Mechanism of Prostaglandin Biosynthesis in Rabbit Kidney Medulla

A RATE-LIMITING STEP AND THE DIFFERENTIAL STIMULATORY ACTIONS OF L-ADRENALINE AND GLUTATHIONE

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Microsomal prostaglandin synthase (EC 1.14.99.1) from rabbit kidney medulla was assayed with [5,6,8,9,11,12,14,15-³H]- and [1-¹⁴C]-arachidonic acid as the substrate. The ratios of prostaglandin F_{2α} to prostaglandin E₂ and to prostaglandin D₂ were determined by both ³H and ¹⁴C labelling. When ³H was used as a label the ratios were much higher than with ¹⁴C labelling indicating that the removal of hydrogen at C-9 or C-11 was the rate-limiting step in the biosynthesis of prostaglandin E₂ or prostaglandin D₂. This finding shows that the octatriitated arachidonic acid is not the appropriate substrate marker for studying the regulation of the synthesis of different prostaglandins by various agents. When the enzyme assay was carried out in the presence of SnCl₂, which was capable of accumulating exclusively prostaglandin F_{2α} at the expenses of prostaglandin E₂ and prostaglandin D₂, the addition of L-adrenaline to the microsomal fraction either alone or with reduced glutathione equally stimulated the formation of prostaglandin F_{2α}, whereas the addition of reduced glutathione to the microsomal fraction either alone or with L-adrenaline produced no additional effect. These results suggest that endoperoxide is formed as the common intermediate for the biosynthesis of three different prostaglandins in rabbit kidney medulla, and that L-adrenaline stimulates the synthesis of endoperoxide, whereas reduced glutathione facilitates the formation of prostaglandins from endoperoxide.

Although the organization and mechanism of prostaglandin synthase (EC 1.14.99.1) have been partially elucidated in seminal vesicles (Wlodawer & Samuelsson, 1973; Miyamoto *et al.*, 1974), little information on these aspects has been obtained in other systems. This is mainly because much less enzyme activity is found in other tissues. We have characterized prostaglandin synthase from rabbit kidney medulla (Tai *et al.*, 1976) and found that it differed in some respects from that isolated from seminal vesicles (Samuelsson, 1969; Foss *et al.*, 1971).

Prostaglandin synthase of rabbit kidney medulla catalyses the formation of prostaglandin E₂ (11 α ,15 α -dihydroxy-9-oxoprostano-5-*cis*,13-*trans*-dienoic acid), prostaglandin F_{2α} (9 α ,11 α ,15 α -trihydroxyprosta-5-*cis*,13-*trans*-dienoic acid) and prostaglandin D₂ (9 α ,15 α -dihydroxy-11-oxoprostano-5-*cis*,13-*trans*-dienoic acid) from arachidonic acid. The enzyme complex was largely localized in the microsomal fraction and this fraction possessed low intrinsic enzyme activity by itself. However, the microsomal enzyme activity could be stimulated by the addition of either boiled soluble fraction, phenolic compounds or GSH (reduced glutathione). Simultaneous addition of a phenolic compound and GSH produced synergistic stimulation of the enzyme activity. The stimulation was reflected in the synthesis of all three prostaglandins.

Our earlier observation that rabbit kidney-medulla prostaglandin synthase catalyses the transformation of [5,6,8,9,11,12,14,15-³H]arachidonic acid predominantly into ³H-labelled prostaglandin F_{2α} rather than ³H-labelled prostaglandin E₂ was quite puzzling to us (Tai & Hollander, 1972). Similar findings were reported in rat stomach (Pace-Asciak *et al.*, 1968), rat kidney (Pace-Asciak, 1975) and bovine seminal vesicles (Flower *et al.*, 1973) when octatriitated arachidonic acid was used as the substrate. These observations appeared to be in conflict with the report that most animal tissues contained predominantly prostaglandin E₂ instead of prostaglandin F_{2α} as determined by bioassay (Karim *et al.*, 1968). Recently we found that if [1-¹⁴C]-arachidonic acid was used as the substrate, rabbit kidney-medulla prostaglandin synthase catalyses predominantly the formation of [1-¹⁴C]prostaglandin E₂ (Tai *et al.*, 1976). These studies indicated that the elimination of ³H from C-9 of the octatriitated substrate to form heptatriitated prostaglandin E₂ showed a kinetic isotope effect. In the present paper I give evidence that endoperoxide is also formed as the common intermediate for the biosynthesis of three different prostaglandins in rabbit kidney medulla, and that the removal of hydrogen at C-9 or C-11 is the rate-limiting step in the biosynthesis of prostaglandin E₂ or prostaglandin D₂. Further, I demonstrate the

differential stimulatory action of a phenolic compound and GSH in this two-step biosynthesis of prostaglandins from arachidonic acid. A preliminary account of these studies has been presented in abstract form (Tai, 1975).

Experimental

Materials

all-*cis*-Eicosa-5,8,11,14-tetraenoic acid (arachidonic acid), GSH, L-adrenaline, 5-hydroxytryptamine (serotonin) and 3,4-dihydroxyphenethylamine (dopamine) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [$1\text{-}^{14}\text{C}$]Arachidonic acid (58 mCi/mmol) and [5,6,8,9,11,12,14,15- ^3H]arachidonic acid (80 Ci/mmol) were respectively purchased from Applied Science Laboratories, State College, PA, U.S.A., and New England Nuclear, Boston, MA, U.S.A. Precoated silica-gel G plates (without fluorescent indicator) were obtained from Brinkman Instruments, Westbury, NY, U.S.A. Frozen rabbit kidney medulla was purchased from Pel-Freeze Biologicals, Rogers, AR, U.S.A. Prostaglandin E_2 , prostaglandin D_2 and prostaglandin $\text{F}_{2\alpha}$ were gifts from Dr. Udo Axén and Dr. John Pike of the Upjohn Co., Kalamazoo, MI, U.S.A.

Methods

Preparation of microsomal fraction from rabbit kidney medulla and methods of enzyme assay were carried out as previously described (Tai *et al.*, 1976).

Briefly, the assay mixture contained 0.1 M-Tris/HCl buffer, pH 8.0, 1 mM-GSH and 1 mM-L-adrenaline when indicated, 60 nmol of labelled arachidonic acid and microsomal fraction as indicated in the Table and Figure legends in a final volume of 1 ml. After incubation at 37°C for 5 min, the reaction mixture was acidified and extracted with 2×3 ml of ethyl acetate. The organic layer was dried under N_2 and the residue was spotted on silica-gel G t.l.c. plates and developed to a height of 12.5 cm with the organic layer of ethyl acetate/acetic acid/iso-octane/water (11:2:5:10, by vol.) as the solvent system. The plates were divided into four bands, prostaglandin $\text{F}_{2\alpha}$, prostaglandin E_2 , prostaglandin D_2 and arachidonic acid plus hydroxy fatty acids after co-chromatography with the authentic compounds and detection with I_2 vapour. Each band was transferred to a scintillation vial and the radioactivity was determined by liquid-scintillation counting as previously described (Tai *et al.*, 1976).

Results and Discussion

Studies on the seminal vesicular system (Hamberg & Samuelsson, 1973; Nugteren & Hazelhof, 1973) indicated that different prostaglandins were formed from the common intermediate, endoperoxide, either by removal of hydrogen at C-9 or C-11 and subsequent isomerization of the bicyclic ring to form prostaglandin E_2 or prostaglandin D_2 or by reductive cleavage of the endoperoxide without concomitant loss of hydrogen to form prostaglandin $\text{F}_{2\alpha}$ as shown in Scheme 1.

Scheme 1. Conversion of arachidonic acid into prostaglandin E_2 , prostaglandin $\text{F}_{2\alpha}$ and prostaglandin D_2 via an endoperoxide intermediate

Table 1. Radioactivity ratios of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to prostaglandin E_2 (PGE_2) and to prostaglandin D_2 (PGD_2) with [5,6,8,9,11,12,14,15- 3H]- and [1- ^{14}C]-arachidonic acid as substrate

The microsomal fraction (1.2mg of protein) was incubated in 1.0ml of 0.1 M-Tris/HCl buffer, pH8.0, with [5,6,8,9,11,12,14,15- 3H]- and [1- ^{14}C]-arachidonic acid (104000 d.p.m. of 3H and 74400 d.p.m. of ^{14}C , 60nmol) in the presence of 1 mM of the indicated compounds at 37°C for 5 min. Extraction of lipids and measurement of the radioactivity of each prostaglandin were carried out as described in the Experimental section. The 3H radioactivity of prostaglandin E_2 and prostaglandin D_2 was corrected by a factor of 8/7 since one 3H atom was lost during their formation. The result of a representative experiment is shown.

is shown.

		³ H		¹⁴ C			
	Prostaglandin	d.p.m.	$\frac{PGF_{2\alpha}}{PGE_2}$	$\frac{PGF_{2\alpha}}{PGD_2}$	d.p.m.	$\frac{PGF_{2\alpha}}{PGE_2}$	$\frac{PGF_{2\alpha}}{PGD_2}$
Microsomal fraction + L-adrenaline	F _{2α}	3906			1297		
	E ₂	1952	2.00	5.36	3595	0.36	1.22
	D ₂	727			1062		
Microsomal fraction + L-adrenaline + GSH	F _{2α}	9413			3063		
	E ₂	4914	1.92	4.94	5843	0.52	1.18
	D ₂	1906			2604		
Microsomal fraction + serotonin	F _{2α}	2309			687		
	E ₂	1629	1.42	4.94	2944	0.23	0.93
	D ₂	467			739		
Microsomal fraction + serotonin + GSH	F _{2α}	4896			1888		
	E ₂	2613	1.87	3.83	3556	0.54	1.29
	D ₂	1279			1465

Fig. 1. Thin-layer radiochromatogram of lipids formed on incubation of arachidonic acid with microsomal fraction of rabbit kidney medulla in the absence (a) and presence (b) of $SnCl_2$

The microsomal fraction (1.2mg of protein) was incubated in 1.0ml of 0.1 M-Tris/HCl buffer, pH8.0, with [1- ^{14}C]-arachidonic acid (104000 d.p.m., 60nmol) and L-adrenaline (1 μ mol) at 37°C for 5 min. $SnCl_2$ when present was added at the concentration of 1.0mg/ml. Extraction of lipids was as described in the Experimental section. T.l.c. was first carried out in the system of benzene/dioxan/acetic acid (80:20:1, by vol.) to a height of 15 cm for better resolution of hydroxy fatty acids and arachidonic acid. The plate was air dried and further developed to a height of 10 cm in the system of ethyl acetate/acetic acid/isooctane/water (11:2:5:10, by vol.) for resolution of different prostaglandins. Strips (0.5 cm) of the chromatogram were scraped off the plate and the radioactivity was determined by liquid-scintillation counting. Shaded spots indicate positions of authentic prostaglandin (E_2 , $F_{2\alpha}$, D_2) markers.

If the prostaglandin synthase of the rabbit kidney medulla were to follow the same mechanism to form different prostaglandins, the removal of hydrogen at C-9 or C-11 labelled with 3H would then show the kinetic isotope effect if the removal of these hydrogen atoms was the rate-limiting step in the formation of prostaglandin E_2 or prostaglandin D_2 . Accordingly, the formation of labelled prostaglandin $F_{2\alpha}$ which does not involve any loss of 3H will be favoured if different prostaglandins originate from the same labelled intermediate. The radioactivity ratios of prostaglandin $F_{2\alpha}$ to prostaglandin E_2 and prostaglandin $F_{2\alpha}$ to prostaglandin D_2 will then be respectively higher for multi-tritium-labelled substrate having 3H at C-9 or C-11 than the corresponding ratios obtained with ^{14}C -labelled substrate. Table 1 shows that when a mixture of [5,6,8,9,11,12,14,15- 3H]- and [1- ^{14}C]-arachidonic acid was used as substrate, the radioactivity ratios of prostaglandin $F_{2\alpha}$ to prostaglandin E_2 and prostaglandin $F_{2\alpha}$ to prostaglandin D_2 for 3H were indeed much higher than the corresponding ratios for ^{14}C in each instance. These results suggest that the rabbit kidney-medulla prostaglandin synthase also catalyses the transformation of arachidonic acid into a common transitory intermediate and that the removal of hydrogen atom at C-9 or C-11 of this intermediate indeed is the rate-limiting step in the formation of prostaglandin E_2 or prostaglandin D_2 . These findings thereby discourage the use of commercially available [5,6,8,9,11,12,14,15- 3H]arachidonic acid as the substrate marker in studying the regulation of the ratio of

Table 2. *Differential stimulatory actions of L-adrenaline and GSH on prostaglandin synthase of rabbit kidney medulla*

The microsomal fraction (1.2 mg of protein) was incubated in 1.0 ml of 0.1 M-Tris/HCl buffer, pH 8.0, with [$1\text{-}^{14}\text{C}$]arachidonic acid (74400 d.p.m., 60 nmol) in the presence of 1 mM of the indicated compounds at 37°C for 5 min. SnCl_2 when present was added at the concentration of 0.4 mg/ml. Extraction and determination of prostaglandins were as described in the Experimental section. The result of a representative experiment is shown.

	Prostaglandin (nmol/5 min)							
	- SnCl_2				+ SnCl_2			
	$\text{F}_{2\alpha}$	E_2	D_2	Total	$\text{F}_{2\alpha}$	E_2	D_2	Total
Microsomal fraction	0.58	1.37	0.54	2.49	7.05	0.47	0.02	7.54
Microsomal fraction + GSH	1.75	2.62	0.86	5.23	6.79	0.50	0.09	7.38
Microsomal fraction + L-adrenaline	3.28	7.85	2.47	13.60	8.92	2.07	0.41	11.40
Microsomal fraction + L-adrenaline + GSH	6.88	10.48	3.97	21.33	10.55	0.93	0.17	11.65

different prostaglandins biosynthesized in various tissues.

To ascertain whether the identity of this intermediate is the same endoperoxide as that found in the seminal vesicular system, the reaction was incubated in the presence of a peroxide-reducing agent such as SnCl_2 which could convert exclusively the endoperoxide intermediate into prostaglandin $\text{F}_{2\alpha}$ (Hamberg & Samuelsson, 1973; Chan *et al.*, 1975). Fig. 1 shows that incubation in the presence of SnCl_2 indeed shifted all the prostaglandin production into prostaglandin $\text{F}_{2\alpha}$ exclusively. This result strongly suggests that the transformation of arachidonic acid into prostaglandins catalysed by rabbit kidney-medulla prostaglandin synthase is also via an endoperoxide intermediate and that this intermediate is trapped as prostaglandin $\text{F}_{2\alpha}$ when incubated in the presence of SnCl_2 . The ability of SnCl_2 to terminate the reaction at the stage of endoperoxide by converting it exclusively into prostaglandin $\text{F}_{2\alpha}$ can be conveniently used for assaying the enzyme components of the prostaglandin synthase that catalyse the formation of endoperoxide.

As indicated above, either GSH or a phenolic compound is an activator of rabbit kidney-medulla prostaglandin synthase. Simultaneous addition of both types of activators in the incubation mixture resulted in the synergistic stimulation of the enzyme activity. In view of the two-step reactions leading to the formation of prostaglandins from arachidonic acid, it would be expedient at this point to locate the stimulatory action of these two different types of activators. Table 2 shows that in the absence of SnCl_2 , as demonstrated previously (Tai *et al.*, 1976), GSH itself is a poorer activator than L-adrenaline and that a combination of both activators produced synergistic stimulation of the formation of prostaglandins. However, in the presence of SnCl_2 all products were converted into prostaglandins $\text{F}_{2\alpha}$ as expected. Addition of L-adrenaline to the microsomal fraction or to the microsomal fraction plus GSH

stimulated the formation of endoperoxide measured as prostaglandin $\text{F}_{2\alpha}$ equally, whereas the addition of GSH to the microsomal fraction or to the microsomal fraction plus L-adrenaline produced no additional effect. Similar observations were obtained with the activator pairs of GSH and serotonin as well as GSH and dopamine. The higher amount of the total prostaglandins formed in the presence of SnCl_2 than in its absence, both in the microsomal fraction and in the microsomal fraction plus GSH, was probably due to the total endoperoxide formed in the system being reduced to prostaglandin $\text{F}_{2\alpha}$. However, the slightly lower yield of total prostaglandins in the presence of SnCl_2 than in its absence after the addition of L-adrenaline might be attributed to some interference by SnCl_2 in the L-adrenaline activation of the enzyme activity. Nevertheless, these studies indicated that L-adrenaline stimulated the formation of endoperoxide from arachidonic acid, and GSH facilitated the formation of prostaglandins from endoperoxide. Accordingly, the synergistic stimulation of rabbit kidney-medulla prostaglandin synthase activity by the combination of L-adrenaline or other phenolic compounds and GSH can be attributed to the ability of GSH in pulling the endoperoxide-forming reaction to the right by converting endoperoxide into prostaglandins. Stimulation by GSH in the formation of all three prostaglandins indicated that GSH exerted less specific activation in rabbit kidney system than in bovine or sheep seminal-vesicular systems. In the latter two systems GSH appeared to activate quite specifically the endoperoxide isomerase component which catalyses the formation of prostaglandin E_2 (Foss *et al.*, 1971; Lands *et al.*, 1971).

Although GSH has also been implicated in the conversion of 15-peroxy-endoperoxide into 15-hydroxy-endoperoxide by the action of glutathione peroxidase (Lands *et al.*, 1971), the presence of peroxide-reducing agent in the assay system probably interferes with the function of this enzyme. Therefore the function of GSH under such assay conditions

may be restricted to the stimulation of the step leading to the formation of prostaglandins from endoperoxide.

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