Altered Response of Stressed Rats to Hypercholesterolemic Diets

By FAWZI DABORA* and MICHAEL M. CLAY

Rats were stressed for 2 weeks by restriction of movement, then fed a diet containing cholesterol, cholic acid, and calciferol for 2 weeks. The prestressed rats developed higher levels of plasma cholesterol and atherosclerosis than did unstressed controls. Plasma cholesterol increased in prestressed rats when the diet was supplemented with 250, 500, and 2000 U.S.P. units of calciferol. The degree of atherosclerosis was not directly related to the degree of hypercholesterolemia in all cases.

ENVIRONMENTAL "STRESS" is one of the factors being studied in the elucidation of the mechanisms of atherosclerosis. Increases in serum cholesterol levels have been reported in students in response to the "stress" posed by examinations (1–4). Page *et al.* (5), however, failed to find a causal relationship between changes in plasma levels of cholesterol, lipoprotein, or triglycerides and short-term "stresses" (tournament bridge playing and performance of surgery).

The contradictory results obtained in human studies may be due to nonuniform definitions of the state referred to as "stress" as well as to the varying periods of exposure to an unpleasant environment. Also, human studies are limited by lack of knowledge of prestudy experience and inability to study blood vessels for evidence of atherosclerosis.

After 14 days of partial restriction of movement of rats, Gabel and Clay (6) reported that systolic blood pressures were elevated. Further studies in this laboratory have demonstrated that peripheral plasma corticosterone levels were sharply increased by restriction of movement in rats. Page and Brown (7) reported that rats fed a diet containing 4% cholesterol, 2% cholic acid, and radioactive iodine for 6 months failed to develop atherosclerosis although plasma cholesterol levels increased.

The present study represents an attempt to induce the deposition of fat in the aorta of the rat by combining several of the treatments used by the above authors.

MATERIALS AND METHODS

Male Wistar strain rats were used. The rats were sacrificed by decapitation and blood was collected upon completion of each treatment. Plasma total cholesterol was assayed with Bloor's modification of the Liebermann-Burchard reaction, and plasma cholesterol esters were assayed by the same method after precipitation of free cholesterol with digitonin. Liver total lipids were determined gravimetrically after extraction with alcohol-ether. The thoracic aortas were removed, stripped free of adventitia, and fixed in 10% formalin. After longitudinal splitting, the flattened vessels were stained with Sudan IV and counterstained with hematoxylin.

Effects of Stress and/or High Cholesterol Diet.—The combined effect of three factors, stress, excessive cholesterol ingestion, and added dietary calciferol, were studied. Five groups of eight rats (140–215 Gm.) were subjected to five test conditions.

Group *1*.—Normal diet,[1] "stress free."
Group *2*.—Normal diet; restriction of movement for 14 days.
Group *3*.—Normal diet; "stress free" for 14 days, followed by a special diet,[2] "stress free" for 14 days.
Group *4*.—Normal diet; restriction of movement for 14 days, followed by the special diet, "stress free" for 14 days.
Group *5*.—Normal diet; complex stress: restriction of movement, heat (31 ±2°), and bright light for 14 days, followed by the special diet, "stress free" for 14 days.

Restriction of movement was imposed by housing a single rat in a clear plastic cage measuring 5.5 × 2.5 × 2.5 inches. The dimensions permitted the animal limited movement. Food and water were supplied at all times *ad libitum*.

Effects of Graded Calciferol Dosage.—Thirty-two rats (190–250 Gm.) were maintained on the normal diet with restriction of movement for 14 days. They were then divided into four equal groups and maintained for 14 days under "stress free" conditions on Turtox vitamin D deficient diet (General Biological Supply House Inc.), supplemented with 4% cholesterol and 2% cholic acid. Group *1* was fed the above diet only. Groups *2*, *3*, and *4* were fed the above diet supplemented with 250, 500, and 2000 U.S.P. units calciferol per 15 Gm. of food, respectively.

RESULTS

The results of the study on the effects of stress and/or high cholesterol diet are summarized in Table I. Group *2* (stressed) differed from Group *1* (unstressed) only in the reduction of food intake by about 50% and the presence of minute sudanophilic aortic areas in the former group. Feeding the special diet to Groups *3*, *4*, and *5* resulted in significantly elevated plasma total cholesterol, plasma cholesterol esters, and per cent liver lipids when compared with the values of Group *2* ($p = <0.01$ in each instance). Plasma total cholesterol, plasma cholesterol esters, and per cent liver lipids of Group *4* (restriction stress) and Group *5* (complex stress) were higher after the 14 days on the special diet than those of Group *3* (unstressed). The differences were significant for Group *4* only for plasma total cholesterol ($p = <0.02$) and per cent liver lipids ($p = <0.001$). Only the difference in per cent liver lipids of Group *5* ($p = <0.01$) was significant.

Aortic sudanophilia was present in seven of eight rats of Group *3*, seven of eight of Group *4*,

Received June 3, 1963, from the Division of Pharmacology and Animal Sciences, College of Pharmacy, Columbia University, New York, N. Y.
Accepted for publication February 18, 1964.
Abstracted from a thesis submitted by Fawzi Dabora in partial fulfillment of Master of Science degree requirements.
Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.
* Research Fellow, Ciba Pharmaceutical Products, Inc., Summit, N. J.

[1] The normal diet consisted of Wayne Lab-Blox pellets (Allied Mills).
[2] The special diet consisted of powdered Wayne Lab-Blox pellets to which was added 4% cholesterol, 2% cholic acid, and 250 U.S.P. units of calciferol per 15 Gm. of diet. The calciferol content of Wayne Lab-Blox is 444.5 U.S.P. units per 100 Gm.

TABLE I.—EFFECTS OF STRESS AND CHOLESTEROL DIET

Treatment (Group)	Plasma Total Cholesterol, mg./100 ml.	Plasma Cholesterol Esters, mg./100 ml.	Total Liver Lipids, % wet wt.
1. Stress free, 14 days	84 ± 9[a]	55 ± 6	6.10 ± 0.46
2. Restriction, 14 days	88 ± 9	57 ± 10	5.65 ± 0.74
3. Stress free, 14 days; cholesterol, 14 days	279 ± 111	200 ± 102	10.25 ± 1.83
4. Restriction, 14 days; cholesterol, 14 days	382 ± 78	250 ± 72	13.34 ± 2.07
5. Triple stress, 14 days; cholesterol, 14 days	304 ± 80	238 ± 69	12.40 ± 0.91

[a] Standard deviation.

TABLE II.—EFFECT OF GRADED DOSES OF CALCIFEROL

U.S.P. units[a] Added Calciferol (Group)	Plasma Total Cholesterol, mg./100 ml.	Plasma Cholesterol Esters, mg./100 ml.	Total Liver Lipids, % wet wt.
1. 0	240 ± 103[b]	170 ± 64	15.36 ± 7 60
2. 250	293 ± 46	204 ± 24	13.56 ± 0.74
3. 500	348 ± 84	235 ± 75	11.73 ± 0.66
4. 2000	440 ± 115	257 ± 75	12.86 ± 1.76

[a] Seven of eight surviving rats in each group. [b] Standard deviation.

and six of seven surviving rats of Group 5. In Group 3, numerous small zones occurred in five rats, while generalized distribution occurred in two rats. In Group 4, numerous small zones occurred in one rat, while generalized distribution occurred in six rats. In Group 5, generalized distribution occurred in six rats.

The results of the study on the effects of graded calciferol dosage are summarized in Table II. Plasma total cholesterol and plasma cholesterol esters increased with each increment in calciferol dosage. Increases from group to group were significant only in the comparison between per cent total liver lipids of Groups 2 and 3 ($p = <0.001$) and plasma total cholesterol and per cent total liver lipids of Groups 3 and 4 ($p = <0.05$ and <0.02, respectively).

The aortas of five of the rats of the four experimental groups failed to show evidence of atherosclerosis. Although the aortas of the remaining rats showed varying degrees of atherosclerosis, there was no consistent pattern of atherosclerosis from group to group. Furthermore, there was no relationship between degree of hypercholesterolemia and severity of atherosclerosis between individual rats of each group.

DISCUSSION

Changes in fat metabolism, similar to those described here, have been reported elsewhere in rats only after thyroidectomy or thyroid depression (8). The temperature in the restriction cages was about 30°, although room temperature was maintained at 21–22°. The effect of elevated environmental temperature and restriction of movement upon thyroid gland activity is not known.

The differences reported among the rats maintained on four levels of calciferol cannot be attributed to different levels of food or cholesterol intake. Mean daily food intake ranged from 12.5 to 13.7 Gm. among the four groups. Mean daily food intake among the three cholesterol fed groups of the stress study were also relatively uniform, ranging from 18.1 to 18.8 Gm.

Page *et al.* (7) suggested that resistance of rats with normal or depressed thyroids to an atherogenic diet containing 4% cholesterol and 2% cholic acid was due to lack of vascular tissue response. The data presented here suggest that stress can overcome aortic resistance. Hauss *et al.* (9) have reported increased connective tissue metabolic activity in various organs of the rat, including the aorta after imposition of stress. They reported a similar finding in excised human atherosclerotic aortas. Similar stress induced changes in the aortic wall might predispose the tissue to the deposition of fat when a diet rich in cholesterol is subsequently fed.

Rats subjected to a triple stress showed more severe atherosclerosis but lower levels of hypercholesterolemia than did rats subjected to a single stress. When comparing individual rats within each group, there was no relationship between degree of hypercholesterolemia and atherosclerosis. Similar findings were made in the rats treated with graded doses of calciferol.

The mechanism whereby calciferol increased plasma free cholesterol and cholesterol esters is obscure. It has been suggested (9) that arteriosclerosis with calcium deposition in the vessel wall might be a precursor to fat deposition.

SUMMARY AND CONCLUSIONS

Prestressed rats showed higher plasma total cholesterol, plasma cholesterol esters, and per cent total liver lipids as well as intensity of aortic sudanophilia than did unstressed rats after 2 weeks on a cholesterol rich diet. The intensity of the plasma changes were accentuated by providing increased calciferol intake. Stress-induced alterations in aortic connective tissues and its mucopolysaccharides may play a role in fat deposition in the aorta when a diet rich in cholesterol is subsequently administered. The degree of atherosclerosis was not directly related to the degree of hypercholesterolemia in all cases.

REFERENCES

(1) Dreyfuss, F., and Czaczkes, J. W., *A. M. A. Arch. Internal Med.*, **103**, 708(1959).
(2) Grundig, S. M., and Griffin, A. C., *J. Am. Med. Assoc.*, **171**, 1794(1959).
(3) Thomas, C. B., and Murphy, E. A., *J. Chronic Diseases*, **8**, 661(1958).
(4) Wertlake, P. T., Wilcox, A. A., Haley, M. I., and Peterson, J. E., *Proc. Soc. Exptl. Biol. Med.*, **97**, 163(1958).
(5) Page, I. H., Moinuddin, M., and Lewis, L. A., *J. Atheroscler. Res.*, **2**, 66(1962).
(6) Gabel, L. P., and Clay, M. M., *Federation Proc.*, **20**, 209(1961).
(7) Page, I. M., and Brown, H. B., *Circulation*, **17**, 99 (1958).
(8) Fellios, L. C., *J. Exptl. Med.*, **104**, 539(1956).
(9) Hauss, W. H., Junge-Huelsing, G., and Hollaender, H. J., *J. Atheroscler. Res.*, **2**, 50(1962).