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# Evidence against the formation of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ following inhalation of prostaglandin $D_2$ in man

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**1** There is evidence that an important step in the metabolism of prostaglandin $D_2$ ($PGD_2$) involves 11-keto-reduction and that such a conversion might account for the reported increase in plasma concentrations of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ in allergic asthmatic subjects challenged with inhaled allergen.

**2** Plasma concentrations of immunoreactive 13,14-dihydro-15-keto-$PGF_{2\alpha}$ were measured by specific radioimmunoassay both before and after inhalation of $PGD_2$ and $PGF_{2\alpha}$ in 7 normal and 7 asthmatic men.

**3** In both groups of subjects, $PGF_{2\alpha}$ produced an approximate two fold increase in plasma concentrations of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ that was maximal 5–7 min after inhalation. There was no significant difference in response between the normal and asthmatic subjects.

**4** In contrast, $PGD_2$ failed to produce a change in plasma 13,14-dihydro-15-keto-$PGF_{2\alpha}$ concentration in either group.

**5** These results provide evidence that the conversion of $PGD_2$ to $PGF_{2\alpha}$ with subsequent metabolism to 13,14-dihydro-15-keto-$PGF_{2\alpha}$ is unlikely to occur when $PGD_2$ is released from mast cells in the airways.

## Introduction

There is accumulating evidence that prostaglandin $D_2$ ($PGD_2$) is an important mediator in the pathogenesis of the airway obstruction seen in bronchial asthma. Antigen challenge of human lung fragments and immunological or ionophore-dependent activation of dispersed human lung cells results in the synthesis and release of large amounts of $PGD_2$ from mast cells (Schulman *et al.*, 1981; Lewis *et al.*, 1982; Holgate *et al.*, 1984). When administered to man by inhalation, $PGD_2$ is a potent bronchoconstrictor agent with asthmatic subjects exhibiting an enhanced response (Hardy *et al.*, 1984).

As local hormones, prostaglandins undergo extensive inactivation close to their sites of release, usually by oxidation of the secondary alcohol function at $C_{15}$. However at present little is known about the metabolic fate of $PGD_2$. Despite being a poor substrate for type I prostaglandin-15-hydroxydehydrogenase (PGDH, E.C. 1.1.1.41) (Sun *et al.*, 1976), in the rat isolated perfused lung, $PGD_2$ is subjected to carrier-mediated uptake and metabolism (Robinson & Hoult, 1982). Intravenous infusion of $PGD_2$ in cynomolgus monkey and in man results in the urinary excretion of a mixture of metabolites with either $PGD_2$ or $PGF_{2\alpha}$ ring structures (Ellis *et al.*, 1979; Oates *et al.*, 1984). Furthermore, following intravenous infusion of $PGD_2$ in normal man, both 15-keto-$PGF_{2\alpha}$ and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ have been tentatively identified in plasma by gas chromatography/mass spectrometry (Barrow *et al.*, 1984), although the stereochemistry of the ring hydroxyl groups was not assigned in these studies. Thus it has been suggested that the increased plasma concentrations of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ observed after allergen challenge of asthmatic subjects (Gréen *et al.*, 1974) may represent release of $PGD_2$ from activated bronchial mast cells (Oates *et al.*, 1984). However, recent investigations in a patient with mastocytosis suggest that the major route of $PGD_2$ metabolism may in fact be by the formation of the 11β-hydroxyl epimer of $PGF_{2\alpha}$ (Roberts &

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Liston, 1985) and it is conceivable that this may have been incorrectly identified in previous studies. To investigate further whether $PGD_2$ undergoes 11-keto-reduction to $PGF_{2\alpha}$ with subsequent inactivation to 13,14-dihydro-15-keto-$PGF_{2\alpha}$ as an important component of its metabolism, we have measured plasma concentrations of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ before and after inhalation of a single concentration of $PGD_2$ or $PGF_{2\alpha}$.

## Methods

### *Study protocol*

Seven normal males (age ± s.e.mean, 27 ± 1 years) and seven males with mild allergic asthma (26 ± 1 years) were studied. The subjects attended the laboratory on three separate occasions at least seven days apart to inhale either sodium phosphate buffer, $PGD_2$ or $PGF_{2\alpha}$. All subjects gave their informed consent to the investigation which was approved by the Southampton University Hospitals Ethical Committee.

On each study day an intravenous Venflon cannula was inserted into an antecubital vein under light local anaesthesia. At 5 min intervals before nebulization, two blood samples were withdrawn for the measurement of baseline levels of 13,14-dihydro-15-keto-$PGF_{2\alpha}$. The mean of these two results was used in the subsequent analysis. Airway calibre was measured as specific airways conductance (sGaw) which was automatically computed by an Apple II microcomputer interfaced to a constant volume pressure compensated whole body plethysmograph (Shah *et al.*, 1980).

$PGF_{2\alpha}$ or $PGD_2$ at a concentration of 250 $\mu g\,ml^{-1}$ in 150 mM sodium phosphate vehicle, pH 7.4, were nebulized from an initial volume of 1 ml. Sodium phosphate buffer was used as placebo. All the agents were nebulized from an Inspiron Mini-Neb nebuliser driven by compressed air at a flow rate of 8 $l\,min^{-1}$. The aerosol generated was inhaled through a valve and mouthpiece during tidal breathing for 1 min. This technique nebulizes 0.2–0.3 ml of the starting volume, of which 10–15% is delivered to the lung.

### *Blood collection and extraction*

Blood samples (10 ml) were withdrawn at frequent intervals for 45 min after inhalation and immediately anticoagulated with 3.15% trisodium citrate solution (9:1 v:v). Cell-free plasma was prepared by immediate centrifugation at 2500 *g* and 4°C for 25 min. The plasma samples were acidified to pH 3.0 with 2 M hydrochloric acid and loaded onto $C_{18}$ reversed phase Sep-Paks that had been conditioned by successive 20 ml washes with methanol and distilled water. After sample loading the Sep-Paks were washed with 5 ml distilled water and the prostanoids eluted with 10 ml analytical grade diethyl ether. The ethereal extracts were reduced to dryness under a stream of nitrogen and resuspended in 200 µl of radioimmunoassay buffer (composition (mM): NaCl 155, $Na_2HPO_4$ 80, $NaH_2PO_4$ 34, $NaN_3$ 0.1%, gelatin 0.1%, pH 7.4) and stored at −20°C before assay. Recovery of [$^3H_7$]-13,14-dihydro-15-keto-$PGF_{2\alpha}$ from plasma through the extraction procedure was 68.7 ± 2.4% ($n = 10$) and results were corrected for this. Extraction of plasma samples spiked with unlabelled $PGF_{2\alpha}$ metabolite yielded comparable results. In all cases plasma was extracted immediately after sampling to avoid breakdown of prostanoids on storage in biological media.

### *Radioimmunoassay*

Plasma extracts (0.1 ml) were incubated for 16 h at 4°C with 0.1 ml of antiserum and 10,000 d.p.m. of 13,14-dihydro-15-keto-[5,6,8,9,11,12,14-(n)-$^3$H]-$PGF_{2\alpha}$, sp.act. 100 Ci $mmol^{-1}$. Bound tracer was measured after addition of 0.5 ml dextran coated charcoal and centrifugation at 4°C and 2000 *g* for 15 min. The supernatant fractions were decanted into plastic vials and liquid scintillation spectrometry performed in a Packard 300 CD instrument after addition of 4.5 ml Optiphase RIA scintillant. Assay limit of detection was 10 pg per tube, inter-assay variation was 10.0 ± 0.9% ($n = 5$) and intra-assay variation was 6.0 ± 0.5% ($n = 5$). Cross reactivities of the antiserum were as follows: 13,14-dihydro-15-keto-$PGE_2$ < 0.18%; $PGE_2$ < 0.01%; $PGF_{2\alpha}$ < 0.01%; 6-keto-$PGF_{1\alpha}$ < 0.01%; thromboxane $B_2$ < 0.01%, $PGD_2$ < 0.05%, 11β-epi-$PGF_{2\alpha}$ < 0.06%, 11β-epi-13,14-dihydro-15-keto-$PGF_{2\alpha}$ < 0.1%.

### *Instrumental analysis*

The methyl ester, methoxime, trimethylsilyl ether derivative of $PGD_2$ (ME-MO-TMS-$PGD_2$) was prepared for mass spectrometric analysis. The methoxime was formed by overnight reaction at room temperature with 100 µl of 5 mg $ml^{-1}$ methoxyamine hydrochloride dissolved in anhydrous pyridine. The methyl ester was formed by treatment with 1 ml ethereal diazomethane for 10 min. The trimethylsilyl ether derivative was prepared by treatment with 25 µl of *bis*(trimethylsilyl)trifluoroacetamide followed by overnight reaction at room temperature. Mass spectrometry was performed in the electron impact mode using an MS-30 instrument operated at 70 eV.

Proton magnetic resonance spectra of $PGD_2$ were recorded in deuterochloroform on a Bruker AM 360 MHz instrument. Resonance signals were assigned relative to tetramethylsilane ($\delta = 0.00$).

*Statistical analysis*

Airway response is expressed as the percentage change in sGaw from the mean baseline value. Changes in plasma levels of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ were analysed by the Wilcoxon signed rank test unless otherwise stated. Data are presented in the form of mean ± s.e.mean.

*Materials*

The following were purchased as indicated: Nebulisers (C.R.Bard International, Sunderland), $C_{18}$ Sep-Paks (Waters Associates Harrow), Venflon Cannulae (Everett Medical, Mitcham, Surrey), Deuterochloroform (Aldrich, Gillingham, Dorset), methoxyamine hydrochloride, *bis*(trimethylsilyl)trifluoroacetamide and derivatization grade pyridine (Regis, Morton Grove, Illinois, U.S.A.). $PGF_{2\alpha}$ (Prostin $F_2$) was obtained from Upjohn, Crawley, Sussex and $PGD_2$ was purchased from Salford Ultrafine Chemicals and Research Ltd., Salford. All other unlabelled eicosanoids were generous gifts from the Upjohn Co., Kalamazoo, Michigan, U.S.A. [$^3H_7$]-13,14-dihydro-15-keto-$PGF_{2\alpha}$ was purchased from Amersham International, Amersham, Buckinghamshire. Antiserum to the $PGF_{2\alpha}$ metabolite was obtained from Bioanalysis, Cardiff. All solvents and other reagents were obtained from Fisons PLC, Loughborough, Leicestershire.

## Results

*Confirmation of the identity of the inhaled prostaglandin $D_2$*

As $PGD_2$ may undergo dehydration and isomerization reactions under facile conditions, mass spectrometry and high field proton magnetic resonance spectrometry were performed to confirm the identity of the material used in these studies. The mass spectrum of ME-MO-TMS-prostaglandin $D_2$ contained fragment ions at the following m/z values consistent with the structure of authentic prostaglandin $D_2$: 539 [$M^+$molecular ion], 524 [M-15 loss of $.CH_3$]; 508 [M-31, loss of $.OCH_3$]; 468 [M-71, loss of $.C_5H_{11}$]; 418 [M-121, loss of $(CH_3)_3$ SiOH with $.OCH_3$]; 398 [M-141; loss of $.CH_2CH{:}CH(CH_2)_3COOCH_3$]; 378 [M-(90 + 71)], 199 [$CH{:}CHCH(OSi(CH_3)_3)(CH_2)_4CH_3$] and 173 [$(CH_3)_3SiO^+$: $CH(CH_2)_4CH_3$].

Examination of the 360 MHz n.m.r. spectrum of $PGD_2$ revealed the presence of the following signals; $\delta = 0.95-0.8$ (3H,t) from the $C_{20}$ methyl group, $\delta = 4.55-4.50$ (1H,m) from the $C_9$ proton, $\delta = 4.25-4.15$ (1H, dd) from the $C_{15}$ proton, $\delta = 5.70-5.40$ (4H, m) alkene protons and $\delta = 2.86-2.78$ (1H, dd, (J = 7.4 Hz, 12.4 Hz)) for the $C_{12}$ proton. The latter signal is confirmatory of $PGD_2$ as dehydration or facile isomerization results in its loss.

*Airway studies*

The time courses of the bronchoconstrictor effects of inhaled prostaglandins $D_2$ and $F_{2\alpha}$ in this study have been described in detail elsewhere (Hardy *et al.*, 1984). A brief summary of these data is included here for completeness. In the seven normal subjects, inhalation of placebo or 250 μg ml$^{-1}$ $PGF_{2\alpha}$ had no significant effect on sGaw. Inhalation of $PGD_2$ produced a small 16 ± 4% fall in sGaw 3 min after inhalation ($P < 0.01$) which gradually returned to baseline within 10 min. In contrast, both $PGF_{2\alpha}$ and $PGD_2$ produced marked falls in sGaw of 33 ± 8% ($P < 0.01$) and 75 ± 5% ($P < 0.001$) which were maximal 3 min after inhalation. In the case of $PGF_{2\alpha}$ sGaw had returned to baseline within 30 min, whereas the greater response evoked by $PGD_2$ resulted in sGaw still being reduced to 60 ± 9% of baseline at this time point ($P < 0.005$).

*Prostaglandin metabolite measurements*

On the $PGF_{2\alpha}$ study days there were no significant differences in plasma 13,14-dihydro-15-keto-$PGF_{2\alpha}$ concentrations in the normal and asthmatic subject groups (Table 1). Following inhalation of $PGF_{2\alpha}$ in the normal subjects there was a rise in plasma 13,14-dihydro-15-keto-$PGF_{2\alpha}$ concentration which reached a peak of 106.3 ± 17.2 pg ml$^{-1}$, 7 min after inhalation ($P < 0.05$). A slightly smaller rise occurred in the asthmatic subjects, that reached a peak value of 88.4 ± 14 pg ml$^{-1}$ after 5 min ($P < 0.05$). There was no statistically significant difference between the two

**Table 1** Plasma concentrations of immunoreactive 13,14-dihydro-15-keto-$PGF_{2\alpha}$ ($KH_2F_{2\alpha}$) in seven normal and seven asthmatic subjects following inhalation of $PGF_{2\alpha}$

| | *Plasma $KH_2F_{2\alpha}$ (pg ml$^{-1}$)* | |
|---|---|---|
| *Time after inhalation* (min) | *Normal* | *Asthma* |
| Baseline | 55.8 ± 11.1 | 50.6 ± 9.1 |
| 1 | 88.7 ± 15.6* | 57.4 ± 11.2 |
| 3 | 96.1 ± 13.4* | 74.1 ± 9.0* |
| 5 | 94.8 ± 13.3* | 88.4 ± 14.0* |
| 7 | 106.3 ± 17.2* | 69.5 ± 8.6 |
| 9 | 87.6 ± 18.4 | 82.1 ± 4.8* |
| 20 | 74.1 ± 12.2 | 66.0 ± 9.2* |
| 30 | 90.5 ± 5.6* | 59.0 ± 7.9 |
| 45 | 63.0 ± 9.2 | 59.0 ± 9.8 |

*$P < 0.001-0.05$ with respect to baseline.

**Table 2** Plasma concentrations of immunoreactive 13,14-dihydro-15-keto-$PGF_{2\alpha}$ ($KH_2F_{2\alpha}$) in seven normal and seven asthmatic subjects following inhalation of $PGD_2$

| | *Plasma* $KH_2F_{2\alpha}$ (pg $ml^{-1}$) | |
|---|---|---|
| *Time after inhalation* (min) | *Normal* | *Asthma* |
| Baseline | 39.0 ± 3.4 | 27.9 ± 3.3 |
| 1 | 38.2 ± 3.3 | 34.4 ± 5.1 |
| 3 | 39.8 ± 5.3 | 30.6 ± 5.5 |
| 5 | 38.1 ± 6.3 | 34.4 ± 4.8 |
| 7 | 38.7 ± 6.5 | 29.9 ± 3.7 |
| 9 | 34.2 ± 4.9 | 30.3 ± 4.3 |
| 20 | 31.1 ± 5.7 | 31.4 ± 3.9 |
| 30 | 40.8 ± 7.1 | 30.0 ± 3.8 |
| 45 | 41.3 ± 9.9 | 30.6 ± 4.4 |

subject groups at any time point when the plasma concentrations of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ were compared. In addition, there was no significant difference when the areas under the concentration-time course curve for the two groups were considered.

In the normal subjects on the $PGD_2$ study days the baseline level of the $PGF_{2\alpha}$ metabolite was 39.0 ± 3.4 pg $ml^{-1}$. There was no significant change from this value over a 45 min period following inhalation of $PGD_2$ (Table 2). The corresponding baseline concentration in the asthmatic subjects was 27.9 ± 3.3 pg $ml^{-1}$ which was significantly lower ($P < 0.05$) than the corresponding value of 50.6 ± 9.1 pg $ml^{-1}$ in this group on the $PGF_{2\alpha}$ inhalation day. However, this concentration did not change significantly after inhalation of $PGD_2$ (Table 2).

## Discussion

In this study we have investigated changes in plasma concentrations of immunoreactive 13,14-dihydro-15-keto-$PGF_{2\alpha}$ following inhalation of either $PGF_{2\alpha}$ or $PGD_2$. In both groups of subjects inhalation of a single concentration of $PGF_{2\alpha}$ was associated with a rapid increase in plasma levels of 13,14-dihydro-15-keto-$PGF_{2\alpha}$. There was no significant difference between the two groups in the magnitude of the increase or its duration. In contrast, inhalation of $PGD_2$ did not produce changes in the concentration of the $PGF_{2\alpha}$ metabolite. A corollary of these observations is that the reported increase in plasma 13,14-dihydro-15-keto-$PGF_{2\alpha}$ levels after inhaled allergen provocation (Gréen *et al.*, 1974) is not a direct consequence of prostaglandin release due to contraction of airway smooth muscle (Platshon & Kaliner, 1978) as we failed to detect changes in this metabolite despite producing appreciable bronchoconstriction measured by falls in sGaw.

At present, relatively little is known about the metabolic disposition of $PGD_2$. It is a poor substrate for purified $NAD^+$-dependent PGDH from rhesus monkey lung (Sun *et al.*, 1976) or human placenta (Hoult & Robinson, unpublished observations), although it is transported into lung tissue by the pulmonary prostaglandin carrier system (Robinson & Hoult, 1982). The metabolism of $PGD_2$ by type II nicotinamide adenine dinucleotide phosphate-dependent PGDH has been reported to occur in swine brain (Watanabe *et al.*, 1980) and human platelets (Watanabe *et al.*, 1982), but these reactions may be of little physiological importance due to the nature of the co-factor and lack of specificity of the type II enzyme (Chang & Tai, 1981).

Ellis and co-workers (1979) have reported that intravenous infusion of $PGD_2$ into the cynomolgus monkey results in the excretion of a large number of metabolites in which the 3-hydroxycyclopentanone ring of $PGD_2$ had been converted to a cyclopentane-1,3-diol ring, although the stereochemistry of the ring hydroxyl groups was not assigned. A similar metabolic transformation also occurs in man in systemic mastocytosis, a disease in which there is an increased production of $PGD_2$ by an abnormal mast cell population (Roberts *et al.*, 1980; Oates *et al.*, 1984). Such patients excrete elevated amounts of urinary $PGD_2$ metabolites, many of which are those with F-ring structures (Oates *et al.*, 1984). Furthermore, the tentative identification of both 15-keto-$PGF_{2\alpha}$ and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ in plasma following intravenous infusion of $PGD_2$ into normal volunteers supports the possibility that the metabolism of $PGD_2$ may proceed via $PGF_{2\alpha}$ (Barrow *et al.*, 1984).

It has been suggested that an 11-keto-reductase enzyme may facilitate these reactions (Hensby, 1974), but the tissue distribution of this enzyme in man is not known. The activity of an 11-keto-reductase enzyme has been reported in sheep whole blood (Hensby, 1974) but is not present in human whole blood or plasma (Robinson and Holgate unpublished data). Substantial amounts of the enzyme have been observed in rat lung (Watanabe *et al.*, 1981) and rabbit liver (Reingold *et al.*, 1981; Wong, 1981), from which it has been partially purified (Wong, 1981). However, although rabbit hepatic tissue contains 11-keto-reductase activity, experiments with perfused liver show that extracellular $PGD_2$ may not have access to the enzyme (Reingold *et al.*, 1981). Furthermore, only in the case of sheep blood (Hensby, 1974) has the identity of the reaction product been positively confirmed as having the stereochemistry of $PGF_{2\alpha}$ by the formation of the *n*-butylboronate derivative. This is important because recent studies in man have demonstrated the exclusive formation of the 11β-hydroxy epimer from $PGD_2$ (Roberts & Liston, 1985) and thus it is necessary to re-evaluate previous investigations.

Our present data argue against the 11-keto-reductase-dependent metabolism of $PGD_2$ to $PGF_{2\alpha}$ when administered by inhalation as there was no increase in plasma concentrations of immunoreactive 13,14-dihydro-15-keto-$PGF_{2\alpha}$ after inhalation. It is important to note that the antiserum used in these studies shows minimal cross-reaction with 11$\beta$-$PGF_{2\alpha}$ and its 13,14-dihydro-15-keto-metabolite. Moreover, it is unlikely that the $PGD_2$ failed to be absorbed into the circulation as we have demonstrated elsewhere that inhalation of this prostanoid produced a statistically significant positive chronotropic response in both normal and asthmatic subjects (Hardy *et al.*, 1984). We felt it inappropriate to measure plasma concentrations of $PGD_2$ or $PGF_{2\alpha}$ themselves as the quantitative analysis of primary prostanoids *in vivo*, particularly by radioimmunoassay, often leads to erroneous values. In contrast prostaglandin metabolite measurement is more reliable. The mean baseline value in the seven normal subjects derived from the two study days was $47.4 \pm 6.3$ pg ml$^{-1}$, compared to $39.3 \pm 5.8$ pg ml$^{-1}$ in the asthmatic subjects, and these are in good agreement with measurements made by mass spectrometry (Gréen *et al.*, 1984, Hubbard & Watson, 1976) and are compatible with the calculated rate of $PGF_{2\alpha}$ synthesis in man (Samuelsson & Gréen, 1974).

In summary we have been unable to show that a major metabolic step in the degradation of $PGD_2$ administered by inhalation is initial 11-keto-reduction to $PGF_{2\alpha}$. These results do not exclude the possibility that 11-keto-reduction of $PGD_2$ to 11$\beta$-epi-$PGF_{2\alpha}$ may occur following this route of administration.

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