PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 179, 13–20 (1985)

Acute Effects of Filipin on the Plasmatic, Hepatic, and Biliary Cholesterol of the Rat (42057)

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Abstract. Twenty-one male Wistar rats, 13 weeks old, were fed *ad libitum* hyperlipidic diets (28% fats) loaded with cholesterol (1.2%) for 5 weeks. One group of 11 rats was fed saturated fats (diet group "S") and another group of 10 rats was fed polyunsaturated fats (diet group "PU"). On the day they were sacrificed 10 of the rats were injected intravenously with 1 mg of filipin. Contrary to the rats in diet group "PU," the rats in diet group "S" treated with filipin presented certain characteristics that were not found in the nontreated group: They provided evidence of biliary cholostase accompanied by a decline in the level of secretion of bile salts and phospholipids into bile. The concentrations of both free and esterified cholesterol in plasma fell and the amount of (esterified) hepatic cholesterol rose, although there was no change due to the filipin in the amounts of hepatic phospholipids. Explanatory hypotheses for these phenomena were considered, first, at the site of plasma membranes where filipin binds selectively to the cholesterol in the membrane, causing a disruption which probably disturbs the absorbance of circulating lipoproteins, especially that of hepatocyte cells, particularly in diet group "PU." Second, the effects of filipin on subcellular membranes seem to disturb the secretion of lipids and lipoproteins into bile and plasma, especially in diet group "S." Last, at the intracellular level, filipin appears to have a blocking effect on the organelles involved in biliary lipid secretion. The activity of certain enzymes such as cholesterol esterase may also be blocked, particularly in diet group "S," which would explain the accumulation of esterified cholesterol in liver.

Filipin, an antibiotic polyene, is a drug that binds selectively and specifically with free cholesterol (1–5). *In vitro,* this drug interacts with vesicles containing phospholipids (3, 6) and with the surface of various categories of lipoproteins (HDL, high-density lipoproteins; LDL, low-density lipoproteins) (1, 2). *In vivo,* filipin is stored on the cholesterol contained in the membranes of bacteria (6) and pluricellular organisms (7), causing the deformation (8) of these membranes.

Filipin has therefore been used to study the structure of plasma membranes and those of subcellular organelles (8) where the amount of cholesterol is much higher than in intracellular membranes (8, 9). Evidence has shown that lower cholesterol levels represent greater fluidity in membranes (7, 10, 11). Moreover, in the intracellular medium filipin accumulates on lysosomes instead of gap junctions, tight junctions, and coated pits (9). Since the secretion of the lipoprotein biliary complex may involve such organelles (12), it appeared interesting to study the influence of filipin on the secretion of lipids into bile.

Previous works (submitted for publication) have shown that after several weeks a hyperlipidic diet loaded in cholesterol and saturated fats results in the stimulation of the biliary cholesterol flow and that polyunsaturated fats have the opposite effect. The purpose of the present study is to examine the influence of filipin on the metabolic regulation of plasma and biliary cholesterol by rats which have been given the above-mentioned hyperlipidic diets and acute injections of the drug.

Materials and Methods. *Animals.* Twenty-one male Wistar rats (raised by Iffa-Credo, Arbresle, France) 11 weeks old were placed at random by pairs in standard animal house cages for a 2-week period of adaptation. The rats were fed *ad libitum* with a standard diet mixture (UAR No. A03, Villemoisson sur Orge, France). The rats were then divided into two groups. The first batch (11 rats) was

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0037-9727/85 $1.50

fed a hyperlipidic diet ("S") containing lard (28%) and cholesterol (1.2%). The second batch (10 rats) was fed under similar conditions a diet ("PU") containing safflower oil (28%) and cholesterol (1.2%). These two diets (containing 24.5% protein, 38% glucides, 1% vitamins—UAR mixture No. 200, 7.5% mineral salts—UAR mixture No. 205b) were administered in the form of flour for 5 weeks. Drinking water consisted of tap water provided *ad libitum*. The rats were sacrificed at 18 weeks, at an average weight of 507 ± 10 g.

Physiological techniques and sampling. The fed rats were anesthetized with sodium pentobarbital (5 mg/100 g of body wt) and a biliary fistula was placed in the bile duct of each animal. After 1 hr of bile sampling, each batch of rats was divided into two groups:

	Diet S	Diet PU
Nontreated rats	6	5
Rats treated with filipin	5	5

The rats in the treated groups were injected intravenously (femoral vein) for 15 min with a 1-mg dose of filipin complex (U-5956/Upjohn Company, Kalamazoo, Mich.) previously solubilized in 30 μl ethanol and 0.5 ml of 0.9% sodium chloride. The rats in the nontreated groups were injected with the same solution without the filipin. Bile was collected every hour for 5 hr, weighed, and then stored frozen. At the end of this period the rats were sacrificed by an aortal puncture of blood which was collected on heparin. The liver was immediately removed, weighed, and stored frozen.

Titration. First the bile samples were titrated for phospholipids (13), bile salts (14), and cholesterol (15). The activity of β glucuronidase was also calculated (16). The filipin content in the bile was studied using a Jobin-Yvon spectrophotometer which displays a 513 nm fluorescent emission in the presence of filipin.

Second, the blood samples were titrated for triacylglycerols (17), total cholesterol, and free cholesterol (18), total protein (19), albumin (20), and phospholipids (13). Enzyme activity was also calculated for alkaline phosphatase (21) and the transaminase enzymes, alanine amino transferase (22) and aspartate amino transferase (22).

Last, livers were thawed and then homogenized using an Ultraturrax instrument in a buffer composed of saccharose/Tris (1 m*M*/0.25 *M*) (23). The homogenate was titrated for total protein (19), triacylglycerols (17), total cholesterol and free cholesterol (18), phospholipids (13) and albumin (20). Enzyme activity was observed for alanine amino transferase (22), aspartate amino transferase (22), and alkaline phosphatase (21). Results were compared by variance analysis.

Results. Results concerning the secretion of lipids and bile enzymes as well as biochemical parameters drawn from plasma and liver data were established by comparison of the treated and nontreated rat groups. The influence of the different diets (S, saturated fats and PU, polyunsaturated fats) on the rats treated with filipin was also analyzed.

Biliary secretion. Bile flow did not vary significantly between the treated and nontreated rats, as was also the case between the treated rats that had been given either diet S or diet PU (Fig. 1).

For both diets there was almost no difference in the amount of cholesterol contained in the biliary secretions of the treated rats and the nontreated rats, whether expressed as a concentration or as a total amount. This rate was quite close to the one displayed by the treated rats in both diet group S and diet group PU.

Filipin brought about a slight decrease in the secretion rate of bile salts (expressed as a concentration) in both diet groups and had the same effect on the secretion rate of phospholipids in diet group S. In diet group PU the bile salt and phospholipid rates (in concentration and total amount) of the treated animals were significantly lower than in diet group S (Table I). As a result, the bile salts/phospholipids/cholesterol ratio (molar ratio) are lower for the treated rats than for the nontreated ones, especially for those in diet group PU (respectively, 48/8/1 and 50/7/1 for the (S) diet, 29/5/1 and 40/5/1 for the (PU) diet, at the sixth hour).

The activity of β glucuronidase, which marks the secretion of lysosomes, is only slightly different in the bile of the treated and nontreated rats in either diet group (Fig. 1).

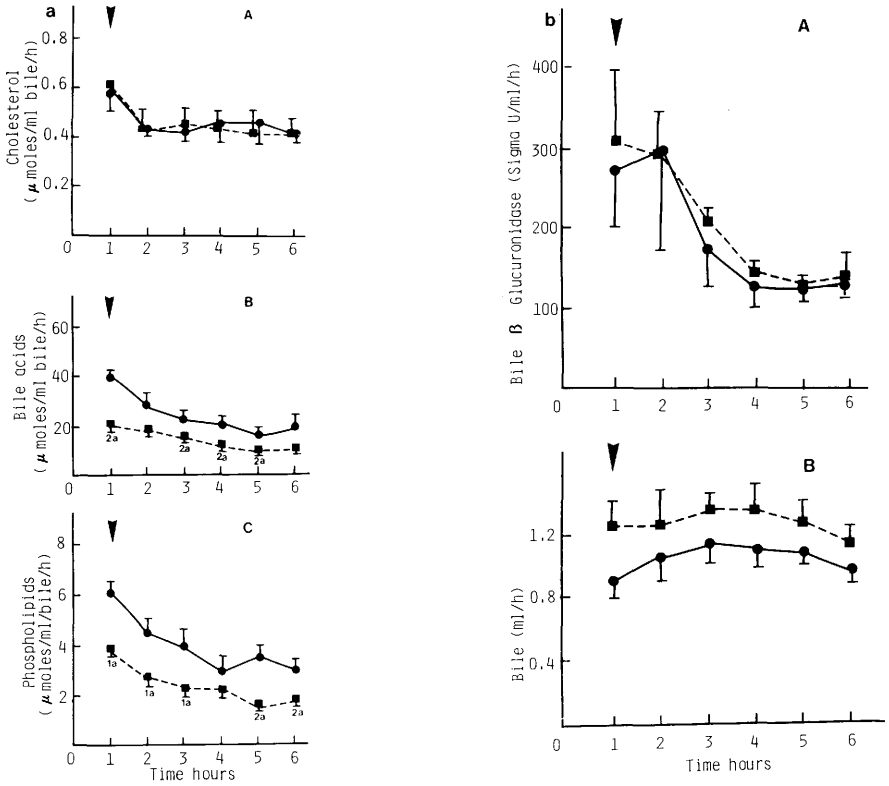

FIG. 1. Compared effects of diets (S) and (PU) on the bile secretion of filipin-treated rats: (a) cholesterol (A), bile acids (B), phospholipids (C); (b) bile β glucuronidase (A), bile flow (B). Results are expressed as means ± SEM.: $n = 5$, diet (S), ●; $n = 5$, diet (PU) ■. Differences were analyzed with analysis of variance and are designated with 1, 2 when significantly different: 1, $P < 0.05$; 2, $P < 0.01$. a, Diet (S) vs diet (PU).

For the rats treated with filipin, the value reached in diet group S was quite similar to that shown for diet group PU. Evidence of filipin in the bile of the treated rats was detected, however, as a fluorescent emission was present at a wavelength of 513 nm, which is characteristic of this antibiotic and its metabolites.

Plasma biochemical data (Fig. 2). At the end of the experiment the total cholesterol level in plasma was significantly lower for rats treated with filipin than for the non-treated rats in diet group S. This difference had an effect on the rates of free cholesterol and esterified cholesterol. In diet group PU this difference was not displayed.

In the filipin-treated rats, the level of hypercholesterolemia induced by diet S decreased to a level close to that of diet group PU.

The other parameters (phospholipids: 1.7 mmoles/liter; protein: 65 g/liter; albumin: 500 μmoles/liter; alkaline phosphatase: 250 I.U./liter; aspartate amino transferase: 450 I.U./liter; alanine amino transferase: 350 I.U./liter) were not affected by the type of diet administered or by the filipin.

Liver biochemical data (Table II). In diet group S filipin brought about an increase in the esterified cholesterol and in the rate of activity of alanine amino transferase. No other differences were observed between the treated and nontreated animals (particularly for the phospholipids).

Average values for total liver of diet group S were as follows: liver weight: 19.5 g; protein: 2.7 g; triacylglycerols: 560 μmoles; phospholipids: 585 μmoles; albumin: 44 μmoles; aspartate amino transferase: 3200 I.U.; alkaline phosphatase: 30 I.U. Average values for total

TABLE I. AMOUNTS OF BILIARY LIPIDS SECRETED PER HOUR (μmole)

	Sampling hour					
	1	2	3	4	5	6
Cholesterol						
Diet (S)						
Nontreated rats ($N = 6$)	0.54 ± 0.07	0.62 ± 0.11	0.49 ± 0.09	0.46 ± 0.05	0.48 ± 0.06	0.53 ± 0.08
Filipin-treated rats ($N = 5$)	0.53 ± 0.09	0.46 ± 0.06	0.47 ± 0.03	0.49 ± 0.05	0.47 ± 0.06	0.39 ± 0.01
Diet (PU)						
Nontreated rats ($N = 5$)	0.42 ± 0.08	0.36 ± 0.08	0.42 ± 0.07	0.46 ± 0.06	0.45 ± 0.09	0.40 ± 0.07
Filipin-treated rats ($N = 5$)	0.83 ± 0.19	0.55 ± 0.10	0.61 ± 0.11	0.54 ± 0.05	0.50 ± 0.11	0.45 ± 0.07
Bile salts						
Diet (S)						
Nontreated rats ($N = 6$)	24.8 ± 4.4	24.7 ± 2.8	21.2 ± 1.7	20.0 ± 1.5	23.1 ± 2.3	19.4 ± 3.3
Filipin-treated rats ($N = 5$)	37.3 ± 4.4	30.7 ± 6.9	25.8 ± 3.0	22.8 ± 3.6	17.2 ± 1.8	19.0 ± 6.1
Diet (PU)						
Nontreated rats ($N = 5$)	19.2 ± 3.7	19.0 ± 2.1	20.6 ± 2.7	20.1 ± 2.7	14.8 ± 1.3	15.6 ± 4.2
Filipin-treated rats ($N = 5$)	25.8 ± 6.3	23.0 ± 5.9	18.2 ± 1.2*,b	15.5 ± 2.5	12.3 ± 2.0	11.9 ± 1.5
Phospholipids						
Diet (S)						
Nontreated rats ($N = 6$)	4.56 ± 0.50	4.15 ± 0.39	4.25 ± 0.55	3.65 ± 0.45	3.31 ± 0.49	3.66 ± 0.91
Filipin-treated rats ($N = 5$)	5.63 ± 0.96	4.59 ± 0.50	4.56 ± 0.93	3.31 ± 0.44	3.89 ± 0.50	2.99 ± 0.30
Diet (PU)						
Nontreated rats ($N = 5$)	3.20 ± 0.11*,b	4.71 ± 1.90	3.05 ± 0.46	2.66 ± 0.36	2.25 ± 0.41	2.19 ± 0.39
Filipin-treated rats ($N = 5$)	5.45 ± 1.04*,a	3.31 ± 0.55	2.73 ± 0.18	2.55 ± 0.28	1.97 ± 0.23**,b	2.00 ± 0.36*,b

Note. Results are expressed as means ± SEM. Differences were analyzed with analysis of variance and are designated with asterisks when significantly different: $*P < 0.05$; $**P < 0.01$. For the same diet: [a]nontreated versus filipin-treated rats. For the nontreated rats or for the filipin-treated rats: [b](S) versus (PU).

liver of diet group PU were as follows: liver weight: 23 g; protein: 4.20 g; triacylglycerols: 260 μmoles; phospholipids: 630 μmoles; albumin: 45 μmoles; aspartate amino transferase: 5000 I.U.; phosphatase alcaline: 50 I.U.

The significant differences observed between diet group S and diet group PU among the nontreated animals were the same as those observed among the two groups treated with filipin.

Discussion. Contrary to the results found in diet group PU in which only slight differences appeared between the treated and nontreated animals, the results in diet group S showed that filipin caused an accumulation of esterified cholesterol in liver which is accompanied by a corresponding decline in the cholesterol concentration present in plasma and bile. Another interesting feature was the dissociation observed between cholesterol and phospholipids during the bile secretion.

The various causes for the effects of filipin may be localized, on one hand, at membranes, especially plasma membranes and those that contribute to hepatic secretions (of bile and blood). On the other hand, filipin may also produce effects at the subcellular

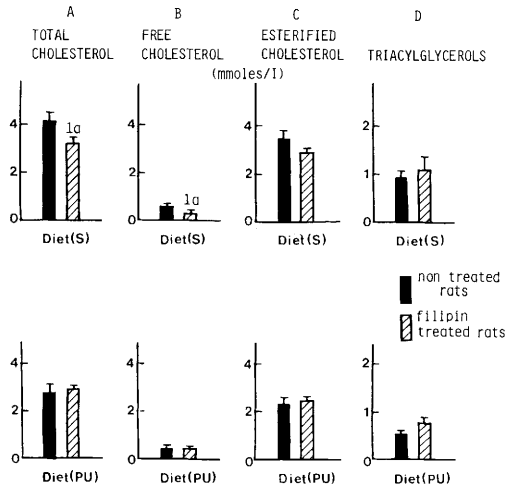

FIG. 2. Effect of filipin on plasma lipids. Results are expressed as means ± SEM. For the filipin-treated rats: $n = 5$, diet (S); $n = 5$, diet (PU). For the nontreated rats: $n = 6$ diet (S); $n = 5$ diet (PU). Differences were analyzed with analysis of variance and are designated with 1 when significantly different: 1 $P < 0.05$. a, Filipin-treated vs nontreated rats (for the same diet).

level by intervening directly in the enzyme activities of cholesterol metabolism or by blocking subcellular organelles.

Certain authors have shown that filipin storage also occurs on the free cholesterol of circulating lipoproteins such as HDL (1) and LDL (2). It is therefore probable that for either diet group S or diet group PU, which naturally show relatively high levels of LDL and HDL, respectively, lipoproteins may very well be capable of carrying filipin injected intravenously.

Since other investigators (9) have studied the intracellular transfer of LDL to fibroblasts using filipin as a trace agent, we may presume that during our tests the cholesterol transported by circulating lipoproteins penetrated freely into the hepatocyte and that it may still be exchanged between seric lipoproteins and plasma membranes (8), especially those of the hepatocytes.

Effects of filipin on plasma membranes. Filipin is a polyene with "membrane disruptive" antibiotic properties which are made possible by the ability of this substance to accumulate selectively and specifically on membrane cholesterol (4, 9) distributed unevenly along the length of the membrane (7), especially along plasma membranes which are particularly rich in cholesterol (8, 9)—low cholesterol rates being associated with high membrane fluidity (10).

Plasma membranes enriched within bile ducts have a higher phospholipid/cholesterol

TABLE II. EFFECT OF FILIPIN ON LIVER COMPOSITION

Diet	(S) Nontreated rats ($N = 6$)	(S) Filipin-treated rats ($N = 5$)	(PU) Nontreated rats ($N = 5$)	(PU) Filipin-treated rats ($N = 5$)
Total cholesterol				
μmole/g liver	35.1 ± 1.0	44.2 ± 10.9	18.5 ± 3.2**,b	16.3 ± 1.2**,b
μmole/liver	693.4 ± 32.3	813.9 ± 176.1	402.2 ± 75.7**,b	409.5 ± 59.1*,b
Free cholesterol				
μmole/g liver	20.3 ± 2.3	19.4 ± 1.7	12.4 ± 1.3*,b	12.5 ± 0.7**,b
μmole/liver	400.5 ± 5.7	366.9 ± 28.2	267.5 ± 20.0	310.2 ± 36.2
Esterified cholesterol				
μmole/g liver	14.8 ± 2.6	25.7 ± 9.3	6.1 ± 2.4*,b	3.8 ± 0.8*,b
μmole/liver	292.9 ± 58.8	463.8 ± 161.9	134.7 ± 57.9	98.9 ± 25.6*,b
Alanine Amino Transferase				
I.U./liver	986 ± 127	1398 ± 97*,a	995.7 ± 137.8	1175.9 ± 136.2
I.U./g liver	50.2 ± 7.5	74.4 ± 6.9*,a	45.5 ± 5.4	48.8 ± 5.9*,b
I.U./g protein	0.72 ± 0.02	0.56 ± 0.07	0.24 ± 0.03	0.28 ± 0.03**,b

Note. Results are expressed as means ± SEM. Differences were analyzed by analysis of variance and are designated with asterisks when significantly different: *$P < 0.05$; **$P < 0.01$. For the same diet: [a]nontreated versus filipin treated rats. For the nontreated rats or for the filipin treated rats: [b](S) versus (PU).

ratio than plasma membranes derived from sinusoidal surfaces (24–26), even though the cholesterol content of bile canaliculi is higher than that of sinusoidal membranes.

Moreover, the membrane transport of lipids, which depends on the Na^+, K^+-ATPase system (10), may be inhibited to a great extent by the influence of cholesterol when it interacts with the calcium pump (8).

The presence of unsaturated fatty acids in membranes may have an effect on the ability of membranes to incorporate cholesterol (8)—a result which was also noted in studies on unicellular organisms (6). Therefore, due to the probable increase of fatty acid insaturation within membranes of the animals in diet group PU, more cholesterol would be stored on these membranes than in the case of diet group S, causing the cholesterol rate in circulating fluids and secretions (blood and bile) to fall, as was the case for diet group PU where these rates were lower than those of diet group S.

Filipin may cause modifications in plasma membranes by disturbing cholesterol exchanges through these membranes. These changes may appear more often in diet group PU than in diet group S. Thus, in the case of diet group PU, filipin would not cause any remarkable variations in the level of plasma cholesterol, contrary to the case of diet group S, since intermembrane cholesterol exchanges would be "frozen."

Effects of filipin at the subcellular level. Presuming that the drug can freely penetrate into the intracellular compartment after being absorbed into the plasma membrane, different probable effects of this antibiotic may be described.

According to certain investigators (9), filipin may be considered as a cytochemical marker specific to subcellular membranes containing cholesterol. As for plasma membranes, there seems to exist a direct relationship between the disruption caused by the polyene and the cholesterol level measured within the membrane (9). These authors conclude that the endocytose vesicle membranes that may act during intracellular catabolism of LDL (8)—such as lysosome membranes—contain cholesterol levels greater than those detected in the membranes of other subcellular particles (such as mitochondria or endoplasmic reticulum).

In the case of diet group S, where a large amount of LDL has already been synthesized, catabolism of these lipoproteins by endocytose vesicles and/or hepatic lysosomes would therefore be aggravated in comparison to diet group PU. Under these conditions, filipin present in the hepatocyte may have a distinctive blocking effect of endocytose vesicle membranes and lysosomes, and probably on the Golgi apparatus as well (8) which seems to have membranes susceptible to cholesterol storage.

This hypothesis would explain the inhibiting effect of filipin on the biliary secretion of cholesterol and also the accumulation of cholesterol caused by the drug through insufficient secretion of lipoproteins by the hepatic pole of secretion.

One of the consequences of this condition would be the rise in the concentration of intrahepatic cholesterol which occurs in spite of the fact that both of the hyperlipidic diets are great inhibitors of cholesterol synthesis (27).

At the subcellular level, the effects of filipin may also be attributed to the blocked activity of hydrolases such as alkaline phosphatase (28) or that of cholesterol esterase, especially hepatic esterase, although to our knowledge, the latter has not yet been demonstrated. Cholesterol esterase is located either in the cytoplasm or in lysosomes and displays optimum activity at neutral and acid pH levels, respectively, for these two types of subcellular fractions.

Another direct consequence would be the blocking of biliary cholesterol secretion which appears at similar rates in both diet group S and diet group PU. It is therefore important to examine the respective roles of hepatic cholesterol, which constitutes various types of membrane and also stored cholesterol, in the biliary and plasmatic secretion processes. On one hand, it is necessary to study the nutritive contribution and the entero-hepatic cycle of cholesterol which is carried to liver by various types of lipoproteins. On the other hand, endogenous synthesis should also be examined, with special attention placed on cholesterol synthesis by liver through smooth reticulum.

Given the two hyperlipidic diet used in this experiment, it is probable that, aside from a modulation in the form of cholesterol storage within the cell, especially within the hepatocyte, a finer analysis of the regulation process would involve the cholesterol content of the various plasma and subcellular membrane types. Lipoprotein absorption (by plasma membranes) is probably affected by the change in diet which raises the question of the "sensitivity" of lipoprotein membrane receptors to cholesterol. This phenomenon is also related to problems of cholesterol influx and efflux between the plasma and cells. According to Fielding (29), one possible process of cholesterol absorption, which is most likely quite active in our two cholesterol-loaded diets, involves the transfer of cholesterol esters to the cell without absorption of lipoproteins, or with absorption of lipoproteins followed by a "retroendocytose" of the lipoproteins whose sterols are partially depleted.

The authors are grateful for the technical assistance of Patrick Garzino and for the secretarial help of Mrs. M. Roussey and M. Bonneil. The authors wish to thank the Upjohn Company, Kalamazoo, Michigan, for the generous gift of filipin.

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Received October 2, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted January 7, 1985.