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Lipolysis-induced partitioning of free fatty acids to lipoproteins: effect on the biological properties of free fatty acids

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Abstract Free fatty acids (FFA) released during the lipolysis of triglyceride (TG)-rich lipoproteins in vivo are generally believed to be bound to serum albumin. When hypertriglyceridemic (HTG) sera were lipolyzed in vitro by purified bovine milk lipoprotein lipase (LpL), there was an 11- to 18-fold increase in serum FFA levels, and a major portion (> 80%) of the FFA in serum was partitioned to lipoprotein fractions. The greatest portion (33%) of FFA in lipolyzed HTG serum was associated with newly formed flocculent remnants that banded just below low density lipoproteins (LDL) in the density gradient tube. Very low density lipoprotein (VLDL), LDL, and high density lipoprotein (HDL) fractions in lipolyzed HTG serum contained 18- to 29-times more FFA molecules than those in prelipolysis serum. Analysis of the fatty acyl chain composition of FFA in lipolyzed HTG serum showed that the extent of partitioning of saturated FFA into the lipoprotein fractions relative to that of polyunsaturated FFA was about 4.5- to 11-times greater than that partitioned into the free protein fraction; most (84%) of FFA partitioned into flocculent remnants were saturated fatty acids. In vivo lipolysis of TG-rich lipoproteins in HTG subjects, induced by heparinization, resulted in only a small (2.8-fold) increase in serum FFA and little or no increase in the partitioning of FFA to lipoproteins. However, in vitro incubation of the postheparin serum at 37°C for 90 min resulted in a 2.9- to 6.8-fold increase in the serum FFA level and the partitioning of > 66% of total serum FFA into lipoprotein fractions. Studies of the interaction of various plasma fractions from control and in vitro lipolyzed HTG serum with cultured mouse peritoneal macrophages (MPM) showed that FFA partitioned to lipoprotein fractions were highly cytotoxic to cultured MPM, whereas FFA partitioned to albumin at a 10 × greater concentration were not cytotoxic. The cytotoxic potencies of FFA bound to lipoproteins and albumin were further compared after in vitro incorporation of FFA (oleic acids) into LDL and to albumin. FFA bound to LDL but not to albumin were cytotoxic to cultured MPM; the cytotoxicity of FFA bound to LDL was more closely related to the FFA to LDL-cholesterol molar ratio than to the total FFA concentration in the culture dish. The ability of FFA bound to LDL and albumin to induce foam cell formation was studied in THP-1 monocyte-derived macrophages, which were less susceptible to cytotoxicity produced by FFA bound to LDL than MPM. With a sublethal dose, FFA bound to LDL induced a significantly greater accumulation of cellular TG than did the same amount of FFA bound to albumin. The data from this study suggest that the partitioning of an excess amount of FFA to lipoproteins, which could occur locally at the site of lipolysis in vivo, may be atherogenic.—**Chung, B. H., G. A. Tallis, B. H. S. Cho, J. P. Segrest, and Y. Henkin.** Lipolysis-induced partitioning of free fatty acids to lipoproteins: effect on the biological properties of free fatty acids. *J. Lipid Res.* 1995. **36:** 1956–1970.

Supplementary key words triglyceride-rich lipoproteins • macrophages • cytotoxicity • foam cells

Triglyceride (TG)-rich chylomicrons and VLDL are the major transport vehicles of dietary and endogenously synthesized fat in circulating blood (1, 2). An initial step in the catabolism of TG-rich lipoproteins involves the hydrolysis of the TG moiety of the particles by LpL bound to vascular endothelium, resulting in the release of FFA and glycerol (3, 4). The lipolytically generated FFA are important sources of energy for certain cells and are substrates for lipid biosynthesis (5), but these FFA have also been implicated as a causal factor in atherogenesis (6, 7). Injury to arterial endothelium and foam cell formation are key events in the

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Abbreviations: FFA, free fatty acid; TG, triglyceride; HTG, hypertriglyceridemia; LpL, lipoprotein lipase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; MPM, mouse peritoneal macrophages; VAP, vertical autoprofiler; apo, apolipoprotein

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pathogenesis of atherosclerosis (8), and FFA released during lipolysis of TG-rich lipoproteins may be involved in both events. Zilversmit (6) hypothesized that the liberation of FFA during LpL-mediated hydrolysis of TG-rich lipoproteins on the arterial endothelium may be responsible for local endothelial cell injury, allowing cholesterol-rich remnant lipoproteins to enter the arterial wall. Hennig, Shasby, and Spector (9) showed that exposing cultured endothelial cells to FFA increased the transfer of macromolecules across the endothelial monolayers. The harmful effect of high FFA concentration on other cells has been reported (10). In vitro studies have shown that enrichment of the culture medium with FFA can induce foam cell formation in various cultured cells including macrophages (11–14). Laughton et al. (15) showed that sera from patients with coronary heart disease caused greater accumulation of neutral lipids in cultured arterial smooth muscle cells than did sera from control subjects. The serum factor that most strongly correlated with neutral lipid accumulation was the FFA concentration, whereas the total serum cholesterol and TG did not show any significant association.

Under normal conditions, FFA in circulating blood are mostly associated with albumin which is known to have both high and low affinity binding sites for FFA (16, 17). It has been shown that FFA bind to lipoproteins when plasma FFA concentration is increased by heparinization (18, 19) or when the plasma albumin concentration is decreased in certain pathological states, such as in nephrotic syndrome (20). Cistola and Small (21) recently demonstrated that the binding of FFA to lipoproteins occurs in plasma having a normal FFA to albumin molar ratio.

Most in vitro studies measuring biological properties of FFA in cultured cells have been performed with FFA bound to albumin where the FFA/albumin molar ratio ranged from 3 to 6 or higher (9, 22–24), even though in normal human subjects the FFA/albumin molar ratio ranges from 0.5 to 1.5 (15). The metabolic properties of FFA bound to lipoproteins are not well defined with respect to either FFA or lipoproteins. We have previously reported that in vitro lipolysis of HTG serum caused the whole serum or lipoprotein fractions but not the free protein fraction in lipolyzed serum to become cytotoxic to cultured MPM and endothelial cells (25, 26) or disruptive to the endothelial cell barrier function (27). Because high levels of FFA associated with the free protein fraction of lipolyzed serum were not cytotoxic to cultured macrophages, we initially suggested that FFA may not be the cytotoxic factor (25). The present study demonstrates that FFA are indeed a responsible cytotoxic factor associated with lipolyzed HTG serum, but that this cytotoxic effect of FFA in lipolyzed HTG serum is dependent on the degree of the partitioning of FFA to the lipoprotein fractions.

METHODS

Materials

HTG sera were obtained from the Alabama Regional Blood Center, Birmingham, AL 2–8 days after the collection of the blood. Lipoprotein cholesterol profiles of these sera, analyzed by the vertical autoprofiler (VAP) method (28), showed that they were either type IV or type V hyperlipidemic sera. Fresh fasting and postprandial lipemic sera were obtained from two normolipidemic volunteer subjects (52- and 53-year-old Asian males), who were previously identified as markedly HTG in response to fatty meals. Fasting and postprandial lipemic serum were obtained from these subjects after an overnight fast and 5 h after feeding the subjects a meal rich in fat. The meal, which was prepared at the research kitchen of the UAB General Clinical Research Center, consisted of 15% calories from proteins, 20% calories from carbohydrates, and 65% calories from fat (polyunsaturated fat to saturated fat (P/S) ratio = 2.66) and contained 600 mg of cholesterol. The meal was calculated on the basis of 50 g of fat/m^2 body surface. Lipoprotein cholesterol and TG profiles of fasting and postprandial serum were examined by the

TABLE 1. Changes in serum triglycerides (TG) and free fatty acid (FFA) concentrations after in vitro lipolysis of hypertriglyceridemic whole serum by purified bovine milk lipoprotein lipase

Subjects	Pre-Lipolysis			Post-Lipolysis	
	Cholesterol	TG	FFA	TG	FFA
	mg/dl		*mM*	*mg/dl*	*mM*
A	192	542	1.01	96	14.6
B	159	735	1.19	172	16.7
C	182	546	0.95	139	11.1
D	220	1,123	1.49	470	17.8
E	202	1,007	0.89	384	16.1
Mean ± SD	191 ± 23	790 ± 265	1.10 ± 0.24	252 ± 164	15.2 ± 2.6

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ipoprotein cholesterol autoprofiler method, described previously (28), after density gradient ultracentrifugal separation of lipoproteins in plasma by using a swingout rotor (Sorvall AH 650 rotor). LpL was isolated from raw bovine milk and purified by the heparin-agarose affinity chromatographic method described by Iverius and Ostlund-Lindqvist (29). Unstimulated macrophages were harvested from the peritoneal cavity of CD1 white male mice (Charles River Breeding Laboratory, Wilmington, MA) according to the method of Edelson and Cohn (30) and plated in 24-well culture dishes having glass cover slips. THP-1 human monocytic leukemia cells were kindly provided by Drs. Gianturco and Bradley at the University of Alabama at Birmingham. Oleic acid and paraoxon were purchased from Sigma Chemical Co., St. Louis, MO, and fatty acid-depleted bovine serum albumin was purchased from Boehringer Mannheim Biochemical Co., Indianapolis, IN. Cell culture media and supplies were purchased from both Sigma and Fisher Chemical Co. (Fairlawn, NJ).

In vitro and in vivo lipolysis of TG-rich lipoproteins and the enrichment of lipoproteins and albumin with FFA

In vitro lipolysis of TG-rich lipoproteins in HTG serum and/or in fasting and postprandial serum from normolipidemic subjects was achieved by adding purified LpL into whole serum (10 μl/ml serum) and incubating the mixtures at 37°C for 90 min. Serum incubated with heat-inactivated LpL was used as a control. In vivo lipolysis of TG-rich lipoproteins was achieved by intravenous injection of sodium heparin (50 unit/kg) into HTG subjects, all of whom gave informed consent. Postheparin blood samples were collected 30 and 60 min after injection of heparin in blood collection tubes with or without the addition of paraoxon (diethyl *p*-nitrophenyl phosphate), an inhibitor of LpL (4 μl/10 ml blood). Blood samples were centrifuged at 3000 rpm for 15 min at 4°C to separate the serum from red cells. A portion of postheparin serum was incubated at 37°C for 2 h to allow further in vitro lipolysis of TG-rich lipoproteins by endogenous lipolytic enzymes in postheparin serum. The extent of lipolysis in whole serum or in the VLDL fraction was estimated by measuring the TG concentration using an enzymatic assay kit (Boehringer Mannheim Reagent set #348292, Biodynamics/BMC Co., Indianapolis, IN) after the removal of free glycerol by dialysis. The lipolysis-induced change in the serum lipoprotein cholesterol profiles and absorbance profiles was examined by the VAP method (28) and by measuring the absorbances of the effluents from density gradient tubes at 280 nm. FFA-enriched LDL or albumin was prepared by slowly injecting 0.25-5.0 μM FFA in 20 μl ethanol into LDL or albumin solution (2.0 ml) using a Hamilton micro syringe while the solution was stirred. The mixtures of FFA-LDL or FFA-albumin were then dialyzed against buffered saline (0.01 M Tris, 0.15 M NaCl, pH 7.2) and filtered through 0.45 μm membranes (Millipore Corp., Milford, MA). Total cholesterol, TG, and FFA in whole serum and isolated lipoproteins and/or free protein fractions were measured by enzymatic assay kits (Boehringer Mannheim reagent set #1082, #12408, and #13905, Boehringer Mannheim Biochemical Co.). The albumin concentration in the free protein fraction was measured by the bromcresol green reagent set (Sigma Chemical Co.).

Level and composition of FFA associated with various plasma fractions

Pre- and postlipolysis samples of HTG serum were subjected to density gradient ultracentrifugation (31), and the major lipoprotein fractions (VLDL, LDL, and HDL) and plasma free protein fraction were quantitatively fractionated. Flocculent remnants that were collected along with LDL during fractionation were further separated from LDL by low speed centrifugation (6000 rpm for 30 min) after dialysis. All flocculent remnants but not LDL were pelletable by this centrifugation step. Levels of FFA and/or cholesterol in each plasma fraction were measured by using enzymatic assay kits (Boehringer Mannheim reagent set #1082 and #13905). In

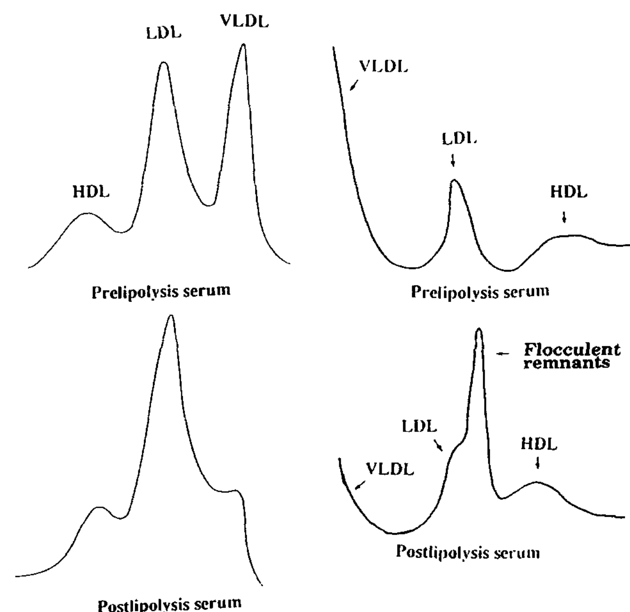

Fig. 1. Lipoprotein cholesterol profiles and absorbance profiles of control and in vitro lipolyzed samples of serum from a HTG subject. The lipoprotein cholesterol profiles (left) of control HTG serum (top) and lipolyzed HTG serum (bottom) were analyzed by the VAP. The density gradient separation of serum samples for absorbance profiles (right) was done in a Sorvall vertical rotor (TV 850), which holds 34 ml size centrifuge tubes, and the fractionation of serum samples in the density gradient tubes were done by the upward flow method.

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TABLE 2. Distribution of cholesterol (CH) and free fatty acids (FFA) among various plasma fractions of pre- and post-lipolysis HTG serum, and ratios of FFA to lipoproteins or albumin

Plasma Fractions	FFA		CH			
	mM	%	mg/dl	%	FFA/CH	FFA/Albumin
					mol/mol	
Pre-lipolysis						
VLDL	0.15 ± 0.07	13.4	96 ± 32	50.3	0.06 ± 0.02	
LDL	0.14 ± 0.11	13.1	75 ± 17	39.0	0.07 ± 0.02	
HDL	0.07 ± 0.06	5.9	20 ± 6	10.7	0.13 ± 0.09	
Free protein	0.74 ± 0.13	67.6				1.42 ± 0.50
Post-lipolysis						
VLDL	2.07 ± 1.33	13.6	74 ± 33	38.3	1.08 ± 0.46	
LDL	3.12 ± 0.89	20.5	93 ± 25	48.3	1.30 ± 0.70	
HDL	2.13 ± 0.61	14.1	22 ± 7	11.5	3.80 ± 0.99	
Flocculent remnants	4.97 ± 1.32	32.8	4 ±1	1.9	48.25 ± 14.83	
Free protein	2.88 ± 0.61	19.0				5.31 ± 2.70

Values are mean ± SD (n = 5).

certain experiments, fatty acid-depleted bovine serum albumin was added into tubes containing FFA-enriched lipoprotein fractions to give a molar ratio of FFA to albumin of 2, and the mixtures were then incubated at room temperature for 16 h. The levels of FFA associated with lipoproteins and/or albumin were measured after density gradient ultracentrifugal separation of lipoproteins from albumin, and the ratio of FFA to lipoprotein-cholesterol or FFA to albumin was then calculated.

In order to examine the composition of FFA in lipolyzed serum, total lipids were extracted from each plasma fraction by the method of Folch, Lees, and Sloane Stanley (32). The lipid extracts were applied to Silica gel thin-layer chromatographic plates, and FFA

TABLE 3. Fatty acyl chain composition of FFA associated with VLDL, LDL, HDL, flocculent remnants, and free protein fractions isolated from a post-lipolysis sample of hypertriglyceridemic serum, and percent distribution of saturated, monounsaturated, and polyunsaturated FFA in lipolyzed HTG serum among various plasma fractions

Fatty Acids	Plasma Fractions				
	VLDL	LDL	Flocculent Remnants	HDL	Free Proteins
A:			*% of total*		
14:0	1.8	1.6	2.0	1.8	1.3
16:0	14.2	24.8	62.7	11.5	6.2
16:1n-7	2.3	1.6	0.2	3.2	2.9
18:0	5.1	8.4	18.8	3.5	1.2
18:1n-9	50.2	44.8	13.8	60.5	41.7
18:1n-7	3.5	3.7	1.0	4.0	3.8
18:2n-6	20.7	13.8	1.2	14.9	38.0
18:3n-3	0.9	0.4	trace	0.3	2.9
20:3n-6	0.3	0.2	0.1	0.1	0.3
20:4n-6	0.9	0.7	0.2	0.2	1.5
22:6n-3	0.1				0.2
Saturated	21.1	34.8	83.5	16.8	8.7
Monounsaturated	56.0	50.1	15.0	67.7	48.4
Polyunsaturated	22.9	15.1	1.5	15.5	42.9
P/S ratio	1.08	0.43	0.02	0.92	4.93
B:			*% distribution among plasma fractions*		
Saturated FFA	8.8	22.7	58.4	4.8	5.3
Monounsaturated FFA	20.5	27.9	9.0	17.4	25.2
Polyunsaturated FFA	18.5	18.5	2.1	9.3	51.6

Values are average area % of duplicated analyses. The plasma fractions were fractionated from the lipolyzed HTG serum sample containing 12.7 mM FFA. Distribution of FFA among VLDL, LDL, flocculent remnants, HDL, and free proteins was 15.8%, 24.3%, 26.1%, 11.0%, and 22.8% of total serum FFA, respectively.

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TABLE 4. Change in levels of FFA on FFA-enriched lipoproteins isolated from post-lipolysis HTG serum after their treatment with fatty acid-poor albumin

Lipoprotein Fraction	Pre-Treatment		Post-Treatment	
	FFA Level	FFA/CH	FFA Level	FFA/CH
	mM	*mol/mol*	*mM*	*mol/mol*
VLDL	2.65	1.14	0.23	0.10
LDL	2.44	1.22	0.10	0.05
HDL	2.53	4.08	0.12	0.21
Flocculent remnant	2.54	46.25	0.59	11.20

FFA-enriched lipoproteins and flocculent remnants were incubated with and without fatty acid-poor bovine serum albumin at room temperature for 16 h. The molar ratio of FFA to albumin in the mixtures was adjusted to 2. The density of the incubated samples was adjusted to 1.21 g/ml, and the density-adjusted samples were ultracentrifuged at 49,000 rpm for 36 h in an AH 650 Sorvall swingout rotor. FFA to lipoprotein-cholesterol molar ratios of the lipoprotein fraction (upper 2.5 ml) were then determined.

was separated from other lipids by developing the plates in petroleum ether–diethyl ether–acetic acid 85:15:1 (v/v/v). Fatty acid methyl esters were prepared and analyzed by a Hewlett-Packard model 5790A gas chromatograph (Hewlett Packard Co., Avondale, PA) equipped with a fused silica capillary column and dual flame ionization detectors. Identification of methyl esters of fatty acids was accomplished by comparing retention times of the peaks of the samples with authentic fatty acid standard purchased from Nu-Chek Prep. (Elysian, MN).

Interaction of various serum fractions from pre- and postlipolysis HTG serum and FFA-enriched LDL or albumin with cultured macrophages

For measuring the cytotoxic potency of lipoproteins, 1–2 × 10^6 MPM were plated in 24-well culture dishes having glass cover slips. After the incubation of the culture dishes in a cell culture incubator for 4 h, non-adhering cells were removed by washing the culture dishes 3 × with buffered saline. A 1-ml serum-free culture medium (Medium 199) containing control and lipolyzed serum, fractionated lipoprotein and free protein fractions, or FFA-enriched LDL or albumin was added to the culture dishes; the levels of FFA in the culture medium ranged from 2 to 100 μM (2–100 nmol FFA/dish). The culture dishes were incubated for 18 h at 37°C in a humidified incubator with 5% CO_2. After incubation, the culture medium was removed, and each well was washed 2 × with 1 ml of buffered saline. MPM on the cover slips were then stained with 0.4% trypan blue solution (Sigma Chemical Co.) for 1–2 min. After washing away excess trypan blue with buffered saline, the cover slips were mounted on glass slides, and the numbers of stained (dead) and non-stained (viable) cells in several microscopic fields were counted (33). A minimum of 100 cells was counted. Cytotoxicity was expressed as the percentage of dead cells relative to a control dish (medium only).

To measure the ability of FFA bound to albumin or LDL to induce foam cell formation, THP-1 monocyte-derived macrophages cultured in 6-well cell culture

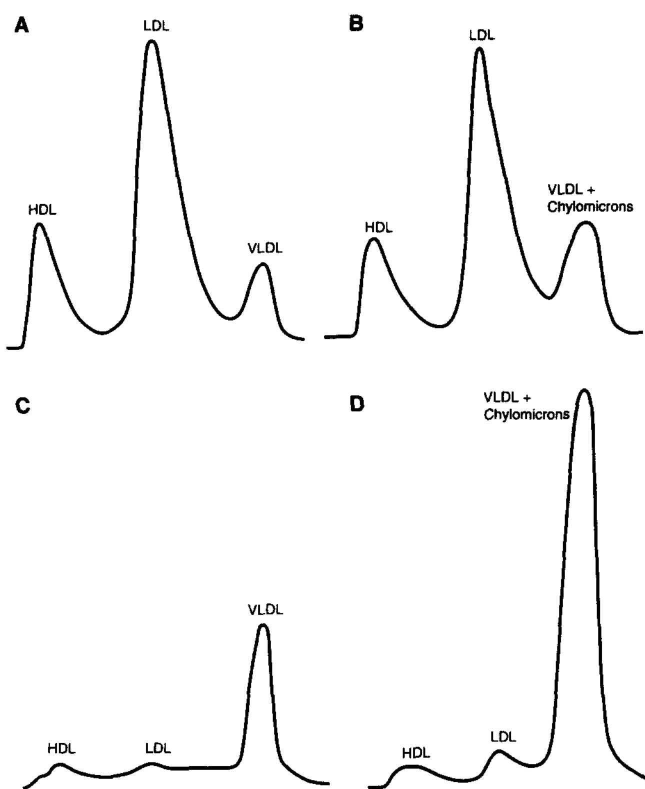

Fig. 2. Lipoprotein cholesterol and TG profiles of fasting and postprandial lipemic plasma from a normolipidemic subject (subject A) who exhibited a brisk postprandial lipemic respons. Profiles A–D are lipoprotein cholesterol profiles of fasting plasma (A) and postprandial plasma (B), and lipoprotein TG profiles of fasting plasma (C) and postprandial lipemic plasma (D). The levels of cholesterol associated with the VLDL, LDL, and HDL density fractions of fasting plasma (profile A) and postprandial plasma (profile B) were 28, 138, and 42 mg/dg (profile A) and 46, 132, and 39 mg/dl (profile B), respectively. The levels of TG associated with the VLDL, LDL, and HDL density fractions of fasting plasma (profile C) and postprandial lipemic plasma (profile D) were 128, 17, and 12 mg/dl (profile C), and 379, 44, and 33 mg/dl (profile D), respectively.

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TABLE 5. Extent of partitioning of free fatty acids to the lipoprotein (d < 1.21 g/ml) fraction of serum after in vitro lipolysis of fasting and postprandial lipemic sera from normolipidemic subjects with a brisk chylomicron response

Sample	Fasting Serum: Serum Level TG	Fasting Serum: Serum Level FFA	Fasting Serum: FFA on d <1.21 g/ml Fraction %	Fasting Serum: FFA on d <1.21 g/ml Fraction FFA/CH	Postprandial Serum: Serum Level TG	Postprandial Serum: Serum Level FFA	Postprandial Serum: FFA on d <1.21 g/ml Fraction %	Postprandial Serum: FFA on d <1.21 g/ml Fraction FFA/CH
	mg/dl	*mM*		*mol/mol*	*mg/dl*	*mM*		*mol/mol*
Pre-lipolysis								
Subject A	161	0.69	12.3	0.015	555	0.92	19.0	0.031
Subject B	157	0.59	11.6	0.013	456	1.19	20.2	0.043
Post-lipolysis								
Subject A		4.28	32.2	0.253		15.6	78.6	2.12
Subject B		4.59	33.1	0.268		10.6	63.8	1.07

Levels of TG in post-lipolysis samples were not determined.

plates were incubated with albumin, LDL, FFA-albumin, or FFA-LDL complexes. To produce THP-1 macrophages, suspension cultures of THP-1 monocytes (5–6 × 10^6 cells) grown in RPMI 1640 culture medium containing 10% fetal bovine serum were added to each well of the 6-well cell culture plates. The differentiation of monocytes into macrophages was induced by adding phorbol ester (phorbol-1,2 myristate-1,3 acetate, Sigma Chemical Co.) at 50 ng/ml culture medium. After overnight (16 h) incubation, nonadhered monocytes were removed by aspirating the culture medium and washing the culture dishes once with RPMI 1640 culture medium. Dishes were then incubated with fresh complete culture medium (RPMI medium containing 10% fetal bovine serum) until day 4 when the culture medium was replaced with RPMI 1640 plus 10% complete serum replacement medium (CPSR-1 medium, Sigma Chemical Co.); dishes were then incubated for a further 24 h. The culture medium was replaced with 6 ml serum replacement RPMI 1640 medium containing control LDL (1.8 mg LDL-cholesterol), FFA-LDL complexes (1.8 mg LDL-cholesterol containing 2 μM FFA, molar ratio of FFA/LDL cholesterol = 0.44), control albumin (28.5 mg), or FFA-albumin complexes (28.5 mg containing 2 μM FFA, molar ratio of FFA/albumin = 4.7). After the incubation of culture dishes for 24 h, the culture medium was removed by aspiration and the cells in the culture plates were washed 4 × with phosphate-buffered saline (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4). Cellular lipids were extracted 2 × with 2 ml of hexane–isopropanol mixtures 3:2 (v/v) and once with 2 ml hexane. The solvent extracts were pooled and aliquoted into three portions (0.6 ml, 1.8 ml, and 3.6 ml) in order to assay TG, total, and unesterified cholesterol, respectively. After evaporation of the solvent, TG, total cholesterol, and unesterified cholesterol were measured by enzymatic assay kits (Boehringer Mannheim reagent set #348292, #12048 and #139050). Cellular proteins were dissolved in 2 ml 0.1 N NaOH, and 0.05–0.25 ml aliquots were taken to assay the protein content (34).

RESULTS

Lipolysis-induced changes in the lipoprotein profile and changes in the concentration and distribution of FFA in the lipoprotein fractions of HTG serum

Table 1 shows the change in the serum levels of TG and FFA after in vitro lipolysis of HTG sera, and **Fig. 1** shows the lipoprotein cholesterol profiles (left) and the

TABLE 6. Change in serum free fatty acid concentration after heparin-induced in vivo lipolysis and/or in vitro incubation of 30 min postheparin serum

Subjects	Preheparin Serum TG	Preheparin Serum FFA	Postheparin Serum (+Paraoxon) FFA	Postheparin Serum (-Paraoxon) FFA	Postheparin Serum Incubated at 37°C for 90 min FFA
	mg/dl		*mM*		
1	505	0.89	1.10	1.20	3.23
2	940	0.38	1.39	2.27	9.50
3	740	0.24	2.25	2.65	9.46
4	549	0.76	1.68	1.93	7.37
Mean ± SD	683 ± 199	0.56 ± 0.30	1.60 ± 0.49	2.01 ± 0.61	7.39 ± 2.94

(Column group header: Levels of TG and/or FFA)

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TABLE 7. Distribution of FFA among VLDL, LDL, HDL, and free protein fractions of preheparin serum, 30 min postheparin serum, and in vitro incubated postheparin serum

	Level (mM) and Distribution (%) of FFA among Plasma Fractions							
	VLDL		LDL		HDL		Free Protein	
Samples	mM	%	mM	%	mM	%	mM	%
Preheparin serum	0.08	18.9	0.02	4.4	0.04	8.1	0.31	68.6
Postheparin serum	0.13	7.7	0.05	2.9	0.11	6.5	1.41	82.9
Incubated postheparin serum	2.05	19.3	3.59	33.7	2.48	23.3	2.53	23.7

The control postheparin serum was obtained from 30 min postheparin blood collected in tubes containing paraoxon and was placed in an ice bath. The post-incubated postheparin serum was obtained after 90 min incubation at 37°C of the postheparin serum containing no paraoxon.

280 nm absorbance profiles (right) of pre- and post-lipolysis samples of serum from an HTG subject. In vitro lipolysis of HTG sera with purified LpL resulted in the hydrolysis of 58–82% of TG and an 11- to 18-fold increase in the FFA level (Table 1). Sixty seven to 90% of TG associated with lipoproteins in the VLDL density were hydrolyzed and/or removed from the VLDL density region after in vitro lipolysis (data not shown). In vitro lipolysis of HTG sera resulted in the removal of about 70% VLDL cholesterol mass from the VLDL density region; most of the displaced VLDL cholesterol was now associated with the IDL–LDL density regions (Fig. 1, left). After in vitro lipolysis, the peak center of HDL shifted to the lower density regions of the density gradient tube (Fig. 1, left).

The absorbance profiles of pre- and post-lipolysis samples of HTG serum (Fig. 1, right) showed that in vitro lipolysis resulted in a significant decrease in the VLDL absorbance peak and the appearance of a new large absorbance peak on the dense side of the LDL peak. The new absorbance peak was comprised of white flocculent material. The flocculent remnants were usually collected with LDL during fractionation but could be separated from LDL by low speed centrifugation (6000 rpm for 30 min in a Sorvall OTD-2 low-speed centrifuge) after dialysis. The flocculent remnants, but not LDL, were easily pelletable by the above centrifugation step.

Table 2 shows the distribution of FFA among various serum fractions of pre- and post-lipolysis samples of HTG serum and molar ratios of FFA to lipoprotein cholesterol or to albumin. In pre-lipolysis serum, a major portion (> 67%) of FFA was associated with the free protein (albumin) fraction while the remaining FFA was distributed among VLDL, LDL, and HDL (Table 2). In post-lipolysis HTG serum, a major portion (81%) of

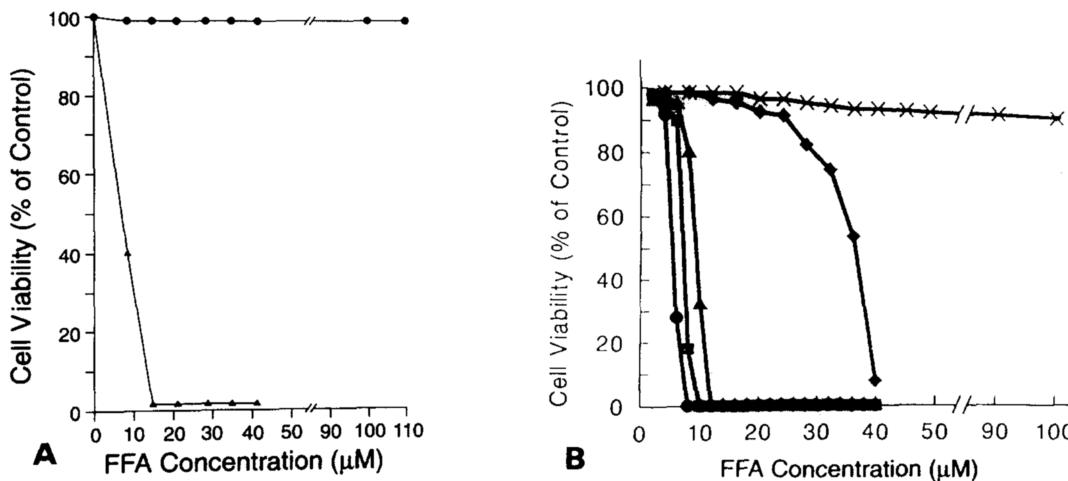

Fig. 3. A: Effects of the levels of FFA in pre- and post-lipolysis samples of HTG serum on the viability of cultured mouse peritoneal macrophages. Cultured MPM in duplicate was incubated with increasing amounts of FFA in control HTG serum (● - ●) and in vitro lipolyzed HTG serum (▲ - ▲) for 18 h, and then the viability of cells was determined as described above. Each value is a mean of duplicated determination. FFA levels of pre- and post-lipolysis HTG serum were 0.92 mM and 14.9 mM, respectively. B: Effects of the FFA concentration associated with various plasma fractions of post-lipolysis HTG serum on the viability of mouse peritoneal macrophages. Cultured MPM in duplicate was incubated with increasing amounts of FFA associated with VLDL (● - ●), LDL (■ - ■), HDL (▲ - ▲), flocculent remnant (◆ - ◆), and free protein (x - x) fractions of lipolyzed HTG serum for 18 h, and then the viability of cells was determined as described above. Each value is an mean of duplicated determination. The FFA to lipoprotein cholesterol molar ratios of VLDL, LDL, flocculent remnants, and HDL were 1.42, 2.26, 36.8, and 5.02, respectively.

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serum FFA was associated with the plasma lipoprotein fraction (Table 2); the concentration of FFA associated with the serum-free protein (albumin) fraction was about 4 × that of pre-lipolysis sera but only represented about 19% of the total serum FFA (Table 2). The largest portion (32.8%) of FFA in post-lipolysis HTG serum was associated with the newly formed flocculent remnants that contained only a small fraction (1.9%) of serum total cholesterol (Table 2). VLDL, LDL, and HDL in HTG serum became markedly enriched with FFA; the molar ratio of FFA to lipoprotein-cholesterol was 18–29 × greater in post-lipolysis than in pre-lipolysis serum (Table 2). The molar ratio of FFA to albumin in post-lipolysis samples of HTG serum was about 3.7 × greater than that in pre-lipolysis serum (Table 2). The extent of lipolysis-induced enrichment of lipoproteins with FFA was much greater for HDL (29 ×) than for VLDL (18 ×) or LDL (19 ×), and the FFA to cholesterol molar ratio of the flocculent remnants was much greater than that of HDL (Table 2). We have previously characterized the composition and morphology of flocculent remnants formed during in vitro lipolysis of HTG serum (35). These particles were shown to be multilamellar liposome-like vesicles, markedly enriched with FFA (> 80% of total mass); they were rich in surface lipid components of TG-rich lipoproteins (unesterified cholesterol and phospholipid) and poor in neutral core lipids and contained apoA-I and apoCs but no detectable apoB (35). The compositional and morphological characteristics of flocculent remnants suggest that they may represent the surface remnants of TG-rich lipoproteins.

We have further characterized the fatty acyl chain composition of FFA associated with various lipoprotein and plasma free protein fractions obtained from a post-lipolysis sample of HTG serum (**Table 3A**). Saturated FFA, monounsaturated FFA, and polyunsaturated FFA constituted 37.4%, 43.5%, and 19.1% of total FFA in lipolyzed HTG serum, respectively; the distribution of these FFA among various plasma fractions is further summarized in Table 3B. The P/S ratios of FFA associated with various plasma fractions (Table 3A) indicate that the degrees of partitioning of saturated FFA into lipoprotein fractions relative to that of polyunsaturated FFA are about 4.5- to 11-fold greater than that partitioned into the plasma free protein fraction. The free protein fraction, which carries 22.8% of total serum FFA, contained 51.6% of total polyunsaturated FFA in lipolyzed serum (Table 3B). FFA partitioned into flocculent remnants are mostly saturated fatty acids, and thus, the flocculent remnant fraction contained 58.4% of total saturated FFA in lipolyzed serum (Table 3A and B). Monounsaturated FFA are partitioned somewhat more uniformly among various plasma fractions than saturated or polyunsaturated FFA (Tables 3A and B).

We found that the treatment of FFA-enriched lipoproteins with fatty acid-poor albumin at FFA to albumin molar ratios of 2 in the mixture removed more than 90% of the FFA associated with VLDL, LDL, and HDL, thereby lowering the FFA to lipoprotein cholesterol molar ratios of lipoproteins in post-lipolysis serum to about the same ratio as those in pre-lipolysis serum (**Table 4**). The removal of FFA from the flocculent remnants by albumin treatment was more difficult compared to their removal from lipoproteins. The flocculent remnants retained a considerable amount (23%) of FFA after treatment with albumin (Table 4).

We also examined the extent of the partitioning of FFA to lipoproteins following in vitro lipolysis of fasting and postprandial lipemic serum from two normolipidemic subjects with a brisk chylomicron response. Postprandial lipemia caused 3.0- to 3.5-fold increases in the level of plasma TG mainly due to an increase of TG in the VLDL density fraction (**Fig. 2**). In vitro lipolysis of fasting normolipidemic serum caused a significant increase in the levels of serum FFA (**Table 5**); however, the levels of FFA and the extent of partitioning of FFA into lipoprotein fractions in the lipolyzed fasting serum were much lower than those in lipolyzed HTG serum (Tables 1, 2, and 5). The major portion (> 66%) of FFA in post-lipolysis fasting serum was associated with the serum free protein (albumin) fraction (Table 5), and little or no flocculent remnants were detected in the

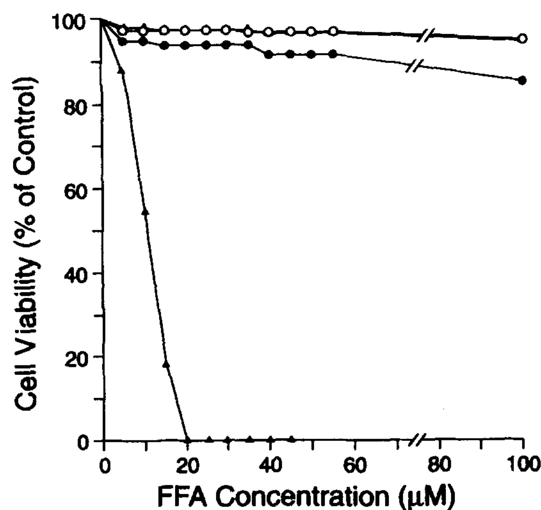

Fig. 4. Effect of the levels of FFA in pre- and post-lipolysis samples of fasting and postprandial serum from a normolipidemic subject. Cultured MPM in duplicate was incubated with increasing amounts of FFA in pre-lipolysis samples of fasting serum (○ - ○) and postprandial serum (△ - △), and post-lipolysis samples of fasting serum (● - ●) and postprandial serum (▲ - ▲) for 18 h, and then the viability of cells was determined as described above. Each value is a mean of duplicated determination. FFA levels of prelipolysis samples of fasting serum and postprandial serum and post-lipolysis samples of fasting and postprandial serum were 0.69 mM and 0.92 mM (pre-lipolysis serum) and 4.28 mM and 15.6 mM (post-lipolysis serum), respectively.

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density gradient tubes of this sample (data not shown). In vitro lipolysis of postprandial lipemic serum resulted in an increase of serum FFA level similar to that of post-lipolysis samples of HTG serum and in the partitioning of a major portion (63–78%) of FFA in serum to the lipoprotein fractions (Tables 1, 2, and 5). Flocculent remnants were detectable in the post-lipolysis samples of postprandial sera (data not shown). This study demonstrates that lipolysis-induced partitioning of FFA to lipoproteins occurs not only in sera from HTG subjects but also in postprandial sera from normolipidemic subjects.

Change in FFA concentration of lipoprotein fractions after in vivo lipolysis in HTG subjects and after in vitro incubation of postheparin serum

Table 6 shows the changes in FFA concentrations after heparinization (in vivo lipolyisis) and in vitro incubation of postheparin serum. In order to prevent the continuing in vitro lipolysis of TG-rich lipoproteins in postheparin blood, a portion of the postheparin blood was collected in tubes containing an inhibitor of LpL (paraoxon). The levels of FFA in 30 min postheparin sera collected in tubes containing paraoxon were about 2.8 × greater than those of preheparin sera (Table 6). When postheparin blood was collected without paraoxon, the FFA concentrations were about 3.6 × greater than those in preheparin sera even though the blood was spun in a refrigerated centrifuge (below 5°C), and the samples were handled in an ice bath. This indicates that significant in vitro lipolysis occurred in the

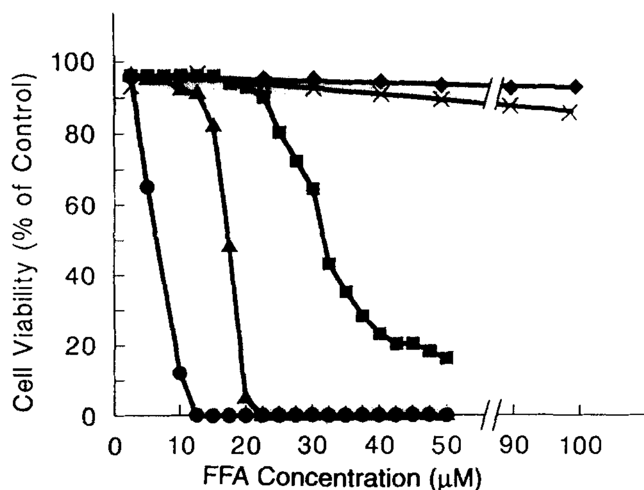

Fig. 5. Viability of MPM after incubation with LDL and/or albumin containing various FFA concentrations. Cultured MPM in duplicate was incubated with increasing amounts of FFA-enriched LDL having FFA to LDL-cholesterol molar ratio of 0.11 (◆ - ◆), 0.23 (■ - ■), 0.43 (▲ - ▲), or 0.61 (● - ●) and FFA-enriched albumin (FFA/albumin molar ratio = 4.7) (x - x) for 18 h, and then the viability of cells was determined as described above. Each value is a mean of duplicated determination.

postheparin sera when the activity of endogenous LpL was not inhibited. The FFA concentration in postheparin (in vivo lipolyzed) sera was much lower than that of in vitro lipolyzed HTG sera, and thus, the partitioning of FFA to lipoproteins was negligible when compared to that of in vitro lipolyzed HTG sera (Tables 1, 2, 6, and 7). However, in vitro incubation of postheparin sera without paraoxon resulted in a significant increase in FFA concentrations (Table 6) and in the partitioning of a major portion (76%) of FFA in serum to lipoprotein fractions (**Table 7**).

Interaction of pre- and post-lipolysis HTG sera and lipoproteins with cultured MPM

The cytotoxic potency of FFA associated with control and in vitro lipolyzed sera and their lipoprotein and free protein (albumin) fractions was examined in cultured MPM after the standardization of serum, lipoprotein, and free protein fractions according to their FFA concentration. **Fig. 3-A** shows that the culture medium having low concentrations of FFA associated with post-lipolysis HTG serum (14 μM FFA or 14 nmol FFA/dish) was cytotoxic to cultured MPM, killing 100% of the cells, whereas the culture medium having the high concentration of FFA associated with pre-lipolysis HTG serum (up to 120 μM FFA or 120 nmol FFA/dish) was not cytotoxic. Measurement of the cytotoxic potencies of various serum fractions separated from post-lipolysis HTG serum showed that culture medium having 8–12 μM FFA partitioned to various lipoprotein fractions was cytotoxic, whereas concentrations of up to 100 μM FFA partitioned to plasma free proteins (albumin) were not cytotoxic (Fig. 3-B). The cytotoxic potency of FFA partitioned to flocculent remnants was considerably lower than that of FFA partitioned to lipoproteins (Fig. 3-B).

Lipoproteins from pre-lipolysis HTG sera normally contained a small amount of FFA (Table 2). When lipoprotein fractions from pre-lipolysis sera were added into a culture medium at FFA concentration of 30–90 μM, a cytotoxic effect of FFA on cultured MPM was not observed (data not shown). Interaction of pre- and post-lipolysis fasting and postprandial sera from normolipidemic subjects showed that FFA in the post-lipolysis samples of fasting sera, in which FFA were partitioned mostly to albumin, were not cytotoxic to cultured MPM (Table 5 and **Fig. 4**). However, FFA in the post-lipolysis samples of postprandial lipemic sera, in which FFA were largely partitioned to lipoprotein fractions, were cytotoxic to cultured MPM (Table 5 and Fig. 4). The above data indicate that excessive partitioning of FFA to lipoproteins increases the cytotoxic potency of FFA. We observed that postheparin HTG sera samples collected without the addition of paraoxon (LpL inhibitor) were occasionally cytotoxic to cultured

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MPM, but that the postheparin sera collected with paraoxon were not cytotoxic. In vitro incubation of most postheparin HTG sera collected without paraoxon at 37°C for 90 min, which resulted in the partitioning of a major portion of serum FFA into lipoprotein fractions (Table 7), usually caused the serum to be cytotoxic to cultured MPM (data not shown).

Biological properties of FFA complexed in vitro to LDL or albumin

We have shown previously (27) that in vitro lipolysis of HTG sera resulted in a significant increase in the concentration of all FFA species that were present in the pre-lipolysis sera. Oleic acid was the most abundant FFA species in lipolyzed HTG sera (Tables 3A and B). To further examine the biological properties of FFA bound to lipoproteins and albumin, FFA (oleic acid)-LDL complexes having various molar ratios of FFA to LDL-cholesterol and FFA-albumin complexes having higher FFA to albumin molar ratios than those in normal plasma were produced in vitro. Their interactions with MPM and THP-1 monocyte-derived macrophages were then studied to assess the ability of FFA to produce cytotoxicity or to induce foam cell formation. **Fig. 5** shows that a low concentration of FFA (12–40 μM) on FFA-LDL complexes having FFA to LDL-cholesterol molar ratios 0.23, 0.43, and 0.61 was cytotoxic to MPM, whereas up to 100 μM FFA on FFA-LDL complexes was not cytotoxic when the molar ratio of FFA to LDL-cholesterol was reduced to 0.11. This demonstrates that the cytotoxic potency of FFA bound to LDL increases as the density of FFA molecules on LDL particles increases. Up to 100 μM of FFA bound to albumin with FFA/albumin molar ratios of 4.7 was not cytotoxic to MPM (Fig. 5).

We further studied the potency of FFA bound to either LDL or albumin to induce foam cell formation in cultured macrophages. As a small concentration of FFA bound to lipoproteins was cytotoxic to cultured MPM, it was difficult to measure the ability of FFA bound to lipoprotein to affect the cellular lipid levels because the cells died before the accumulation of significant lipid. We have previously reported that MPM and human monocyte-derived macrophages were very susceptible to the cytotoxic effect of lipolyzed HTG sera or lipoproteins, but that other macrophage cell lines (J744 macrophages) were less susceptible to this cytotoxic effect; lipolyzed HTG serum was not cytotoxic to human skin fibroblast, cardiac myocytes, or smooth muscle cells (25). We used THP-1 monocyte-derived macrophages to measure the ability of FFA to induce foam cell formation because LDL containing a moderate amount of FFA (molar ratio of FFA to LDL cholesterol = 0.44) was not cytotoxic to this cell line. However, LDL isolated from post-lipolysis HTG sera, which had a FFA to LDL-cho-

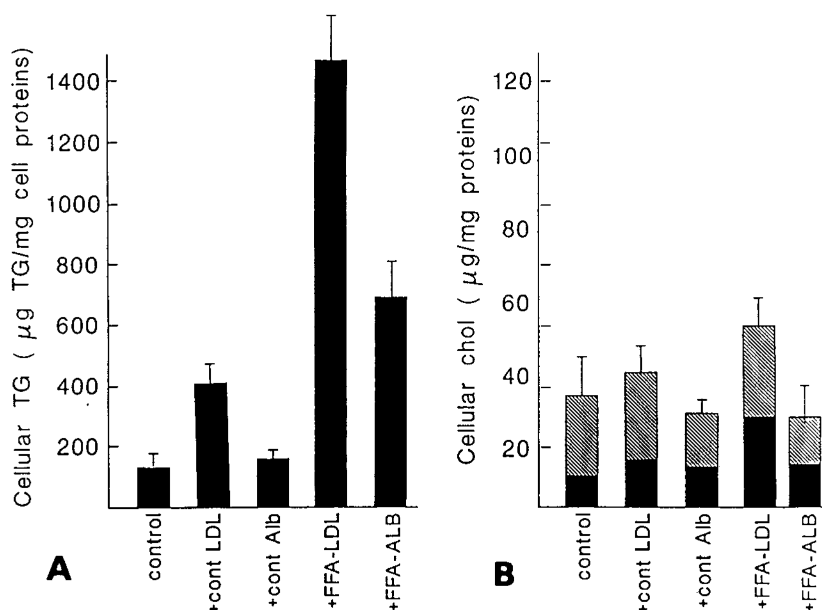

Fig. 6. Cellular TG and cholesterol in THP-1 monocyte-derived macrophages incubated with control and FFA-enriched LDL or albumin. Control LDL (1.8 mg cholesterol), FFA-enriched LDL (2 μM FFA–1.8 mg cholesterol; FFA to LDL-cholesterol molar ratio = 0.43), control albumin (28.5 mg) and FFA-enriched albumin (2 μM FFA–28 mg albumin; FFA to albumin molar ratio = 4.7) were added to a well of 6-well culture plate containing 6 ml culture medium. A control dish contained medium only. After 18 h incubation, levels of cellular TG, total, and unesterified cholesterol and proteins were measured as described in the Methods section. The ratios of cellular TG to protein (A) and cholesterol to protein (B) were then determined. Esterified cholesterol (■) levels were calculated by subtracting the levels of unesterified cholesterol (▨) from the levels of total cholesterol. Values are means ± SD of two separate experiments performed in triplicate.

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lesterol molar ratio > 1.20, was cytotoxic to the THP-1 monocyte-derived macrophages (data not shown). **Fig. 6** shows cellular TG and cholesterol levels of THP-1 monocyte-derived macrophages incubated with control LDL and albumin and FFA-albumin and FFA-LDL complexes. FFA complexed either to LDL or to albumin markedly increased the cellular TG (Fig. 6A). When the net increase of cellular TG induced by FFA complexed to LDL or albumin was calculated, FFA bound to LDL was significantly more effective ($P < 0.01$) than FFA bound to albumin in increasing cellular TG (1030 μg vs. 525 μg TG/mg cell proteins) (Fig. 6A). The cholesterol levels of cells incubated with LDL were not significantly different from those of control cells, whereas cells incubated with FFA-LDL complexes contained significantly higher levels of cholesterol than the control cells ($P < 0.05$) (Fig. 6-B). The increase in cellular cholesterol induced by FFA-LDL was largely due to an increase in cholesteryl ester (Fig. 6-B). The cholesterol content of macrophages incubated with FFA-LDL complexes was higher than that of cells incubated with LDL, but this difference was not statistically significant. FFA-albumin complexes did not influence cellular cholesterol concentration.

DISCUSSION

The concentration of albumin in human plasma is about 0.63 mM (16), and one molecule of albumin has the capacity to bind about 10 molecules of long chain fatty acids (36). Thus, theoretically, albumin in normal serum could bind about 6.3 mM FFA, which is the concentration of FFA released during the complete lipolysis of 2.1 mM TG or about 185 mg TG/dl. Although the level of albumin concentration in normotriglyceridemic subjects would be sufficient to clear the FFA released during the catabolism of TG-rich lipoproteins, it is uncertain whether albumin in HTG subjects is able to clear these FFA when rapid lipolysis of TG-rich lipoproteins occurs in vivo. This study demonstrates that when rapid and extensive lipolysis of TG-rich lipoproteins occurs in sera from HTG subjects or in postprandial lipemic sera from normolipidemic subjects, albumin in serum binds only a small portion (14–35%) of those FFA generated by lipolysis. As a consequence, the majority of FFA bind to lipoproteins resulting in 18- to 30-fold increases in the levels of FFA on lipoprotein particles. The association of FFA with lipoproteins after in vitro lipolysis of HTG sera reflects FFA levels that exceed the binding capacity of albumin. Our data show that the FFA to albumin molar ratio in post-lipolysis HTG sera in which more than 80% of FFA are associated with the lipoprotein fraction is about 5.3. This indicates that albumin in circulating blood is able to bind a much smaller number of FFA molecules than the numbers determined in vitro (36), or that the partitioning of FFA to lipoproteins occurs without fully saturating the FFA binding sites on albumin.

A number of previous reports showed that the percent of plasma FFA associated with lipoproteins, measured on the d < 1.21 g/ml plasma fraction, ranged from 5 to 21% in normolipidemic subjects and from 24 to 31% in HTG subjects (18, 19). Our study showed that lipoprotein fractions in pre-lipolysis samples of HTG serum contained a substantial portion (21–30%) of serum FFA while the serum had an FFA/albumin ratio of 1.4. This association of FFA to lipoproteins at a low FFA/albumin molar ratio, as seen in pre-lipolysis HTG serum, suggests that lipoproteins can bind a certain amount of FFA with a binding affinity similar to that of albumin. It has been suggested that, based on the concept of three high affinity fatty acid binding sites, the binding of FFA lipoproteins will not occur until the FFA to albumin molar ratio in plasma exceeded 3 (16); however, a recent study by Cistola et al. (21) indicates that the threshhold FFA to albumin molar ratio for binding of FFA to lipoproteins in human circulation is 1, rather than 3.

Scow, Desnuelle, and Verger (37) have shown that FFA released from TG-rich lipoproteins by lipolysis initially spread throughout the interface between the remnants and water. When albumin was added after lipolysis, FFA at the interface were desorbed immediately by albumin in the aqueous subspace. It is uncertain whether the transfer of lipolytically generated fatty acids from remnants to other lipoproteins occurs in the same manner as the transfer of FFA to albumin. The transfer of lipolytically generated FFA to LDL and HDL could occur by the transfer of FFA-surface remnant complexes as it has been shown that the lipolysis of TG-rich lipoproteins in serum resulted in the transfer of surface components from TG-rich lipoproteins to both LDL and HDL (38). The greater increase of FFA in HDL compared with LDL is consistent with the role of HDL as the primary acceptor of lipolytic surface remnants enriched with FFA.

We found that the greatest portion of FFA generated during in vitro lipolysis was associated with flocculent remnants (Table 2) that had compositional characteristics similar to the surface remnants of TG-rich lipoproteins (35). It has been shown that FFA may form fatty acid soaps at physiologic pH (39). Flocculent remnants may possibly be soaps that solubilize apolipoproteins, albumin, and other lipoprotein lipid components. Soaps and acid-soap complexes, but not FFA, may form bilayered lamellar structures under certain conditions (39). We found that the removal of FFA from flocculent remnants by fatty acid-poor albumin was considerably

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more difficult than its removal from FFA-enriched LDL and HDL (Table 4). This suggests that flocculent remnants bind FFA with greater affinity than do lipoproteins or probably albumin. Spooner et al. (40) showed that phospholipid bilayers have a high affinity for FFA such that a net transfer of FFA from albumin to phospholipid bilayers may occur provided that the latter is sufficiently large. Because lipolytic surface remnants may have a structure resembling the phospholipid bilayer (41), the association of a large amount of FFA with these remnants may be related to their structure. Ashbrook et al. (36) have reported previously that the binding affinity of FFA to albumin in phosphate buffer (pH 7.4) increased as the acyl chain length of FFA increased; with the 18-carbon chain FFA, oleic acids bound more tightly than stearic acids while linoleic acids bound less tightly than stearic acids. The compositional data of FFA partitioned to various plasma fractions (Table 3) showed that plasma free protein contained proportionally more linoleic acids and less oleic acids and stearic acids when compared with those bound to lipoprotein fractions. These data suggest that the presence of lipoproteins as secondary acceptor of FFA in the lipolysis mixtures may alter the relative binding affinity of FFA to albumin.

Our data showed that the association of FFA with lipoproteins increased the potency of FFA to produce the cytotoxicity, whereas the association of FFA with free proteins (albumin) prevented the cytotoxic effect of FFA to cultured MPM. This suggests that the binding of FFA by albumin may be an important mechanism of preventing the toxic effect of FFA in vivo. The toxic effect of FFA to cells is well known and has been suggested to be caused by the presence of excessive fatty acids in an unesterified form that are able to alter the fluidity of cell membranes or adversely affect the important membrane-bound enzymes, receptors, or the transport system (17). The mechanism of the increase in the cytotoxic potency of FFA by its association with lipoproteins is unknown, but it may be related to the physical state of FFA on the lipoproteins and/or the rate and extent of transport of FFA from lipoprotein-FFA complexes to cells. Parks et al. (42) showed that at physiological pH, FFA bound to albumin are fully ionized, while about 50% of FFA bound to phospholipid vesicles is unionized. The physical state of FFA on lipoproteins would be similar to that bound on phospholipid vesicles. It is possible that the increase in the cytotoxic potency of FFA by its association with lipoproteins may be the result of an increase in the level of unionized FFA as the unionized FFA will be more hydrophobic than ionized FFA and thus will penetrate the hydrophobic cell membrane bilayers more readily than ionized FFA. Because FFA bound to lipoproteins are readily removable by albumin, the FFA binding affinity of lipoproteins is lower than that of albumin, and thus, the transfer of FFA molecules from FFA-lipoprotein complexes to cells may occur more readily than from the FFA-albumin complexes to cells. Although plasma lipoproteins and cell membranes are known to have a high affinity for FFA (43, 44), the binding kinetics of FFA to lipoproteins and cell membranes have not been fully compared. It has been reported that the uptake of FFA by cultured cells or binding of FFA to lipoproteins does not appreciably occur until the FFA to albumin molar ratio exceeds 3 or 4 (16, 17). This suggests that the binding affinities of FFA to lipoproteins and cell membranes relative to that of albumin may be about equal.

Our data show that lipoproteins in pre-lipolysis samples of HTG contained a small amount of FFA, but a large amount of this FFA was not cytotoxic to MPM. The cytotoxic effect of FFA bound to lipoproteins began to appear when the lipoprotein particles reached a threshhold FFA concentration (Fig. 5). It is probable that FFA associated with lipoproteins may become cytotoxic when the binding affinity of FFA for lipoproteins is sufficiently low such that dissociation of FFA from lipoproteins occurs readily in the presence of cell membranes. The cytotoxic potencies of lipoprotein-bound FFA to cultured MPM were closely related to the molar ratio of FFA to lipoprotein cholesterol (Fig. 5); however, the cytotoxic potency of FFA associated with flocculent remnants, which had a much (11–41 ×) higher molar ratio of FFA to cholesterol than any other lipoprotein fraction in the lipolyzed HTG serum, was much less than that of FFA associated with lipoprotein fractions (Table 2 and Fig. 3). The low cytotoxic potencies of FFA bound to flocculent remnants as compared to FFA associated with lipoproteins may be due to the differences in the composition of FFA on the particles and/or the binding affinities of FFA to the particles. A number of studies have shown that saturated fatty acids were much less disruptive than unsaturated fatty acids to cultured endothelial cells (45) and red blood cells (46). We have observed recently that at the same level, all long chain (C16–C18) unsaturated FFA bound to lipoproteins were much more cytotoxic than saturated FFA bound to cultured MPM (B. H. Chung, and T. A. Tallis, unpublished data). It is likely that an abnormally high proportion of saturated fatty acids relative to that of unsaturated fatty acids on flocculent remnants (Table 3) may be a factor responsible for the low cytotoxic potencies of FFA bound to the flocculent remnants. The binding affinity of FFA to flocculent remnants is higher than that of FFA to lipoproteins (Table 4); thus, cytotoxic potencies of FFA bound to flocculent remnants will be lower than those of lipoprotein-bound FFA because the FFA with the high binding affinity will not be readily available to cells.

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We have shown in this study that FFA bound to lipoproteins are significantly more effective in inducing cellular accumulation of TG than the same amount of FFA bound to albumin (Fig. 6). The increase in potencies of FFA to promote cellular TG accumulation by the association of FFA to lipoproteins is also likely due to a lower binding affinity of FFA to lipoproteins than to albumin. Bergstraesser and Bates (12) have shown that the progressive increases in the FFA to albumin molar ratio, which would progressively lower the binding affinity of FFA to albumin, linearly increased the TG accumulation in cultured macrophages and smooth muscle cells. A number of studies have shown that supplementation of culture medium with FFA resulted in statistically significant increases in the levels of esterified cholesterol in various cultured macrophages (47–49). Our data showed that the cellular cholesterol content of macrophages incubated with FFA-LDL complexes was higher than that of cells incubated with LDL (Fig. 6) with borderline statistical significance ($P = 0.10$). It is reasonable to suppose that the binding of FFA to lipoproteins would increase the potency of lipoproteins to promote cellular cholesterol accumulation as provision of both cholesterol and fatty acid substrates for cellular acyl-CoA:cholesterol acyltransferase (ACAT) could lead to greater cholesteryl ester accumulation than would either substrate alone. Rumsey et al. (49) have shown recently that FFA supplementation into culture medium of J774 macrophages increased both LDL receptor activity and cholesteryl ester accumulation. These investigators hypothesized that the FFA delivered to cells first increased cellular ACAT activity, which would cause a decrease in an intracellular free cholesterol pool, signaling a need for increased LDL receptor activity.

Hayashi et al. (50) showed that the enrichment of LDL with FFA increased the uptake and degradation of LDL by human monocyte-derived macrophages via a receptor other than LDL receptors. It is well known that the enrichment of lipoproteins with FFA alters the electrophoretic mobility of lipoproteins (18–20). We found that LDL isolated from lipolyzed HTG serum or LDL enriched with FFA in vitro had much faster electrophoretic mobility than that of control LDL (data not shown). Because cultured macrophages, including THP-1 monocyte-derived macrophages, express scavenger receptors, which recognize LDL particles with an altered surface charge (51, 52), the modification of the surface charge of LDL by FFA could make them more susceptible to the uptake by macrophage scavenger receptors. Further studies remain to clarify the role of FFA in inducing cellular cholesterol accumulation.

We have previously reported that in vitro lipolyzed serum and its isolated lipoproteins were cytotoxic to cultured endothelial cells and/or able to disrupt the endothelial barrier function (27). As injury of arterial endothelium is the earliest event in atherogenesis (8), cytotoxicity produced by FFA partitioned to lipoproteins in vivo could be an important factor responsible for the initiation of the atherogenic process. Although we demonstrated in this study that rapid and extensive in vitro lipolysis of TG-rich lipoproteins in serum caused the partitioning of a major portion of FFA into lipoproteins and that the lipoprotein-bound FFA increased the atherogenic potency of FFA as well as lipoproteins, it is uncertain whether such partitioning of FFA to lipoproteins occurs physiologically during normal lipolysis of TG-rich lipoproteins in vivo. Earlier studies by Gordon (18) and Herbst et al. (19) showed that heparinization altered the electrophoretic mobility of LDL due to the enrichment with FFA. However, it was not clear whether such changes reflected the true effect of in vivo lipolysis or were instead an artifactual effect due to ongoing in vitro lipolysis. A recent study by O'Meara et al. (53) showed that the average level of plasma FFA in postheparin serum from HTG subjects was 6.3, a level that would be sufficiently high enough to cause partitioning of FFA into lipoproteins. Zambon, Hashimoto, and Brunzell (54) recently demonstrated that in vitro artifactual elevation of plasma FFA occurs in postheparin serum unless specific steps are taken to avoid in vitro lipolysis. Our data showed that the level of FFA in in vivo lipolyzed HTG serum (postheparin serum) is much lower than that in in vitro lipolyzed serum, and little or no partitioning of FFA into lipoproteins occurred when ongoing in vitro lipolysis in postheparin blood was inhibited by adding an inhibitor of LpL to the blood collection tube; however, extended in vitro incubation of postheparin HTG serum resulted in the partitioning of a large amount of FFA into lipoproteins (Table 6), which caused the sera to become cytotoxic to MPM (data not shown).

In summary, the present study reveals that lipolysis-induced partitioning of FFA into lipoproteins increases the cytotoxic potencies of FFA and ability of FFA and/or lipoproteins to induce foam cell formation. Lipolysis of TG-rich lipoproteins in vivo occurs locally at the endothelial surface and probably in close proximity to macrophages that have been shown to secrete LpL (55, 56). As the levels of FFA or FFA/albumin molar ratio at the site of lipolysis would be expected to be higher than in circulating blood, there is a possibility of partitioning of an excess amount of FFA into lipoproteins locally, which is sufficient to alter the endothelial cell function. However, the atherogenicity of FFA associated with lipoproteins remains uncertain until FFA concentrations can be measured at the arterial wall surface.

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