ON THE OCCURRENCE OF PROSTACYCLIN METABOLITES IN PLASMA AND VASCULAR TISSUE AS DETERMINED RADIOIMMUNOLOGICALLY

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1. Introduction

Circulating prostaglandins (PG) of the E and F series are rapidly inactivated, mainly in the lung [1], by the enzymes 15-hydroxy-PG-dehydrogenase and PG-Δ^{13}-reductase [2]. Thus, levels of the 15-keto-13,14-dihydro metabolites in plasma reflect more accurately PG biosynthesis than levels of the primary PG [2]. On the other hand, prostacyclin (PGI_2) has been suggested to be a circulating hormone [3,4], as its biological activity is not significantly diminished during passage through the lung [5], probably because it is not a substrate for the PG transport system across the cell membranes [6]. In aqueous solution PGI_2 is hydrated to the biologically much less active 6-keto-$PGF_{1\alpha}$ [7], which is only slowly metabolized by 15-hydroxy-PG-dehydrogenase and PG-Δ^{13}-reductase [8,9]. Contrary to 6-keto-$PGF_{1\alpha}$, PGI_2 has a high affinity for 15-hydroxy-PG-dehydrogenase of several organs [9–11] including vascular tissue [12] in vitro. While in the rat in vivo in one study [13] no metabolism of 6-keto-$PGF_{1\alpha}$ via the 15-hydroxy-PG-dehydrogenase pathway was observed, in other studies using different experimental conditions considerable oxidation of the C-15 hydroxyl group and reduction of the Δ^{13} double bond of exogenous PGI_2 [6,14] and to a smaller extent of 6-keto-$PGF_{1\alpha}$ [14] have been shown to occur. The stable metabolites of PGI_2 resulting from these enzyme reactions followed by hydration are 6,15-diketo-$PGF_{1\alpha}$ and 6.15-diketo-13,14-dihydro-$PGF_{1\alpha}$. We describe here radioimmunoassays for these two PGI_2 metabolites and determinations of the occurrence of these compounds in rat vascular tissue incubated in vitro as well as in rat and human plasma.

2. Materials and methods

PG and PG metabolites were a generous gift of Dr J. Pike, The Upjohn Co., Kalamazoo, MI. The haptens 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ were made immunogenic by coupling to bovine serum albumin (BSA, A grade, Calbiochem, San Diego, CA) by the method in [15]. BSA (7.5 mg) was dissolved in 2.5 ml distilled water and 4 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Ott Chemical Co., Muskegon, MI) was added. Then 3 mg hapten, dissolved in 0.2 ml dimethylformamide plus 1.7 ml 0.9% NaCl was added dropwise, the solution was stirred at room temperature for 1 h, then incubated overnight. The conjugate was dialyzed exhaustively against distilled water.

Rabbits were immunized by the schedule in [16] and blood was taken by puncture of the ear artery 10–14 days after booster injections. The blood was collected into 7.5% (v/v) of 77 mM sodium EDTA plus 0.1 mM indomethacin (final conc.) to inhibit synthesis of PG and thromboxanes (TX) by blood cells. The antiplasma was immediately separated from the blood cells by centrifugation at 4°C (1000 × g, 15 min).

The labelled ligands for both PGI_2 metabolite radioimmunoassays were prepared by incubation of 5 μCi [9-^{3}H(N)]PGI_2 (spec. act. 10.0 Ci/mmol, New England Nuclear Co., Dreieichenhain) as substrate with 1.0 ml 100 000 × g supernatant of guinea pig lung homogenate as enzyme source and 1 mM NAD as coenzyme in 1.1 ml total vol. The lung of one guinea pig was homogenized in 30 ml 0.1 M ice-cold potassium phosphate buffer (pH 7.4) containing 1 μg/ml indomethacin in order to prevent synthesis of PG

and TX during the enzyme preparation procedure. Incubations were performed at 37°C for 4 min for the synthesis of the tritiated ligand for the radioimmunoassay of 6,15-diketo-$PGF_{1\alpha}$ and for 60 min for the synthesis of the tritiated ligand for the radioimmunoassay of 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$. After acidification to pH 3.5 the incubation mixtures were extracted twice with 10 ml ethyl acetate. The combined extracts were evaporated and purified by thin-layer chromatography (solvent system: ethyl acetate/H_2O/isooctane/acetic acid, 110:100:50:20, by vol., upper phase, developed twice). The zones containing the radioactivity co-chromatographing with the authentic standards were eluted with methanol and used as tracers in the radioimmunoassays. An antiplasma against 6,15-diketo-$PGF_{1\alpha}$ bound 50% of the homologous tracer at a final dilution of 1:2000, while an antiplasma against 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ bound 50% of its homologous tracer at a final dilution of 1:60 000.

Radioimmunoassays were performed as in [16] for the determination of other PG, using either charcoal suspension or, in the case of plasma, the double antibody method with goat anti-rabbit γ-globulin (Calbiochem, San Diego, CA) as precipitating antibody to separate free and antibody-bound fractions of antigen. Charcoal-adsorbed plasma [16] was used as a PG-free reference plasma pool.

Rat thoracic and abdominal aorta (100 mg wet wt) was taken from ether-anesthetized animals (200–300 g). The tissue was cut into small pieces, washed and incubated in 2.0 ml Krebs-Henseleit bicarbonate buffer (pH 7.4) gassed with 95% O_2–5% CO_2, at 37°C for various time intervals. Aliquots of the incubates were analyzed in the radioimmunoassays for 6-keto-$PGF_{1\alpha}$ as well as for the PGI_2 metabolites. To further characterize the immunoreactive PGI_2 metabolites minced aortic tissue (200 mg) was incubated in 0.2 ml Krebs-Henseleit bicarbonate buffer and a 0.1 ml aliquot of the incubate was spotted onto a thin-layer plate (Kieselgel 60, Merck, Darmstadt) and chromatographed twice along with authentic standards using the above solvent system. After development the zones corresponding to the PGI_2 metabolites were eluted with methanol and rechromatographed. Then the area of the plate, where the material from the incubate had been developed, was cut into 1 cm zones from origin to solvent front. The zones were eluted with 1.0 ml methanol. The methanol was evaporated and the residues were analyzed in the radioimmunoassays for 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$.

Blood from healthy volunteers (age 19–30 years, 3 male, 5 female) was drawn by venipuncture from the antecubital vein. Blood from ether-anesthetized rats was taken from the carotid artery. The blood samples were collected into a mixture of sodium EDTA and indomethacin as described for the antiplasma obtained from immunized rabbits. The plasma was immediately separated from blood cells by centrifugation and the samples were stored frozen (−20°C) until assayed.

3. Results and discussion

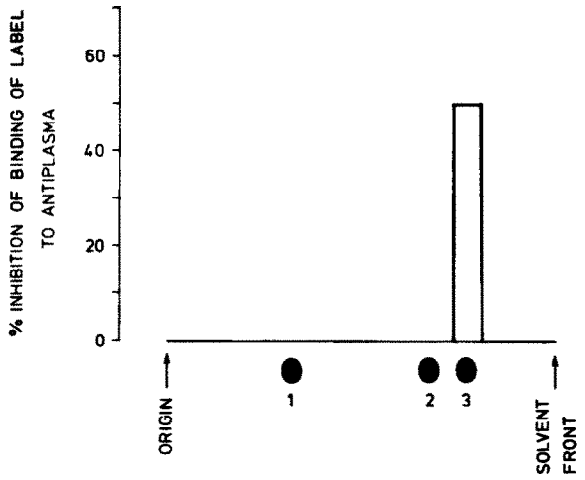
The specificity of the antibodies against 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ is shown in table 1. Both radioimmunoassays recognize the 15-keto group as an immunodominant part of the hapten molecules. In addition, the presence or absence of the Δ^{13} double bond in the PGI_2 metabolite molecules can be determined by the simultaneous use of both radioimmunoassays. The relative cross-reaction of related PG metabolites also shows that compounds with the immunodominant 15-keto group have a high affinity for the antibodies. However, the significant cross-reaction by 15-keto-PGE_2 and 15-keto-$PGF_{2\alpha}$ does not invalidate the radioimmunoassays, as these compounds are usually rapidly converted to the corresponding 15-keto-13,14-dihydro metabolites [2].

Table 2 shows, that a whole cell preparation of rat aorta releases in a time-dependent manner not only large amounts of immunoreactive 6-keto-$PGF_{1\alpha}$, the hydration product of PGI_2, but also significant quantities of immunoreactive PGI_2 metabolites. The PGI_2 metabolites formed during the incubation were additionally characterized by thin-layer chromatography. These experiments demonstrated that the immunoreactive PGI_2 metabolites co-chromatograph exclusively with the corresponding authentic standards. An example is shown in fig.1. These results validate the data obtained by direct radioimmunoassay of the incubates and exclude the possibility, that cross-reacting 6-keto-$PGF_{1\alpha}$ accounts for the immunoreactivity determined in the radioimmunoassays for 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$.

Although 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ can be formed from 6-keto-$PGF_{1\alpha}$ [8,9], PGI_2 has a much higher affinity for the

Table 1
Specificity of the radioimmunoassays for 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$

Inhibitor (ng)	50% displacement of labelled ligand in radioimmunoassay for:			
	6,15-diketo-$PGF_{1\alpha}$	Relative cross-reaction (%)	6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$	Relative cross-reaction (%)
6,15-diketo-$PGF_{1\alpha}$	0.30	100	3.90	5.6
6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$	1.32	22.7	0.22	100.0
15-keto-PGE_2	2.17	13.8	7.50	2.9
15-keto-$PGF_{2\alpha}$	2.87	10.5	35.00	0.6
15-keto-13,14-dihydro-$PGF_{2\alpha}$	14.50	2.1	2.60	8.5
15-keto-13,14-dihydro-PGE_2	16.50	1.8	17.50	1.3
6-keto-$PGF_{1\alpha}$	21.50	1.4	46.00	0.5
PGE_2	260.00	0.1	>500.00	<0.04
$PGF_{2\alpha}$	>500.00	<0.06	>500.00	<0.04
PGD_2	>500.00	<0.06	>500.00	<0.04
TXB_2	>500.00	<0.06	>500.00	<0.04



15-hydroxy-PG-dehydrogenase then has 6-keto-$PGF_{1\alpha}$ [9–12]. We therefore assume that the metabolites are formed from PGI_2 rather than from 6-keto-$PGF_{1\alpha}$. Using the cytoplasmic fraction of bovine mesenteric vessels as enzyme source and exogenous tritiated PGI_2 or 6-keto-$PGF_{1\alpha}$ as substrate [12] it was found that

Fig.1. Thin-layer chromatographic characterization (solvent system: ethyl acetate/H_2O/isooctane/acetic acid, 110:100:50:20, by vol., upper phase) of immunoreactive 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ released from a whole cell preparation of rat aorta. The immunoreactive material co-chromatographs as a single peak with the authentic standard. Spot (1) 6-keto-$PGF_{1\alpha}$, (2) 6,15-diketo-$PGF_{1\alpha}$, (3) 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$.

Table 2
Time-dependent release of immunoreactive 6-keto-$PGF_{1\alpha}$, 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ from a whole cell preparation of rat aorta

Incubation time (min)	6-keto-$PGF_{1\alpha}$	6,15-diketo-$PGF_{1\alpha}$	6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$
3	644 ± 157	51 ± 7	25 ± 3
10	2607 ± 492	134 ± 7	79 ± 9

Values are given in pg/mg wet wt and are the mean ± SEM of 6 expt

only [^{3}H]PGI_2 was metabolized extensively, while most of the 6-keto-[^{3}H]$PGF_{1\alpha}$ was recovered unchanged. Under the conditions used by these authors with exogenous substrate, added NAD or NADP as cofactors and a short incubation time (max. 2 min) the reaction product formed from PGI_2 was identified as 6,15-diketo-$PGF_{1\alpha}$. We find with a whole cell preparation of rat aorta as enzyme source, endogenous substrate and endogenous enzyme cofactors and longer incubation periods specific inhibition of the antigen–antibody reaction in both the radioimmunoassay for 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ (table 2). Obviously a mixture of both PGI_2 metabolites is formed under our experimental conditions. The conditions used in [12] might have delayed the expression of PG Δ^{13}-reductase activity [17].

The levels of the PGI_2 metabolites in unextracted normal rat (n = 10) and human (n = 8) plasma were found to be below the detection limits of the radioimmunoassays for 6,15-diketo-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-$PGF_{1\alpha}$ (25 pg/ml and 45 pg/ml, respectively). However, various amounts of exogenous PGI_2 metabolites (50 pg/ml–10 ng/ml) added to rat or human plasma were recovered quantitatively indicating that the failure to detect measurable amounts of these compounds in normal plasma is not due to non-specific factors interfering with the assay method, e.g., binding to plasma proteins. Our results suggest that the low or even undetectable levels of PGI_2 and 6-keto-$PGF_{1\alpha}$ [18–20] found in peripheral plasma under normal unstimulated conditions are obviously not caused by extensive metabolism to the 6,15-diketo and 6,15-diketo-13,14-dihydro derivatives. The possible formation of these PGI_2 metabolites in vivo during adminstration of exogenous PGI_2 or under conditions of stimulated endogenous PGI_2 synthesis is presently under investigation.

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