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Identification of 6-oxo-prostaglandin E_1 as a naturally occurring prostanoid generated by rat lung

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1 The spontaneous release of prostanoids from rat isolated perfused lungs was studied after acid/organic extraction of perfusates by bioassay, radioimmunoassay, thin layer and high performance liquid chromatographic methods and by gas chromatography-negative ion mass spectroscopy (g.c.-n.i.m.s.).

2 An acid/organic extractable anti-aggregatory vasodilator prostaglandin which inhibited the twitch response of the field-stimulated guinea-pig vas deferens was released from the Krebs-perfused rat lung in nanogram amounts similar to those of other detected prostanoids. Parallel biological assay suggested that this prostaglandin had very closely similar pharmacological activity to authentic 6-oxo-prostaglandin E_1 (6-oxo-PGE_1), a metabolite of prostacyclin (PGI_2) generated by the action of the enzyme 9-hydroxyprostaglandin dehydrogenase (9-PGDH).

3 6-oxo-PGE_1 was identified conclusively in extracts of rat lung perfusate by thin layer chromatography, high performance liquid chromatography and g.c./m.s. combined with bioassay (inhibition of platelet aggregation), and its covalent structure was defined by g.c. negative ion chemical ionization mass spectroscopy.

4 The rank order of spontaneous release of prostanoids (measured by radioimmunoassay) from the perfused rat lung was 6-oxo-$PGF_{1\alpha}$ > thromboxane B_2 (TXB_2) > PGE_2 > 6-oxo-PGE_1 (measured biologically) > $PGF_{2\alpha}$. Release of all five prostanoids was inhibited by indomethacin, but only that of 6-oxo-PGE_1 was inhibited by naringenin.

5 Rat lung 100,000 *g* cytosolic supernatants contained 9-PGDH activity capable of removing 9β-tritium from labelled prostacyclin and forming an acid/organic extractable 6-oxo-PGE_1-like anti-aggregatory substance. This 9-PGDH activity was inhibited by naringenin (IC_{50} 10.3 μM).

6 The relevance of these findings to the possible physiological role of 6-oxo-PGE_1 in the lung is discussed, and we propose that 6-oxo-PGE_1 should be accorded the status of a physiologically relevant, naturally occurring metabolite of arachidonic acid.

Introduction

6-oxo-Prostaglandin E_1 (6-oxo-PGE_1), a stable prostanoid with potent prostacyclin-like properties and thus of possible therapeutic interest, was first identified as a product of 9-hydroxyprostaglandin dehydrogenase (9-PGDH)-dependent metabolism of prostacyclin (PGI_2) in perfused rabbit liver (Wong *et al.*, 1980b). Since then, 9-PGDH enzymes capable of forming 6-oxo-PGE_1 from PGI_2 or, less commonly, 6-oxo-$PGF_{1\alpha}$, have been found in several tissues, notably kidney and platelets (Chang & Tai, 1982; Griffiths & Moore, 1983a; Griffiths *et al.*, 1985; Wong *et al.*, 1980a; Griffiths & Moore, 1983b). 6-oxo-PGE_1 shares many similar pharmacological properties with prostacyclin, and is often equipotent or more potent (for reviews see Moore & Griffiths, 1983; Moore & Hoult, 1985). For example, it is vasodilator and hypotensive (Quilley *et al.*, 1979; Van Dam *et al.*, 1981; Tod & Cassin, 1981), bronchodilator (Spannhake *et al.*,

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1981), inhibits platelet aggregation (Wong *et al.*, 1980b; Miller *et al.*, 1980; Whittle *et al.*, 1981; Berry & Hoult, 1983), and induces renal release of renin (Jackson *et al.*, 1981; McGiff *et al.*, 1982) and of erythropoietin (Nelson *et al.*, 1983).

The acceptance of 6-oxo-PGE_1 as an important physiologically relevant endogenous mediator ranking alongside other bioactive prostanoids (e.g. PGE_2, $PGF_{2\alpha}$, PGI_2, thromboxane A_2 (TXA_2) and PGD_2) requires proof of its generation from endogenous arachidonic acid in intact tissues. In this paper we have used the classical pharmacological technique of parallel quantitative bioassay on three independent test systems together with chromatographic methods to show that the rat isolated perfused lung continuously releases a 6-oxo-PGE_1-like substance in quantities similar to those of the above-mentioned prostanoids, and that the rat lung contains 9-PGDH activity capable of converting prostacyclin to 6-oxo-PGE_1. We have confirmed the identity of this lung-derived material unequivocally as 6-oxo-PGE_1 with the aid of a novel and sensitive mass spectrometric procedure. Some of these results have been communicated in preliminary form (Griffiths & Moore, 1983c).

Methods

Preparation and perfusion of rat isolated lungs

Male Wistar rats weighing 250 g were stunned and exsanguinated. The lungs were removed and perfused at 5 ml min^{-1} with warmed (37°C), oxygenated (95% O_2, 5% CO_2) Krebs solution (composition, mM: NaCl 65.3, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25, $CaCl_2$ 1.9 and glucose 11.1) via a cannula inserted into the pulmonary artery. Perfusate was collected every 10 min in 50 ml fractions, acidified with one or two drops of concentrated formic acid and extracted using Waters C-18 reverse phase Sep-Pak cartridges. Prostanoids were eluted with 10 ml ethyl acetate which was evaporated to dryness under a stream of air at 30°C. Extraction efficiencies obtained using radiolabelled prostanoid tracers (6 determinations) were PGE_2 85.3%, $PGF_{2\alpha}$ 90.8%, 6-oxo-$PGF_{1\alpha}$ 95.0% and TXB_2 85.0%, or measured biologically by inhibition of adenosine 5'-pyrophosphate (ADP)-induced human platelet aggregation (6 determinations) were PGE_1 88.0% and 6-oxo-PGE_1 84.0%, and, accordingly, the measured values in the reconstituted extracts have not been corrected.

Bioassay for 6-oxo-PGE_1-like activity

Dried rat lung perfusate extracts were resuspended in an appropriate volume (50–500 µl) Krebs solution and compared with authentic 6-oxo-PGE_1 using three biological assay preparations. For purposes of calibration and comparison, other biologically active prostanoids were also tested in these preparations, as indicated in the text.

Vasodilator activity was assessed as the ability to decrease mean arterial blood pressure (MABP) in anaesthetized rats. For these experiments, male Wistar rats of 300–350 g were anaesthetized by intraperitoneal injection of urethane (1.2 g kg^{-1}) and cannulae inserted retrogradely into the jugular vein (for injection of drugs) and into the carotid artery for measurement of blood pressure using a Bell & Howell pressure transducer coupled to a Devices pen recorder. At least 4 doses of extract or authentic prostanoid were injected into each of 6 rats.

To determine the platelet anti-aggregatory activity of the extract, 20 ml blood was obtained by clean venipuncture from non-medicated healthy human volunteers and anti-coagulated with 3.8% (w/v) trisodium citrate. Platelet-rich and platelet-poor plasma (PRP and PPP) were prepared by differential centrifugation (Moore, 1982). Aggregation was performed using 0.1 ml aliquots of PRP warmed to 37°C and stirred at 1100 rev min^{-1} in a Payton dual channel aggregometer, model 300BD. To determine the anti-aggregatory activity of extracts, aliquots (0.5–10 µl) were preincubated with PRP for 1 min before addition of 4–10 µM ADP and the degree of inhibition determined 3 min later by comparison with the control ADP response. Inhibition of ADP-induced aggregation by authentic 6-oxo-PGE_1 was also measured.

The effect of lung perfusate extract on contractions of the field-stimulated guinea-pig vas deferens was determined. Dunkin Hartley guinea-pigs (450 g) were killed by a blow to the head and exsanguinated. Vasa deferentia (epididymal end) were removed, cleared of connective tissue and mounted in 20 ml organ baths containing warmed and well-oxygenated Mg-free Krebs solution. Contractions were recorded using Grass FT03 force transducers connected to a Devices pen recorder. Preparations were field stimulated using platinum ring electrodes connected to an SRI square wave stimulator (0.1 Hz, 1 ms, 150 V). Extract or authentic 6-oxo-PGE_1 was added to the bath and kept in contact with the tissue for 1 min. Responses were measured in mg tension developed per twitch.

Chromatography

For thin layer chromatography (t.l.c.), dried residues were resuspended in 15 µl methanol and 5–10 µl applied onto plastic-backed silica gel-coated t.l.c. sheets (Kodak type 13181), together with 5 µg authentic 6-oxo-PGE_1, $PGF_{2\alpha}$, TXB_2, 6-oxo-$PGF_{1\alpha}$, PGE_1 and PGE_2 applied to separate channels. After development to a distance of 10 cm in ethyl acetate, acetone, acetic acid (90:10:1 v/v) the chromatogram was cut

into ten 1 cm sections from which prostanoids were eluted into 2 ml methanol. After evaporation of solvent, samples were redissolved in Krebs solution and assayed for platelet anti-aggregatory activity as described above.

For high performance liquid chromatography (h.p.l.c.), extracts were resuspended in 0.1 ml 0.034 M H_3PO_4/acetonitrile (2:1 v/v) and 0.05 ml injected onto a Partisil ODS-2 reverse phase column. Prostanoids were separated by isocratic elution with the same solvent at 0.85 ml min^{-1} and samples were collected at 1 min intervals and extracted twice into equal volumes of ethyl acetate. After evaporation of the solvent, samples were redissolved in Krebs solution and assayed for platelet anti-aggregatory activity or, alternatively, derivatized for gas chromatography/mass spectrometry (g.c./m.s.) as described below.

For g.c. negative ion chemical ionization m.s. (g.c.-n.i.c.i.m.s.), extracts prepared from the perfusate of 25–30 rat lungs were used. After preliminary purification by h.p.l.c. and derivatization by sequential reaction with methoxyamine hydrochloride in pyridine, pentafluorobenzyl bromide and bis-(trimethylsilyl) trifluoroacetamide (BSTFA), extracts were analysed using either a Finnigan 4000 or 4500 automated gas chromatograph mass spectrometer as described by Barrow *et al.* (1982). The n.i.c.i. mass spectrum of authentic 6-oxo-PGE$_1$ was obtained in like manner.

Radioimmunoassay

Dried residues from extracts of 50 ml lung perfusate were resuspended in 1 ml distilled water and 2 µl and 5 µl aliquots radioimmunoassayed for PGE$_2$, PGF$_{2\alpha}$, 6-oxo-PGF$_{1\alpha}$ and TXB$_2$ by a double antibody method (Dighe *et al.*, 1975) using antibodies kindly supplied by Dr F.D.C. Lytton. The sensitivity of these assays was 10–20 pg, and the coefficients of variation between and within assays were as follows (percentages): PGE$_2$ – 3.2, 11.6; PGF$_{2\alpha}$ – 12.6, 10.2; 6-oxo-PGF$_{1\alpha}$ – 17.5, 9.5 and TXB$_2$ – 15.7, 8.9. Percentage cross-reactivities against PGE$_2$, PGF$_{2\alpha}$, 6-oxo-PGF$_{1\alpha}$, TXB$_2$ and 6-oxo-PGE$_1$ for anti-PGE$_2$ were 100, 1.0, 0.7, 2.4 and 4.5, respectively; for anti-PGF$_{2\alpha}$ were 0.5, 100, 2.3, 0.3 and 0.2, respectively; for anti-6-oxo-PGF$_{1\alpha}$ were 4.0, 1.1, 100, 1.4 and 8.4, respectively; and for anti-TXB$_2$ were 0.2, 0.2, 0.2, 100 and 0.2, respectively.

Effect of drugs on prostanoid release from perfused lung

In some experiments rat isolated lungs were perfused for 10 min with Krebs solution containing either the cyclo-oxygenase inhibitor, indomethacin (20 µM), or naringenin (100 µM), a selective inhibitor of the enzyme 9-PGDH.

Assay of 9-PGDH activity in rat lung 100,000 g cytosolic fractions

Cell-free 100 000 *g* supernatants were prepared from homogenized rat lungs and incubated with 1 µg ml^{-1} prostacyclin containing 0.05 µCi [9β-^{3}H]-prostacyclin tetramethylammonium salt as previously described (Griffiths & Moore, 1983a). Conversion to E-type prostaglandins by loss of tritium label at the 9β-position was measured by comparing the acid/organic-extractable radioactivity at timed intervals with that in samples extracted at zero time on ice; presence of 6-oxo-PGE$_1$ was also measured by resuspending part of the extract in 0.9% w/v NaCl solution and testing its platelet anti-aggregatory activity as described above.

Statistics

Results show mean ± s.e.mean with the number of observations shown in parentheses. Statistically significant differences between groups were determined using Student's unpaired *t* test.

Materials

PGI$_2$ sodium salt (stored at 1 mg ml^{-1} in 0.1 M NaOH, – 20°C) and 6-oxo-PGE$_1$ were gifts from Wellcome Research Laboratories, Beckenham, Kent, and the Upjohn Company, Kalamazoo, U.S.A., respectively. All other prostanoids were obtained from Sigma (London) Ltd, Poole, Dorset and dissolved in methanol at 1 mg ml^{-1} and kept at – 20°C until used. Indomethacin and naringenin (Sigma) were dissolved in 0.5% (w/v) Na_2CO_3 solution. Radiolabelled prostanoids were obtained as follows: the multitritiated [^{3}H$_n$] prostanoids used for radioimmunoassays had specific activities of 140–180 Ci mmol^{-1} and were from Amersham International, whereas the [9β-^{3}H]-prostacyclin (sp. act. 12 Ci mmol^{-1}) was from New England Nuclear (Dupont).

Results

Pharmacological activity of rat lung perfusate extracts

Analysis of extracted lung perfusates on the three bioassay test systems showed that they possessed a profile of biological activity very closely similar to that of authentic 6-oxo-PGE$_1$ (Figure 1a). Two series of experiments using 6 lungs in each case were performed. In the first set of experiments the estimated rate of release of 6-oxo-PGE$_1$-like material was 0.91 ± 0.18 ng min^{-1}, $n = 9$ (measured as the ability to inhibit ADP-induced human platelet aggregation), or 0.95 ± 0.13 ng min^{-1}, $n = 9$, measured as the fall in

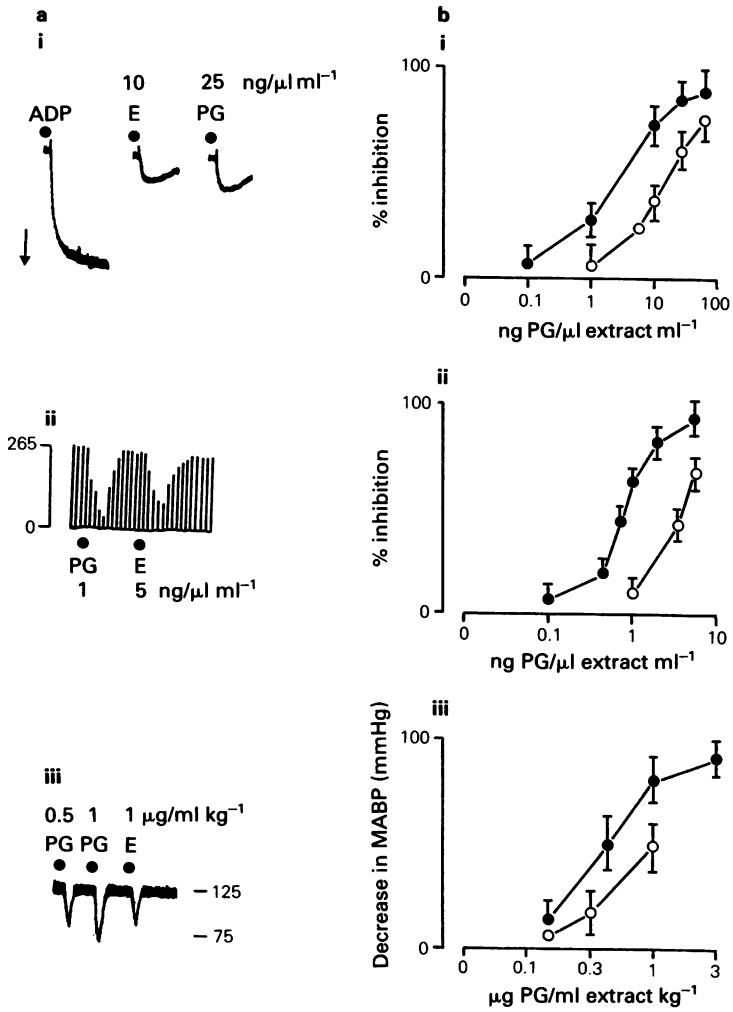

Figure 1 Biological activity of extracted lung perfusate. (a) Representative traces comparing the effects of authentic 6-oxo-prostaglandin E_1 (6-oxo-PGE_1; labelled PG in figure) with extracted rat lung perfusate (E) on ADP-induced human platelet aggregation (i, direction of light transmittance shown by arrow), contraction of the field-stimulated guinea-pig vas deferens (ii) and rat blood pressure (iii). (b) Dose-response curves comparing the biological effects of 6-oxo-PGE_1 (PG, ●) with lung perfusate (○) (expressed as μl or ml volume) in each of the three bioassays. Data points show mean and vertical lines s.e. mean for 12 observations (platelet, vas deferens) or 6 observations (blood pressure).

mean arterial blood pressure after intravenous injection in the anaesthetized rat. In the second set of experiments, the rate of release was 0.41 ± 0.10 ng min^{-1}, $n = 12$ (measured using the inhibition of twitch responses to field stimulation of the guinea-pig isolated vas deferens preparation), compared to 0.36 ± 0.06 ng min^{-1}, $n = 12$, assayed on platelet aggregation inhibition. The 'within experiment' results are not significantly different. The identity of the material in the rat lung perfusate extracts as 6-oxo-PGE_1 was further supported by the parallelism of the extract curves and those of authentic 6-oxo-PGE_1 in all three bioassay systems (Figure 1b), whereas other possible prostanoid candidates can be excluded as follows.

The observed biological activity cannot be accounted for by any other single prostaglandin because PGE_1, PGE_2 and PGI_2, although vasodepressor, do not all inhibit platelet aggregation (PGE_2), inhibit twitches of the field-stimulated guinea-pig vas deferens (PGD_2) or reduce blood pressure in the anaesthetized rat (PGD_2). Furthermore, any PGI_2 released

Table 1 Effect of drugs on prostanoid release from rat isolated lungs

	6-oxo-PGE$_1$	*6-oxo-PGF$_{1\alpha}$*	*PGE$_2$*	*PGF$_{2\alpha}$*	*TXB$_2$*
Control	0.7 ± 0.1	4.2 ± 1.2	1.0 ± 0.5	0.1 ± 0.02	2.5 ± 0.2
Indomethacin (20 μM)	0.2 ± 0.05*	1.1 ± 0.3*	0.2 ± 0.05*	0*	0.1 ± 0.1*
Control	0.8 ± 0.1	2.7 ± 0.5	0.7 ± 0.4	0.1 ± 0.05	0.6 ± 0.1
Naringenin (100 μM)	0.3 ± 0.1*	3.1 ± 0.6	0.6 ± 0.1	0.1 ± 0.03	0.5 ± 0.1

Results show prostanoid release (ng min^{-1}) from 4 to 6 rat lungs. Perfusate was collected in 10 min portions (50 ml) over the period 30–40 min after starting the experiment (control), and then indomethacin or naringenin was added to the Krebs reservoir and perfusate collected 20 min later, viz. 60–70 min after starting the experiment. * $P < 0.05$ compared to rate in control period.

from the lung is rapidly hydrolysed during the extraction procedure and appears as 6-oxo-PGF$_{1\alpha}$ which has minimal biological activity in all the assays used. However, this does not rule out the possibility that a mixture of prostaglandins in the lung eluate contributes to the biological activity we have observed. For this reason, extracts combined from 6 lungs were assayed not only against 6-oxo-PGE$_1$ but also against other prostaglandins with similar biological activity in each bioassay preparation. In this set of experiments the rate of release of 6-oxo-PGE$_1$-like material as ng min^{-1} and estimated against authentic 6-oxo-PGE$_1$ was 0.87 ± 0.11 (anti-aggregatory assay), 0.78 ± 0.06 (vas deferens assay) and 0.80 ± 0.14 (vasodepressor assay) (ratio = 1.1:1.0:1.0). In contrast, when the samples were assayed against authentic PGE$_1$ and PGE$_2$ (in parentheses) the estimated rate of release was 17.1 ± 1.6 (no value for PGE$_2$ which potentiates ADP-induced human platelet aggregation), 2.12 ± 0.67 (6.91 ± 1.2) and 2.46 ± 0.8 (5.2 ± 1.3). The ratio of biological activity for PGE$_1$ and PGE$_2$ (in parentheses) for the three assays was thus 8.1:1.0:1.2 (0:1.3:1.0). Assayed in terms of PGD$_2$ the lung released 9.4 ± 0.9 ng min^{-1} anti-aggregatory activity. Since PGD$_2$ has little effect on the twitch response of the guinea-pig isolated vas deferens preparation and is pressor in the anaesthetized rat it was not possible to assay lung extracts in terms of PGD$_2$ using these methods. Thus, calculation of the index of discrimation for 6-oxo-PGE$_1$, PGE$_1$ and PGE$_2$ provides further evidence for the identification of 6-oxo-PGE$_1$ in extracted rat lung perfusates. Moreover, it is quite clear that PGD$_2$, PGE$_1$ and PGE$_2$ cannot account for the observed profile of biological activity. These conclusions are reinforced by chromatographic and mass spectrometric evidence as explained in subsequent sections.

Release of other prostanoids from perfused rat lungs

Although released in nanogram quantities, 6-oxo-PGE$_1$ is not the most abundant cyclo-oxygenase metabolite generated by the rat perfused lung under basal perfusion conditions. The rate of release of four other major prostanoids was determined by radioimmunoassay and showed that the rank order of release was 6-oxo-PGF$_{1\alpha}$ > TXB$_2$ > PGE$_2$ > PGF$_{2\alpha}$ with rates of release (expressed as ng min^{-1}, $n = 8$, in perfusate collected over 10 min duration 30 min after starting perfusion) of 3.1 ± 0.6, 1.94 ± 0.2, 0.6 ± 0.2 and 0.45 ± 0.09, respectively.

We have also studied the effect on pulmonary prostanoid release of drugs which selectively interfere with arachidonic acid metabolism (Table 1). Perfusion of lungs with indomethacin, a potent cyclo-oxygenase inhibitor, very substantially reduced the pulmonary efflux of all five prostanoids as expected, whilst naringenin, a selective inhibitor of 9-PGDH (Moore *et al.*, 1983), reduced the release of 6-oxo-PGE$_1$ by more than 90% without affecting the release of the other four prostanoids.

The two experiments in Table 1 also confirm the general pattern of prostanoid abundance described above; together with similar experiments on rats of Peers & Hoult (1985) and averaging results from (n) animals, the rates of basal release (ng min^{-1}) are 6-oxo-PGF$_{1\alpha}$ – 3.31 ± 0.15 (28), TXB$_2$ – 2.08 ± 0.16 (28), PGE$_2$ – 0.73 ± 0.04 (28), 6-oxo-PGE$_1$ – 0.64 ± 0.04 (35) (measured by bioassay) and PGF$_{2\alpha}$ – 0.22 ± 0.04 (28).

Chromatographic identification of 6-oxo-PGE$_1$

Direct supportive physicochemical evidence for the identity of the biologically active material in pulmonary perfusates as 6-oxo-PGE$_1$ was obtained using thin layer and reverse phase high performance liquid chromatography (Figure 2). In each case, the biologically active material (assayed in terms of inhibition of ADP-induced platelet aggregation) appeared as a single peak comigrating with authentic 6-oxo-PGE$_1$. No anti-aggregatory activity was detected comigrating with any other known prostanoid, in agreement with our conclusions from parallel bioassay.

In order to obtain conclusive evidence for the

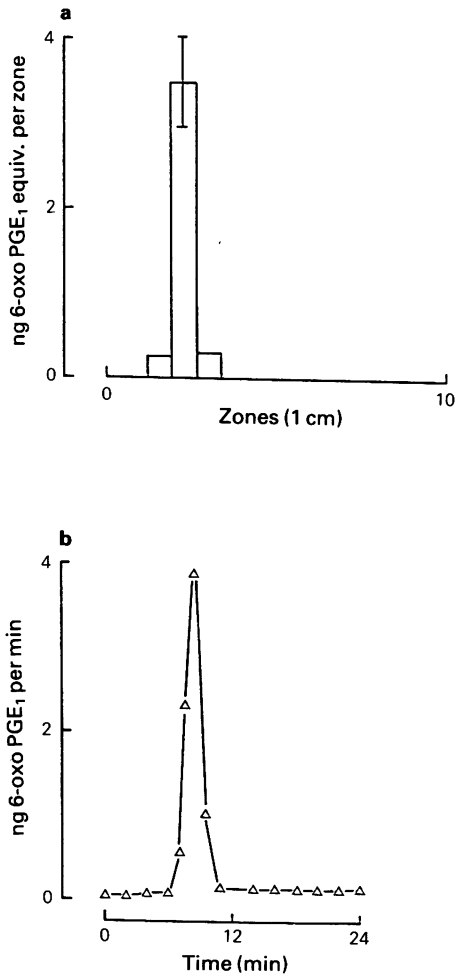

Figure 2 Chromatography of prostaglandin-like material from extracted lung perfusates. Thin layer chromatography (a) was performed as described in Methods. Biological activity of eluted sections from the chromatogram was determined as ability to inhibit ADP-induced human platelet aggregation and expressed as ng 6-oxo-prostaglandin E_1 (6-oxo-PGE_1) equivalents per 1 cm section. R_F values of some authentic prostaglandin standards are as follows: 6-oxo-$PGF_{1\alpha}$ 0.12, $PGF_{2\alpha}$ 0.15, thromboxane B_2 (TXB_2) 0.23, 6-oxo-PGE_1 0.24, PGE_1 0.34, PGE_2 0.35, PGD_2 0.44. Eluate from the h.p.l.c. column (b) was extracted and bioassayed for anti-aggregatory activity. Results show ng 6-oxo-PGE_1-like biological activity per 1 min collection period. Retention times for prostaglandins were 6-oxo-$PGF_{1\alpha}$ 9.3 min, 6-oxo-PGE_1 10.6 min, TXB_2 14.6 min, $PGF_{2\alpha}$ 20.5 min, PGE_1 25.4 min, PGD_2 27.6 min and PGE_2 32.8 min. Extraction efficiencies for 6-oxo-PGE_1 at 1 $\mu g\,ml^{-1}$ through the t.l.c. and h.p.l.c. procedures were 51.1 ± 5.6% and 57.8 ± 5.1% ($n = 6$), respectively.

identity of 6-oxo-PGE_1 as the biologically active principle in the lung perfusates, we turned to a sensitive mass spectrometric assay. For these experiments, perfusates from several rat lungs were pooled, extracted and the 6-oxo-PGE_1-like material purified by h.p.l.c. Half of the purified material was retained and bioassayed against authentic 6-oxo-PGE_1 for its ability to inhibit ADP-induced human platelet aggregation. An aliquot of the remainder was derivatized for g.c.-n.i.c.i.m.s. as described in Methods.

The partial n.i.c.i. mass spectrum of the methoxime-trimethylsilyl ether-pentafluorobenzyl ester (MOX-TMS-PFB) derivative of the biologically active substance in rat lung perfusate exhibited an intense fragment ion $(M\text{-}PFB)^-$ at m/z 569 (Figure 3a), and little further fragmentation occurs. This ion corresponds to dimethoximated, di-methylsilylated 6-oxo-PGE_1 carboxylate anion. The spectrum was indistinguishable from that of the authentic prostanoid (Figure 3b). Further, the gas chromatography retention times of perfusate-derived synthetic 6-oxo-PGE_1 derivatives were identical ($t_r = 11.7$ min for the major methoxime isomer on SE54), and were different from other prostanoids.

Derivatization using deuterated reagents provided further evidence that intact rat lungs release 6-oxo-PGE_1. When the h.p.l.c. purified lung perfusate extract was derivatized with $^2H_3$-methoxyamine hydrochloride, the mass of the fragment ion $(M\text{-}PFB)^-$ so obtained was increased by 6 mass units (from m/z 569 to m/z 575), indicating the presence of two carbonyl functions per molecule. Similarly, derivatization with $^2H_3$-BSTFA resulted in a shift of the major $(M\text{-}PFB)^-$ fragment ion to m/z 587, defining the presence of two hydroxyl groups (Figure 3c and d). In each case authentic 6-oxo-PGE_1 analysed immediately after the naturally occurring substance behaved similarly. Importantly, in a g.c./m.s. quantitative study of fractions from h.p.l.c. purification of rat lung material, the rise and fall of 6-oxo-PGE_1 determined spectrometrically correlated well with biological activity in tests from the same h.p.l.c. fractions, further strengthening the covalent structural assignment.

Assay of 9-PGDH activity in rat lung 100,000 g cytosolic fractions

Rat lung 100,000 *g* supernatant (protein concentration 15.1 ± 0.6 mg ml^{-1}, $n = 6$) converted radiolabelled prostacyclin to nonradioactive metabolite(s) as determined by the loss of radioactivity method (Figure 4a). The rate of utilization of PGI_2 measured over the first 30 min incubation (0.27 ± 0.03 ng mg protein^{-1} min^{-1}, $n = 8$) matched the rate of formation of 6-oxo-PGE_1 assayed after acid/organic extraction for its ability to inhibit ADP-induced human platelet

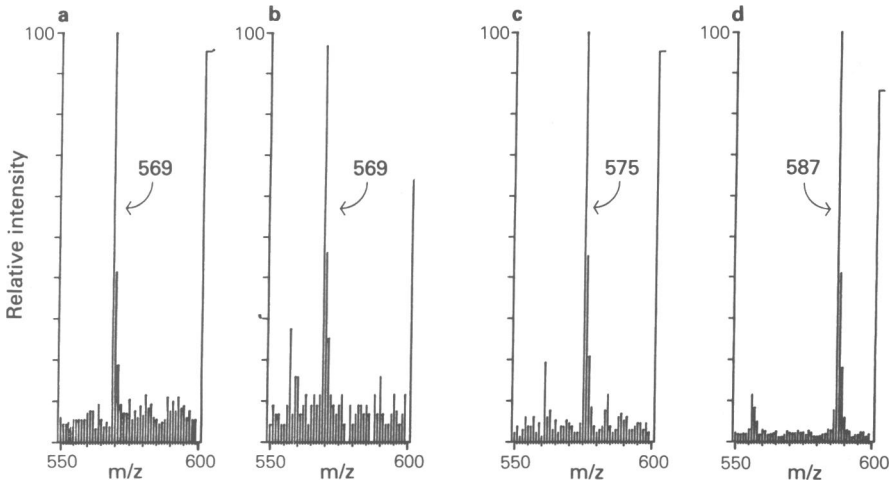

Figure 3 Partial negative ion chemical ionization (n.i.c.i.) mass spectra of (a) rat lung 6-oxo-prostaglandin E_1 (6-oxo-PGE_1) (methoxime-trimethylsilyl ether-pentafluorobenzyl ester; MOX/TMS/PFB derivative); (b) authentic 6-oxo-PGE_1 (MOX/TMS/PFB derivative). In both cases an intense $(m\text{-}PFB)^-$ fragment ion corresponding to the MOX_2 TMS_2 carboxylate anion of 6-oxo-PGE_1 was observed at m/z 569. (c) Rat lung 6-oxo-PGE_1 ($^2H_3$-MOX/TMS/PFB derivative). The fragment ion shifts to m/z 575, indicating the presence of 2 carbonyl functions; (d) rat lung 6-oxo-PGE_1 (MOX/$^2H_9$ TMS/PFB derivative). The 18 mass unit shift to m/z 587 arises from 2 hydroxyl groups in the molecule. Note that methoximation of 6-oxo-PGE_1 results in the formation of two pairs of syn/anti isomers with different gas chromatography retention times but identical mass spectra.

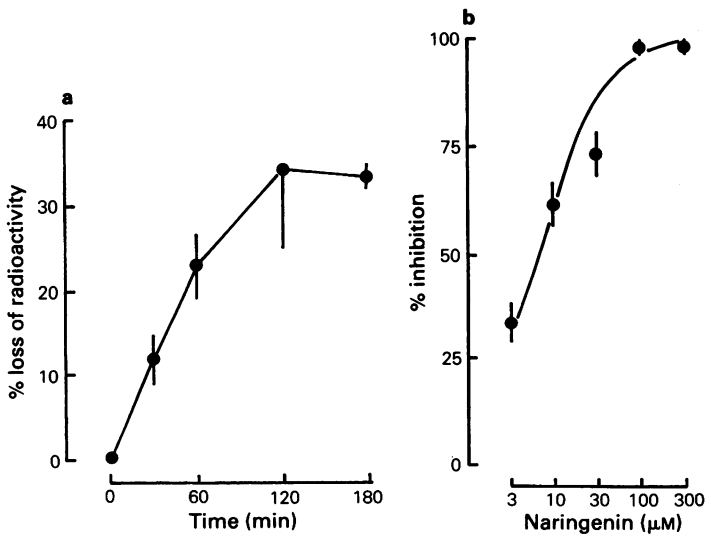

Figure 4 Presence of 9-hydroxyprostaglandin dehydrogenase (9-PGDH) activity in rat lung 100,000*g* cytosolic fraction and its inhibition by naringenin. (a) Conversion of radiolabelled prostaglandin I_2 (PGI_2) to 6-oxo-PGE_1 assessed by loss of acid/organic extractable radioactivity. (b) Inhibition of 6-oxo-PGE_1 formation by naringenin. Results show mean, with vertical lines indicating s.e.mean, of data from 8 lungs.

aggregation (0.28 ± 0.05 ng mg protein^{-1} min^{-1}, $n = 8$). At zero time there was no acid/organic extractable anti-aggregatory activity (complete loss of PGI_2 and formation of 6-oxo-$PGF_{1\alpha}$ in amounts insufficient to affect platelet function). Thus the loss of radioactivity method may be assumed to be a valid index for the conversion of PGI_2 to 6-oxo-PGE_1.

Formation of 6-oxo-PGE_1 plateaued after 120 min incubation ($34.6 \pm 6.7\%$ PGI_2 converted, $n = 8$). The inclusion of NAD^+ ($19.2 \pm 3.1\%$ converted, $n = 8$) and $NADP^+$ ($9.5 \pm 4.7\%$ PGI_2 converted, $n = 8$) inhibited the oxidation of PGI_2 to 6-oxo-PGE_1 (for both $P < 0.01$). No loss of radioactivity was detected following incubation for up to 120 min in Tris-HCl buffer at pH 8.2 or in rat lung 100,000 g cytosolic fraction boiled for 2 min. Naringenin inhibited the formation of 6-oxo-PGE_1 from PGI_2 in a dose-dependent manner (Figure 4b), and the concentration required for 50% inhibition (IC_{50}) of rat lung 9-PGDH activity was 10.3 ± 0.8 μM, $n = 8$.

Discussion

These experiments show that nanogram quantities of 6-oxo-PGE_1 are continuously released from the rat isolated perfused lung. This prostaglandin has a wide range of potent biological actions in the body, as described in the Introduction and in the reviews cited therein. We propose that 6-oxo-PGE_1 should now be accorded the status of a naturally occurring and potentially physiologically important endogenous metabolite of arachidonic acid.

The cellular source of 6-oxo-PGE_1 in the lungs is not known, but our experiments suggest strongly that its formation depends upon the actions of cyclo-oxygenase and 9-PGDH, i.e. that prostacyclin is an intermediate, and thus that its source is likely to be vascular endothelial cells. Indeed, a recent study has shown that cultured rabbit coronary microvessel endothelial cells contain 9-PGDH (Gerritsen & Cheli, 1983), and blood vessels have been shown to be capable of 9-PGDH-catalysed transformation of F-series prostaglandins (Wong *et al.*, 1977). Furthermore, we have also shown that 100,000 g cytosolic supernatants from homogenized rat lungs effectively convert radiolabelled prostacyclin to a non-radioactive 9β-tritium-depleted substance (Figure 4) characterized biologically (platelet aggregation inhibition) as 6-oxo-PGE_1. In separate experiments (data not shown), this biologically active material from the rat lung cytosolic incubations was found to cochromatograph with 6-oxo-PGE_1 in both t.l.c. and h.p.l.c. systems.

Platelets also contain an NAD^+-dependent 9-PGDH enzyme capable of converting PGI_2 or 6-oxo-$PGF_{1\alpha}$ to 6-oxo-PGE_1, either *in vitro* (Wong *et al.*, 1980a) or in intact platelets (Quilley *et al.*, 1980; Griffiths & Moore, 1983b; Berry & Hoult, 1983). Thus it appears that there is a widespread system in both the vasculature and formed elements of the blood with the potential for generating not only prostacyclin, the implications of which have been widely discussed, but also of 6-oxo-PGE_1.

The continuous basal release of 6-oxo-PGE_1 from rat lung is in the nanogram range, easily detectable by the bioassays we have used, and is similar in magnitude to that of PGE_2, but less than that of prostacyclin or thromboxane A_2. However, as with prostacyclin, the amounts are likely not to be sufficient to affect platelet function. This is also in line with preliminary estimates of the amount of 6-oxo-PGE_1 in the blood of rabbit (less than 70 pg ml^{-1}, Haslam & McLenaghan, 1981), humans (<9 to 30 pg ml^{-1}, Jackson *et al.*, 1982) and rat (up to 52 pg ml^{-1} in 2 out of 8 samples tested, Pace-Asciak & Micallef, 1984), and suggests that, like prostacyclin, the compound has a local hormonal rather than circulating hormone-like function. It is also possible that measurements of 6-oxo-PGE_1 levels in blood may be serious underestimates of total production because of its transformation to as yet unknown catabolites (see Moore & Hoult, 1985, for further discussion). There is also the possibility that the lung may be capable of releasing pulses of 6-oxo-PGE_1 in response to appropriate pathophysiological triggers, and this may affect the lungs, heart and associated vasculature since 6-oxo-PGE_1 not only inhibits platelet aggregation at subnanogram levels (Moore & Griffiths, 1983), but also stimulates fibrinolysis (Korbut *et al.*, 1983; Hussaini & Moore, 1985) and is bronchodilator (Spannhake *et al.*, 1981). In both of the latter effects 6-oxo-PGE_1 is considerably more potent than its precursor, prostacyclin.

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References

BARROW, S.E., WADDELL, K.A., ENNIS, M., DOLLERY, C.T. & BLAIR, I.A. (1982). Analysis of picogram concentrations of 6-oxo-prostaglandin $F_{1\alpha}$ in biological fluids. *J. Chromatogr.*, **239**, 71–80.

BERRY, C.N. & HOULT, J.R.S. (1983). 6-keto-prostaglandin E_1: its formation by platelets from prostacyclin and resistance to pulmonary degradation. *Pharmacology*, **26**, 324–330.

CHANG, D.G-B. & TAI, H.H. (1982). A radioimmunoassay for 6-keto-PGE$_1$. *Prostaglandins leukotrienes Med.*, **8**, 11–20.

DIGHE, K.K., EMSLIE, H.A., HENDERSON, L.K., RUTHERFORD, F. & SIMON, L. (1975). The development of antisera to prostaglandin B_2 and F_2 and their analysis using solid-phase and double antibody radioimmunoassay methods. *Br. J. Pharmac.*, **55**, 503–514.

GERRITSEN, M.E. & CHELI, C.D. (1983). Arachidonic acid and prostaglandin endoperoxide metabolism in isolated rabbit coronary microvessels and isolated and cultivated coronary microvessel endothelial cells. *J. clin. Invest.*, **72**, 1658–1671.

GRIFFITHS, R.J. & MOORE, P.K. (1983a). Formation of 6-keto-prostaglandin E_1 in mammalian kidneys. *Br. J. Pharmac.*, **79**, 149–155.

GRIFFITHS, R.J. & MOORE, P.K. (1983b). Conversion of prostaglandin to 6-oxo-prostaglandin E_1 by rat, rabbit, guinea-pig and human platelets. *Br. J. Pharmac.*, **80**, 395–402.

GRIFFITHS, R.J. & MOORE, P.K. (1983c). Spontaneous release of a 6-keto-PGE$_1$ like substance from isolated perfused rat lungs. *Br. J. Pharmac.*, **80**, 442P.

GRIFFITHS, R.J., BERRY, C.N., HOULT, J.R.S. & MOORE, P.K. (1985). Age-dependent changes in the synthesis and catabolism of 6-oxo-PGE$_1$ and other prostanoids by the rat kidney *in vitro*. *Biochem. Pharmac.*, **34**, 223–228.

HASLAM, R.J. & McCLENAGHAN, M.D. (1981). Measurement of circulating prostacyclin. *Nature*, **292**, 364–365.

HUSSAINI, I. & MOORE, P.K. (1985). Fibrinolytic effects of prostanoids in the rat. *Br. J. Pharmac. Proc. Suppl.*, (in press).

JACKSON, E.K., GOODWIN, R.P., FITZGERALD, G.A., OATES, J.A. & BRANCH, R.A. (1982). Assessment of the extent to which exogenous prostaglandin I_2 is converted to 6-keto-prostaglandin E_1 in human subjects. *J. Pharmac. exp. Ther.*, **221**, 183–187.

JACKSON, E.K., HERZER, W.A., ZIMMERMAN, J.B., BRANCH, R.A., OATES, J.A. & GERKENS, J.F. (1981). 6-keto-prostaglandin E_1 is more potent that prostaglandin I_2 as a renal vasodilator and renin secretagogue. *J. Pharmac. exp. Ther.*, **216**, 24–27.

KORBUT, R., BYRSKA-DANEK, A. & GRYGLEWSKI, R.J. (1983). Fibrinolytic activity of 6-oxo-PGE$_1$. *Thromb. Haemostas.*, **50**, 893.

McGIFF, J.C., SPOKAS, E.G. & WONG, P.Y-K. (1982). Stimulation of renin release by 6-oxo-prostaglandin E_1 and prostacyclin. *Br. J. Pharmac.*, **75**, 137–144.

MILLER, O.V., AIKEN, J.W., SHEBUSKI, R.J. & GORMAN, R.R. (1980). 6-keto-prostaglandin E_1 is not equipotent to prostacyclin (PGI_2) as an anti-aggregatory agent. *Prostaglandins*, **20**, 391–396.

MOORE, P.K. (1982). Prazosin potentiates the effect of prostacyclin on platelets. *Thromb. Haemostas.*, **47**, 76.

MOORE, P.K. & GRIFFITHS, R.J. (1983). 6-keto-prostaglandin E_1. *Prostaglandins*, **26**, 509–517.

MOORE, P.K., GRIFFITHS, R.J. & LOFTS, F.J. (1983). The effect of some flavone drugs on the conversion of prostacyclin to 6-oxo-prostaglandin E_1. *Biochem. Pharmac.*, **32**, 2813–2817.

MOORE, P.K. & HOULT, J.R.S. (1985). Formation and inactivation of 6-keto-PGE$_1$, a prostacyclin metabolite of possible physiological relevance. In *Prostacyclin: New Basic and Clinical Studies*, ed. Kharasch, N. & Watkins, G.L., Los Angeles (in press).

NELSON, P.K., BROOKINS, J. & FISHER, J.W. (1983). Erythropoietic effects of prostacyclin (PGI_2) and its metabolite 6-keto-prostaglandin E_1. *J. Pharmac. exp. Ther.*, **226**, 493–499.

PACE-ASCIAK, C. & MICALLEF, S. (1984). Catabolism of 6-keto prostaglandin E_1 by rat kidney homogenates. *Can. J. Biochem. cell Biol.*, **62**, 709–714.

PEERS, S.H. & HOULT, J.R.S. (1985). Inhibition by ethanol and mepacrine of phospholipase-dependent prostaglandin release from the isolated perfused rat lung. *Eur. J. Pharmac.*, (in press).

QUILLEY, C.P., McGIFF, J.C., LEE, W.H., SUN, F.F. & WONG, P.Y-K. (1980). 6-keto-PGE$_1$: A possible metabolite of prostacyclin having platelet anti-aggregatory effects. *Hypertension*, **2**, 524–528.

QUILLEY, C.P., WONG, P.Y-K. & McGIFF, J.C. (1979). Hypotensive and renovascular actions of 6-keto-prostaglandin E_1, a metabolite of prostacyclin. *Eur. J. Pharmac.*, **57**, 273–276.

SPANNHAKE, E.E., LEVIN, G.L., HYMAN, A.L. & KADOWITZ, P.J. (1981). 6-keto-PGE$_1$ exhibits more potent bronchodilatory activity in the cat than its precursor, PGI_2. *Prostaglandins*, **21**, 266–275.

TOD, M.C. & CASSIN, S. (1981). Effects of 6-keto-prostaglandin E_1 on perinatal pulmonary vascular resistance. *Proc. Soc. exp. biol. Med.*, **166**, 148–152.

VAN DAM, J., FITZPATRICK, T.M., FRIEDMAN, L.S., RAMWELL, P.W., ROSE, J.C. & KOT, P.A. (1981). Cardiovascular responses to 6-keto-prostaglandin E_1 in the dog. *Proc. Soc. exp. biol. Med.*, **166**, 76–79.

WHITTLE, B.J.R., MONCADA, S. & VANE, J.R. (1981). Biological activity of some metabolites and analogues of prostacyclin – In Medical Chemistry Advances, ed. De Las Heras, F.G. & Vega, S. pp. 141–160. Oxford: Pergamon Press.

WONG, P.Y-K., LEE, W.H., CHAO, P.H-W., REISS, R.F. & McGIFF, J.C. (1980a). Metabolism of prostacyclin by 9-hydroxyprostaglandin dehydrogenase in human platelets. *J. biol. Chem.*, **225**, 9021–9024.

WONG, P.Y-K., MALIK, D., DESIDERIO, J.C., McGIFF, J.C. & SUN, F.F. (1980b). Hepatic metabolism of prostacyclin (PGI_2) in the rabbit: Formation of a potent novel inhibitor of platelet aggregation. *Biochem. biophys. Res. Commun.*, **93**, 486–494.

WONG, P-Y.K., TERRAGNO, D.A., TERRAGNO, N.A. & McGIFF, J.C. (1977). Dual effects of bradykinin on prostaglandin metabolism: Relationship to the dissimilar vascular actions of kinins. *Prostaglandins*, **13**, 1113–1115.

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