The Pathogenesis of Neurogenic Hypercholesterolemia: IV. Abnormal Metabolism of Chylomicronous Cholesterol[1] (37022)

MEYER FRIEDMAN, SANFORD O. BYERS, AND STEPHEN ELEK
(With technical assistance of Warren Hayashi, Clarence Omoto, and Roberta Neuman)

Harold Brunn Institute, Mount Zion Hospital and Medical Center, San Francisco, California 94115; and University of Southern California, School of Medicine, Los Angeles, California 90033

Following our initial observation (1) that bilateral electrolytic injury of the hypothalamic ventromedial nuclei, the fornices and the medial portion of the lateral hypothalamic area of the rat promptly induces a chronic, diet-sensitive hypercholesterolemia, we have attempted to determine the "target" organ(s) mediating this cholesterol elevation and the character of the "message" sent (or failing to be sent) to such target organ(s) from the damaged brain area. In our first study (2) concerning these problems, we found that the chronic hypercholesterolemia did not occur because of any obstruction in biliary flow, a change which Gutstein, Schneck and Appleton (3) believed was responsible for the type of *acute* hypercholesterolemia which they observed in their rats immediately following feeding and unilateral stimulation of the lateral hypothalamus. In our second study (4), we observed that despite slight histological changes in the thyroid and moderate to severe histological changes in the pituitary gland, this form of neurogenic hypercholesterolemia appeared to be independent of the functions of these two glands. It also was independent of the adrenal, testes, and the hormone, insulin.

In our most recent study (5) concerning this problem, we observed that the liver of the hypothalamus-lesioned rat did differ in its handling of cholesterol somewhat from that of the normal rat. Thus, although the liver of the lesioned rat appeared to synthesize cholesterol (as indicated by its rate of incorporation of Na acetate-^{3}H) as efficiently as the normal rat liver, it did not appear to be able to remove labeled cholesterol already circulating in the plasma nearly as rapidly as the liver of the normal rat. This lag in removal was particularly observed in respect to labeled cholesterol of exogenous provenance.

This last finding, of course, suggested to us that following induction of our diencephalic lesion in the rat, its liver failed to remove chylomicronous cholesterol at its previous rate. In order to determine whether this lag in hepatic removal of chylomicronous cholesterol was due solely to some intrinsic change in the liver of this type of lesioned animal or to some change in the physicochemical structure of its cholesterol-bearing chylomicrons, studies utilizing chylomicron-containing lymphs obtained from both normal rats and from rats bearing the hypothalamic lesions were done. These studies were facilitated by the fact that almost all cholesterol absorbed by the rat travels with the lymph chylomicrons (6). In addition, studies concerning the hepatic rate of uptake of chylomicronous triglyceride and the plasma heparin-activated lipoprotein-lipase activity of rats with hypothalamic lesions were done. The results of these various studies are reported below.

I. Methods. A. The rate of disappearance from plasma of normal rats of intestinal lymph ^{3}H-cholesterol obtained from hypothalamus-injured and normal rats. Six male rats (Long-Evans strain) 12 wk of age and weighing between 275 and 315 g were subjected to hypothalamic injury as previously described (1). One week later, after prior

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starvation for 16 hr, these 6 rats were given by stomach tube, 1 ml of cottonseed oil containing 0.116 mCi of cholesterol-1α, 2α,-^{3}H (New England Nuclear Co., Boston, MA.) Six normal rats were also given the labeled cholesterol in oil. In addition, 4 normal rats were given only 1 ml of cottonseed oil. Intestinal lymph was collected from these 16 rats for 8 hr after initial feeding. Our previously described collection technique (7) was modified in this instance by using a flushing solution (for backflow through the catheter leading from the lymph duct) which consisted of 17 g/liter of EDTA and 8 g/liter of NaCl, adjusted to pH 7.2. This solution flowed retrograde through the catheter at a rate of 0.3 ml/hr, mixing with the lymph in the catheter and preventing clotting. The EDTA was added because of its demonstrated capability (8) to preserve the physicochemical integrity of the chylomicrons. All the lymph samples were analyzed for their cholesterol concentration (9) and their radioactivity. The pooled lymph of the normal rats who received the labeled cholesterol, while containing approximately the same cholesterol concentration as the pooled lymph of the rats with lesions, did exhibit slightly more radioactivity. Accordingly, this lymph was diluted with sufficient intestinal lymph of the control rats (that had received no labeled cholesterol) to lower its radioactivity to that observed in the lymph of the rats with lesions. Thus, the pooled lymph obtained from the rats bearing lesions contained 47 mg of cholesterol/100 ml and 17.9 $\times$ 10^2 dpm/ml. The pooled lymph obtained from the normal rats (when corrected by addition of nonradioactive lymph) contained 49 mg of cholesterol/100 ml and 17.6 $\times$ 10^2 dpm/ml. These two lymph pools were stored at 4° until used. Almost all cholesterol absorbed by the rat travels with the lymph chylomicrons (6), which, therefore, are tagged by the ^{3}H-cholesterol fed.

Forty-eight hours after this lymph had been collected, 14 previously starved (12 hr) normal rats were anesthesized with ether, operated upon and an indwelling polyethylene catheter was inserted into the right iliofemoral vein. The rats were than placed in restraining cages. Six hours later, the animals having recovered from their anesthesia, 8 were injected (via the catheter) with 2 ml of the radioactive lymph sample previously obtained from the rats bearing lesions. The remaining 6 were injected with the pooled lymph sample obtained from the normal rats. Blood samples then were obtained 4, 8, 12, 16, 20 and 32 min following such injections and measured for their content of radioactivity. This was done by pipetting 0.1 ml of plasma into 10 ml of scintillation cocktail composed of toluene and ethanol in ratio of 7:3 and containing 3.5% (w/v) of Omnifluor (New England Nuclear Co., Boston, MA). The radioactivity was measured in a Packard Model 3003 scintillation counter. Quench corrections were calculated after addition of known quantities of toluene-^{3}H.

B. Rate of disappearance from plasma and accumulation in liver of intestinal lymph ^{3}H-cholesterol in hypothalamus-injured and normal rats. Ten normal male rats, after previous starvation (16 hr) were given ^{3}H-cholesterol suspended in 1 ml of cottonseed oil. Their intestinal lymph was collected for 8 hr, pooled, measured for its radioactivity and stored at 4° until used.

Forty-eight hours after this collection, 12 rats which 1 wk previously had been subjected to hypothalamic injury and 12 normal rats were vein-cannulated, placed in restraining cages, injected with lymph and bled at 2, 4, 8, 12, 16 and 32 min exactly as described in Method A except that each rat received 1.5 ml of the pooled normal intestinal lymph containing 670,000 dpm/ml. The initial sample also was analyzed for its plasma cholesterol concentration.

Immediately after the last bleeding, the rats were killed and the liver was perfused with normal saline to rid it of residual blood. It was then removed, weighed and analyzed for its content of total cholesterol and radioactivity. A weighed sample of liver was dissolved in 6 ml of 10% methanolic NaOH by heating for 3 hr or more at 60°. Sufficient chloroform (12 ml) was then added to give final volumes of chloroform:methanol in ratio 2:1; the mixture was shaken, allowed to

TABLE I. The Rate of Disappearance from Plasma of Normal Rats of Intestinal Lymph ^{3}H-Cholesterol Obtained from Hypothalamus-Injured and Normal Rats.

No. rats	Av wt (g)	After injection of intestinal lymph containing ^{3}H-cholesterol-bearing chylomicrons (dpm/0.1 ml of plasma) (min): 4	8	12	16	20	32
		A. Normal rats injected with lymph from hypothalamus-injured rats					
8	280	938	858	667	539	444	198
Range		(746–1088)	(752–971)	(449–816)	(318–697)	(213–638)	(111–278)
SE mean		±45.2	±28.9	±46.1	±44.7	±43.0	±18.1
		B. Normal rats injected with lymph from normal rats					
6	284	807	714	642	531	433	204
Range		(479–1046)	(482–900)	(444–781)	(397–595)	(261–460)	(134–321)
SE mean		±90.6	±76.4	±53.8	±57.3	±47.6	±32.6

stand at least 1 hr, and then 9 ml water were added and the mixture was shaken and allowed to separate into layers. Appropriate volumes of the chloroform layers were evaporated and assayed for cholesterol (9) and for radioactivity by solution in scintillation cocktail and measurement in a Packard Model 3003 scintillation counter.

C. Rate of disappearance from plasma and accumulation in liver of intestinal lymph ^{3}H-tripalmitin in hypothalamus-injured and normal rats. Five normal rats, after previous starvation (16 hr), were given glyceryl tri (palmitin-9,10-^{3}H) (Nuclear Chicago Co., Des Plaines, IL) suspended in 1 ml of cottonseed oil. Their intestinal lymph was collected, pooled and stored at 4°. The pooled sample was found to contain 1.5×10^8 dpm/ml. Analysis of this pooled lymph by thin layer chromatography revealed that its triglyceride component contained 94% of the total radioactivity of the lymph. This indicated that the chylomicrons carrying almost all of this same triglyceride were almost completely responsible for the radioactivity exhibited by the pooled lymph. The thin layer chromatography was carried out by the method of Brown and Johnston (10) and developed with 90:20:2:3 (v/v) *n*-hexane: diethyl ether:acetic acid:methanol to assay the percentage of radioactivity in diglycerides, phospholipids and material at the origin. A second silica gel G chromatogram was developed with benzene to assay radioactivity in cholesterol esters and triglyceride. The separations were verified with authentic tripalmitin and cholesterol palmitate.

Forty-eight hours after this collection, 8

TABLE II. The Rate of Disappearance from Plasma of Intestinal Lymph ^{3}H-Cholesterol in Hypothalamus-Injured and Normal Rats.

No. rats	Av wt (g)	Av plasma cholesterol (mg/100 ml)	After injection of intestinal lymph containing ^{3}H-cholesterol-bearing chylomicrons (dpm × 10^2/0.1 ml plasma) (min): 2	4	8	12	16	32
			A. Hypothalamic rats					
12	313	143[a]	63.1	55.4[a]	49.5[a]	45.5[a]	41.6[a]	31.1[a]
Range		(86–240)	(54.7–70.8)	(49–66.1)	(41.4–58.9)	(38.9–55.3)	(27.5–53.8)	(19.7–47.9)
SE mean		±12.4	±1.9	±1.9	±1.6	±1.5	±2.1	±2.5
			B. Normal rats					
12	331	80	58.1	49.5	40.1	32.0	24.8	12.9
Range		(51–130)	(46.6–68.9)	(39.5–58.8)	(31.6–52.4)	(19.5–40.3)	(13.4–33.8)	(7.6–21.7)
SE mean		±7.3	±2.5	±2.1	±1.8	±2.0	±1.8	±1.2

[a] Significantly ($p < .001$) different than corresponding control value.

TABLE III. Accumulation in Liver of Radioactivity from Intravenously Injected Donor Lymph.[a]

Av liver wt (g)	Cholesterol (mg/liver)	dpm: Per mg cholesterol	dpm: Per total liver ($\times 10^5$)
I. Lymph from donor rats fed cholesterol-1α,2α,-^{3}H			
A. Hypothalamic rats			
10.8 (8.1–14.1) ±0.59	361.5 (187–623) ±35.3	938[b] (156–441) ±112.9	3.03[c] (0.7–3.8) ±0.25
B. Normal rats			
11.8 (6.8–18.2) ±0.91	344.2 (223–484) ±23.4	1315 (1010–1660) ± 67.7	4.45 (2.9–5.3) ±0.24

	Total liver lipid (dpm $\times 10^4$)	Percentage of total radioactivity dose in liver)
II. Lymph from donor rats fed glyceryl tri (palmitin-9,10,-^{3}H)		
A. Hypothalamic rats		
11.4 (8.8–15.4) ±0.7	5.2 (2.9–8.5) ±0.7	3.9 (2.3–6.2) ±0.2
B. Normal rats		
11.7 (10.8–12.7) ±0.4	4.7 (2.3–6.4) ±0.5	3.6 (1.9–4.9) ±0.2

[a] All animals were killed 32 min after injection.

[b] Significantly different from control value: $p < .05$.

[c] $p < .001$.

rats which had been subjected to the hypothalamic injury 1 wk previously and 9 normal rats were injected with lymph, bled, *etc.*, exactly as described in Method A except that each rat received 0.1 ml of the pooled lymph sample containing tripalmitin-^{3}H. The liver was perfused free from blood with normal saline and a sample was dissolved in methanolic NaOH and assayed for cholesterol and radioactivity in the same manner as described in Method B.

D. The heparin-induced plasma lipoprotein-lipase activity of hypothalamus-injured and normal rats. Sixteen rats with hypothalamic lesions (2 wk following operation) and an equal number of normal rats, after 16 hr of starvation, were injected intravenously with 2 units of heparin. A blood sample was obtained 3 min later. Lipoprotein lipase was assayed by the method of Schotz *et al.* (11). Lymph obtained from rats fed glyceryl tri (palmitin-9,10-^{3}H) was used as substrate for the enzyme by incorporating it into the following mixture: 6 ml of normal rat serum, 2 ml of 1% bovine albumin (adjusted to pH 8.0) and 15 ml of 0.2 *M* Tris HCl buffer (pH 8.0) was mixed with 1 ml of lymph containing 1.5×10^8 dpm/ml.

For each assay 0.2 ml of postheparin plasma from a test rat was added to 0.8 ml of the above substrate mixture and incubated for 20 min at 37°. The reaction was stopped by addition of isopropanol–H_2SO_4 and the released fatty acids were extracted and volume was corrected as directed by Schotz *et al.* (11); and radioactivity was counted in Aquasol (New England Nuclear Co., Boston, MA). Counts were corrected by subtraction of the value of the unincubated blank. Lipoprotein-lipase activity is proportional to the amount of fatty acid released.

II. Results. A. The disappearance from plasma of normal rats of labeled cholesterol administered as intestinal lymph obtained from hypothalamus-injured and normal rats. As Table I illustrates, normal rats cleared their blood of the labeled cholesterol contained in intestinal lymph obtained from rats with hypothalamic lesions as rapidly as they cleared the labeled cholesterol contained in the lymph of normal animals. The intravascular half-time for lymph from rats with lesions was 13.2 min, that for normal lymph, 14.6 min.

B. The disappearance from plasma and accumulation in liver of labeled cholesterol from normal intestinal lymph in the hypothalamus-injured and normal rat. The rate of disappearance of intravenously injected labeled cholesterol contained in intestinal lymph was observed (see Table II) to be retarded significantly in the hypothalamic rat. The intravascular half-time in normal rats was 11.2 min, in the hypothalamic rats it was 31 min. This retardation occurred throughout the postinjection period.

TABLE IV. The Rate of Disappearance from Plasma of Intestinal Lymph ^{3}H-Tripalmitin in Hypothalamus-Injured and Normal Rats.

No. rats	Av wt (g)	Av plasma cholesterol (mg/100 ml)	After injection of intestinal lymph containing ^{3}H-tripalmitin chylomicrons (dpm × 10^3/0.1 ml of plasma) (min): 2	4	8	12	16	32
			A. Hypothalamic rats					
8	309	139[a]	92.0	50.0	25.2	15.8	12.8	9.0[b]
Range		(96–184)	(69–119)	(28.2–81.6)	(12.4–45.6)	(10.6–26.2)	(8.8–19.0)	(7.0–9.8)
SE mean		±10.9	±6.8	±7.0	±5.0	±2.4	±1.4	±0.6
			B. Normal rats					
9	316	90	93.8	50.0	26.8	13.0	9.8	7.0
Range		(66–105)	(70.2–131.0)	(26.2–75.8)	(14.2–38.0)	(9.0–24.2)	(7.8–15.2)	(6.0–9.8)
SE mean		± 4.5	±8.6	±8.8	±3.8	±2.4	±1.2	±0.6

[a] Significantly more than corresponding control value: $p < .001$; [b] $p < .05$.

As might be expected from the results of the plasma studies, the amount of labeled cholesterol, 32 min following the injection of the lymph, was significantly less in the livers of the lesioned rats than in those of the normal rats (see Table III).

C. The disappearance from plasma and accumulation in liver of labeled triglyceride from normal intestinal lymph in the hypothalamus-injured and normal rats. As Table IV demonstrates, the rate of disappearance from the plasma of the labeled triglyceride contained in intestinal lymph was not significantly different in the rats with hypothalamic lesions from that observed in the normal rats; the intravascular half-times were 4.0 and 4.1 min respectively. The findings of the liver studies (see Table III) also suggest that the fat-handling capacities of the rats bearing hypothalamic lesions were not grossly impaired because the hepatic accumulation of labeled fatty acids in the total liver lipid was essentially the same in both types of rats. In both type of rats the total amount of labeled lipid in the liver 32 min after its administration was less than 5% of the total dose given.

D. The heparin-induced lipoprotein-lipase activity of the hypothalamus-injured and normal rats. The postheparin lipoprotein-lipase activity (resulting in release of labeled free fatty acids from tripalmitin in lymph) of the plasma obtained from rats with hypothalamic lesions appeared significantly greater (see Table V) than that of the plasma taken from normal rats.

III. Discussion. The present studies indicate that the liver of the rat bearing lesions in the hypothalamus, previously found (5) to be defective in removing endogenous lipoprotein cholesterol from its plasma, also appears to be faulty in removing chylomicronous cholesterol. Moreover, this relative inefficiency of the liver of the rat with lesions to remove chylomicronous cholesterol from plasma was due to some intrinsic hepatic defect and not to some physicochemical change in their chylomicrons.

On the other hand, the triglyceride component of the chylomicrons of our rats with lesions appeared to be removed from their plasma as rapidly as it was from that of the

TABLE V. Lipoprotein-Lipase Activity in Hypothalamus-Injured and Normal Rats.

	Av wt (g)	Av plasma cholesterol (mg/100 ml)	FFA release (dpm × 10^5)
Hypothalamic rats (16)	312	124[a]	6.8[a]
Range		(88–184)	(3.8–8.9)
SE mean		±8.8	±0.4
Normal rats (16)	328	75	4.5
Range		(43–101)	(2.1–6.4)
SE mean		±4.7	±0.3

[a] Significantly ($p < .05$) more than corresponding control value.

control rats. This normal rate of fat removal could be due to the fact that triglyceride of the chylomicron, unlike its cholesterol component, is removed by extrahepatic tissues (12–14).

It is of interest to note that our hypercholesterolemic rats appeared to generate more lipoprotein lipase in response to heparin than our normal rats. Therefore, the hypercholesterolemia of these animals probably is not related to any defect in their circulating lipolytic enzyme content.

Is the retardation observed earlier (5) in the rate of hepatic removal of endogenous lipoprotein cholesterol and the lag now observed in the liver's removal of exogenous or chylomicronous cholesterol from plasma principally responsible for the hypercholesterolemia which develops so rapidly in our animals following the specific hypothalamic injury? In view of the fact that in our earlier studies (5) we were not able to note any change in the respective rates of hepatic and intestinal synthesis of cholesterol or in the rate of intestinal absorption of cholesterol in these rats with hypothalamic lesions, we suspect that the slower clearance of cholesterol from plasma of this type of rat may be important in the genesis and maintenance of the chronic hypercholesterolemia observed. We speculate that the hypothalamic lesion may affect the circadian rhythm in the activity of hepatic cholesterol-7α hydroxylase (15) in such fashion as to slow the catabolism of cholesterol. We plan to investigate this possibility in the near future.

Summary. Intestinal lymph was collected from both normal and hypothalamus-injured rats given ^{3}H-cholesterol or ^{3}H-tripalmitin. It was found that chylomicronous cholesterol obtained from the hypothalamus-injured rat was removed from the blood and accumulated in the liver of the normal rat at the same rate as chylomicronous cholesterol collected from normal rats. However, the liver of the hypothalamus-lesioned rat removed normal chylomicronous cholesterol from plasma at a much slower rate than the liver of the normal rat. Chylomicronous triglyceride, however, was removed from plasma at a normal rate by the lesioned rat.

The heparin-induced lipoprotein-lipase activity of the plasma of hypothalamus-injured rats was significantly greater than that of the plasma of normal rats.

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