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Prostaglandin D_2 is a novel repressor of IFNγ induced indoleamine-2,3-dioxygenase via the DP1 receptor and cAMP pathway

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ABSTRACT

Expression of elevated levels of Indoleamine 2,3-dioxygenase (IDO) is well established as a mechanism of cancer induced immunosuppression. Pharmacological inhibition of IDO activity is thus a promising alternative in the treatment of cancer. Previously we demonstrated that cyclooxygenase derived metabolites of arachidonic acid inhibited the interferon-gamma mediated induction of IDO in both THP-1 cells and human monocytes. Here we identified that of the five primary prostanoids produced by COX-1/COX-2, only PGD_2 displayed significant repressor activity. PGD_2 inhibited IDO activity with an IC_{50} of 7.2 μM in THP-1 cells and 5.2 μM in monocytes. PGD_2 caused a significant decrease in both IDO mRNA and protein. Using receptor specific agonists, PGD_2 was found to act via the DP1 receptor, while the CRTH2 receptor was not involved. A DP1 antagonist significantly reduced the activity of PGD_2, while CRTH2 agonists were ineffective. PGD_2 increased intracellular cAMP levels and exogenous N^6-cAMP was also found to be highly inhibitory. The effects of PGD_2 via cAMP were blocked by Rp-cAMP indicating involvement of PKA. PGD_2 also stimulated CREB phosphorylation, a PKA dependent transcription factor. This is the first report demonstrating that PGD_2, a prostanoid typically associated with allergy, can inhibit IDO activity via the DP1/cAMP/PKA/CREB pathway. Our findings suggest that PGD_2 and its derivatives may form the basis of novel repressors of IFNγ-mediated IDO expression.

1. Introduction

Cancer cells have developed multiple mechanisms to evade the immune response. One mechanism is an elevation in the level of the enzyme, Indoleamine 2, 3-dioxygenase (IDO, EC 1.13.11.52) [1]. IDO catalyses the first and rate limiting step in the metabolism of the essential amino acid L-Tryptophan (L-Trp), through the Kynurenine (Kyn) pathway, initially forming N-formyl Kynurenine [2]. IDO is now accepted as being an inducible negative regulator of T cell viability, proliferation and activation [3]. IDO has been proposed to suppress T cell functions by two mechanisms. Firstly, depletion of L-Trp causes an increase in the amount of uncharged transfer RNA [4,5], which leads to the activation of the general control non-derepressible kinase (GCN2) [6]. GCN2 enables T-cells to sense and respond to the stress of depleted L-Trp. This activation leads to inhibition of T-cell proliferation and induces T-cell anergy [4]. Secondly, increased levels of L-Trp metabolites e.g. Kyn, induces T cell apoptosis and cell cycle arrest [7,3]. These two mechanisms lead to a decrease in T cell infiltration in the tumour micro-environment and hence tumour evasion. Several studies have now shown that aberrant activation of IDO is strongly associated with a poor clinical prognosis in a variety of cancers [8,9]. It is based on this activity, that a range of IDO specific inhibitors are currently being trialled as potential cancer therapeutics[10].

Recently we investigated the biological activity of the fatty acid, arachidonic acid (AA) to modulate IDO activity. We demonstrated that unidentified cyclo-oxygenase (COX) derived metabolite(s) of AA inhibited the IFNγ/STAT1-mediated induction of IDO in THP-1 cells and human monocytes [11]. Upon its release from membrane phospholipids, AA undergoes oxidation by COX-1/COX-2 (prostaglandin (PG) endoperoxide H synthase) to PGG_2, which is reduced to PGH_2 [12]. PGH_2 is then converted into five principle bioactive prostanoids, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and Thromboxane A_2 (TXA_2) [13]. These PGs are further metabolised into a broad range of derivatives that display varying biological activities in both physiological and pathological conditions. However, the exact role of each metabolite is unknown.

The biological activity of one of these PGs, PGD_2 in relation to IFNγ mediated induction of IDO activity has not been reported. PGD_2 has traditionally been associated with inflammation, allergy, atopy and cancer [13]. PGD_2 is principally produced by mast cells and initiates IgE-mediated acute allergic responses [14]. After antigen challenge, PGD_2 is released into the airways causing

Abbreviations: IDO, Indoleamine 2,3-dioxygenase; Kyn, Kynurenine; IFNγ, Interferon-γ; PKA, Protein kinase A; DP1, Prostanoid DP receptor; CRTH2, Chemoattractant receptor homologous molecule expressed on Th2 cells

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bronchoconstriction; and during acute allergic responses is released into the skin, causing atopic dermatitis [15,16]. PGD_2 is also produced by dendritic cells (DCs) and T helper cells confirming its role in regulating the local immune response [17]. Other biological actions of PGD_2 include facilitating platelet aggregation [18], smooth muscle relaxation, contraction [19], vasodilatation and vasoconstriction [20]. PGD_2 has also been shown to inhibit human ovarian tumour cell proliferation [21].

PGD_2 binds to two receptors, prostanoid DP receptor 1 (DP1) and chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2) [22–24]. DP1 binds to both PGD_2 and PGJ_2 with high affinity, to the synthetic agonist BW245C and to the partial agonist BW868C [25]. The signalling pathway associated with PGD_2 is known; DP1 is a Gsα coupled receptor leading to increases in intracellular cyclic AMP (cAMP) [26]. In contrast, CRTH2 is a Gsi coupled receptor, inhibiting intracellular cAMP and increasing intracellular calcium [27]. CRTH2 also binds the PGD_2 metabolite DK-PGD_2 but not BW245C [23].

To the best of our knowledge, no study has examined the ability of PGD_2 to modulate the IFNγ induced activity of IDO. In this study we have characterised the effects of PGD_2 towards IFNγ induced IDO activity in both THP-1 cells and human monocytes. PGD_2 was found to inhibit IDO activity in a dose dependent manner, in both cell types with a concomitant decrease in *INDO1* mRNA and IDO protein expression. The effects were due to PGD_2 binding to DP1, activating cAMP production, which in turn activated Protein kinase A (PKA) and led to the phosphorylation of the transcription factor CREB that inhibited IDO activity.

2. Materials and methods

2.1. Reagents

Recombinant human IFNγ (IFNγ) was from PeproTech (Rocky Hill, NJ, USA). N^6-cAMP, (4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid (BW245C), MK 0524, 3-isobutyl-1-methylxanthine (IBMX) and Rp-cAMP were from Cayman Chemical (Ann Arbor, Michigan, USA). Forskolin, L-tryptophan, L-kynurenine, *p*-dimethylaminobenzaldehyde (Ehrlich's reagent), dithiothreitol, penicillin, streptomycin, β-mercaptoethanol, trichloroacetic acid and foetal bovine serum were from Sigma Chemical Company (Saint Louis, Missouri, USA). All other reagents were of the highest grade available.

2.2. Prostaglandins

PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, TXB_2, 15(R)-15 methyl PGD_2, PGJ_2, 15-deoxy $\Delta^{12,14}$ PGJ_2 and 13,14 dihydro-15-keto PGD_2 were all from Cayman Chemical (Ann Arbor, Michigan, USA) and were prepared as 20 mM stocks in 100% ethanol and stored at −80 °C.

2.3. Cell culture

Human acute monocytic cells, THP-1 were maintained in RPMI-1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 μM β-ME at 37 °C in a humid atmosphere of 5% CO_2. Monocytes were isolated from healthy volunteers. Mononuclear cells were purified from peripheral blood by density gradient centrifugation [28] and monocytes enriched by adherence to plastic culture plates. Cells were treated with individual prostaglandin (0–10 μM for 30 min) and 1000 IU/ml of IFNγ for 72 h (unless stated otherwise). All work was carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association. Ethical approval was granted by the University of South Australia Human Research Ethics Committee and informed consent was obtained from all participants.

2.4. Presentation of prostaglandins to cells

THP-1 or monocytes were washed in RPMI-1640 medium lacking serum and suspended in the same medium supplemented with L-Trp (100 μM) at 4×10^5/well. A total of 100 μl of cells were plated into a 24 well flat bottom plate. To this was added 100 μl of PG (0–10 μM) or diluent (never ≥ 0.01%) for 30 min at 37 °C/5% CO_2 in a humidified atmosphere. The cells were then stimulated with 200 μl of IFNγ (1000 IU/ml) prepared in RPMI-1640 medium containing 10% FCS supplemented with L-Trp (100 μM) and incubated at 37 °C/5% CO_2 in a humidified atmosphere for 24–72 h. Samples were collected and the level of Kyn quantified using a colorimetric based assay.

2.5. Kynurenine quantification

Kynurenine was quantified as described previously [2,11] with minor modifications. To 100 μl of supernatant was added 40 μl of 30% (w/v) trichloroacetic acid, centrifuged (16,000 g × 5 min), 100 μl of supernatant was transferred to a 96 well flat bottom plate and an equal volume of *p*-dimethylaminobenzaldehyde (4%, w/v) added. After 10 min, the absorbance was measured at 492 nm using a Labsystem Multi scan Ascent plate reader (model 354) (Helsinki, Finland) using the supplied Ascent software (version 2.4.1). This assay was used to detect Kyn as well as other pyrole containing molecules. This assay is high throughput and we have previously demonstrated that the results from this assay agree strongly with a HPLC based assay in determining in vitro Kyn levels [11].

2.6. Modulation of cAMP signalling

Intracellular levels of cAMP were modulated using the pharmacological agents Forskolin (100 μM, 30 min) or N^6-cAMP (0.5 mM, 30 min) while PKA activity was inhibited with Rp-cAMP (50 μM, 60 min). After pre-treatment, cells were treated with PGD_2 (10 μM) for an additional 30 min and then stimulated with IFNγ (1000 IU/ml) for 0.5–72 h. Samples were collected and the level of Kyn quantified as described above.

2.7. Measurement of intracellular cAMP levels

THP-1 cells (4×10^6) were pre-treated with IBMX (0.25 mM, 10 min), then treated with PGD_2, $PGF_{2\alpha}$ (both at 10 μM) or diluent for 10 min. Cells were collected, lysed in 0.1 M HCl and assayed for changes in cAMP levels according to the manufacturer's instructions (Cayman Chemical, Kit no, 581001). Data are expressed as the mean ± SEM pmol/ml cAMP from 6 separate experiments.

2.8. Protein isolation

Proteins were extracted from cells using lysis buffer (20 mM HEPES, pH 7.4, 0.5% (v/v) Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml benzamidine and 100 KIU aprotinin). Cells were disrupted by sonication, centrifuged and the supernatant transferred to a fresh tube for protein analysis. The total protein concentration was measured according to the method of Lowry [29].

2.9. Western blotting

Samples were analysed as described previously [11]. Briefly, 100 μg of protein was subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane and blocked in TBS containing 5% (w/v) skim milk and 0.1% (v/v) Tween 20 for 1 h at room temperature. The membrane was incubated with a