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EFFECT OF PHENOBARBITAL AND CHLORCYCLIZINE ON THE DEVELOPMENT OF ATHEROMATOSIS IN THE CHOLESTEROL-FED RABBIT*

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Abstract—The administration of phenobarbital inhibited the increase in serum concentration of cholesterol and phospholipids and the development of atheromata in the aorta of the cholesterol-fed rabbit. The protein concentration of liver microsomes increased significantly in cholesterol-fed rabbits given phenobarbital, and phenobarbital stimulated the activity of liver microsomal enzymes that hydroxylate testosterone in the 6β-, 7α- and 16α-positions. The administration of chlorcyclizine to cholesterol-fed rabbits did not inhibit the increase in serum cholesterol or the development of atheromata in the aorta, and this treatment did not stimulate the hydroxylation of testosterone by liver microsomes.

CHLORCYCLIZINE and a number of other diarylalkylpiperazines have been reported to lower serum cholesterol (CL)† in the rat and mouse.[1–3] Our results show that chlorcyclizine also reduces markedly the serum triglycerides (TG) and phospholipids (PL) in the mouse and the serum TG in the rat.[4] Phenobarbital has similar activity in the mouse, but is much less potent than chlorcyclizine.[4] The purpose of this investigation was to determine if chlorocyclizine and phenobarbital could inhibit the development of atheromata in the aorta of the cholesterol-fed rabbit and to determine if these drugs altered the ability of rabbit liver microsomes to hydroxylate steroids.

METHODS

Male New Zealand white rabbits were fed 100 g daily of Rockland Rabbit Ration.‡ During the first 4 weeks, groups of rabbits were fed drug-free diet or diet containing 0·09 per cent phenobarbital, 0·03, 0·06, or 0·12 per cent chlorcyclizine. After the first

* A preliminary report of this study was presented before the American Society for Pharmacology and Experimental Therapeutics, Washington, D.C. (August 1967).

† The following designations are used: CL, cholesterol; TG, triglyceride(s); PL, phospholipid(s).

‡ Special diets were prepared by Nutritional Biochemical Corp. Rockland Rabbit Ration was ground to a powder to facilitate the addition to the diet of either cholesterol (1%), phenobarbital (0·09%) or chlorcyclizine (0·03, 0·06, or 0·12%), or the combination of cholesterol with either phenobarbital or chlorcyclizine. After the appropriate drug addition, the diets were repelleted. Drug-free diet consists of Rockland Rabbit Ration which was ground and repelleted without the addition of any drug. Rabbits were fed 100 g of repelleted diet daily, The body weight of the rabbits averaged 2·4 kg at the beginning of the experiments and 4·0 kg at the end of the experiments. The approximate average daily intake of drug was 40, 30, 20 and 10 mg/kg for animals fed 0·12%, 0·09%, 0·06% and 0·03% of drug in the diet respectively.

4 weeks, diets containing phenobarbital and chlorcyclizine were replaced with diets containing 1 per cent cholesterol in addition to either phenobarbital or chlorcyclizine. One group of rabbits was fed drug-free diet for the first 4 weeks and this diet was replaced with repelleted diet containing 1 per cent cholesterol for the remainder of the experiment. In some experiments, one group of rabbits was fed drug-free and cholesterol-free diet throughout the experimental period, and these animals served as untreated controls. Venous blood was obtained from the ear at regular intervals after an overnight fast, and the serum was analyzed for CL, TG and PL. At the end of each experiment, the animals were weighed, bled and then subjected to autopsy. The aortas were removed, cleaned of adhering tissue and opened longitudinally to expose the intimal surface for visual grading of the plaques. In addition, sections of the aortas were examined for histological changes. A normal aorta was scored zero. Grade 1 indicated that there were small focal deposits with slight thickening of the intima. Grade 2 indicated that there were more extensive deposits with greater intimal thickening. Grade 3 indicated extensive deposits with considerable intimal thickening, calcification and necrosis. In early experiments, a consistent correlation was developed between visual grading of the aorta and the severity of the tissue changes found by examining sections of the aorta microscopically after staining with oil red-O, hematoxylin and eosin, and Van Gieson's stain.

The serum and homogenates of liver and aorta were extracted as previously described,[4] and the extracts were analyzed for cholesterol using the Technicon Autoanalyzer procedure N-24a. Triglycerides were determined by the procedure of Kessler and Lederer,[5] and the phospholipid content was determined by the method of Zilversmit and Davis.[6] Protein was determined by the method of Sutherland *et al.*[7]

The metabolism of testosterone-4-^{14}C and *p*-nitroanisole was examined *in vitro*, using microsomes from freshly removed livers as the source of enzyme, and an NADPH-generating system, as was previously described.[8–10] For the hydroxylation of testosterone-4-^{14}C, microsomes from 200 mg of liver were incubated with 900 mμ moles testosterone-4-^{14}C in 0·1 ml of methanol at 25° for 15 min in a final volume of 2·5 ml. Each of the following were added to the incubation mixture in 0·1 ml of 0·05 M Tris (hydroxymethyl) aminomethane (pH 7·4): 18 μmoles glucose 6-phosphate, 2·5 μmoles triphosphopyridine nucleotide and 2 units glucose 6-phosphate dehydrogenase. In addition, incubation mixtures contained 0·5 ml of a suspension of microsomes in 0·1 M phosphate buffer (pH 7·4), 5 μmoles $MgCl_2$ (0·05 ml), 0·7 ml Tris buffer (0·05 M, pH 7·4) and 0·85 ml water. At the end of the incubation period, a 2-ml portion of each incubation mixture was extracted with 30 ml methylene chloride by shaking for 20 min. Twenty ml of the methylene chloride extract was reduced to dryness with nitrogen. The residue was redissolved in a small volume of methanol, chromatographed to separate the hydroxylated metabolites, and the metabolites were quantitated as previously described.[8, 9]

For the dealkylation of *p*-nitroanisole to *p*-nitrophenol, liver microsomes equivalent to 333 mg liver were incubated 10 min at 37° with 36 μmoles *p*-nitroanisole in the presence of an NADPH-generating system. The final volume of the incubation mixture was 2·2 ml. The reaction was stopped by adding a solution of trichloroacetic acid to obtain a final concentration of 5%, and the protein was removed by centrifugation for 10 min at 9000 *g*. The concentration of *p*-nitrophenol in 2 ml of the supernatant

was determined by adding 1 ml of 0·75 N NaOH and determining the optical density at 420 mμ.

RESULTS

Effect of phenobarbital and chlorcyclizine on the serum concentration of cholesterol, triglycerides and phospholipids in the cholesterol-fed rabbit. The change in serum concentration of CL and PL in the cholesterol-fed rabbit given either phenobarbital (30 mg/kg/day) or chlorcyclizine (20 mg/kg/day) is shown in Fig. 1. Administration of

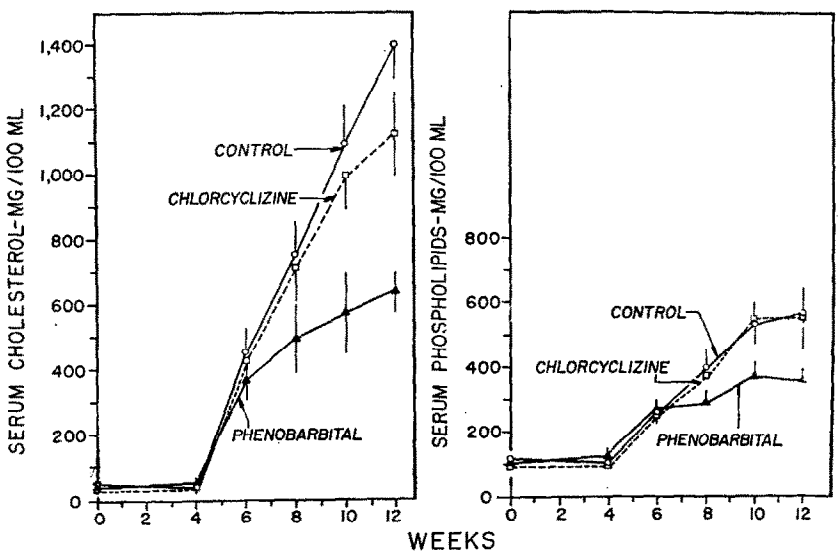

FIG. 1. The effect of phenobarbital and chlorcyclizine administration on the serum concentration of cholesterol and phospholipids in the cholesterol-fed rabbit. Control rabbits were fed drug-free diet for the first 4 weeks and diet containing 1% cholesterol for the remainder of the experiment. Other rabbits were fed diet containing 0·90% phenobarbital (dose, 30 mg/kg/day) or 0·06% chlorcyclizine (dose, 20 mg/kg/day) throughout the experiment. Cholesterol (1%) was added to the diet of chlorcyclizine- or phenobarbital-treated rabbits after the first 4 weeks and the combination of cholesterol with either chlorcyclizine or phenobarbital was fed for the remainder of the experiment. Each value represents the mean and S.E. of six to ten rabbits.

phenobarbital or chlorcyclizine for 4 weeks to rabbits receiving a cholesterol-free diet did not alter the serum concentrations of CL or PL. The serum concentrations of CL and PL increased when the rabbits were placed on a diet containing 1 per cent cholesterol, and this increase was inhibited by phenobarbital, but not by chlorcyclizine administration. The TG concentration of serum in all groups was 50–80 mg/100 ml prior to feeding cholesterol and rose to 150–180 mg/100 ml during cholesterol feeding. No significant difference in the serum concentration of TG occurred with any treatment.

Changes in the aorta of cholesterol-fed rabbits given either phenobarbital or chlorcyclizine. Atheromata were localized in the ascending aorta and in the aortic arch of animals fed 1 per cent cholesterol in the diet for 2 months. The major microscopic findings in these areas were the presence of lipid staining material and a disrupted

internal elastica; in some instances there were necrosis and calcification of the media. There was a reduction in the severity of plaque formation and a significant inhibition of the deposition of CL in the aorta of phenobarbital-treated rabbits (Table 1, experiment 1). A second experiment was conducted in which chlorcyclizine was evaluated for its ability to inhibit the development of atheromata in the cholesterol-fed rabbit (Table 1, experiment 2). No inhibition of the formation of atheromata occurred in the aorta of cholesterol-fed rabbits given chlorcyclizine, nor was there an inhibition of the deposition of CL.

Liver changes in the cholesterol-fed rabbit given either phenobarbital or chlorcyclizine. There was a significant increase (30–50%) in the weight of livers of rabbits fed CL for

TABLE 1. EFFECT OF PHENOBARBITAL AND CHLORCYCLIZINE ON THE INDUCTION OF ATHEROMATA IN THE AORTA OF THE CHOLESTEROL-FED RABBIT*

Experiment	Addition to diet and daily dose of drug (mg/kg)	No. of aorta	Atheromata (score ± S.E.)	Cholesterol (mg/g aorta ± S.E.)
1	None	3	0	2·0 ± 0·1
	Cholesterol (1%)	7	2·0 ± 0·2	11·8 ± 6·5
	Cholesterol (1%) and phenobarbital (30)	7	0·6 ± 0·2†	4·1 ± 1·5†
2	None	4	0	2·4 ± 0·2
	Cholesterol (1%)	10	1·6 ± 0·3	10·9 ± 1·7
	Cholesterol (1%) and chlorcyclizine (40)	11	1·3 ± 0·2	9·3 ± 1·0
	Cholesterol (1%) and chlorcyclizine (20)	10	1·6 ± 0·2	10·6 ± 1·3
	Cholesterol (1) and chlorcyclizine (10)	9	1·7 ± 0·4	12·1 ± 2·5

* The atheromata of the aortas were graded visually using the criteria described in Methods. The first 5 cm of each aorta, measured from the point of attachment to the heart, was analyzed for cholesterol. The results were compared statistically using the analysis of variance method in conjunction with Duncan's multiple range test.

† The atheromata score and the cholesterol concentration in the aorta of rabbits given 30 mg/kg/day of phenobarbital are significantly lower than that of cholesterol-fed rabbits ($P < 0.01$).

2 months, which was not affected by the concomitant administration of either phenobarbital or chlorcyclizine (Table 2). The concentration of protein in liver did not change significantly with any treatment, although it was slightly lower in cholesterol-fed rabbits than in untreated controls. The concentration of CL in liver increased significantly (3- to 6-fold) as a result of feeding CL ($P < 0.01$). A somewhat lower deposition of CL was observed in the liver of cholesterol-fed rabbits given 40 mg/kg/day of chlorcyclizine, but not in animals given lower doses of chlorcyclizine or 30 mg/kg/day of phenobarbital.

Effect of phenobarbital and chlorcyclizine on the metabolism in vitro *of testosterone-4-^{14}C and p-nitroanisole.* Attempts to study the hydroxylation of CL by rabbit liver microsomes were not successful. Therefore, testosterone-4-^{14}C was used as a model steroid substrate to determine if phenobarbital or chlorcyclizine administration increased the activity of hydroxylating enzymes in liver microsomes. The metabolism of testosterone-4-^{14}C to 6β-, 7α- and 16α-hydroxytestosterone was determined *in vitro* using liver microsomes from cholesterol-fed rabbits and cholesterol-fed rabbits given

TABLE 2. EFFECT OF PHENOBARBITAL AND CHLORCYCLIZINE ON THE LIVER OF THE CHOLESTEROL-FED RABBIT*

Experiment	Addition to diet and daily dose of drug (mg/kg)	No. of rabbits	Body wt. at autopsy (kg ± S.E.)	Liver wt. (g ± S.E.)	Protein (mg/g liver ± S.E.)	Cholesterol (mg/g liver ± S.E.)
1	None	3	3·5 ± 0·2	83 ± 4	212 ± 8	4·5 ± 0·2
	Cholesterol (1%)	7	3·3 ± 0·1	131 ± 8	182 ± 6	19·6 ± 3·1
	Cholesterol (1%) and phenobarbital (30)	7	3·2 ± 0·1	153 ± 8	223 ± 30	17·8 ± 3·7
2	None	4	3·6 ± 0·1	80 ± 6	251 ± 8	3·7 ± 0·1
	Cholesterol (1%)	9	3·4 ± 0·1	122 ± 9	230 ± 11	23·3 ± 2·6
	Cholesterol (1%) and chlorcyclizine (40)	11	3·3 ± 0·1	117 ± 8	246 ± 6	15·4 ± 1·8†
	Cholesterol (1%) and chlorcyclizine (20)	10	3·5 ± 0·1	143 ± 9	225 ± 14	21·3 ± 1·5
	Cholesterol (1%) and chlorcyclizine (10)	9	3·6 ± 0·1	140 ± 10	214 ± 6	19·5 ± 2·2

* The statistical methods are described in Table 1.
† The concentration of cholesterol in the liver of rabbits fed 40 mg/kg/day of chlorcyclizine is significantly lower than that in the liver of cholesterol-fed rabbits ($P < 0.05$).

TABLE 3. EFFECT OF PHENOBARBITAL ON HYDROXYLATION OF TESTOSTERONE-4-^{14}C BY LIVER MICROSOMES FROM RABBITS FED CHOLESTEROL*

Addition to diet and daily dose of drug (mg/kg)	Microsomal protein (mg/g liver ± S.E.)	Hydroxylated metabolites of testosterone-4-^{14}C formed/200 mg wet wt. liver (mμmoles ± S.E.)		
		6β-OH	16α-OH	7α-OH
Cholesterol (1%)	17·9 ± 0·8	107 ± 13	35 ± 3	8 ± 1
Cholesterol (1%) and phenobarbital (30)	31·3 ± 0·9†	164 ± 18‡	96 ± 14†	17 ± 2†

* One group of rabbits was fed 1% cholesterol for 2 months without pretreatment. A second group was fed 30 mg/kg of phenobarbital daily for 1 month and drug administration was continued for 2 additional months during which these rabbits were fed a diet containing 1% cholesterol. Liver microsomes equivalent to 200 mg liver were incubated with 900 mμmoles testosterone-4-^{14}C in the presence of an NADPH-generating system as described in Methods. Each value represents the average and standard error obtained from 6–7 livers.
† $P < 0.01$.
‡ $P < 0.05$.

TABLE 4. EFFECT OE PHENOBARBITAL ON THE DEALKYLATION OF *p*-NITROANISOLE TO *p*-NITROPHENOL BY LIVER MICROSOMES FROM UNTREATED AND CHOLESTEROL-FED RABBITS*

Addition to diet and daily dose of drug (mg/kg)	No. of livers assayed	Microsomal protein (mg/g liver ± S.E.)	*p*-Nitrophenol formed (μmoles/g liver/hr ± S.E.)
None	3	18·7 ± 0·5	53·4 ± 3·1
Phenobarbital (30)	5	34·9 ± 1·9†	77·2 ± 5·6‡
Cholesterol (1%)	6	22·3 ± 1·1	48·1 ± 7·1
Cholesterol (1%) and phenobarbital (30)	6	34·1 ± 1·7†	70·0 ± 3·7‡

* Liver microsomes equivalent to 333 mg liver were incubated with 36 μmoles *p*-nitroanisole in the presence of an NADPH-generating system. The results were compared statistically using the analysis of variance method in conjunction with Duncan's multiple range test. The results in the phenobarbital-treated groups were significantly higher than in cholesterol-fed or untreated rabbits.
† $P < 0.01$.
‡ $P < 0.05$.

phenobarbital. Administration of phenobarbital to the rabbits increased the concentration of protein in liver microsomes, and the metabolism of testosterone to hydroxylated metabolites was enhanced (Table 3). Administration of phenobarbital to rabbits also stimulated the metabolism of *p*-nitroanisole to *p*-nitrophenol by liver microsomes (Table 4). Studies to determine the effect of chlorcyclizine administration on testosterone hydroxylation by liver microsomes from cholesterol-fed rabbits were inconclusive. Administration of 20mg/kg of chlorcyclizine daily for 3 months in one experiment had no effect on the 6β-, 7α and 16α-hydroxylation of testosterone by rabbit liver microsomes, whereas decreased rates of hydroxylation were observed in a second experiment.

DISCUSSION

The administration of phenobarbital inhibited the development of atheromata in the aorta of the cholesterol-fed rabbit. The deposition of cholesterol in the aorta increased significantly and to the same extent in cholesterol-fed rabbits given chlorcyclizine and in cholesterol-fed controls, while it was significantly lower in cholesterol-fed rabbits given phenobarbital. The increase in the serum concentration of cholesterol and phospholipids was less in cholesterol-fed rabbits given phenobarbital than in either cholesterol-fed rabbits or in cholesterol-fed rabbits given chlorcyclizine. It seems likely that the less severe atheromata found in the aorta of the cholesterol-fed rabbit given phenobarbital is related to the serum concentration of cholesterol not being maintained at a high enough concentration for a sufficiently long period.[11, 12] The lower serum concentration of cholesterol found in phenobarbital-treated rabbits is not related to a decrease in cholesterol intake as determined by food consumption. There was no apparent change in intestinal absorption of the food in phenobarbital-treated rabbits as measured by body weight gain, but the possibility that these animals absorbed only a portion of the dietary cholesterol was not ruled out. Myasnikov[13] also reported that phenobarbital inhibits atheromata formation in the rabbit. However, no quantitative data on the serum and tissue concentration of cholesterol or lipids were given in this report. Myasnikov suggested that the inhibitory effect of phenobarbital on atheromata formation was related to either a depression of the central nervous system or a reduction in blood pressure. In our experiments, there was no obvious depression of the central nervous system in phenobarbital-treated rabbits, but it is not possible to rule out more subtle effects. The mechanism by which phenobarbital prevents the rise of serum cholesterol and inhibits the formation of atheromata in the aorta of the cholesterol-fed rabbit is not known. Attempts were made to determine whether phenobarbital enhances the metabolism of cholesterol, thereby accelerating its removal from the body. Although it was not possible to demonstrate appreciable hydroxylation of cholesterol *in vitro* by rabbit liver microsomes, it was found that treatment of rabbits with phenobarbital enhanced the liver microsomal hydroxylation of testosterone in the 6β-, 7α- and 16α-positions. Studies by others have shown that administration of phenobarbital to rats stimulates the liver microsomal 7α-hydroxylation of cholesterol, an early step in the conversion of cholesterol to bile acids,[14, 15] and this treatment also enhanced bile flow.[16]

In contrast to the results obtained with phenobarbital, the administration of chlorcyclizine to cholesterol-fed rabbits did not increase the hydroxylation of testosterone by liver microsomes, and chlorcyclizine did not inhibit the increase in serum

cholesterol or the formation of atheromata in the aorta of the cholesterol-fed rabbit. It is of interest that the development of atheromata in cholesterol-fed rabbits is enhanced by exposing the animals to carbon monoxide,[17] a potent inhibitor of enzymes in liver microsomes that hydroxylate cholesterol and testosterone.[9, 18] It was recently shown that the aorta from several species metabolizes testosterone and estrogens *in vitro*, but it is not known whether cholesterol is metabolized by the aorta or whether phenobarbital or other drugs change the activity of enzymes in the aorta that metabolize cholesterol.[19]

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