Restraint of cholesterol accumulation in tissue pools associated with drastic short-term lowering of serum cholesterol levels by clofibrate or cholestyramine in hypercholesterolemic swine

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Abstract In this study, young growing swine were made hypercholesterolemic (~300 mg/dl) by feeding milk and eggs for 7 wk. They were then divided into three groups (untreated, clofibrate-treated, and cholestyramine-treated), and the diet was continued for an additional 3–4 wk. A cholesterol balance study was carried out in the terminal week. When the swine were killed, the total carcass content of cholesterol was determined, as well as contents of individual tissues. Both drugs caused a 50% reduction in serum cholesterol levels. The total carcass cholesterol contents were significantly lower in both treatment groups than in the untreated group. The difference was due largely to lower concentrations in the plasma and in bulk tissues.

[4-^{14}C]Cholesterol was fed 7 days before the animals were killed, and specific activities of cholesterol in individual tissues were determined terminally. These gave a broad spectrum of values in tissues (excluding central nervous system) ranging rather evenly from 33% of plasma specific activity in the aorta to 100% in some tissues.

The balance data suggest that cholestyramine reduces the enterohepatic bile acid pool and cholesterol absorption but increases fecal output of bile acids and total body cholesterol synthesis. The balance data, limited to the terminal week, give no indication of the mode of action of clofibrate. Even synthesis was not significantly lower than in the untreated swine.

Supplementary key words cholesterol balance · cholesterol degradation · cholesterol absorption · cholesterol synthesis · sitosterol · chromium oxide

Clofibrate and cholestyramine are two of the most common hypocholesterolemic drugs in use at the present time. The rationale for giving such drugs is that by lowering serum cholesterol levels it is expected that tissue stores of cholesterol will be lowered, including, hopefully, stores in arterial tissue. In man there is no satisfactory direct way to determine the effect of these drugs on tissue levels of cholesterol. However, there are studies in man using indirect methods that suggest tissue pools of cholesterol are reduced by the drugs (1–3).

The principal aims of the experiment reported here were to determine the effect of short-term treatment of hypercholesterolemic swine with clofibrate or cholestyramine on serum and tissue levels of cholesterol and to try to relate these to some of the parameters of cholesterol balance.

MATERIALS AND METHODS

Experimental animals

10 male Yorkshire swine approximately 8 wk old, having an average weight of 9.7 kg, were housed individually in slat-bottomed cages. All animals were fed 630 g of a commercial mash diet daily for 1 wk prior to the experiment. Approximately 1000 ml of water was given daily throughout the experiment. An additional four swine kept in a similar manner were used for an evaluation of a sampling method for tissue cholesterol.

Experimental design

At the end of 1 wk on the mash diet, all 10 animals were given a high cholesterol diet supplemented with chromium oxide and plant sterol as markers for fecal flow and cholesterol loss, respectively (4). All swine received the milk–cholesterol diet for the entire period of the experiment. After 50 days on the diet, swine were divided into three groups. One group was left untreated, a second group was given 2 g of clofibrate with each daily feeding, and the third group was given 12 g of cholestyramine with each feeding. 7 days before being killed, all animals re-

ceived a single dose of [4-^{14}C]cholesterol in the diet. During this 7-day period, daily blood samples and total fecal collections were obtained.

Diets, drugs, and markers

The basic hypercholesterolemic diet was composed of whole milk powder, poached fresh eggs, and nonnutritive fiber plus mineral and vitamin supplements (5). The daily caloric intake was 2805 kcal, 21% from protein, 55% from fat, and 24% from carbohydrate. The cholesterol intake from the milk and eggs was 2230 mg daily. In two swine, crystalline cholesterol was substituted for the egg source but, because results with them were indistinguishable from those with the swine fed eggs, this difference in source will be disregarded for simplicity of presentation. 300 mg of chromium oxide and 682 mg of plant sterol were added daily to the diets. The sources of supplementary plant sterols were Cytellin (Lilly) and peanut oil. Cytellin is composed of 57.4% sitosterol, 35.8% campesterol, and 6.8% stigmasterol. Peanut oil sterols have a composition of 74.4% sitosterol, 17.0% campesterol, and 8.6% stigmasterol.

All additives and drugs were mixed with approximately 100 g of whole milk powder with added water, and the remainder of the diet was given after this was consumed. Swine consumed all of the diet daily under strict observation. All feedings were done at 9 a.m.

Isotopes

A single dose of 44 μCi of [4-^{14}C]cholesterol (sp act 47.2 mCi/mmole; Schwarz BioResearch) was administered orally to all animals, 7 days prior to killing, by adding to an initial portion of the diet.

Fecal collection

Total feces were collected in daily lots, starting with the day of oral isotope administration; they were treated as described previously (5).

Analysis of neutral and acid steroids in feces, intestinal contents, and gallbladder bile

Seven daily dried aliquots of feces from each swine were pooled proportionally according to daily output. 1 g of pooled fecal powder was subjected to analysis as described below. Total radioactivity was determined after combustion.

Fecal neutral and acid steroids were extracted separately, and their masses and radioactivities were measured as described previously (5), using methods adapted from those described by Miettinen, Ahrens, and Grundy (6) and Grundy, Ahrens, and Miettinen (7). Silylation of neutral steroids in preparation for gas–liquid chromatography was carried out using Sil-Prep (3:1:9 mixture of hexamethyldisilazane–trimethylchlorosilane–pyridine; Applied Science Laboratories, Inc.); DMF-Sil-Prep (40:1:40 mixture of hexamethyldisilazane–trimethylchlorosilane–dimethylformamide; Applied Science) was used for acid steroids. After neutral steroid silylation, the content of the tube was dried under nitrogen and steroids were redissolved in chloroform before analysis. The chromium oxide was determined on daily fecal samples and intestinal contents.

The contents of large and small intestines at the time the animals were killed were collected separately, homogenized, and dried, and the aliquots of dry powder were subjected to analysis as described above.

Concentrations and radioactivities of cholesterol and bile acids in gallbladder bile were determined by the extraction procedure outlined above for fecal neutral and acid steroids.

Total body cholesterol analysis

At the end of the experiment, swine were killed by an intravenous injection of pentobarbital sodium (Nembutal), and the carcasses were dissected into organs, tissues, blood, and other fluids. Each part was weighed, and the cholesterol content of triplicate samples was determined by Leffler's method (8) after extraction of the tissue by the procedure of Folch, Lees, and Sloane Stanley (9). All radioactivity measurements were made with a Packard Tri-Carb liquid scintillation spectrometer, model 3375, as described previously (5). For the measurement of fecal radioactivity, an aliquot (approx. 100 mg) of dried feces was oxidized and resulting carbon dioxide trapped using a Tri-Carb sample oxidizer, model 305 (Packard Instrument Co.).

After the experiment was completed, a question was raised regarding the validity of the sampling technique. To answer this question, four swine, two on the mash diet and two on the hypercholesterolemic diet, were killed, and organs, tissues, and fluid were sampled individually as described above. The remainder (including bone but excluding the brain and spinal cord) was homogenized in a commercial vertical cutter–mixer (Hobart); eight samples were obtained from each homogenate. In the mash-fed pair, values obtained by sampling were 99.9 and 91.4%, respectively, of values obtained by homogenization. In the hypercholesterolemic pair, values by sampling were 102.6 and 91.8%, respectively, of homogenization values. Although average values are somewhat higher with the homogenization method, the differences do not appear to be great enough to warrant serious concern. However, with proper equipment the homogenization method is simpler and probably the method of choice.

Serum and tissue cholesterol were measured by the method of Leffler (8), using free cholesterol (Sigma Chemical Co.) as a standard. All cholesterol measurements were expressed as free cholesterol.

TABLE 1. Serum cholesterol levels, body cholesterol contents, body weights, and cholesterol balance data

	Treatment			*P* Values		
	Untreated (A)	Clofibrate (B)	Cholestyramine (C)	A vs. B	A vs. C	B vs. C
No. in groups	4	3	3			
Days on high cholesterol diet	81	79	72			
Days on drugs	0	28	23			
Serum Cholesterol Level						
Average for 30 days pretreatment (mg/dl)	268 ± 42[a]	286 ± 44	338 ± 79	n.s.[b]	n.s.	n.s.
Average for treatment period (mg/dl)	296 ± 33	146 ± 29	156 ± 22	0.02	0.02	n.s.
Difference, pretreatment to treatment (%)	+14 ± 9	−49 ± 7	−51 ± 8	0.005	0.005	n.s.
Last pretreatment value (mg/dl)	256 ± 23	299 ± 26	326 ± 77	n.s.	n.s.	n.s.
Terminal treatment value (mg/dl)	298 ± 29	124 ± 24	148 ± 33	0.005	0.02	n.s.
Difference, last pretreatment to terminal (%)	+16 ± 4	−58 ± 4	−55 ± 24	0.001	0.05	n.s.
Body Cholesterol Content and Body Weight						
Initial content (mg)	6,526 ± 672	7,120 ± 1,085	6,838 ± 739	n.s.	n.s.	n.s.
Initial body weight (kg)	10.3 ± 1.1	11.3 ± 1.7	10.8 ± 1.2	n.s.	n.s.	n.s.
Terminal content (mg)	36,264 ± 1,555	31,555 ± 311	28,790 ± 209	0.03	0.005	0.001
Terminal body weight (kg)	43.7 ± 0.7	45.1 ± 0.9	42.6 ± 1.0	n.s.	n.s.	n.s.
Gain in total cholesterol (mg)	29,739 ± 1,284	24,435 ± 1,382	21,952 ± 846	0.03	0.005	n.s.
Terminal cholesterol/weight (mg/kg)	830 ± 30	700 ± 17	676 ± 16	0.01	0.005	n.s.
Average retention (mg/day)	363 ± 21	307 ± 17	304 ± 11	0.05	0.05	n.s.
Cholesterol Balance Data for Last 7 Days and Enterohepatic Bile Acid Pool						
Total steroid excretion (mg/day)	2,007 ± 162	1,943 ± 109	2,820 ± 225	n.s.	0.05	0.05
Acid steroid excretion (mg/day)	461 ± 70	392 ± 122	698 ± 143	n.s.	n.s.	n.s.
Neutral steroid excretion (mg/day)	1,546 ± 223	1,552 ± 215	2,122 ± 347	n.s.	n.s.	n.s.
Endogenous neutral steroid excretion (mg/day)	605 ± 136	596 ± 90	579 ± 289	n.s.	n.s.	n.s.
Dietary cholesterol intake (mg/day)	2,165	2,186	2,101			
Dietary cholesterol absorbed (mg/day)	1,224 ± 63	1,230 ± 120	556 ± 58	n.s.	0.001	0.005
Dietary cholesterol unabsorbed (mg/day)	941 ± 92	956 ± 143	1,545 ± 57	n.s.	0.005	0.02
Retention (mg/day)	330 ± 29	273 ± 28	268 ± 12	n.s.	0.01	n.s.
Synthesis (mg/day)	300 ± 97	198 ± 75	1,115 ± 234	n.s.	0.05	0.025
Enterohepatic bile acid pool (mg)	1,818 ± 226	1,824 ± 163	1,038 ± 112	n.s.	0.03	0.02

[a] Standard error.
[b] Not significant.

Calculation of various parameters of sterol balance

Fecal excretion of neutral steroids and bile acids was calculated using sitosterol to correct for loss and chromium oxide to determine transit times, utilizing methods previously described by many workers (4, 5). In addition, we made corrections for loss on the basis of percentage recovery of isotope, and the results indicate general agreement between this method and the sitosterol correction method.

Determinations of cholesterol absorption and fecal excretion of endogenous cholesterol were made using the combined chromatographic and isotopic data and methods of calculation similar to those previously described (5).

Average daily retention of cholesterol in the body over the course of the experiment was made by subtracting the amount of cholesterol estimated to be in the body at the beginning of the experiment from that present at the end. The estimate of initial content was made by multiplying body weight in kilograms by average values for milligrams of cholesterol per kilogram in similar swine already established (5) with the same methods used in this experiment (632 mg/kg).

An estimate of synthesis can be made for only the terminal 7 days of the experiment because these are the only days for which excretion data are available. We know dietary input and fecal output and can estimate urinary output of cholesterol derivatives from radioactivity in the urine. We measured the terminal plasma volume and can safely assume that the volume was the same 7 days previously in proportion to body weight. Because we know the changes in cholesterol concentration in plasma, we can calculate the total changes in plasma for the week. We measured the total amount of cholesterol in the tissues terminally and calculated the milligrams per kilogram of body weight. Multiplying this figure by the body weight 7 days previously gives an approximation of the amount present at that time, and then, by subtracting this from the terminal content, changes in the tissues for the week can be determined. Use of the terminal milligrams of cholesterol per kilogram of body weight to determine cholesterol content 7 days previously is not entirely accurate because change in concentration is usually taking place. However, one can readily show that the rate of change per day in concentration of cholesterol in the tissues is too

small to introduce a serious error. For example, the rate of change in milligrams of cholesterol per kilogram of body weight in the untreated group was only 2.5 mg/kg per day during the entire 79 day diet period. The only other significant item not accounted for is loss through the skin. In the absence of concrete data, we have assigned this loss an arbitrary value of 50 mg/day. The surface area of these young swine is about half that of human adults, who have been shown to lose approximately 100 mg of cholesterol daily from the skin (10).

Statistical evaluation

The significance of the difference between various means was tested by Student's t test. Significance was ascribed if P was ≤ 0.05.

RESULTS

All swine remained in good health and grew normally throughout the experiment. Changes in serum cholesterol levels, total body cholesterol concentrations and contents, cholesterol balance data for the terminal 7 days, and enterohepatic bile acid pools are shown in **Table 1.** In both treated groups, average serum cholesterol levels during the treatment period as well as terminal serum cholesterol levels dropped significantly. No significant difference was observed between clofibrate- and cholestyramine-treated groups in their terminal serum cholesterol levels. In the untreated group, the serum cholesterol levels remained essentially the same as pretreatment levels with slight elevation terminally.

Body weight gains in all swine were similar and the average increase was to approximately four times the original weight. Naturally, all swine gained in total body cholesterol through growth, but both treated groups gained significantly less than the untreated group. The terminal body cholesterol concentration (mg/kg body wt) and the average daily retention during the entire experiment were significantly less in both groups of drug-treated swine than in untreated swine. Although values were generally lower in the cholestyramine group than in the clofibrate group, the differences were not statistically significant.

Total fecal excretion of neutral and acidic steroids combined are increased in the cholestyramine-treated group compared with the untreated group, but no increase was observed in the clofibrate-treated group. Bile acid excretion values were higher in the cholestyramine group than in the untreated and clofibrate groups, but the differences were not statistically significant, due largely to individual variations. This may have been due to the fact that the balance data obtained were only for the terminal 7 days of treatment. Absorption of cholesterol was markedly decreased in the cholestyramine group but no change was observed in the clofibrate group. Endogenous excretion of neutral steroids was variable, and there were no differences among the groups. Daily retention of cholesterol, though significantly lower in both the cholestyramine and the clofibrate groups than in the untreated group for the entire diet period, was significantly lower in the terminal 7 days only in the cholestyramine group. Synthesis remained low in both clofibrate and untreated groups, but a marked increase was observed in the cholestyramine group.

Amounts of bile acid in the gallbladder and small intestine were significantly lower in the cholestyramine group than in the two other groups.

Individual tissue concentrations and total contents of cholesterol are given in **Table 2.** Cholesterol concentrations of tissues were generally lower in both treated groups than in the untreated group.

In **Table 3** the specific activities of cholesterol in the tissues are given in relation to the serum cholesterol specific activity. These data are consistent with the idea that there are multiple pools of cholesterol in the body exchanging with the plasma at different rates. Note that the specific activity of cholesterol in the aorta is low, suggesting a slower rate of exchange with the plasma than any of the tissues except brain and spinal cord.

DISCUSSION

The most important observation in this study is that in hypercholesterolemic swine both cholestyramine and clofibrate are capable of excluding excess cholesterol from most body tissues almost as rapidly as from the plasma. Both serum and tissue levels of cholesterol were at near normal levels after 3–4 wk of treatment. One must be cautious, however, in extrapolating these results to man. The rapid drop of serum cholesterol values to approximately 50% of pretreatment levels is much greater than that usually seen in man (4, 11). However, we know from other studies that swine vary in their responses to clofibrate. We have been looking for swine less responsive than those in the current study, and in an experiment now in progress with clofibrate we have swine whose serum cholesterol levels dropped only about 20%. The effect of this amount of change in serum levels will be the subject of a subsequent report.

In the cholestyramine-treated swine, in which balance studies were carried out in the third week of treatment, the reason for the change in serum and tissue levels appears to be decreased absorption coupled with increased fecal excretion of acidic steroids. The decreased absorption probably was due to a marked decrease in the size of the gallbladder–small intestine bile acid pool. Cholesterol syn-

TABLE 2. Average cholesterol content and concentration in various body tissues

	Untreated		Clofibrate		Cholestyramine	
	total mg	*mg/g wet wt*	*total mg*	*mg/g wet wt*	*total mg*	*mg/g wet wt*
Muscles	12,017	0.56 ± 0.04[a]	11,519	0.51 ± 0.04	10,016	0.49 ± 0.05
Blood	4,808	2.28 ± 0.44	2,698	1.28 ± 0.34	2,789	1.35 ± 0.31
Skin	3,959	1.24 ± 0.14	3,239	1.10 ± 0.10	3,556	1.08 ± 0.12
G.I. tract, total	3,712		3,094		3,108	
Esophagus	45	1.19 ± 0.09	37	1.00 ± 0.03	33	0.96 ± 0.09
Stomach	594	2.54 ± 0.18	435	1.86 ± 0.32	494	2.00 ± 0.26
Duodenum	73	2.28 ± 0.25	60	2.00 ± 0.42	66	1.89 ± 0.32
Jejunum	938	2.43 ± 0.21	743	1.98 ± 0.08	929	1.90 ± 0.27
Ileum	1,121	2.26 ± 0.59	974	2.14 ± 0.32	785	1.91 ± 0.32
Colon	830	1.64 ± 0.19	760	1.53 ± 0.12	744	1.48 ± 0.14
Rectum	94	1.95 ± 0.25	93	1.61 ± 0.37	77	1.60 ± 0.16
Liver	3,157	4.75 ± 1.30	2,511	3.00 ± 0.48	1,783	2.48 ± 0.22
Bones	3,108	0.44 ± 0.05	2,572	0.36 ± 0.09	2,341	0.34 ± 0.06
Adipose tissue	2,771	0.71 ± 0.10	2,978	0.76 ± 0.11	2,729	0.64 ± 0.10
Brain	1,304	14.47 ± 2.01	1,343	14.75 ± 2.62	1,392	15.88 ± 2.15
Spinal cord	1,155	33.55 ± 4.13	1,080	32.02 ± 3.88	1,149	35.60 ± 5.78
Lungs	1,057	3.90 ± 0.43	1,183	3.09 ± 0.54	1,025	3.45 ± 0.20
Kidneys	449	3.75 ± 0.29	569	3.23 ± 0.09	500	3.44 ± 0.31
Testes	449	2.24 ± 0.39	533	2.51 ± 0.30	325	2.26 ± 0.50
Spleen	367	4.61 ± 0.81	267	3.84 ± 0.67	296	4.01 ± 0.43
Heart	203	1.24 ± 0.14	207	1.06 ± 0.10	139	1.13 ± 0.09
Pancreas	133	2.13 ± 0.33	115	1.96 ± 0.18	115	1.89 ± 0.13
Urinary bladder	40	1.58 ± 0.27	33	1.35 ± 0.08	35	1.44 ± 0.21
Aorta	23	1.04 ± 0.17	21	0.90 ± 0.14	19	0.87 ± 0.09
Adrenals	13	4.55 ± 0.70	10	3.67 ± 0.52	12	3.65 ± 0.65
Thyroid	4	1.17 ± 0.17	4	1.13 ± 0.13	4	1.16 ± 0.13
Total content[b]	36,264		31,555		28,790	
mg/kg body wt[b]		830		700		676

[a] Standard error.
[b] Excluding central nervous system.

thesis rates were greatly increased, as is expected with cholestyramine, allowing a new state of balance between input and output to develop.

Cholesterol balance studies carried out in the clofibrate group in the fourth week of treatment did not show why the serum and tissue cholesterol levels were reduced. None of the parameters were significantly different from those of the untreated controls. Obviously, there must have been an excess of output over input at some in the first 3 wk of treatment in order to have achieved the observed result. These observations lead to the concern that the beneficial effect of clofibrate on cholesterol balance may have been short-lived and that by the fourth week the swine are in a transition period back toward their previous state. Longer-term experiments are needed to provide the answer on this point.

The suggestion that clofibrate may affect at least some tissue pools directly (1) is not borne out by our available data. There are, however, several interesting features in Table 3. First of all, the degree of equilibration of various tissues with plasma, as presumably indicated by terminal relative specific activities, affords little support for any specified number of pools: there are no values below 0.33 (not counting brain and spinal cord), but above that the values are more or less uniformly spread over the entire range.

There is a distinct effect of treatment on the degree of equilibration: it increases in a majority of the compartments, and perhaps more with cholestyramine than with clofibrate. However, an attempt to correlate this clearly with an effect of treatment in reducing certain compartments (or a lack of effect with others) is doomed to disappointment. Thus, the fairly active adipose tissue (relative specific activity with clofibrate, 0.71; with cholestyramine, 0.90) contributes as little to the reduction (or perhaps more properly restraint) of body cholesterol as the inert brain.

Cross comparison of total contents of the various compartments directs our attention elsewhere. Clofibrate treatment lowered body cholesterol by about 5000 mg. Blood contributed 2100 mg to this difference, and most of the balance came from muscle, skin, liver, and bone in amounts between 500 and 700 mg. This suggests that the drug either facilitates removal of cholesterol from the blood or prevents its entry in the first place and that the mobile cholesterol of the tissue compartments then simply follows the "gradient" so established. In this perspective, the increased equilibration of tissue compartments with

TABLE 3. Ratios of average specific activities of cholesterol in tissues at autopsy to that of serum 7 days after isotope feeding

	Untreated	Clofibrate	Cholestyramine
Serum[a]	1.00	1.00	1.00
Bile	1.28	1.18	1.31
Lungs	1.02	1.05	1.31
Spleen	1.02	1.06	0.96
Thyroid	0.98	1.01	1.30
Heart	0.98	1.17	1.29
Adrenals	0.88	0.95	1.20
Bones[b]	0.85	1.00	0.97
Liver	0.84	0.97	0.97
Pancreas	0.82	0.93	1.13
Duodenum	0.79	0.74	0.98
Ileum	0.77	1.00	0.97
Testes	0.70	0.71	0.99
Jejunum	0.70	0.93	0.86
Adipose tissue	0.58	0.71	0.90
Esophagus	0.66	0.68	1.00
Colon	0.68	0.67	0.85
Kidneys	0.58	0.57	0.81
Stomach	0.54	0.65	0.69
Muscles	0.52	0.71	0.79
Rectum	0.50	0.60	0.69
Urinary bladder	0.47	0.50	0.69
Skin	0.44	0.44	0.48
Aorta	0.33	0.37	0.36
Brain	0.02	0.01	0.01
Spinal cord	<0.01	<0.01	<0.01

[a] Terminal serum specific activities (dpm/mg cholesterol): untreated, 1372; clofibrate, 1131; cholestyramine, 599.
[b] Includes marrow.

plasma is reasonable as an indication of increased turnover between them, but the motive force must be located elsewhere.

Paradoxically enough, it is with cholestyramine treatment that compartments such as muscle and liver decrease by amounts comparable with blood. We have no real explanation of this point at the moment. The serum cholesterol with cholestyramine treatment initially decreases much more sharply than with clofibrate; and it may be that tissue compartments are thereby more affected.

What is clear from these results, aside from the need for more factual information, is that the direct mechanism of action in swine of cholestyramine is well understood, that the indirect consequences are less well understood, and that the mode of action of clofibrate is not really understood at all.

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