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# THE METABOLISM OF PROSTAGLANDIN $D_2$

## EVIDENCE FOR THE SEQUENTIAL CONVERSION BY NADPH AND $NAD^+$ DEPENDENT PATHWAYS

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**Abstract**—Cell-free 100,000 *g* supernatants from human lung converted $PGD_2$ into a product which on HPLC was indistinguishable from 9α,11β-$PGF_2$. The rate of reaction was relatively slow (0.30 ± 0.03 pmol/min/mg protein). In the presence of $NAD^+$, 9α,11β-$PGF_2$ was itself metabolized at a rate of 1.46 ± 0.3 pmol/min/mg protein. The product of this reaction was less polar than the substrate and eluted with an HPLC retention time similar to that seen in our previous study where it was identified by GC/MS as being 15-keto-9α,11β-$PGF_2$. There was no evidence for the formation of 13,14-dihydro-15-keto-9α,11β-$PGF_2$. The sequence of metabolism of $PGD_2$ was further established in cell-free supernatants prepared from guinea-pig liver and kidney which have previously been shown to be rich in both 11-ketoreductase and 15-hydroxy-prostaglandin dehydrogenase (PGDH) activities. Reactions contained both $NAD^+$ and a NADPH-regenerating system and demonstrated the sequential metabolism of $PGD_2$ to 9α,11β-$PGF_2$ and ultimately 13,14-dihydro-15-keto-9α,11β-$PGF_2$. Under these conditions the rate of the C-15 oxidation reaction was slower than that of 11-ketoreduction. These observations provide further support for our previous suggestions that the 11-ketoreduction of $PGD_2$ is followed by a PGDH-type reaction, and that these reactions are likely to occur sequentially *in vivo* provided that the appropriate cofactors are present.

The biological activities of prostaglandin $D_2$ ($PGD_2$) have attracted considerable interest following the demonstration of its release from immmunologically activated mast cells [1–3] and during pulmonary anaphylactic shock [4]. It is now known that this prostanoid possesses pharmacological actions which suggest a role as an inflammatory mediator [5]. The metabolism of $PGD_2$ has been studied in man and experimental animals partly as an aid to the identification of suitable metabolites for its quantification *in vivo*. To date 25 urinary metabolites of $PGD_2$ have been identified following intravenous injection into a normal volunteer [6]. The majority of these metabolites had a ring system characteristic of F- rather than D-series prostaglandins, but they differed from previously identified compounds in having a β-orientation of the hydroxyl group at C-11. This observation suggested that the major route of metabolism for $PGD_2$ in normal man was probably by 11-ketoreduction to produce 9α,11β-$PGF_2$, a compound which itself possesses potent biological activity [6, 7], particularly on airways smooth muscle [8].

Although identifying the major elimination products, profiling of urinary metabolites does not necessarily indicate their sequence of formation or provide evidence that endogenously-generated material is dealt with in a similar fashion. We have presented evidence for the likely initial events in the metabolism of $PGD_2$ following its administration by inhalation or intravenous infusion in normal volunteers [9]. These studies suggest that after the reduction of $PGD_2$ to 9α,11β-$PGF_2$, further metabolism probably occurs by oxidation at C-15 and reduction at $\Delta^{13}$, although we were unable to prove the sequence of these reactions *in vivo*. These findings are consistent with our previous investigations using cell-free supernatants prepared from animal organs rich in PG-metabolising enzymes, in which we presented evidence that the further transformations of 9α,11β-$PGF_2$ could be mediated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and $\Delta^{13}$-reductase [10]. However, it was not established in our previous work whether these individual reactions could act in the appropriate sequence to produce the metabolites identified *in vivo*.

The aims of the present experiments were two-fold. In view of the bronchoconstrictor activities of both $PGD_2$ and 9α,11β-$PGF_2$, we firstly wished to ascertain whether $PGD_2$ or 9α,11β-$PGF_2$ were metabolised in human lung. Secondly, we aimed to establish the sequence of these reactions using cell-free organ systems which we had previously identified as being able to metabolize both prostanoids.

### MATERIALS AND METHODS

*Chemicals*. The following were obtained as indicated: $NAD^+$, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (from Baker's yeast) and dithiothreitol from the Sigma Chemical Co. (Poole, U.K.). Unlabelled $PGD_2$ and 9α,11β-$PGF_2$ were obtained from Salford Ultrafine Chemicals and Research Ltd (Manchester, U.K.). Authentic 13,14-dihydro-15-keto-9α,11β-$PGF_2$ was a generous gift of Dr Gordon Bundy of the Upjohn Co. (Kalamazoo, MI). [5,6,8,9,12,14,15-(n)-$^3$H]-

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$PGD_2$ (sp. act. 130 Ci/mmol) was purchased from Amersham International (Aylesbury, U.K.). Tritiated 9α,11β-$PGF_2$ was synthesized biosynthetically as described previously [9, 10].

*Prostaglandin metabolism in cell-free supernatants.* Fresh, grossly normal lung was obtained from patients aged 60–75 years undergoing resection of bronchial carcinoma. Liver and kidneys were removed from male Dunkin Hartley strain guinea-pigs (400–500 g). Organs were homogenized in 4 vol. of 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 1 mM cysteine, 0.1 mM dithiothreitol and 4 mM $MgCl_2$, and 100,000 *g* supernatants prepared by ultracentrifugation as described previously [10]. Cytosolic supernatants were stored at −20° and used within 2 weeks of preparation. The protein concentration of the supernatants was measured by the Coomassie Blue method of Bradford, using bovine serum albumin as standard [11].

Reactions contained 2 μg of unlabelled prostaglandin and 0.1 μCi tritiated analogue per millilitre of incubation mixture. The precise composition of the reactions was as described previously unless otherwise indicated [10]. Cofactor concentration was 4 mM for $NAD^+$ and consisted of a regenerating system for NADPH. The regenerating system comprised 0.1 mM NADPH, 0.5 U glucose-6-phosphate dehydrogenase and 10 mM glucose-6-phosphate. In time course studies, reactions were performed in a single tube and 0.2 ml aliquots were removed at timed intervals and added to an ice-cold mixture of 0.2 ml 1 M formic acid and 0.2 ml ethanol. These samples were extracted twice with ethyl acetate, the organic phase evaporated to dryness under nitrogen and processed as described below.

*High performance liquid chromatography.* High performance liquid chromatographic (HPLC) separation of prostaglandins was performed with a Spectra-Physics SP 8700 ternary solvent delivery system with UV monitoring of authentic standards at 195 nm using a Spectra-Physics SP 8440 or Kratos SF 783 absorbance detector. The mobile phase consisted of 0.017 M orthophosphoric acid: far UV grade acetonitrile (67.2:32.8 v:v) at a flow rate of 1 ml/min. The stationary phase comprised a Nucleosil 5 $C_{18}$ column (12.5 × 0.46 cm) connected in series with a μBondapak $C_{18}$ column (25 × 0.46 cm). In some experiments a single column was used for analyses. The dried extracts were reconstituted in mobile phase and injected together with a cocktail of 5–10 μg of appropriate unlabelled standards via a Rheodyne 7125 injector. Column effluent was collected automatically using a Gilson model 202 fraction collector, and the radioactivity present in each fraction measured by liquid scintillation counting (Packard 300CD or 2000CA) after the addition of 4 ml Opti-Fluor (Canberra-Packard, Pangbourne, U.K.).

*Data presentation.* All data are presented as the mean ± SE mean of N experiments. Significance of differences were evaluated by Student's t-test. Retention times on HPLC are normalized to those of the appropriate substrate; see Ref. 10 for further details.

## RESULTS

### *NADPH-dependent metabolism of $PGD_2$ in human lung*

In the presence of the NADPH-generating system, metabolism of $PGD_2$ proceeded slowly in the 100,000 *g* supernatant fraction prepared from human lung. The enzymatic reaction rate was 0.30 ± 0.03 pmol/min/mg protein (N = 4 lungs) and was linear over 60 min using the reaction conditions stated (Fig. 1). Analysis by HPLC of the extracted reaction mixture demonstrated the time-dependent formation of a product which coeluted with the retention time indicated by authentic 9α,11β-$PGF_2$. In the presence of $NAD^+$ or absence of NADPH, $PGD_2$ was not metabolized enzymatically (data not shown), although in all reactions in which residual $PGD_2$ was present there was evidence of non-enzymatic dehydration to less polar product(s). No further enzymatic transformations of the 9α,11β-$PGF_2$ product were detected in these experiments.

### *$NAD^+$-dependent metabolism of 9α,11β-$PGF_2$ in human lung*

In nine out of ten lung preparations tested, 9α,11β-$PGF_2$ was slowly metabolized in the 100,000 *g* cytosolic supernatant fraction. The rate of reaction was 1.46 ± 0.3 pmol/min/mg protein, and was linear over 60 min with the conditions employed (Fig. 2). The major metabolite formed in this reaction had a relative retention time of 1.33 ± 0.01 (9α,11β-$PGF_2$ = 1.00, N = 15 determinations) on the Nucleosil 5 $C_{18}$ column, consistent with the formation of a less polar 15-keto metabolite. A minor product was evident in some experiments, this having a relative retention time of 1.74 ± 0.02. We have presented mass spectral evidence elsewhere that the major metabolite formed from 9α,11β-$PGF_2$ in human lung cytosolic supernatants is 15-keto-9α,11β-$PGF_2$ [10].

### *Sequence of $PGD_2$ metabolism in guinea-pig organs*

In order to establish the sequence of $PGD_2$ metabolism in the presence of both $NAD^+$ and NADPH we employed cytosolic supernatants of guinea-pig liver and kidney, these being preparations which we have shown previously to be rich in both $PGD_2$ 11-ketoreductase and 15-PGDH. Reactions were terminated at various time-points and the relative proportions of $PGD_2$ and its metabolites determined by HPLC. Figure 3 illustrates that in both organ systems $PGD_2$ underwent rapid conversion to 9α,11β-$PGF_2$ and that this was followed by the slower formation of a metabolite which coeluted with 13,14-dihydro-15-keto-9α,11β-$PGF_2$. In the guinea-pig kidney, the rate of formation of 9α,11β-$PGF_2$ determined from the linear phase of the reaction was 39.3 ± 3.4 pmol/min/mg protein, compared to 58.3 ± 20.9 pmol/min/mg in the liver. The sequential metabolism to the further metabolite was slow, being 2.2 ± 0.4 pmol/min/mg in the kidney compared to 2.1 ± 1.0 pmol/min/mg in the liver (N = 3 in all cases).

In three experiments to study the metabolism of 9α,11β-$PGF_2$ in guinea-pig liver in the presence of $NAD^+$ it was found that the substrate underwent time-dependent conversion to relatively small amounts of material chromatographically indistinct from 13,14-dihydro-15-keto-9α,11β-$PGF_2$. In addition, a second product was also formed in a time-dependent manner. This second metabolite coeluted with the retention time of $PGD_2$ and was also found

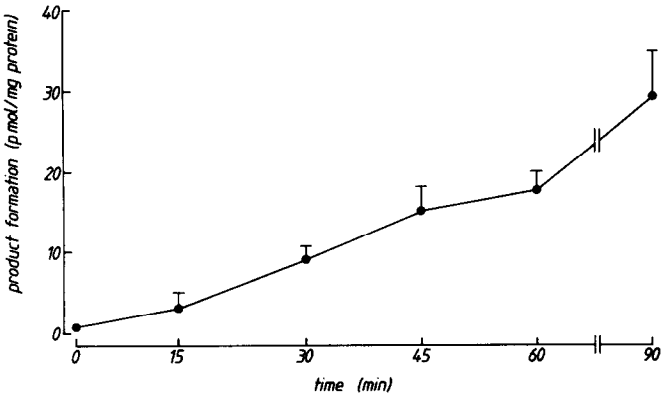


Fig. 1. Time course of NADPH-dependent $PGD_2$ metabolism in the 100,000 *g* supernatant from human lung. Data are mean ± SE mean in four lungs.

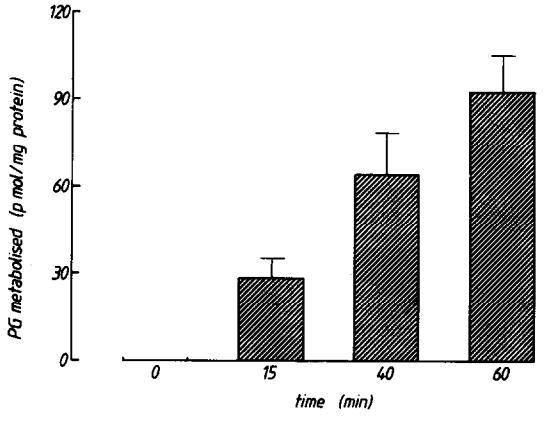


Fig. 2. Time course of $NAD^+$-dependent 9α,11β-$PGF_2$ metabolism in the 100,000 *g* supernatant from human lung. Data are mean ± SE mean in nine lungs.

in incubations performed with guinea-pig kidney cytosol preparations (data not shown). Similar results were obtained when the incubations were repeated using a further three liver cytosolic supernatants. After incubation for 60 min the prostanoid composition of the reaction was 9α,11β-$PGF_2$, 16.2 ± 8.8%; 13,14-dihydro-15-keto-9α,11β-$PGF_2$, 21.9 ± 9.8%; and $PGD_2$-like material, 61.8 ± 7.3%. However, formation of the $PGD_2$-like material was abolished by performing the reaction in the presence of $NAD^+$ and the NADPH-regenerating system. At the end of these reactions the composition was 9α,11β-$PGF_2$, 36.1 ± 13.2% and 13,14-dihydro-15-keto-9α,11β-$PGF_2$, 50 ± 14.7%. For both compounds these amounts are significantly ($P < 0.05$) different from the amounts found in the $NAD^+$-containing reactions. In comparison, when $PGD_2$ was the substrate with combined cofactors, 9α,11β-$PGF_2$ comprised 33.7 ± 19.3% and the 13,14-dihydro-15-keto metabolite 66.3 ± 19.3% at the end of the reaction (N = 3). Further evidence for the for-

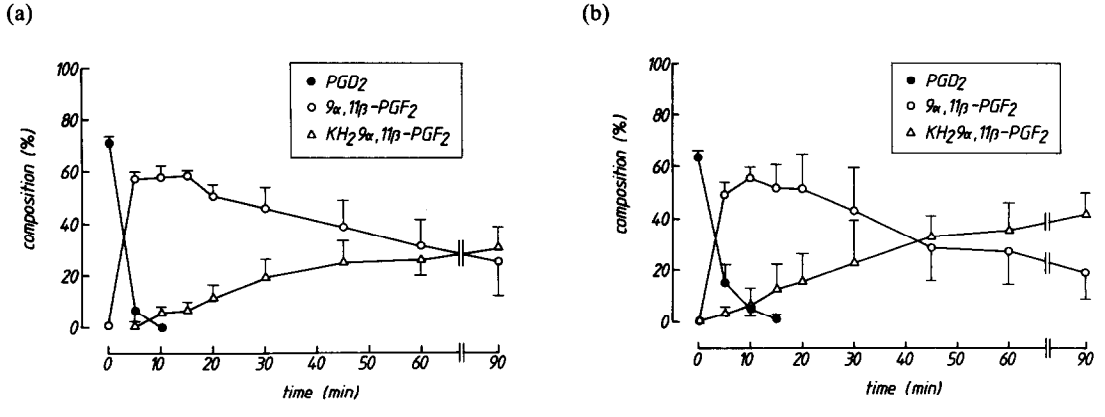


Fig. 3. Time course of $PGD_2$ metabolism in the 100,000 *g* supernatant from guinea-pig liver (a) and guinea-pig kidney (b). Both reactions were performed in the presence of $NAD^+$ and NADPH regenerating system. Symbols: $PGD_2$ (●——●); 9α,11β-$PGF_2$ (○——○); 13,14-dihydro-15-keto-9α,11β-$PGF_2$ (△——△). All data are mean ± SE mean in three organ preparations and are plotted to show the percentage composition of the chromatograms at each time point.

mation of $PGD_2$ from $9\alpha,11\beta$-$PGF_2$ was obtained by performing an incubation in the absence of radiolabelled $9\alpha,11\beta$-$PGF_2$, but the presence of unlabelled substrate and $NAD^+$. After 20 min incubation the amount of immunoreactive $PGD_2$ detectable in the reaction mixture had risen from 0.6 ng/ml to 21.9 ng/ml. These observations suggest that under suitable *in vitro* conditions $PGD_2$ 11-ketoreductase catalyses a reverse reaction from $9\alpha,11\beta$-$PGF_2$.

## DISCUSSION

In these experiments we have demonstrated that under conditions which favour the activity of $PGD_2$ 11-ketoreductase, 15-PGDH and $\Delta^{13}$-reductase in guinea-pig liver and kidney cytosols, there is a sequential metabolism of $PGD_2$ to $9\alpha,11\beta$-$PGF_2$ and, ultimately, 13,14-dihydro-15-keto-$9\alpha,11\beta$-$PGF_2$. Under the conditions employed the rate of metabolism via the 15-PGDH pathway was slower than the 11-ketoreduction of $PGD_2$. These observations provide a more formal confirmation of our previous suggestion, which was based on the separate study of each reaction, that the 11-ketoreduction of $PGD_2$ is followed by a PGDH-type reaction [10].

The activities of 11-ketoreductase and 15-PGDH were found to be low in human lung when compared to values obtained in animal organs. In the case of the 11-ketoreductase pathway the rate of reaction was approximately one tenth that reported by Seibert and colleagues [12]. Although such a discrepancy might be accounted for by kinetic considerations, this is an unlikely explanation because Seibert and colleagues were able to detect significant metabolism of $PGD_2$ even at substrate concentrations lower than the one used in our study. In view of the use of Seibert *et al.* of a single lung specimen from an organ transplant donor who did not smoke, it is possible that other factors such as age, smoking history or the presence of lung disease in the patients may account for these differences, but these ideas await further evaluation. In contrast to the experiments of Seibert *et al.* [12], we were able to demonstrate the $NAD^+$-dependent metabolism of $9\alpha,11\beta$-$PGF_2$ in this tissue, the activity being similar to those found in rabbit, rat and guinea-pig lung cytosols when using the same experimental conditions [10]. These rates of $NAD^+$-dependent metabolism are approximately one third to one half of those obtained with $PGF_{2\alpha}$ as substrate [10]. We also attempted to perform in human lung cytosol preparations the combined cofactor studies described for the guinea-pig. However, our attempts to do this were frustrated by the relatively low 11-ketoreductase activity present in human lung coupled with a relatively fast loss of 15-PGDH activity on storage of these cytosols. We cannot also exclude the possibility, albeit seemingly unlikely, that human lung 11-ketoreductase is inhibited by $NAD^+$.

In our previous study, in which incubations were sampled at a single time point, GC/MS provided tentative evidence for the product of $9\alpha,11\beta$-$PGF_2$ metabolism in human lung being 15-keto-$9\alpha,11\beta$-$PGF_2$. In neither our previous report nor the present time course studies were significant amounts of the 13,14-dihydro-15-keto-$9\alpha,11\beta$-$PGF_2$ metabolite formed. In human lung cytosols it was also of interest to note that the rate of metabolism of $9\alpha,11\beta$-$PGF_2$ was four times faster than the metabolism of $PGD_2$ by 11-ketoreductase. This observation, although surprising in view of the data in guinea-pig tissues, is consistent with our previous study in normal young men in whom the plasma concentrations of 13,14-dihydro-15-keto-$9\alpha,11\beta$-$PGF_2$ were shown to exceed those of $9\alpha,11\beta$-$PGF_2$ shortly after intravenous or inhaled administration [9]. The reasons for these interspecies differences in the relative rates of 11-ketoreduction and C-15 oxidation are not known, but there are established species differences in the tissue distribution of these enzymes [10]. Furthermore, it is likely that the affinity of $PGD_2$ for membrane uptake carriers will also be a major factor in regulating the profile and rate of metabolism *in vivo*.

In conclusion, these studies show that in the presence of both $NAD^+$ and NADPH sequential reactions occur to give rise to $9\alpha,11\beta$-$PGF_2$ and then products typical of the 15-PGDH metabolic pathway. These pathways, and other factors, will thus regulate the formation of this biologically active $PGD_2$ metabolite.

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