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PGD_2 metabolism in plasma: Kinetics and relationship with bioactivity on DP1 and CRTH2 receptors

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ABSTRACT

Prostaglandin (PG)D_2, an important mediator in allergic diseases, is rapidly transformed in plasma to active metabolites that bind and activate two distinct receptors, DP1 and CRTH2. Since the rate of PGD_2 degradation and the bioactivity of the resulting metabolites are still unclear, the aim of our study was to analyze the kinetics and biological effects of PGD_2 metabolites formed in plasma. Eosinophil shape change was taken as a parameter of chemotactic activation mediated by CRTH2 whereas inhibition of platelet aggregation served as a measure of DP1 activity. PGD_2 was degraded in plasma with an apparent half-life of approximately 30 min, accompanied by a loss of potency in inhibiting platelet aggregation as well as inducing eosinophil stimulation. Incubation of PGD_2 in plasma for 120 min caused an increase in the IC_{50} for platelet aggregation by a factor of 6.5 and an increase of the EC_{50} for eosinophil shape change by a factor of 7.2. However, tandem mass spectrometry analysis showed that incubation of PGD_2 in plasma for 120 min resulted in clearance of PGD_2 of more than 92%, which was mirrored by a continuous formation of Δ^{12}-PGD_2 and Δ^{12}-PGJ_2, whereas only small amounts of 15d-PGD_2 and 15d-PGJ_2 were detected. Interestingly, a rapid degradation of PGD_2 was also observed in serum, which was not prevented by pepsin digestion of serum preceding the addition of PGD_2. Therefore, despite extensive non-enzymatic metabolization of PGD_2 in plasma, its biological activity with respect to DP1 and CRTH2 is maintained through the formation of bioactive metabolites.

1. Introduction

Prostaglandin (PG)D_2 is the major arachidonic acid derived cyclooxygenase metabolite produced by mast cells in response to IgE-dependent stimuli and might play a prominent pro-inflammatory role in various diseases such as allergic rhinitis [1], bronchial asthma [2], allergic conjunctivitis [3], and atopic dermatitis [4].

PGD_2 is rapidly metabolized enzymatically to 11-epi-$PGF_{2\alpha}$ or 13,14 dihydro-15-keto-PGD_2 (dk-PGD_2) or non-enzymatically in aqueous solution to PGJ_2 [5]. The apparent half-life of PGD_2 in blood has been reported to be 0.9 min [6], and PGD_2 is

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rapidly metabolized in plasma *in vitro* to PGJ_2, Δ^{12}-PGJ_2, Δ^{12}-PGD_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and 15-deoxy-$\Delta^{12,14}$-PGD_2 (15d-PGD_2), which have been proposed to arise from catalytic conversion of PGJ_2 by albumin [7–9]. Alternatively, PGD_2 can be dehydrated in aqueous solution to PGJ_2, 15d-PGJ_2 and 15d-PGD_2, while formation of Δ^{12}-PGJ_2 from PGJ_2 in any case depends on albumin [10].

Although the relative importance of this non-enzymatic pathway *in vivo* is unknown, there is evidence that J-type prostanoids are synthesized *in vivo*. This is based on observations that Δ^{12}-PGJ_2 is present in human body fluids and PGD_2 appears to be the natural precursor of PGJ_2 and its derivatives, since *in vivo* administration of PGD_2 leads to a large increase in Δ^{12}-PGJ_2 levels in urine [11].

PGD_2 exerts its effects by activating two distinct G-protein-coupled receptors, the DP (also named DP1) receptor and the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2, also named DP2), respectively [12,13]. DP1 is positively linked to adenylyl cylclases via $G_{\alpha s}$ proteins, and CRTH2 activation of $G_{\alpha i}$ proteins stimulates phosphoinositide 3-kinase, mitogen-activated protein kinases and phospholipase C [14]. Moreover, CRTH2 can also couple with G_q, since eosinophil shape change responses to PGD_2 are insensitive to pertussis toxin [15]. While PGD_2 and PGJ_2 bind with comparable affinity to DP1 and CRTH2, dk-PGD_2, Δ^{12}-PGJ_2, Δ^{12}-PGD_2, 15d-PGJ_2 and 15d-PGD_2 have been described to bind preferentially to CRTH2 [16–18]. PGD_2 as well as its metabolites dk-PGD_2, PGJ_2, Δ^{12}-PGD_2, Δ^{12}-PGJ_2, 15d-PGD_2 and 15d-PGJ_2, have been shown to be potent eosinophil activators with respect to chemotaxis, actin polymerization, L-selectin shedding and CD11b up-regulation [13,17–20]. It seems that all these effects are mediated by CRTH2. In contrast, the PGD_2-induced inhibition of platelet aggregation is known to be a consequence of DP1 receptor activation [21]. In previous experiments we have shown that Δ^{12}-PGJ_2 is a highly potent chemoattractant and stimulator of respiratory burst in human eosinophils, with the same efficacy as PGD_2, PGJ_2 or 15d-PGJ_2 [22]. Moreover, we have observed that Δ^{12}-PGJ_2 can induce the release of mature eosinophils from bone marrow and primes eosinophils for activation by other chemoattractants. Therefore, we hypothesized that PGD_2 and/or its metabolites might exert a hormone-like action, being released at sites of allergic inflammation and signaling for the mobilization of eosinophils from the bone marrow into the circulation [22]. Although it might be expected that PGD_2 is metabolized in the blood to limit a systemic response, the rate of PGD_2 degradation and the biological activity of the resulting metabolites is unclear.

The aim of our study hence was (i) to investigate the velocity of PGD_2 metabolism in plasma, (ii) to identify the most relevant metabolites and (iii) to compare the biological activity of the endogenous metabolites with respect to CRTH2 and DP1 activation. To this end, eosinophil shape change was taken as a parameter indicative of chemotactic activation mediated by CRTH2, while DP1 agonistic activity of human plasma incubated with PGD_2 was determined as inhibition of platelet aggregation. Liquid chromatography tandem mass spectrometry (LC/MS/MS) was employed to identify the metabolites formed in plasma.

2. Material and methods

2.1. Materials

All laboratory reagents were from Sigma (Vienna, Austria), unless specified. Dulbecco's modified phosphate buffered saline PBS (with or without Ca^{2+} and Mg^{2+}) was from Invitrogen (Vienna, Austria). Assay buffer was made up from Dulbecco's PBS with 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} supplemented with 0.1% BSA, 10 mM HEPES and 10 mM glucose, pH 7.4). Collagen was obtained from Probe&Go (Endingen, Germany). CellFix and FACS-Flow were from Becton Dickinson (Vienna, Austria). Fixative solution was prepared by diluting Cellfix 1/10 in distilled water and 1/4 in FACS-Flow. PGD_2, its metabolites and ramatroban were from Cayman (Ann Arbor, MI). ZK138357 was kindly provided by Prof. G. Stock (Schering, Berlin, Germany).

2.2. Incubation of PGD_2

In order to investigate the velocity of PGD_2 metabolism, a series of dilutions of the prostanoid was prepared in saline, human albumin (fatty acid free, 1%) or plasma and incubated at 37 °C up to 120 min. After different periods of incubation, concentration–response curves were performed with regard to collagen-induced platelet aggregation, eosinophil shape change and eosinophil chemotaxis. The concentrations of PGD_2 and metabolites in the samples were determined by LC/MS/MS. For these experiments PGD_2 was added to the samples (final concentration 10 μM). Similarly, the kinetics of PGD_2 conversion products, PGJ_2, Δ^{12}-PGJ_2, Δ^{12}-PGD_2, 15d-PGD_2 and 15d-PGJ_2 in plasma were tested by incubating their dilutions for up to 120 min.

The degradation of PGD_2 was also investigated in serum, which, in contrast to plasma, is devoid of platelets. To this end, whole blood samples were allowed to clot in glass vials at room temperature in the absence of an anticoagulant for 1 h and then centrifuged. For the inactivation of potential enzymatic pathways involved in the metabolism of PGD_2, pepsin digestion of serum was performed [23]. Acidified serum (pH 2.0) was incubated with 2% pepsin at 37 °C for 1 h. Control serum was acidified and incubated for 1 h without pepsin. Samples were readjusted to pH 7.0 thereafter, and PGD_2 was added at a concentration of 500 nM. The serum was incubated at 37 °C for 120 min. In additional samples, PGD_2 was added to serum (with or without pepsin) after 120 min incubation. Dilutions of the samples were then tested in the eosinophil shape change assay. Responses to serum alone, PGD_2 incubated in serum for 0 min and PGD_2 incubated in serum for 120 min were compared with and without pepsin digestion.

2.3. Preparation of human leukocytes

Blood was sampled from healthy volunteers according to a protocol approved by the Ethics Committee of the Medical University of Graz and processed as described previously [22]. Mixed peripheral blood leukocytes (eosinophils, neutrophils, basophils, monocytes and lymphocytes) were obtained by dextran sedimentation of citrated whole blood.

Polymorphonuclear leukocytes (eosinophils and neutrophils) were prepared by Histopaque gradients. In some experiments, eosinophils were further purified to near homogeneity by negative magnetic selection using an antibody cocktail from StemCell Technologies (Vancouver, Canada). Resulting populations of eosinophils were typically >97%, with the majority of contaminating cells being neutrophils.

2.4. Leukocyte shape change assay

Eosinophil and neutrophil shape change was assayed as described previously [24,25]. Polymorphonuclear leukocytes were resuspended in assay buffer at 5×10^6 cells/ml. 50 μl aliquots were mixed with 50 μl of agonists (in saline or plasma, incubated for different time periods) and stimulated for 4 min at 37 °C. In order to stop the reaction, samples were transferred to ice and fixed with 250 μl of fixative solution. Samples were immediately analyzed on a FACSCalibur flow cytometer (Becton Dickinson) and eosinophils were monitored according to their autofluorescence in FL-1 and FL-2, whilst neutrophils were identified by their lack of autofluorescence. Shape change responses in eosinophils and neutrophils were quantified as percent of baseline forward scatter.

2.5. Chemotaxis assays

Purified eosinophils were suspended in assay buffer at 2×10^6/ml and 50 μl of the suspension was placed onto the top of a 48-well microBoyden chemotaxis chamber with 5 μm pore-size polycarbonate filter (NeuroProbe Inc., Gaithersburg, MD), with 30 μl of increasing concentrations of agonists in the bottom well of the plate. Baseline migration was determined in wells containing only assay buffer. The plates were incubated at 37 °C in a humidified CO_2 incubator for 1 h, and the membrane carefully removed. Cells that had migrated to the lower chamber were enumerated by flow cytometry counting for 30 s as previously described [15].

2.6. Platelet aggregation

Human platelet-rich plasma and platelet-poor plasma were prepared from citrated whole blood by centrifugation. Platelet aggregation was recorded at 37 °C with constant stirring (1000 rpm), in a four-channel Aggrecorder II aggregometer (KDK Corp., Kyoto, Japan) as described [26]. Platelet aggregation was measured as the increase in light transmission for 5 min, starting with the addition of collagen (1.25–10 μg/ml) as pro-aggregatory stimulus. $CaCl_2$ at a final concentration of 1 mM was added 2 min before collagen. To record inhibition of collagen-induced aggregation, iloprost, or PGD_2 or its metabolites were added 2 min before collagen. In some experiments, the DP1 antagonist ZK138357 or the CRTH2 antagonist ramatroban were added 5 min before PGD_2. Data were expressed as percent of maximum light transmission, with non-stimulated platelet-rich plasma being 0% and platelet-poor plasma 100%.

2.7. Mass spectrometry

Sample analysis was performed by using liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS). The LC–MS/MS system consisted of an API 4000 triple-mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative ESI mode, an Agilent 1100 binary HPLC pump and degasser (Agilent, Böblingen, Germany) and an HTC Pal autosampler (Chromtech, Idstein) fitted with a 100 μl LEAP syringe (Axel Semrau GmbH, Sprockhövel, Germany). A cooling stack was used to store the samples at 4 °C in the autosampler. An inlet valve was used to truncate non relevant signals (10-port, VICI Valco Instruments, Houston, USA). High purity nitrogen for the mass spectrometer was produced by a NGM 22-LC/MS nitrogen generator (cmc Instruments, Eschborn, Germany).

For the chromatographic separation a Synergi Hydro-RP column and precolumn were used (150 mm × 2 mm I.D., 4-μm particle size and 80 Å pore size from Phenomenex, Aschaffenburg, Germany). A linear gradient was employed at a flow rate of 300 μl/min. Mobile phase A was water/formic acid (100:0.0025, v/v, pH 4.0) and mobile phase B was acetonitrile/formic acid (100:0.0025, v/v). Sample solvent was methanol/water (50:50, v/v). Total run time was 18 min and injection volume of samples was 20 μl. Retention times of PGD_2, Δ^{12}-PGD_2, Δ^{12}-PGJ_2, 15d-PGJ_2, 15d-PGD_2 were 9.2 min, 8.9 min, 11.2 min, 13.5 min and 12.1 min, respectively.

All samples were diluted 1:100 with methanol/water (50:50, v/v) containing two deuterated internal standards [$^2H_4$]-PGD_2 and [$^2H_4$]-15d-PGJ_2. 20 μl of the samples were injected into the LC-MS/MS-system and measured against a freshly prepared calibration curve.

The mass spectrometer was operated in the negative ion mode with an electrospray voltage of −3300 V at 450 °C. Multiple reaction monitoring (MRM) was used for quantification. The mass transitions used were *m/z* 351.1 → *m/z* 271.2 for PGD_2 and Δ^{12}-PGD_2, *m/z* 333.1 → *m/z* 232.9 for Δ^{12}-PGJ_2, *m/z* 315.1 → *m/z* 271.1 for 15d-PGJ_2, *m/z* 333.1 → *m/z* 271.1 for 15d-PGD_2, *m/z* 355.1 → *m/z* 275.1 for [$^2H_4$]-PGD_2 and *m/z* 319.2 → *m/z* 275.2 for [$^2H_4$]-15d-PGJ_2.

All quadrupoles were working at unit resolution. Quantitation was performed with Analyst Software V1.4 (Applied Biosystems, Darmstadt, Germany) using the internal standard method (isotope-dilution mass spectrometry). Ratios of analyte peak area and internal standard peak area (*y*-axis) were plotted against concentration (*x*-axis) and calibration curves for each prostaglandin were calculated by least square regression with 1/concentration^2 weighting.

2.8. Statistics

Data are shown as mean ± S.E.M. for *n* observations. IC_{50} and EC_{50} were calculated with Sigma Plot Ligand binding module (SPSS; Chicago; IL, USA). Comparisons of groups of data were performed using two-way Anova, followed by Holm-Sidak method (Sigma Stat; SPSS; Chicago; IL, USA). Probability values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Inhibition of platelet aggregation

To determine the velocity by which PGD_2 is degraded, aliquots of saline, human albumin (1%) in saline and plasma were incubated for 120 min at 37 °C and PGD_2 was added to these aliquots at various time points. Freshly diluted PGD_2 in plasma caused a concentration-dependent inhibition of collagen-induced platelet aggregation with an IC_{50} which was not different from PGD_2 freshly diluted in saline (Table 1). When incubated in plasma, the apparent potency of PGD_2 to inhibit collagen-induced platelet aggregation markedly decreased as compared with the corresponding values obtained after incubation in saline. The results showed that after 60 and 120 min incubation in plasma, the apparent IC_{50} of PGD_2 was increased by a factor of 1.9 and 6.5, respectively (Fig. 1B, Table 1). In contrast, incubation of PGD_2 in saline or human albumin (1%) did not significantly influence the inhibitory potency of PGD_2 in this assay (Fig. 1A and C; Table 1).

In order to determine the receptor involved in the inhibition of collagen-induced platelet aggregation caused by PGD_2 and/or its metabolites formed in plasma, antagonists of the DP1 and CRTH2 receptors were employed. The DP1 receptor antagonist ZK138357 caused concentration-dependent reversal of PGD_2-induced inhibition of platelet aggregation (Fig. 1F). Moreover, even at concentrations up to 100 μM it did not significantly reduce the effect of the prostacyclin receptor (IP) agonist iloprost (data not shown). In experiments using PGD_2 incubated for 120 min in plasma, the apparent potency of ZK138357 was similar to that determined with freshly diluted PGD_2 (Fig. 1F). In contrast, the CRTH2 receptor antagonist ramatroban (1 μM) had no significant effect on PGD_2-induced inhibition of collagen-induced platelet aggregation (data not shown).

The PGD_2 metabolite PGJ_2 likewise inhibited the collagen-induced platelet aggregation, although 10–30-fold higher concentrations were required than for PGD_2 (Fig. 1D). Incubation of PGJ_2 in plasma resulted in a very rapid decrease of its inhibitory potency (Fig. 1D). While the PGD_2 metabolites Δ^{12}-PGJ_2, 15d-PGJ_2, and 15d-PGD_2 had no effect at concentrations up to 1 μM, Δ^{12}-PGD_2 retained an inhibitory effect on collagen-induced platelet aggregation which was comparable to PGJ_2. The inhibitory potency of Δ^{12}-PGD_2 was rapidly decreased by incubation in plasma (Fig. 1E).

3.2. Eosinophil shape change and chemotaxis

Aliquots of saline and plasma were incubated for 120 min at 37 °C, PGD_2 was added to these aliquots at various time points, and shape change responses of human eosinophils were determined thereafter by flow cytometry as measure of CRTH2 activation. PGD_2 diluted in saline or in plasma, in a concentration-dependent manner, induced shape change in eosinophils but not neutrophils (EC_{50} and $CI_{95\%}$ are shown in Table 2). Incubation in plasma for up to 120 min resulted in an apparent rightward shift of the concentration–response curve (Fig. 2A). The EC_{50} was increased 2.7 times at 30 min and 9.6 times at 60 min of incubation in plasma; thereafter no further loss of potency was observed. In contrast, incubation with plasma of the PGD_2 metabolites PGJ_2, Δ^{12}-PGD_2 and Δ^{12}-PGJ_2 as well as 15d-PGD_2, 15d-PGJ_2, resulted in only a small decrease of their potency to induce shape change in human eosinophils (Table 2; Fig. 2B–F).

Table 1 – Effect of PGD_2 on collagen- induced platelet aggregation

Incubated in		0 min	30 min	60 min	90 min	120 min
Collagen-induced platelet aggregation						
PGD_2	Saline	3.7^{-8} (2.0^{-9}–6.8^{-7})	3.6^{-8} (8.5^{-10}–1.5^{-6})	2.7^{-8} (1.2^{-9}–5.9^{-7})	2.0^{-8} (8.8^{-10}–4.4^{-7})	2.2^{-8} (1.5^{-10}–3.2^{-6})
PGD_2	Plasma	1.7^{-8} (3.2^{-9}–9.1^{-8})	3.8^{-8} (3.9^{-9}–3.7^{-7})	3.3^{-8} (2.7^{-10}–4.0^{-6})	2.9^{-7} (1.1^{-9}–7.9^{-5})	1.1^{-7} (9.8^{-9}–1.2^{-6})
PGD_2	Albumin	2.4^{-8} (4.6^{-9}–1.2^{-7})	2.7^{-8} (4.1^{-10}–1.8^{-6})	2.2^{-8} (4.0^{-9}–1.2^{-7})	9.3^{-9} (2.2^{-10}–3.9^{-7})	1.8^{-8} (5.4^{-11}–6.0^{-6})

IC_{50} ($CI_{95\%}$) of PGD_2 incubated in saline, plasma or human albumin (1%) for different time periods, with respect to inhibition of collagen-induced platelet aggregation. n = 4–18.

Table 2 – Effect of PGD_2 and PGD_2 metabolites on eosinophil shape change and chemotaxis

	Incubation in				
	Saline 0 min	Plasma 0 min	Plasma 30 min	Plasma 60 min	Plasma 120 min
Eosinophil shape change					
PGD_2	3.5^{-10} (1.3^{-10}–9.9^{-10})	2.5^{-10} (5.2^{-11}–1.2^{-9})	6.7^{-10} (2.2^{-10}–2.0^{-9})	2.4^{-9} (8.1^{-10}–6.9^{-9})	1.8^{-9} (5.2^{-10}–6.1^{-9})
PGJ_2	1.1^{-8} (5.1^{-9}–2.2^{-8})	6.6^{-9} (3.1^{-9}–1.4^{-8})	1.4^{-8} (6.6^{-9}–3.2^{-8})	1.9^{-8} (1.2^{-8}–3.0^{-8})	2.1^{-8} (1.1^{-8}–3.8^{-8})
Δ^{12}-PGJ_2		2.8^{-8} (1.3^{-8}–6.3^{-8})	1.6^{-8} (5.9^{-9}–4.3^{-8})	2.1^{-8} (8.1^{-9}–5.2^{-8})	3.0^{-8} (1.3^{-8}–6.8^{-8})
Δ^{12}-PGD_2		2.5^{-9} (1.1^{-9}–5.5^{-9})	7.4^{-9} (3.2^{-9}–1.7^{-8})	4.5^{-9} (2.0^{-9}–9.9^{-9})	5.5^{-9} (2.3^{-9}–1.3^{-8})
15d-PGJ_2		1.2^{-8} (1.8^{-9}–7.7^{-8})	1.4^{-8} (3.2^{-9}–6.3^{-8})	1.9^{-8} (3.5^{-9}–1.1^{-7})	2.7^{-8} (6.7^{-9}–1.1^{-7})
15d-PGD_2		2.2^{-9} (4.3^{-10}–1.1^{-8})	2.9^{-9} (9.4^{-10}–8.9^{-9})	2.1^{-9} (7.1^{-10}–6.2^{-9})	2.7^{-9} (7.3^{-10}–1.0^{-8})
Eosinophil chemotaxis					
PGD_2		5.8^{-9} (2.1^{-9}–1.6^{-8})	3.4^{-8} (8.3^{-9}–1.4^{-7})	6.5^{-8} (1.5^{-8}–2.8^{-7})	7.0^{-8} (1.5^{-8}–3.3^{-7})

EC_{50} ($CI_{95\%}$) of PGD_2 and metabolites incubated in saline, or for different time periods in plasma, determined in eosinophil shape change and chemotaxis assays. n = 4–6.

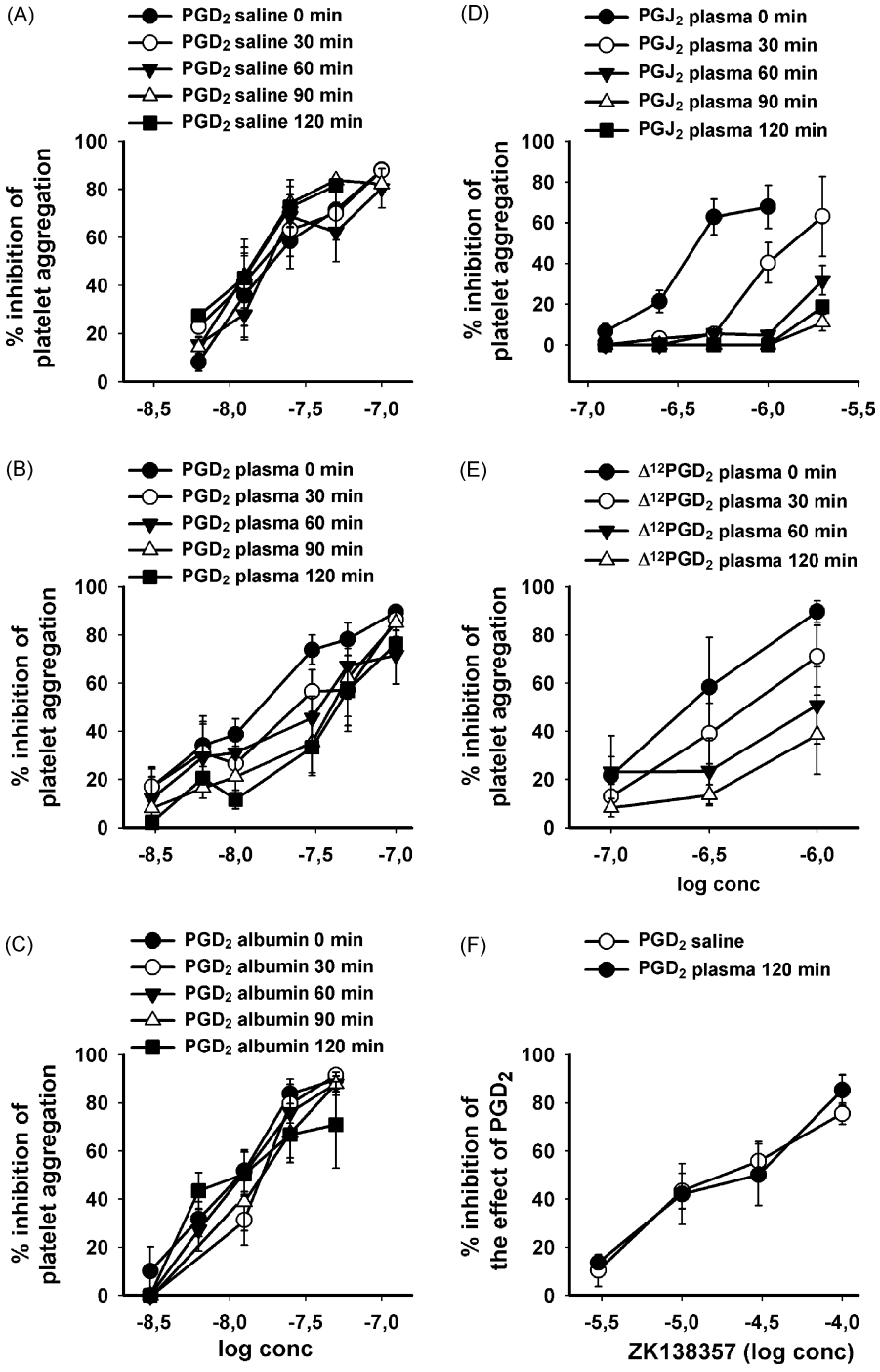

Fig. 1 – Effects of incubation of PGD_2 in saline (A), in plasma (B) or human albumin (C) and of PGJ_2 (D) or Δ^{12}-PGD_2 (E) in plasma for up to 120 min on collagen-induced platelet aggregation. The incubation of PGD_2, and PGJ_2 as well as Δ^{12}-PGD_2 in plasma caused a loss in potency of the prostanoids at inhibiting platelet aggregation. The DP1 receptor antagonist ZK138357 attenuated the inhibitory effect of PGD_2, irrespectively whether it had been pre-incubated for 120 min in plasma or not (saline), with the same potency (F). Values are means ± S.E.M., n = 4–18.

In order to demonstrate that the eosinophil shape change induced by plasma-incubated PGD_2 was still mediated by CRTH2, the CRTH2 antagonist ramatroban was used. Ramatroban, in a concentration-dependent manner, inhibited the effect of PGD_2, as well as that of PGD_2 incubated in plasma for 120 min (Fig. 3A and B). Similarly, ramatroban inhibited the eosinophil shape change responses as induced by the PGD_2 metabolite Δ^{12}-PGJ_2 (Fig. 3C).

In additional experiments, we investigated how the incubation with plasma altered the chemotactic activity of PGD_2 on purified human eosinophils. Comparable to the effects observed in eosinophil shape change, incubation of

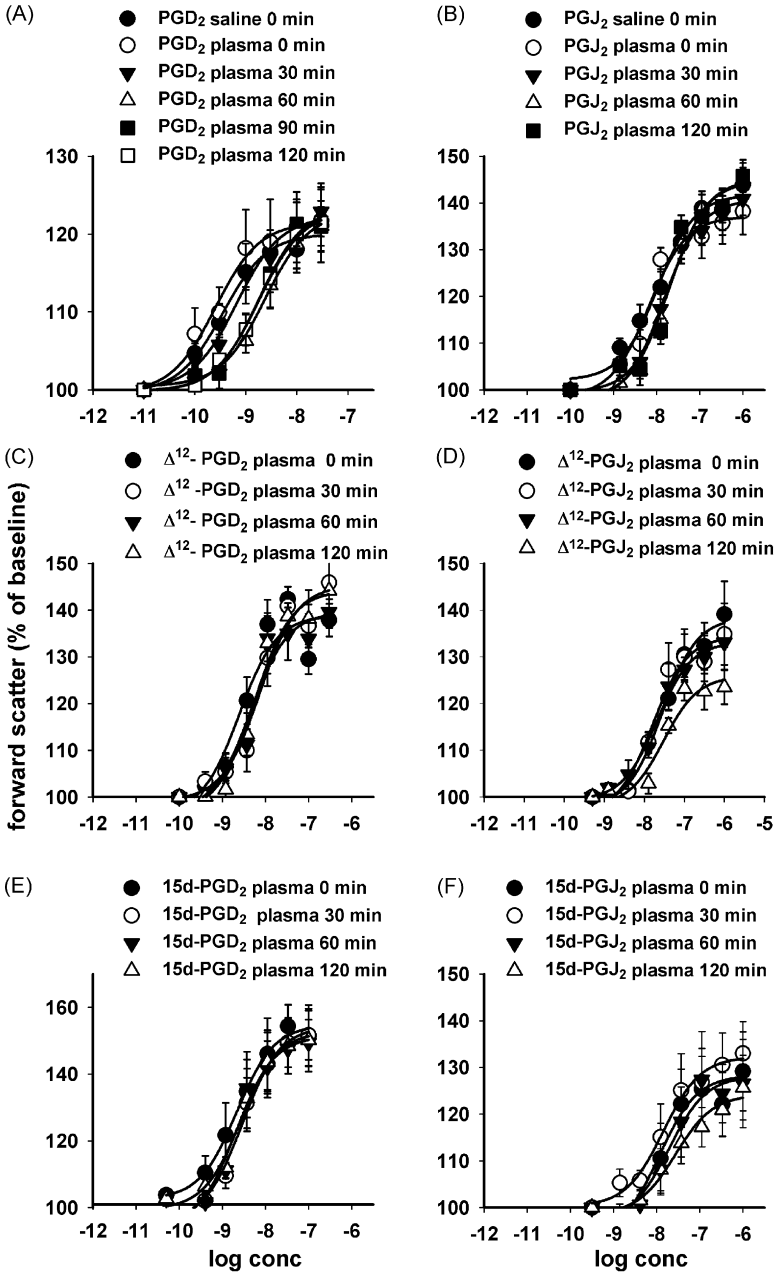

Fig. 2 – Comparison of the potencies of PGD_2 (A), PGJ_2 (B), Δ^{12}-PGD_2 (C), Δ^{12}-PGJ_2 (D), 15d-PGD_2 (E) and 15d-PGJ_2 (F) incubated in saline or plasma for up to 120 min at inducing shape change responses in human eosinophils. Incubation of PGD_2 in plasma caused an apparent rightward shift of the concentration–response curve. Shape change was analyzed by flow cytometry as increase in forward scatter and expressed as percent of baseline forward scatter. Values are mean ± S.E.M., $n = 4–6$.

PGD_2 in plasma for up to 120 min, resulted in an up to 20-fold rightward shift of the concentration–response curve and corresponding increases of EC_{50} values (Fig. 4, Table 2).

3.3. *Formation of PGD_2 metabolites in plasma and by albumin*

In order to determine the metabolites of PGD_2 that are formed in plasma, we employed mass spectrometry analysis. Aliquots of saline, albumin in saline and plasma were incubated for 120 min at 37 °C and PGD_2 was added to these aliquots at various time points. Two-way analysis of variance showed significant differences whether PGD_2 was incubated in saline, albumin or plasma. These differences increased over time. A significant decrease of the PGD_2 concentration was already observed after 30 min incubation in plasma, as compared to the concentration measured when no incubation took place. After 120 min of incubation, a reduction by more than 90% ($n = 3$) was observed (Fig. 5A). Incubation in albumin, caused a smaller, but significant reduction of PGD_2 from 60 min

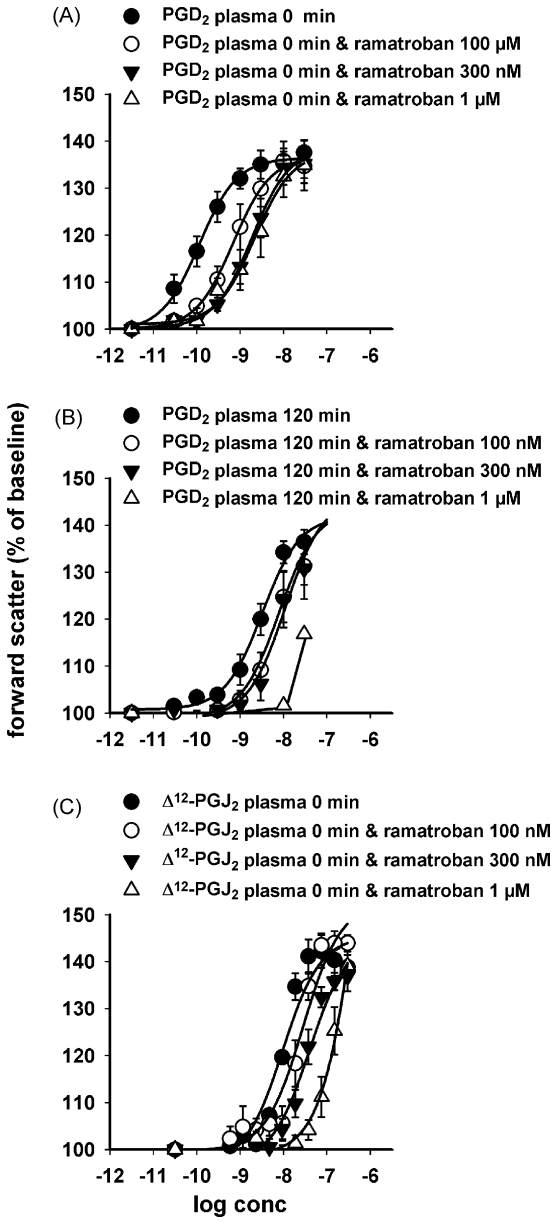

Fig. 3 – Effects of the CRTH2 antagonist ramatroban on eosinophil shape change induced by PGD_2 freshly diluted in plasma (A), by PGD_2 incubated in plasma for 120 min (B) and by Δ^{12}-PGJ_2 freshly diluted in plasma (C). Ramatroban caused an apparent rightward shift of the concentration–response curves. Shape change was analyzed by flow cytometry as increase in forward scatter and expressed as percent of baseline forward scatter. Values are mean ± S.E.M., n = 4–10.

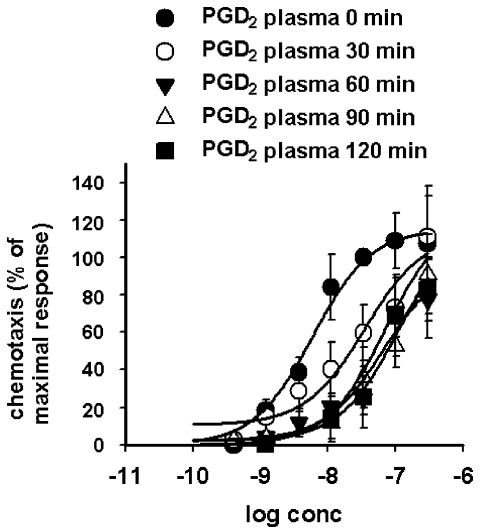

Fig. 4 – Comparison of the potencies of PGD_2 incubated in plasma for up to 120 min on the chemotactic response of human eosinophils. Incubation of PGD2 in plasma caused an apparent rightward shift of the dose response curve. Eosinophils that had migrated to the lower wells of the microBoyden chemotaxis chamber were enumerated by flow cytometry and data were expressed as percent of the maximal response to PGD_2 (usually 30 nM). Values are mean ± S.E.M., n = 7.

onwards, and after 120 min of incubation an approximately 40% loss of PGD_2 was measured. In contrast, no decrease of the PGD_2 concentration was found, when incubation took place in saline. The main metabolites that were formed from PGD_2 in plasma during the 120 min incubation were Δ^{12}-PGD_2 and Δ^{12}-PGJ_2 (21.9 ± 3.2% and 24.1 ± 3.6%, respectively, of the PGD_2 concentration measured in plasma samples without incubation; n = 3; Fig. 5B and C). Only a small portion of PGD_2 was metabolized to 15d-PGD_2 and 15d-PGJ_2 (5.4 ± 0.1% and 4.9 ± 1.3%, respectively; n = 3). Significant effects of time and treatment were found for the formation of metabolites of PGD_2 ($P < 0.05$). Incubation of PGD_2 in albumin also caused a smaller but significant increase of Δ^{12}-PGD_2 (Fig. 5B). No significant formation of Δ^{12}-PGJ_2, 15d-PGD_2 or 15d-PGJ_2 was detected when PGD_2 was incubated in albumin (Fig. 5C–E).

3.4. *Inactivation of serum enzymes*

Since the metabolism of PGD_2 incubated in plasma was more pronounced than when incubated in albumin, we investigated whether enzymes in plasma may contribute to this effect. Pepsin digestion was performed in serum instead of plasma, since a very strong shape change response in human eosinophils was already observed with plasma alone that had been subjected to pepsin digestion. Therefore, serum was acidified to pH = 2, incubated with 2% pepsin at 37 °C for 1 h and then neutralized to pH = 7.0. Control serum was acidified and incubated in the absence of pepsin. Thereafter, PGD_2 (500 nM) or its vehicle was incubated in pepsin-digested serum or control serum for 120 min, or PGD_2 was added to incubated pepsin digested serum or control serum just before the stimulation of eosinophils. Incubation of PGD_2 in control serum for 120 min caused a rightward shift of the PGD_2 concentration–response curve in eosinophil shape change (Fig. 6A), while control serum alone showed only a small effect on shape change. Pepsin digestion did not prevent the rightward shift of the PGD_2 concentration–response curve in eosinophil shape change after incubation of PGD_2 in serum

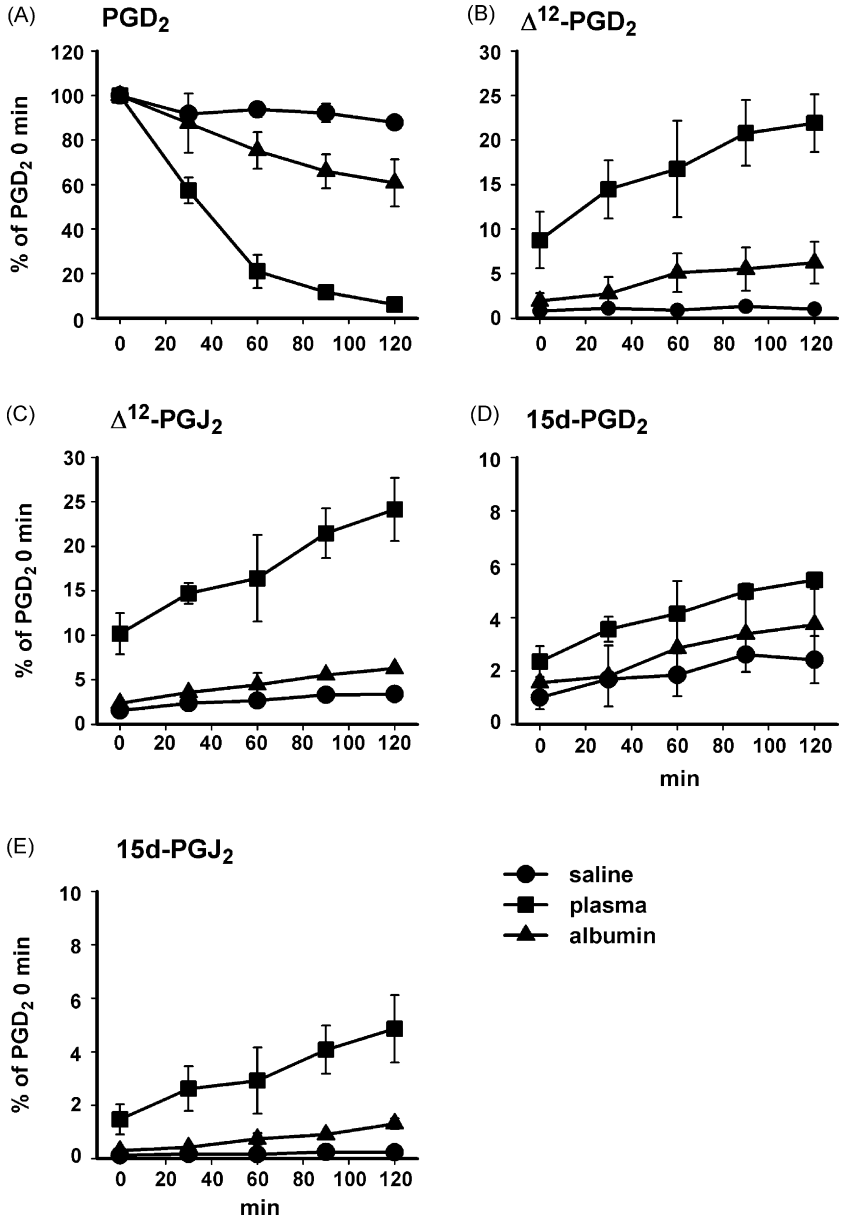

Fig. 5 – Formation of PGD_2 metabolites in saline, plasma or human albumin as measured by mass spectrometry. A significant decrease in the concentrations of PGD_2 was obtained after incubation in plasma or, to a lesser degree, albumin (A). Increased concentrations of Δ^{12}-PGD_2 were found when PGD_2 was incubated either in plasma or albumin, however, at higher concentrations when incubated in plasma (B). Concentrations of Δ^{12}-PGJ_2, 15d-PGD_2 and 15d-PGJ_2 (C–E) were only increased when PGD_2 was incubated in plasma. Data were expressed as percent of the PGD_2 concentration measured in samples without incubation. Values are mean ± S.E.M., $n = 3$.

(Fig. 6B). Therefore, it appears that serum enzymes play only a little role in catalyzing the degradation of PGD_2 and its conversion to its metabolites.

4. Discussion

In the present study we have analyzed the kinetics and biological effects of PGD_2 metabolites formed endogenously in plasma. Our findings demonstrate that PGD_2 is degraded in plasma with an apparent half-life of approximately 30 min, which is accompanied by a loss of potency in inhibiting collagen-induced platelet aggregation and in eosinophil stimulation. However, the degradation of PGD_2 is counterbalanced by the formation of metabolites, which are biologically active and more stable than the parent compound.

The effect of PGD_2 on platelet aggregation has been described to be mediated by the DP1 receptor [21]. The DP1 receptor antagonist ZK138357, while having no effect on iloprost-induced inhibition of platelet aggregation, concentration-dependently counteracted the effect of PGD_2 as well as that of PGD_2 incubated in plasma for 120 min. This provides

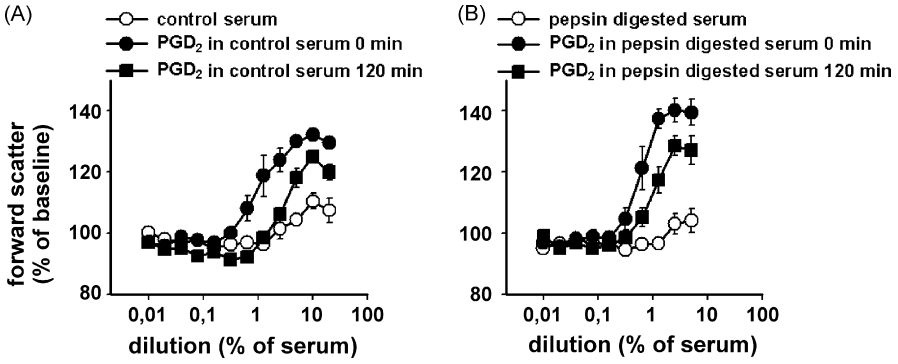

Fig. 6 – Pepsin digestion of serum does not eliminate PGD_2 metabolism. Serum was prepared from human blood. Pepsin digested serum was prepared by addition of 2% pepsin to acidified serum (pH = 2.0) and incubation at 37 °C for 1 h followed by neutralization to pH = 7.0. Control serum was incubated at pH = 2.0 for 1 h without pepsin and thereafter neutralized. In (A), PGD_2 (500 nM) was added to control serum. After incubation at 37 °C for 0 or 120 min, aliquots were added to eosinophil shape change assays. As control, aliquots of control serum (incubated at 37 °C for 120 min) in the absence of PGD_2 were added to eosinophil shape change assays. In (B), PGD_2 (500 nM) was added to pepsin digested serum. After incubation at 37 °C for 0 or 120 min, aliquots were added to eosinophil shape change assays. As control, aliquots of pepsin digested serum (incubated at 37 °C for 120 min) in the absence of PGD_2 were added to eosinophil shape change assays. Shape change was analyzed by flow cytometry as increase in forward scatter and responses were expressed as percent of baseline. Values are mean ± S.E.M., $n = 5$.

evidence that after incubation of PGD_2 in plasma, the inhibitory effect of the metabolites is still accomplished via activation of DP1 receptors. In contrast, the CRTH2 receptor antagonist ramatroban did not alter the effect of PGD_2 on platelet aggregation, further substantiating the role of the DP1 receptor alone in this response. After 120 min of incubation of PGD_2 in plasma, the IC_{50} of PGD_2-induced inhibition of platelet aggregation was increased by a factor of 6.5, suggesting degradation of PGD_2. However, mass spectrometry analysis showed that incubation in plasma for 120 min caused a clearance of PGD_2 in the samples by approximately 92%, which was more pronounced than had been expected from the inhibition of platelet aggregation. Therefore, we speculated that metabolites of PGD_2, which are formed in plasma, might also be capable of inhibiting the aggregation of platelets via DP1 receptor activation.

It is known that PGD_2 undergoes spontaneous dehydration in aqueous solutions to form PGJ_2 [5], which is then further transformed to Δ^{12}-PGJ_2 and 15d-PGJ_2 in the presence of albumin [7,8]. A conversion of PGD_2 in aqueous solution to 15d-PGJ_2 and 15d-PGD_2, in the absence of albumin, has also been described [10]. Mass spectrometry analysis provided evidence that the main metabolites formed from PGD_2 in plasma after incubation for 120 min were Δ^{12}-PGD_2 and Δ^{12}-PGJ_2 with only minor amounts of 15d-PGJ_2 and 15d-PGD_2 being formed. It has been shown that PGJ_2 is formed in plasma [8] and that PGJ_2 is an intermediate product that is converted to Δ^{12}-PGJ_2 and 15d-PGJ_2 in the presence of albumin [10]. Of the PGD_2 metabolites, only PGJ_2 and Δ^{12}-PGD_2 showed inhibitory effects on collagen-induced platelet aggregation, however the potency was 30 and 13 times lower than that of PGD_2, respectively. While PGJ_2 is known to bind and activate the DP1 as well as the CRTH2 receptor [16,18,19,22] and Δ^{12}-PGD_2 has been found to be a potent CRTH2 agonist [17], this is the first report that Δ^{12}-PGD_2 can activate the DP1 receptor. Incubation of PGJ_2 and Δ^{12}-PGD_2 in plasma caused a rapid loss of platelet aggregation inhibiting bioactivity, with more than 10-fold rightward shifts of the concentration–response curves after 120 min. This suggests that PGJ_2 and Δ^{12}-PGD_2 are further degraded in plasma to metabolites devoid of DP1 receptor activity, presumably Δ^{12}-PGJ_2. Therefore, it appears that PGJ_2 as well as Δ^{12}-PGD_2 are being generated continuously and compensate for the degradation of PGD_2 in plasma as to the biological activity.

The possible alterations of the CRTH2 activity of PGD_2 following plasma incubation were investigated in the eosinophil shape change assay. Stimulation of eosinophils by chemoattractants, including PGD_2, results in changes in the cell shape, which is reflected by increases of light scattering in flow cytometry [22]. PGD_2 caused a concentration-dependent shape change in eosinophils, but not neutrophils, and this was prevented by the CRTH2 antagonist ramatroban. Incubation of PGD_2 in plasma caused a rightward shift of the concentration–response curve, which amounted to a 7-fold increase in EC_{50} values. Interestingly, the maximum shift was observed after 60 min already and no further loss of potency was observed thereafter. When the chemotactic response of eosinophils was determined using the microBoyden chemotaxis chamber, we also found a similar loss of potency for PGD_2 after incubation in plasma for up to 120 min. Since less than 10% of PGD_2, which had been originally added, could be found in plasma after 120 min, it can be assumed that the metabolites formed in plasma accounted for the activation of eosinophils. The CRTH2 antagonist ramatroban concentration-dependently inhibited the shape change of eosinophils induced by plasma-incubated PGD_2, confirming that this effect was likewise mediated by CRTH2. Our results show that several PGD_2 metabolites caused eosinophil shape change with different potencies: 15d-PGD_2 = Δ^{12}-PGD_2 > PGJ_2 > 15d-PGJ_2 = Δ^{12}-PGJ_2. Incubation of these metabolites in plasma did not produce appreciable loss in their respective potencies,

suggesting that (i) either the metabolites are stable (ii) or they are further metabolized to bioactive compounds as assumed for PGJ_2 and Δ^{12}-PGD_2.

Interestingly, incubation of PGD_2 in saline or human albumin did not cause a loss of potency in inhibiting platelet aggregation. Mass spectrometry analysis showed that about 40% of PGD_2 disappeared after incubation in albumin for 120 min, and a negligible loss was found after incubation in saline. Incubation in albumin caused significant increase of Δ^{12}-PGD_2, but the formation of other metabolites was not significantly elevated. The finding that the effect of PGD_2 on platelet aggregation was not attenuated by incubation in albumin, despite the loss of PGD_2 as determined by mass spectrometry, may be due to the continuous formation of the intermediate products PGJ_2 and Δ^{12}-PGD_2 as described above. This demonstrates that albumin is less efficient than plasma in the degradation of PGD_2, and constituents other than albumin are also involved in catalyzing the conversion of PGD_2 to Δ^{12}-PGJ_2. These results seem to contradict previous reports which demonstrated profound albumin-mediated metabolism of PGD_2 [8,10]. However, in our experimental setup, the incubation time was limited to 120 min, whereas previous findings were based on incubation times of 24 h. It seems, therefore, that the degradation of PGD_2 in plasma in the first 120 min is more pronounced than by incubation in albumin or the dehydration observed in aqueous solution. In order to exclude an enzymatic step in this accelerated degradation process, we used pepsin digestion to inactivate the enzymes in serum [23]. However, no evidence for a major role of serum enzymes was obtained, since pepsin digestion did not affect the rightward shift of the concentration–response curve in eosinophil shape change which occurred when PGD_2 was incubated in serum for 120 min. This corroborates previous findings showing that dehydration/isomerization of PGD_2 is unrelated to serum enzymes [8] [7]. Moreover, the involvement of cellular elements in plasma, such as platelets, seems unlikely, since the degradation of PGD_2 could also be observed in serum.

Taken together, our results show that PGD_2 is extensively metabolized in human plasma, but its biological activity with respect to DP1 and CRTH2 is maintained through the formation of bioactive metabolites, such as PGJ_2 and Δ^{12}-PGD_2 at the DP1 receptor, or Δ^{12}-PGD_2 and Δ^{12}-PGJ_2 at the CRTH2 receptor. The resulting metabolites, Δ^{12}-PGJ_2 in particular, appear to be stable in plasma for hours and can be found also in the urine in humans [11]. The *in vivo* half-life of PGD_2 in the circulation was described to be 0.9 min [6]. However, that study determined the clearance of PGD_2 from blood after intravenous bolus injection, and the obtained data hence predominantly reflect the redistribution of PGD_2 into tissues. Therefore, the kinetics of endogenous PGD_2 metabolism *in vivo* are still unclear. Our *in vitro* results disregard the circulation of blood and the metabolism of PGD_2 that may take place in liver or lung. Even so, dk-PGD_2 and 9α,11β-PGF_2, metabolites of PGD_2 formed enzymatically *in vivo* [27], have also been found to be potent CRTH2 agonists [20]. Therefore, our data further support the hypothesis that PGD_2 and its metabolites might play important roles in the circulation under pathophysiological conditions, including inhibition of platelet aggregation in vascular disease or mobilization from bone marrow and priming of eosinophils in allergic responses.

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REFERENCES

[1] Naclerio RM. The pathophysiology of allergic rhinitis: impact of therapeutic intervention. J Allergy Clin Immunol 1988;82:927–34.
[2] Murray JJ, Tonnel AB, Brash AR, Roberts II LJ, Gosset P, Workman R, et al. Release of prostaglandin D2 into human airways during acute antigen challenge. N Engl J Med 1986;315:800–4.
[3] Proud D, Sweet J, Stein P, Settipane RA, Kagey-Sobotka A, Friedlaender MH, et al. Inflammatory mediator release on conjunctival provocation of allergic subjects with allergen. J Allergy Clin Immunol 1990;85:896–905.
[4] Charlesworth EN, Kagey-Sobotka A, Schleimer RP, Norman PS, Lichtenstein LM. Prednisone inhibits the appearance of inflammatory mediators and the influx of eosinophils and basophils associated with the cutaneous late-phase response to allergen. J Immunol 1991;146:671–6.
[5] Fukushima M. Prostaglandin J2—anti-tumour and anti-viral activities and the mechanisms involved. Eicosanoids 1990;3:189–99.
[6] Suzuki F, Hayashi H, Hayaishi O. Transport of prostaglandin D2 into brain. Brain Res 1986;385:321–8.
[7] Kikawa Y, Narumiya S, Fukushima M, Wakatsuka H, Hayaishi O. 9-Deoxy-delta 9, delta 12-13,14-dihydroprostaglandin D2, a metabolite of prostaglandin D2 formed in human plasma. Proc Natl Acad Sci USA 1984;81:1317–21.
[8] Fitzpatrick FA, Wynalda MA. Albumin-catalyzed metabolism of prostaglandin D2. Identification of products formed in vitro. J Biol Chem 1983;258:11713–8.
[9] Atsmon J, Freeman ML, Meredith MJ, Sweetman BJ, Roberts II LJ. Conjugation of 9-deoxy-delta 9,delta 12(E)-prostaglandin D2 with intracellular glutathione and enhancement of its antiproliferative activity by glutathione depletion. Cancer Res 1990;50:1879–85.
[10] Shibata T, Kondo M, Osawa T, Shibata N, Kobayashi M, Uchida K. 15-Deoxy-delta 12,14-prostaglandin J2. A prostaglandin D2 metabolite generated during inflammatory processes. J Biol Chem 2002;277:10459–66.
[11] Hirata Y, Hayashi H, Ito S, Kikawa Y, Ishibashi M, Sudo M, et al. Occurrence of 9-deoxy-delta 9,delta 12-13, 14-dihydroprostaglandin D2 in human urine. J Biol Chem 1988;263:16619–25.
[12] Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. J Biol Chem 1995;270:18910–6.
[13] Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med 2001;193:255–61.
[14] Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther 2004;103:147–66.

[15] Stubbs VE, Schratl P, Hartnell A, Williams TJ, Peskar BA, Heinemann A, et al. Indomethacin causes prostaglandin D(2)-like and eotaxin-like selective responses in eosinophils and basophils. J Biol Chem 2002;277: 26012–20.
[16] Sawyer N, Cauchon E, Chateauneuf A, Cruz RP, Nicholson DW, Metters KM, et al. Molecular pharmacology of the human prostaglandin D2 receptor, CRTH2. Br J Pharmacol 2002;137:1163–72.
[17] Gazi L, Gyles S, Rose J, Lees S, Allan C, Xue L, et al. Delta12-prostaglandin D2 is a potent and selective CRTH2 receptor agonist and causes activation of human eosinophils and Th2 lymphocytes. Prostaglandins Other Lipid Mediat 2005;75:153–67.
[18] Monneret G, Li H, Vasilescu J, Rokach J, Powell WS. 15-Deoxy-delta 12,14-prostaglandins D2 and J2 are potent activators of human eosinophils. J Immunol 2002;168: 3563–9.
[19] Monneret G, Gravel S, Diamond M, Rokach J, Powell WS. Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. Blood 2001;98:1942–8.
[20] Sandig H, Andrew D, Barnes AA, Sabroe I, Pease J. 9alpha,11beta-PGF2 and its stereoisomer PGF2alpha are novel agonists of the chemoattractant receptor, CRTH2. FEBS Lett 2006;580:373–9.
[21] Giles H, Leff P, Bolofo ML, Kelly MG, Robertson AD. The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. Br J Pharmacol 1989;96:291–300.
[22] Heinemann A, Schuligoi R, Sabroe I, Hartnell A, Peskar BA. Delta12-prostaglandin J2, a plasma metabolite of prostaglandin D2, causes eosinophil mobilization from the bone marrow and primes eosinophils for chemotaxis. J Immunol 2003;170:4752–8.
[23] Polet H, Levine L. Metabolism of prostaglandins E, A, and C in serum. J Biol Chem 1975;250:351–7.
[24] Heinemann A, Hartnell A, Stubbs VE, Murakami K, Soler D, LaRosa G, et al. Basophil responses to chemokines are regulated by both sequential and cooperative receptor signaling. J Immunol 2000;165:7224–33.
[25] Hartnell A, Heinemann A, Conroy DM, Wait R, Sturm GJ, Caversaccio M, et al. Identification of selective basophil chemoattractants in human nasal polyps as insulin-like growth factor-1 and insulin-like growth factor-2. J Immunol 2004;173:6448–57.
[26] Bohm E, Sturm GJ, Weiglhofer I, Sandig H, Shichijo M, McNamee A, et al. 11-Dehydro-thromboxane B2, a stable thromboxane metabolite, is a full agonist of chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2) in human eosinophils and basophils. J Biol Chem 2004;279:7663–70.
[27] Liston TE, Oates JA, Roberts II LJ. Prostaglandin D2 is metabolized in humans to 9 alpha,11 beta-prostaglandin F2, a novel biologically active prostaglandin. Adv Prostaglandin Thromboxane Leukot Res 1985;15:365–7.