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FURTHER STUDIES ON THE ENZYMATIC CONVERSION OF PROSTAGLANDIN ENDOPEROXIDE INTO PROSTACYCLIN BY PORCINE AORTA MICROSOMES

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Summary

A simple, rapid radiochemical assay for prostacyclin synthesis has been used to characterize the enzyme in arterial walls which converts prostaglandin endoperoxides to prostacyclin. The enzyme displays a broad pH optimum, and catalyses a rapid conversion of saturating concentrations of the endoperoxide at 37°C. Hydroperoxides of several unsaturated fatty acids are potent inhibitors of the enzyme, and act in a time dependent manner. The isomerase which converts prostaglandin endoperoxides to prostaglandin E_2 or D_2 was not detected in the arterial wall.

Introduction

Recently, a novel prostaglandin with potent anti-aggregatory and vasodilator properties was detected when prostaglandin endoperoxides were incubated with blood vessels [1—4]. This compound (initially called prostaglandin X) was unstable, having a half-life of approx. 10 min at room temperature in aqueous media at pH 7.6 [5]. The degradation product was stable but possessed little biological activity [1]. Prostaglandin X was subsequently identified as (5z)-9-deoxy-6,9α-epoxy-Δ^5 prostaglandin $F_{1\alpha}$ and was renamed prostacyclin or PGI_2 (it has also been called prostaglandin I_2). The stable degradation product was identified as 6-keto prostaglandin $F_{1\alpha}$ [5].

In this paper we report the development of a simple radiochemical assay for prostacyclin synthesis and describe some of the characteristics of this novel enzyme.

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Materials

[1-^{14}C]arachidonic acid (60 mCi/mmol) was purchased from the Radiochemical Centre, Amersham; the purity was found by thin layer chromatography to be greater than 98%. Unlabelled arachidonic acid, soybean lipoxidase (Type 1), *p*-hydroxymercuribenzoate and reduced glutathione were obtained from Sigma Chemical Co. and prostaglandins $F_{2\alpha}$ and E_2 from Cambrian Chemicals Ltd. Authentic 6-keto prostaglandin $F_{1\alpha}$ was synthesized by Dr. N. Whittaker (Wellcome Research Laboratories, Beckenham). Activated silicic acid (Unisil, 100—200 mesh) was purchased from Clarkson Chemical Co.; "Uniplate" thin layer chromatography plates, pre-coated with Silica Gel G (250 μm) from Anachem; Analar grade solvents from BDH and bis(trimethylsilyl) trifluoroacetamide containing 1% trimethyl chlorosilane from the Pierce Chemical Company.

Porcine aortic microsomes were prepared as previously described [1,3] and the protein concentration was determined by the method of Lowry et al. [6].

[1-^{14}C]prostaglandin H_2 was prepared from [1-^{14}C]arachidonic acid using an acetone/pentane preparation of ram seminal vesicles as a source of enzyme [7].

Preparation of fatty acid hydroperoxides

ω-6 hydroperoxides of arachidonic acid, γ-linolenic acid, α-linolenic, linoleic and dihomo-γ-linolenic acids were prepared from the parent fatty acids by incubation with soybean lipoxidase according to the method of Hamberg and Samuelsson [8]. The products were purified by high pressure liquid chromatography using a Waters liquid chromatograph fitted with a 3.9 × 30 cm column packed with μ-Porasil. The eluting solvent, *n*-hexane/propan-2-ol/acetic acid (994/5/1; [9]) was pumped through the column at 4 ml/min.

The ω-10 hydroperoxide of γ-linolenic acid (i.e. 9-hydroperoxy linolenic acid) was prepared by a method based on the report by Funk et al. [9]. The fatty acid was incubated with soybean lipoxidase at pH 7.0 and 25°C for 2 min. After acidification of the aqueous phase the products were extracted with diethyl ether and subsequently purified by high pressure liquid chromatography using the same system as described above. The method produced almost equal amounts of the ω-6 and ω-10 hydroperoxides but these isomers were clearly resolved during high pressure liquid chromatography. The ω-10 hydroperoxide of linoleic acid was formed by auto-oxidation of the fatty acid [10] and was purified by high pressure liquid chromatography.

Preliminary identification of the compounds as hydroperoxides was made by thin layer chromatography. The hydroperoxides of the unsaturated fatty acid were chromatographed on thin layer plates which were developed in *n*-hexane/diethyl ether/acetic acid (60 : 40 : 1). The compounds (R_F 0.36) were visualized under ultra-violet light, or by spraying with a solution of freshly prepared ferrous thiocyanate.

Further confirmation of structure was supplied by gas liquid chromatography/mass spectrometry. The hydroperoxides were reduced to the corresponding hydroxy acid by treatment with sodium borohydride in methanol. Methylation of the free acids with diazomethane was followed by trimethylsilylation of the hydroxyl groups by reaction with bis(trimethylsilyl) trifluoro-

acetamide containing 1% trimethyl chlorosilane. The derivatives were injected into a VG 70707 gas chromatograph/mass spectrometer equipped with a 5-ft glass column packed with 1% OV 1 on Gas Chrom Q which was operated at 220°C and a helium carrier gas flow rate of 30 ml/min. Chemical ionization (isobutane) mass spectrometry was recorded at an ionizing voltage of 150 eV.

In all cases the structures were confirmed by the presence of a weak M + 1 ion and an intense (M + 1) − 90. In addition, the ω-6 isomers of arachidonic, γ-linolenic, dihomo-γ-linolenic and linoleic acids had intense ions at *m/e* 173; the comparable β-cleavage ion was also observed but it was less intense i.e. *m/e* 335 for 15-hydroxyarachidonic acid, *m/e* 309 for 13 hydroxy-γ-linolenic, *m/e* 337 for 15-hydroxydihomo-γ-linolenic acid, *m/e* 311 for 13-hydroxylinoleic acid. A comparable spectrum was obtained for 13-hydroxy-α-linolenic acid except that an intense ion was observed at *m/e* 171 (cf. *m/e* 173) and the β-cleavage product had *m/e* 311.

Confirmation of the ω-10 isomers of hydroxy-γ-linolenic acid and linoleic acid was obtained by the presence of an intense *m/e* 225 ion; the β-cleavage product was less intense (*m/e* 257 for 9-hydroxy-γ-linolenic and *m/e* 259 for 9-hydroxylinoleic acid).

The methyl esters of the above hydroperoxides were synthesized by reaction of the acid with etheral diazomethane. The purity was checked by thin layer chromatography in *n*-hexane/diethyl ether (70 : 30).

The pure hydroperoxides in ethanol were quantified by measurement of their ultraviolet absorption at 234 nM ($\epsilon_{ethanol}$ = 30 000 [8]). They were stored in ethanol at −20°C; decomposition was negligible after a 2-week storage under these conditions.

Methods

Enzyme assays

In an initial series of experiments the effects of pH, enzyme, substrate concentration and reaction times were investigated (see below), but thereafter enzyme assays were performed as follows; Aortic microsomes (2 mg) were gently resuspended in buffer (50 mM Tris, pH 7.5) with the aid of a glass/Teflon homogenizer. Drugs or inhibitors were added as required and the suspension was incubated with [1-^{14}C]prostaglandin H_2 (1.43 nmol; 3.18 Ci/mol) in a total volume of 1 ml. After 3 min incubation at room temperature (20—22°C), the reaction was terminated by the addition of 0.1 ml citric acid (0.5 M) which gave a final pH of 3—3.5. Saturated sodium chloride (0.5 ml) was added to prevent emulsion formation and the mixture was immediately extracted twice with 2.5 ml ethyl acetate. The combined organic phases were evaporated under nitrogen, the residue was dissolved in 50 μl chloroform/methanol (2 : 1) and quantitatively applied to a thin layer chromatography plate together with authentic prostaglandin E_2, prostaglandin $F_{2\alpha}$ and prostaglandin D_2 and 6-keto prostaglandin $F_{1\alpha}$. The plate was developed to a distance of 20 cm in isooctane/ethyl acetate/acetic acid/water (5 : 11 : 2 : 10; upper phase, [11]). Areas of radioactivity on the plate were located with a Panax RTLS-1A radiochromatogram scanner and for quantitative determinations radioactivity in the zones corresponding to 6-keto prostaglandin $F_{1\alpha}$,

prostaglandin E_2, prostaglandin D_2 and prostaglandin $F_{2\alpha}$ was estimated by conventional liquid scintillation counting techniques. Remaining areas on the plate were divided into 2-cm bands and treated similarly. After correction for counting efficiency (by external standardization) and subtraction of background the amount of 6-keto prostaglandin $F_{1\alpha}$ formed was calculated and expressed as pmol 6-keto prostaglandin $F_{1\alpha}$ synthesised per mg microsomes per 3 min. The reaction is not linear for this length of time (see below) and so this figure does not strictly speaking constitute a rate.

Localization of prostacyclin-synthesizing enzyme

Aortic microsomes (2 mg ≡ 1.2 mg protein) and high speed supernatant (containing 15 mg protein) were separately incubated with [1-^{14}C]prostaglandin H_2 for 10 min at 22°C. The products were extracted and separated as described above and the conversion by each fraction estimated.

pH and type of buffer

Aortic microsomes were suspended in phosphate (100 mM) or Tris buffers (50 mM) of varying pH. Each suspension was incubated with [1-^{14}C]-prostaglandin H_2 for 3 min at room temperature (22°C). The production of 6-keto prostaglandin $F_{1\alpha}$ was determined at each pH.

Time course

Aortic microsomes were suspended in 50 mM Tris buffer and incubated with [1-^{14}C]orostaglandin H_2 for varying times at 0—4, 22 and 37°C. The radioactive products were extracted and resolved by thin layer chromatography as described above and the production of 6-keto prostaglandin $F_{1\alpha}$ calculated.

Substrate concentration

Varying amounts of [1-^{14}C]prostaglandin H_2 (0—15 nmol) were incubated with aortic microsomes for 3 min at 20°C. The percentage conversion of endoperoxide to 6-keto prostaglandin $F_{1\alpha}$ was determined and the mass of 6-keto prostaglandin $F_{1\alpha}$ synthesized was calculated.

Effect of hydroperoxides of unsaturated fatty acids

15-hydroperoxy arachidonic, 15-hydroperoxy dihomo-γ-linolenic, 13-hydroperoxy-γ-linolenic, 9-hydroperoxy-γ-linolenic, 13-hydroperoxy α-linolenic, 13-hydroperoxy linoleic and 9-hydroperoxy linoleic acids and methyl esters (final concentration range $0.1—25 \cdot 10^{-6}$ M) were separately added to a suspension of aortic microsomes. After a variable pre-incubation time [1-^{14}C]-prostaglandin H_2 was added. Incubation was continued for 3 min at 20°C after which 6-keto prostaglandin $F_{1\alpha}$ was calculated in the usual way.

Effect of other compounds

Reduced glutathione (final concentration range $10^{-3}—10^{-6}$ M) and *p*-hydroxy mercuribenzoate (final concentration range $1 \cdot 10^{-5}—5 \cdot 10^{-3}$ M) were pre-incubated for 5 min with a suspension of aortic microsomes. [1-^{14}C]-prostaglandin H_2 was added and incubation continued for 3 min at 20°C.

Results

The principle of the radiochemical assay described in this paper depends upon the non-enzymatic degradation of prostacyclin to 6-keto prostaglandin $F_{1\alpha}$; which because of the instability of prostacyclin at the pH used for extraction (pH 3), can be assumed to be quantitative. The thin layer chromatography system utilized gives good resolution of the products (Fig. 1) enabling precise

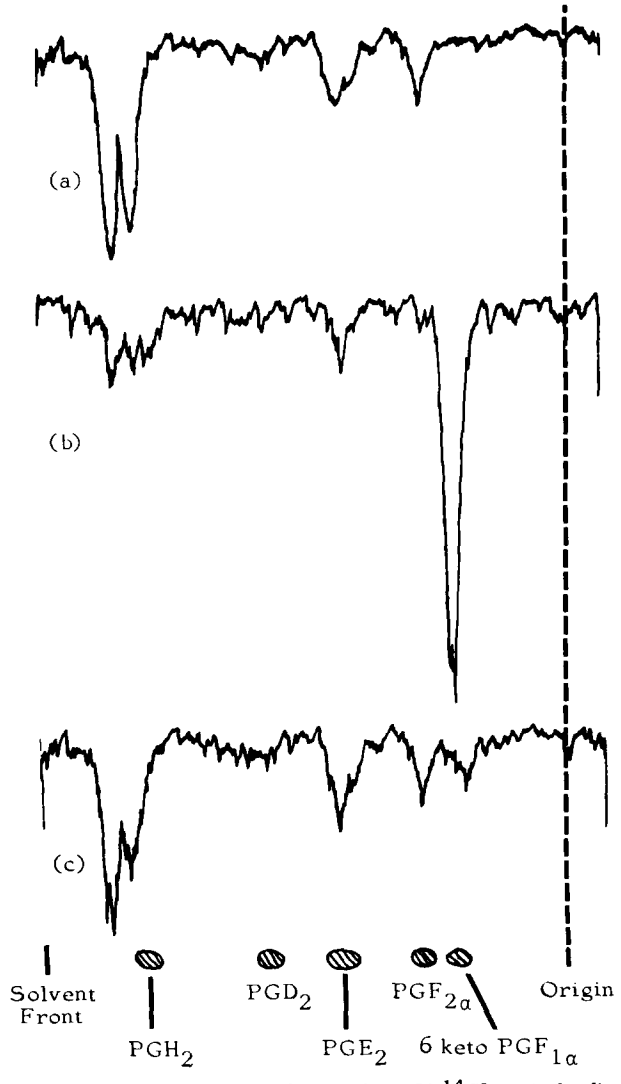

Fig. 1. Radioactive products formed from [1-^{14}C]prostaglandin H_2 by aortic microsomes. The products were resolved by thin layer chromatography as detailed in the text and the developed plate scanned with a Panax radiochromatogram scanner (full scale deflection 30 cpm). Panel (a), [1-^{14}C]prostaglandin H_2 incubated with boiled microsomes. Panel (b), [1-^{14}C]prostaglandin H_2 incubated with microsomes. Panel (c), [1-^{14}C]prostaglandin H_2 incubated with microsomes and 15-hydroperoxyarachidonic acid (5 μM). The position of authentic PG standards is shown at the bottom of the figure. The least polar zone of radioactivity is probably 12-hydroxyheptadecatrienoic acid.

quantitation of the amount of 6-keto prostaglandin $F_{1\alpha}$ produced. From the subcellular fractionation it seems that the prostacyclin-synthesizing enzyme primarily resides in the 105 000 × *g* pellet (microsomes); a low conversion (9 pmol 6-keto prostaglandin $F_{1\alpha}$/mg protein) was observed in the high speed supernatant whereas 900 pmol 6-keto prostaglandin $F_{1\alpha}$/mg protein were produced by the microsomal fraction. The activity of the high speed supernatant may be related to incomplete precipitation of microsomes. The main products observed after incubation of prostaglandin H_2 with high speed supernatant were a hydroxy acid (probably 12-hydroxyheptadecatrienoic acid), prostaglandin D_2, prostaglandin E_2 and prostaglandin $F_{2\alpha}$, a profile compatible with non-enzymatic breakdown of endoperoxide (Fig. 1).

pH and type of buffer

No significant difference was observed between phosphate and tris buffers. Enzymatic activity was relatively insensitive to changes in pH (Fig. 2), maximum synthesis occurring in the range 6.8—7.5. However, this experiment reflects not only the enzymatic activity of the system but also the pH sensitivity of prostacyclin. Prostacyclin is unstable at acidic pH but is relatively stable above pH 7.5, thus the reaction equilibrium may be modified at different pH values (even through prostacyclin does not seem to exert an inhibitory effect, see below). For example, at pH 4.7 the enzymatic activity is higher than might be expected because of rapid decomposition of prostacyclin to 6-keto

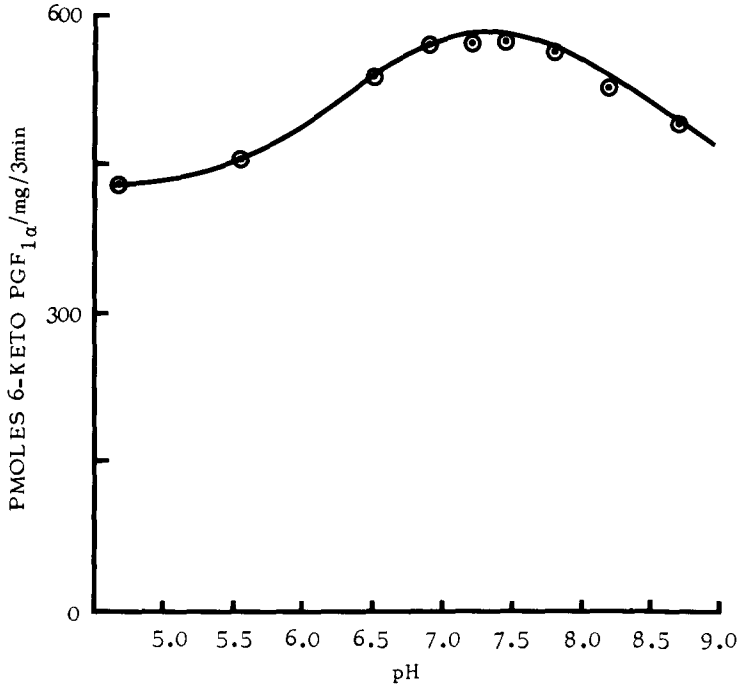

Fig. 2. The effect of pH on the synthesis of 6-keto prostaglandin $F_{1\alpha}$ by aortic microsomes. Various proportions of tris acid and tris base were mixed to give a range of pH values. Each point is the mean of two observations.

prostaglandin $F_{1\alpha}$, perhaps favouring a greater synthesis of prostacyclin. The reverse would be true at alkaline pH.

Time course and linearity of the enzyme reaction

The amount of prostacyclin produced (as estimated by the 6-keto prostaglandin $F_{1\alpha}$ production) at varying times of incubation at different temperatures is shown in Fig. 3. At room temperature (22—23°C) the reaction was maximal after 5 min (linear for 1 min). At 37°C the reaction was complete within 1 min (linear for 30 s) whilst at 0—4°C the maximum production was not observed until 16 min (linear for 4 min). It is interesting to note that the maximum amount of 6-keto prostaglandin $F_{1\alpha}$ produced at different temperatures varied. Thus, a yield of over 80% was observed at 22 and 37°C but only 58% at 0—4°C. This probably reflects a difference in the thermodynamic decomposition of the endoperoxide. After 64 min at 0—4°C, considerable amounts of prostaglandin D_2, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ were detected. Thus, the endoperoxide degrades non-enzymatically more rapidly at 0—4°C than at 22 and 37°C, relative to the enzymatic activity at the respective temperatures, thus limiting the synthesis of prostacyclin at 0—4°C.

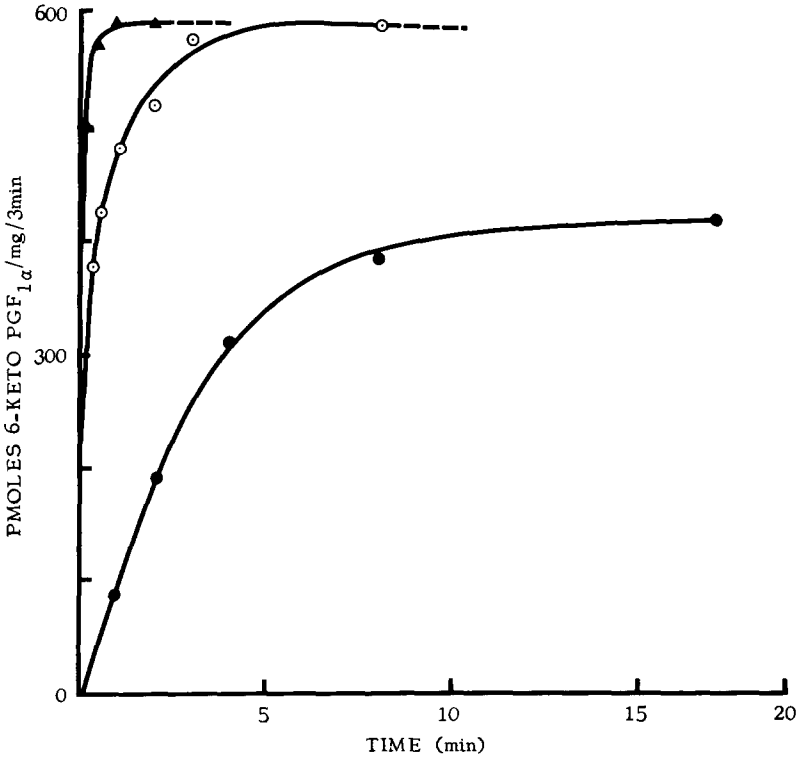

Fig. 3. The effect of time and temperature on the synthesis of 6-keto prostaglandin $F_{1\alpha}$ by aortic microsomes. ▲———▲, 37°C; ○———○, 22°C; ●———●, 0—4°C. Each point is the mean of two observations.

Substrate concentration

The conversion of prostaglandin H_2 to prostacyclin by 2 mg aortic microsomes was approximately linear to concentration of 3 μM prostaglandin H_2; 10 μM was a saturating concentration (Fig. 4). This result agrees well with data obtained by bioassay [1].

Effect of unsaturated fatty acid hydroperoxides

15-Hydroperoxyarachidonic acid was previously demonstrated to be a potent inhibitor of prostacyclin synthesis by bioassay [2]. The data obtained in the present study confirmed this finding (Fig. 5). The IC_{50} obtained ($0.96 \cdot 10^{-6}$ M or 0.32 μg/ml) was comparable to that reported earlier [2]. As the production of 6-keto prostaglandin $F_{1\alpha}$ was decreased with increasing concentration of 15-hydroperoxyarachidonic acid there was only a low conversion of prostaglandin H_2 to prostaglandin E_2 (Fig. 1); most of the radioactivity was associated with unidentified hydroxy acids (probably 12-hydroxyheptadecatrienoic acid). The amount of prostaglandin E_2 formed after treatment with 15-hydroperoxy arachidonic acid was not greater than that obtained in a boiled enzyme control (Fig. 1). Since 15-hydroperoxyarachidonic acid does not inhibit the endoperoxide isomerase (prostaglandin H_2 to prostaglandin E_2) of ram seminal vesicles at concentrations which inhibit prostacyclin synthesis

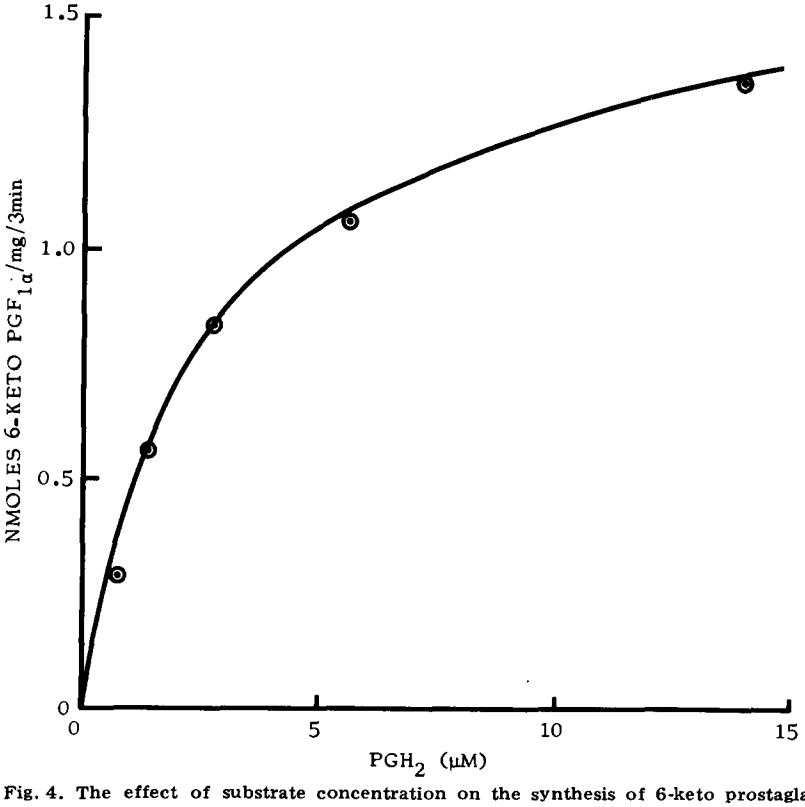

Fig. 4. The effect of substrate concentration on the synthesis of 6-keto prostaglandin $F_{1\alpha}$ by aortic microsomes. Each point is the mean of two observations.

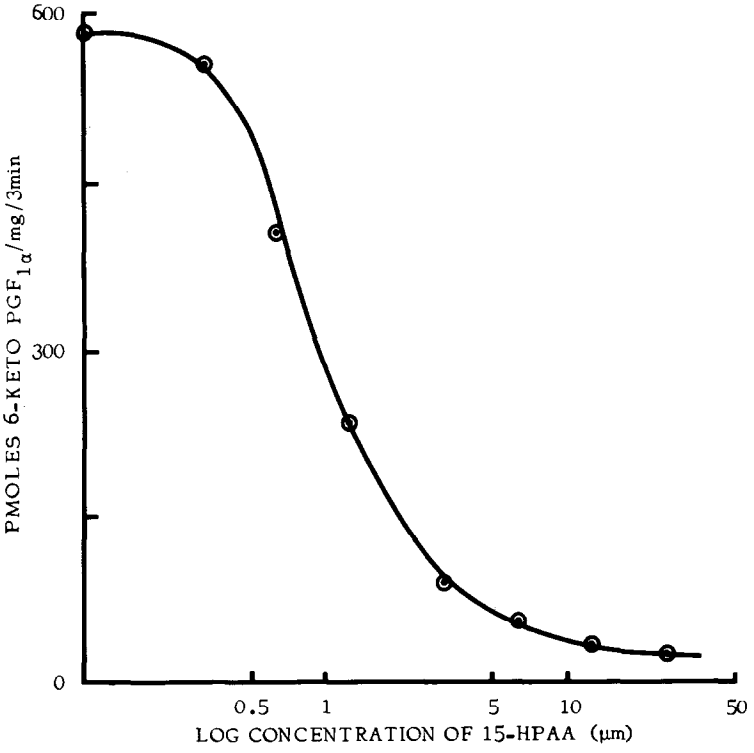

Fig. 5. Inhibition by 15-hydroperoxyarachidonic acid (15-HPAA) of 6-keto prostaglandin $F_{1\alpha}$ synthesis by aortic microsomes. Each point is the mean of two observations. The calculated IC_{50} is $0.96 \cdot 10^{-6}$ M (0.32 μg/ml).

[12] it would appear that this endoperoxide isomerase is absent from aortic microsomes.

The degree of inhibition achieved by 15-hydroperoxyarachidonic acid was dependent on the time of pre-incubation with the aortic microsomes (Fig. 6). Inhibition was maximal after 10 min preincubation and this time was routinely used to determine the IC_{50} values. Hydroperoxides of other fatty acids and methyl esters were also potent inhibitors of prostacyclin synthesis (Table I). The potency of ω-6 and ω-10 hydroperoxides was very similar indicating that inhibition of this enzyme is a general property of hydroperoxides. The inhibitory activity of the methyl esters of the hydroperoxides was very comparable to that of the free acids, suggesting that the mechanism of action is not dependent on a carboxylic acid group.

Effect of other compounds

Glutathione inhibited the conversion of prostaglandin H_2 to 6-keto prostaglandin $F_{1\alpha}$ but only at high concentrations ($>10^{-5}$ M) (Fig. 7). This inhibitory activity is an order of magnitude less than that observed in ram seminal vesicle microsomes [12]. In fact, the concentration of glutathione which inhibited the production of 6-keto prostaglandin $F_{1\alpha}$ caused a non-enzymatic conversion of prostaglandin H_2 to prostaglandin E_2, prostaglandin

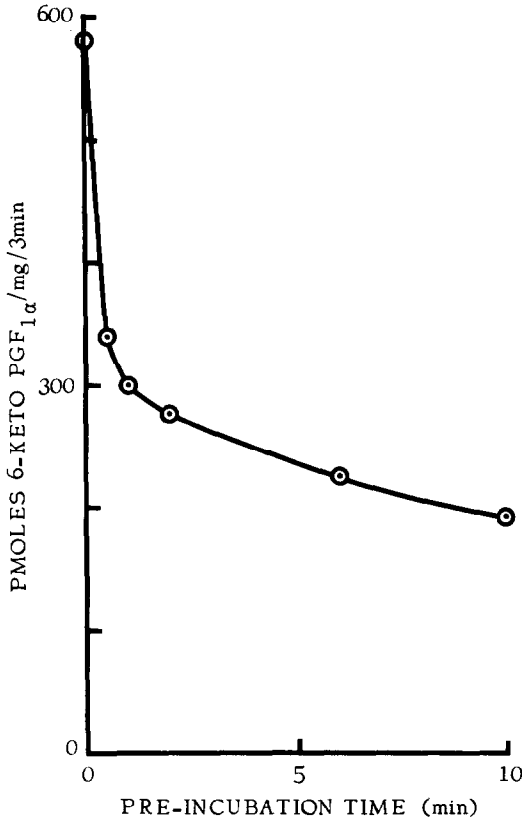

Fig. 6. The inhibitory effect of 15-hydroperoxy arachidonic acid is a time-dependent phenomenon. This graph shows that preincubation of the hydroperoxide with the enzyme markedly effects the final inhibition achieved. Each point is the mean of two observations.

TABLE I

INHIBITION BY LIPID HYDROPEROXIDES OF 6-KETO PROSTAGLANDIN $F_{1\alpha}$ FORMATION BY AORTIC MICROSOMES

Parent acid				Hydroperoxide		
C-number	Number of double bonds	Trivial name	Carboxyl function	Hydroperoxide position	Trivial prefix	IC_{50} (μM)
18	2	linoleic	free acid	ω-6	13-hydroperoxy	1.2
			methyl ester	ω-6	13-hydroperoxy	1.9
			free acid	ω-10	9-hydroperoxy	1.3
18	3	α-linolenic	free acid	ω-6	13-hydroperoxy	1.4
18	3	γ-linolenic	free acid	ω-6	13-hydroperoxy	1.4
			methyl ester	ω-6	13-hydroperoxy	1.9
			free acid	ω-10	9-hydroperoxy	1.5
20	3	dihomo-γ-linolenic	free acid	ω-6	15-hydroperoxy	1.4
			methyl ester	ω-6	15-hydroperoxy	2.2
20	4	arachidonic	free acid	ω-6	15-hydroperoxy	1.1
			methyl ester	ω-6	15-hydroperoxy	1.7

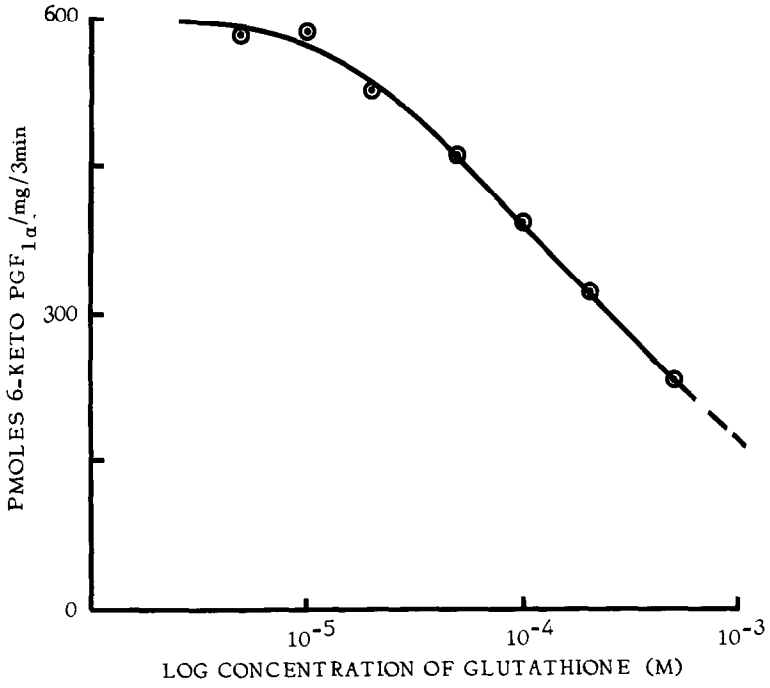

Fig. 7. Inhibition by reduced glutathione of 6-keto prostaglandin $F_{1\alpha}$ formation by aortic microsomes. The calculated IC_{50} for glutathione was 0.2 mM. Each point is the mean of two observations.

$F_{2\alpha}$, prostaglandin D_2 and 12-hydroxy heptadecatrienoic acid. This provides further evidence that aortic microsomes contain little or no endoperoxide E_2 isomerase. *p*-Hydroxymercuribenzoate ($1 \cdot 10^{-5}$—$5 \cdot 10^{-3}$ M), indomethacin (50 μM), or prostacyclin itself (40 μM) did not modify the amount of 6-keto prostaglandin $F_{1\alpha}$ produced.

Discussion

The assay described in the present report provides a practical means of quantifying prostacyclin synthesis. The results obtained using this assay agree well with those obtained previously by bioassay [1]. Under optimal conditions, 80—85% of added prostaglandin H_2 was converted to prostacyclin, a figure which could not be estimated by bioassay and therefore previous reports [1—4] had assumed 100% conversion. 6-keto prostaglandin $F_{1\alpha}$ was never formed spontaneously from the endoperoxide, thus, unlike the other prostaglandins such as prostaglandin E_2, prostaglandin $F_{2\alpha}$ or prostaglandin D_2 (but similar to thromboxane A_2) there is an absolute requirement for an enzymatic reaction to take place.

We have confirmed that 15-hydroperoxy arachidonic acid was a potent inhibitor of prostacyclin synthesis. The pathological significance of these observations has already been discussed at length [3,13]. The present study reveals that other hydroperoxides besides 15-hydroperoxyarachidonic acid also inhibit prostacyclin synthesis. The similarity of potency of positional isomers

suggests that this is a general property of hydroperoxides of unsaturated fatty acids. Other enzymes are also sensitive to hydroperoxides of fatty acids (for example, isocitrate dehydrogenase enzyme, [14]) and it has been suggested that this inhibition is due to the oxidation of -SH groups by the hydroperoxide. Because of the high titre of other -SH groups in the aortic microsomes used in the present study, it could not be conclusively demonstrated that inactivation of the prostacyclin-synthesizing enzyme proceeded by this mechanism (unpublished results). However, the time-dependent nature of the inhibitory effect does indeed suggest that oxidation of some function in the enzyme may occur.

The methyl esters of the unsaturated fatty acid hydroperoxides were also potent inhibitors of prostacyclin synthesis and therefore it is probable that other esters of these hydroperoxides would also be effective. Cutler and

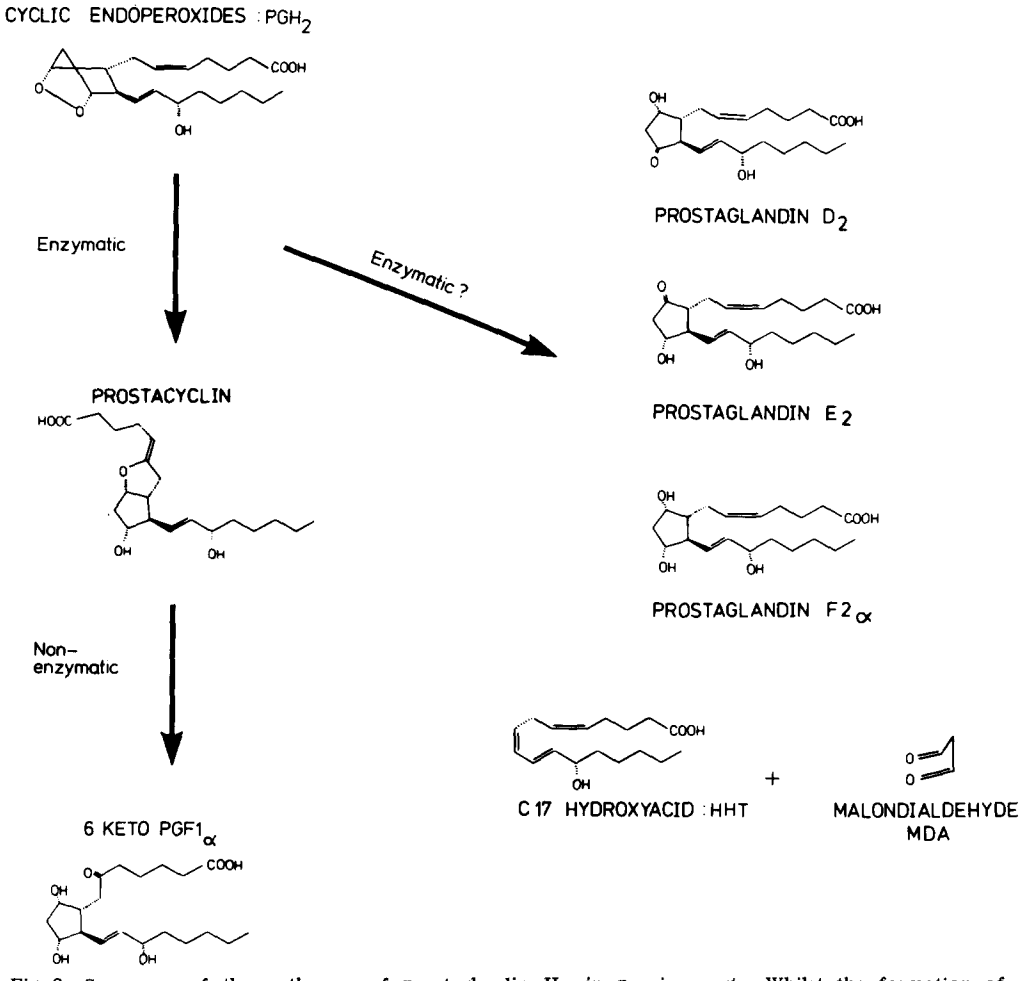

Fig. 8. Summary of the pathways of prostaglandin H_2 in porcine aorta. Whilst the formation of prostacyclin is enzymatic, it is not possible to determine whether prostaglandin E, F and D or 12 hydroxyheptadecatrienoic acid and malondialdehyde are generated enzymatically: The study reported here suggests that they are not.

Schneider [15] demonstrated that subcutaneous administration of linoleate hydroperoxide into rats caused an increased incidence of myocardial fibrosis and produced medial thickening in the coronary vessels. Similar results were observed in rabbits, and intimal plaques containing deposits of lipid and fibrinogen were observed in the aorta. These authors showed that neither the administration of purified linoleate nor that of the secondary oxidation products caused comparable effects. Harland et al. [16,17] isolated cholesterol linoleate hydroperoxides from advanced atherosclerotic plaques of human aortas and identified these as a mixture of 9 and 13 isomers similar to those produced by auto-oxidation. The concentration of these compounds in tissues increased with the severity of the disease; they were not detected in arterial tissue affected only by 'fatty streaks'. It is attractive to speculate that the toxic effects of added hydroperoxide were attributable to inhibition of prostacyclin synthesis and that the presence of hydroperoxides in atherosclerotic plaque gives rise to some of the symptoms associated with atherosclerosis.

The findings that glutathione does not affect the production of 6-keto prostaglandin $F_{1\alpha}$ except at high concentrations and that inhibition of prostacyclin synthesis by 15-hydroperoxyarachidonic acid does not cause a parallel increase in prostaglandin E_2, both suggest that the endoperoxide isomerase (prostaglandin H_2 to prostaglandin E_2) is absent or only present in low concentrations in the aorta.

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