

BBA 55980

COFACTORS IN THE BIOSYNTHESIS OF PROSTAGLANDINS F_{1α} AND F_{2α}

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(Received August 23rd, 1971)

SUMMARY

Cu²⁺, added to enzyme preparations from vesicular gland stimulates the production of prostaglandins F. This is further enhanced by addition of dithiols, which presumably provide reducing equivalents to convert the intermediate endoperoxide to the diol, F, derivative. The increased yield of prostaglandins F is accompanied by decreased amounts of prostaglandins E, confirming the concept of a common intermediate in the biosynthesis of the two types of prostaglandin.

INTRODUCTION

Prostaglandins are synthesized from eicosapolyenoic acids in a variety of tissues¹. HAMBERG AND SAMUELSSON² have reported that the prostaglandins produced by homogenates of sheep vesicular gland are mostly of the E type. Other reports have indicated the formation of about equal amounts of prostaglandin E and prostaglandin F compounds³⁻⁵.

The favorable effect of glutathione in combination with an antioxidant such as propylgallate or hydroquinone on the biosynthesis of prostaglandin E, has been consistently documented^{4,6}. The addition of NADH, NADPH, ATP, albumin or metal ions, however, resulted in no stimulation of prostaglandin E₁ synthesis by the particulate fraction of sheep vesicular gland⁴. SAMUELSSON^{6,7} has also reported a stimulatory effect of synthetic tetrahydropteridine, tetrahydrofolic acid and reduced glutathione on the ability of washed microsomes to synthesize prostaglandins. Although cofactors for the production of prostaglandin E have been described, a selective enhancement of the biosynthesis of prostaglandin F at the expense of other prostaglandin-like materials has not been reported.

The last common intermediate in the conversion of unsaturated fatty acids into prostaglandin E and prostaglandin F compounds has been proposed to be a cyclic endoperoxide⁸. In order to convert the endoperoxide to prostaglandin F (rather

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than prostaglandin E) some source of additional electrons is necessary which could be possibly supplied *in vivo* by a large number of available reductants. Hypothesizing that a metastable diradical resulting from a homolytic cleavage of the O-O bond might favor prostaglandin F production, we investigated the effect of metal ligand complexes and reductants on the biosynthesis of prostaglandins by acetone powder preparations of sheep vesicular gland. This approach led to the demonstration that copper-dithiol complexes (particularly that of dihydrolipoamide) can serve in the selective formation of prostaglandin F derivatives.

EXPERIMENTAL

Preparation of acetone powder

A homogenate of sheep vesicular gland was prepared by homogenizing 20 g of minced tissue with a Dounce ball-type homogenizer in 30 ml of 0.25 M sucrose solution (pH 7.4) which was 1 mM in EDTA. The pH of the homogenate was adjusted to 7.4 with dropwise additions of 10% Tris base and centrifuged at $7000 \times g$ for 10 min. The supernatant was made 1 mM in ascorbate and the pH was adjusted to 5.2 with 0.5 M citric acid. This supernatant was centrifuged at $25000 \times g$ for 15 min and the supernatant discarded. The pellet was added with trituration and agitation to 500 ml of anhydrous acetone at -20 to -30° . The suspension was filtered and washed with 3 200-ml portions of cold (-20°) acetone. The solid material was immediately dried *in vacuo* over P_2O_5 in a precooled desiccator. The yield of acetone powder varied from 300–500 mg from 20 g of tissue.

Incubations, isolation and assay

A typical incubation mixture contained 10 nmoles of ammonium arachidonate, 0.01 μ C ammonium [$1-^{14}C$]arachidonate, 0.1 ml of 10 mM metal salts, 0.1 ml of 10 mM thiol and 0.7 ml of 0.1 M Tris buffer (pH 9.0). The incubation was initiated by the addition of 2 mg of powder suspended in 0.1 ml of Tris-HCl buffer (pH 9.0) and left standing with occasional shaking at room temperature. The incubations were stopped by the addition of 7.0 ml of chloroform-methanol (1:1, v/v). The suspension was centrifuged to remove the precipitated protein and the supernatant was poured into tubes containing 3.0 ml of chloroform and 1.6 ml of 1% formic acid and mixed. The resultant lower phase was evaporated to dryness with the aid of a stream of N_2 , the residue dissolved in a small volume of chloroform and quantitatively transferred to a silica gel G thin-layer plate. The thin-layer plate was developed to 15 cm in benzene-dioxane-acetic acid-formic acid (82:14:1:1, by vol.) and air dried for 15 min. A line was then scratched across the layer of silicic acid, 7.5 cm above the origin and the plate developed to that line with acetone-methylene chloride (60:40, v/v). The standards were visualized with the aid of iodine vapor and the plate was sectioned into regions corresponding to arachidonic acid, hydroxy acids, prostaglandin E and prostaglandin F with appropriate unoccupied intermediate bands. After spontaneous loss of the adsorbed iodine, the sections were scraped into scintillation vials, suspended in 6 ml of toluene scintillation fluid⁹ and assayed for radioactivity in a Packard Model 2211 liquid scintillation spectrometer. The amounts of radioactive materials produced were estimated by subtracting the values obtained in control incubations without enzyme from the experimental results.

RESULTS

Identification of prostaglandin F_{2α} and prostaglandin E₂

Radioactive prostaglandin E₂ and prostaglandin F_{2α} produced by the incubation of [¹⁴C]arachidonate with acetone powder was purified and isolated by eluting the silicic acid from the corresponding region of a thin-layer plate with methanol. When rechromatographed on silica gel G with benzene-dioxane-acetic acid (20:20:1, by vol.), practically all of the radioactivity chromatographed with the same mobility as prostaglandin E₁ and prostaglandin F_{1α}, respectively (see Fig. 1). Prostaglandin E₁ and prostaglandin F₁ were used as standards because they were conveniently available and exhibited the same R_F values as prostaglandin E₂ and prostaglandin F_{2α} in this chromatographic system¹⁰.



PGE	Standards	PGF
513	front	459
1157		1518
22226	PGE 	1402
442		161
488	PGF 	17797
70		2064
79		334
88	origin	249
(25379)	totals	(24286)

Fig. 1. Thin-layer chromatography of isolated radioactive prostaglandin E₂ and prostaglandin F_{2α}. Unlabeled prostaglandin E₁ and prostaglandin F_{1α} were used as reference on silica gel G with benzene-dioxane-acetic acid (20:20:1; by vol.) as the developing solvent. PGE, prostaglandin E; PGF, prostaglandin F.

The products of a copper-dithiol catalyzed incubation were treated with NaBH₄ in methanol, separated, and their chromatographic mobility compared to products of an identical incubation not treated with NaBH₄. It was found that NaBH₄ treatment did not significantly change the R_F of the radioisotope. However, when a similar experiment was run on the products formed in the presence of glutathione, the NaBH₄ treatment converted 91% of the isotope in the prostaglandin E₂ region to products migrating in the prostaglandin F_{2α} region.

Using eicosatrienoic and arachidonic acid as substrates in copper-dithiol incubations, the purified product from the prostaglandin F regions was isolated. In each case, the trimethylsilyl ether derivatives of the methyl esters showed a single peak upon gas-liquid chromatography with a retention time corresponding to that of the anticipated prostaglandin F_{2α} derivative, and with no evidence of the β isomer. The mass spectra of these derivatives obtained on an LKB 9000 showed a fragmentation pattern

TABLE I

FRAGMENTATION PRODUCTS OF THE PROSTAGLANDIN F DERIVATIVES PRODUCED IN COPPER-DITHIOL SYSTEMS

Mass spectral data on the trimethylsilyl derivatives of the methyl esters of prostaglandin $F_{1\alpha}$ and prostaglandin $F_{2\alpha}$ obtained from either eicosatrienoic acid (20:3 (*n*-6)) or arachidonic acid (20:4 (*n*-6)) as described in EXPERIMENTAL.

Fragment	<i>m/e</i>	Relative abundance	
		20:4 derivative	20:3 derivative
M-15	573		0.6
	572	0.2	1.2
	571	0.4	2.2
	570	1.0	
	569	2.4	
M-90	498		2.5
	497	0.5	6.7
	496	2.0	16.2
	495	5.4	
	494	13.7	
M-(90+71)	427		4.7
	426	1.0	11.3
	425	6.1	32.9
	424	9.8	
	423	27.0	

TABLE II

THE EFFECT OF Cu^{2+} AND DITHIOTHREITOL ON PROSTAGLANDIN SYNTHESIS

Incubations were run for 5 or 10 min at 20° after the addition of 2 mg acetone powder suspended in 0.2 ml of 0.1 M Tris-HCl (pH 9.0) to tubes containing tracer levels of ammonium [$1-^{14}C$]arachidonate in the absence of any cofactor or the presence of 0.5 mM $CuSO_4$ or in the presence of both 0.5 mM $CuSO_4$ and 1.0 mM dithiothreitol in 0.8 ml of 0.1 M Tris (pH 9.0). Extracts were then assayed as described in EXPERIMENTAL. The values for prostaglandin E_2 and for prostaglandin $F_{2\alpha}$ are expressed as the percentage of products formed and the extent of oxidation as a percentage of the initial ^{14}C total radioactivity which no longer chromatographed as free arachidonic acid.

Incubation	<i>E</i>	<i>F</i>	<i>F/E</i>	Extent oxidized %
5 min	21.4	7.8	0.36	55.4
10 min	19.2	12.0	0.63	66.9
0.5 mM Cu^{2+}				
5 min	10.8	26.9	2.49	65.2
10 min	10.8	26.7	2.47	70.2
0.5 mM Cu^{2+} + 1.0 mM dithiothreitol				
5 min	9.7	47.8	4.93	59.0
10 min	7.7	42.9	5.57	64.1

identical to that reported earlier¹¹ for prostaglandin $F_{1\alpha}$ with the exception that for the prostaglandin $F_{2\alpha}$ derivative the *m/e* values were two units less than for the prostaglandin $F_{1\alpha}$ derivative (see Table I).

Influence of added copper and dithiols

As demonstrated in Table II the incubation of acetone powder prepared from sheep vesicular gland with arachidonic acid in the presence of 1 mM copper led to the formation of 2.5 times more prostaglandin F than prostaglandin E. The ratio of prostaglandin F to prostaglandin E (*F/E* ratio) produced in 10 min changed from 0.6, in the

TABLE III

THE EFFECT OF Cu^{2+} AND DITHIOHREITOL LEVELS ON PROSTAGLANDIN SYNTHESIS

Incubations were run for 2 and 4 min using 1 mg of acetone powder in a manner similar to that described in Table I. Equal amounts of CuSO_4 and dithiothreitol dissolved in 0.9 ml Tris-HCl (pH 9.0) were present in the concentrations indicated.

Time	Concentration of Cu^{2+} and dithiothreitol (mM)	E	F	F/E	Extent oxidized (%)
2 min	0	16.6	5.7	0.34	17.4
4 min	0	18.1	7.1	0.39	28.1
2 min	0.5	18.8	15.2	0.81	45.3
4 min	0.5	20.9	22.2	1.06	45.4
2 min	1.0	21.3	20.3	0.95	28.1
4 min	1.0	20.6	25.5	1.24	37.3
2 min	3.0	11.3	19.4	1.72	43.4
4 min	3.0	15.4	33.3	2.16	48.0
2 min	5.0	12.9	10.0	2.6	24.1
4 min	5.0	33.6	31.3	3.13	31.0
2 min	10.0	9.8	31.8	3.24	24.5
4 min	10.0	10.6	33.6	3.17	38.7

TABLE IV

EFFECT OF DIFFERENT COPPER:DITHIOL RATIOS UPON PROSTAGLANDIN $\text{F}_{2\alpha}$ SYNTHESIS

Incubations were run for 2 and 4 min as described in Table I, but with varied levels of CuSO_4 and dithioglycerol as indicated.

Incubation	E	F	F/E	Extent Oxidized (%)
<i>3 mM Cu²⁺ + 1 mM dithioglycerol</i>				
2 min	8.2	21.1	2.6	38.8
4 min	8.0	24.7	3.1	52.2
<i>2 mM Cu²⁺ + 1 mM dithioglycerol</i>				
2 min	7.3	25.0	3.4	38.8
4 min	6.8	25.1	3.7	54.5
<i>1 mM Cu²⁺ + 1 mM dithioglycerol</i>				
2 min	10.1	28.3	2.8	37.5
4 min	9.7	26.8	2.8	50.7
<i>1 mM Cu²⁺ + 2 mM dithioglycerol</i>				
2 min	6.6	36.8	5.6	50.0
4 min	7.3	36.2	5.0	62.7
<i>1 mM Cu²⁺ + 3 mM dithioglycerol</i>				
2 min	10.5	50.0	4.8	14.3
4 min	5.7	53.7	9.4	29.6

absence of any cofactors, to 2.5 in the presence of 1 mM copper. Further enhancement of the production of prostaglandin F was found with added dithiothreitol. Under these conditions, the F/E ratio reached a value of 5.6. Regardless of the relative amounts of prostaglandin F and prostaglandin E produced, the overall extent of oxidation of substrate was comparable in either the absence or presence of cofactors.

The optimum level of cofactor was investigated using equimolar Cu^{2+} and dithiothreitol concentrations of 0, 0.5, 1.0, 3.0, 5.0 and 10.0 mM (see Table III). The F/E ratio obtained with 2-min incubations varied from 0.34 in the absence of any

TABLE V

CONDITIONS FOR PROSTAGLANDIN $F_{2\alpha}$ PRODUCTION

Incubations were run for 3, 6, 9 and 20 min by the introduction of 4 mg of acetone powder prepared from sheep vesicular gland suspended in 0.2 ml of 0.1 M Tris (pH 9.0) into tubes containing tracer amounts of ammonium [^{14}C]arachidonate, 10 nmoles of non-labeled arachidonate, 1 mM CuSO_4 , and 3 mM dithioglycerol in a total volume of 0.8 ml of 0.1 M Tris (pH 9.0). The incubations were run and assayed as described in EXPERIMENTAL.

	Prostaglandin $F_{2\alpha}$ formed (nmoles)			
	3 min	6 min	9 min	20 min
Hydroxy acids	1.1	1.5	2.2	2.2
Prostaglandin E_2	0.4	0.3	0.4	0.4
Prostaglandin $F_{2\alpha}$	3.3	4.0	3.8	5.0
Ratio F/E	8.2	13.3	9.5	12.5

cofactor to 3.24 in the presence of 10 mM Cu^{2+} and 10 mM dithiothreitol. Although the ratio of F/E increased with increased amounts of added cofactors, the amount of prostaglandin F produced per unit time reached a maximum of 33% when 3.0 mM $\text{Cu}_{2\alpha}$ and 3.0 mM dithiothreitol were used. The extent of oxidation of substrate then appeared to drop off as concentrations of cofactors above 3.0 mM were used.

Next the relative amounts of Cu^{2+} and dithiol (in this case dithioglycerol) were varied to determine the optimal ratio for production of prostaglandin F. Table IV demonstrates that the F/E ratio was highest when 1 mM Cu^{2+} and 3 mM dithioglycerol was used. However, the extent of substrate oxidation in 2–4 min was again much lower with this level of reductant than that observed at lower dithioglycerol concentrations.

As the experimentation progressed, we considered that larger amounts of protein were necessary for the production of larger amounts of product in the presence of the higher, more inhibitory, levels of dithiol. This led us to determine the amounts of prostaglandin F produced at different times by 4 mg of protein in the presence of 1 mM Cu^{2+} and 3 mM dithioglycerol. Table V shows that after 20 min of incubation, 50% of the oxidized arachidonic acid chromatographed as prostaglandin F. A very high F/E ratio (12.5) was obtained under these conditions.

The use of Zn^{2+} , Cd^{2+} , Mg^{2+} , Mn^{2+} , $\text{K}_4\text{Fe}(\text{CN})_6$, $\text{K}_3\text{Fe}(\text{CN})_6$, Ca^{2+} , Co^{2+} and Fe^{2+} in place of Cu^{2+} in incubations of the acetone powder with 1 mM dithioglycerol showed no enhancement in the production of prostaglandin F. When CuCl was added under the same conditions, however, it showed an enhancement similar to that exhibited by the cupric salt.

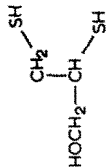
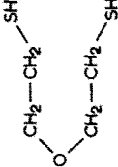
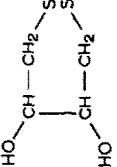
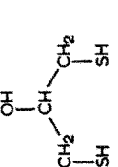
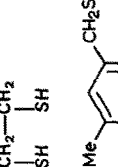
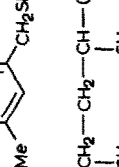

Relative effectiveness of different dithiols

Other thiols were assayed for their ability to enhance prostaglandin $F_{2\alpha}$ production in the presence of Cu^{2+} . Table VI illustrates data obtained in the presence of 1 mM Cu^{2+} using 2 mg of acetone powder and 1 mM dithiol for those dithiols that showed the greatest enhancement in the production of prostaglandin $F_{2\alpha}$ whereas dihydro-lipoamide exhibited the largest average rate as well as having the largest amount of substrate oxidized. 1,4-Dimercaptobutane and 1,6-dimercaptohexane completely inhibited oxygenation of arachidonate whereas 1,9-dimercaptononane, 2,3-dimercapto-succinic acid, 3,4-dimercaptotoluene, 2-mercaptoethanol, mercaptoethane and mer-

TABLE VI

PROSTAGLANDIN F₂₂ SYNTHESIS IN THE PRESENCE OF Cu²⁺ AND SELECTED DITHIOLS

Acetone powder (2 mg) was incubated for 2 and 6 min with 10 nmoles of ammonium arachidonate containing ¹⁴C tracer in the presence of 1 mM CuSO₄ and the indicated dithiol (1 mM) in a final volume of 1.0 ml of 0.1 M Tris (pH 9.0). The incubations were run in the manner described in EXPERIMENTAL. The nmoles prostaglandin F produced were determined from the counts/min at the appropriate region of the thin-layer plate. The nmoles of total substrate oxidized (Prod.) was determined from the loss of counts from the substrate region and the increase of counts/min in all other regions of the thin-layer plate.

Dithiol	2-min incubation		6-min incubation		Av. Prod. as Prostaglandin F (%)	Av. (nmoles/min)
	Prostaglandin F (nmoles)	Prod. (nmoles) · 100	Prostaglandin F (nmoles)	Prod. (nmoles) · 100		
	0.43	1.45	29.7	0.78	28.4	0.23
	0.66	2.64	25.0	1.09		
	0.42	1.64	25.6	0.74		
	0.61	2.72	22.4	1.08	27.0	0.215
	0.53	1.58	33.5	0.89	42.7	0.32
	1.28	2.55	50.2	2.09		
	0.96	2.69	35.7	1.50	31.7	0.43
	1.32	4.25	31.1	1.54	28.4	0.18
	0.42	1.50	28.0	0.61		
	0.62	2.03	30.5	0.68	28.4	0.18
	0.45	1.55	29.0	0.68		
	0.52	1.93	26.9	1.00	28.4	0.21
	1.56	4.16	37.5	1.91		
	1.75	5.41	32.3	2.31	33.8	0.60

captobenzene produced 0.4–1.5 nmoles of prostaglandin $F_{2\alpha}$ in 6 min, under the conditions described in Table VI. 1,2-Dimercaptobutane and 2,5-dimercapto-1,3,5-thiadiazole produced 1.7 nmoles of prostaglandin $F_{2\alpha}$.

DISCUSSION

The enhancement of prostaglandin F production by copper–dithiol complexes is demonstrated by experiments involving the utilization of [^{14}C]arachidonic acid and [^{14}C]eicosatrienoic acid. The decrease in the formation of prostaglandin E_2 which accompanies the enhancement of prostaglandin $F_{2\alpha}$ in the presence of the copper–dithiol complex is in agreement with the report of NUGTEREN *et al.*⁴ who found a pronounced inhibition of prostaglandin E synthesis by preincubation with certain metals including Cu^{2+} . However, those authors did not describe a concomitant rise in the amount of prostaglandin F produced. The present results lend further support to the idea of an intermediate endoperoxide common to both prostaglandin E and prostaglandin F biosynthetic routes⁸. The high F/E ratio serves to demonstrate the high efficiency with which the endoperoxide is converted to prostaglandin F by dithiols. We have previously shown a parallel capacity of glutathione to favor prostaglandin E synthesis at the expense of the other cyclic derivatives¹².

In the absence of Cu^{2+} , dithiothreitol and dihydrolipoamide inhibit the oxidation of substrate. This inhibitory effect may be due to a reductive removal of an obligatory intermediate.

Previous attempts to develop conditions favoring the reductive cleavage of the endoperoxide with tetrahydrofolate led to an increase in the amounts of both prostaglandin E and prostaglandin F with an F/E ratio of 0.7 (ref. 6). Recently, SIH *et al.*¹³ described a stimulation of prostaglandin F synthesis by epinephrine, but this was also accompanied by increases in other prostaglandin derivatives, so that in all cases the yield of prostaglandin F was less than that for prostaglandin E. The overall stimulation observed may have been related to the reported non-specific activation of the system by a variety of phenolic derivatives⁴.

The copper–dithiol reagents may favor an artifactual non-enzymic reaction somewhat analogous to that described for the Cu^{2+} -catalyzed decomposition of H_2O_2 (ref. 14). In this case the intermediate endoperoxide that had been stereoselectively formed would still be reduced to form only prostaglandin $F_{2\alpha}$ derivatives, as was observed. However, the fact that dihydrolipoamide was more effective than the many other dithiols tested raises the possibility of an enzyme-catalyzed reduction in our incubation mixtures and of enzymic regulation between the E and F-type hormones *in vivo*. Nevertheless, dihydrolipoamide is, in itself, an „artificial” electron donor since the active lipoate is normally covalently bound to protein. REICHARD¹⁵ and LAVRENT *et al.*¹⁶ have shown that an added dithiol can serve in three ways to facilitate deoxynucleotide formation. It served as a direct electron donor at high concentrations, but it also reduced thioredoxin and stabilized the BI protein at lower levels.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the National Science Foundation (GB-8243).

Assistance with the gas chromatograph-mass spectrograph was provided by Dr. C. C. Sweeley with a supporting grant RR 00480 from the U.S. Public Health Service.

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Biochim. Biophys. Acta, 260 (1972) 203-211