# Omega-3 Triglycerides Modify Blood Clearance and Tissue Targeting Pathways of Lipid Emulsions†

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ABSTRACT: Omega-3-rich (n-3) triglycerides (TG) are increasingly recognized as having modulating roles in many physiological and pathological conditions. We questioned whether the catabolism of lipid emulsions would be changed after enrichment with fish oil (n-3) TG as compared to enrichment with omega-6-rich soy oil (n-6) TG. Phospholipid-stabilized emulsions of n-3 TG and n-6 TG were labeled with [$^3$H]cholesteryl oleoyl ether and administered by bolus injection to wild-type (WT) mice, mice lacking the low-density lipoprotein receptor (LDL-R) (LDL-R −/−), and apolipoprotein E (apoE) knockout mice (apoE −/−). The effects of exogenous apoE, heparin, Triton WR 1339, and lactoferrin on catabolism of emulsions were also assayed. n-3 TG emulsions were cleared faster from blood and had different extrahepatic tissue targeting compared to n-6 TG emulsions. In apoE −/− and LDL-R −/− mice, blood clearance of n-6 TG emulsions slowed with decreased liver uptake, but no changes were observed in n-3 TG emulsion clearance and tissue uptake compared to WT mice. In WT mice, addition of exogenous apoE to the emulsion increased liver uptake of n-6 TG emulsions but had no impact on n-3 TG emulsions. Pre-injection of heparin increased and Triton WR 1339 and lactoferrin decreased blood clearance of n-6 TG emulsions with little or no effect on n-3 TG emulsions. Liver uptake of n-6 TG emulsions increased after heparin injection and decreased after Triton WR 1339 injection, but uptake of n-3 TG emulsions was not changed. These data show that the catabolism of n-3 TG emulsions and the catabolism of n-6 TG emulsions occur via very different mechanisms. Removal of chylomicron-sized n-6 TG emulsions is modulated by lipoprotein lipase (LPL), apoE, LDL-R, and lactoferrin-sensitive pathways. In contrast, clearance of chylomicron-sized n-3 TG emulsions relies on LPL to a very minor extent and is independent of apoE, LDL-R, and lactoferrin-sensitive pathways.

Soy oil or safflower oil-based phospholipid-stabilized triglyceride (TG)[1] emulsions consisting of mainly omega-6-rich (n-6) TG are widely used therapeutically in intravenous nutrition support, as a means of providing a calorie dense energy supply and essential fatty acids, as well as vehicles for delivery of water-insoluble agents (*1*−*3*). Recently, there has been therapeutic interest in utilization of fish oil (FO)-containing emulsions consisting of mainly omega-3-rich (n-3) TG (*1*, *2*). In particular, two major n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to be active in a number of biological processes, including retinal and brain development, immune function, blood clotting, and prevention of cardiac arrhythmias (*4*−*6*). Thus, n-3 TG emulsions made from FO are likely to be increasingly used not only for nutrition support but also for modification of a number of biological and pathological processes.

Traditionally, lipid emulsions have been thought to be a part of clearance and targeting pathways similar to that of endogenous triglyceride-rich particles (TGRP), i.e., chylomicrons and very low-density lipoproteins (VLDL). After infusion, emulsions acquire apolipoproteins such as apolipoprotein CII (apoCII) and apolipoprotein E (apoE) in the circulation, and after intravascular lipolysis by lipoprotein lipase (LPL), emulsion remnants, similar to chylomicron remnants, are cleared by liver (*7*−*10*). However, more recent studies have suggested that lipid emulsions are cleared from blood with less lipolysis and release of free fatty acids than chylomicrons, and that substantial amounts of emulsions can be cleared as intact whole particles by different tissues (*11*, *12*). Of interest, in animal models while some lipolysis occurred during clearance of n-6 TG emulsions made from

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[1] Abbreviations: apoE, apolipoprotein E; [$^3$H]CEt, [1α,2α(n)-$^3$H]-cholesteryl oleoyl ether; FCR, fractional catabolic rate; FFA, free fatty acid; FO, fish oil; HSPG, heparan sulfate proteoglycans; LCT, long-chain triglyceride; LDL-R, low-density lipoprotein receptor; LPL, lipoprotein lipase; LRP, LDL-R-related protein; MCT, medium-chain triglyceride; SD, standard deviation; SEM, standard error of the mean; TG, triglyceride; TGRP, triglyceride-rich particles; VLDL, very low-density lipoprotein; WT, wild type.

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soy oil (Intralipid) particles in the fasted state, the level of lipolysis was markedly diminished and lipolysis was hardly involved in emulsion particle clearance in the fed state (*12*). Moreover, it has been recognized that emulsions are preferentially targeted to extrahepatic tissues compared to chylomicrons (*12*). Nevertheless, the liver still clears more emulsions from blood than other tissues. ApoE is important in directing and increasing clearance of TGRP and model emulsions by the liver via apoE-specific receptors, particularly in hepatocytes (*9*, *13*−*17*). Thus, while not directly studied, it is possible that apoE-dependent pathways such as the LDL receptor (LDL-R), LDL-R related protein (LRP), and heparin sulfate proteoglycans (HSPG) could all contribute to clearance of n-6 TG emulsions from blood.

The TG composition of lipid emulsions can have marked effects on their metabolism. For example, lipid emulsions with added medium-chain triglyceride (MCT) emulsions are cleared faster from blood than pure long-chain triglyceride (LCT) (n-6 TG) emulsions (*18*, *19*). This corresponds to the 4−5-fold higher rates of lipolysis by LPL of MCT-containing emulsions in in vitro systems as compared to the rates of LCT emulsions (*20*). In contrast, in vitro the level of LPL-mediated hydrolysis of emulsions made from FO is markedly decreased compared to that for emulsions made from soy oil (*21*). This suggests that intravascular lipolysis might have no, or a very limited, role in the in vivo clearance of lipid emulsions enriched with FO.

We questioned whether clearance and tissue delivery of n-6 TG and n-3 TG emulsions would occur by similar or different pathways. To address this, we compared blood clearance and tissue uptake of n-6 TG and n-3 TG emulsions in wild-type (WT) mice, LDL-R knockout (LDL-R −/−), and apoE knockout (apoE −/−) mice. Nonfasted mice were used for most experiments to parallel more what occurs during therapeutic situations where lipid emulsions are always infused with concomitant glucose and amino acids (i.e., essentially a nonfasting state). We also measured the influences of exogenous apoE, compounds that alter in vivo TG lipolysis (heparin and Triton WR 1339) (*9*, *22*), and an inhibitor of apoE-mediated TGRP clearance pathways (lactoferrin) (*9*, *14*). Our data show that n-3 TG emulsion particles are cleared faster from blood, have different tissue targeting, and utilize different metabolic pathways compared to n-6 TG emulsions.

## EXPERIMENTAL PROCEDURES

*Materials.* [1α,2α(n)-$^3$H]Cholesteryl oleoyl ether (TRK 888) was purchased from Amersham Pharmacia Biotech. Avertin (2,2,2-tribromoethanol) (T-4840-2) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Bovine lactoferrin and Triton WR 1339 (tyloxapol) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Heparin sodium was obtained from Elkins-Sinn, Inc. (Cherry Hill, NJ). Recombinant human apoE3 prepared in *Escherichia coli* was provided by Biotechnology General, Ltd., and has been shown to exhibit the same in vitro cell binding and in vivo properties as native human plasma apoE3 (*23*). The human normal fibroblast cell line, HS-68, was purchased from ATCC (Rockville, MD) and was grown in culture as described previously (*24*, *25*). Cell cultivation materials, including glutamine, penicillin, streptomycin, and fetal or standard calf serum, were purchased from Gibco/Life Technology Inc. (Grand Island, NY).

*Lipid Emulsions.* Phospholipid-stabilized emulsions of n-6 and n-3 TG (20 g of TG/100 mL) were kindly provided by B. Braun Melsungen AG (Melsungen, Germany) and prepared with soy oil and fish oil TG, respectively, and egg yolk phospholipid according to standard procedures as previously described in detail (*20*). Each emulsion contained 20 g of TG, emulsified by 1.2 g of egg yolk lecithin, and 2.5 g of glycerol/100 mL. The emulsion lipids were mixed in doubly distilled water (30 g of water and 20 g of oil) and dispersed by means of an Ultra-Turrax (Janke and Kunkel KG, Staufen, West Germany) for 10 min; water was added to give a final volume of 100 mL, and emulsions were dispersed for an additional 10 min. Subsequently, the dispersion was homogenized by ultrasound in a cooling cell with a Labsonic 2000 homogenizer for 10 min at an energy input of 200 W. The emulsions were then sealed in 5 mL vials under $N_2$, and thereafter kept at 4 °C. Mean particle sizes were determined by laser spectroscopy, and both were similar in size and homogeneity with mean diameters between 290 and 300 nm. n-3 and n-6 TG emulsions contained 1.42 ± 0.25 and 1.10 ± 0.04% (mean ± standard deviation for three measurements) of total TG as FFA, respectively, i.e., a range of 0.006−0.009 mM, concentrations too low to significantly affect emulsion metabolism. The relative fatty acid composition (by weight) of emulsion TG determined by gas−liquid chromatography was as follows: n-6 TG (LCT) emulsion, 0.1% C14:0, 10.1% C16:0, 0.1% C16:1, 4.3% C18:0, 23.8% C18:1, 53.9% C18:2(n-6), 5.8% C18:3(n-3); 1.7% C20:0, and 0.4% C20:4(n-6); and n-3 TG (FO) emulsion, 6.2% C14:0, 12.6% C16:0, 1.3% C18:0, 6.8% C18:1, 1.4% C18:2(n-6), 0.2% C18:3(n-6), 1.3% C18:3(n-3), 4.7% C18:4(n-3), 1.4% C20:1, 2.6% C20:4(n-6), 34.4% C20:5(EPA,n-3), 1.8% C22:4, 4.1% C22:5, and 20.7% C22:6(DHA,n-3). The main difference in fatty acid composition between the two emulsions was that the n-6 TG emulsion contained more than half of its TG as linoleic acid and the n-3 TG emulsion contained more than half of its TG as EPA and DHA.

We labeled each emulsion with nondegradable [$^3$H]-cholesteryl oleoyl ether ([$^3$H]CEt) (0.2 mCi/100 mg of TG) to trace emulsion particle catabolism using previously described methods (*26*, *27*). In a small glass vial, the desired amount of [$^3$H]CEt and 20−50 μL of 100% of ethanol were added, and the vial was rotated to coat the vial wall evenly under $N_2$ gas until the solvent was evaporated completely. Immediately upon the vial becoming dry, 150 μL of the emulsion was added to the vial. The vial was mixed vigorously and allowed to sit for 30 min. After the same procedure, another two portions of emulsion were added to a total volume of 500 μL. The emulsion was sonicated three times on ice for 20 s each at a power setting of 40 W using a Branson Sonifier Cell Disruptor (model W185) (Branson Scientific, Inc., Plainview, NY) to incorporate the [$^3$H]CEt into the core of the emulsion particles. To remove the small amounts of titanium released from the sonifier probe, the labeled emulsions were centrifuged in an Eppendorf centrifuge at 14000*g* for 15 min at 4 °C. The resulting emulsions were stored at 4 °C under $N_2$ and used for experiments within 7 days, a period during which no physiochemical changes in emulsion particles occur.

To determine whether the [$^3$H]CEt had been incorporated into the two emulsions similarly and to demonstrate that emulsion particles were not changed during sonication, small aliquots of each emulsion were centrifuged in 7.5 cm long capillary tubes. After centrifugation, the tubes were cut into seven sections (1 cm each), and TG and phospholipid concentrations were measured in each section of the sonicated and unsonicated emulsions. For sonicated emulsions, the amount of radioactivity present in each section was counted by liquid scintillation spectrometry (Beckman LS 1800 liquid scintillation counter). TG concentrations were assayed by an enzymatic procedure using the triacylglycerol GPO-PAP kit (Boehringer Mannheim BmbH). The phospholipid level was measured according to the Bartlet procedure (*28*). All emulsions, whether sonicated or not, had exactly the same TG/phospholipid ratios in the corresponding sections of the capillary tube. Also, >90% of the total [$^3$H]-CEt used was recovered in the top fraction after centrifugation. [$^3$H]CEt incorporated into lipid emulsions remains with the emulsion core. Note that there is no exchange of emulsion neutral lipids with other lipoproteins in the absence of cholesteryl ester transfer protein (*29*) and that rodents do not have cholesteryl ester transfer protein.

*Animals.* Wild-type (WT) C57BL/6J mice (7−8 weeks old, female) were purchased from Jackson Laboratory (Bar Harbor, ME) and allowed to recover from transportation for 2−4 weeks before the experiments. Stock homozygous apoE −/− (*30*) and LDL-R −/− (*31*) C57BL/6J mice aged 12 weeks were kindly provided by J. Breslow (Rockefeller University, New York, NY). Sibling mating was used to breed mice homozygous for the null mutations. All the mice were maintained in the house of the Animal Facilities of Columbia University in a 12 h light/12 h dark cycle. They had access to standard pellet rodent diets and water ad libitum. For all experiments, 10−12-week-old female mice weighing 18−23 g were used. To compare the effects of feeding versus fasting states on emulsion catabolism, mice were fasted 16−24 h. The plasma cholesterol levels of homozygous C57BL/6J apoE −/− and LDL-R −/− mice were 473.1 ± 53.2 mg/dL ($n = 3$) and 178.6 ± 16.4 mg/dL ($n = 4$), respectively. These values were higher than that of WT mice (69.3 ± 11.5 mg/dL) ($n = 5$). In contrast, plasma TG levels were 101.2 ± 33.2 and 87.7 ± 11.6 mg/dL for apoE −/− and LDL-R −/− mice, respectively, not significantly different from that for WT mice (60.5 ± 15.0 mg/dL).

*Determination of the Rates of Blood Clearance and Tissue Uptake of Emulsions.* Mice were anesthesized by intraperitoneal injection of Avertin (125 mg/kg) and received 50 μL of emulsion solution (diluted with 0.9% NaCl) containing 400 μg of TG (nonsaturating dose) by a bolus injection via the femoral vein. In some studies, lactoferrin, heparin, or Triton WR 1339 was injected 2, 1, and 15 min, respectively, before emulsion injection (*9*, *14*, *22*, *32*, *33*). To investigate the role of exogenous apoE in emulsion removal, emulsions were incubated with apoE (20/1 TG/apoE ratio, w/w) for 30 min at 25 °C prior to injection, which allowed sufficient time for apoE equilibrium and even binding of the surface of emulsion particles (*34*). Emulsion clearance in blood was assessed by measuring the level of [$^3$H]CEt in retro-orbital blood (20 μL), which was drawn at 0.5, 2, 5, 10, 15, and 25 min by heparinized capillary tubes following emulsion injection. Mice were sacrificed immediately by injection of an overdose of Avertin (500 mg/kg) after the final blood sample collection at 25 min. Organs and tissues (heart, lung, liver, kidney, spleen, pancreas, intestine, peritoneal fat, muscle, bone, brain, and adrenal) were dissected after perfusion with 0.9% NaCl-containing heparin (2 units/mL) and kept on ice. After surface residual blood had been washed away, the tissues were weighed and homogenized using a Polytron Tissue Disruptor (Power Gen 125, Fisher Scientific), and a chloroform/methanol mixture (2/1, v/v) was used to extract lipids in tissue homogenates as described by van Bennekum et al. (*26*). The amount of radioactivity in blood and tissue samples was measured by liquid scintillation spectrometry.

The amount of radioactivity in blood was expressed as the percent (%) of the injected dose remaining in the whole blood. The whole blood volume for each mouse was calculated as 4.9% of its body weight. Fractional catabolic rates (FCR) were calculated on the basis of first-order linear kinetics during the first 10 min after emulsion injection (*35*). Tissue uptake was expressed as % of recovered injected dose per organ except for adipose tissue and bone uptake, which were expressed as % of recovered injected dose per gram (g) of tissue. The recovered injected dose was the sum of the amount of radioactivity from all organs and tissues assayed, including blood at the end of experiments. Values are expressed as means ± SD for blood clearance and FCR, whereas means ± SEM were used in comparing tissue uptakes.

*Cell Uptake of Lipid Emulsions in Vitro.* The experiments were performed according to the procedures described previously (*24*, *25*). Human normal fibroblasts (HS-68) were plated at a density of $2.5 \times 10^6$ cells/well in 12-well plates. Cells were grown in a humidified incubator (5% $CO_2$) at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, glutamine (292 μg/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). After the cells had reached >80% confluence, the growth medium was replaced with DMEM containing 10% lipoprotein-deficient serum (LPDS) to upregulate LDL-R for 2 days before experiments. Cells were incubated with the emulsions (250 μg of TG/mL) with and without prior incubation with apoE (20/1 TG/apoE ratio, w/w) (*34*) in DMEM containing 1% bovine serum albumin for 4 h at 37 °C. In other experiments where apoE was preincubated with n-6 and n-3 emulsions, essentially all the apoE associated with the emulsions as determined by Sepharose CL-2B column chromatography (*34*). Lactoferrin (2 mg/mL) was also added to the medium to inhibit apoE-mediated emulsion uptake in some experiments. At the end of experiments, cells were washed and solubilized in 0.1 N NaOH to determine the amount of cell-associated [$^3$H]CEt, which was then normalized for cell protein (*24*, *25*). Cell uptake of emulsions was determined on the basis of the amount of TG calculated from the amount of associated [$^3$H]CEt.

*Statistics.* One-way analysis of variance (ANOVA) and two-tailed *t* tests were used to determine statistical significance at the $p < 0.05$ level.

## RESULTS

*Differences in Blood Clearance and Tissue Uptake between n-6 TG and n-3 TG Emulsions.* Blood clearance of

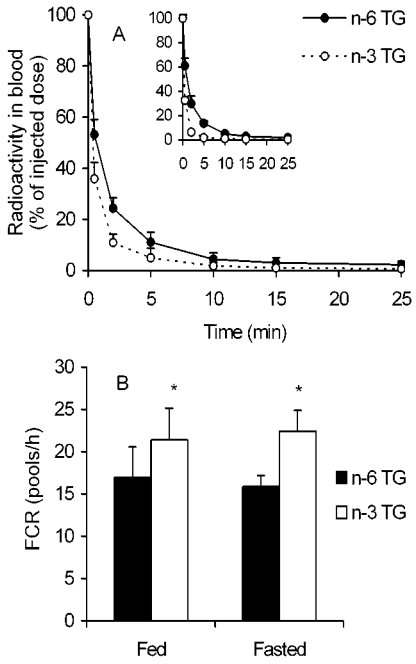


FIGURE 1: Blood clearance of lipid emulsions of n-6 TG and n-3 TG in mice. C57BL/6J mice were injected with [$^3$H]CEt-labeled emulsions (400 $\mu$g of TG/mouse), and blood clearance was determined as described in Experimental Procedures. Emulsion particles remaining in blood were calculated as % of the injected dose. Fractional clearance rates (FCR) were calculated from clearance curves on the basis of first-order linear kinetics during the first 10 min after emulsion injection: (●) n-6 TG emulsions and (○) n-3 TG emulsions. (A) Clearance curves in fed mice; inset, fasted mice. (B) FCR of emulsions in fed and fasted mice. Values are means ± SD from one experiment typical of four others ($n$ = 8−10 in each group for each experiment). Asterisks denote significant differences between n-6 TG and n-3 TG emulsions ($p$ < 0.01).

n-6 TG emulsions and n-3 TG emulsions in WT C57BL/6J mice are compared in Figure 1. n-3 TG emulsion particles were cleared faster than n-6 TG emulsion particles in the fed state (Figure 1A). Similar results were observed in fasted mice (Figure 1A, inset). At each time point, fewer n-3 TG emulsion particles remained in blood than n-6 TG emulsions. This is consistent with the calculated FCR (Figure 1B) which demonstrated n-3 TG emulsions had a higher FCR than n-6 TG emulsions in both the fed and fasted states (21.4 ± 3.8 vs 17.0 ± 3.2 pools/h and 22.4 ± 2.4 vs 15.9 ± 1.3 pools/h, respectively, $p$ < 0.01).

An important question was whether the procedure required for radiolabeling the emulsion would change particle properties. To address this, we injected the original untreated nonradiolabeled emulsions into mice and compared their blood clearance to those for radiolabeled emulsion particles. To accomplish this, we needed to inject higher doses of emulsions to measure clearance by the increase and decrease of plasma TG levels immediately after, and following, injection. We injected 4 mg of TG/mouse of nonradiolabeled emulsions and compared clearance with that for the radiolabeled emulsions with the same amount of TG injected, and calculated FCR. Differences in FCR between the radiolabeled and nonradiolabeled were not significant at 4.3 ± 2.6% for n-6 TG emulsions and 5.6 ± 3.8% for n-3 TG emulsions ($n$ = 4 for each group). Importantly, when the blood clearance curves are extrapolated back to time 0 min, there was no difference between labeled and unlabeled emulsions in their TG values immediately postinjection. This indicates that there was no rapidly removed pool of aggregated or changed emulsion particles after radiolabeling. Thus, our radiolabeling procedure did not affect the biological and physical properties (see Experimental Procedures) of the emulsions.

Tissue uptake of emulsion particles are shown in Table 1. More n-6 TG emulsions were taken up by heart, adipose tissue, and muscle than n-3 TG emulsions in the fed state, whereas more n-3 TG emulsions were taken up by lung in both fasted and fed states. Liver uptake of the two emulsions in both the fed and fasted states were similar. When the fed with fasted states are compared, feeding decreased hepatic uptake and increased peripheral uptake of both emulsions, and the increased peripheral uptake primarily resided in adipose tissue and muscle with n-6 TG emulsions and in lung with n-3 TG emulsions. No changes in heart uptake were observed between the fasted and fed states in both n-6 TG and n-3 TG emulsions. Of interest, brain took up more n-3 TG emulsions than n-6 TG emulsions. Experiments with the high infusion dose of both emulsions (4 mg of TG/mouse) were also carried out, and very similar tissue distributions were obtained (data not shown). Taken together, these data suggest that TG composition in lipid emulsions did affect their blood clearance and tissue targeting. We next questioned whether the catabolic mechanisms for n-3 and n-6 TG emulsions are similar or different. To minimize effects of lipolysis (*12*), fed mice were used in all the following experiments.

*Different Effects of LPL on Catabolism of n-6 TG and n-3 TG Emulsions.* Interaction with LPL is the key step in the catabolism of TGRP. Thus, we used both heparin (releases LPL into plasma) and Triton WR 1339 (blocks LPL action) to compare the effects of LPL on lipid emulsion removal. Preinjection of heparin (1 min before emulsion injection) accelerated blood clearance of both n-6 and n-3 TG emulsions, whereas Triton WR 1339 (15 min before emulsion injection) slowed blood clearance of the two emulsions (Figure 2). However, the effects of heparin and Triton WR 1339 on n-3 TG emulsion blood clearance were much lower in magnitude than that on n-6 TG emulsion clearance.

Figure 3 depicts the effects of preinjection of heparin and Triton WR 1339 on tissue uptake of emulsion particles. Liver uptake of n-6 TG emulsions increased 2-fold with preinjection of heparin and decreased 42.4% with preinjection of Triton WR 1339 compared to control ($p$ < 0.01), but no changes were found in the liver uptake of n-3 TG emulsions with either heparin or Triton WR 1339. Preinjection of heparin or Triton WR 1339 decreased the uptake in many extrahepatic tissues, but again changes were greater with n-6 TG than with n-3 TG emulsions. Note that after Triton WR 1339 injection much more n-6 TG emulsion than n-3 TG emulsion remained in blood after 25 min.

*Different Effects of ApoE on Catabolism of n-6 TG and n-3 TG Emulsions.* ApoE is a key ligand mediating hepatic clearance of TGRP via LDL-R, LRP, and HSPG pathways (*13*−*17*). Thus, we examined whether apoE influences blood clearance and hepatic uptake of n-6 TG and n-3 TG emulsions in WT and apoE −/− mice. Exogenous apoE, preincubated with emulsions and then injected into WT mice, did not change blood clearance of both emulsions compared to that of mice given emulsions without incubation with apoE

Table 1: Tissue Uptake of n-6 TG and n-3 TG Emulsions in C57BL/6J Mice[a]

| | fed | | fasted | |
|---|---|---|---|---|
| | n-6 TG emulsion | n-3 TG emulsion | n-6 TG emulsion | n-3 TG emulsion |
| heart | 14.48 ± 1.49 | 2 43 ± 0.48[b] | 12.72 ± 0.14 | 2.81 ± 0.03[b] |
| lung | 1.64 ± 0.19 | 25.08 ± 2.54[b] | 2.33 ± 0.06 | 8.61 ± 0.22[b,c] |
| liver | 44.51 ± 3.39 | 47.41 ± 3.18 | 70.85 ± 3.61[c] | 74.47 ± 1.38[c] |
| kidney | 2.53 ± 0.31 | 3.18 ± 0.41 | 1.46 ± 0.01[c] | 2.17 ± 0.04[c] |
| spleen | 6.20 ± 0.60 | 6.01 ± 0.56 | 3.02 ± 0.05[c] | 5.55 ± 0.12[b] |
| adipose tissue | 15.38 ± 3.09 | 3.12 ± 0.75[b] | 1.79 ± 0.05[c] | 2.13 ± 0.11 |
| muscle | 11.65 ± 1.40 | 5.44 ± 1.26[b] | 7.75 ± 0.08[c] | 4.19 ± 0.10[b] |
| brain | 0.02 ± 0.00 | 0.05 ± 0.01[b] | 0.02 ± 0.00 | 0.03 ± 0.00[b,c] |
| adrenal | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.07 ± 0.01 | 0.05 ± 0.01 |

[a] The mice that were used were the mice described in the legend of Figure 1. The organs and tissues of each mouse were obtained 25 min after the final blood sample was taken. [$^3$H]CEt activity in tissues was extracted with a chloroform/methanol mixture (2/1, v/v). Tissue uptake was expressed as % of recovered injected dose. Values are means ± SEM from one experiment typical of four others ($n$ = 8−10 in each group). [b] Significant differences ($p < 0.05$) between n-6 TG and n-3 TG emulsions with the same feeding conditions. [c] Significant differences ($p < 0.05$) between fed and fasted conditions with the same emulsions.

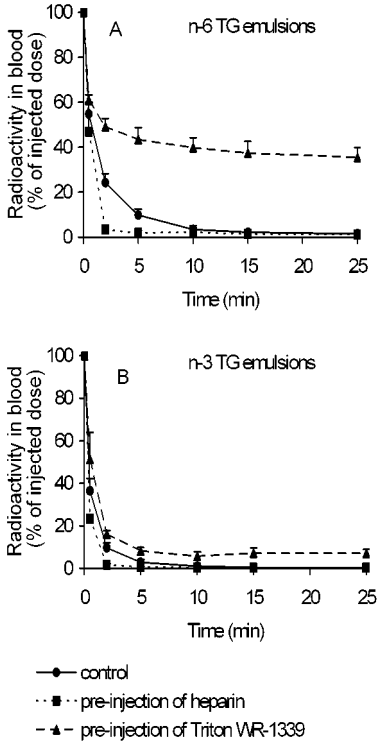


FIGURE 2: Effects of heparin and Triton WR 1339 on blood clearance of n-6 TG and n-3 TG emulsions in mice. C57BL/6J mice were injected with [$^3$H]CEt-labeled emulsions (400 $\mu$g of TG/mouse) after preinjection of heparin (10 units/mouse) (1 min before) and Triton WR 1339 (10 mg/mouse) (15 min before), and then blood clearance was assessed as described in Experimental Procedures. Results are from one experiment typical of two others. Values are means ± SD with 5−8 ($n$) in each group: (●) control, (■) preinjection of heparin, and (▲) preinjection of Triton WR 1339. (A) n-6 TG emulsions. (B) n-3 TG emulsions.

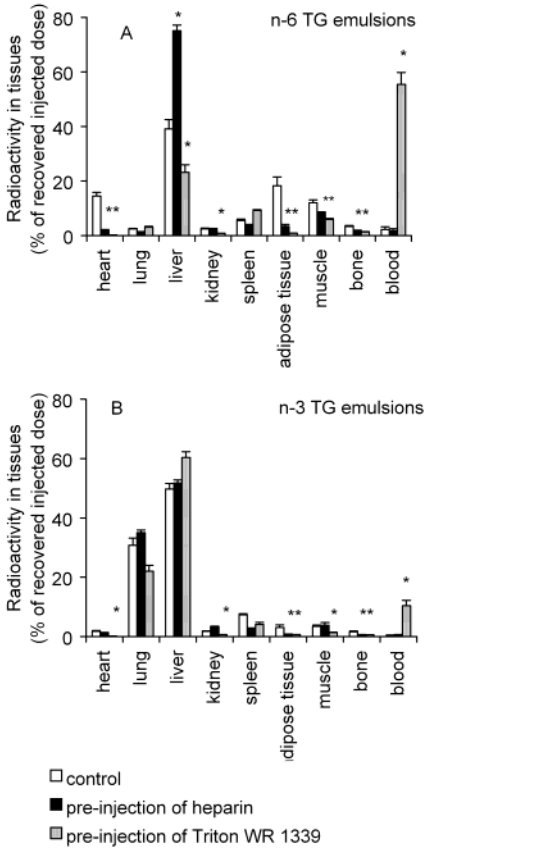


FIGURE 3: Effects of heparin and Triton WR 1339 on tissue uptake of n-6 TG and n-3 TG emulsions in mice. Organs and tissues of each mouse described in Figure 2 were obtained 25 min after the final blood sample was taken. The amount of radioactivity in tissues was determined as described in Experimental Procedures. Tissue uptake was expressed as % of recovered injected dose: (A) n-6 TG emulsions and (B) n-3 TG emulsions. Values are means ± SEM ($n$ = 5−8 for each group). Asterisks denote significant differences compared to control mice which received a preinjection of saline ($p < 0.05$).

(Figure 4). In apoE −/− mice, in the absence of endogenous apoE, blood clearance of n-6 TG emulsions was initially decreased (Figure 4A), but no changes were noted in n-3 TG emulsion clearance (Figure 4B).

Tissue uptakes are shown in Figure 5. In WT mice, exogenous apoE, preincubated with emulsions before injection, increased liver uptake of n-6 TG emulsions by 55.2% ($p < 0.01$). In apoE −/− mice, the level of liver uptake of n-6 TG emulsions was ~40% of that of WT ($p < 0.01$), but adipose tissue uptake was 3-fold higher (25 min, Figure 5A). In contrast, deficiency in endogenous apoE or preincubation with exogenous apoE did not affect tissue uptake of n-3 TG emulsions (Figure 5B).

*Roles of LDL-R and Lactoferrin-Sensitive Pathways in Catabolism of n-6 TG and n-3 TG Emulsions.* LDL-R, LRP, and HSPG are important pathways for chylomicrons and their remnant catabolism (*13*−*17*). We investigated the role of LDL-R on n-6 and n-3 TG emulsion removal. Also, lactoferrin (2 mg/mouse), a compound that inhibits LRP- and

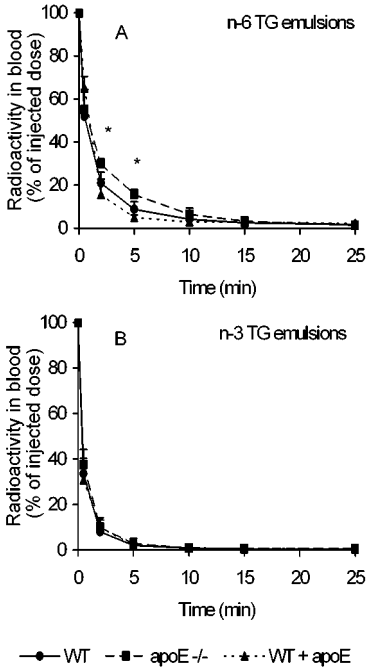


FIGURE 4: Effects of apoE on blood clearance of n-6 TG and n-3 TG emulsions in mice. Apo E −/− and WT mice were given [$^3$H]-CEt-labeled emulsions (400 $\mu$g of TG/mouse) intravenously. In experiments with added apoE, emulsions were incubated with apoE (20/1 TG/apoE, w/w) for 30 min at room temperature before injection. Blood clearance was assayed as described in Experimental Procedures: (●) WT, (■) apoE −/−, and (▲) WT treated with emulsions preincubated with exogenous apoE. (A) n-6 TG emulsions. (B) n-3 TG emulsions. Values are means ± SD of a representative experiment ($n$ = 4−6 in each group). Asterisks denote $p < 0.05$ between apoE −/− and WT mice.

HSPG-mediated TGRP clearance (*9*, *14*), was injected into mice (2 min before emulsion injection) to determine its effect on lipid emulsion clearance. As shown in Figure 6, deficiency in LDL-R decreased blood clearance of n-6 TG emulsions (FCR = 13.3 ± 1.1 pools/h) compared to that of WT mice (FCR = 17.5 ± 3.0 pools/h) ($p < 0.05$). Preinjection of lactoferrin into WT and LDL-R −/− mice also slowed n-6 TG emulsion clearance (FCR = 10.1 ± 0.8 and 8.9 ± 0.8 pools/h) with stronger effects than in the absence of LDL-R alone. Still, in all three groups, most n-6 TG emulsion particles were cleared from blood, with only ∼7% of the injected dose remaining 25 min after injection. In contrast, LDL-R deficiency or preinjection of lactoferrin did not affect the blood clearance of n-3 TG emulsions.

Figure 7 demonstrates that, in the absence of LDL-R, heart and liver uptake of n-6 TG emulsions decreased 61 and 37%, respectively ($p < 0.05$), and adipose tissue uptake increased 1.6-fold compared to that of WT mice ($p < 0.001$). Preinjection of lactoferrin into both WT mice and LDL-R −/− mice led to 3.5- and 1.5-fold increases in uptake of n-6 TG emulsions by adipose tissue, but to 60 and 70% reductions in heart uptake, respectively ($p < 0.01$). No significant changes in tissue uptake of n-3 TG emulsions were found in LDL-R deficiency or with lactoferrin injection (Figure 7B).

*Effects of ApoE and Lactoferrin on Cell Uptake of n-6 TG and n-3 TG Emulsions in Human Fibroblasts in Vitro.* To determine if the in vivo effects of apoE and lactoferrin on emulsion clearance were due to potential interaction with cell-mediated clearance pathways, in vitro cell culture

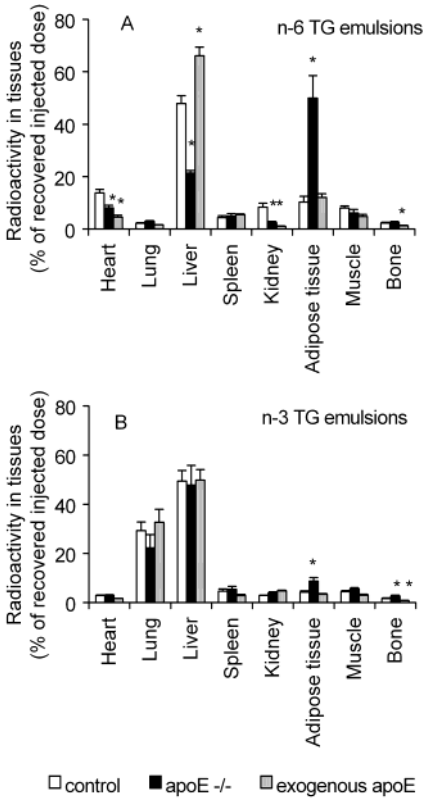


FIGURE 5: Effects of apoE on tissue uptake of n-6 TG and n-3 TG emulsions in mice. Organs and tissues of each mouse described in the legend of Figure 4 were obtained 25 min after the final blood sample was taken. The amount of radioactivity in tissues was measured as described in Experimental Procedures. Tissue uptake was expressed as % of the recovered injected dose. (A) n-6 TG emulsions. (B) n-3 TG emulsions. Values are means ± SEM ($n$ = 4−6). Asterisks denote $p < 0.05$ compared to WT mice.

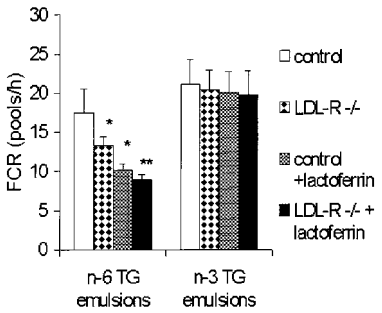


FIGURE 6: Effects of LDL-R deficiency and lactoferrin on blood clearance of n-6 TG and n-3 TG emulsions in mice. Bovine lactoferrin (2 mg/mouse) was injected into mice 2 min before [$^3$H]-CEt-labeled emulsions (400 $\mu$g of TG/mouse) were administered. The amount of radioactivity in blood was assayed, and FCR were calculated as in Experimental Procedures. Results are from one experiment typical of two others. Values are means ± SD ($n$ = 5 or 6 in each group). Asterisks denote $p < 0.05$ compared to WT mice. Two asterisks denote $p < 0.05$ compared to LDL-R −/− mice.

experiments were performed using human fibroblasts, a cell line in which we have previously characterized apoE-mediated TGRP uptake (*25*, *34*). As expected, apoE increased n-6 TG emulsion uptake more than 15-fold; however, apoE had an effect on n-3 TG emulsion uptake that was much lower in magnitude, and addition of lactoferrin almost abolished the effects of apoE on emulsion uptake (Figure 8). These data suggest that apoE had little or no effect on

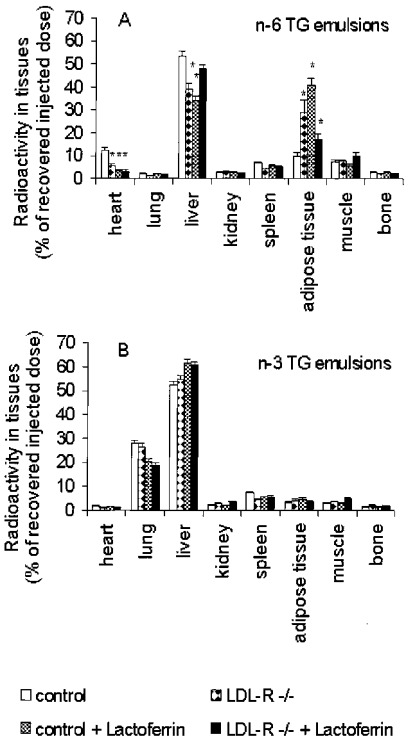


FIGURE 7: Effects of LDL-R deficiency and lactoferrin on tissue uptake of n-6 TG and n-3 TG emulsions in mice. Values for organs and tissues of each mouse described in the legend of Figure 6 were obtained 25 min after the final blood sample was drawn. The amount of radioactivity in tissues was measured as described in Experimental Procedures. Tissue uptake was expressed as % of recovered injected dose. (A) n-6 TG emulsions. (B) n-3 TG emulsions. Values are means ± SEM ($n = 5$ or 6 for each group). Asterisks denote $p < 0.05$ compared to WT mice.

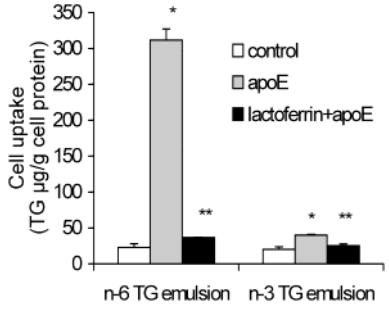


FIGURE 8: Effects of apoE and lactoferrin on n-6 TG and n-3 TG emulsion uptake by human fibroblasts. Human fibroblasts were incubated with lipid emulsions (250 $\mu$g of TG/mL) for 4 h at 37 °C in the absence or presence of lactoferrin and apoE. In experiments with apoE, the emulsions were incubated with apoE (20/1 TG/apoE, w/w) before injection as described in Experimental Procedures. To determine the effects of lactoferrin on apoE-mediated emulsion uptake, lactoferrin (2 mg/mL) was also added to the medium. Results represent means ± SD of triplicate incubations from one experiment typical of two others. One asterisk denotes significant differences between the absence and presence of apoE. Two asterisks denote significant differences between the absence and presence of lactoferrin in the presence of apoE.

modulating cell uptake of n-3 TG emulsion compared to n-6 TG emulsion, in keeping with our in vivo findings (Figures 4 and 5).

## DISCUSSION

The effects of TG composition, comparing MCT to LCT, on the catabolism of lipid emulsions have been previously studied (*18*, *19*), but the effects of n-3 TG on emulsion catabolism are still poorly defined. In this study, we investigated the effects of n-3 TG on the catabolism of phospholipid-stabilized emulsions. n-3 TG emulsion particles were cleared faster from blood and had different profiles of tissue uptake compared to n-6 TG emulsions, suggesting different catabolic pathways contribute to the faster clearance of n-3 TG than of n-6 TG emulsions.

Hultin et al. (*12*) found that a phospholipid-stabilized emulsion, very similar to the n-6 TG emulsion used in our studies, was removed from blood with little preceding lipolysis and taken up by both hepatic and peripheral tissues as essentially nonlipolyzed intact particles in the fed state. They also found that fasting increased the level of lipolysis and increased core particle removal by liver (*12*). Consistent with their findings, we demonstrated that fasting increased liver uptake of both emulsion particles with concomitant decreased extrahepatic tissue uptake, and that more emulsion particles were targeted to extrahepatic tissues in the fed state. Although we did not double-label the emulsions (with core and TG labels), the differences in tissue uptake of n-6 and n-3 TG emulsion particles between the fasted and fed states are in agreement with the previous studies indicating much less lipolysis in fed rats (*12*).

Of relevant interest, the metabolism of n-6 TG emulsion (Intralipid) has been studied by using electron microscopy and full-sized emulsion droplets were observed trapped in the space of Disse, and intact large emulsion droplets were present in hepatocytes next to mitochondria (*36*). This demonstrated that large particles, with little or no preceding lipolysis, can penetrate the fenestrated endothelium and enter liver cells intact for intracellular degradation. Also, Savonen et al. (*37*) demonstrated that the core label of intact chylomicrons was distributed similarly to both liver and peripheral tissues in a mink model that lacks LPL as in normal mink. Thus, tissue uptake of intact whole particles with little hydrolysis can be a major pathway for lipid emulsion clearance in vivo.

Our current findings are consistent with another study in our laboratory that showed that remnant-size n-3 tridocosahexaenoin emulsions are cleared faster than remnant-size n-6 triolein emulsions and trioctanion/triolein (1/1, w/w) emulsions (*38*).[2] Also, n-3 TG reduces plasma TG levels in rat and mice by accelerating chylomicron and VLDL particle clearance (*39*, *40*), an effect not associated with differences in lipolysis (*40*). In humans, addition of 10% fish oil TG to MCT/LCT intravenous lipid emulsions enhanced elimination of infused TG, a difference not related to a higher rate of hydrolysis, but rather to a more efficient uptake of emulsion particles (*41*). Therefore, addition of fish oil n-3 TG to conventional emulsions can change blood clearance and, as we now demonstrate, specific tissue uptakes. In addition to n-3 fatty acid inhibition of endogenous TG synthesis (*42*),

[2] In our previous paper (*27*), comparing clearance of fish oil-containing emulsions and LCT- and MCT-containing emulsions, we noted slower blood clearance of pure fish oil emulsions than of other emulsions. However, these earlier experiments were carried out with emulsions made from a fish oil preparation that is no longer available. Since then, in all fish oil-containing emulsions that were tested, either provided by a commercial manufacturer or produced in our laboratory, faster clearance of fish oil emulsions is consistently observed, with very similar results as demonstrated herein.

this faster removal of n-3 TG-containing particles might be a mechanism contributing to lower plasma TG levels during high-fish oil diets in humans and animals (*43*−*45*).

Differences in blood clearance between n-6 and n-3 TG emulsions are not likely influenced by different levels of lipolysis for the following reasons. First, phospholipid-stabilized emulsions undergo little in vivo lipolysis in the fed state (*12*), and second, in vitro lipolysis studies with LPL showed much lower, not higher, lipolytic activities on n-3 TG than on n-6 TG emulsions (*21*). In addition, since the particle sizes of the two emulsions used herein were the same, this factor did not contribute to the differences in clearance. Likely, the observed differences in apparent catabolism reflect differences in physiochemical particle properties induced by n-3 TG, which would affect their interaction at cell surfaces.

Since little lipolysis of emulsions occurs in the fed state, LPL may mainly functions as a "bridge" protein (*46*−*49*) to mediate emulsion particle uptake. In our study, heparin greatly increased clearance of n-6 TG emulsions with decreased extrahepatic tissue uptake and increased hepatic uptake. This is consistent with previous data showing that the catabolism of endogenous LPL released by intravenous lipid infusion versus heparin is different (*50*, *51*). LPL released by lipid emulsion (Intralipid) injection is removed by adipose tissue, heart, and red muscle. Alternately, LPL released by heparin is mainly taken up by liver (*51*), where we suggest it acts to increase n-6 TG emulsion particle uptake as a bridging molecule.

Our data showed that Triton WR 1339 greatly impaired clearance of n-6 TG emulsions, in keeping with previous findings that Triton WR 1339 inhibited lipase action and impaired TGRP blood clearance (*22*, *52*). Still, its influence on emulsion clearance in the current study may be more ascribed to its action on preventing LPL from binding to emulsions (*53*) and blocking the bridge function of LPL due to little lipolysis of emulsions in the fed state. Again, the different effects of heparin and Triton WR 1339 on the catabolism of n-6 TG versus n-3 TG emulsions are presumably caused by their different affinities of binding to LPL or Triton WR 1339 rather than by differences in lipolysis. Clearly, LPL-mediated particle removal is not a major route for the n-3 TG emulsions. This might be one reason LPL-rich tissues such as heart, adipose tissue, and muscle took up substantially less n-3 TG than n-6 TG chylomicron-sized emulsions.

ApoE has a critical role in the catabolism of triglyceride-rich lipoproteins, mediating their clearance via the LDL-R, LRP, and HSPG pathways (*13*−*17*). Addition of purified apoE increased plasma clearance and liver uptake of chylomicrons and TGRP (*54*, *55*). This is in keeping with our results which show that preincubation of apoE with emulsions increased liver uptake of n-6 TG emulsion particles. In the current study, apoE knockout or LDL-R deficiency slowed blood clearance of n-6 TG emulsions and decreased liver uptake. This might be ascribed to competition of the elevated plasma level endogenous TG-rich lipoproteins (*16*) for common clearance pathways with emulsion particles, in addition to the loss of apoE and LDL-R. However, apoE and LDL-R did not affect removal of n-3 TG emulsions, indicating that different pathways contribute to the clearance of n-3 TG emulsions compared to n-6 TG emulsions. Also, in vitro cell culture studies showed much greater effects of apoE on cell uptake of n-6 TG than n-3 TG emulsions.

LRP and HSPG, together with LDL-R, are important mediators for TGRP removal (*13*−*17*, *56*, *57*). Lactoferrin has been demonstrated to impair TGRP clearance and decrease liver uptake (*9*, *14*, *32*, *33*) by inhibiting and blocking interaction of apoE with LRP and HSPG (*58*). Consistent with those data, we demonstrated that lactoferrin inhibited n-6 TG emulsion clearance in vivo, and it also blocked apoE-mediated particle uptake in vitro. The impaired clearance of n-6 TG emulsions after preinjection of lactoferrin might have been offset, to some extent, by the increased association with adipose tissue. Possibly, adipose tissue acts as a "buffer" system to clear emulsion particles when normal removal pathways are impaired. In contrast, clearance of n-3 TG emulsions was not affected by lactoferrin.

In summary, particle TG composition had a major influence on the catabolism of chylomicron-sized lipid emulsions. The removal of n-6 TG emulsions is dependent on LPL-, apoE-, LDL-R-, and lactoferrin-sensitive pathways, whereas the clearance of n-3 TG emulsions only relies on LPL to a very minor extent and is independent of apoE-, LDL-R-, and lactoferrin-sensitive pathways. The observed differences in apparent catabolism may reflect differences in physicochemical properties associated with different TG. The mechanisms responsible for n-3 TG emulsion removal still need to be elucidated. Studying these clearance mechanisms should also contribute to a better understanding of the carriage and metabolism of n-3 TG-rich particles in humans on diets high in fish oil.

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