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ORGAN SELECTIVE CONVERSION OF PROSTAGLANDIN D_2 TO 9α,11β-PROSTAGLANDIN F_2 AND ITS SUBSEQUENT METABOLISM IN RAT, RABBIT AND GUINEA PIG

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Abstract—Cell-free 100,000 *g* supernatants from liver, kidney, lung and caecum of rat, rabbit and guinea-pig were compared for their ability to transform prostaglandins $F_{2\alpha}$, D_2, E_2 and 9α,11β-prostaglandin F_2 (11epi-$PGF_{2\alpha}$) to metabolic products. Experiments utilized multitritiated substrate PGs, with assessment of biotransformation by TLC, HPLC and GC/MS. $PGF_{2\alpha}$ was converted via the sulphasalazine analogue-inhibitable NAD^+-dependent 15-hydroxy-prostaglandin dehydrogenase pathway (15-PGDH), with high activity (>5 pmol/min/mg protein) in all 12 systems except rat and rabbit liver (e.g. guinea-pig kidney and rat caecum both 64 pmol/min/mg; rat liver 0.3 pmol/min/mg), forming 15-keto and 13,14-dihydro-15-keto metabolites as determined by TLC, HPLC and GC/MS. Prostaglandin D_2 was not transformed in similar fashion in NAD^+- or $NADP^+$-supplemented incubations in any of the 12 cytosolic systems. However, PGD_2 was converted to a single product identified by TLC, HPLC and GC/MS as 9α,11β-PGF_2 in certain of the systems when supplemented with an NADPH regenerating system, with high activity in guinea-pig kidney (55.0 pmol/min/mg), guinea-pig liver (27.5 pmol/min/mg) and rabbit liver (13.7 pmol/min/mg) and less than 5 pmol/min/mg in 8 of the remaining 9 systems. This stereospecific 11-ketoreductase of rabbit and guinea-pig liver was stable to 10 min heating at 50°, dialysis, storage at −20° and repeated freeze/thawing but was not inhibited by sulphasalazine analogues. The 11-ketoreductase had a markedly different tissue profile from PGE_2 9-ketoreductase, which was shown to convert PGE_2 stereospecifically to 9α,11α-prostaglandin F_2 ($PGF_{2\alpha}$) and was present at highest activity in rabbit liver and kidney. Evidence was obtained that 9α,11β-PGF_2 was actively transformed by the sulphasalazine-inhibitable 15-PGDH pathway at approximately one third of the rate of $PGF_{2\alpha}$ with high activity in several cytosolic systems (e.g. rat caecum, guinea-pig liver and kidney), suggesting that further transformation *in vivo* of this biologically active product of PGD_2 metabolism could be initiated by this route.

Prostaglandin (PG) D_2 has a characteristic tissue distribution [1–6] and profile of potent biological actions [7–12], and thus an understanding of its metabolism is of importance. In the cynomolgus monkey, PGD_2 was found to be principally transformed to urinary metabolites with the 9,11-diol substitution of F-series prostaglandins [13]. Furthermore, an 11-ketoreductase enzyme capable of forming "PGF_2" from PGD_2 in rat lung [14], bovine lung [15] and rabbit liver [16] was subsequently identified, but only in the case of bovine lung and sheep blood [15, 17] was the stereochemistry of the 9,11-hydroxyl functions assigned. Later studies in man by Liston and Roberts [18] showed that although PGD_2 is metabolised to compounds with F-ring structures, the majority of these compounds do not have the co-planar geometry of the 9,11-hydroxyls in $PGF_{2\alpha}$ (9α,11α,15(*S*)-trihydroxyprosta-5(*Z*),13(*E*)-dienoic acid), but instead have 9α,11β-geometry. The same workers [19] showed that human liver cytosol contains an 11-ketoreductase activity which stereospecifically converts PGD_2 directly to 9α,11β-PGF_2. This metabolite has now been identified as the principal product of PGD_2 produced by the 11-ketoreductase enzyme present in rabbit liver [20] and the PGF synthase/11-ketoreductase complex of bovine lung [18].

We and others have shown that 9α,11β-PGF_2 possesses potent biological activity different from that of either PGD_2 or $PGF_{2\alpha}$ [8, 19–22]. It has thus been suggested that the overall biological profile of PGD_2 could reflect contributions made by 9α,11β-PGF_2 [8]. Secondly, the urinary metabolites of PGD_2 in both human and non-human primates [13, 18] suggest that 9α,11β-PGF_2 is itself subjected to further enzymatic transformations, most likely initiated by oxidation of the C15(*S*) hydroxyl function by 15-hydroxyprostaglandin dehydrogenase (15-PGDH), analogous to the principal reaction which is responsible for the inactivation of PGE_2 and $PGF_{2\alpha}$.

The aim of this study was to compare the activity of the PGD_2-11-ketoreductase enzyme (11-KR) in tissues of the rat, rabbit and guinea pig, and to compare this with the analogous PGE_2-9-ketoreductase enzyme (9-KR) [23–25]. In addition we have determined whether 9α,11β-PGF_2 can be further metabolised *in vitro* by the 15-PGDH pathway. For this purpose the liver, lung and caecum

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and kidney of three species were used, and $PGF_{2\alpha}$ was used as the reference substrate for 15-PGDH activity.

MATERIALS AND METHODS

Chemicals

The following were purchased as indicated: NAD^+, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (from Baker's yeast), dithiothreitol, rutin, quercetin, indomethacin, PGE_2 and $PGF_{2\alpha}$ from the Sigma Chemical Co. (Poole, Dorset, U.K.). [5,6,8,9,12,14,15-(*n*)-^{3}H]PGD_2 (sp.act. 131 Ci/mmol); [5,6,8,11,12,14,15-(*n*)-^{3}H]PGE_2 (sp.act. 160 Ci/mmol); [9β-^{3}H]$PGF_{2\alpha}$ (sp.act. 14.8 Ci/mmol) from Amersham International plc (Aylesbury, Bucks., U.K.). Phenanthrenequinone and 4-nitrobenzaldehyde were from the Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and sodium borohydride from Fisons plc, Loughborough, Leics., U.K. Sulphasalazine and its analogues Ph CK35A (homosalazine) and pH CL12A (see Ref. 26 for structures) were gifts from Pharmacia AB, Uppsala, Sweden. Unlabelled PGD_2 and 9α,11β-PGF_2 were obtained from Dr F. Scheinmann, Salford Ultrafine Chemicals and Research, Manchester, U.K. Prostaglandin metabolites were generous gifts of Dr G. L. Bundy and Dr J. E. Pike, Upjohn Co., Kalamazoo, MI, U.S.A.

Synthesis of [5,6,8,9,12,14,15-(n)-^{3}H]9α,11β-*PGF_2*

This was prepared by reduction of 1 mCi [$^3H_7$]PGD_2 in methanol solution with 1 mg sodium borohydride for 60 min. The excess borohydride was then destroyed by addition of water and the products extracted at pH 3 into 5 vol. of ethyl acetate. This yielded 9α,11α-PGF_2 and 9α,11β-PGF_2 in a 9:1 ratio and which were resolved by high performance liquid chromatography (HPLC) as described below. The products were extracted from the mobile phase with ethyl acetate, dried with magnesium sulphate and stored at −20° in ethyl acetate. In later experiments [$^3H_7$]PGD_2 was incubated with rabbit or guinea-pig liver cytosol preparations supplemented with an NADPH-regenerating system to produce [$^3H_7$]9α,11β-PGF_2 with greater efficiency. The reaction product was purified by TLC and HPLC as described below. Specific activity of preparations was >130 Ci/mmol.

Prostaglandin inactivation in cytosolic supernatants

Liver, caecum, lung and kidney were removed from male New Zealand White rabbits (3 kg), Sprague–Dawley rats (350 g) and Dunkin Hartley guinea pigs (750 g). Organs were homogenised in 4 vol. of ice-cold 50 mM phosphate buffer, pH 7.4 containing 1 mM EDTA and cysteine, and centrifuged at 4° for 15 min at 3000 *g*, and then for 45 min at 100,000 *g*. Pellets were discarded. These cytosolic supernatants were kept at −20° for up to 12 weeks with no appreciable loss of activity, even if thawed up to 3 times. The protein concentration of the supernatants was measured with Folin-Ciocalteau reagent using bovine serum albumin as standard.

Samples containing 100 μl supernatant and 100 μl phosphate buffer containing substrate, tracer and cofactors were incubated for various times up to 60 min, and the reaction stopped by addition of 0.2 ml ethanol and 0.2 ml 1 M formic acid. The samples were then extracted twice with 1 ml ethyl acetate. Recovery of substrate PG and metabolites was comparable and in the range 78–85%. In routine studies the solvent was evaporated, the residues dissolved in 15 μl methanol and applied to silica gel thin layer chromatography (TLC) plates of two types: Kodak plastic backed sheets, type 13181, layer thickness 0.1 mm (Kodak, Liverpool, U.K.) or Merck aluminium foil-backed sheets, type 5554, layer thickness 0.2 mm (BDH Chemicals, Poole, Dorset, U.K.) and developed in one of the following solvents. Solvent A: (composition by volume) ethyl acetate 90, acetone 10, glacial acetic acid 1.5; Solvent B: the organic phase of ethyl acetate 90, 2,2,4-trimethylpentane 50, acetic acid 20, water 100; Solvent C: ethyl acetate 80, formic acid 1.

In all experiments the substrate concentration was 2 μg/ml with 0.1 μCi tritiated prostaglandin unless otherwise indicated, together with 4 mM NAD^+ (for 15-PGDH experiments) or 0.1 mM NADPH, 0.5 U glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, 4 mM $MgCl_2$ and 0.1 mM dithiothreitol (for 9-KR and 11-KR experiments). In some experiments, potential enzyme inhibitors were also added as indicated.

Table 1. Separation of prostaglandins by thin layer and high performance liquid chromatography

Compound	Solvent A foil	Solvent A plastic	Solvent B foil	Solvent B plastic	Solvent C foil	HPLC
$PGF_{2\alpha}$	0.25	0.17	0.16	0.24	0.24	0.690 ± 0.001 (124)
15-Keto-$PGF_{2\alpha}$	0.54	0.30	0.30	0.41	0.55	0.890 ± 0.001 (111)
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	0.64	0.34	0.41	0.54	0.63	1.270 ± 0.001 (101)
9α,11β-PGF_2	0.24	0.22	0.15	0.31	0.21	0.560 ± 0.001 (124)
13,14-Dihydro-15-keto-9α,11β-PGF_2	0.75	0.55	0.46	0.63	0.71	1.120 ± 0.002 (14)
$PGF_{2\beta}$ (9β,11α)	0.15	0.14	0.09	0.19	0.14	0.510 ± 0.001 (14)
PGE_2	0.45	0.31	0.25	0.40	ND	0.850 ± 0.001 (111)
PGD_2	0.81	0.48	0.40	0.52	ND	1.000 ± 0.00 (124)

The table shows R_f values of the relevant compounds using freshly prepared solvents in the case of thin layer separations, or the relative retention time in the case of liquid chromatographic separations (PGD_2 = 1.000, 50.78 ± 0.41 min). The R_f values are typical data from 8–12 experiments. The relative retention times are shown as the mean ± SEM of (n) experiments. ND = not determined. Analytical details are given in the text.

Quantification of PG metabolism

In routine experiments the extent of PG metabolism was measured by TLC as described above with a 40–60 min development time for foil-backed plates and 70–90 min for plastic backed plates. Table 1 lists the R_f values of the relevant PGs in the various solvents. These systems were chosen so as to optimise the separation of the various products and to allow initial corroboration of the suspected product identities. After chromatography, the positions of radioactive peaks were verified by scanning the plates using a Panax model RTLS-1A radiochromatogram scanner, and the relevant portions of the chromatograms sectioned and counted for radioactivity. After corrections for blanks, and for results obtained using boiled enzyme controls, results were expressed as a percentage of the total radioactivity detected in the regions of interest in each chromatogram.

High performance liquid chromatography

High performance liquid chromatographic separation of prostaglandins was performed with a Spectra-Physics SP8700 ternary solvent delivery system with ultra-violet (UV) detection at 195 nm using a Spectra-Physics SP8440 absorbance detector. The mobile phase employed 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 consisted of a Nucleosil 5 C_{18} column (12.5 × 0.46 cm) connected in series with a μBondapak C_{18} column (25 × 0.46 cm). Table 1 illustrates the relative retention indices of standard prostanoids in this assay system. Samples, reconstituted in mobile phase and containing unlabelled prostaglandin standards, were injected via a Rheodyne 7125 loop injector and column effluent collected automatically using a Gilson Model 202 fraction collector. Radioactivity present in each fraction was measured by liquid scintillation spectrometry (Packard 300 CD) after addition of 4 ml Opti-Fluor (Canberra-Packard, Pangbourne, Berks., U.K.).

Gas chromatography/electron impact mass spectrometry

Prior to structural analysis by GC/MS the samples, together with appropriate standards, were derivatized as described.

(a)*Methoximation.* Keto functions were converted to *O*-methoxime derivatives by treating dried extracts with 100 μl methoxyamine hydrochloride (5 mg/ml in dry redistilled pyridine) for 60 min at 60°. Pyridine was then removed in a stream of dry nitrogen.

(b) *Esterification.* Carboxyl functions were converted to methyl esters by addition of 500 μl ethereal diazomethane to the extract after reconstitution in 250 μl methanol.

(c) *Trimethylsilylation.* Hydroxyl groups were converted to the corresponding trimethylsilyl ether derivatives by addition of 25 μl bis(trimethylsilyl)trifluoroacetamide and 25 μl pyridine and allowing reaction for 60 min at 60°.

GC/MS analyses were performed in the electron impact mode using a Varian-MAT 44S mass spectrometer interfaced to a Varian 3700 gas chromatograph. Samples (1 μl) were introduced by injection with a 30:1 split ratio onto a 10 m DB-5 fused silica capillary column (0.32 mm i.d.) with helium as carrier gas (1 ml/min). Injector temperature was 240° and separations were performed isothermally. Ioniser temperature was 200° and electron energy 55 eV.

RESULTS

Distribution of enzyme activities

The activities of the three enzymes (15-PGDH, 9-KR and 11-KR) in the selected organs are shown in Table 2. These results are based on radio-TLC quantification of prostaglandin metabolism. Also included in Table 2 are data for the inactivation of $9\alpha,11\beta$-PGF_2 in NAD^+ supplemented incubations.

Table 2 illustrates that there are striking variations

Table 2. Distribution of 15-PGDH, 11-KR and 9-KR enzyme activity in 100,000 g supernatants prepared from organs of guinea-pig, rat and rabbit

	15-PGDH $PGF_{2\alpha}$	11-KR PGD_2	9-KR PGE_2	15-PGDH $9\alpha,11\beta$-PGF_2
Guinea-pig				
Liver	33.6 ± 2.8	27.5 ± 0.9	2.0 ± 0.1	8.1 ± 0.1
Caecum	9.3 ± 0.5	8.0 ± 0.1	0.2 ± 0.1	2.3 ± 0.6
Lung	21.8 ± 1.3	3.9 ± 0.1	0	6.9 ± 0.1
Kidney	63.5 ± 0.3	55.0 ± 0.8	3.9 ± 0.4	29.8 ± 0.5
Rat				
Liver	0.3 ± 0.0	0.7 ± 0.2	0	0.1 ± 0.0
Caecum	64.0 ± 0.2	0.8 ± 0.3	6.2 ± 0.1	15.2 ± 0.1
Lung	7.4 ± 0.1	1.5 ± 0.4	0.4 ± 0.2	1.5 ± 0.1
Kidney	5.0 ± 0.1	0.8 ± 0.2	0.3 ± 0.1	4.1 ± 0.2
Rabbit				
Liver	3.2 ± 0.1	13.7 ± 0.2	13.4 ± 0.3	5.7 ± 0.2
Caecum	10.6 ± 0.7	4.1 ± 0.2	1.3 ± 0.5	6.4 ± 1.5
Lung	12.5 ± 0.1	1.4 ± 0.4	0.6 ± 0.3	3.6 ± 0.1
Kidney	7.5 ± 0.2	1.1 ± 0.2	14.2 ± 0.1	2.5 ± 0.3

Results expressed in terms of the rate of enzyme activity in pmol/min/mg protein, and show mean values ± SEM for at least 4 to 6 tests.

in the tissue distribution of the enzyme activities metabolising the four substrates. This is true whether the comparison is made between the same organ of different species, or between the four organs of one species. For example 15-PGDH activity is very high (defined as a specific activity of >20 pmol/min/mg) in liver, lung and kidney of guinea-pig and in rat caecum, but very low (<2 pmol/min/mg) in rabbit and rat liver. In contrast, 11-KR activity is high in guinea-pig kidney and liver tissues of rabbit and guinea-pig, but very low in all four rat organs and in rabbit lung and kidney. Rabbit liver and kidney were the only organs in which appreciable 9-KR activity was detected. The enzyme was essentially absent in lung cytosols from all three species and from the rat organs tested, with the exception of rat caecum (Table 2). PGD_2 was not metabolised in any of the organ systems in the presence of either NAD^+ or $NADP^+$. After 60 min incubation, 70–90% of the radioactivity was recovered as unchanged PGD_2, with the remainder eluting as a less polar peak on TLC or HPLC. A similar peak was found in incubations performed in buffer alone or with heat-inactivated cytosols (data not shown).

That 15-PGDH, 9-KR and 11-KR are distinct enzymes, as demonstrated in previous studies by their different physical and catalytic properties following purification (e.g. Refs. 16, 23–25, 27–29), is confirmed by the failure of PGD_2 to be metabolised by cytosols containing 15-PGDH and by the lack of correlation between the activities of 11-KR and 9-KR ($r = 0.07$, N = 12, $P > 0.8$, unweighted least squares linear regression) and between 15-PGDH and 9-KR ($r = 0.03$, N = 12, $P > 0.9$). However, there was a significant correlation between 15-PGDH activity (assayed with $PGF_{2\alpha}$) and 11-KR ($r = 0.60$, N = 12, $P < 0.05$).

Identity of prostaglandin metabolites

Four of the cytosolic systems were chosen for further analysis by radio-TLC and, where necessary, HPLC and GC/MS. They were guinea-pig liver (rich in 15-PGDH and 11-KR activities, 9-KR demonstrable), rabbit liver (low 15-PGDH activity, rich in 9-KR and 11-KR), rabbit kidney (rich in 9-KR activity, 15-PGDH demonstrable but 11-KR absent) and rat caecum (rich in 15-PGDH, 9-KR demonstrable but 11-KR absent). For these experiments the radio-TLC analyses were performed using at least three combinations of chromatography sheet and solvent. When instrumental analyses by HPLC or GC/MS were also performed additional samples were run in parallel to those analyzed by radio-TLC.

(a) 15-*PGDH pathway*. The principal products of $PGF_{2\alpha}$ metabolism by 15-PGDH co-chromatographed with 15-keto-$PGF_{2\alpha}$ and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ ($KH_2F_{2\alpha}$), although there were differences in the ratios of these products in different organs. In guinea-pig and rabbit liver the sole product detected by radio-TLC was $KH_2F_{2\alpha}$. This was confirmed in separate experiments in which the extracted incubation mixture was analyzed by HPLC and GC/MS. A single product was identified with a GC retention time identical to that of authentic $KH_2F_{2\alpha}$. Examination of the mass spectrum revealed the following fragment ions: 541 [M^+]; 510 [M-31, loss of $.OCH_3$]; 451 [M-90, loss of $(CH_3)_3SiOH$]; 420 [M-121, loss of $(CH_3)_3SiOH + .OCH_3$]; 380 [M-161, loss of

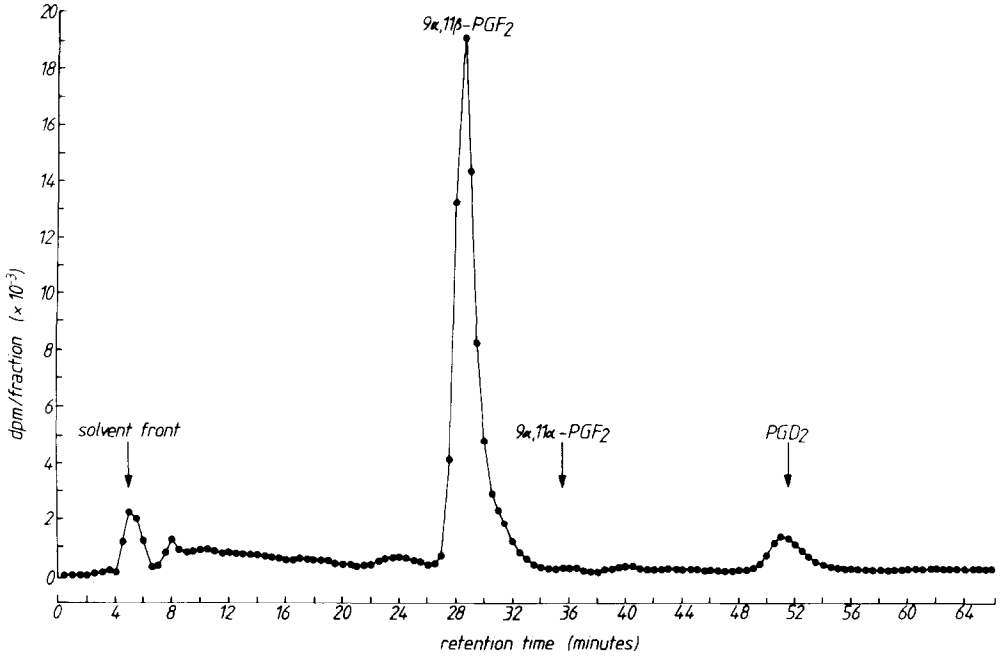

Fig. 1. Separation by HPLC of the product formed by the NADPH-dependent metabolism of PGD_2 in cell free fractions of guinea-pig kidney. Markers indicate the retention time of authentic standards. The sole metabolite was 9α,11β-PGF_2, retention time 28.5 min. Relatively little unchanged PGD_2 (retention time 51.5 min) was detectable after incubation for 60 min.

$(CH_3)_3SiOH + .C_5H_{11}$]; 330 [M-211, loss of 2 × $(CH_3)_3SiOH + .OCH_3$]; 307 (base peak). These fragments were found in the same relative abundance in derivatized authentic $KH_2F_{2\alpha}$.

In contrast, in rabbit kidney incubations both 15-keto-$PGF_{2\alpha}$ and $KH_2F_{2\alpha}$ were present in roughly equal proportions, whereas in rat caecum 15-keto-$PGF_{2\alpha}$ was the dominant product.

(b) PGD_2 11-*KR pathway*. From the TLC experiments it was apparent that a single metabolite was formed from PGD_2 when incubated in the presence of NADPH in cytosolic preparations from all 4 guinea-pig tissues and in rabbit liver and rabbit caecum (Table 2). On plastic backed sheets it was possible to resolve $PGF_{2\alpha}$ from its 11β epimer and this provided tentative evidence for the identity of the metabolite as 9α,11β-PGF_2. In all cases separation of the products of these incubation mixtures by HPLC also revealed a single metabolite peak in addition to a residual amount of PGD_2. An example is illustrated in Fig. 1. This metabolite eluted with authentic [$^3H_7$]9α,11β-PGF_2 prepared by sodium borohydride reduction of tritiated PGD_2 and the retention time was identical to that predicted from unlabelled 9α,11β-PGF_2 after correction for the isotope effect. It should be emphasised that the conditions employed here achieve excellent baseline resolution between the 11α and 11β epimers (Table 1). In experiments where little or no metabolism of PGD_2 occurred (e.g. buffer blanks or heat-inactivated cytosols) there was evidence of chemical dehydration of the PGD_2. The dehydration product(s) was not detected in cytosol preparations containing highly active 11-KR enzyme activity.

Structural identify of the 11-KR dependent metabolite was further confirmed by GC/MS in the case of guinea-pig, liver, kidney and rabbit liver cytosols. The key fragment ions were: 584 [M^+]; 513 (loss of $.C_5H_{11}$); 494 (loss of $(CH_3)_3SiOH$); 443 (loss of $.CH_2CH{:}CH(CH_2)_3COOCH_3$); 423 (loss of $(CH_3)_3SiOH$ and $.C_5H_{11}$); 404 (loss of 2 × $(CH_3)_3SiOH$); 333 (loss of 2 × $(CH_3)_3SiOH$ and $.C_5H_{11}$; 307; 217; 191 and 199 (CH:CHCH(OSi$(CH_3)_3$)$(CH_2)_4CH_3$). An example is shown in Fig. 2. In all cases the GC retention times, fragmentation patterns and relative abundances of the fragments were identical to those of authentic 9α,11β-PGF_2.

(c) *PGE_2 9-KR pathway*. Coelution of the single major product of these incubations with authentic 9α,11α-PGF_2 ($PGF_{2\alpha}$) was achieved in three TLC systems, when using rabbit kidney, rabbit liver and rat caecum supernatants. Additional experiments using chicken liver cytosol also resulted in the formation of a similar product. Separation by HPLC of the product formed in these organs revealed a peak at a retention time of 34.12 ± 0.35 min confirming the identify of this material as $PGF_{2\alpha}$. The retention time for the 9β,11α-PGF_2 ($PGF_{2\beta}$) epimer in these experiments was 24.53 ± 0.24 min (Fig. 3). In addition, a minor peak (comprising 0.8–7.1% total radioactivity) was eluted on the acetonitrile gradient in some samples. This peak, which was present in greater abundance (13–18%) in buffer blanks or in heat-inactivated cytosols, is presumably a dehydration product of PGE_2.

(d) *Metabolism of 9α,11β-PGF_2 by the 15-PGDH pathway*. In the presence of 4 mM NAD^+ 9α,11β-PGF_2 was itself metabolised in cytosol preparations (Table 2). Although the initial rates of reaction were lower than those for $PGF_{2\alpha}$ (with the exception of rabbit liver, Table 2), the transformation of 9α,11β-PGF_2 was particularly efficient in those organs rich in 15-PGDH activity (Fig. 4). There was a significant linear correlation between 15-PGDH activity measured with $PGF_{2\alpha}$ as substrate and the ability of the same 12 cytosolic supernatants to metabolise 9α,11β-PGF_2 ($r = 0.89$, $P < 0.001$, $N = 12$).

In radio-TLC experiments presumptive evidence for the identity of the metabolites as the 15-keto and 13,14-dihydro-15-keto compounds was obtained by comparison of the scans with those obtained using $PGF_{2\alpha}$ as substrate as well as by co-chromatography of the presumptive 13,14-dihydro-15-keto-9α,11β-PGF_2 metabolite with an authentic standard. The metabolites formed in the guinea-pig liver, guinea-pig kidney and rat caecum cytosolic systems were characterized further using HPLC. In rat caecum

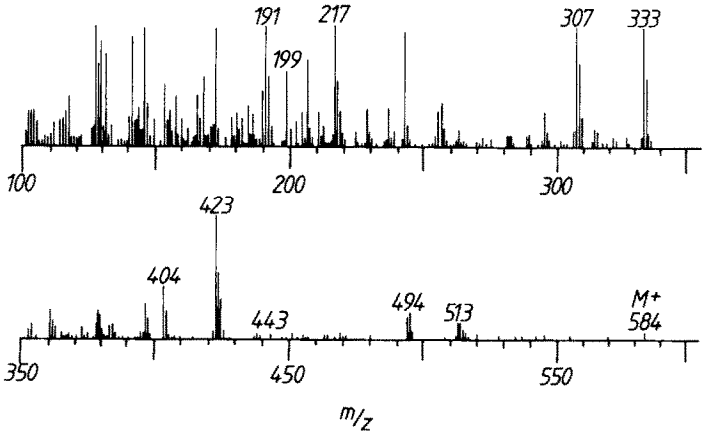

Fig. 2. Electron impact mass spectrum of the PGD_2 metabolite formed in guinea-pig liver cytosol. The GC retention time and fragmentation pattern are consistent with the structure of 9α,11β-PGF_2 as the methyl ester tris-trimethylsilyl ether derivative.

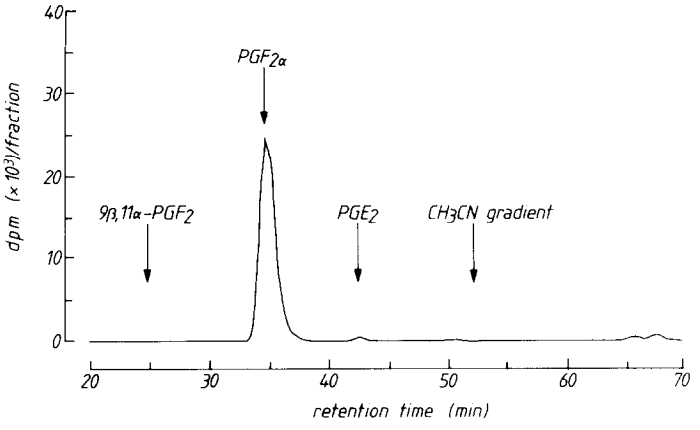

Fig. 3. Separation by HPLC of the metabolite formed by the NADPH-dependent metabolism of PGE_2 in cell free fractions of rabbit liver. The sole enzymatic metabolite was identified as $PGF_{2\alpha}$-PGF_2.

34.4% of the recovered radioactivity eluted with 9α,11β-PGF_2 (relative retention = 1.00), whereas 44.5% eluted as a peak with a relative retention of 1.33 (metabolite I), and 7.4% (metabolite II) coeluted with 13,14-dihydro-15-keto-9α,11β-PGF_2 (relative retention 1.93). Similar results were obtained with guinea-pig liver: 54.9% of this incubation mixture was 9α,11β-PGF_2, 24.7% appeared as metabolite I and 4.7% as metabolite II. However, greatest conversion was observed in the guinea-pig kidney cytosol, with 56.0% of the radioactivity coeluting with 13,14-dihydro-15-keto-9α,11β-PGF_2 as the sole metabolite.

Inhibitor studies

Further information about these enzymatic transformations of the prostaglandins was obtained using enzyme inhibitors. Sulphasalazine and two related azobenzene analogues, homosalazine and Ph CL12A, which inhibit the breakdown of classical prostaglandins by a direct action on 15-PGDH [26, 30, 31], were effective inhibitors of the metabolism of $PGF_{2\alpha}$ in these experiments (Table 3). The relative inhibitory potencies correspond to those already established [26]. All three compounds also inhibited the biotransformation of 9α,11β-PGF_2, lending further support to the view that this reaction is facilitated by 15-PGDH. However, the sulphasalazine analogues only weakly inhibited 9-KR and 11-KR, indicating their specificity.

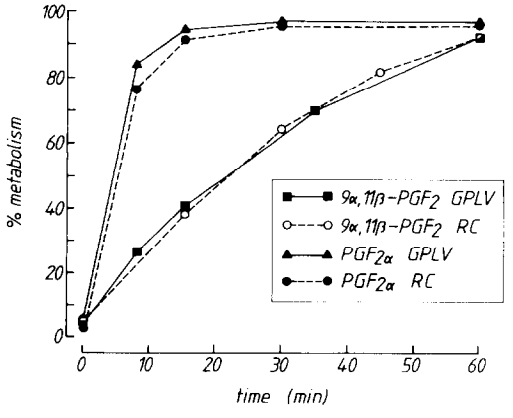

Fig. 4. The time course of the NAD^+-dependent metabolism of $PGF_{2\alpha}$ and 9α,11β-PGF_2 in guinea-pig liver and rat caecum.

In the case of indomethacin and the flavonoids quercetin and rutin, the compounds had little or no inhibitory activity on the four enzyme reactions tested. The exception to this was rutin which produced moderate inhibition of 9-KR. Most notably there was little effect on 11-KR.

Phenanthrenequinone and 4-nitrobenzaldehyde are effective substrates for carbonyl reductase. Phenanthrenequinone (10 μM) inhibited 11-KR more effectively than 9-KR and was more potent than 4-nitrobenzaldehyde which only inhibited 11-KR (Table 3).

Other physical properties of 11-KR activity in cytosolic systems were also investigated (Table 3). The enzyme possesses greater thermal stability than is the case for 15-PGDH and is unaffected by overnight dialysis or repeated freeze/thawing after storage at −20°. The rabbit liver enzyme shows similar catalytic activity at 45° as at 37°, but activity is less at 20° and 10° and absent at 0° (data not shown). Omission of the NADPH cofactor markedly reduced 11-KR activity in rabbit or guinea-pig liver (Table 3), as is the case for the relevant cofactors of the other enzymes. In dialysed preparations, enzyme activity could be restored by addition of NADPH (2 mM; 62 ± 3% conversion of PGD_2 in rabbit liver in 60 min, N = 4) or with the NADPH generating system (NADPH 0.1 mM, 74 ± 2% conversion), but not by 2 mM NADH, NAD^+ or $NADP^+$. There is thus a strict cofactor requirement for PGD_2 11-KR.

DISCUSSION

In this study we have described the organ distribution of the cytosolic 11-keto-reductase enzyme activity responsible for the initial NADPH-dependent metabolism of PGD_2. The enzyme was present at high activity in all of the guinea-pig organs tested, with the exception of the lung. The richest sources

Table 3. Inhibition by drugs and various other treatments of 15-PGDH, 11-KR and 9-KR enzyme activity in 100,000 *g* supernatants prepared from organs of guinea-pig, rat and rabbit

Experimental condition	15-PGDH $PGF_{2\alpha}$	15-PGDH $9\alpha,11\beta$-PGF_2	11-KR PGD_2	9-KR PGE_2
Inhibitors at 50 μM				
Sulphasalazine	45.3 ± 5.6	37.9 ± 5.0	9.3 ± 3.2	0
Homosalazine	72.9 ± 2.6	40.1 ± 5.8	8.8 ± 4.0	3.8 ± 2.2
Ph CL 12A	87.7 ± 1.6	64.8 ± 5.9	14.1 ± 6.3	3.3 ± 3.3
Indomethacin	8.5 ± 6.6	13.3 ± 7.1	0	8.1 ± 3.0
Quercetin	1.5 ± 0.6	13.2 ± 6.0	10.3 ± 4.9	9.8 ± 4.9
Rutin	1.7 ± 0.5	4.9 ± 4.9	2.7 ± 1.6	33.1 ± 0.9
Inhibitors at 100 μM				
Phenanthrenequinone	1.8 ± 0.8	12.6	43.6 ± 11.4	25.3 ± 1.7
4-Nitrobenzaldehyde	1.0 ± 0.2	20.3 ± 6.0	14.5 ± 7.5	0
Experimental manipulations				
Omission of cofactor	n.t.	n.t.	87.6 ± 0.4	100
Overnight dialysis	n.t.	n.t.	0	0
Heating 50°C, 10 min	60.5 ± 7.4	74.0 ± 7.4	14.9 ± 2.4	49.2 ± 18.3
Boiling, 3 min	100	100	100	100

Results expressed as percentage inhibition of conversion after comparison with reaction in control tubes, and show mean values ± SEM for at least 3 to 12 tests. These tests were carried out using enzyme preparations derived from different animal or tissue sources (as in Table 2), but results were comparable regardless of the enzyme source and are thus pooled for clarity.

of the enzyme activity were liver and kidney. The enzyme activity was also present in the rabbit, although most activity was found in the liver. Very low enzyme activities were detected in the rat organs tested. This map of enzyme activity distinguishes PGD_2 11-KR from the 9-KR enzyme which metabolizes PGE_2 and excludes the possibility that these activities are alternate expressions of a single protein. The formation of $PGF_{2\alpha}$, and not $9\beta,11\alpha$-PGF_2, by 9-KR was clearly demonstrated in our experiments, suggesting that the prostaglandin ketoreductase enzymes have their own unique stereoselectivities. Previous reports have suggested that 9-KR is a carbonyl reductase enzyme with a broad range of possible substrates and can be inhibited by compounds such as phenanthrenequinone and 4-nitrobenzaldehyde, as well as by indomethacin and flavonoids [32–37]. In our studies phenanthrenequinone was also an effective inhibitor of 11-KR, suggesting that this enzyme may also have a relatively broad specificity. Interestingly, the 11-KR protein purified from bovine lung also serves as a 9,11-endoperoxide reductase in producing $PGF_{2\alpha}$ from PGH_2 [15]. Whether other 11-KR proteins also perform such a reaction is not known, although it should be noted that there is a large molecular weight difference between the bovine lung and rabbit liver enzymes [15, 16].

Although 11-KR activity was originally identified in rabbit liver, the product of reaction was incorrectly identified as $PGF_{2\alpha}$ [16, 38]. A similar PGD_2 11-KR is present in human liver, and in this case the product was identified as being $9\alpha,11\beta$-PGF_2 [19]. Subsequent re-examination of the rabbit liver metabolite indicated that it too was $9\alpha,11\beta$-PGF_2. Our studies have extended these observations by finding other organ systems rich in the PGD_2 11-ketoreductase enzyme. Based on TLC, HPLC and GC/MS identification the initial product of PGD_2 metabolism under these conditions is $9\alpha,11\beta$-PGF_2, a compound which itself possesses appreciable biological activity on airway or vascular smooth muscle [8, 21, 22] and which is an inhibitor of platelet aggregation [20]. Our studies clearly predicate the β, as opposed to α, geometry of the C-11 hydroxyl function, but we cannot formally comment on the stereochemistry at other positions in the molecule. In view of the facile isomerisation of PGD_2 at Δ^{13} it is possible that the resultant PGD_2 isomers may also undergo metabolism by 11-KR to produce the corresponding isomers of $9\alpha,11\beta$-PGF_2. Further experiments will be necessary to evaluate this possibility, although we would expect to have resolved any major structural isomers that might have been formed in the present studies.

Studies investigating the metabolism of PGD_2 in man following either intravenous or inhaled administration reveal a complex pattern of metabolites. In the plasma we have identified both $9\alpha,11\beta$-PGF_2 and 13,14-dihydro-15-keto-$9\alpha,11\beta$-PGF_2 as metabolites of PGD_2 following either route of administration [39]. Roberts and colleagues have characterized the urinary metabolites of PGD_2 in normal man [18]. A total of 25 metabolites were identified, of which 23 had a cyclopentane-1,3-diol or F ring structure. Out of 15 metabolites in which the ring hydroxyl stereochemistry was assigned, 13 compounds had $9\alpha,11\beta$-geometry. These studies suggest that 11-ketoreduction is the principal route of PGD_2 metabolism, a view consistent with the absence of enzymatic metabolism in our NAD^+-supplemented cytosol preparations and it being a poor substrate for purified 15-PGDH ([40] Hoult and Robinson, unpublished observations). However, the finding in plasma and urine of PGF ring metabolites which had undergone C15 oxidation and Δ^{13}reduction [18] suggests that $9\alpha,11\beta$-PGF_2 may itself be an effective substrate for 15-PGDH as demonstrated in the

present study. Moreover, in human lung we have tentatively identified by GC/MS and HPLC both 15-keto-9α,11β-PGF_2 and 13,14-dihydro-15-keto-9α,11β-PGF_2 as metabolites of 9α,11β-PGF_2 [41], and the dihydro-keto metabolite of 9α,11β-PGF_2 is rapidly formed in the isolated perfused rat lung [42].

Our HPLC analyses showed the presence of two metabolites from 9α,11β-PGF_2 and these have relative retention values identical to the human lung metabolites which have been characterized by GC/MS. That the metabolism of 9α,11β-PGF_2 is indeed mediated by PGDH is supported by several lines of experimental evidence. Firstly, there was a significant correlation between the distribution of 15-PGDH activity and 9α,11β-PGF_2 biotransformation. Secondly, the azobenzene inhibitors of 15-PGDH attenuated both reactions. Thirdly, the formation of 13,14-dihydro-15-keto-9α,11β-PGF_2 and the NAD^+ dependence of the reaction is consistent with sequential 15-PGDH/Δ^{13}-reductase metabolism. It is possible that we have underestimated the initial rate of metabolism of 9α,11β-PGF_2 because formation of the 15-keto metabolite will result in loss of tritium from the molecule. Furthermore, it is possible that the 13,14-dihydro-15-keto metabolite may form an 11,15-hemiacetal, although the fact that we accounted for most of the radioactivity argues against this.

Thus, in conclusion we have mapped the distribution of PGD_2 11-KR and shown the enzyme to be distinct from another ketoreductase enzyme capable of metabolising a related prostaglandin. The product of reaction, 9α,11β-PGF_2, was itself further metabolised by 15-PGDH to produce metabolites tentatively identified as 15-keto- and 13,14-dihydro-15-keto,9α,11β-PGF_2. Further proof of these events will, of course, require studies with purified enzymes.

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