Clearance from plasma of triacylglycerol and cholesteryl ester after intravenous injection of chylomicron-like lipid emulsions in rats and man

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Chylomicrons transport fat and cholesterol via lymphatic vessels from the intestine into the bloodstream. The understanding of the metabolism of chylomicrons in man has been slowed by the difficulty of obtaining lymph chylomicrons for experimental studies. Acceptable methods for the study of chylomicron clearance in man are required, because the metabolism of chylomicrons may be abnormal in diseases such as diabetes mellitus. Metabolism of chylomicrons may also play a role in the development of atherosclerosis. In the present work, lipid emulsions were used as a physical model of chylomicrons. Triacylglycerol-rich lipid emulsions labelled with tracer amounts of radioactive triolein and cholesteryl oleate were prepared by sonication and purified by density gradient ultracentrifugation, then injected into unanaesthetized rats and normal human subjects. Plasma radioactivities were measured for 30 min in rats

and 90 min in human subjects. Rat lymph chylomicrons were also injected into rats for comparison with the clearance of the lipid emulsions. The plasma clearance data for triacylglycerols and cholesteryl esters were fitted with a kinetic model using the SAAM/CONSAM programs. Multiple studies analysis of the individual studies in each group was used to obtain estimates of the parameter average values and variabilities. The plasma residence times of the lipid labels were obtained from the fitted clearance data. Our results suggest that information about chylomicron metabolism in man can be obtained by analysis of the plasma clearance data following the injection of suitably labelled chylomicron-like lipid emulsions. Our data provide a baseline for comparisons with individuals having abnormalities of lipid metabolism or risk factors for arteriosclerosis.

INTRODUCTION

Lipids are transported from the intestine via lymphatics into the bloodstream predominantly in the form of spherical particles called chylomicrons, which are composed of triacylglycerols, cholesteryl esters, cholesterol, phospholipids and small amounts of several apolipoproteins. Entry of chylomicrons into the bloodstream is followed by rapid metabolism by the enzyme lipoprotein lipase acting at the endothelial surface of capillaries in sites such as muscle and adipose tissue. Hydrolysis of particle triacylglycerols by lipoprotein lipase is incomplete, proceeding only until 70–90% of triacylglycerols have been hydrolysed. Residual triacylglycerols and the whole of the particle cholesteryl ester remain with the particle core to form a chylomicron remnant particle. The clearance of labelled triacylglycerols therefore reflects the sum of two processes, lipolysis and particle uptake, while clearance of labelled cholesteryl ester reflects tissue uptake from the plasma of remnant particles in various states of delipidation.

The second phase of chylomicron remnant metabolism requires the presence of apolipoprotein E as a ligand [1,2] for binding by apolipoprotein B100-E [low-density lipoprotein (LDL)] receptors [3–5] and possibly by other receptors. The role of LDL receptors in the clearance of remnants from plasma was recently clarified by studies with Watanabe Heritable Hyperlipidaemic (WHHL) rabbits, in which LDL receptors are defective. The removal of remnants from the plasma of WHHL rabbits was greatly decreased, consistent with the dominant role of LDL receptors in remnant removal [3,4]. The plasma levels of retinyl esters were greatly increased in WHHL rabbits after a meal containing vitamin A, consistent with impaired remnant catabolism [6]. The

relatively large size of chylomicron remnants prevents their trans-capillary passage and uptake by most tissues. Most remnants are removed by the liver, where the large pores of the hepatic sinusoids permit their passage [7].

Lipid emulsions that model lymph chylomicrons in size and lipid composition provide a means to avoid the difficulties otherwise associated with the measurement of chylomicron catabolism in man [8]. The apolipoproteins CII and E, that are needed for physiological clearance of the lipid emulsions, readily transfer from the plasma. Here we describe the removal from the plasma of lipids after injection of labelled emulsions into normal individuals. A kinetic model using the SAAM/CONSAM software was used to assist the interpretation of clearance data in rats and in man. Multiple studies analysis of the data from many individuals gave estimates of the errors of the model parameters.

MATERIALS AND METHODS

Details of the preparation of emulsions and of the injection studies in unanaesthetized rats have been given previously [8]. Briefly, mixtures of triolein, cholesteryl oleate, egg yolk lecithin and cholesterol were sonicated, then the crude emulsions were purified by ultracentrifugation to obtain emulsion particles with diameters of approx. 150 nm. Before use in human studies the emulsions were sterilized by passage through 220 nm Millipore filters with pre-filter AP-15; they were then shown to be sterile by aerobic and anaerobic culture and also shown to be pyrogen-free by the Limulus amoebocyte lysate test (Mallinkrodt Inc., St. Louis, MO, U.S.A.) and by intravenous injection into rabbits, kindly done at Squibb Laboratories, Sao Paulo, Brazil.

Radioactive labels for triolein and cholesteryl oleate were

present in all emulsions to trace the metabolic events of interest. In rats, emulsions containing approx. 2 mg of lipid in a volume of 0.3 ml were injected into the bloodstream of non-fasted conscious animals prepared with cannulas in the left common carotid artery and the left jugular vein. Blood samples of 0.35 ml were taken at intervals up to 30 min after injection and replaced with equal volumes of 0.15 M NaCl [9]. For studies in man, injection was into an antecubital vein and 8 ml of blood was taken from the opposite arm at intervals over 90 min from a cannula perfused between samples with sterile 0.15 M NaCl solution. Each patient was injected with a pulse of emulsion containing 7 μ Ci of glycerol tri-[9,10(n)- 3 H]oleate and 3 μ Ci of cholesteryl [1- 14 C]oleate (Amersham), corresponding to 4.2 mg of triolein and 0.12 mg of cholesteryl oleate mass. The procedures for human studies were approved by the Ethical Committee of the Internal Medicine Department and by the Medical School Administration of the University of Sao Paulo. All subjects gave written informed consent. All subjects were normolipidaemic, of normal weight and free of conditions such as diabetes or thyroid disease. Subjects with excessive alcohol consumption or undergoing therapy with anticoagulant, antihypertensive or hypolipidaemic drugs were excluded. Female subjects were not taking contraceptive steroidal agents. All tests were done in the morning after a 12–14 h fast.

Rat lymph chylomicrons were obtained from rats prepared with a mesenteric lymph fistula. Milky lymph was collected after injection of Intralipid via a gastrostomy tube inserted at the time of lymph surgery. Labelled triolein and cholesterol were incorporated into the Intralipid. Lymph was collected at room temperature into vessels containing gentamycin, EDTA, phenylmethanesulphonyl fluoride and reduced glutathione [4]. Chylomicrons were isolated from the lymph by density gradient ultracentrifugation as previously described, and were injected into rats within 48 h. The chylomicron diameter was about 200 nm. Clearance studies were as described above for emulsions.

Kinetic modelling

A model was developed with data from individual rats and humans using CONSAM [10] in the MS-DOS environment on 80386-based microcomputers. The parameters of the plasma triolein and cholesteryl oleate model were fitted directly to the radioactivity data. The parameters obtained from each individual were then analysed by the SAAM code 16 multiple studies model [11] to obtain the average values and variances.

RESULTS

Plasma clearances

Plasma triacylglycerol and cholesteryl ester radioactivities in rats injected with chylomicrons are shown in Figure 1. As described below, in all studies the data from each individual were separately fitted by the model. The individual parameters were then used for a multiple studies analysis to provide averaged parameters for the population [11]. In this and subsequent figures, the curves shown are those generated for the averaged model. Figure 2 shows the plasma triolein and cholesteryl oleate radioactivities in rats injected with emulsions, together with the computed curve for the average response of the population. There is reasonable agreement between the data for emulsions and lymph chylomicrons. In all rats injected with either emulsions or chylomicrons, the cholesteryl ester radioactivity in the plasma (expressed as a percentage of the injected dose in the plasma

volume) remained higher than the triacylglycerol radioactivity for the duration of the experiment. Recycling was indicated by an upturn of the curves for triolein label after about 14 min, and in rats injected with emulsion, but not with chylomicrons, there was an increase in plasma cholesteryl oleate label after about 20–25 min (Figures 1 and 2). Evidence of recycling was consistent with previous findings with rat chylomicrons [12].

Figure 3 shows the plasma triolein and cholesteryl oleate radioactivities in normal human subjects injected with emulsions. The individual data from each subject are plotted together with the curves generated from the mathematical model, as in previous figures. The amount of triolein radioactivity (as a percentage of the injected dose) was slightly higher at early times than cholesteryl oleate radioactivity, but after 8 min the curves crossed and thereafter cholesteryl oleate radioactivity remained higher than triolein radioactivity. Recycling was indicated by an increase in plasma triolein label after about 45 min.

Figure 1 Plasma triacylglycerol and cholesteryl ester radioactivities in non-fasted rats injected with chylomicrons

In this and subsequent figures, the curves shown are those generated by the mathematical model. The plotted points are the data based on the averaged model from replicated studies in five rats. $\square$, Cholesteryl ester; $\triangle$, triacylglycerol.

Figure 2 Plasma triolein and cholesteryl oleate radioactivities in non-fasted rats injected with emulsions

As described for Figure 1, the plotted points are the data from the averaged model from replicated studies in 12 rats. $\square$, Cholesteryl oleate; $\triangle$, triolein.

Figure 3 Plasma triolein and cholesteryl oleate radioactivities in five normal fasted human subjects injected with emulsions

In this figure the individual data from each subject are plotted together with the curves generated from the mathematical model as in previous figures. $\square$, Cholesteryl oleate; $\blacktriangle$, triolein.

Figure 4 Kinetic model for clearance of radioactivity from plasma in rats and in man

Compartments 1, 2, 11 and 12 are within the plasma, where they are summed as the sample compartments 21 and 22 as shown. Compartments 1 and 11 represent the injected material. Turnover from compartments 1 and 11 occurs into compartments 2 and 12 respectively, and also into chains of connected delay compartments outside the sampled pool with allowance for return of labels to the plasma. $P(6)$ and $P(16)$ are the rate constants for transfer of label between individual elements of the chains (see Table 1). Irreversible loss occurs from compartments 2 and 12. $P(36)$ and $P(46)$ are the turnovers from compartments 11 [i.e. $L(16,11) + L(12,11)$] and 1 [i.e. $L(6,1) + L(2,1)$] respectively. The rate constants are given in Table 1.

Model development

The general modelling approach was described by Boston and Weber [13]. The model used in the present studies is shown in Figure 4. Compartments 21 and 22 represent the triacylglycerol and cholesteryl ester plasma radioactivities respectively. Compartments 1, 2, 11 and 12 are within the plasma, where they are summed as the sample compartments 21 and 22 as shown. Compartments 1 and 11 represent the injected material. Loss from compartments 1 and 11 occurs into compartments 2 and 12 respectively, and also into delay chains of connected compartments 6–8 and 16–18 respectively outside the sampled pool, and return of labels to the plasma is allowed [$L(8,1)$ and $L(18,11)$]; see

Table 1 Parameters of the kinetic model fitted to the data for emulsions injected into man and rats and for lymph chylomicrons injected into rats

The data are the average parameters from multiple studies analyses (reference [11]), with S.E.M. values derived from the same analyses. Abbreviations: CM, chylomicrons; CE, cholesteryl ester; EM, chylomicron-like emulsions; L , the rate constant for flow of label between compartments as defined in Figure 4; P , parameter as defined in text; TG, triacylglycerol. *Significantly different from the corresponding rat EM data ($P < 0.01$).

Parameter (min^{-1})	Human EM ($n = 5$)	Rat EM ($n = 12$)	Rat CM ($n = 5$)
$L(0,2)$	0.089 ± 0.010	0.454 ± 0.043	0.504 ± 0.063
$P(6) = L(7,6)$ etc.	0.024 ± 0.005	0.317 ± 0.014	0.235 ± 0.013
$P(46) = L(2,1) + L(6,1)$	0.333 ± 0.071	1.200 ± 0.048	0.959 ± 0.076
$L(2,1)$	0.089 ± 0.064	0.883 ± 0.046	0.724 ± 0.075
$L(6,1)$	0.244 ± 0.030	0.317 ± 0.014	0.235 ± 0.013
$L(0,12)$	0.038 ± 0.010	0.247 ± 0.032	0.140 ± 0.014
$P(16) = L(17,16)$ etc.	0.021 ± 0.006	0.187 ± 0.044	0.283 ± 0.084
$P(36) = L(12,11) + L(16,11)$	0.386 ± 0.090	0.563 ± 0.092	1.270 ± 0.341
$L(12,11)$	0.086 ± 0.087	0.376 ± 0.081	0.987 ± 0.330
$L(16,11)$	0.300 ± 0.023	0.187 ± 0.044	0.283 ± 0.084
Residence time for TG (min)	9.03 ± 0.17	2.35 ± 0.028	$2.21 \pm 0.019^*$
Residence time for CE (min)	12.85 ± 0.229	3.85 ± 0.045	$4.90 \pm 0.056^*$
CE/TG Ratio	1.42 ± 0.037	1.64 ± 0.027	$2.22 \pm 0.032^*$

Table 1]. Irreversible loss from plasma occurs from compartments 2 and 12.

Table 1 shows the parameters of the kinetic model fitted to the clearance data for emulsions injected into man and rats and for lymph chylomicrons injected into rats. The data shown are those from the multiple studies analysis of the individuals in each group. In this procedure (SAAM model code 16; see [11]), the method of maximum likelihood is used to obtain parameter and covariance matrix estimates for the population based on the individual studies. The errors of the parameters are derived from the multivariate normal distributions. In Table 1, $P(6)$ is the triacylglycerol turnover rate per delay element and $P(16)$ is the cholesteryl ester turnover rate per delay element. $P(36)$ and $P(46)$ are constraints set to determine the turnover rates from compartments 11 [$= L(16,11) + L(12,11)$] and 1 [$= L(6,1) + L(2,1)$] respectively. $L(0,2)$ was consistently greater than $L(0,12)$. In rats, the flows from compartments 1 and 11 were predominantly into compartments 2 and 12 respectively [i.e. $L(2,1) > L(6,1)$ etc.], whereas in the human subjects most flow from compartments 1 and 11 was into compartments 6 and 16 respectively [i.e. $L(2,1) < L(6,1)$ etc.].

Overall flux rates were greater in rats than in man. Residence times were calculated from the areas under the fitted curves until upturns were apparent at 14 min in rats and at 45 min in the human studies. The residence times in plasma for triacylglycerol and cholesteryl ester radioactivities were similar at about 2.3 min and 4 min in rats injected with emulsions and lymph chylomicrons respectively. The differences in residence time between the emulsions and lymph chylomicrons were significantly different ($P < 0.01$). Both were shorter than the residence times of about 4 min for triacylglycerol and about 9 min for cholesteryl ester previously calculated for chylomicrons in a different strain of rats [12]. However, the ratio of the residence times (about 2) was similar in the present study to that in previous findings, where this ratio was called the 'index of delipidation' [12]. In rats the ratio for chylomicrons was 35% higher than for emulsions ($P < 0.01$). In the human studies the absolute residence times of both lipids were about 4-fold greater than in rats, but the ratios

or indices of delipidation in rats and man injected with emulsions were in reasonable agreement, at 1.64 and 1.42 respectively.

DISCUSSION

Many factors affect chylomicron clearance, the most important being the activity of lipoprotein lipase and the availability of its activator, apolipoprotein CII. There is also evidence of regulation by the activity of hepatic lipase and by endocrine factors. Clearance of chylomicron remnants requires apolipoprotein E, LDL receptors and possibly other receptors. More precise knowledge of the factors regulating the clearance of chylomicron remnants in man is unavailable because direct measurement has not been possible. For example, a possible role of chylomicron remnants in the aetiology of atherosclerosis is uncertain. It is clear that plasma cholesterol measured in the fasting state is a poor predictor of the risk of coronary heart disease, since about one-half of heart attacks occur in individuals with plasma cholesterol in the normal range [14]. Current concepts are unable to account for the occurrence of atherosclerosis in individuals with low concentrations of plasma cholesterol in the fasted state. Unrecognized problems with clearance of chylomicron remnants could contribute to these apparent paradoxes. In normolipidaemic men the clearance of postprandial lipoproteins in patients with coronary arteriosclerosis was delayed compared with controls [15].

A significant barrier to elucidation of the role of chylomicron remnants in human disease has been the lack of available methods for measurement of chylomicron catabolism. It is not possible to distinguish reliably the remnants in plasma from their precursor chylomicron particles. Sequential flotation is inefficient, because remnants overlap in size and density with the precursor chylomicrons. Size-exclusion chromatography also fails because remnants overlap with chylomicrons. Remnants appear to contain no unique component to permit their separation by chromatography or electrophoresis, although a separation was achieved by ion-exchange chromatography with selected media [16]. However, the separation described by Felts [16] is cumbersome, requiring an initial ultracentrifugation to remove albumin and other serum proteins. A fraction enriched in remnants was recently obtained from whole human plasma by immunoaffinity chromatography, using a monoclonal antibody to apolipoprotein B100 that presumably bound to an epitope beyond the B48 C-terminal equivalent site of the B100 molecule [17].

Changes in plasma triacylglycerols in the post-prandial state during steady intestinal delivery of fat were used by Grundy and Mok [18] to measure chylomicron triacylglycerol clearance in man, but this approach gives only indirect information about remnant clearance because most chylomicron triacylglycerols are cleared by lipoprotein lipase. Plasma contents of retinyl esters in man after meals containing vitamin A have been used to describe the effects on chylomicron remnant clearance of hyperlipidaemia [19], apolipoprotein E genotype [20,21], exercise [22], hepatic lipase activity [23], diet [24,25], age [26] and inhibition of cholesterol biosynthesis [27]. Interpretations are nevertheless uncertain, because plasma data are affected not only by clearance but also by the kinetics of absorption of vitamin A from the intestinal lumen. A refinement of this approach avoided the problem of the kinetics of intestinal absorption, but added the extra difficulty of collecting post-prandial plasma from study subjects before re-injection after a few days [28,29]. There is also doubt about the adequacy of retinyl esters as markers for chylomicron remnants [30]. The interpretation of plasma clearance of lymph chylomicrons is further complicated because

chylomicrons vary in size and chemical composition depending on the load and nature of the dietary fat. Lipids derived from a fatty meal are combined to a variable extent with endogenously derived lipids.

To circumvent such difficulties we have developed methods for studying lipoprotein metabolism using radiolabelled lipid emulsion particles of defined size and composition [8]. While the emulsions and chylomicrons injected into rats behaved similarly in their overall patterns of clearance (Figures 1 and 2), agreement was not exact. In fact, as shown in Table 1, the residence times for both triacylglycerol and cholesteryl ester differed significantly. These differences possibly arise because the chylomicrons are more heterogeneous in composition and size than the model emulsion particles. The emulsions are prepared from four pure lipids, while all lipid classes in the chylomicrons are complex mixtures due to the processes of intestinal absorption and formation in the mucosal cells. The lymph chylomicrons had average diameters of approx. 200 nm, while the emulsion particles had diameters of approx. 150 nm. Chylomicron size affects clearance from plasma, with slower removal of larger chylomicrons [31]. However, with emulsions, smaller particles were removed more slowly [32].

Before use in human studies, the emulsions were sterilized by passage through 220 nm-pore-size Millipore filters. While this is necessary for ethical reasons, it is possible that filtration has some effect on emulsion size or composition. This possibility was not tested in the current experiments. Emulsions are used for intravenous nutrition of human patients and also have been used for assessment of lipid clearance. The intravenous fat tolerance test (IVFTT) [33] provides no information about remnant clearance because the injected emulsion contains no marker for remnants, and neither does it contain cholesterol, so physiological clearance is impossible [32]. Nevertheless, the IVFTT continues to be used for studies of plasma triacylglycerol metabolism [34–36].

In chylomicrons the lipid core contains mostly triacylglycerols, 1–2% cholesteryl esters and some dissolved cholesterol, while the surface contains phospholipids, interdigitated cholesterol molecules, apolipoproteins and approx. 2% triacylglycerols and cholesteryl esters. In our previous studies in rats, emulsions containing the small amounts of cholesterol typical of natural chylomicrons and very low density lipoproteins were metabolized like the natural lipoproteins [8]. Emulsions have proved useful in studies of disease processes, for example insulin deficiency [37], and in defining the roles of lipid constituents in lipoprotein clearance [9,32,38].

On the one hand, emulsions offer advantages for the study of metabolism of chylomicrons because the chemical composition and particle size can be controlled and readily varied. Possible immunological problems or unrepresentative clearance due to heterologous apolipoproteins injected with natural lipoproteins are avoided because of the absence of apolipoproteins on emulsions. Injected lipid emulsions must gain from the plasma of the individual recipient the apolipoproteins CII and E necessary for their catabolism by lipoprotein lipase and by receptor-mediated organ uptake. Clearance of chylomicron-like emulsions is unaffected by the absence of apolipoprotein B48, which appears to play no part in the clearance of chylomicrons from the plasma. On the other hand, in rats the clearance of emulsions differs significantly from that of chylomicrons. Figures 1 and 2 show differences in the upturns of the curves, and Table 1 shows that the residence times are significantly different. Until the reasons for these differences are established, caution is required in extrapolating findings with emulsions to chylomicrons. Nevertheless, our data provide a baseline for future comparisons with

clearances in human subjects with defined abnormalities of lipid metabolism.

Modelling of chylomicron clearance using the SAAM/CONSAM programs was previously applied to studies in rats [12]. Georgopoulos and Phair [39] modelled the post-prandial clearance from plasma of lipoproteins and found delayed clearance of remnants in men with diabetes mellitus. Cortner and co-workers [40] modelled the plasma data of absorbed retinyl esters in man to infer values for chylomicron remnant clearance rates. Cortner et al. calculated that the half-life for removal of remnants in normal individuals was 14.1 ± 9.7 min. This compares with the residence time for emulsion cholesteryl oleate of 12.9 ± 0.2 min in our study (Table 1), corresponding to a half-life of 8.9 ± 0.2 min ($\ln 2 \times$ residence time). The calculation of residence times is sensitive to assumptions concerning the nature of the clearance. The error increases when recycling is evident.

In previous work chylomicron clearance was affected by fasting in rats [12]. In the present study human subjects were fasted to eliminate variability in absorption kinetics and endocrine and circulatory responses to a meal. It is possible that half-lives may differ post-prandially. Additional studies are needed to address this possibility.

When combined with injections of lipid emulsion, models of lymph chylomicrons, kinetic analysis and quantitative measurement of chylomicron remnant clearance in human subjects will enable the influence of specific variables such as nutritional and hormonal status to be assessed. We found that the same mathematical model accurately described the triacylglycerol and cholesteryl ester clearance data from human and rat emulsion studies and from rat chylomicron studies. Residence times were readily obtained to facilitate comparisons between individuals. The combination of the techniques of injecting chylomicron-like emulsions and of mathematical modelling of the plasma clearance data improves the evaluation of chylomicron metabolism in human subjects, and could increase our understanding of the possible roles of chylomicron remnants in diseases such as diabetes and atherosclerosis.

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