Biphasic Effects of Low-Molecular-Weight and Conventional Heparins on Chylomicron Clearance in Rats

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Chylomicrons labeled in vivo with [14C]triglycerides and [3H]retinyl esters were injected in rats at a series of times after administration of conventional unfractionated heparin (UFH), low-molecular-weight heparin (LMWH), or saline. In saline controls the clearance of both chylomicron triglycerides and retinyl esters seemed to follow exponential courses, with half-lives of about 5 and 10 minutes, respectively. Five minutes after administration of LMWH or UFH, the triglyceride clearance rates were dramatically increased and were associated with an increased appearance of the radiolabel in circulating free fatty acids (FFAs). The clearance of [3H]retinol radioactivity, ie, chylomicron particles, was also enhanced 5 minutes after heparin injection. From 75% to 90% disappeared from the circulation within the first 5 minutes. Their continued disappearance was much slower, with a slope similar to that of the saline-treated rats. Hence, it was as if a new, rapid exponent had been added to the disappearance curve that accounted for most of the particle clearance. Injection of chylomicrons 1 hour after the heparins resulted in substantially slower clearance compared with saline-treated controls of both triglyceride and retinol radioactivity in rats given a high dose of LMWH or a low dose of either heparin. Appearance of label in plasma FFAs was also decreased, suggesting that impaired lipolysis was responsible, at least in part, for the impeded chylomicron clearance. Four and 24 hours after heparin injection all studied parameters of chylomicron clearance had returned to normal. These data showed that the response of lipoprotein catabolism to heparin is biphasic: immediately after heparin injection, lipolysis and particle removal are greatly accelerated; later, both aspects of chylomicron catabolism are temporarily, but markedly, decreased. (Arterioscler Thromb. 1993;13:1397-1403.)

KEY WORDS • triglycerides • retinol esters • lipoprotein lipase • lipolysis • particle clearance • accelerated clearance • retarded clearance

Injection of heparin is known to acutely accelerate lipolysis of triglyceride-rich lipoproteins in plasma. This is because heparin releases two lipases from endothelial binding sites into the circulating blood: lipoprotein lipase (LPL), which hydrolyzes mainly triglyceride-rich lipoproteins, chylomicrons, and very-low-density lipoproteins, and hepatic lipase (HL), which acts on remnants formed by the action of LPL and on high-density lipoproteins. In the absence of heparin, chylomicrons and very-low-density lipoproteins bind transiently to endothelium binding–lipolysis sites, where LPL hydrolyzes some of the particle's triglycerides, and the fatty acids are taken up and used by adjacent tissue cells. Several rounds of binding and lipolysis transform the particle to a triglyceride-depleted remnant that is recognized by receptors, mainly on liver cells, and is then internalized and degraded. The degree of lipolysis of the particles has been shown to modulate their clearance from the plasma; chylomicron remnants are cleared more rapidly than nascent chylomicrons from the circulation. Recently, it has been suggested that LPL remaining on the remnant particles may act as a receptor ligand and enhance particle binding. Hence, LPL may have roles in both steps of chylomicron catabolism.

In the accompanying article we present evidence that release of LPL into the blood by heparin leads to accelerated uptake and catabolism of the enzyme in the liver and a temporary depletion of functional LPL. This raised the question of what effect LPL depletion might have on the metabolism of triglyceride-rich lipoproteins. Liu et al report that 1 hour after injection of a low-molecular-weight heparin (LMWH) in rats, clearance of an injected fat emulsion was retarded. In the present study we used chylomicrons with retinyl esters as core label and oleic acid as label for the triglyceride moiety. This enabled us to follow both lipolysis of triglycerides and particle clearance after injection of the chylomicrons to rats over a 24-hour period after administration of LMWH or unfractionated heparin (UFH) at two clinically relevant doses. The results confirmed that both lipolysis and particle clearance were retarded during the period of LPL depletion. An unexpected finding was that particle clearance was dramatically enhanced during the early phase after heparin injection.
Materials

The heparins were prepared and the study was set up as described in the accompanying article. In the present study, however, the experimenter did not know which rat received which preparation. The code was broken only after all experiments and analyses had been completed. Radiochemicals ([1-14C]oleic acid and [11,12(n)-3H]retinol) were from Amersham Sweden, Solna, Sweden.

Chylomicron Preparation

Rats weighing 250 to 300 g were maintained on standard chow and tap water. Under anesthesia, the thoracic duct was cannulated with PE-50 tubing and a catheter was inserted in the stomach. After surgery, the rats were given, through the stomach catheter, 2 mL Intralipid 20% (Kabi-Pharmacia Hospital Care, Stockholm, Sweden) into which trace amounts of [14C]oleic acid and [3H]retinol had been incorporated by brief sonication. The lymph was collected at room temperature for 5 hours in sterile tubes with EDTA (0.01%) and gentamicin (0.01%), layered under 0.9% NaCl containing EDTA (pH 7.4), and centrifuged for 30 minutes at 30,000 rpm in a Beckman SW-50 rotor at 15°C. The chylomicrons were resuspended and then washed under the same conditions as for the isolation. More than 93% of 14C and 3H were found in triglycerides and retinol esters, respectively, after thin-layer chromatography.

Animal Procedures

The handling of rats and the injection of heparins are described in the accompanying article. Chylomicrons (10 mg triglyceride/kg body wt) were injected in an exposed jugular vein 5 minutes, 1 hour, 4 hours, or 24 hours after the heparins or saline (controls). Ten minutes before injection of the chylomicrons, the animals had been anesthetized. Blood samples (200 μL) were collected in tubes containing 2 mL isopropanol/heptane/1 M H2SO4 (40:10:1, vol/vol/vol) at the indicated times. Sample volumes were determined by weighing the tubes before and after the blood was added. Calculation of the percentage of the injected dose of chylomicrons remaining in the plasma was based on the estimate that blood volume constituted 5.5% of the body weight. After adding 1.2 mL heptane and 1.2 mL water, 1 mL of the upper phase (total lipids) was added to the same volume of alkaline ethanol. Aliquots were counted for the determination of the radioactivity associated with triglycerides and retinol esters (upper phase) or with free fatty acids (alcoholic phase). All animal procedures were approved by the local Animal Ethics Committee.

Statistics

Statistical methods are described in the accompanying article.

Results

In the present experiments chylomicrons labeled in vivo with [14C]triglyceride and [3H]retinol were injected in fasted rats at a series of times after administration of a solution containing either UFH, LMWH, or saline. To avoid any bias, the nature of the solution was unknown to the experimenter, and the code was broken only after all analyses and calculations were finished. We first consider the results obtained in the saline-treated rats as a basis for considering the effects of the heparin preparations.

Clearance of chylomicron triglycerides seemed to follow an exponential course, with a half-life of about 5 minutes (Fig 1, top). Label appeared in the plasma free fatty acids (FFAs) (Fig 1, top), demonstrating that some of the chylomicron fatty acids recirculated in plasma after hydrolysis. Triglyceride clearance appeared to be somewhat faster at 4 hours and somewhat slower at 24 hours compared with at 5 minutes or 1 hour after the saline injection (3% and 10%, respectively, of the injected dose remained in plasma at 20 minutes compared with 5% to 6%), but these differences did not reach statistical significance.

More than 93% of the retinol label in the injected chylomicrons was in esters. Since these are not hydrolyzed by LPL or HL, their clearance signifies removal of the particles from circulation. The disappearance seemed to follow an exponential course, but the fractional catabolic rate was slower than for triglycerides (Fig 1, left), corresponding to a half-life of about 10 minutes. There were slight differences between rats at different times after the saline injection. These differences were similar to the triglyceride clearance but were not statistically significant. Extrapolation of the disappearance curves gave intercepts on the ordinate corresponding to 60% to 80% of the injected dose. The same values were obtained on extrapolation of the curves for triglyceride disappearance. For these calculations, the blood volume was directly measured in corresponding rats. Hence, it is unlikely that the low intercepts were due to underestimation of the blood volume. The intercepts therefore indicate that some of the chylomicrons disappeared almost instantaneously from the circulating blood, which would be the case if the distribution volume were larger than the plasma volume or if a fraction of the chylomicrons distributed to binding sites directly available from the blood.

Five minutes after administration of LMWH or UFH the triglyceride clearance rates were dramatically increased; more than 90% of the injected dose disappeared within the first 3 minutes compared with about 50% in the saline-treated controls (Fig 2, top left). The rapid disappearance of triglycerides was accompanied by a peak of radioactivity in circulating FFAs, which at 2 minutes contained 7% to 9% of the injected label (top center). The corresponding figure in rats not given heparin was below 2%. The high FFA radioactivity presumably reflects rapid triglyceride hydrolysis by heparin-released lipases in the circulation. At 5 minutes, FFA radioactivity had decreased to values similar to those in the saline-treated controls. At this time the substrate for the circulating lipases had been depleted; only about 3% of the labeled triglycerides remained in plasma. Interestingly, the triglyceride clearance rates
and the appearance of label in FFAs were similar for LMWH and UFH and did not differ between the high and low doses of heparin (Fig 2, top).

The clearance of [\textsuperscript{3}H]retinol radioactivity, ie, chylomicron particles, was also enhanced 5 minutes after heparin injection (Fig 2, top right). From 75% to 90% of the retinol radioactivity disappeared from the circulation within the first 5 minutes compared with about 50% in the controls. The continued disappearance was much slower, with a slope similar to that in the saline-treated rats. Hence, it was as if a new rapid exponent had been added to the disappearance curve that accounted for most of the particle clearance. Using the 1- and 2-minute time points to extrapolate the rapid exponent of the disappearance curves to the ordinate gave intercepts around 100% in the heparin-treated rats. Extrapolation of the 10- to 20-minute part of the curves gave intercepts for the slow component of 20% or below. This contrasts to the observations in saline-treated rats, in which only the slower exponent could be resolved and extrapolated to 60% to 80% of the injected radioactivity.

In the heparin-treated rats, the initial disappearance tended to be faster after the high heparin doses and faster after LMWH compared with UFH (not significant). This contrasts to the observations for triglyceride clearance, which occurred at indistinguishable rates after the high or the low dose of either LMWH or UFH.

From the data on triglyceride and retinol ester radioactivity, the degree of lipolysis of the particles remaining in plasma (lipolysis index) was calculated for each time (Fig 3, top left). This plot illustrates that lipolysis was greatly accelerated in the heparin-treated rats compared with the saline-treated controls. The lipolysis indexes tended to be somewhat lower for the rats given UFH at the low dose, but were similar for the other three groups of heparin-treated rats. These data show that the particles remaining in plasma after 5 minutes (ie, when the slower exponent dominated the disappearance curves) had lost 70% or more of their triglycerides, indicating that the particles in plasma had been rapidly lipolyzed. The remaining particles had a composition typical for so-called chylomicron remnants. Fig 3 (top right) shows the percent of injected particles remaining in plasma (from the retinol radioactivity) as a function of the lipolysis index. The relation between particle clearance and lipolysis index differed between the groups, but for all groups half of the particles had disappeared from plasma while the index was still above 0.7.

Injection of chylomicrons 1 hour after the heparins resulted in similar triglyceride clearance in rats given the high dose of UFH as in control rats, but slower clearance in rats given the high dose of LMWH or the low dose of either heparin (Fig 2, center). The differences were substantial: 20 minutes after injection of the chylomicrons, 4% to 5% of [\textsuperscript{14}C]triglycerides and [\textsuperscript{3}H]retinol esters. Blood samples were drawn at the indicated times and radioactivity in triglycerides (TG; top, solid lines), FFAs (top, dashed lines), and retinol esters (RE; bottom) was determined. Data are mean±SEM of six rats except for 5 minutes where n=3. △ indicates 5 minutes, ●, 1 hour, ◀, 4 hours, and ▼, 24 hours.

**FIG 1.** Line graphs showing clearance of chylomicrons and appearance of label in plasma free fatty acids (FFA) in saline-treated control rats. First, saline was injected in a tail vein of unanesthetized rats. Ten minutes before the designated times the rats were anesthetized and injected with chylomicrons (10 mg triglyceride/kg body wt) containing [\textsuperscript{14}C]triglycerides and [\textsuperscript{3}H]retinol esters. Blood samples were drawn at the indicated times and radioactivity in triglycerides (TG; top, solid lines), FFAs (top, dashed lines), and retinol esters (RE; bottom) was determined. Data are mean±SEM of six rats except for 5 minutes where n=3. △ indicates 5 minutes, ▲, 1 hour, ●, 4 hours, and ◀, 24 hours.
clearance, i.e., similar rates for rats given the high dose of UFH as for controls, but slower disappearance rates for rats given the low dose of either heparin or the high dose of LMWH (Fig 2, right center). The differences between doses were substantial, e.g., after 20 minutes 41% and 37% of the particles (i.e., retinol label) remained in plasma of rats given the low dose of LMWH and UFH, respectively, but only 25% and 19% remained in plasma of rats given the high dose of the heparins. The lipolysis index for the particles remaining in plasma were higher at most times for all groups of rats that had received heparin compared with the controls, indicating that lipolysis was slower (Fig 3, bottom left). The plot of percent of injected particles remaining in plasma versus lipolysis index suggests that particles were removed from the plasma at similar rates of lipolysis for all groups and shows that about half of the particles disappeared from the plasma while the lipolysis index remained above 0.7 (Fig 3, bottom right). The relation between particle clearance and lipolysis index was similar for all groups.

Four hours (Fig 2, bottom) and 24 hours (data not shown) after heparin injection all parameters studied were similar to those in controls. Previous experiments had shown that at this time the injected heparin had disappeared from the plasma, and LPL and HL activities had returned to control levels.

Discussion

This study showed that the response of lipoprotein catabolism to heparin is biphasic. It is well-known that immediately after heparin injection lipolysis of triglyceride-rich lipoproteins is enhanced; this is the "clearing reaction." Our present data showed that particle removal was also greatly accelerated. Later, in our experiments at 1 hour, both lipolysis and particle removal were slowed down. This was a temporary reaction, as by 4 hours all parameters had returned to control values.

The proposed pathway for chylomicron catabolism is a two-step process in which the particles are first partly delipidated by LPL-mediated lipolysis, and the remnant particles are then recognized by specific receptors. Our results in the saline-treated controls agreed with previous studies. Both triglycerides and retinol esters disappeared at essentially exponential rates, and the rate was higher for triglycerides than for retinol esters.

Fig 2. Line graphs showing clearance of chylomicrons 5 minutes (top), 1 hour (center), and 4 hours (bottom) after injection of the heparins. Same protocol as in Fig 1 except that anesthetized rats received 50 U (△) or 250 U (▲) low-molecular-weight heparin (LMWH; dashed lines) per kg body wt, 50 U (●) or 250 U (●) unfractionated heparin (UFH; solid lines) per kg body wt, or saline (○; dotted line). Panels show radioactivity in triglycerides (TG; left), free fatty acids (FFA; center), and retinol ester (RE; right). Data are mean±SEM of six rats.
tion of the particles disappeared almost instantaneously from the circulation, a finding that, to our knowledge, has not been considered previously. Our supporting data are that the clearance curves for both triglycerides and retinol esters extrapolated to only 60% to 80% of the injected dose. A likely interpretation is that a fraction of the particles bound to endothelial binding-lipolysis sites. That this must happen is inherent in the available data. The lipase is not present in the blood, but rather is bound to the endothelium. The delipidation, a very rapid process, requires that the particles spend appreciable time in contact with the lipase. For instance, it can be calculated from the known properties of the enzyme\(^2\) that hydrolysis of 50% of the triglycerides of a chylomicron with 1 million triglyceride molecules in 5 minutes (the observed half-life) would require the constant action of 3 to 4 LPL molecules (dimers). Sufficiently rapid hydrolysis could also be accomplished if the particle spent a quarter of the time at the endothelium and was acted on by 12 to 16 LPL molecules. It could not, however, be accomplished if the particle spends virtually all its time circulating in the blood. Hence, the observed margination of 20% to 40% of the particles is, in fact, to be expected.

The immediate response to heparin was enhanced lipolysis and enhanced particle removal. A major factor in the accelerated lipolysis must be that after release into the circulating blood, LPL has immediate access to all lipoprotein particles. In contrast, while bound at the endothelium the enzyme has access only to those particles that have adsorbed to endothelial binding-lipolysis sites. For this argument to be valid there must be as many or more LPL molecules as chylomicron particles. We can estimate this relation as follows. Plasma LPL activity 5 minutes after injection of the high dose of either heparin was 300 to 350 mU/mL. Assuming that the specific activity of rat LPL is 400 U/mg (the value for bovine LPL under the present assay conditions), plasma LPL concentration would be about 7 nmol/L (assuming the enzyme is dimeric). The initial concentration of injected chylomicron triglycerides was about 350 μmol/L. Hence, the molar ratio of triglycerides to LPL was about 50 000. If each chylomicron contained 1 million triglyceride molecules, then there were 20 LPL molecules per chylomicron. The low dose of the heparins gave lower plasma LPL activity, but even with LMWH there would be about 7 LPL molecules per chylomicron. This relation supports the hypothesis that it is easier to assemble most of the LPL molecules on chylomicron particles in the circulating blood than to assemble the LPL molecules on a fraction of the chylomicron particles while these are temporarily attached at endothelial binding-lipolysis sites.
Particle clearance was also greatly enhanced 5 minutes after heparin injection. The suggested mechanisms for chylomicron remnant uptake involve binding to cell surface heparan sulfate, followed by transfer to the low-density lipoprotein receptor or to the low-density lipoprotein receptor-related protein. Heparin successfully competes for all these interactions. Hence, they are unlikely candidates for the heparin-stimulated particle removal. No other obvious candidate is presently defined. LPL is known to be removed by the liver even in the presence of the heparin concentrations relevant here. In postheparin plasma LPL travels on lipoproteins. Hence, one possible candidate for the heparin-induced chylomicron removal would be the sites taking up LPL.

The heparin-enhanced removal was evident immediately after injection of the chylomicrons; 75% or more of the particles disappeared within the first 5 minutes. The particles remaining after this time had lost more than 70% of their triglycerides, and hence had the characteristics of so-called chylomicron remnants. They were then removed at a fractional catabolic rate similar to that observed for particle removal in the saline-treated controls. Our data therefore indicated that removal of remnant particles was not greatly influenced by heparin. This is in accord with a study by Berr et al., who showed that heparin does not affect the removal of chylomicron remnants in humans.

One hour after injection of heparin the rapid exponent for chylomicron catabolism seen at 5 minutes was no longer apparent, i.e., the shape of the disappearance curves was again similar to those in the saline-treated controls. In rats given the low dose of either heparin both lipolysis and particle removal were slower than in controls. The conclusion that lipolysis was slower is based on lower levels of radioactivity in plasma FFAs and on a slower decrease of the lipolysis index. That particle removal was slower is evident from the slow disappearance of retinol radioactivity. The differences were substantial. Ten minutes after injection about 15% of triglycerides and 35% of retinol radioactivity remained in circulation in the saline-treated rats. In rats given the low dose of either heparin the corresponding figures were 40% and 60%. Expressed as half-lives, the saline-treated rats cleared triglycerides with a half-life of 5 minutes and retinol esters with a half-life of about 10 minutes. In rats given the low dose of either heparin the values had increased to 11 minutes and 18 minutes. The slower disappearance of triglycerides is in accord with a previous study by Liu et al.. The most apparent mechanism is that a partial depletion of LPL limits lipolysis and that the slower particle removal is secondary to this. Compared with rats given the low dose of the heparins, clearance of both triglycerides and retinol esters was more rapid in rats given the higher doses of the heparins. In these rats LPL activity in plasma remained elevated at 1 hour. Hence, they represented an intermediate situation compared with the 5-minute groups, in which both heparin and LPL values were high in the blood, or the 1-hour groups given the low doses of heparin, in which heparin had disappeared virtually completely, and LPL values had returned to control levels.

A previous study indicates that LMWH might lead to a period of depletion of endothelial LPL and decreased capacity for catabolism of triglyceride-rich lipoproteins. The present study was designed to test if and for how long this happens with clinically relevant doses of heparin. The results showed that the period of decreased catabolism is not unique to LMWH. A similar decrease in chylomicron clearance was seen after UFH administration. Hence, the difference between the heparins is in the time-frame and perhaps in the degree of the decrease. In a study by Liu et al., a narrow-sized fraction of heparin fragments was used, corresponding mainly to decasaccharides. In contrast, the present study used a commercial LMWH preparation. This preparation is rather polydisperse, which has pharmacokinetic advantages. There is an overlap of around 25% between the molecule size range in the two heparin preparations. It is likely that this overlap blunted the differences between the two. Hence, caution should be exercised in extrapolating the present results to other LMWH preparations.

We have recently found that both types of heparin cause a transient reduction of available LPL activity. This depletion seems to correlate with the retarded chylomicron clearance observed here. The depletion and the retardation were both seen 1 hour after heparin injection. At 1 hour no differences were observed between the two heparins given at the low dose, but at the high dose chylomicron clearance tended to be faster after UFH than after LMWH. This correlates to a higher LPL activity remaining in the plasma of the rats that received UFH at 5 minutes there were no differences in triglyceride clearance between the doses or types of heparin, but particle clearance was somewhat more enhanced after LMWH. This does not correlate with plasma LPL levels, which were higher with UFH compared with LMWH, and raises the question of whether heparin influences chylomicron catabolism by mechanisms other than through plasma LPL activity. Interaction with cell surface heparan sulfate is probably important for guiding the lipoproteins to dedicated sites for interaction and catabolism, not only “binding-lipolysis” sites but also specific receptors, i.e., “binding-internalization” sites. Heparin would certainly be expected to interfere with lipoprotein–heparan sulfate interactions. Moreover, the receptor binding sites on apolipoproteins B and E also bind heparin, and in vitro binding of lipoproteins to the low-density lipoprotein receptor as well as to low-density lipoprotein receptor-related protein is effectively dominated by heparin.

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