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Selective inhibition of prostaglandin D_2 biosynthesis in human mast cells to overcome need for multiple receptor antagonists: Biochemical consequences

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Abstract

Background: The major mast cell prostanoid PGD_2 is targeted for therapy of asthma and other diseases, because the biological actions include bronchoconstriction, vasodilation and regulation of immune cells mediated by three different receptors. It is not known if the alternative to selectively inhibit the biosynthesis of PGD_2 affects release of other prostanoids in human mast cells.

Objectives: To determine the biochemical consequences of inhibition of the hematopoietic prostaglandin D synthase (hPGDS) PGD_2 in human mast cells.

Methods: Four human mast cell models, LAD2, cord blood derived mast cells (CBMC), peripheral blood derived mast cells (PBMC) and human lung mast cells (HLMC), were activated by anti-IgE or ionophore A23187. Prostanoids were measured by UPLC-MS/MS.

Results: All mast cells almost exclusively released PGD_2 when activated by anti-IgE or A23187. The biosynthesis was in all four cell types entirely initiated by COX-1. When

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pharmacologic inhibition of hPGDS abolished formation of PGD_2, PGE_2 was detected and release of TXA_2 increased. Conversely, when the thromboxane synthase was inhibited, levels of PGD_2 increased. Adding exogenous PGH_2 confirmed predominant conversion to PGD_2 under control conditions, and increased levels of TXB_2 and PGE_2 when hPGDS was inhibited. However, PGE_2 was formed by non-enzymatic degradation.

Conclusions: Inhibition of hPGDS effectively blocks mast cell dependent PGD_2 formation. The inhibition was associated with redirected use of the intermediate PGH_2 and shunting into biosynthesis of TXA_2. However, the levels of TXA_2 did not reach those of PGD_2 in naïve cells. It remains to determine if this diversion occurs *in vivo* and has clinical relevance.

KEYWORDS
asthma, basic mechanisms, IgE, mast cells

1 | INTRODUCTION

PGD_2 is the major eicosanoid released from activated mast cells.[1,2] It has potent biological effects including bronchoconstriction via the TP receptor,[3,4] vasodilation via the DP_1 receptor,[5] induction of eosinophilic lung inflammation[6] as well as activation and recruitment of group 2 innate lymphoid cells (ILC-2) and Th2 cells via the DP_2 receptor,[7] also called CRTH2 receptor.[8-10] It is therefore a current interest in targeting PGD_2 for treatment of asthma and allergic diseases.[8] For effective inhibition of PGD_2 there is accordingly a need to block several receptors that mediate pro-inflammatory biological responses, in particular in asthma where bronchoconstriction via the TP receptor is a fundamental component of the clinical presentation. We hypothesised that inhibition of the biosynthesis of PGD_2 might represent an attractive alternative interventional strategy requiring only one drug. We therefore performed this in-depth study of the mechanistic consequences of selective inhibition of the biosynthesis of PGD_2 in human lung mast cells (HLMC), as well as in three other human mast cell models.

The first step in the biosynthesis of PGD_2 is shared with all other prostanoids, namely the sequential oxygenation of arachidonic acid into the endoperoxide intermediates PGG_2 and PGH_2[11] (Figure 1). The reaction is catalysed by the prostaglandin-endoperoxide synthases usually described as the cyclo-oxygenase (COX) isoenzymes, COX-1 and COX-2. The next step, the formation of PGD_2 from PGH_2, is in mast cells catalysed by the haematopoietic PGD synthase (hPGDS).[12] Although it has been reported that COX-1 exclusively catalyses formation of PGD_2 in HLMC,[13] this has not been replicated nor studied in other human mast cells.

The primary aim of this study was to determine the effects of inhibition of the synthesis of PGD_2 in human mast cells at the level of the hPGDS. The overall consequences of such an intervention on the formation of other COX products has previously not been assessed in human mast cells. It has been speculated that inhibition of one enzyme distal of COX might increase the biosynthesis of other prostanoids, with such shunting of the intermediate into other pathways possibly leading to unwanted consequences.[14] There is however no published study of whether or not such shunting occurs in human mast cells.

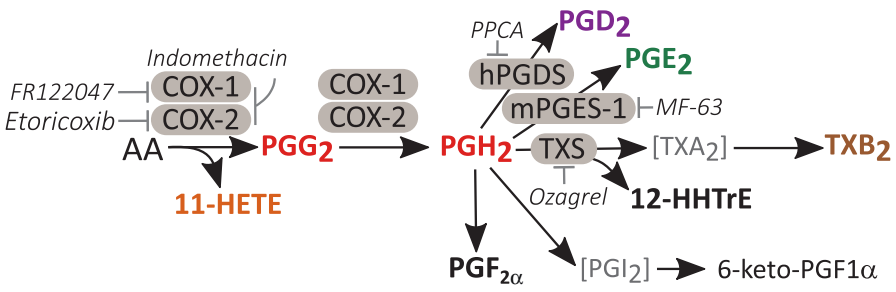

FIGURE 1 The COX pathway. Arachidonic acid is a substrate for different pathways with enzymes giving rise to numerous metabolites. The COX pathway is the main arachidonic acid utilizing pathway of mast cells. The enzymes inhibited and the inhibitors used in this study are shown. 6-keto-PGF1α, 6-keto Prostaglandin F1α; AA, arachidonic acid; COX, cyclo-oxygenase; hPGDS, hematopoietic prostaglandin D synthase; mPGES-1, microsomal prostaglandin E synthase 1; TXS, thromboxane-A synthase; PGD_2, Prostaglandin D_2; PGE_2, Prostaglandin E_2; PGG_2, Prostaglandin G_2; PGH_2, Prostaglandin H_2; PGI_2, 15-deoxy-D12,14-Prostacyclin; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; 11-HETE, 11-Hydroxyeicosatetraenoic acid.

We therefore investigated biosynthesis of PGD_2 in four different human mast cell models. Specifically, cultured human mast cell leukaemia cell line LAD2, cord blood derived mast cells (CBMC), peripheral blood derived mast cells (PBMC) and human lung mast cells. Although there is no clinically used inhibitor of hPGDS, there are experimental compounds that target this enzyme.[14] The candidate drug HQL-79 was originally designed as an anti-histamine but found to also be a PGDS inhibitor.[15] The compound PPCA (2-Phenyl-pyrimidine-5-carboxylic acid (2,3-dihydro-indol-1-yl)-amide),[16] which belongs to the group of pyrimidine hydrazides,[17] showed more potent activity than HQL-79[9] and was selected for this study.

2 | METHODS

2.1 | Mast cell cultures

LAD2 cells (kindly provided by Dr. Kirshenbaum and Dr. Metcalfe, NIH) were grown in StemSpan medium containing 100 ng/ml of human stem cell factor (SCF, Invitrogen).[18] CBMC and PBMC were developed from $CD34^+$ progenitors isolated from cord blood and peripheral blood using CD34 MicroBead kit (Miltenyi Biotec) as previously described.[19] Culture was deemed mature when >90% stained positive for the mast cell-specific protease tryptase. In some experiments, mast cells were primed with IL-4 (10 ng/ml, Peprotech) for 3 days (LAD2) or 4 days (CBMC) before experiments.

With permission of the regional ethical review board in Stockholm (reference Nos. 2010/181-31/2 and 2018/1819-31/1), macroscopically healthy human lung tissue was obtained by consent from patients undergoing lobectomy and HLMC isolated as described.[20] The obtained cell suspension was cultured overnight (without SCF) and mast cells isolated by CD117 positive selection using a Micro Bead kit and MACS column (Miltenyi Biotec). Cells were recovered in RPMI-1640 medium with 100 ng/ml SCF for 4–7 days before the experiment.

2.2 | Activation of mast cells in vitro

Mast cells were sensitized with IgE (1 μg/ml, Calbiochem) in complete culture media for 20–24 h, washed with PBS and resuspended in PIPES buffer (Sigma-Aldrich) with 0.2% bovine serum albumin (BSA, Sigma-Aldrich). Cells (0.1×10^6/mL) were stimulated with 2 μg/ml of anti-human IgE (Sigma-Aldrich) or calcium ionophore A23187 (0.5–1 μM; Sigma-Aldrich) for 30 min at 37 ˚C, 5% CO_2. Enzyme inhibitors, including indomethacin (non-selective COX inhibitor, 10 μM, Sigma-Aldrich), FR-122047 (selective COX-1 inhibitor, 1 μM, Cayman Chemicals), etoricoxib (selective COX-2 inhibitor, 1 μM, Merck Research Laboratories), PPCA (hPGDS inhibitor, 0.1–10 μM, was custom synthesised by Cayman Chemical Company, Inc), MF-63 (mPGES-1 inhibitor, 10 μM, Anthem BioSciences), and Ozagrel (thromboxane synthase inhibitor, 1 μM, Cayman Chemicals), were added 15 min before stimulation (final DMSO concentration of less than 0.1%). Viability after incubation with inhibitors was determined by trypan blue staining. Incubations of PGH_2, 50 ng/ml (Larodan) was stopped by the addition of 4 volumes of 5 mg/ml $SnCl_2$ in ethanol as in.[21] Supernatants were collected and stored at −80°C until analysis. After activation, the viability of the CBMC and HLMC was evaluated by flow cytometry. Neither stimulation nor inhibitors effected viability. Purity of the lung mast cells was also evaluated at this point and the overall purity was 77%–95% with a median of 89%. Level of activation was assessed by the degranulation marker CD63 which under basal conditions in HLMCs was at a median of 1% (range 0.8%–16%) and after IgE cross-linking at a median of 67% (range 39%–74%) and for CBMC the basal median was 2% (range 2%–3%) and increased to 42% (range 16%–47%) after stimulation.

2.3 | Flow cytometry

Lung mast cells were stained using BD Horizon™ Fixable Viability Stain 450 and antibodies against CD45-V500 (BD Biosciences, clone HI30), CD14-APC-Cy7 (Biolegend, clone M5E2), CD117-APC (BD Biosciences, clone 104D2), CD63 –PeCy7 (BD Biosciences, clone H5C6) and mast cells were gated as live, CD45+, CD14–, CD117 high and activation was determined by CD63 translocation to the membrane. CBMCs were stained with Viability stain and anti CD63. FlowJo software was used for flow cytometry data analysis.

2.4 | Measurement of mediators

Release of β-hexosaminidase and PGD_2 (using the Cayman Chemicals methoxylation kit) were initially measured with enzyme immunoassays (EIA) as previously described.[22] The extended profiling of COX-metabolites involved solid phase extraction (SPE) and analysis by ultra-performance liquid chromatography coupled to tandem mass spectrometry UPLC-MS/MS as described.[23] Prior to extraction, an internal standard mix containing 42 deuterated standards was added to each sample. Quantification was based on 11-point calibration curves. The UPLC-MS/MS method is described in detail in reference.[23]

2.5 | Western blot analysis

Western blot was performed as described previously.[24] Antibodies against the following proteins were used: COX-1 (1:1000, rabbit anti-ovine, Cayman Chemicals), COX-2 (1:200, rabbit anti-human, Cayman Chemicals) and hematopoietic-type PGDS (hPGDS, 1:600, rabbit, Cayman Chemicals). A horseradish peroxidase conjugated secondary antibody (1:2000, anti-rabbit) was added for 1 h at room temperature and protein bands were visualized with an enhanced chemiluminescence system (LumiGLO).

2.6 | Statistical analyses

Data are expressed as mean ± *SEM* from at least three separate experiments. Statistical analyses between two groups were performed with student *t*-test, and *p*-value of less than .05 was considered statistically significant.

3 | RESULTS

3.1 | COX-1 initiates biosynthesis of PGD_2

First we examined the initial step in the PGD_2 biosynthesis, that is, the dependence on the COX isozymes, in primary cultured mast cells from cord blood (CBMC) and peripheral blood (PBMC) as well as the LAD2 cell line (Figure 2A). As it was previously shown only in human lung mast cells (HLMC),[13] we wanted to confirm also in these other mast cell models that PGD_2 production is solely COX-1 dependent. Indeed, pre-treatment with the selective COX-1 inhibitor, FR-122047 consistently abolished PGD_2 release in the three models, irrespectively of whether stimulated with anti-IgE or calcium ionophore A23187, whereas the selective COX-2 inhibitor etoricoxib had no effect on PGD_2 release. Stimulation by calcium ionophore A23187, gave a higher release of PGD_2 than anti-IgE in all three cell types. CBMC and PBMC released higher levels of PGD_2 in response to both stimuli than LAD2.

In line with the results obtained with the pharmacologic inhibitors, western blot experiments only found expression of COX-1 in the studied mast cells. As shown for the LAD2 cells, COX-2 was not detected even after a prolonged exposure of the western blot gel (Figure 2B).

To optimize the IgE-receptor mediated activation of LAD2 cells they were primed with IL-4 for 72 h to increase the expression of IgE-receptor.[25] This priming resulted in enhancement of PGD_2 release induced by anti-IgE stimulation (Figure 2C), but maintained responsiveness to pharmacologic inhibitors as in un-primed cells. The IL-4 exposure did not lead to an induction of COX-2 protein expression (not shown).

3.2 | Inhibition of the biosynthesis of PGD_2 at the level of hPGDS affects the formation of other prostanoids

The selected hPGDS inhibitor PPCA dose-dependently (0.1–10 μM) inhibited the anti-IgE induced release of PGD_2 in LAD2 cells (Figure 3A). Release of β-hexosaminidase release was not affected by the two lowest doses of PPCA, but there was around 30% inhibition at the highest dose (10 μM) (Figure 3B). The concentration of 1 μM of PPCA was therefore used in subsequent experiments.

The influence of PPCA on the release of all COX products (Figure 1) was next determined by the use of UPLC-MS/MS.[23] In order to optimise the receptor activation of CBMCs they were primed in the presence of IL-4 followed by anti-IgE (Figure 4A), LAD2 cells were activated by ionophore (Figure 4B), and the HLMC with the addition of anti-IgE.

In the absence of inhibitors, the levels of PGD_2 were more than 10 times higher than any other COX product (Figure 4A for CBMC, Figure 4B for LAD2, and Table 1 for HLMC) following the stimulation. The rank order for the other primary prostanoids was $TXB_2 > PGE_2 > PGF_{2\alpha}$. The PGI_2 metabolite 6-keto-PGF1α was found to be detected only in HLMC.

Furthermore, the UPLC/MS-MS analysis confirmed the COX-1 dependency of the PGD_2 biosynthesis (Figure 4). Thus, FR-122047 and indomethacin efficiently inhibited production of PGD_2, whereas COX-2 inhibition by etoricoxib had no effect. Indomethacin and

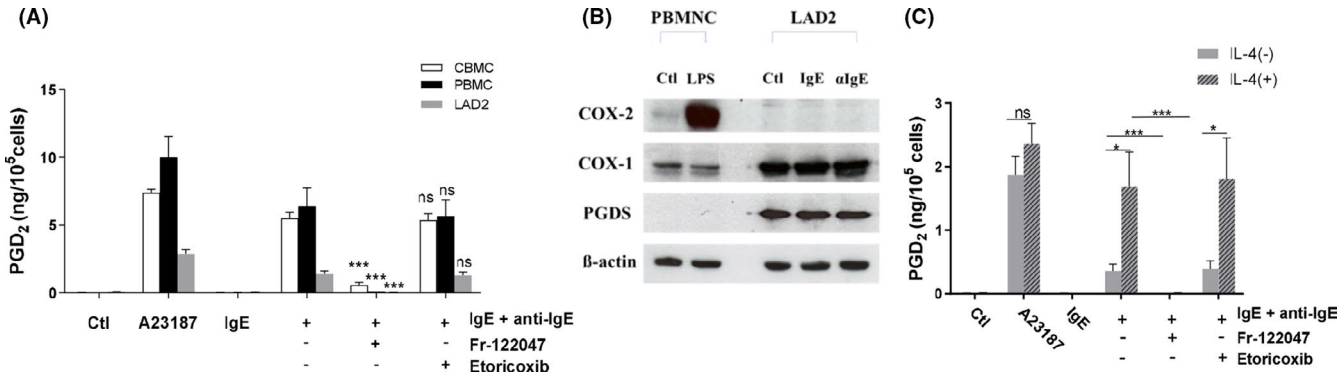

FIGURE 2 Stimulation of cultured human mast cells with anti-IgE and effect of COX inhibitors. Cells were pre-treated with inhibitor for 15 min and stimulated for 30 min. (A) PGD_2 release in response to stimulation of CBMC, PBMC and LAD2, with A23187 or anti-IgE as indicated and the effect of selective COX-1 inhibitor (FR-122047, 1 μM) and selective COX-2 inhibitor (etoricoxib, 1 μM). (B) LAD2 cell expression of COX-1 and COX-2 isozymes, LPS-induced COX-2 expression of peripheral blood mononuclear cells (PBMNC) shown as control. (C) Effect of IL-4 priming (10 ng/ml for 72 h) on PGD_2 biosynthesis in LAD2 cells stimulated with A23187 (0.5 μM) or anti-IgE (2 μg/ml) and the effect of selective COX-1 inhibitor and selective COX-2 inhibitor on PGD_2 production after stimulation with anti-IgE. Here and in following figures values are expressed as mean ± *SEM* $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$, ns: not significant

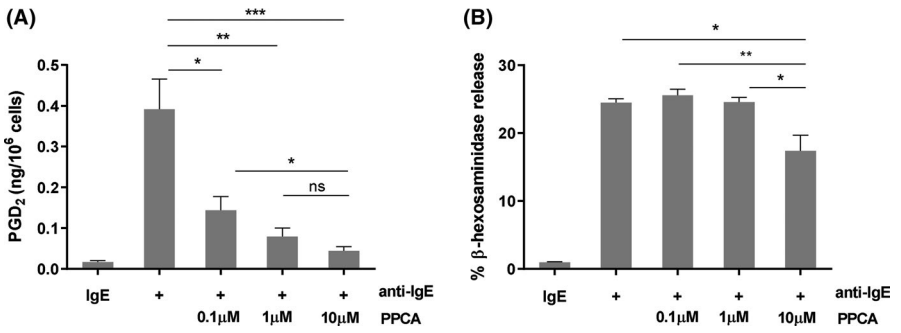

FIGURE 3 Effect of the hPGDS synthase inhibitor PPCA on mast cell mediator release. LAD2 cells were incubated with PPCA (0.1, 1 and 10 μM) for 15 min and then stimulated by anti-IgE for 30 min. Effect on (A) PGD_2 and (B) β-hexosaminidase release. Data from 3 different experiments in duplicates

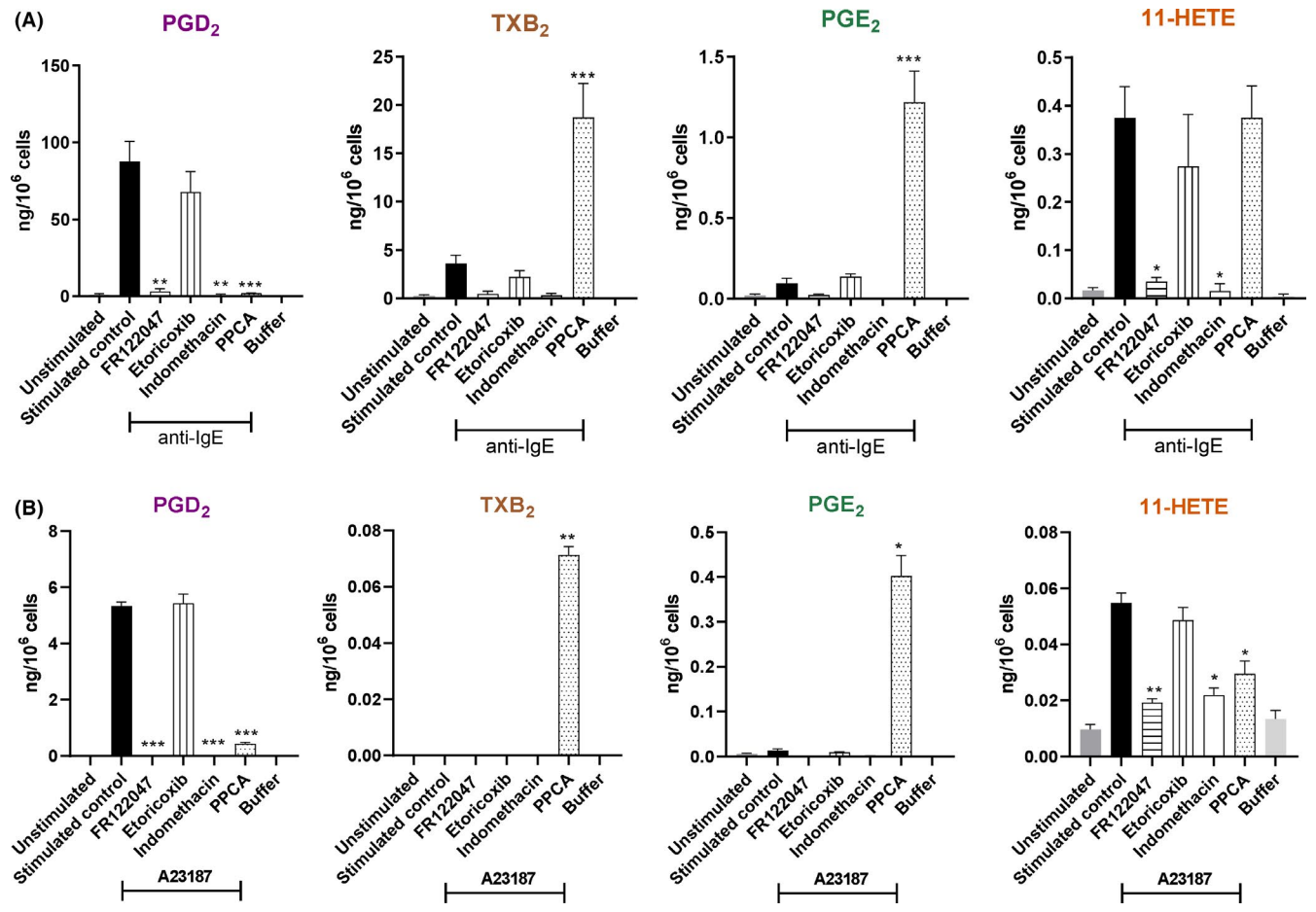

FIGURE 4 Prostanoid levels after enzyme inhibition. (A) and (B) Cells were stimulated for 30 min after 15 min of preincubation with inhibitors. UPLC-MS/MS was used to quantify release of lipid mediators. The main prostanoids affected by the inhibitors are presented. The non-prostanoid 11-HETE is shown as COX-dependent mediator not derived from PGH_2 but formed in parallel withPGG_2 by the cyclooxygenation of arachidonic acid. (A) CBMC stimulated by anti-IgE (values from 9 donors for controls and PPCA treatment and 3 donors for the other inhibitors) and (B) LAD2 cells stimulated by A23187 (1 μM) for 30 min after 15 min preincubation with inhibitor (data from 3 separate experiments, statistics relative to stimulated control)

FR-122047 also inhibited formation of PGE_2, TXB_2 and 11-HETE after stimulation, again indicating no COX-2 activity in the studied mast cells.

Next, the UPLC/MS-MS analysis documented essentially arrested release of PGD_2 after the PPCA treatment (Figure 4 and Table 1). In contrast, most other monitored COX products increased irrespective of the trigger used to activate the different mast cell preparations. The exception was 11-HETE which, as expected, remained unaffected by PPCA in the CBMC (Figure 4A) and HLMC (Table 1). Chiral column analysis showed enantiomeric excess of the *R*-isomer of 11-HETE (not shown), confirming its origin in the 11-oxygenation reaction of COX-1, proximal to the reaction where hPGDS catalyses biosynthesis of PGD_2 from PGH_2 (see Figure 1). The level of 11-HETE was too low in the LAD2 cells to allow for chiral analysis and there was also significant formation of 11-HETE in that model by autooxidation (Figure 4B, see buffer and unstimulated).

TABLE 1 Release of lipid mediators by HLMC (ng/10^6 cells ± *SEM* after 30 min of IgE-receptor cross-linking

	Stimulated control	HPGDS inhibition by PPCA	*p*-value
PGD_2	51 ± 6.55	1.6 ± 0.22	<.0001
TXB_2	4.4 ± 0.72	24.8 ± 2.72	.0001
12-HHTrE	0.6 ± 0.1	3.1 ± 0.51	.0012
PGE_2	0.3 ± 0.09	1.8 ± 0.16	<.0001
$PGF_{2\alpha}$	0.1 ± 0.06	0.2 ± 0.12	.0547
6-keto-$PGF_{1\alpha}$	0.01 ± 0.003	0.07 ± 0.02	.0148
11-HETE	0.7 ± 0.17	0.7 ± 0.21	.3015

Note: Data from 9 donors and 7 donors for stimulated control and PPCA treated, respectively.

In both HLMC (Table 1) and CBMC (Figure 4A), TXB_2 became the main COX product after the inhibition of hPGDS, followed by 12-HHTrE, which also is formed by the thromboxane-A synthase (TXS), and PGE_2. Quantitatively, TXB_2 release by HLMC was elevated to about 50% of PGD_2 levels in un-inhibited cells (Table 1). In the CBMC, release of TXB_2 after PPCA corresponded to about 25% of PGD_2 in the control stimulation (approximately 20 vs. 80 ng/10^6 cells; Figure 4A). The level of PGE_2 after hPGDS inhibition increased more than 10-fold in the CBMC but was still only about 1.5% of the quantity of PGD_2 formed in the intact cell. Likewise, the level of PGE_2 in HLMC was about 4%–5% of the level of PGD_2 in the unperturbed cell.

In the LAD2 cells, PGE_2 was the major COX product after hPGDS inhibition (Figure 4B). The amount of PGE_2 formed in the LAD2 cells

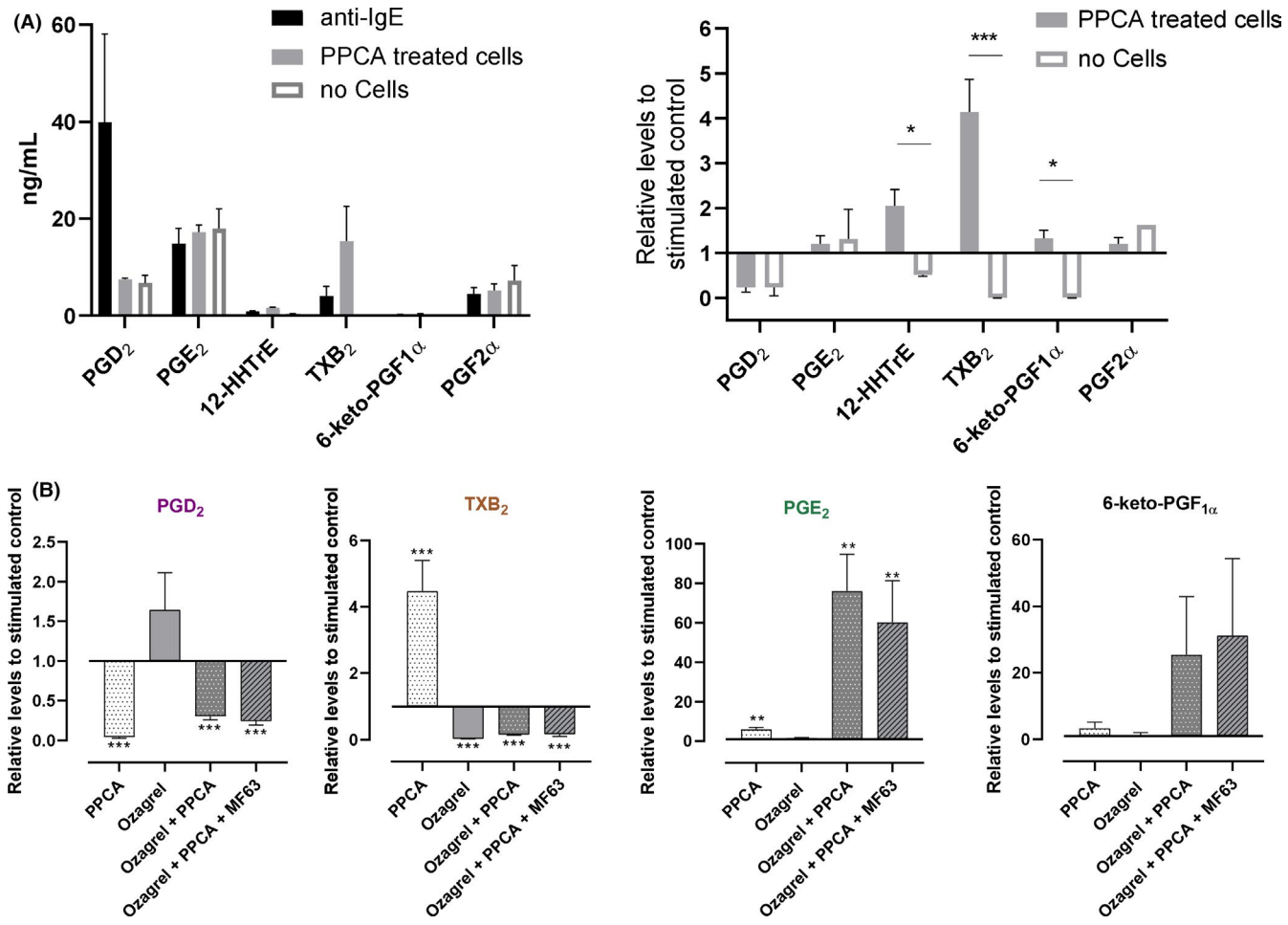

FIGURE 5 Inhibition of PGH_2 dependent enzymes causes a redirection in the biosynthesis of prostanoids. Supernatant analysed by UPLC-MS/MS. (A) HLMC were treated with or without hPGDS inhibitor (PPCA) and then exposed to 2 μg/ml anti-IgE and 50 ng/ml PGH_2 for 5 min. The reaction was stopped by the addition of 5 parts 5 mg/ml $SnCl_2$ in ethanol. This was done in parallel to a no cells control to show what mediators are produced from PGH_2 in buffer. Absolute values (left) and relative values in relation to stimulated control (right). Data are from two donors. (B) HLMC were pre-incubated with the hPGDS inhibitor PPCA (1 μM), the thromboxane synthase inhibitor ozagrel (1 μM) and the mPGES-1 inhibitor MF-63 (1 μM) for 15 min as indicated by the axis labels and stimulated by addition of 2 μg/ml anti-IgE for 30 min. Because of variability in control responses between donors, normalized values are shown. Bars begin at $y = 1$. Data from 2–3 donors

after the inhibition increased 40-fold, however, the absolute amount corresponded to less than 10% of the quantity of PGD_2 released in the control (Figure 4B).

There are two theoretical explanations of the increase in non-PGD_2 COX products after inhibition of hPGDS. Either PGD_2 itself mediates an inhibition of the release of other COX products (presumably by loss of DP- or TP-receptor activation), or the pathway intermediate PGH_2 accumulates and is redirected for use by the other distal enzymes (Figure 1). The first possibility was tested by adding 100 ng/ml of PGD_2 to HLMC while stimulating the cells, pre-incubated with the hPGDS inhibitor PPCA. However, the previously observed increase in PGH_2 products remained the same. This very clear experiment supported the hypothesis that the changed profile of COX products after inhibition of the hPGDS most likely was due to an alternative use of PGH_2. The view that PGD_2 signalling does not modulate the release of other mast cell mediators was also supported by experiments where the influence of PPCA on release of cysteinyl-leukotrienes CysLTs (LTC_4 + LTD_4 + LTE_4) was determined. Accordingly, the levels of CysLTs in HLMC activated by IgE cross-linking remained unchanged after treatment of PPCA (72 ± 11 in controls and 63 ± 10 ng/ml after PPCA; mean ± *SEM*, *n* = 3).

3.3 | Metabolism of exogenous PGH_2 in human lung mast cells

We next tested the hypothesis that the increased levels of TXB_2 and PGE_2 seen after PPCA treatment were due to PGH_2 being used in other biosynthetic pathways. The study was performed in tissue matured HLMC preparations where the increase in TXB_2 was greater than in the three other mast cell models. The protocol included a control arm with buffer only and no cells. Synthetic PGH_2 together with anti-IgE was added to HLMCs and after 5 min $SnCl_2$ was added to reduce any remaining PGH_2 to $PGF_{2\alpha}$.[21] Because the absolute amounts of metabolites ranged over two orders of magnitude, the left panel of Figure 5A shows absolute amounts and the right panel relative changes compared to control.

In the control challenged HLMC (Figure 5A), PGD_2 was the major product recovered (about 60% of metabolites), followed by PGE_2 (25%), TXB_2 (6%), 12-HHTrE (2%), 6-keto-$PGF_{1\alpha}$ (<1%), and about 5% $PGF_{2\alpha}$ representing mostly unmetabolized PGH_2. However, PGE_2 was detected in equal amounts whether cells were present or not, indicating that it was formed non-enzymatically from PGH_2. In contrast, TXB_2 was only detected in the presence of cells, in line with the prerequisite of TXS catalytic activity for its generation.[26]

Following PPCA pre-treatment, there was a drastic reduction in the PGD_2 levels after challenge with PGH_2 (Figure 5A). The low level of PGD_2 remaining after PPCA was similar as in the absence of cells, indicating a minor formation of PGD_2 via spontaneous degradation of PGH_2 during the 5-min incubation. The most prominent shift in metabolism of PGH_2 after PPCA was for TXB_2 which increased nearly 5-fold (from 5.8 ± 0.7% of metabolites in control to 30.4 ± 5.8% after PPCA; Figure 5B). There was also an increase in 12-HHTrE, the metabolite formed in parallel to TXA_2 by the action of TXS. Of interest, the prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ also increased to a small degree but was not produced in the absence of cells.

Because $SnCl_2$ reduced remaining PGH_2 to $PGF_{2\alpha}$, the levels of $PGF_{2\alpha}$ of around 5 ng/ml indicates that 90% of the PGH_2 (which was added at 50 ng/ml), was consumed by enzymatic activity as well as non-reductive isomerization into several COX-metabolites.

3.4 | Interventions with endogenous metabolism of PGH_2

Adding of PGH_2 showed that when hPGDS was inhibited, a significant proportion of PGH_2 was used for biosynthesis of TXA_2, which is measured as TXB_2. The experiments also suggested that PGE_2 was entirely formed non-enzymatically.

It was therefore decided to test if selective interference with utilization of endogenous PGH_2 by in particular TXS and hPGDS would produce reciprocal effects on the biosynthesis of TXA_2 and PGD_2 in the HLMC. First, the inhibition of PGD_2 and the enhancement of TXB_2 by PPCA as well as the increase of PGE_2 and the tendency for increased level of 6-keto-$PGF_{1\alpha}$. was replicated (Figure 5B). Second, adding the TXS inhibitor ozagrel, the opposite response was observed, PGD_2 release increased further, while the release of TXA_2 was abolished (Figure 5B). There were no effects of ozagrel on the level of PGE_2 or 6-keto-$PGF_{1\alpha}$. Third, when both PGD_2 and the TXB_2 were inhibited by combined treatment with ozagrel and PPCA, there was a substantial increase in PGE_2, and also a suggestive increase in 6-keto-$PGF_{1\alpha}$ that did not reach significance due to larger variability in this analyte.

Finally, when the mPGES-1 inhibitor MF-63 was added to the combination of ozagrel and PPCA, the anti-IgE elicited increase in PGE_2 was not prevented. Although in theory the two other PGE synthases[27] might be involved, the experiments with exogenous PGH_2 (Figure 5A) favour the interpretation that PGE_2 in HLMC is formed as a non-enzymatic degradation product of the labile PGH_2 (half-life at 37°C, 5 min).

4 | DISCUSSION

The study used pharmacologic inhibitors and a sensitive and selective mass spectrometry-based method to explore the metabolic effects of inhibiting the hPGDS enzyme that catalyses formation of PGD_2 in four human mast cell models. First, the study confirmed that PGD_2 is by far the most prominent COX product released by human mast cells activated by anti-IgE or ionophore, with other metabolites only being produced in comparatively small amounts. Second, we replicated and extended the previous data in HLMC[13] and demonstrate, consistently across all four human mast cell models, that endogenous biosynthesis of PGD_2 is only catalysed by COX-1. The results in this cellular study are also in agreement

with our previous *in vivo* studies in humans, where treatment with COX-2 inhibitors did not change urinary levels of the PGD_2 metabolites in asthmatics nor healthy controls, whereas non-selective COX inhibitors did.[28,29] It is important to recognise that human mast cells do not show COX-2 protein induction as seen in murine mast cells.[30]

Furthermore, the hypothesis that inhibition of hPGDS might lead to shunting of the intermediate PGH_2 into formation of other prostanoids was confirmed. As commonly discussed in review papers, the concept of PGH_2-shunting into other primary PG products appears intuitive, but would depend on distal enzymes and their biosynthesis activity or capacity. However, when the literature is scrutinized, there are only a handful papers that actually have documented that shunting occurs in different cells, and there is no such publication in human mast cells. Shunting of PGH_2 has thus been observed in response to inhibition of thromboxane synthase during blood clotting, where it mainly led to increased levels of PGE_2 and some increase in PGD_2 and $PGF_{2\alpha}$.[31-33] Also, mPGES-1 deficient murine macrophages produced more TXB_2 and 6-keto-$PGF_{1\alpha}$ than their wild type counter parts[34] although surprisingly the same was not observed during mPGES-1 inhibition in human monocytes.[35] To our knowledge the present study is the first to demonstrate PGH_2 transfer in human mast cells. This was done both by analysis of the profile of endogenous COX products after inhibition of the hPGDS, and by monitoring the transformation of exogenously supplied PGH_2. The mass spectrometry methods used, including chiral chromatography, made it possible to study the involved mechanisms with unprecedented precision as already discussed in the result section.

Accordingly, when hPGDS was inhibited by PPCA, TXB_2 became the major prostanoid followed by increased levels of PGE_2 and PGI_2. The further analysis including experiments with no cells, however, indicates that PGE_2 was formed non-enzymatically, which presumably explains the relatively low levels observed in comparison with the levels of PGD_2 in the intact cell. In contrast, cells were required for the biosynthesis of TXA_2 (measured as TXB_2), and its sibling 12-HHTrE also formed by the TXS, as well as PGI_2 (measured as 6-keto-$PGF_{1\alpha}$). In further support of the transfer of PGH_2 to other pathways, the amounts of PGD_2 was increased when TXS was inhibited.

The question that naturally arises is whether or not this redirected use of the precursor PGH_2 when hPGDS is inhibited introduces an unwanted effect leading to adverse reactions. We believe that this study cannot provide a final answer to that concern. However, the absolute amounts of the other COX products that appeared when hPGDS was inhibited were much lower than the original amounts of PGD_2 synthesised. The only potential issue would be if the formation of TXA_2 increases significantly, because it is also a bronchoconstrictor via the TP receptor.[4] Increased levels of PGE_2 and PGI_2 would if anything be expected to have anti-asthmatic effects such as inhibition of mast cells and other immune competent cells as well as causing bronchodilatation.[36,37] Of interest in this context is our observation that inhibition of the hPGDS did not increase the IgE-dependent release of the potent CysLT bronchoconstrictors.

It is not known why the naïve mast cells generate almost exclusively PGD_2, but factors such as the relative levels of the distal prostanoid synthases in the cell, their substrate affinities and turnover rate, and their proximity to the site of intracellular release of PGH_2 presumably contribute. Capacity of remaining prostanoid producing enzymes determines the outcome after hPGDS inhibition, meaning that PGH_2 becomes available for TXS, PGIS or even non-enzymatic processes, not possible to appreciate unless hPGDS is inhibited. However, in LAD2 cells the relative increase in TXB_2 was low compared to CBMC and HLMC, with a reversed shift in PGE_2:TXB_2 ratio from 1:15 to 6:1 in the CBMC and LAD2 cells respectively, collectively reflecting a lower TXS capacity in LAD2. Obviously, the *in vivo* relevance of the redirection of PGH_2 utilisation we describe in the various human cell models cannot be assessed until safe, selective and bioavailable inhibitors for use in humans are available. From previous animal experiments, there is no clear picture. Although HQL-79 caused a marginal increase in PGE_2 in rat mastocytoma cells,[38] the same drug did not change the amount of PGE_2 or PGF_{2a} in a transgenic mouse model. Likewise, there were no changes in prostanoid synthesis apart from inhibition of PGD_2 in response to HQL-79 in murine ovaries.[39] Beyond allergic inflammation the hPGDS inhibitor TAS-205 has been used in early clinical trials for treating Duchenne muscular dystrophy.[40,41] As a reflection of the treatment excretion of urinary PGD_2 metabolite decreased, while PGE_2 metabolite did not significantly change and the safety profile was deemed favourable, thromboxane was however not monitored. Future *in vivo* studies will be required to demonstrate the biochemical impact and physiological outcomes of hPGDS inhibition in the clinical setting of asthma treatment.

Taken together, this study first demonstrates that biosynthesis of PGD_2 in several different human mast cell models is catalysed by COX-1, and not by COX-2. This confirms one previous report in HLMC[13] and extends the concept by establishing the same initiating pathway in the three other explored human mast cell models. Selective COX-1 inhibition is however not a therapeutic alternative for asthma because of adverse effects including increased bleeding from the gastro-intestinal tract and triggering of asthma attacks in the subpopulation of asthmatics with aspirin-exacerbated respiratory disease (AERD). We next show that selective targeting of hPGDS is an effective means to essentially arrest biosynthesis of PGD_2. The analysis of the consequences of this inhibition of hPGDS for the first time provides evidence that this might lead to redirections of PGH_2 into biosynthesis of other prostanoids. However, the amounts of the other products were relatively low, and it remains unknown if this biochemical *in vitro* effect has consequences *in vivo*. We believe our finding prompts for tests of hPGDS inhibitors in human experimental medicine studies (*eg* the allergen bronchoprovocation setting) to determine if the redirections of PGH_2, we describe have relevance to the *in vivo* situation. Given the potent biological effects of PGD_2 and the prominent role of mast cells in asthma, allergy and other mast cell-driven diseases, we conclude that it appears attractive to focus on hPGDS as one single target, rather than combination therapy against three

different receptors (TP, DP_1 and DP_2/CRTH2). This view is underpinned by the current failure of the selective DP_2 antagonist fevipiprant in severe asthma.[42] If such a development is successful, it would in addition to asthma, and allergy, be beneficial in other mast cell activating disorders including systemic mastocytosis,[43] and in particular when combined with antihistamines and antileukotrienes to block all major mast cell mediators.[37,44]

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

GN, SED, AKJ, JHC, BD, CW and MH contributed to the conception and design of the study. AKJ, JHC, ER, DF and JK contributed to the acquisition of the data and the data analysis. AKJ, JHC, MH, GN and SED participated in the interpretation of data. AK, JHC, GN and SED drafted the manuscript. All authors critically reviewed and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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