in score. The IP^{-/-} and IP^{+/+} mice treated with the IP antagonist had a marked and comparable reduction in their arthritis scores (91 and 93%, respectively), suggesting that COX-2-derived PGI2 plays a major role in the pathophysiology of CAIA (Fig. 8).

**Discussion**

PGs are involved in a range of diverse physiological and pathological processes. Understanding the various roles of PGs is a key to determining effective new therapeutic targets with minimized potential for side effects. It has been shown that PGE_{2} and PGI_{2} are the primary PGs involved in inflammatory pain responses (Ferreira et al., 1978; Portanova et al., 1996). Thus, global inhibition of PG synthesis by NSAIDs and selective COX-2 inhibitor is widely used to treat inflammatory symptoms associated with osteoarthritis and rheumatoid arthritis. There is now emerging evidence that chronic use of COX-2-specific inhibitors and NSAIDs in-
crease the risk of cardiovascular side effects (http://www.fda.gov/cder/drug/infopage/COX2/NSAIDdecisionMemo.pdf) (Bresalier et al., 2005). NSAID and COX-2 inhibitors block the production of PGI2 and PGE2 while sparing COX-1-mediated TXA2 production by platelets to a different degree, depending on their relative selectivity for COX-1 and COX-2. Because PGL2 has vasodilatory and antithrombotic properties, there is considerable interest in evaluating the thera-
induced paw edema. In the latter model, a 50% reduction of the edema was observed, approaching that obtained with COX-2 inhibition. Our results further confirm, at the pharmacological level, the contribution of PGJ2-mediated processes in acute inflammatory reactions previously described in IP−/− mice and with other IP antagonists (Murata et al., 1997; Keitz et al., 2004; Bley et al., 2006).

Having established the efficacy of the IP antagonist in acute rat models, we then focused our efforts on models of chronic inflammation and pain. Administration of MIA, an inhibitor of chondrocyte metabolism, can induce disruption of glycosylation and subsequent cell death. The loss of chondrocytes results in cartilage and histological lesions in the stifle joint similar to that observed in human OA (Kalbhen, 1987). Concomitant with the histological deterioration, OA-like weight-bearing pain also develops in these animals (Bove et al., 2003). Therefore, this model presents an interesting platform for testing anti-inflammatory and analgesic compounds. We showed that 6-keto-PGF1α levels in synovial fluids were up-regulated 3 days after MIA injection and returned to basal levels by day 7. This early up-regulation was also observed in the rat adjuvant-induced arthritis model where 6-keto-PGF1α levels were found to be increased only in the early induction phase (Claveau et al., 2003). We have demonstrated that IP antagonist administration in this model significantly reduced the surrogate pain measurement endpoints on both days 3 and 7. Our results are consistent with a recent study showing the efficacy of a different IP antagonist, at a different time point, 14 days after MIA injection (Bley et al., 2006). Our results also revealed a similar efficacy profile of the IP antagonist to an NSAID and COX-2 inhibitor underlying the importance of COX-2-dependent PGJ2 in the setting of OA-like weight-bearing pain. Based on the presence of 6-keto PGF1α in the joint cavity and previously reported expression of IP receptors in the spinal cord, the mechanism could be one that involves both local inflammatory reactions and/or neuronal pathways (Doi et al., 2002; Oida et al., 1995).

Following the observation that an IP antagonist displays NSAID-like efficacy in both acute and chronic inflammatory pain models, we then focused on dissecting the role of IP receptors in the onset and maintenance phases of chronic inflammation. Prophylactic administration of an IP antagonist significantly reduced the signs of arthritis in arthritic mice. However, when used in a therapeutic mode, no detectable reduction in the arthritis score was obtained. In contrast, therapeutic administration of a COX-2 inhibitor in a rat model of arthritis significantly reduced the development
of inflammation, suggesting that simply inhibiting the PGI2 signaling cascade may not be sufficient for maximal anti-inflammatory effects (Harris et al., 2004).

While preparing this manuscript, a recent publication described that IP receptor-deficient mice displayed a degree of protection against the induction of collagen-induced and collagen antibody-induced arthritis (Honda et al., 2006). These authors detected an approximate 40% reduction in the AUC of the CAIA scores. This relatively mild reduction in arthritis is in contrast to our current findings, revealing an almost complete protection in IP/ mice compared with congenic wild-type controls. Previous reports have underlined phenotypic differences related to prostaglandin-mediated effects depending on the mouse strain studied (Kennedy et al., 1995; Trebino et al., 2005). We also detected differences in 6-keto PGF1α and PGE2 levels in the paws of arthritic C57BL/6 and Balb/c mice. Therefore, this difference in phenotype between the two manuscripts could be explained by the fact that these authors performed their studies using IP/ mice on a DBA/1JNcr genetic background, in contrast to our experiments performed in both C57BL/6 and Balb/c mice.

In conclusion, our findings support the notion that modulation of IP receptor-evoked effects may be critical for the efficacy of NSAIDs and COX-2 inhibitors in both acute and chronic inflammation. These observations further complement our understanding of the relative role of PGE2 and PGI1 in mediating inflammatory responses and also underline some interesting similarities between these two mediators. For example, in the acute caerulein-induced writhing model, disruption of EP1 receptor, mPGES-1, or IP receptors resulted in a similar reduction in pain perception (Stock et al., 2001; Kamei et al., 2004; Ohishi et al., 1999). Herein, we identified a critical role of IP receptors in inflammatory arthritis, not unlike that reported for EP3 receptor-deficient mice (Honda et al., 2006; McCoy et al., 2002). In both acute and chronic inflammation, disruption of either PGE2 or PGI2 pathway had a similar impact. Altogether, these results suggest that the successful development of third generation NSAIDs will probably depend on the comprehensive characterization of individual prostaglandins in inflammation.