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ENZYMIC PATHWAYS OF GLYCERIDE AND PHOSPHOLIPID SYNTHESIS IN AORTIC HOMOGENATES

YECHEZKIEL STEIN, OLGA STEIN
AND BENYAMIN SHAPIRO

Departments of Medicine B, Experimental Medicine and Cancer Research, and Biochemistry, Hadassah University Hospital, Hebrew University–Hadassah Medical School, Jerusalem (Israel)

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SUMMARY

Homogenates were prepared from rabbit and dog aortas and were shown to catalyze the incorporation of [1-^{14}C]linoleic acid into neutral lipids and phospholipids. α-Glycerophosphate, CoA and ATP are required for this process. Triglycerides and diglycerides were the major radioactive components in the neutral lipid fraction. Lecithin, lysolecithin and phosphatidyl ethanolamine made up most of the radioactivity in the phospholipid fraction. An alternative pathway of lecithin synthesis was found in which lysolecithin served as immediate precursor. This reaction is also ATP and CoA dependent. With lysolecithin, as precursor, [1-^{14}C]linoleic acid was confined to the β-position of the lecithin synthesised; with α-glycerophosphate, both the α- and β-positions were labelled. Purified lecithin also promoted incorporation of linoleic acid into phospholipids. This is probably due to its transformation into lysolecithin, which then serves as [^{14}C]fatty acid acceptor.

INTRODUCTION

Considerable difficulties have been encountered in the preparation of homogenates and subcellular fractions of the aorta. This was mainly due to the abundance of elastic and fibrous components in the vessel wall. Hence the study of enzymic processes in general and those dealing with lipid metabolism of the aorta in particular was rather limited, while at the same time the composition of aortic lipids and the morphology of the atherosclerotic changes were investigated quite extensively. Phospholipid synthesis in the intact aortic wall *in vivo* was first demonstrated by ZILVERSMIT *et al.*[1]. In a previous communication from our group[2] it has been shown that aortic slices of several species of mammals incorporate labelled fatty acids into tissue glycerides and phospholipids. In the present study an attempt was made to elucidate some of the enzymic pathways involved in glyceride and phospholipid metabolism of the aortic wall. A procedure for preparing homogenates of aortic tissue facilitated this investigation.

MATERIALS AND METHODS

[1-^{14}C]Linoleic acid, obtained from Amersham (Great Britain) checked for purity by gas–liquid chromatography contained more than 99 % linoleic acid. Non radioactive linoleic acid was obtained from the Hormel Institute, Austin, Minn. Glyceryl monooleate was obtained from California Corp. Biochem. Res. CoA, ATP-Na and α-glycerophosphate were commercial preparations. Dipalmitoyl lecithin was obtained from Mann Research Corporation. Egg lecithin was purified according to HANAHAN *et al.*[3]. It was then treated in ether with *Crotalus adamanteus* venom[4] and the resulting lysolecithin was purified by repeated precipitations with ether. The purity of lysolecithin was checked by thin layer silicic acid chromatography.

Animals

Rabbits of either sex of a locally bred strain weighing 2.5 kg, kept on a diet consisting of fresh vegetables and purina chow, were used. Mongrel dogs of either sex weighing 6–12 kg were kept in single cages and fed purina chow.

Preparation of aortic homogenates

The animals were sacrificed under pentobarbital anaesthesia. The aorta was excised and rinsed in ice-cold KCl–Tris buffer (KCl, 0.154 M–Tris, 0.5 M (pH 7.45), 19:1 and EDTA, 10^{-4} M). The rabbit aortas were carefully freed of any adventitial fat tissue; in the dog aortas the adventitia was removed together with the outer third of the media and only the intima and the inner two thirds of the media were used. The tissues were finely minced with scissors and homogenized with 10 volumes KCl–Tris buffer in an all glass conical homogenizer (Kontes Glass Co. Vineland N.J.) for 1 min. The homogenate was centrifuged at 1000 rev./min for 2 min in a refrigerated MSE-centrifuge. The supernatant was decanted and the sediment resuspended in 5 volumes of KCl–Tris, rehomogenized for 1 min and recentrifuged under the same conditions. The combined supernatants were made up to volume with KCl–Tris to bring the protein concentration to 4–6 mg/ml. This suspension was designated "aortic homogenate" and used as the enzyme source. All the operations were carried out at 0–4°. In several instances the homogenate was lyophilized and stored at —20°.

Extraction and lipid fractionation

At the end of incubation about 10 ml of ethanol–ethyl ether (3:1) were added to the contents of the incubation flasks and the mixture was brought to boiling. The volume was then made up to 25 ml with ethanol–ethyl ether (3:1) mixed thoroughly and after centrifugation 15-ml aliquots were used for the determination of the extent of incorporation of [1-^{14}C]linoleic acid into tissue lipids. The aliquot was evaporated to dryness in a rotary flash evaporator at 40° and the residue was taken up in 3.0 ml petroleum ether 30–60°.

Separation of phospholipids from neutral lipids and free fatty acids was carried out by batch elution from silicic acid[2]. The extent of esterification of the radioactive fatty acids in the neutral lipid fraction was determined by trapping the unesterified fatty acid on MgO–Celite columns[5].

Separation of the neutral lipids in cholesterol ester, tri-, di- and monoglycerides was performed on florisil columns[6]. The phospholipids were separated on thin-layer silicic acid plates using the solvent system[7] of chloroform–methanol–water (65:25:4).

Separation and degradation of [^{14}C]lecithin

For preparation of lecithin from the phospholipids synthesized in aortic homogenates aliquots of the methanol extract were applied to thin-layer silicic acid plates. A small spot served as a marker and the rest was applied in the form af a streak. After separation of the phospholipids only the spot was visualized by exposing it to iodine vapors, while the area of the streak was covered by a glass plate and remained unstained The region of the streak corresponding to the lecithin spot was scraped off and extracted thrice with 3 ml of methanol. The pooled methanol extract was evaporated to dryness, diluted with 0.5 μmole of synthetic dipalmitoyl lecithin and the enzymic degradation of the lecithin to lysolecithin was achieved by the use of snake venom[4].

The products of degradation were rechromatographed on thin-layer silicic acid plates and the radioactivity of the lysolecithin, lecithin and the liberated fatty acids was determined.

The preparation of samples for radioactivity determination was carried out as described previously[2].

Protein was estimated turbidimetrically[8], using bovine serum albumin as standard.

RESULTS

α-Glycerophosphate as acyl-acceptor

Homogenates prepared from rabbit and dog aortas catalyse the incorporation of [1-^{14}C]linoleic acid into tissue lipids (Table I). Under the experimental conditions used, both neutral lipids and phospholipids became labelled, the latter to a somewhat greater extent. The absolute dependence of these processes upon ATP and CoA is demonstrated in Table II. The enzymic system is quite stable and withstands lyophilization.

The role of the α-glycerophosphate as the fatty acid acceptor was further investigated, using varying concentrations and trying its substitution with other possible precursors. As seen in Table III only minimal synthesis of neutral lipids was obtained when α-glycerophosphate was omitted from the incubation medium. With

TABLE I

INCORPORATION OF [1-^{14}C]LINOLEIC ACID INTO LIPIDS OF AORTIC HOMOGENATES

The incubation medium consisted of 0.25 μmole [1-^{14}C]linoleic acid with specific activity of 3.7 μC/μmole, 10 μmoles ATP-Na, 0.2 mg CoA, 20 μmoles K-phosphate buffer (pH 7.45), 10 μmoles $MgCl_2$, 10 μmoles Na-α-glycerophosphate and 2 ml of aortic homogenate, containing 8–12 mg protein. Final volume 3.0 ml, incubated in air at 37° with shaking for 60 min.

Expt. No.	*Fatty acids incorporated (mμmoles/10 mg protein)*			
	Dog		*Rabbit*	
	Neutral lipids	*Phospholipids*	*Neutral lipids*	*Phospholipids*
1	7.1	11.0	4.4	13.8
2	6.0*	7.0*	5.4	11.5
3	5.8	8.3	7.7	15.8

* Lyophilized preparation.

rising concentrations of added α-glycerophosphate increasing amounts of [1-^{14}C]-linoleic acid were incorporated into both lipid fractions. On the other hand considerable incorporation of [1-^{14}C]linoleic acid into phospholipids was found even in the absence of any added precursor. Of the other substances tested only monoolein has been shown to stimulate synthesis of neutral lipids. A direct esterification of the monoglyceride seems likely, since added glycerol was found to be completely inactive.

Lysolecithin as acyl-acceptor

The relatively high rate of incorporation of linoleic acid into phospholipids without added α-glycerophosphate as well as previous findings[2] suggested the existence of an alternative pathway of phospholipid synthesis. This has been investigated further with the use of lysolecithin as the fatty acid acceptor. As shown in Table IV, rather minute quantities of lysolecithin stimulate the rate of phospholipid synthesis to a considerable extent. The incorporation of linoleic acid into phospholipids increased with the concentration of lysolecithin up to 80 mμmoles/ml. When this concentration of lysolecithin was exceeded, inactivation of the enzyme systems took place. As expected, addition of lysolecithin had no effect on the incorporation of [1-^{14}C]linoleic acid into neutral lipids. The esterification of lysolecithin to lecithin has been shown to be ATP and CoA dependent (Table V).

TABLE II

COFACTOR DEPENDENCE OF [1-^{14}C]LINOLEIC ACID INCORPORATION INTO LIPIDS OF AORTIC HOMOGENATES WITH α-GLYCEROPHOSPHATE AS FATTY ACID ACCEPTOR

Conditions of incubation	*Fatty acid incorporated (mμmoles/10 mg protein)*			
	Dog		*Rabbit*	
	Neutral lipids	*Phospholipids*	*Neutral lipids*	*Phospholipids*
Complete system	6.0	9.0	5.5	12.0
ATP omitted	0.3	1.3	0.1	1.1
CoA omitted	0.4	2.2	0.1	1.3

TABLE III

INCORPORATION OF [1-^{14}C]LINOLEIC ACID INTO LIPIDS OF RABBIT AORTIC HOMOGENATE AT VARIOUS CONCENTRATIONS OF α-GLYCEROPHOSPHATE AND IN THE PRESENCE OF ALTERNATIVE PRECURSORS

Conditions of incubation as in Table I.

Precursor added (μmoles/ml)	*Fatty acid incorporated (mμmoles/10 mg protein)*	
	Neutral lipids	*Phospholipids*
None	0.3	5.7
α-Glycerophosphate 0.5	0.8	8.1
α-Glycerophosphate 1.0	2.3	9.6
α-Glycerophosphate 2.0	3.1	10.3
α-Glycerophosphate 4.0	4.4	13.8
Glycerol 4.0	0.6	7.6
Glucose 4.0	0.7	6.4
Monoolein 1.5	3.1	7.2

TABLE IV

EFFECT OF VARYING CONCENTRATIONS OF LYSOLECITHIN ON LIPID SYNTHESIS IN RABBIT AORTIC HOMOGENATES

Conditions of incubation as in Table I. α-Glycerophosphate has been substituted by lysolecithin.

Lysolecithin added (mμmoles/ml)	*Fatty acid incorporated (mμmoles/10 mg protein)*			
	Neutral lipids		*Phospholipids*	
	I	*II*	*I*	*II*
None	0.6	1.2	3.4	3.0
8	0.2	—	6.3	—
16	0.2	—	15.2	—
32	0.2	—	19.7	—
80	0.2	0.7	45.7	60.0
160	—	0.3	—	31.0
320	0	0	0	0.1
960	0	0	0	0

TABLE V

COFACTOR DEPENDENCE OF [1-^{14}C]LINOLEIC ACID INCORPORATION INTO LIPIDS OF AORTIC HOMOGENATES, WITH LYSOLECITHIN AS FATTY ACID ACCEPTOR

Conditions of incubation as in Table IV.

Conditions of incubation	*Fatty acid incorporated (mμmoles/10 mg protein)*			
	Dog		*Rabbit*	
	Neutral lipids	*Phospholipids*	*Neutral lipids*	*Phospholipids*
Complete system with lysolecithin	0.4	30.0	0.2	47.0
ATP omitted	0.1	2.3	0.1	3.0
CoA omitted	0.3	4.7	0.1	6.3

TABLE VI

COMPARISON OF ALTERNATIVE PATHWAYS OF LIPID SYNTHESIS IN AORTIC HOMOGENATES

Conditions of incubation as in Table I.

Species	*Fatty acid acceptor added (μmoles)*	*Fatty acid incorporated (mμmoles/10 mg protein)*					
		Neutral lipids			*Phospholipids*		
		I	*II*	*III*	*I*	*II*	*III*
Rabbit	None	0.4	1.2	0.3	3.4	3.0	5.7
	α-Glycerophosphate 10	5.5	14.0	4.4	12.0	15.0	13.8
	Lysolecithin 0.25	0.2	0.2	0.5	45.7	47.0	31.0
	α-Glycerophosphate 10 + Lysolecithin 0.25	0.1	0.8	1.0	52.3	80.0	55.7
Dog	None	—	2.5	—	—	8.0	—
	α-Glycerophosphate 10	6.0	7.0	6.0*	9.0	11.0	7.0*
	Lysolecithin 0.25	0.4	0.2	0.7	30.0	33.0	37.0

* Lyophilized preparation.

TABLE VII

EFFECT OF TIME ON INCORPORATION OF [1-^{14}C]LINOLEIC ACID INTO LIPIDS OF DOG AORTIC HOMOGENATES

Conditions of incubation as in Table I.

Time of incubation (min)	*Fatty acid incorporated (mμmoles/10 mg protein)*			
	α-Glycerophosphate added (10 μmoles)		*Lysolecithin added (0.25 μmoles)*	
	Neutral lipids	*Phospholipids*	*Neutral lipids*	*Phospholipids*
5	1.1	5.2	0	6.7
15	1.5	7.5	0.1	17.6
30	5.8	9.1	0.4	29.5
60	5.8	8.3	0.4	29.5
120	5.1	7.0	0.4	27.0

TABLE VIII

SEPARATION OF NEUTRAL LIPIDS SYNTHESIZED IN AORTIC HOMOGENATES INCUBATED WITH [1-^{14}C]LINOLEIC ACID IN THE PRESENCE OF α-GLYCEROPHOSPHATE

Isolation of neutral lipids and separation as in METHODS.

Species		*Percentage radioactivity incorporated into*			
		Cholesterol ester	*Triglycerides*	*Diglycerides*	*Monoglycerides*
Rabbit	1	2	50	46	2
	2	2	72	25	1
	3	2	52	45	1
Dog	1	4	52	44	0
	2	5	79	16	2
	3	6	66	27	1

TABLE IX

SEPARATION OF PHOSPHOLIPIDS SYNTHESIZED IN AORTIC HOMOGENATES INCUBATED WITH [1-^{14}C]LINOLEIC ACID

Isolation of phospholipids and separation as in METHODS.

Species	*Labelled compound isolated*	*Fatty acid acceptor added*					
		None		*α-Glycerophosphate*		*Lysolecithin*	
		Radioactivity (%)					
		I	*II*	*I*	*II*	*I*	*II*
Rabbit	Lysolecithin	6	9	12	8	1	1
	Lecithin	86	79	78	79	97	96
	PEA*	8	12	10	13	2	3
Dog	Lysolecithin	4	—	4	8	1	1
	Lecithin	92	—	92	84	97	98
	PEA	4	—	4	8	2	1

* PEA, phosphatidyl ethanolamine.

Comparison of both precursors

In the next experiments a comparison was made of the extent of incorporation of linoleic acid into lipids with either α-glycerophosphate or lysolecithin or both as the fatty acid acceptors. As seen in Table VI much more linoleic acid was incorporated into phospholipids in the presence of lysolecithin, than with α-glycerophosphate. Upon addition of both α-glycerophosphate and lysolecithin the incorporation was stimulated even further and at the same time there was a marked inhibition of neutral lipid formation. The difference in the extent of incorporation of linoleic acid into phospholipids when either lysolecithin or α-glycerophosphate were present is evident at all time intervals examined. Table VII shows that the incorporation of linoleic acid into both phospholipids and neutral lipids, with all precursors used is maximal after 30 min and no marked changes occur during subsequent 90 min of incubation.

Analysis of products

The neutral lipids synthesized in the presence of α-glycerophosphate were separated on florisil columns and the distribution of the label is shown in Table VIII. In both species examined most of the radioactivity was recovered in the diglyceride and triglyceride fractions. Separation of the labelled phospholipids showed that lecithin was the major component formed. This was especially evident when lysolecithin was used as the fatty acid acceptor. When α-glycerophosphate served as precursor some radioactivity was also found in lysolecithin and phosphatidyl ethanolamine (Table IX).

Positional analysis of [1-^{14}C]linoleic acid in lecithin

It seemed of interest to investigate the distribution of the labelled fatty acid in the lecithin molecule, synthesized either from endogenous substrate, α-glycerophosphate or from lysolecithin. Following incubation with each of the above mentioned substrates the phospholipids were isolated and the lecithin was separated as described in METHODS. The site of the radioactive fatty acid in the lecithin was ascertained by subjecting it to the action of snake venom. The free fatty acid produced represents

TABLE X

DISTRIBUTION OF [1-^{14}C]LINOLEIC ACID IN THE α- AND β-POSITION OF LECITHIN SYNTHESIZED IN AORTIC HOMOGENATES

Isolation of lecithin and degradation procedure as in METHODS.

Species	*Precursor added*	*Radioactivity (%) of lecithin in (after lysis with venom)*			
		Fatty acid		*Lysolecithin*	
		I	*II*	*I*	*II*
Rabbit	None	67	—	33	—
	α-Glycerophosphate	56	52	44	48
	Lysolecithin	94	97	6	3
Dog	None	76	—	24	—
	α-Glycerophosphate	66	86	34	14
	Lysolecithin	96	99	4	1

that in the β-position of lecithin, while the lysolecithin retains the fatty acid in the α-position[9]. It is evident from Table X that when lysolecithin was used as the fatty acid acceptor, the labelled fatty acid was found in the β-position of the lecithin. In the presence of α-glycerophosphate, the labelled fatty acid is equally distributed between the α- and β-position of the lecithin molecule when rabbit homogenates were used. In dog homogenates the distribution is slightly altered with 66 % of the fatty acid in the β-position.

TABLE XI

INCORPORATION OF [1-^{14}C]LINOLEIC ACID INTO PHOSPHOLIPIDS OF RABBIT AORTIC HOMOGENATE WITH LECITHIN AS PRECURSOR

Conditions of incubation as in Table I.

Precursor added (μmoles)	*Fatty acid incorporated (mμmoles/10 mg protein)*	
	Neutral lipids	*Phospholipids*
α-Glycerophosphate 10	6.2	13.0
Lecithin 1.3	0.7	40.0
Lysolecithin 0.25	0.3	44.7
Lecithin 1.3 + lysolecithin 0.25	0.5	70.0
Lecithin 1.3 without ATP and CoA	0.4	2.3

The finding of the reactions leading to formation of lecithin through lysolecithin in the aortic homogenate prompted an investigation of the endogenous origin of lysolecithin. A typical experiment is presented in Table XI. It is evident that the addition of purified lecithin to the incubation mixture resulted in an extensive incorporation of the labelled fatty acid into phospholipids, but not into neutral lipids. The effects of lecithin and lysolecithin were found to be additive and the reaction was ATP and CoA dependent.

DISCUSSION

The fibrous structure of aorta has presented many a problem to investigators trying to prepare homogenates or subcellular fractions in order to learn about enzymic processes of this tissue[10]. After repeated trials using homogenizers of various makes, like the Potter-Elvejhem, Waring blendor, Virtis homogenizer etc. the conical all-glass Kontes homogenizer was found to be the most suitable. A very fine and even homogenate was obtained, which could be pipetted easily and was quite adequate for the investigation of enzymic reactions.

It soon became evident that glyceride and phospholipid synthesis in these homogenates proceeds through a pathway similar to that described for the liver, in which α-glycerophosphate served as the glycerol precursor[11,12]. In contrast to the findings in the liver system, glycerol did not serve as acceptor. This observation implies that the rabbit aortic homogenate does not possess appreciable glycerokinase activity. Similar to the findings in kidney, intestinal mucosa and liver[13], monolein served as precursor of glycerides in the aortic homogenate system. The possibility that an alternative pathway of phospholipid synthesis may be operative in the aortic tissue was first considered on the basis of results obtained with aortic slices[2]. In the absence of glucose, dog aortic slices incorporated only minimal amounts of [1-^{14}C]-

linoleic acid into neutral lipids, whereas the phospholipids became labelled quite extensively. With the use of homogenates it was now found that α-glycerophosphate was indispensable for triglyceride but not for phospholipid synthesis. Since most of the labelled phospholipid formed, in the absence of added precursor, was lecithin, it was suspected that endogenous lysolecithin served as precursor. Such a reaction was first described by LANDS[14,15] in lung- and liver-subcellular fractions. The acylation of added lysolecithin was ATP and CoA dependent. The release by phospholipase A of more than 95% of the labelled fatty acid from the lecithin synthesized in the presence of lysolecithin, would mean that the entire lysolecithin molecule has served as the precursor of lecithin. The optimal concentrations of lysolecithin in the aortic homogenate system were lower by a factor of 10 than those found by LANDS for liver homogenates[15]. Moreover, the amounts optimal for the liver system proved highly toxic for the aorta homogenates probably owing to lower protein and/or cholesterol content of the latter preparation. These substances are known to exhibit protective properties against hemolytic activity of lysolecithin[16].

The stimulating effects of α-glycerophosphate and lysolecithin on the incorporation of [1-^{14}C]linoleic acid into aortic lipids were additive. However, under such conditions, the reaction was found to shift entirely towards phospholipid synthesis. The interpretation of this finding is still lacking.

[1-^{14}C]Linoleic acid was found only in the β-position of the lecithin obtained with lysolecithin as acceptor, but it was nearly equally distributed in both α- and β-positions of the molecule when α-glycerophosphate served as precursor. The distribution of the [1-^{14}C]linoleic acid in lecithin synthesized in the absence of added precursors indicated that it originated at least in part from endogenous lysolecithin. The presence of lysolecithin in aortic tissue was demonstrated previously by ZILVERSMIT *et al.*[17] and could be confirmed in the present study. Lysolecithin is not formed directly during phospholipid synthesis, according to the pathway described by KENNEDY *et al.*[18], but could originate as a product of degradation of lecithin. Indeed, incubation of aortic homogenates with linoleic acid in the presence of purified lecithin, resulted in extensive incorporation of the fatty acid into lecithin. This finding suggests the presence of phospholipases in the aortic wall, and these enzymic reactions are under current investigation.

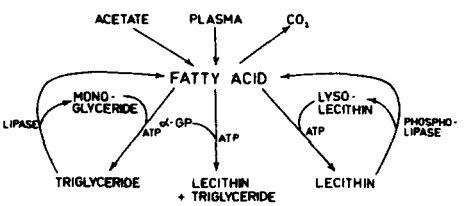

Fig. 1. Tentative scheme of triglyceride and lecithin metabolism in the aortic wall.

In the light of present data and on the basis of observations reported by others it might be possible to formulate a tentative scheme of lipid metabolism in the aortic wall (Fig. 1). Aortic fatty acids may be derived either by *in situ* synthesis from acetate[19] or from the unesterified fatty acids bound to serum albumin[2]. The fatty acids undergo esterification to either glycerides or phospholipids through the pathways described

above. These synthetic reactions require the addition of high-energy phosphate and CoA. On the other hand, the lytic reactions causing degradation of glycerides[20] and phospholipids are not ATP or CoA dependent. The relative importance of each pathway and its derangement under pathologic conditions leading to increased lipid synthesis and accumulation await further investigation.

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