Biochemical Pharmacology, Vol. 24, pp. 2263–2267. Pergamon Press, 1975. Printed in Great Britain.

EFFECT OF A HYPERCHOLESTEROLAEMIC DIET AND A SINGLE INJECTION OF POLYUNSATURATED PHOSPHATIDYL CHOLINE SOLUTION ON THE ACTIVITIES OF LIPOLYTIC ENZYMES, ACYL-CoA SYNTHETASE AND ACYL-CoA CHOLESTEROL ACYL-TRANSFERASE IN RABBIT TISSUES

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(*Received* 31 *January* 1975; *accepted* 20 *May* 1975)

Abstract—In rabbits fed a high cholesterol diet for 12 weeks the following were found: in serum, a decrease in lipase and no changes in phospholipase A and cholesterol esterase activities; in the aorta, decreased cholesterol esterase and acyl-CoA cholesterol acyltransferase (ACAT) activities and no changes in phospholipase A, lipase and acyl-CoA synthetase (ACS) activities; in the liver, increased phospholipase A, cholesterol esterase and ACAT activities, and decreased lipase and ACS activities. A single injection of polyunsaturated phosphatidyl choline in 4% sodium deoxycholate (EPL solution) in control and hypercholesterolaemic rabbits resulted in different effects on the enzyme activities. The stimulating, diet-independent and heparin-like effect of the drug on lipase consisted of increased activities of the enzyme in serum and liver and decreased activity in the aorta. Diet-dependent changes of the enzyme activities were as follows: in serum, decreased phospholipase A activity in control and increased phospholipase A activity in hypercholesterolaemic diet; in the aorta, decreased cholesterol esterase and elevated cholesterol ester synthesis/hydrolysis ratio in control and increased phospholipase A activity, decreased ACS activity and lowered cholesterol ester synthesis/hydrolysis ratio in hypercholesterolaemic diet; in the liver, increased phospholipase A and ACAT activities and decreased ACS activity with the ratio of cholesterol ester synthesis/hydrolysis elevated in control and this ratio unchanged in hypercholesterolaemic diet. Possible mechanisms of the enzyme activity changes are discussed.

Lipolytic enzyme activities are markedly changed in aortas of experimental animals given atherogenic diets containing large amounts of fat with [1] or without added cholesterol [2]. The response of the enzymes to hyperlipidaemia is believed to determine the different accumulation of lipids, mainly cholesterol esters, and favour the production of arterial lesions [2].

Aortic atherosclerosis was reduced in rabbits fed a high cholesterol [3] or a semi-synthetic diet containing 20% beef tallow without added cholesterol [2] by injections of polyunsaturated phosphatidyl choline solution in 4% sodium deoxycholate (EPL solution). The drug altered enzyme activities by exerting a stabilizing or normalizing effect on the aortic enzymes and serum phospholipase A†, and a stimulating effect by enhancing serum and liver lipase activities in rabbits fed the semi-synthetic diet [2].

There are no data regarding the enzyme activities and effects of the EPL solution in rabbits given a high cholesterol diet. It seemed of interest, therefore, to test whether the enzyme activities might be changed and stimulated by the drug in such animals. For this purpose, the activities of lipolytic enzymes, acyl-CoA synthetase (ACS) and acyl CoA cholesterol acyltransferase (ACAT) of serum, aorta and liver were examined in rabbits fed a cholesterol-rich diet and after a single injection of EPL solution.

MATERIALS AND METHODS

Experimental design

Male New Zealand White rabbits, which were approximately 24 weeks of age and 3 kg of body weight at the start of the experiment, were used. The animals were given a commercial cubed diet (Groups A and B) or the cubed diet with added 1% cholesterol and 5% corn oil (Groups C and D) and water *ad lib.*, for 12 weeks. The animals were injected in the marginal ear vein with 2 ml of either saline (Groups A and C) or the polyunsaturated phosphatidyl choline solution in 4% sodium deoxycholate (EPL solution, Nattermann, Germany). Blood was taken from the other ear vein, before and 10 min after the injection, chilled and centrifuged at 4° to obtain serum. Subsequently, within a further 10 min, the animals were killed with an injection of 5 ml Nembutal® and the aorta and liver removed.

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† Phospholipase A—phosphatide acyl-hydrolase (EC 3.1.1.4), lipase—glycerol-ester hydrolase (EC 3.1.1.3), cholesterol esterase—sterol-ester hydrolase (EC 3.1.1.13), acyl-CoA synthetase—acid: CoA ligase (AMP) (EC 6.2.1.3), cholesterol acyltransferase—acyl-CoA: cholesterol acyltransferase (EC 2.3.1).

Preparation of enzyme extracts

The adventitia and loose connective tissue were carefully stripped off and the aortas opened longitudinally. The aortas and liver specimens were rinsed with 0·25 moles/l. sucrose in 0·01 moles/l. Tris–HCl buffer, pH 7·5, dried with blotting paper and stored until processed further at −20°.

The aortas were sliced by means of scissors and liver specimens minced using a French press. Acetone–butanol powders were then prepared according to Morton [4]. For this purpose the material was immersed in solvent and homogenization followed by means of a Polytron PT 10 homogenizer (Kinematika GmbH, Luzerne, Switzerland) for two periods of 5 sec, and the procedure repeated three times, i.e. at each solvent change.

For the enzyme activity assay, the powders were extracted with 0·001 moles/l. Tris buffer at pH 7·5 containing 0·01 moles/l. 2-mercaptoethanol (12·5 and 25·0 mg of the powder for aorta and liver, respectively, per ml of the extractant) at 4° using the Polytron homogeniser for 3–5 sec. The suspension was spun at 3000 rev/min for 3 min and the supernatant separated from the upper lipoprotein layer and tissue residues. The protein extracts were kept at 4° and used for assay of the enzyme activities. Protein was determined according to Lowry [5].

Assay of enzyme activities

The assay of enzyme activities was carried out using sample-type two-point methods based on measuring concentrations of reaction products extracted from reaction mixtures. Optimum experimental conditions for the lipolytic enzymes (2,21,22), acyl-CoA synthetase (7) and cholesterol acyl-transferase (9), were used.

Lipolytic enzymes. Substrate hydrosols were prepared as previously [2] but lecithin, glyceryl trioleate (Sigma, U.S.A.) and cholesterol (British Drug Houses) were dispersed in 0·1 moles/l. Tris buffer by means of the Polytron homogenizer for several minutes. The substrate hydrosols contained: (1) 3 μmoles of lecithin per ml (pH 7·5 for serum and pH 8·0 for aorta and liver enzyme); (2) 1 μmole glyceryl trioleate and 5 mg of albumin per ml (pH 8·3); and (3) 3 μmoles of cholesteryl oleate + 5 mg albumin + 7·5 μmoles of sodium taurocholate per ml (pH 8·6). The reaction mixtures consisted of 0·5 ml of the appropriate substrate hydrosol, i.e. 1·5 μmoles lecithin or 0·5 μmoles glyceryl trioleate or 1·5 μmoles cholesteryl oleate, and 0·5 ml enzyme preparation containing 0·5–5·0 mg protein in 1 ml total volume. Hydrolysis was carried out for 1 hr at 37° and stopped by adding 5 ml of ethanol–toluene (1:2 v/v) extraction mixture and 0·5 ml 1 N sulphuric acid [6]. After shaking for 3 min and centrifuging for 10 min, 3 ml of supernatant were taken for titration of free fatty acids with 0·025 N NaOH in the presence of thymol blue under nitrogen. In controls, substrate and enzyme preparation were incubated separately and mixed together with the extraction mixture after incubation. Three tests and controls were run in parallel. Results are expressed in nmoles of fatty acids released/60 min per mg of protein.

Acyl-CoA synthetase (ACS) and acyl-CoA cholesterol acyltransferase (ACAT). The palmitoyl CoA synthetase was assayed according to the hydroxamate formation method of Pande and Mead [7]. The reaction mixture consisted of Tris hydroxymethyl methylamine 100 μmoles pH 7·5, potassium palmitate 5 μmoles, hydroxylamine 500 μmoles, KF 25 μmoles ATP 20 μmoles, CoA-SH 1 μmole, 2-mercaptoethanol 5 μmoles and enzyme preparation 0·5 ml in 1 ml total volume. In controls ATP and CoA-SH were omitted. After 1 hr incubation at 37°, the reaction was terminated by addition of 1 ml ethanol and successively 0·5 ml M perchloric acid. In supernatant the palmitoyl hydroxamate formed was determined according to Skidmore and Entenman [8]. ACAT was assayed using a modified procedure of Goodman *et al* [9]. Enzyme preparation (0·5 ml) was incubated with 5 μmoles potassium palmitate, 1 μmole cholesterol, 20 μmoles ATP, 1 μmole CoA-SH, 100 μmoles Tris hydroxymethyl methylamine, pH 7·5, 25 μmoles KF, 4 μmoles $MgCl_2$ and 5 μmoles 2-mercaptoethanol in total volume of 1 ml. In controls ATP and CoA-SH were omitted. After 1 hr incubation at 37°, the reaction was stopped by adding 1 ml ethanol, and followed by 30 min hydroxyaminolysis with 330 μmoles of hydroxylamine in alkaline conditons. 0·5 ml of 2 M perchloric acid was added and the palmitoyl hydroxamate determined as before. Results are expressed in nmoles of fatty acid esterified/60 min per mg of protein.

RESULTS

In rabbits fed the hypercholesterolaemic diet (Group C) the following changes in enzyme activities were found: decreased activity of serum lipase (Table 1, $P < 0·02$), decreased cholesterol esterase and ACAT activities in the aortic wall, and decreased lipase and ACS and increased phospholipase A, cholesterol esterase and ACAT activities in the liver, with unchanged ratios of cholesterol ester synthesis/hydrolysis in aorta and liver (Table 2).

Injection of EPL solution in animals fed the control diet (Group B) resulted in decreased phospholipase A and increased lipase activities in serum (Table 1), decreased lipase and cholesterol esterase activities in the aorta, and increased phospholipase A, lipase and ACAT and decreased ACS activities in the liver, with elevated ratios of cholesterol ester synthesis/hydrolysis in aorta and liver (Table 2).

The following changes resulted from the injection of EPL solution in cholesterol fed animals (Group D): increased activities of serum phospholipase A and lipase (Table 1), increased activities of aortic phospholipase A and liver lipase, and decreased activities of the aortic lipase and ACS with the ratio of cholesterol ester synthesis/hydrolysis lowered in the aorta and unchanged in the liver (Table 2).

DISCUSSION

There are some similarities and differences between the results obtained in rabbits fed the high cholesterol diet (Tables 1 and 2) and those previously demonstrated in animals given the high fat semi-synthetic diet [2]. Decreased activity of cholesterol esterase in the aortic wall is common for both diets. The semi-synthetic diet increased the aortic incorporation of

Table 1. Lipolytic enzyme activities in serum

Group[a]	Diet	Injection[b]		No. of animals	Phospholipase A[c]	Lipase	Cholesterol esterase
					nequiv. hr^{-1}. mg^{-1}		
A	control	saline	before	7	24·4 ± 5·0[d]	16·4 ± 3·8	10·5 ± 2·5
			after		26·0 ± 3·7	18·6 ± 5·0	10·7 ± 4·3
			diff.		1·6 (2·50)[e]	2·2 (1·40)	0·2 (2·24)
B	control	EPL soln.	before	9	27·0 ± 5·0	18·3 ± 5·4	11·0 ± 2·2
			after		7·7 ± 4·9	27·3 ± 7·4	8·9 ± 2·3
			diff.		−19·3 (1·24)‡	9·0 (2·51)†	−2·1 (1·07)
C	cholesterol	saline	before	6	24·8 ± 2·8	11·4 ± 3·2	12·0 ± 1·7
			after		25·2 ± 3·2	11·3 ± 3·1	12·7 ± 1·0
			diff.		0·4 (0·58)	−0·1 (0·72)	0·7 (0·61)
D	cholesterol	EPL soln.	before	7	27·0 ± 5·0	14·0 ± 2·4	13·7 ± 4·2
			after		35·8 ± 8·9	31·0 ± 8·8	17·2 ± 4·7
			diff.		8·8 (2·31)*	17·0 (3·19)†	3·5 (2·99)

[a] Rabbits fed a cubed diet (A,B) or the cubed diet containing 1% of added cholesterol and 5% corn oil (C,D) for 12 weeks.

[b] Single, intravenous injection of 2 ml. EPL soln. contained 200 mg polyunsaturated phosphatidyl choline.

[c] For reaction mixtures see text.

[d] Mean ± standard deviation.

[e] Mean (standard error of the mean). Statistically significant differences according to Student's paired *t*-test are indicated by: *$P \leqslant 0{\cdot}02$; †$P \leqslant 0{\cdot}01$; ‡$P \leqslant 0{\cdot}001$.

free fatty acid into cholesterol esters along with increased rates of hydrolysis of triglyceride and lecithin. The cholesterol diet, however, decreased the rate of cholesterol ester synthesis and failed to alter the rates of hydrolysis of the other esters. The low fat content of the cholesterol diet may explain the unchanged phospholipase and lipase activities.

Furthermore, the semi-synthetic diet increased the activity of serum phospholipase A but had no effect on serum and liver lipase and liver phospholipase A activities. On the other hand, the cholesterol diet had no effect on serum phospholipase A but decreased serum and liver lipase and liver ACS activities, and increased liver phospholipase A, cholesterol esterase and ACAT activities. Substrate induction of the enzymes may account for at least two of their increased activities.

The influence of EPL solution on the phospholipase A, cholesterol esterase, ACS and ACAT activities was different in different tissues and dependent on the kind of diet given to the animals. This is particularly expressed by the different ratios of cholesterol ester synthesis/hydrolysis rates which were significantly lowered, i.e. shifted towards hydrolysis, in the aortic wall in animals fed the experimental diet but elevated in the control ones. Thus, in atherosclerotic animals, EPL solution is having a beneficial effect in that the formation of cholesterol esters is less favoured.

There may be objections to a comparison of the reaction rates because of the marked differences for individual fatty acids, particularly oleic [10] compared with other fatty acids. In these experiments, palmitic acid was used as substrate and it might be unwise to extrapolate the results on cholesterol ester synthetases of oleic and other fatty acids. Nevertheless, the significance of the relative increase in hydrolysis and decrease in syntheiss of cholesterol esters is supported by the results obtained after long-term injections of the drug in rabbits [2] and baboons [11] fed atherogenic diets. It is these effects of EPL solution which may play an important role in its antiatherosclerotic effect by reducing the net synthesis of cholesterol esters.

Thus, the kind and duration of the experimental diet and dosage of the drug may be important for the enzyme activity patterns obtained and more precise resolution could be anticipated if more data over a longer experimental period were available.

On the other hand, lipase activity in the three tissues investigated was stimulated by the drug in different but diet-independent directions. The increase in serum and liver lipase activities and the decrease in aortic lipase activity are in agreement with results obtained after long-term injections of the drug in rabbits fed the semi-synthetic diet [2] and after a single injection of heparin in animals given a control diet [12]. The heparin-like action of the drug on plasma lipids in the course of alimentary lipaemia, i.e. the decrease in concentrations of glycerides and esterified cholesterol shown in serum in both control and atherosclerotic patients [13, 14], can be explained by the enhancing effect on lipolytic enzyme activities.

When the mechanism of action of the polyunsaturated phosphatidyl choline–sodium deoxycholate solution on the enzyme activities is considered, the possibility that either compound in the mixture may be acting cannot be ignored. Various properties of phospholipids [15], involving transacylations between lecithin and cholesterol [16, 17] and cholesterol ester and lysolecithin [18], are possibly of importance. This is supported by decreased activities of the aortic lipase and cholesterol esterase which also were seen after a single injection of the phospholipid prepared as hydrosol, i.e. without sodium deoxycholate, in rabbits fed the control diet (unpublished data). Bile salts, like heparin [12], may act as anions affecting the charge distribution of the enzyme–substrate interface

Table 2. Enzyme activities in organs

Organ	Group[a]	Diet	Injection	No. of animals	Phospholipase A	Lipase	Cholesterol esterase	Acyl-CoA synthetase	Cholesterol acyltransferase	Cholesterol ester synthesis/ hydrolysis
					nequiv. hr^{-1}. mg^{-1}					
Aorta	A	control	saline	8	158·3 ± 35·2[b]	146·8 ± 45·3	97·1 ± 16·0	36·1 ± 5·1	123·0 ± 11·8	1·27 ± 0·30
	B	control	EPL soln.	8	193·9 ± 63·6	49·0 ± 19·6§	54·8 ± 10·3§	29·9 ± 5·9	151·8 ± 35·0	2·77 ± 0·61§
	C	cholesterol	saline	6	185·5 ± 20·8	153·9 ± 24·5	50·9 ± 10·7§	40·3 ± 9·3	65·5 ± 19·8§	1·29 ± 0·31
	D	cholesterol	EPL soln.	7	231·1 ± 34·1‡	89·0 ± 16·0†	60·8 ± 10·2§	28·5 ± 4·6‡	57·3 ± 6·8§	0·93 ± 0·21*
Liver	A	control	saline	8	25·6 ± 4·1	29·9 ± 4·7	21·0 ± 4·3	20·4 ± 4·4	19·7 ± 3·9	0·94 ± 0·25
	B	control	EPL soln.	8	58·8 ± 11·8§	55·9 ± 19·0§	22·0 ± 5·0	15·9 ± 3·1*	32·1 ± 7·7‡	1·46 ± 0·37‡
	C	cholesterol	saline	6	41·5 ± 10·0‡	21·1 ± 4·5‡	37·3 ± 8·0§	14·5 ± 3·9*	28·3 ± 4·0‡	0·76 ± 0·21
	D	cholesterol	EPL soln.	7	47·2 ± 7·2§	35·5 ± 4·3*	39·4 ± 7·3§	14·5 ± 3·5*	29·4 ± 4·9‡	0·75 ± 0·18

[a] For Groups and injections see Table 1.

[b] Mean ± standard deviation. The means were compared by Student's *t*-test. Where the values for means were different from control (A), the statistical significance is indicated by: * $P \leqslant 0{\cdot}05$;

† $P \leqslant 0{\cdot}02$; ‡ $P \leqslant 0{\cdot}01$; § $P \leqslant 0{\cdot}001$.

and as specific cofactors [19] or emulsifying agents. This is indicated by the *in vitro* activating effect of small concentrations of sodium taurocholate on serum lipase in the rabbit (unpublished data) and man [20], and by the inhibitory effect of sodium deoxycholate and other bile salts on the aortic lipase in the pig [21, 22]. It seems, therefore, that both the phospholipid and bile salt are important for the response of the enzyme activities to the injection of the drug.

Acknowledgements—We are grateful to Nattermann & Co. of Cologne for a grant in support of this work.

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