A Possible Mechanism for Accelerated Atherogenesis in Male Versus Female Rats

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Dietary fat and cholesterol enter the circulation as chylomicrons. They are removed from the circulation by attachment to lipoprotein lipase located on the endothelial surfaces. As the result of lipoprotein lipase action, chylomicrons are partially hydrolyzed and then reenter the circulation as remnants, which are rapidly cleared by the liver. We investigated the fate of 3H-retinol- and 14C-cholesterol-labeled chylomicrons injected into male and female rats. The disappearance curves of chylomicrons from the circulation were not significantly different in males and females, which suggests that translocation from plasma to endothelium is similar for both sexes. However, in male rats, the "dwell time" of chylomicrons on the endothelium was significantly prolonged. At 10 and 20 minutes after chylomicron injection, more label was found in the livers of female than male rats. The opposite was true for hearts. Male hearts contained significantly more endothelium-bound chylomicrons when compared with female hearts. This increase in dwell time may allow greater cholesterol deposition in the endothelium of male rats. The more rapid processing of chylomicrons was associated with a 300% greater postheparin lipoprotein lipase in female rats, which suggests a greater enzyme density at chylomicron attachment points on endothelium.

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The death rate from the complications of atherosclerosis accounts for 50% of the overall mortality in most Western countries. It has also been recognized for many years that women live longer than men. In the Framingham Study, Kannel et al. reported that, under 60 years of age, the probability of developing a cardiovascular event is 27.5% for men, but only 10.1% for women. The relative immunity of women to myocardial infarction and sudden death is graphically illustrated by a 20-year lag in the incidence of these complications for women when compared with men. Pathological evidence also supports the marked difference between men and women. The more rapid appearance of cardiovascular complications after menopause suggests a hormonal relationship to the delayed progression of coronary artery disease in women. In the Framingham report of 1976, it was noted that natural or surgical menopause is accompanied by an increase in the incidence of cardiovascular complications, which could not be explained by known risk factors. However, it was noted that there was a small rise in serum cholesterol levels caused by a rise in the lipoprotein fractions, very low density (VLDL) and low density (LDL). This was accompanied by an increase in the ratio of LDL to high density lipoproteins (HDL). That lipoproteins and their altered metabolism are intimately associated with the development of atherosclerosis is firmly established by a voluminous literature. This is particularly evident in inherited forms of dyslipoproteinemia.

Although sex differences have been reported for the metabolism of free fatty acids (FFA), little attention has been devoted to sex differences in the metabolism of chylomicrons (CM). In this study, we compared the metabolism of CM in male and female rats with emphasis on the "dwell time" of CM on endothelial surfaces.

Methods

Animals

Male and female Sprague-Dawley rats (175 to 225 g) were purchased from Bantin and Kingman (Fremont, CA) and were maintained on Purina #5012 rat Chow and water ad libitum. In all experiments comparing males and females, the rats were matched for weight. The rats were fasted overnight before the experiments, but drinking water was freely available. All animal studies were performed in accordance with institutional policies, and all procedures were approved by the Subcommittee on Animal Studies.

Chylomicron Preparation

Chyle was obtained from a thoracic duct fistula in male rats by a modification of the technique of Boltman et al. by using heparin-coated Renathane tubing for cannulation (outside diameter, 0.040 in., Braintree Scientific, Inc., Braintree, MA). Nambudal (50 mg/kg) was used as the anesthesia. The rats were intubated 2 hours before surgery with 1.5 ml of an emulsion of corn oil and milk (1:2, vol/vol), which was dispersed with a 1-cm probe of a Biosonik IV Sonifier (Bromwell, Rochester, NY). After the establishment of lymph
flow and stable animal condition, the rats were intubated with a 2-ml mixture of a corn oil/milk emulsion sonicated together with 0.05 ml of 4-14C-cholesterol, 0.10 ml of 11,12-3H-retinol, and 20 μg retinol carrier. Both radioactive compounds were purchased from Dupont NEN Research Products, Boston, Massachusetts and were specified as 99% pure. The rats were restrained, and chyle was collected in ice tubes for periods up to 18 hours. During this period, the rats were allowed access to water and observed for any discomfort. Each donor rat was injected subcutaneously with 10 ml of 0.15M NaCl to maintain hydration.

CM were isolated by layering 5 ml of chyle under 0.15 M NaCl in ultracentrifuge tubes; they were floated in an SW-40 rotor for 5.10 g each at 10°C in an L2-65B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The CM pellet was isolated with a tube slicer, and the chylomicrons were resuspended in 0.15 M NaCl. Aliquots were taken for triglyceride (TG) and cholesterol determination. The CM used in these studies had 35.7% of the label in free cholesterol and 64.3% of the label in cholesteryl esters as determined by silicic acid chromatography. The 3H-retinol-labeled CM were assumed to have essentially all of the label in retinyl esters as was found by Ross and Zilversmit.

Chylomicron Clearance

Disappearance curves of CM in blood were determined in anesthetized male and female rats restrained in plastic cones. 14C-cholesterol- and 3H-retinol-labeled CM (10 mg TG, 75 μg total cholesterol) were injected via a tail vein. Whole blood samples (0.01 ml) were taken from a tail vein by venous puncture into heparin-coated microhematocrit tubes. The tubes were dropped into 1 ml 0.15 M NaCl, and the lipids were extracted according to the method of Dole.

Samples were taken at various periods up to 15 minutes. For radioactivity determination, the heparinase (3 ml) of the Dole mixture was transferred to counting vials together with 10 ml Bio-Safe II counting cocktail (Research Products International Corp., Mount Prospect, IL).

Recovery of Chylomicron Label by Liver and Heart

Tissue distribution of radiolabels was determined 10, 20, and 30 minutes after the injection of 3H-retinol- and 14C-cholesterol-labeled CM in male and female rats. The animals were anesthetized with methoxyflurane approximately 2 minutes before the desired interval. Their livers were perfused via the portal vein with 20 ml 0.15 M NaCl to remove blood. The hearts were rapidly removed and perfused via the aorta with 10 ml of cold 0.15 M NaCl. Whole livers and hearts were weighed before isotope analysis. The average weight for the livers and hearts was 10 and 0.8 g, respectively. Duplicate 1-g portions of liver and whole hearts were homogenized in 1 ml 0.15 M NaCl in centrifuge tubes using a Polytron homogenizer (Brinkman, Westbury, NY), and 40 ml chloroform-methanol (2:1, vol/vol) was added. Carrier retinol (20 μg) was added immediately before homogenization. The extracts were then centrifuged, and 5 ml aliquots of the chloroform-methanol extracts were taken to dryness in counting vials. The lipids were resolubilized in 1 ml heptane and analyzed for 14C and 3H. The method gave >95% recovery for both 14C-cholesterol and 3H-retinol. All techniques used in the analysis of 3H-retinol were carried out in subdued light. To verify the absence of any contamination with blood in perfused hearts and livers, three male and three female rats were injected via tail veins with 1 μCi125I-labeled albumin (ICN Biochemical, Irvine, CA). After a 10-minute interval, the livers and hearts were extracted as described above, and 125I was determined in extracts and small blood samples. No significant amount of radioactivity was detected in the organ extracts.

Postheparin Lipoprotein Lipase Activity

Lipoprotein lipase (LPL) was determined in male and female rats anesthetized with methoxyflurane. A midline incision was made in the abdominal wall, and heparin (5 U/kg, Evans, Liverpool) was injected into the inferior vena cava. Exactly 5 minutes later, blood was taken from the abdominal aorta and was allowed to clot; serum was obtained. The LPL assay was performed according to the method of Whayne and Felts by using Intralipid as a substrate. Free fatty acids (FFA) were titrated at 0 time and after 60 minutes of incubation at 37°C. One LPL unit is defined as μmole FFA released per ml of serum per hour. When incubated in 1 M NaCl, no net increase in FFA was detected. Indicating that this method specifically determined LPL and not hepatic triacylglycerol lipase.

Analytic Procedures

Triglycerides were determined by the method of Fletcher, and total cholesterol was estimated as described elsewhere. Assays for 14C and 3H were performed in Bio-Safe II counting cocktail in a Beckman radioactivity spectrometer (Model LS-150) fitted with an external standard. The counts were corrected for quenching and crossover between channels with curves established from counting internal standards when monitoring the automatic external standard. 3H was determined in a Packard gamma counter (Packard, Downers Grove, IL). The T1/2 values for disappearance curves were calculated by linear regression analysis with the Lotus Program (Lotus Development Corp., Cambridge, MA). The final disappearance curves were obtained using SigmaPlot (Jandel Scientific, Sausalito, CA) on an IBM personal computer AT. Student's t-test was used to test the significance between means.

Results

The disappearance of CM from the circulation was measured in male and female rats. As seen in Figure 1, male and female rats have similar rates of removal for the 3H-retinol-labeled and 14C-cholesterol-labeled CM. The T1/2 for the cholesterol label is similar to that of retinol, which indicates that there is no significant exchange of the free cholesterol with VLDL or other lipoproteins. By 15 minutes, over 85% of both labels had been removed from the circulation. In addition, there was no rapid disappearance phase before the first time point since all lines extrapolated to the calculated value of the initial CM concentration, assuming that the plasma volume was 4% of the rat weight. When postheparin LPL activity was determined in male and female rats, a large difference was found
between the sexes. The results of this study are shown in Table 1. Heparin releases 300% more LPL in female than in male rats. These data indicate that, in female rats, heparin-releasable LPL does not significantly influence CM disappearance from the circulation. The rate of disappearance of CM from the circulation is probably determined by two factors: the number of binding sites containing LPL and the cardiac output.4 However, at the present, no evidence has been presented to show that these factors are different in male and female rats. The fact that 1 M NaCl in this assay completely inhibited the lipase activity indicates that this assay system determined only LPL and not rat hepatic lipase.

Recovery of \(^{3}H\)-retinol and \(^{14}C\)-cholesterol in heart and liver tissue was determined in rats at 10, 20, and 30 minutes after the injection of labeled CM. Little exchange of core components of CM or chylomicron remnant (RM) takes place with other lipoproteins in the rat, since this species has low lipid transfer protein activity.24 Thus, cholesterol ester and retinol are ideal tracers for the metabolism of CM as they are removed from plasma to endothelium, are converted to RM, are released from the endothelium into the circulation, and are ultimately taken up by the liver. As shown in Figure 2, the recovery of label in hearts showed a remarkable difference between the sexes. At 30 minutes, less than 0.1% of the injected label was recovered in hearts of both male and female rats. No significant differences could be detected at these low amounts of radioactivity. However, at 10 and 20 minutes, the male hearts contained almost double the amount of both labels when compared with female hearts. These results indicate that more CM were bound to endothelial surfaces in male rats than in female rats during the clearance of the injected CM. The opposite pattern was observed for the recovery of injected label in the livers. It can be seen in Figure 3 that at 10- and 20-minute intervals, female livers contained significantly more of the injected dose than did male livers. These results agree with the assumption that the hepatic uptake of CM is slower in male than in female rats due to an increase in time CM resides on the endothelial surfaces. This dwell time or "residence time" probably depends on the rate of the hydrolysis reaction. At 30 minutes, more than 90% of the injected label was found in the livers of rats of both sexes. These results demonstrate a quantitative difference in CM clearance between male and female rats. In female rats, the hydrolysis rate of TG of CM and the formation of RM was faster, producing a shorter dwell time of the CM on the blood vessel walls.

### Discussion

Lymph CM are the major transport proteins for fats and cholesterol absorbed from the gut.25 In normal rats, the metabolism of CM proceeds via a two-step pathway: hepatic and extrahepatic.24 In the first step, CM is partially hydrolyzed by the enzyme, LPL. LPL is located on the capillaries of extrahepatic tissues such as adipose tissue, muscle, and heart. While attached to the enzyme, TG become hydrolyzed to FFA and free glycerol, and a fraction of phospholipid may be hydrolyzed as well. Components may transfer to other lipoprotein species and to albumin as the particle size becomes diminished.26 During the interaction of CM with LPL, there is a dwell time or residence time of the CM particle on the endothelial surface, which has received little attention. At some critical point, the CM particle detaches from the endothelium, and the RM\(^{29}\) transports the circulation and transports to the liver the remaining TG, cholesterol esters, and fat-soluble vitamins (e.g., retinol) of dietary origin. As demonstrated by Redgrave25 and by Feils et al.,26 the removal of RM by the liver is very rapid.

The atherogenicity of CM, which carry all of the dietary cholesterol, has been addressed by Zilversmit.27 He has suggested that atherogenesis is a postprandial phenomenon. He proposed that the interaction of the CM particle with the endothelial LPL is a necessary requirement, since Type I hyperlipemic patients are not prone to the development of premature atherosclerosis. He has also postu-
uated that, at physiological pH, a portion of the released FFA at hydrolysis sites exists as soaps, which could damage the endothelial wall and solubilize the cholesterol. This physical dispersion of cholesterol into micelles could effect the penetration of cholesterol into the endothelium and into the underlying structures. If this process is operative, the dwell time of the particle on the endothelial surface becomes a major determinant of the amount of cholesterol that leaves the attached CM particle and becomes attached to the endothelium.

In this study, we infected female hamster CM into male and female rats and compared the CM metabolism by following the label from circulation to liver uptake. The appearance of radioactivity in the liver is a measure of CM which have: 1) been removed from the circulation by attachment to LPL, 2) been partially hydrolyzed by LPL, 3) reentered the circulation as RMI particles, and 4) been removed by the liver. The label recovered from the heart is an indication of the CM bound to the endothelium. Thus, isotope recovery in the liver and heart gives an insight into a dwell time of CM on endothelial surfaces containing LPL.

As seen in Figure 1, male and female rats have similar rates of removal for the 3H-retinol-labeled and the 14C-cholesterol-labeled CM. Our finding that the rates of disappearance of plasma CM are essentially the same for male and female rats, and the fact that female rats have three times more LPL activity (Table 1), suggests that there may be three times as many LPL molecules per CM binding site in female rats. Thus, heparin-releasable LPL does not appear to significantly influence CM disappearance from the circulation over this range of values. There have been isolated reports in the literature that female rats have more LPL than male rats. Nikkilä et al.28 noted that adipose tissue from fasted women had more LPL than did adipose tissue from men, but this was not true for skeletal muscle. Cryer and Jones29 reported that adipose tissue in female hamsters and mice have higher LPL activity than males.

To the best of our knowledge, there have been no prior systematic studies comparing CM metabolism in males and females in any species. Our data from the recoveries of 3H-retinol and 14C-cholesterol in hearts and livers of male and female rats at 10 and 20 minutes after the CM injection show significant differences between the sexes. More of the CM core constituents remained attached to the endothelium of male hearts. The opposite was true for livers. More label was recovered in female livers when compared with those of males. These findings show a fundamental difference in the initial metabolism of CM on the endothelial surfaces between male and female rats. The differences in the dwell time between the sexes may be related to the way
in which LPL is situated on the endothelium. It may be significant that heparan sulfate chains, to which LPL is attached, exist as clusters on endothelial surfaces, since they are anchored through a covalent linkage to a protein core in the membrane. Current evidence suggests that each proteoglycan molecule may have up to four heparan sulfate chains. Thus, in female rats, the enzyme density (LPL) may be greater for each attachment point for CM. An in vitro model supports this hypothesis. Clark and Quarrport have tested columns of heparin-Sepharose containing attached LPL. More rapid hydrolysis of TG was observed with high and low zones of LPL density than when LPL was evenly distributed throughout the column.

In conclusion, the results reported here demonstrate a quantitative difference in the metabolism of CM between male and female rats and show a marked difference in heparin-releasable LPL. In female rats, the hydrolysis rate for TG of CM and the formation of RM was more rapid, producing a shorter dwell time of CM on blood vessel walls. The significance of these results at this time is not completely clear. However, the longer dwell time in male rats could suggest a basic mechanism in which longer contact of CM with endothelium could lead to greater deposition of cholesterol on blood vessel surfaces in male than female rats. Over time, this mechanism could be the genesis of atherosclerosis. Since female rats appear to have higher plasma HDL, superimposed on this mechanism of cholesterol deposition may be the cholesterol scavenger function of plasma HDL, which would transport a portion of the cholesterol to the liver.

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