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Release of prostaglandin D_2 by murine mast cells: importance of metabolite formation for antiproliferative activity

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PROSTAGLANDIN (PG) D_2, PGJ_2 and Δ^{12}-PGJ_2 are antiproliferative eicosanoids. We investigated the production of PGD_2 by murine bone marrow-derived mast cells (BMMC) taking into consideration metabolism of PGD_2 to PGD_2 and Δ^{12}-PGJ_2. PG-metabolites were quantified by high performance liquid chromatography (HPLC) combined with radioimmunoassay (RIA). Stimulated with calcium ionophore A23187 BMMC released eight-fold more PGJ_2 and Δ^{12}-PGJ_2 than PGD_2. Conversion of endogenously produced PGD_2 to PGJ_2 and Δ^{12}-PGJ_2 proceeded rapidly in contrast to metabolism of exogenously added PGD_2. The antiproliferative potency of these prostaglandins is demonstrated *in vitro*. We conclude that determination of PGD_2 production by mast cells must take into consideration rapid conversion to active derivatives, which may play a significant role in growth regulation.

Key words: Prostaglandin D_2, Prostaglandin J_2, Δ^{12}-Prostaglandin J_2, Cyclopentenone, Anti-proliferative activity, Mast cell

Introduction

Prostaglandin (PG) D_2 and its metabolites PGJ_2 and Δ^{12}-PGJ_2 are potent antiproliferative eicosanoids.[1] The concentrations of PGD_2, PGJ_2 and Δ^{12}-PGJ_2 required for regulation of cell proliferation *in vitro* are in the micromolar range.[1] The high molar activity suggests that these lipid mediators may play a role in the regulation of cell proliferation *in vivo* and that, on the other hand, this principle may be used in anti-tumour therapy. In fact, the related chemically stabilized prostanoid Δ^7-PGA_1 has been proposed for clinical studies.[2] PGD_2 may also represent a component in natural anti-neoplastic defence. Mast cells are regarded as major sources of endogenous PGD_2 production. Murine bone marrow-derived mast cells (BMMC) have been reported to produce 15–120 pmol PGD_2 per million cells during 10–30 min of incubation following different kinds of stimulation.[3,4] However, these apparently low production rates of PGD_2 are unlikely to exert major biological effects *in vivo*.

We hypothesized that the reported PGD_2 production rates underestimate biosynthesis of this mediator, because PGD_2 can be converted to other active metabolites including antiproliferative PGJ_2 and Δ^{12}-PGJ_2. These cyclopentenone prostanoids have been shown to occur in aqueous media, blood plasma and urine.[1]

In the present study PGD_2 and its metabolites were detected and quantified in supernatants from murine mast cells by combined use of high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) analysis. The antiproliferative potencies of PGD_2 and its derivatives PGJ_2 and Δ^{12}-PGJ_2 were evaluated testing the effect of these PGs on [^{3}H]-thymidine incorporation into human myelocytic (HL-60), monocytoid (THP-1, U937) and lymphoid (Burkitt, Raji) cell lines and on MTT-conversion by murine bone marrow-derived mast cells (BMMC) and IL-3-dependent and autonomous mast cell lines.

Materials and Methods

Chemicals

Unlabelled PGD_2, PGJ_2, Δ^{12}-PGJ_2, LTC_4, LTD_4 and LTE_4 were from Paesel & Lorei, Frankfurt, Germany, 5,6,8,9,11,12,14,15-[^{3}H]-PGD_2 (3,7 MBq/ml), 14,15-[^{3}H]-LTC_4, -LTD_4, -LTE_4 (925 kBq/ml), [1-^{14}C]-arachidonic acid (AA) (1,85 MB/qml), PGD_2-RIA-kit, and [methyl-^{3}H]-thymidine ([^{3}H]-T) (37 MBq/ml) were from Amersham-Buchler, Braunschweig, Germany. Calcium-ionophore A23187, indomethacin, 4-hydroxy-2,2,6,6,-tetrametylpiperidine-l-oxyl (HTMP) and gelatine from porcine skin (300 bloom) were from Sigma, St Louis, MO, USA. 3-(4,5-dimethyl-2- thiazolyl)-

2,5-diphenyl-2H-tetrazolium-bromid (MTT) was from Serva, Heidelberg, Germany. Fetal calf serum (FCS) and RPMI 1640 were from Biochrom, Berlin, Germany. The other chemicals used were purchased from Merck, Darmstadt, Germany and were at least of analytical grade.

Cells and cell lines

Primary polyclonal BMMC were generated in cultures of murine (BALB/c) bone marrow cells supplemented with pokeweed mitogen-activated spleen cell-conditioned medium (SCM) as previously described.[5] Briefly, murine bone marrow cells (1×10^5/ml) were grown in RPMI-1640 medium supplemented with 20% FCS, 20% SCM, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin and 10^{-4} M α-thioglycerol. When propagating non-adherent bone marrow cells in this culture medium with weekly refeeding, homogenous populations of mast cells could be obtained within a period of 3 to 8 weeks. Mast cells used for analysis of eicosanoid production were 21–55 days *ex vivo* (33 ± 10 days; mean ± standard deviation (SD)). IL-3-dependent long-term cultured mast cell lines were derived from BMMC.[5] A growth factor independent malignant autonomous mast cell line was derived from an IL-3-dependent long term cultured mast cell line.[6]

A human myeloid leukaemic cell line (HL-60)[7] was cultured in RPMI 1640 containing 15% FCS and 100 U/ml penicillin-streptomycin. Human monocytic (THP-1)[8] and histiocytic (U937)[9] cell lines were cultured in RPMI 1640 with 10% FCS and 100 U/ml penicillin–streptomycin. Human Burkitt[10] and Raji[11] lymphoma cell lines were cultured in RPMI 1640 containing 10% FCS and 100 U/ml penicillin–streptomycin. HL-60-, THP-1-, U937-, Burkitt and Raji lymphoma cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured at 37°C under humidified atmosphere with 5% (mast cells) or 10% CO_2.

Determination of prostaglandin and leukotriene production

For analysis of radioactive AA-metabolites 5×10^7 of BMMC were prelabelled with [^{14}C]-AA (18.5 kBq/ml) for 48 h. Cells were washed twice and resuspended in PBS (10^7 cells/ml) containing gelatine (0.5 mg/ml) or in culture medium (RPMI 1640 with 20% FCS). After a 5-min period of preincubation, cells were incubated with or without calcium ionophore A23187 (0.2 μM) at 37°C. Incubation was stopped by centrifugation (10 min at 4°C and 600 × **g**). For deproteinization supernatant and pellet were seperated and added to 8 volumes of 90% aqueous methanol containing 0.5 mM EDTA and 1 mM HTMP, pH 7.4. HTMP was used for stabilization of lipid mediators. The suspensions were stored at –40°C for at least 12 h and then centrifuged (20 min at – 10°C and 9000 × **g**). Supernatants were evaporated to dryness in a centrifuge under low pressure (Speed Vac, Savant, Farmingdale, NY, USA) and redissolved in 30% aqueous methanol containing trace amounts of unlabelled PGD_2, PGJ_2 and Δ^{12}-PGJ_2.

[^{14}C]-AA-metabolites were seperated by HPLC performed on a C18 Hypersil column (4.6 × 250 mm, 5 μm particles; Shandon, Runkorn, UK) with a C18 precolumn (Waters, Milford, MA, USA). The mobile phase consisted of methanol, water, acetic acid (65:35:0.1 by volume), pH 4.0 adjusted with ammonium hydroxide. The flow rate was 0.4 ml/min. After a 45 min period the methanol concentration in the solvent was gradually increased to 100% Metabolites were detected by liquid scintillation counting (HPLC radioactivity monitor LB 507 A; Berthold, Wildbad, Germany). [^{14}C]-AA-metabolites (PGD_2, PGJ_2 and Δ^{12}-PGJ_2) were identified by comigration with unlabelled standards as detected at 220 nm (Spectrophotometer, Waters, Milford, MA, USA).

Analysis of prostaglandin and leukotriene production by unlabelled mast cells was performed by sequential use of HPLC and RIA. BMMC were washed twice and resuspended in PBS (0.5 mg/ml gelatine) at a concentration of 10^6 cells/ml. Incubation and deproteinization were performed as described above. Samples were redissolved in 30% aqueous methanol containing trace amounts of [^{3}H]-labelled PGD_2 or LTC_4, LTD_4 and LTE_4 standards.

The mobile phase of HPLC for routine prostaglandin analysis consisted of methanol, water, acetic acid (65:35:0.1 by volume), pH 4.0 adjusted with ammonium hydroxide (flow rate: 0.4 ml/min). For confirmation of the identity of endogenously produced PGD_2 metabolites, additional solvent systems were used: acetonitrile, water, acetic acid (40:60:0.1 by volume), flow rate: 0.4 ml/min and acetonitrile, water and acetic acid (50:50:0.1 by volume), flow rate: 0.5 ml/min. The mobile phase for routine leukotriene analysis consisted of methanol, water, acetic acid (65:35:0.1 by volume), pH 5.6 adjusted with ammonium hydroxide (flow rate: 1.0 ml/min). HPLC-fractions were collected in 0.5-min intervals. An aliquot of these fractions was counted for tritium radioactivity to determine retention time of [^{3}H]-standards. Another aliquot was stored at – 20°C under argon for further analysis by RIA.

The commercial [^{3}H]-PGD_2-RIA (Amersham Buchler, Braunschweig, Germany) of HPLC-fractions was performed as described by the manufacturer. We determined cross-reactivies of PGJ_2- and Δ^{12}-PGJ_2 to be 7% and 24%, respectively. The [^{3}H]-cysteinyl leukotriene-RIA was performed as described earlier.[12]

Determination of metabolism of [^{3}H]-PGD_2

Metabolism of [^{3}H]-PGD_2 to [^{3}H]-PGJ_2 and [^{3}H]-Δ^{12}-PGJ_2 was determined by HPLC following incubation of [^{3}H]-PGD_2 (3.7 kBq/ml) for 0 min to 24 h in phosphate buffered saline (PBS) or in culture medium. Controls in PBS were performed with and without BMMC (10^6 cells/ml) and with and without gelatine (0.5 mg/ml). Preparation of samples for HPLC was performed as described for analysis of endogenous PG-production including the deproteinization procedure. Retention times of [^{3}H]-PGD_2 and its metabolites were established as described for analysis of [^{14}C]-AA metabolites.

Proliferation assays

Cells were incubated for 24 h with or without PGD_2, PGJ_2 and Δ^{12}-PGJ_2 at concentrations of 10 nM to 0.1 mM. [^{3}H]-T uptake into cells was used as a parameter of DNA-replication and cell proliferation. After 24 h of incubation in the presence of [^{3}H]-T in microtitre plates (37 kBq/well) cells were collected on glassfibre filters by a cell harvester (Canberra-Packard, Dreieich, Germany) and radioactivity associated with the washed and dried filters was quantified with a Matrix 96 counter (Canberra-Packard, Dreieich, Germany) as β-counts. Alternatively, a colorimetric proliferation assay, the MTT-test, was used as described.[5] Briefly, cultured cells in microtitre plates were incubated for 4 h with MTT. Incubation was stopped with acidified isopropanol. Optical density (OD) was detected in a ELISA-reader (SLT, Salzburg, Austria) at a test wave length of 550 nm and a reference wave length of 690 nm. Results are shown as OD 550–690 values.

Results

Mast cells release PGD_2 that is rapidly converted to PGJ_2 and Δ^{12}-PGJ_2

Following labelling with [^{14}C]-AA and stimulation with calcium ionophore A23187 BMMC released [^{14}C]-AA and small amounts of its metabolites co-migrating on HPLC with LTC_4, PGD_2, PGJ_2 and Δ^{12}-PGJ_2 (not shown). In addition a number of unidentified metabolites were formed.

Release of unlabelled PGD_2, PGJ_2 and Δ^{12}-PGJ_2 by BMMC was quantified by combined use of HPLC and RIA. PGD_2, PGJ_2 and Δ^{12}-PGJ_2 were detected in samples stimulated with A23187 (0.2 μM) for 30 min (Fig. 1). The total concentration of these mediators amounted to 153.6 ± 78.4 pmol/10^6 cells (n = 4) (Fig. 2). The relative quantities of PGD_2, PGJ_2 and Δ^{12}-PGJ_2 were 11 ± 8%, 53 ± 18% and 36 ± 17% (n = 4, mean ± SD), respectively (Figs. 1 and 2). The A23187-stimulated release of PGD_2, PGJ_2 plus Δ^{12}-PGJ_2 was 169% ± 14% of the unstimulated samples (n = 3, mean ± SD) (Fig. 2).

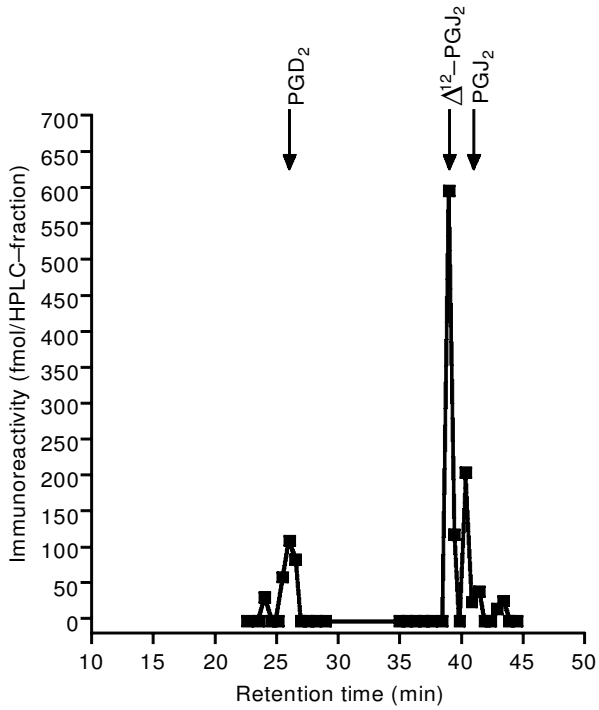

FIG. 1. PGD_2 and its metabolites detected by [^{3}H]-PGD_2-RIA in HPLC fractions of a supernatant of BMMC. BMMC were stimulated with calcium ionophore A23187 (0.2 μM). Supernatants were separated by HPLC and HPLC-fractions were analysed by RIA. Retention times of authentic PGD_2, PGJ_2 and Δ^{12}-PGJ_2 standards are indicated by arrows.

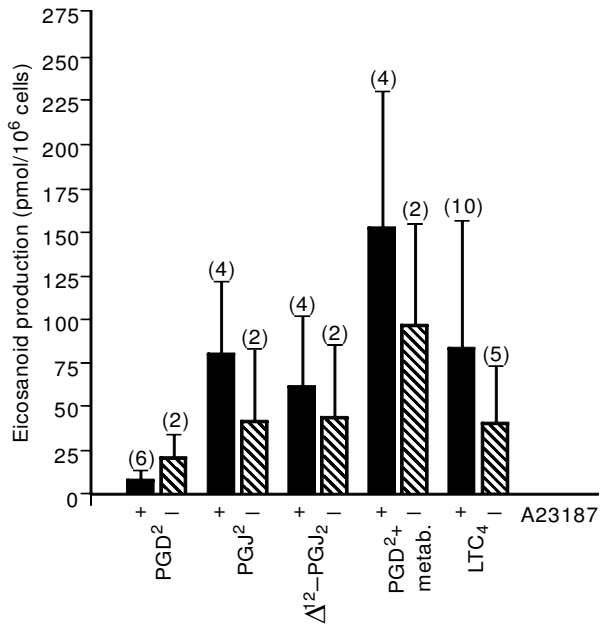

FIG. 2. Endogenous production of PGD_2, PGJ_2, Δ^{12}-PGJ_2 and of total of these prostaglandins (PGD_2 + metab.) and of LTC_4. BMMC were suspended in PBS with (+) or without (–) calcium ionophore A23187 (0.2 μM) for 30 min at 37°C. Results are given as mean ± SD or range of two to 10 independent experiments (number in parenthesis).

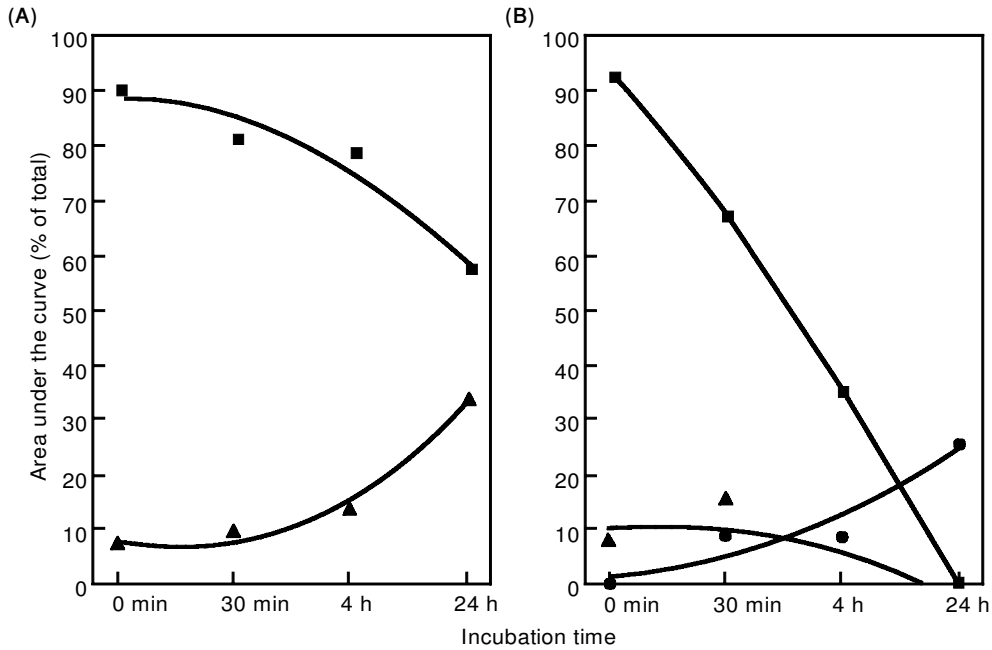

FIG. 3. Metabolism of [^{3}H]-PGD_2 in PBS (A) and culture medium containing FCS (B). After incubation of [^{3}H]-PGD_2 in PBS or culture medium, samples were processed as described in the Methods section. [^{3}H]-PGD_2 and its metabolites were detected by continuous radiodetection in the HPLC eluate. [^{3}H]-PGD_2 (■), [^{3}H]-PGJ_2 (▲) and [^{3}H]-Δ^{12}-PGJ_2 (●) were identified by co-migration with authentic unlabelled standards. [^{3}H]-Δ^{12}-PGJ_2 could only be detected in culture medium containing FCS.

The identity of PGD_2, PGJ_2 and Δ^{12}-PGJ_2 released in the supernatant by BMMC was confirmed by co-migration with authentic [^{3}H]-labelled PGD_2 and authentic unlabelled PGD_2, PGJ_2 and Δ^{12}-PGJ_2 with varying mobile phases and flow rates as described in the Methods section (data not shown).

PGD_2 is spontaneously metabolized to PGJ_2 and Δ^{12}-PGJ_2

PGD_2 was metabolized to PGJ_2 in PBS. Further metabolism to Δ^{12}-PGJ_2 occurred in culture medium containing FCS (Fig. 3). Following 30 min of incubation in PBS or culture medium 19% or 33% of [^{3}H]-PGD_2 were metabolized, respectively. The half-life of PGD_2 was more than 10 h in PBS and 3 h in culture medium. Metabolism of extracellular PGD_2 was not altered in the presence of 10^6 BMMC per ml. Gelatine (0.5 mg/ml) had also no effect on PGD_2 metabolism. The procedures of sample preparation had no influence on recovery or metabolism of PGD_2.

BMMC produce large amounts of LTC_4

Following 30 min of incubation, BMMC produced 85.4 ± 74.0 pmol $LTC_4/10^6$ cells (n = 10) and 42.1 ±

Table 1. Concentrations of PGD_2, PGJ_2 and Δ^{12}-PGJ_2 required for 50% inhibition of cell proliferation (IC_{50}) in selected cell lines (n = 4)

Cell culture	IC_{50} of		
	PGD_2	PGJ_2	Δ^{12}-PGJ_2
HL-60-myelocytic cell line (human)	11 µM	11.5 µM	12 µM
Primary bone marrow-derived mast cells (BMMC) (murine)	10 µM	25 µM	20 µM
IL-3-dependent mast cell line (murine)	4 µM	9 µM	5 µM
Autonomous mast cell line (murine)	4 µM	11 µM	6 µM
THP-1 monocytic cell line (human)	50 µM	50 µM	30 µM
U937 histiocytic cell line (human)	70 µM	80 µM	40 µM
Burkitt lymphoma cell line (human)	2.5 µM	3.5 µM	5 µM
Raji lymphoma cell line (human)	7 µM	7 µM	9 µM

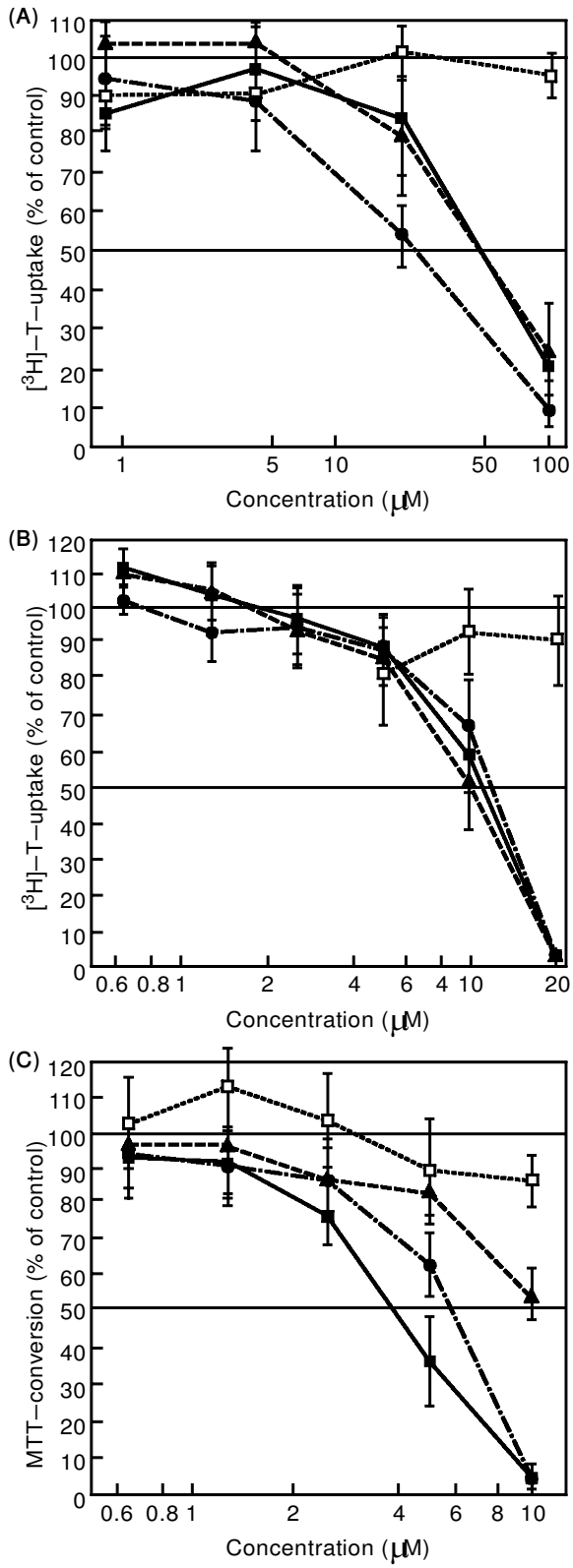

FIG. 4. Modulation of cell proliferation by PGD_2 and by its metabolites. Results obtained in a THP-1 monocytic cell line (human) (A), a HL-60 myeloid leukemic cell line (human) (B) and a murine autonomous mast cell line (C) are demonstrated. Results are expressed in percentage of control of [^{3}H]-T-uptake or MTT-conversion. PGD_2 (■), PGJ_2 (▲) and Δ^{12}-PGJ_2 (●) show distinct antiproliferative activity. Indomethacin (□) had no significant effect on cell proliferation. Experiments were performed in quadruplicates (mean ± SD).

33.8 pmol $LTC_4/10^6$ cells (n = 5; mean ± SD) with and without calcium ionophore A23187 (0.2 μM), respectively. LTC_4 was not metabolized to LTD_4 or LTE_4 under the conditions used.

PGD_2 and its derivatives PGJ_2 and Δ^{12}-PGJ_2 exert strong antiproliferative activity

PGD_2 and its metabolites inhibited cell proliferation in all *in vitro* models tested (Table 1, Fig. 4). The IC_{50} values varied between 2.5 and 80 μM in different cell types. PGD_2, PGJ_2 and Δ^{12}-PGJ_2 were equally effective. At concentrations below 0.5 μM, no significant effect on cell proliferation was observed. Indomethacin (0.1 mM to 10 nM) had no influence on the proliferation of BMMC, TPH-1, U937 or HL-60 cells (Fig. 4). PGD_2 was rapidly metabolized in culture medium to PGJ_2 Δ^{12}-PGJ_2 and other not identified metabolites or conjugates.

Discussion

Our data demonstrate that murine BMMC release PGD_2 and LTC_4, potent eicosanoid mediators. PGD_2 undergoes immediate metabolism to PGJ_2 and Δ^{12}-PGJ_2 during production by BMMC (Fig. 1). This rapid metabolism of PGD_2 is not explained by degradation in extracellular fluid, because the half-life of PGD_2 is more than 10 h in PBS and 3 h in culture medium containing FCS. Mast cells do not contribute to conversion of extracellular PGD_2.

The total of PGD_2, PGJ_2 and Δ^{12}-PGJ_2 produced by stimulated mast cells is about eight-fold the amount of PGD_2 alone (Figs 1 and 2). Separation of PGD_2-metabolites is required prior to RIA, because considerable differences exist in the immunoreactivity of the various PGD_2 metabolites corresponding to very different cross-reactivity profiles in radio-immunologic analysis. Direct radio-immunologic analysis[3] may thus result in gross underestimation of the PGD_2 production and of the PGD_2 mediated antiproliferative potential of BMMC. Stimulation of BMMC with A23187 significantly increased the release of PGD_2, PGJ_2 and Δ^{12}-PGJ_2 in total. This is largely due to an increased release of the latter two metabolites (Fig. 2).

A number of peaks detected in the HPLC chromatogram of supernatant or pellet of [^{14}C]-AA-labelled BMMC have not yet been identified. Some of them may represent further products derived from PGD_2.[1,13–15] Identification of these metabolites requires more efficient procedures to label BMMC with [^{14}C]-AA. The respective experiments are under way.

In contrast to PGD_2 LTC_4 remained stable under the conditions investigated, i.e. in serum-free medium. The amounts of LTC_4 produced in our model of murine BMMC accord with data shown by Razin *et al.*[3] indicating that BMMC represent a reproducible model for analysis of eicosanoid production.

The metabolites PGJ_2 and Δ^{12}-PGJ_2 were about equipotent to PGD_2 in their anti-proliferative activity on myelocytic, mastocytic, monocytic/histiocytic and lymphocytic cells (Table 1, Fig. 4). PGD_2, PGJ_2 and Δ^{12}-PGJ_2 had distinct antiproliferative effects with IC_{50} values ranging from 2.5 to 80 µM. The metabolism of PGD_2 in culture medium (Fig. 3) and the fact that there were no significant differences in the antiproliferative effects of PGD_2, PGJ_2 and Δ^{12}-PGJ_2 in the proliferation assays using incubation times of 24 h (Table 1, Fig. 4) are both in line with the suggestion of Narumiya and Fukushima[1,15], that Δ^{12}-PGJ_2 is the active metabolite of PGD_2 and that PGD_2 may exert no growth inhibition by itself.

The mechanism of action of Δ^{12}-PGJ_2 is not fully understood. There are no surface receptors. The lipid mediator is actively transported into cells through a temperature-dependent transporter on plasma membranes. Δ^{12}-PGJ_2 accumulates in the nuclei, where it binds to thiol groups of nuclear proteins.[16]

Gluthatione depletion enhances antiproliferative activity of PGJ_2 and Δ^{12}-PGJ_2.[14] Recently it has been shown that intracellular glutathione modulates induction of apoptosis by Δ^{12}-PGJ_2.[17]

PGD_2 and its metabolites exerted a more distinct antiproliferative effect in lymphocytic, myelocytic and mast cells than in monocytic or histiocytic cells. Primary murine BMMC were more resistant to growth inhibition by PGD_2, PGJ_2 or Δ^{12}-PGJ_2 than IL-3-dependent and autonomous mast cell lines (Table 1). Indomethacin had no effect on proliferation of BMMC, U937 or HL-60 cells in the proliferation assays used.

We conclude that measurement of PGD_2 disregarding its conversion to metabolites may grossly underestimate PGD_2 production and effects related to these mediators. Our results suggest that the total of PGD_2, PGJ_2 and Δ^{12}-PGJ_2 produced by stimulated mast cells may be expected to exert significant antiproliferative activity. Production of antiproliferative PGD_2 metabolites by BMMC *in vitro* raises the possibility that mast cells exert an anti-proliferative and anti-neoplastic activity by the release of anti-proliferative PGD_2 metabolites *in vivo*.

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