Influence of Theobromine Magnesium Oleate on Formation of Experimental Atheroma[1] (37478)

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Over a century ago cholesterol was first identified as a constituent of atheromatous plaques (1). Later in 1913 Anitschkow and Chalatov (2) demonstrated that dietary cholesterol resulted in atheromatous deposits in rabbit arteries. These early observations were immediately translated as a direct cause-and-effect relationship between dietary cholesterol and atherosclerosis and numerous studies followed which attempted to show this same relationship in spontaneous atherosclerosis of man. Although the results have been disappointingly inconclusive, several important facts have emerged which indicate that cholesterol is in some way linked to the human atherogenic process. The evidence demonstrating that cholesterol is a constant constituent of atheromatous deposits seems indisputable (3). Its presence in arterial plaques could be regarded as fortuitous were it not for the epidemiological studies (4, 5) strongly implicating blood cholesterol levels in human coronary heart disease and the fact that most tissues of the body including the artery wall are capable of synthesizing cholesterol (6).

Although the primary defect of atherosclerosis is unknown, the belief that elevated levels of blood lipid and cholesterol have a causative relationship to this disease has led to the use of both pharmacological and dietary treatments to reduce these substances in blood. Theobromine magnesium oleate (TMO) (magnesium 3,7-dimethylxanthine oleate) is a drug shown to have several effects in the human, *i.e.*, vasodilation, diuresis, as well as that of reducing elevated blood cholesterol in some subjects (7–10). The exact mechanism of action of TMO in reducing blood cholesterol is unclear although it was once theorized to have a solubilizing influence on both cholesterol and lipoprotein, thus preventing the deposition of these substances in the intima and/or aiding in their removal from the artery wall (9). This theory was prompted by the observation of an initial elevation and subsequent decline of serum cholesterol in subjects given TMO (Athemol) as treatment for peripheral vascular disease (10). The investigation reported herein was undertaken in an attempt to explain this phenomenon and the symptomatic improvement reported after administration of TMO to patients with peripheral vascular disease (7) and cerebral vascular disease (9). This study was designed to evaluate the influence of TMO on plasma cholesterol and the formation of atheromatous lipid deposits in the aorta of rabbits fed an atherogenic diet.

Materials and Methods. A total of 45, 10-week-old male white New Zealand rabbits were used in this study. The animals were initially given, *ad libitum,* a standard pelleted rabbit diet for a 10-day period of adjustment and observation. They were then divided into two groups on the basis of paired-weight matching; 22 control and 23 experimental (drug) animals. Each animal was assigned a number (ear tattoo). Throughout the experiment of 12 weeks, both groups received water, *ad libitum,* and the standard rabbit diet supplemented with 1% cholesterol and 5% peanut oil. To insure proper mixing, the standard diet was purchased in meal form and thoroughly mixed with the cholesterol and peanut oil before being pelleted. This procedure was carried out weekly. Heating

[1] Supported by a grant from Meyer Laboratories, Research Division, Fort Lauderdale, Fla.

the peanut oil served to dissolve the cholesterol before both were added to the standard meal.

In addition to the atherogenic diet, the experimental group received 60 mg/kg TMO[2] in divided daily doses and the control group was given a placebo twice daily. The drug, made up in the form of scored 20-mg tablets, and the placebo were administered orally, using a type of "pilling gun." This was constructed using a 5 inch × 8 mm i.d. tygon tube and a solid nylon indwelling plunger (6-mm diameter). The drug or placebo was administered by inserting the pilling gun over the tongue followed by dispensing and manual closure of the mouth.

Throughout the study, daily feed consumption, weekly body weights and monthly plasma cholesterol concentrations were determined for each animal. Using standard procedures, heparinized blood samples were withdrawn directly from the heart without difficulty, and the plasma separated and stored in a frozen state until analyzed. The blood sampling was done on all rabbits in the fasted state at approximately the same time each day. Total plasma cholesterol was determined using the colorimetric method of Liebermann–Watson (11).

At the end of twelve weeks, all animals were sacrificed and their aortas (extending from heart to iliac bifurcation) removed for determining the extent of atheroma formation. The vessels were first stripped of their adventitia and then opened longitudinally, washed free of blood and fixed in a flattened position for 24 hr in 10% neutral-buffered formalin. Following fixation, the arteries were stained for lipids using Herxheimer's Sudan IV stain and the procedure of Holman *et al.* (13) in which the vessels are rinsed briefly in 70% ethanol, immersed and agitated in the dye solution for 15 min, drained, differentiated in 80% ethanol for 20 min and then washed under tap water for one hour. Extent of atheroma formation was determined by two methods: (a) Visual grading for per cent of intimal surface area covered by stained lipid. The grading was done independently by three persons not acquainted with the experimental protocol and the three values obtained for each aorta were averaged. (b) Spectrophotometric determination of quantity of extractable dye in each aorta. For this procedure, the aortas were first dried in a 100° oven, weighed and then homogenized in an excess of 2:1 chloroform–methanol solvent using a motor-driven Potter–Elvejhem glass tissue grinder. The homogenate was filtered and the filtrate made up to 100 ml with the solvent and the optical density of this was determined at 530 nm (absorption maximum for Sudan IV) in a spectrophotometer. The values obtained were converted, using a standard curve, to milligrams of dye per gram dry weight of aorta. The data were analyzed statistically using Student's *t* test.

Results. Of the total of 45 rabbits, five were lost during the course of the experiment; animals 10, 19, and 20 from the control group and animals 35, and 40 from the experimental TMO group. Animal 35 died at the beginning of the second month as a consequence of the blood-collecting procedure. This was the only animal lost with this method of blood sampling. Animal number 10 was sacrificed after 75 days on experiment after developing a thrombosis-induced posterior paralysis. Animals 19 and 20 died after about two months on experiment following a week or more of decline in condition, loss of appetite and severe icterus. Animal 40 developed similar signs and died only a few days before termination of the experiment. All of the animals lost to the experiment were necropsied. Most of the animals within both groups showed some loss in condition after about the second month on experiment and there was visible evidence of lipid deposition around the iris and in the form of subcutaneous nodules over the joints of the feet in many of the rabbits within both groups at that time. Although feed consumption declined slightly in the two groups between the first and third months on the atherogenic diet, both showed a progressive increase in body weight throughout the study. The *t* test showed that the average total feed intake and gain in body weight were not significantly different between the two groups.

[2] TMO supplied by Meyer Laboratories, Inc., Research Division, Fort Lauderdale, Fla.

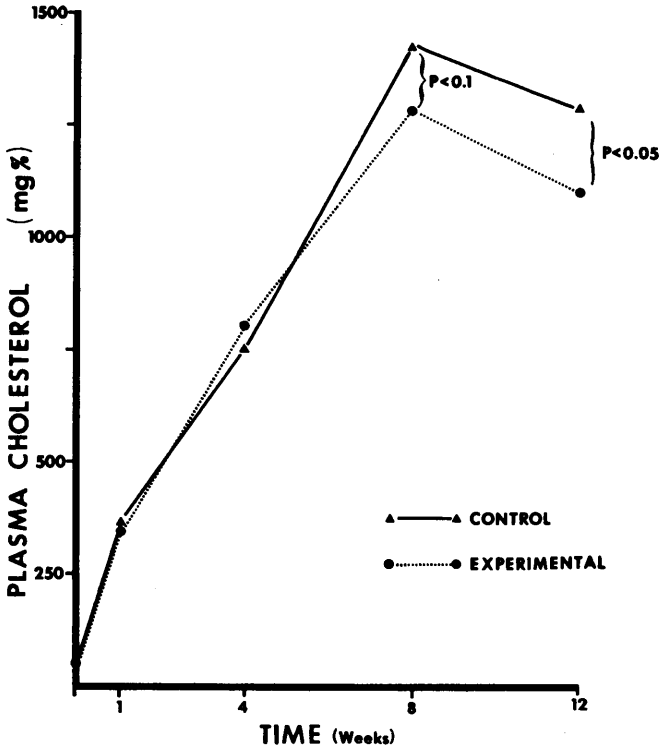

FIG. 1. Mean plasma total cholesterol for control and experimental groups.

The group mean values for plasma total cholesterol at 0, 1, 2, and 3 months following initiation of the experiment are shown in Fig. 1. The atherogenic diet increased the plasma total cholesterol in both groups of rabbits. During the first month the two groups showed a similar increase but by the end of the second month the mean value for the group receiving TMO was lower than that of the control group. This difference increased by the end of the third month and was found to be statistically significant ($p < 0.05$) at that time.

The data on extent of aortic atheroma formation in the two groups of rabbits based on two methods are shown in Table I. In the method of visual grading for per cent of aortic intimal surface covered by lipid, the three independent estimates obtained on each animal were averaged. Although the mean for the experimental group was lower than that for the control group (66.1 vs 77.8%) this difference was not statistically significant at the accepted 5% level. It is recognized, however, that the method of visual grading has several limitations. The subjective nature of the method could be expected to give a wide range of values, as was observed in this study. The method also fails to take into account the depth of the atheromatous lesions which in the present study was noticeably different for the individual aortas. Because of limitations of the visual grading method, we further evaluated the severity of atheroma formation in the two groups of rabbits by determining the quantity of Sudan IV dye deposited in the aorta of each animal. Results in Table I show that the aortas of the experimental group had less dye per gram dry weight than did those of the control group. This difference was highly significant ($p < 0.01$).

Figures 2 and 3 show plots of the final plasma cholesterol versus per cent of aortic quantity of aortic Sudan IV. The control rabbits showed a rather constant, elevated plasma cholesterol level associated with stained atheromatous lesions covering, in most of the animals, between 70 and 100%

TABLE I. Influence of TMO on Atheroma Formation.[a]

Rabbit no.	Aortic intima covered by lipid (%)				Aortic Sudan IV (mg/g dry wt)
	Estimates				
Control group	1	2	3	Average	
1	70	75	65	70.0	5.47
2	40	35	40	38.3	1.53
3	95	98	90	94.3	4.83
4	40	60	55	51.2	2.26
5	95	96	90	93.7	4.87
6	95	90	90	91.7	3.11
7	90	85	80	85.0	5.55
8	75	70	80	75.0	2.48
9	98	99	99	98.7	6.21
11	97	96	95	96.0	4.81
12	90	92	85	89.0	2.69
13	65	70	70	68.3	4.32
14	91	90	90	90.3	2.47
15	99	99	99	99.0	3.75
16	98	95	90	94.3	2.64
17	15	20	30	21.7	2.87
18	80	85	85	83.3	3.21
21	99	99	95	97.7	3.80
22	35	40	45	40.0	2.24
			Group means	77.8 ± 5.5	3.64 ± 0.31
Experimental group					
23	98	95	90	94.3	1.89
24	99	100	100	99.7	2.85
25	55	50	40	48.3	1.65
26	13	15	10	12.7	1.98
27	60	60	70	63.3	2.08
28	65	70	60	65.0	4.29
29	50	60	50	53.3	2.20
30	40	40	35	38.3	2.74
31	55	50	60	55.0	2.01
32	80	90	70	80.0	3.54
33	70	70	75	71.7	2.37
34	11	15	10	12.0	2.12
36	98	99	100	99.0	4.25
37	96	92	90	92.7	1.57
38	97	99	90	95.3	3.52
39	97	95	90	95.0	3.10
41	60	65	70	65.0	2.12
42	93	95	90	92.7	—
43	22	20	15	19.0	1.84
44	60	70	60	63.3	3.74
45	70	70	80	73.3	—
			Group means	66.1 ± 6.1	2.62 ± 0.20

Comparison of group means: $p < 0.01$.

[a] Group means ± SE and individual values.

of their aortic intima. However, the same data plotted for the TMO group shows a dispersion of the points to areas of the graph associated with lower plasma cholesterol levels and less intimal surface covered by lipid. The same general relationships are apparent

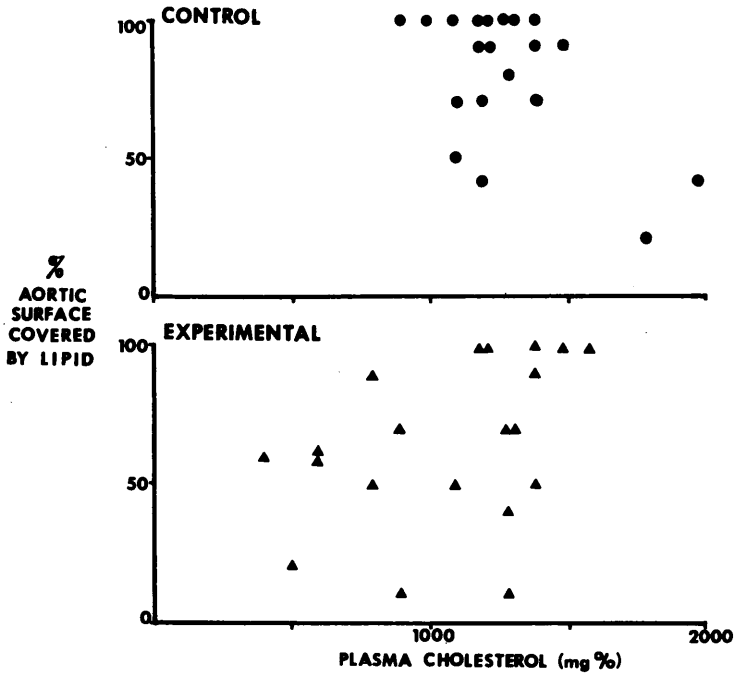

FIG. 2. Final plasma cholesterol versus per cent aortic intimal surface covered by lipid for individual rabbits.

for the two groups regarding plasma cholesterol and quantity of aortic Sudan IV. There was some disparity between the values obtained for aortic Sudan IV and those for per cent of intimal surface lipid for an individual aorta, as might be expected. However, a highly significant positive correlation ($r = 0.484$) between values obtained by the two methods indicates that both probably measured the same parameter, *i.e.*, the severity of atheromatosis.

There was no obvious relationship between average food consumption and the degree of atheromata for individual animals in either the control or experimental groups. The same was true for body weight vs severity of atheroma in the two groups.

Discussion. Almost all of the pharmacologic actions of the methylxanthines, theobromine, theophylline and caffeine have recently been attributed to their effect upon the adenyl cyclase system (14, 15). These pharmacologic effects probably rely upon the purine ring system of the methylxanthines, and its similarity to adenosine compounds such as adenosine triphosphate (ATP) and adenosine cyclic 3′,5′-monophosphate (cyclic AMP). The adenyl cyclase system has been recognized as a sequence of enzymatic reactions which mediate hormonal and neurohumoral control over many physiologic processes (14, 16). In this system, the level of cyclic AMP is regulated by the relative activities of adenyl cyclase, which catalyzes the synthesis of cyclic AMP from ATP, and phosphodiesterase, which converts cyclic AMP to 5′-AMP.

It is generally accepted that the methylxanthines exert their influence upon the adenyl cyclase system by competitive inhibition of phosphodiesterase causing increased levels of cyclic AMP in the tissues. Cyclic AMP is the stimulating agent for lipolysis, gluconeogenesis, steroidogenesis (from cholesterol), and many other enzymatic processes (14). Figure 4 demonstrates the mechanism by which the methylxanthines have been proposed to alter lipolytic activity in adipose tissue (14).

The adenyl cyclase system has not been

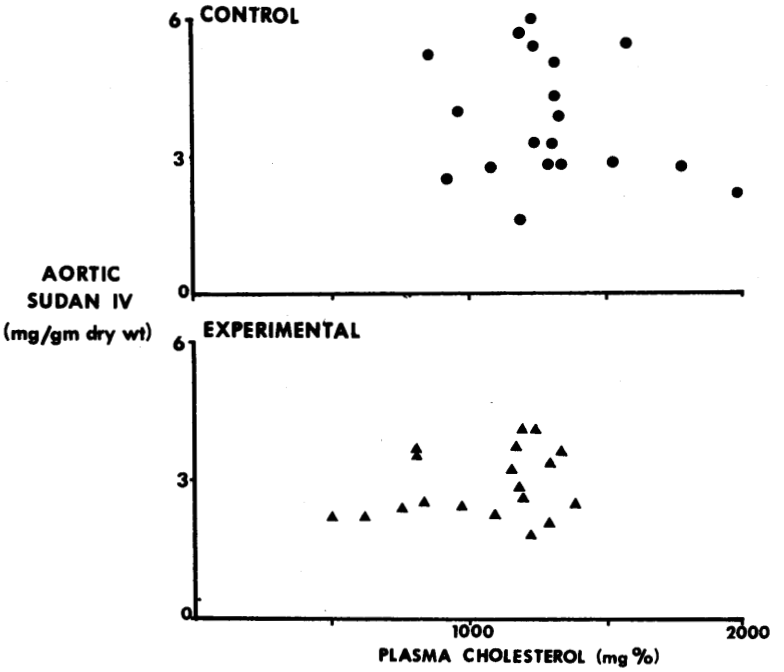

FIG. 3. Final plasma cholesterol versus aortic content of Sudan IV for individual rabbits.

thoroughly examined for function in the arterial wall. However, components of the system, adenyl cyclase (17) and phosphodiesterase (18), have been found in the aorta of dogs. An indication that the adenyl cyclase system is functional in rabbit aorta was found by Clements *et al.* (19). Their results indicated that the adenyl cyclase system regulates some aspects of metabolism in the arterial wall as it does in the liver, heart, brain and other tissues. In the liver and adipose tissue, cyclic AMP controls lipid metabolism by regulating the activity of lipolytic enzymes termed hormone-sensitive lipase, phospholipase and cholesterol esterase (14). Similar enzymes have been identified in the

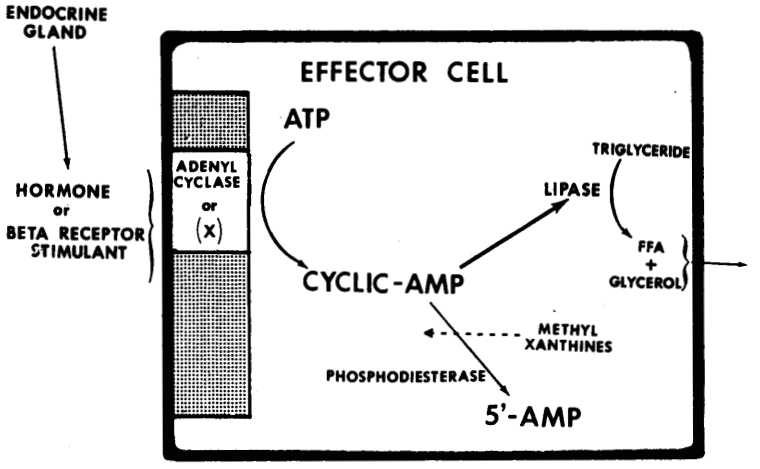

FIG. 4. Physiologic role of cyclic AMP in the control of lipase activity. [Adapted from: Sutherland, Earl W., J. Amer. Med. Assoc. 214 (7), 1281 (1970)].

arterial wall of humans and several other species (20–25). Experiments with animals have shown that the activities of the lipolytic enzymes are increased in proportion to the severity of atherosclerosis (25, 26). Zemplenyi (27) has interpreted these results as an indication that the increased enzymatic activity is an attempt by the artery wall to protect itself against accumulation of potentially injurious triglycerides.

A possible explanation of the results reported in this investigation might involve an effect of TMO upon lipolytic enzymes as shown in Fig. 4. This effect could be mediated through inhibition of phosphodiesterase; thereby causing an increased level of cyclic AMP and subsequent stimulation of the lipolytic enzymes. This action of TMO would cause a more effective catabolism of the lipids which normally enter the arterial wall and thereby prevent their accumulation in atheromatous plaques. This might explain why the group of rabbits receiving TMO in this study had significantly less lipid deposits in the arterial wall than the control group.

Since all the enzymes of the lipolytic pathway and the adenyl cyclase system are found in the arterial wall (17–25), it is possible that the site of action of TMO is in the arterial wall. However, the interrelation of the adenyl cyclase system and lipolysis has been more completely delineated in liver and adipose tissue and an action at these sites might also, indirectly, prevent lipid accumulation in arterial plaques. The results of this investigation do not allow discrimination between these possible sites of action. Also, the data do not allow interpretation of the mechanism of TMO's action on plasma cholesterol level. However, since all lipids are interrelated due to their common synthesis from acetate, it is possible that an effect of TMO upon triglyceride lipase could indirectly affect cholesterol levels. A direct effect is also possible since, in adrenal and ovarian tissue, the adenyl cyclase system controls the conversion of cholesterol to pregnenolone (14). It may be important that the hypocholesterolemic effect of TMO was observed in the present study only after several months of administration.

Sutherland, Robison, and Butcher (32) have predicted that cyclic AMP research "may lead to the more rational and widespread use of theophylline and other phosphodiesterase inhibitors as therapeutic agents." Data presented here, indicating that TMO is capable of inhibiting atherogenesis, might be interpreted as a consequence of its effect upon phosphodiesterase.

However, it is difficult to assume that the results obtained in this artificial system are relevant to the pathogenesis of human atherosclerosis. The major problem is that one cannot prove that the human and rabbit artery will react identically. Another problem is that in order to induce atherosclerotic changes in the rabbit in a reasonable length of time, serum cholesterol levels must be greater than those normally found in rabbits or humans. Nevertheless, the results indicate that studies should now be undertaken in other species and under more physiologic circumstances.

Summary. Forty-five rabbits were fed an atherogenic diet for three months. One group (23 rabbits) received theobromine magnesium oleate (TMO) orally in two equal doses daily (60 mg/kg/day). The control group received a placebo. Plasma cholesterol levels were determined at 0, 1, 2, and 3 months after the rabbits were placed on the atherogenic diet. At the end of three months the animals were sacrificed, their aortas excised and stained for lipids, and the degree of atherosclerosis estimated by two methods; visual grading of per cent of aortic intima covered by lipid and spectrophotometic determination of the quantity of Sudan IV dye extracted from individual aortas.

The experimental group receiving TMO showed less atherosclerosis than did the control group. The mean per cent of aortic intimal area covered by lipid, based on an average of three independent visual estimates was 66.1 for the experimental group as opposed to 77.8 for the control group. The mean quantity of extracted Sudan IV in milligrams per gram dry weight of aorta for the experimental group was 2.62 and for the control group, 3.64 ($p < 0.01$). The atherogenic diet increased the plasma total

cholesterol in both groups of animals and for the first month the mean values were similar in both groups but by the end of the second month the group receiving TMO showed a lower level of plasma cholesterol. This difference between the two groups increased by the end of the three month study and was statistically significant ($p < 0.05$) at that time.

These results have been interpreted as evidence that TMO is capable of impeding atherogenesis. Exactly how this is accomplished is unclear. TMO, through inhibition of phosphodiesterase, could lead to increased levels of cyclic AMP which would activate lipase in either the arterial wall, adipose tissue, or liver. Activation of lipase at one or more of these sites could possibly explain the influence of TMO upon the atherogenic process. Further studies in other species and studies to investigate the possibility of direct activation of arterial lipolytic enzymes by TMO are in progress.

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