Effect of apoE on triglyceride emulsion interaction with hepatocyte and hepatoma G2 cells

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Abstract The uptake and internalization of a triglyceride emulsion by rat hepatocytes in culture less than 24 hr was either inhibited or uninfluenced by apoE. ApoE significantly increased the uptake of these emulsions in later cultures. Specific low density lipoprotein (LDL) binding was similar for hepatocyte monolayers prior to and after 24 hr. Rat hepatocytes in culture for 2 days, which were treated with collagenase, detached and then replated within 1 hr and were apoE-responsive in 2 hr. Heparin inhibited the apoE stimulation in both hepatocytes and hepatoma monolayers. Heparin wash of hepatocytes or hepatoma cells incubated with apoE-[^{14}C]triolein emulsions at 4°C resulted in a considerable loss in radiolabeled cell lipid. A similar wash after 37°C incubations produced little loss suggesting internalization. Hepatocytes had lower affinity but similar apoE-emulsion binding capacity compared to hepatoma cells. Triolein emulsions with apoE were significantly more rapidly metabolized by the hepatocyte than unsupplemented emulsions. The apoE-mediated hepatocyte lipid uptake was inhibited by apoC proteins. High molar ratios of free fatty acid/albumin also suppressed hepatocyte apoE-mediated lipid uptake. Both rat high density lipoprotein (HDL) and LDL inhibited with a potency directly related to their content of apoE. Human LDL and HDL without apoE also inhibited the interaction with less potency than the rat lipoproteins. Human HDL inhibition was diminished after removal of apoC proteins.—**Oswald, B., and S. Quarfordt.** Effect of apoE on triglyceride emulsion interaction with hepatocyte and hepatoma G2 cells. *J. Lipid Res.* 1987. **28:** 798–809.

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It has been proposed from studies of hepatocyte membrane binding (1) that apoE-rich lipoproteins interact with two classes of receptors on the canine hepatocyte sinusoidal membrane. One receptor (apoB, E) interacts specifically with both LDL and apoE-containing lipoproteins. It has been observed (2) primarily in young animals and is readily induced by interventions that produce a decrease in hepatocyte cholesterol. The other receptor interacts specifically with only apoE-containing lipoproteins and not LDL. It is present in adult animals and appears to be relatively refractory to change by various metabolic interventions. Recent progress on the isolation and characterization of hepatocyte membrane apoE-binding proteins has been reported (3, 4). These studies indicate a difference in the molecular properties of canine and rat hepatic membrane apoE-binding proteins. Both the apoE and apoB,E receptors are calcium-dependent and pronase-sensitive, but only the B,E receptor appears to be sensitive to higher ionic strengths.

The relevance of the characterized (3, 4) membrane-associated apoE binding proteins to hepatocyte uptake and subsequent metabolism of apoE-enriched lipoproteins has not been defined. The reported (5) association of rat chylomicron remnants with rat hepatocyte suspensions and cultured monolayers demonstrated properties substantially different from those exhibited in membrane studies (1). Remnant uptake in this system was not calcium-dependent and only partly pronase-sensitive. Isolated rat liver perfusion with either chylomicron or very low density lipoprotein remnants having virtually identical apoprotein contents revealed uptake of the two remnants by receptors with differing metabolic properties (6). These differences between various studies of apoE-mediated hepatic lipoprotein uptake my be reflections of species variation, differences in methodologic approach (homogenate membranes, monolayer culture or isolated hepatic perfusion) or differences in apoprotein compliments of substrate lipoproteins.

Virtually none of the previous investigations have correlated the hepatic apoE binding characteristics with the metabolic fate of the lipoproteins. The present studies were done to correlate the hepatocyte sinusoidal binding of defined apoE-enriched emulsions with the subsequent metabolic events, internalization, and triglyceride degradation. Although the apoE-supplemented phosphatidylcholine-stabilized triglyceride emulsion is not a replica

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; MEM, minimum essential medium; FBS, fetal bovine serum; EGF, epidermal growth factor; NEAA, nonessential amino acids; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

of a remnant, which is considerably more complex with respect to lipid and protein content, the emulsion is used in these studies to develop a more controlled insight into the potentially important role which the E apoprotein has in hepatic remnant assimilation.

EXPERIMENTAL PROCEDURES

Materials

Tri[1-^{14}C]oleoylglycerol (55.5 mCi/mmol) and trioleoyl-[2-^{3}H]glycerol (50 mCi/mmol) and ^{125}I were purchased from Amersham Inc. (Arlington Heights, IL). Sepharose 4B was from Pharmacia Fine Chemicals (AB, Upsala, Sweden); cyanogen bromide was from Pierce Chemical Company (Rockford, IL); collagenase Type IV was from Copper Biomedical (Malvern, PA); nutrient media, fetal bovine serum (FBS), pyruvate, and trypsin-EDTA were from Grand Island Biological Company (Grand Island, NY); and grade I heparin, bovine serum albumin (BSA), epidermal growth factor (EGF), gentamicin sulfate, insulin, trypsin, cycloeximide and tunicamycin were from Sigma Chemical Company (St. Louis, MO). Collagen obtained from a rat tail and rat hepatocellular carcinoma JM-2 were gifts from Dr. Steve Strom (Duke University, Durham, NC). All other chemicals were reagent grade. Hepatoma G2 cells were purchased from American Tissue Culture Collection (Rockville, MD).

Preparation of hepatocytes

Male Sprague-Dawley rats (200–300 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and maintained on standard chow. Hepatocytes were isolated by a modified collagenase perfusion of the rat liver (7). The rat was anesthetized with intraperitoneal (8 mg/100 g) sodium pentobarbital. The inferior vena cava was catheterized and 250 ml of Ca^{2+}-free buffer (7 mM) KCl, (143 mM) NaCl, (4.5 mM) NaOH, and (10 mM) HEPES, pH 7.4, was perfused through the liver at a rate of 12 ml/min. The portal vein was tied off and cut for drainage out of the liver and the superior vena was also tied off. Immediately following the calcium-free buffer, 250 ml of collagenase buffer (same buffer as Ca^{2+}-free buffer plus 0.5 mM $CaCl_2$ and 0.05% collagenase) was perfused through the liver. The liver was removed, placed in 25 ml of Ca^{2+}-free buffer and shaken for 5–10 min at 37°C. Cells freed from the liver were filtered through sterile cotton gauze and then kept at 4°C. The cells were spun in a Sorvall RC4 centrifuge at 4°C for 2 min at a speed of 500 rpm. The supernatant was removed and the cells were washed three times with Ca^{2+}-free buffer. The cells were counted in a hemocytometer and viability was measured by trypan blue exclusion and by media lactate dehydrogenase formation (8).

Cell culture

Approximately 1.2×10^6 hepatocytes were plated per 35-mm tissue culture dish, previously coated with collagen. The culture medium was 1 ml of Eagle's minimum essential medium (MEM) containing nonessential amino acids (NEAA), 10% fetal bovine serum (FBS), 10^{-7}M insulin, and 50 μg of gentamicin sulfate. In some cultures at 1–3 hours after plating the medium was changed to contain epidermal growth factor (EGF) (5 μg/50 ml). Culture medium was subsequently changed every 2–3 days thereafter. Cell cultures were maintained in a 95% O_2–5% CO_2 atmosphere at 37°C. Hepatoma cells were maintained in MEM with NEAA, 10% FBS, and 1 mmol of pyruvate. Suspensions of hepatocytes were assayed for triolein emulsion binding activity immediately after the collagenase isolation. The cells were assayed in MEM with NEAA and 1% BSA at a concentration of 1×10^6 cells/ml of buffer.

Lipoprotein and apoprotein preparation

Human lipoproteins and apoprotein E were prepared from hypertriglyceridemic humans using molecular sieve and heparin-Sepharose chromatography as described (9). All apoE samples were subjected to SDS polyacrylamide gel electrophoresis (10) to establish purity. Lipoprotein and apoprotein concentrations were determined by the methods of Lowry et al. (11) and Bradford (12). The E apoproteins which produced appreciable binding more than fivefold that of an unsupplemented emulsion to cultured hepatocyte monolayers were used in these studies. ApoE was stored at −20°C in either a 5 M urea, 0.1 M NaCl, 2 mM phosphate buffer (pH 7.4) or in 0.15 M NaCl, 2 mM phosphate buffer. Human and rat LDL, d 1.020–1.063 g/ml, and HDL, d 1.10–1.20 g/ml were prepared by standard (13) ultracentrifugation. Treated HDL was prepared by incubating 10 mg of human HDL with 50 mg of phospholipid-stabilized triglyceride emulsion for 2 hr at 37°C. The solution was then centrifuged at 26,600 *g* for 30 min. This solution was divided into top (emulsion) and bottom (treated HDL) and aliquots were taken for analysis by SDS polyacrylamide electrophoresis. The human LDL was labeled with ^{125}I by a modification (14) of the iodine monochloride methods. The apoproteins of both human and rat lipoproteins were qualitatively characterized by SDS electrophoresis with Coomassie blue staining and scanning densitometry.

Preparation of the Triglyceride emulsion

One ml of benzene containing 5 mg of triolein, 4 mg of purified egg lecithin (15), and 1 mg of free cholesterol with 5 μCi tri[1-^{14}C]oleoylglycerol or 25 μCi trioleoyl[2-^{3}H]glycerol or both was dried at 37°C under nitrogen and lyophilized for 2–3 min. The dried lipid was hydrated with 5 ml of 3% glycerol in 0.1 M Tris buffer (pH 8.2)

and sonicated four times for 3 min each at 40–50 watts (4°C) under nitrogen (Branson sonifier, Danbury, CT). Aliquots were removed for scintillation counting and the remaining solution was layered under saline and centrifuged for 30 min at 25,000 rpm in a Ti 50 rotor using a Beckman ultracentrifuge. The isolated triglyceride emulsion was radioassayed in an Intertechnique SL 30 liquid scintillation spectrometer (Dover, NJ). The size distribution of the emulsion was compared to that of rat chylomicrons by chromatography on Sepharose 2B (Pharmacia, Piscataway, NJ).

Binding assays

ApoE was added to the triolein emulsion at various apoE to triglyceride concentrations and equilibrated for 10–15 min at room temperature prior to addition to the cells. The distribution of the apoE on the synthetic emulsion was determined for differing protein:triglyceride ratios by isolation on Sepharose 4B and Bradford (12) protein determinations on the free and emulsion fractions. The apoE-supplemented and unsupplemented emulsions were added to the MEM–1% BSA medium of the prepared cell cultures and incubated at 37°C or 4°C for 60 min in a shaking waterbath in a 95% O_2–5% CO_2 atmosphere. Other apoproteins or lipoproteins were added to the culture immediately after the addition of the radiolabeled emulsions. Subsequently, the cells were washed two times with 2 ml of 7.3 mM phosphate buffer (PBS) (pH 7.4), 0.14 M saline or PBS with heparin at 100 μg/ml. Cell removal from the dishes was accomplished by scraping with a plastic policeman two times in 1 ml of PBS each time, followed by a 1 ml of PBS wash. The combined washes were centrifuged for 2 min at 1500 rpm. The supernatant was removed and the cell pellet was resuspended in 1 ml of distilled water, using a 0.1-ml aliquot of this suspension for protein determinations. The cell suspension was extracted by the method of Dole (16). The organic phase was placed in a scintillation vial and dried under air; the residue was dissolved in 0.05% diphenyloxazole in toluene and radioassayed as described. Determination of triglyceride, either bound or internalized, utilized trioleoyl[2-^{3}H]glycerol or tri[1-^{14}C]oleoylglycerol activity divided by the specific activity of the added triglycerides.

RESULTS

Hepatocytes isolated from rats fed standard chow by collagenase perfusion as described demonstrated greater than 75% viability by standard (17) trypan blue exclusion. No loss of lactic dehydrogenase activity to the media during a 1-hr incubation of the plated cells was observed. The addition of human apoE protein to a lecithin-stabilized triglyceride emulsion at a mass ratio of 3 protein/10 triglyceride did not increase the recovery of the radiolabeled triolein in suspensions of those isolated hepatocytes **(Table 1).** At the 1-hr and 3-hr time intervals after plating, the addition of apoE to the tri[^{14}C]oleoylglycerol emulsion decreased the amount of [^{14}C]triolein in the monolayer culture. At these early plating times specific LDL binding equivalent to that observed at later times was observed **(Fig. 1).**

At 24 and 48 hr after plating, at a time when the recovery of unsupplemented emulsion significantly decreased, the addition of apoE produced a significant enhancement of hepatocyte triolein uptake when compared to respective controls (Table 1). The cell protein content per dish was constant from the inception of plating out to 48 hr. Similar to previous observations (18), a significant change in the morphology of the cells was noted during this time interval. Immediately after plating the cells were rounded with less intercellular contact than at 24 and 48 hr when the cells were spread to more flattened, polygonal shapes with greater cell contact.

Hepatoma G2 cells that were maintained in almost confluent monolayer culture were exposed to the same collagenase concentration employed for the hepatic perfusion **(Table 2)** as well as one-half and twice this concentration for a time period equal to the perfusion. The cells rapidly detached upon exposure to twice the perfusate concentration (1 mg/ml) and replated after the enzyme was removed. At 1 hr after enzyme exposure, without cell detachment (0.25 and 0.50 mg/ml) or with detachment and replating (1 mg/ml), apoE still enhanced the emulsion recovery of the hepatoma cells when compared to the controls. A small increment in the control recovery was seen in each study after enzyme exposure. Similar collagenase treatment of a rat hepatoma line JM2 (19) resulted in cell detachment and a substantial enhancement of unsupplemented recovery and preservation of apoE-induced stimulation. When apoE-responsive rat hepatocytes that had been plated for more than 24 hr were detached by treatment with collagenase, they were also responsive to apoE 2 hr after replating (Table 2). In both hepatoma cells and responsive hepatocytes the apoE-induced increment in triolein recovery was not lost shortly after collagenase treatment and replating nor was the apoE-induced inhibition of the post-perfusion hepatocytes observed.

Ratios of apoprotein E to triglyceride below 0.3 mg of protein/mg of triglyceride were substantially less effective in increasing cellular radiolabeled lipid recovery than those above this level both for hepatocytes and hepatoma cells **(Fig. 2A and B).** Ratios above this did not appreciably increase the uptake of triglyceride. The fraction of added apoprotein that was emulsion-associated as determined by protein assays (11) of molecular sieve (Sepharose 4B) eluates decreased from 0.035 at 0.3/1 (apoE/triglyceride) to 0.01 and less at 0.6/1 and greater. This relatively

TABLE 1. Effects of apoE on triglyceride recovery in rat hepatocytes in suspension and after plating in monolayer culture[a]

Time	Number of Determinations	Control	ApoE
hr		*nmol/triglyceride/mg cell protein*	
Cells in minimal media with bovine serum albumin			
1	6	2.159 ± 0.4	1.573 ± 0.1
3	3	3.117 ± 0.2	2.184 ± 0.04[b]
24	6	0.470 ± 0.02	4.663 ± 0.1[d]
48	5	0.342 ± 0.02	5.910 ± 0.6[d]
Cells in minimal media			
1	3	1.643 ± 0.1	0.307 ± 0.09[c]
24	6	1.058 ± 0.7	2.212 ± 0.2
48	3	0.979 ± 0.08	3.733 ± 8.3[c]
Suspension culture in minimal media[e]		2.112 ± 0.7	2.249 ± 0.3

[a]Values represent means ± SEM. Minimal media (Eagle's) was used without and with 10 mg/ml bovine albumin containing 0.54 mol of fatty acids per mol of albumin. Cells were incubated at 37°C for 1 hr with 25 μg of triolein stabilized with egg lecithin and with 7.5 μg of human apoE as indicated. The cells were washed and processed as described in the text. By standard *t* testing, the apoE was significantly different from respective control at [b]$P < 0.02$, [c]$P < 0.01$, [d]$P < 0.001$.

[e]The cell suspension study consisted of two control determinations and six with apoE in 1 ml of minimal essential media with nonessential amino acids in the absence of albumin.

larger amount of apoE unassociated with lipid at higher apoE/triolein ratios did not appear to inhibit the emulsion uptake by hepatocyte or hepatoma G2 cells.

Incubations of hepatocyte monolayers with increasing concentrations of [^{14}C]triolein emulsion supplemented with apoE demonstrated saturation of hepatocyte-radiolabeled triglyceride recovery at higher concentrations of substrate for the 4°C incubations **(Fig. 3A).** Incubations of hepatocyte monolayers with apoE-supplemented emulsions at 37°C resulted in substantially greater recovery than at 4°C and a similar saturation at higher concentrations. The triglyceride emulsions containing no apoE were recovered in the hepatocytes in minimal amounts when compared to the apoprotein-supplemented emulsions and demonstrated no difference for the 4°C and 37°C incubations.

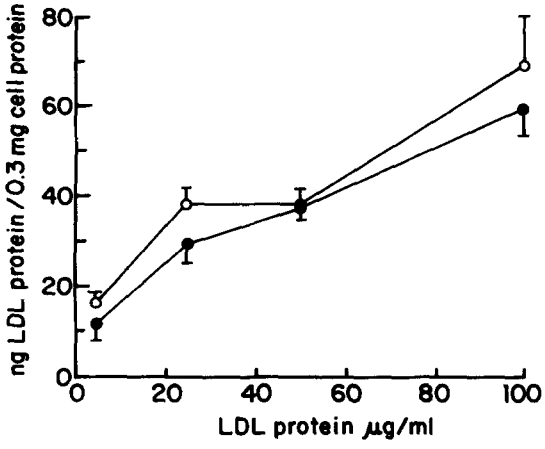

Fig. 1. The specific hepatocyte uptake of human [^{125}I]-labeled LDL by rat hepatocyte monolayers after 3-hr incubations (as described in the text) 1 hr (●) and 24 hr (○) after plating. Unlabeled LDL was added prior to labeled LDL. Specific binding is determined by subtracting nonspecific binding (in the presence of unlabeled LDL) from total binding (without unlabeled LDL).

Concentration increments of triolein emulsions with and without apoE gave similar results for the hepatoma G2 cells (Fig. 3B). The 37°C and 4°C recoveries for the unsupplemented emulsions were the same for each concentration with no suggestion of saturation. The emulsions containing apoE had significantly greater recoveries of cellular triglyceride at each concentration for incubations at 37°C as compared to 4°C. The 4°C and 37°C hepatoma cell incubations indicated saturation at higher substrate contents at both temperatures. Binding analysis of the hepatocyte 4°C apoE–triolein emulsion data (inset Fig. 3A) was single exponential and gave a K_d of 450 μM and 600 μM triolein in two studies and binding capacities of 31 and 39 nmol/mg of cell protein, respectively. The analysis of hepatoma data (inset Fig. 3B) again was single exponential and gave K_ds of 60 μM and 49 μM and binding capacities of 18 and 24 nmol/mg of cell protein, respectively.

The increased cellular recovery of emulsion triglyceride produced by apoE was inhibited by incorporating small amounts of either heparin **(Table 3)** or protamine in the media. As previously indicated, incubations of apoE-supplemented emulsion with hepatoma cells at 4°C pro-

TABLE 2. Hepatoma and hepatocyte monolayer triolein recoveries after exposure to collagenase[a]

	Collagenase Concentration	Time after Collagenase Exposure	Cell Triolein Recovery Control	ApoE
	mg/ml	*hr*	*nmol/mg of cell protein*	
Hepatoma G2	0.25	prior	0.23 ± 0.042	16.18 ± 3.07
Hepatoma G2	0.25	1	0.60 ± 0.01	7.22 ± 0.04
Hepatoma G2	0.25	5	0.42 ± 0.003	11.32 ± 0.33
Hepatoma G2	0.25	48	0.21 ± 0.001	12.31 ± 1.1
Hepatoma G2	0.5	prior	0.071 ± 0.002	10.22 ± 2.1
Hepatoma G2	0.5	1	0.148 ± 0.02	5.42 ± 0.3
Hepatoma G2	0.5	24	0.086 ± 0.002	8.33 ± 0.09
Hepatoma G2	1.0	prior	0.071 ± 0.002	10.22 ± 2.1
Hepatoma G2	1.0	1	0.188 ± 0.03	4.98 ± 0.2
JM2	0.5	prior	0.066 ± 0.003	0.562 ± 0.1
JM2	0.5	4	0.867 ± 0.09	1.58 ± 0.2
JM2	0.5	17	0.235 ± 0.006	2.17 ± 0.4
JM2	0.5	28	0.182 ± 0.02	1.56 ± 0.07
Hepatocytes	0.02	prior	0.579 ± 0.24	10.90 ± 0.55
Hepatocytes	0.02	2	1.90 ± 0.14	29.35 ± 0.62

[a]Values represent means ± SEM of triplicate assays for respective cell lines at the designated times with substrate concentrations in minimal essential medium without albumin. The confluent hepatoma monolayers were exposed to the indicated collagenase concentrations for 15 min after which the cells were washed with 1 ml of PBS. Hepatocytes would not replate at concentrations greater than 20 μg/ml. The incubation was begun after replating in experiments with 1 mg of hepatoma G2, JM2, and hepatocytes. Replating occurred about 1 hr after enzyme exposure. All apoE-enriched emulsions were significantly greater than control at $P < 0.01$ or less, except for the 4-hr JM2, which was at $P < 0.05$.

duced less recovery of the tri[^{14}C]oleoylglycerol than similar incubations at 37°C **(Table 4).** This could be on the basis of the suggested (20) temperature effect on triglyceride-rich lipoprotein interaction with the hepatocyte sinusoidal membrane or due to differences in cellular internalization at the two temperatures. The radiolabeled

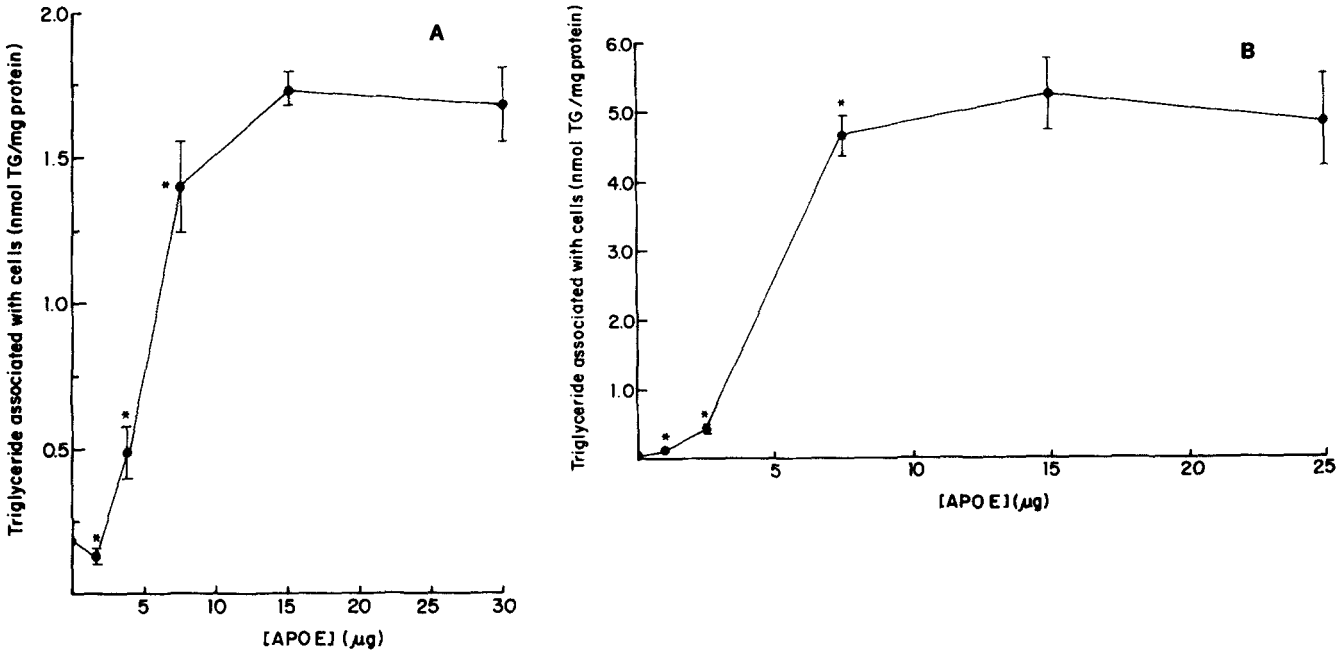

Fig. 2. The recovery of 25 μg of tri[^{14}C]oleoylglycerol in hepatocytes (A) and hepatoma G2 cells (B) after adding different amounts of apoE and incubating with the monolayers as described in the text.

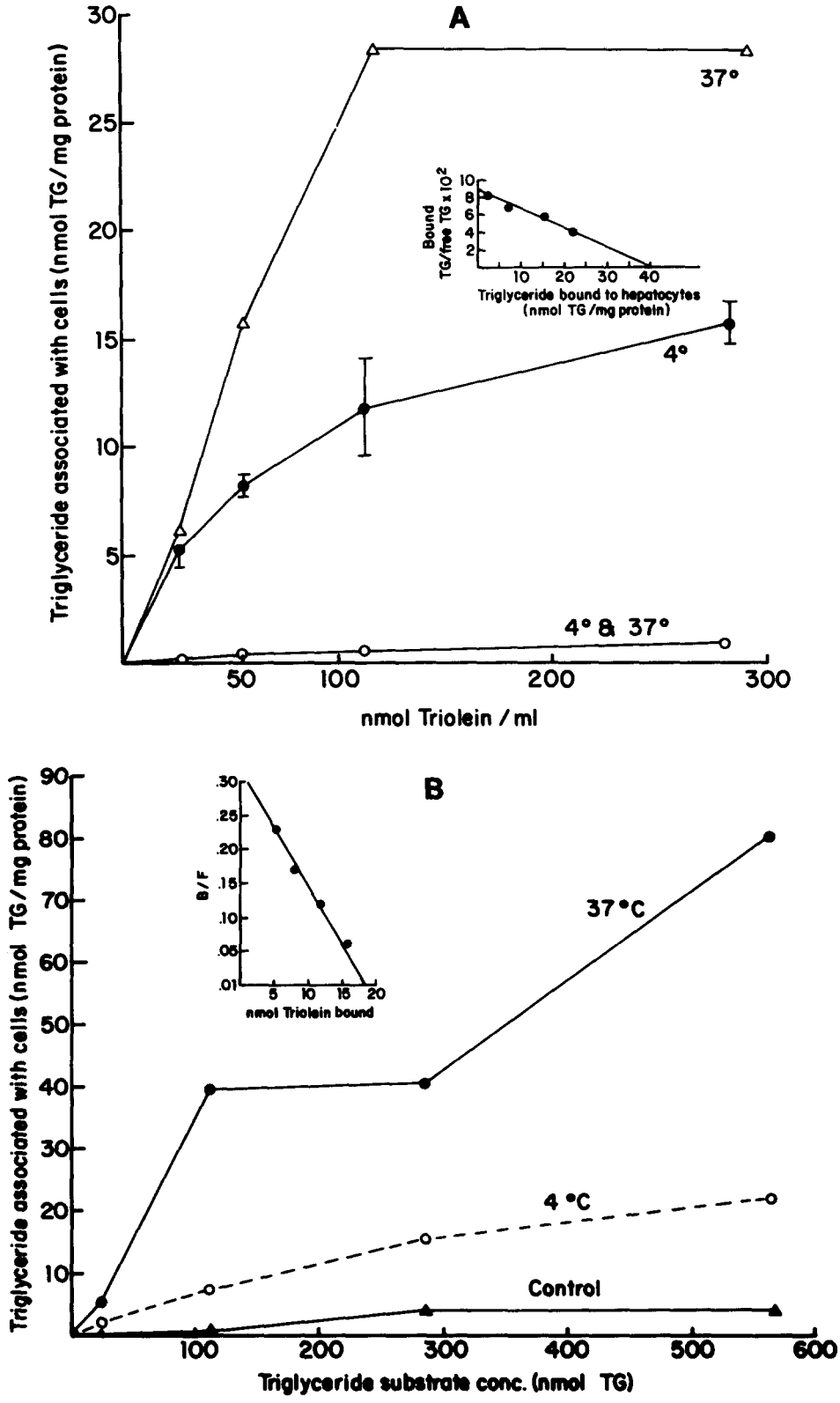

Fig. 3. The recovery of triolein in hepatoma G2 cells (B) and hepatocytes (A) after 1-hr incubations at 4°C and 37°C supplemented with apoE (A: ●, △; B: ○, ●) or unsupplemented (A: ○; B ▲). Inset: the bound/free (B/F) plotted against the bound triolein/mg of cell protein.

TABLE 3. Inhibition of triolein recovery in hepatocytes and hepatoma G2 by heparin and protamine[a]

Substrate	Hepatocytes	Hepatoma
	nmol/cellular /[14C]triglyceride/dish	
Without apoE	0.25 ± 0.01	0.08 ± 0.002
With apoE	1.67 ± 0.12	3.40 ± 0.10
With apoE and protamine (5μg/ml)	0.25 ± 0.02	0.18 ± 0.005
With apoE and heparin (10 μg/ml)	0.49 ± 0.04	0.09 ± 0.002

[a]Means ± SEM of triplicate incubations of egg lecithin-stabilized triolein (28 nmol/ml) added to minimal media containing 10 mg/ml of bovine albumin with a 0.64 molar ratio of fatty acid/albumin.

triglyceride recovered in these cells after a 1-hr incubation at 4°C was substantially removed from the cells by washing twice with 2 ml of PBS containing heparin (100 μg/ml) prior to lipid extraction. Incubation of hepatoma cells with apoE-supplemented emulsion at 37°C did not produce an appreciable loss in cell [^{14}C]triolein recovery after heparin washing. Incubation of unsupplemented emulsion with hepatoma cells produced little radiolabeled triolein recovery at either 4°C or 37°C in these hepatoma incubations and little difference was observed for the heparin wash.

Similar studies of nonreplicating rat hepatocytes 48 hr after plating also demonstrated (Table 4) a substantial loss of radioactivity from the cells incubated at 4°C with apoE-supplemented emulsion after washing with heparin.

TABLE 4. Hepatocyte and hepatoma triolein recoveries after incubations at different temperatures with heparin washing[a]

	Temp.	Substrate	Wash	Cellular Triolein
				nmol/mg protein
Hepatoma G2	4°C	Control	PBS	0.07 ± 0.02
Hepatoma G2	4°C	Control	Heparin	0.04
Hepatoma G2	4°C	ApoE	PBS	2.64 ± 0.62
Hepatoma G2	4°C	ApoE	Heparin	0.75 ± 0.13
Hepatoma G2	37°C	Control	PBS	0.09 ± 0.02
Hepatoma G2	37°C	Control	Heparin	0.08 ± 0.02
Hepatoma G2	37°C	ApoE	PBS	4.14
Hepatoma G2	37°C	ApoE	Heparin	3.81 ± 0.31
Hepatocytes	4°C	Control	PBS	0.15 ± 0.08
Hepatocytes	4°C	Control	Heparin	0.05 ± 0.006
Hepatocytes	4°C	ApoE	PBS	1.93 ± 0.03
Hepatocytes	4°C	ApoE	Heparin	0.48 ± 0.13
Hepatocytes	37°C	Control	PBS	0.22 ± 0.06
Hepatocytes	37°C	Control	Heparin	0.05 ± 0.01
Hepatocytes	37°C	ApoE	PBS	1.90 ± 0.39
Hepatocytes	37°C	ApoE	Heparin	1.40 ± 0.11

[a]Substrate production and incubation conditions are provided in Table 1 and the text. Values represent means ± SEM of triplicate determinations except for the two values without SEM which are duplicates. Hepatocytes were maintained in monolayer culture for 48 hr prior to the study. The cells were incubated for 1 hr and washed twice with either 2 ml of 0.15 M NaCl, 2 mM PO_4 (pH 7.4) (PBS) or PBS containing 100 μg of heparin/ml.

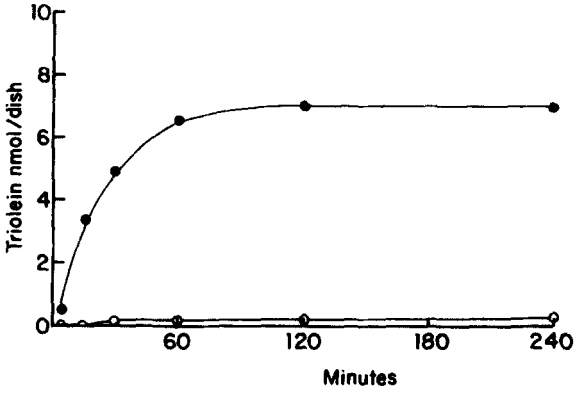

Fig. 4. The time course of hepatocyte triolein recovery after incubations with 25 μg of [^{14}C]triolein (○) and 25 μg of [^{14}C]triolein/7.5 μg of apoE (●) in MEM containing 10 mg of bovine albumin/ml at 37°C.

The incubations at 37°C demonstrated much less of a loss of radiolabel with heparin washing than the respective 4°C study. The unsupplemented emulsion demonstrated little ^{14}C-labeled triglyceride recovery in the PBS-washed plates at 4°C and 37°C and somewhat less after heparin washing.

The time course of [^{14}C]triolein recovery in hepatocyte cells **(Fig. 4),** as well as hepatomas (data not shown), demonstrated maximum recovery of [^{14}C]triolein after 1 hr with half-maximum recovery within 15 min. Using a trioleoyl[2-^{3}H]glycerol emulsion substrate, differences in the recovery of lipid radioactivity were observed between the apoE-supplemented and the unsupplemented substrate **(Fig. 5).** Complete recovery of the tracer in the lipid fraction was obtained for the unsupplemented emulsions at 8 and 12 hr whereas 73 and 58% of the label, respectively, was recovered for the apoE-supplemented emulsions at these times. Similar results were seen at these times when the tracer was in the triglyceride fatty acid (Fig. 5). At 24 hr, appreciable loss of the [^{3}H]triolein was seen for the control substrate with a substantially greater loss for the

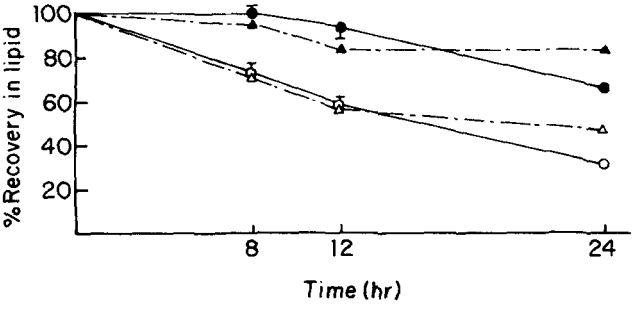

Fig. 5. The percent recovery of triglyceride in cells and media after incubations of emulsions (25 μg of triolein) labeled with trioleoyl[2-^{3}H]glycerol containing no added apoprotein (●) and apoE (○). The same recovery when emissions were labeled with tri[^{14}C]oleoylglycerol with no apoprotein added (▲) or with apoE (△) added.

TABLE 5. The influence of calcium and NaCl concentrations on hepatocyte and hepatoma triglyceride recovery[a]

	Hepatoma G2		Hepatocytes	
	No ApoE	ApoE	No ApoE	ApoE
	nmol/dish × 10^2			
[Calcium]				
1 mM Ca^{2+}, 0.14 M NaCl	3.4 ± 0.35)	72.5 ± 6.2)	4.7 ± 0.3)	18.4 ± 2.2)
Ca^{2+}-free, 0.14 M NaCl	3.0 ± 0.35)	32.2 ± 3.3)		
1 mM Ca^{2+}, 0.14 M NaCl and 60 mM EDTA	8.7 ± 1.9)	13.9 ± 2.1)	5.0 ± 0.3)	6.4 ± 0.2)
[NaCl]				
1.4 mM Ca^{2+}, 0.12 M NaCl			9.2 ± 0.8)	56.7 ± 1.9)
1.4 mM Ca^{2+}, 0.20 M NaCl				11.2 ± 1.2)
1.4 mM Ca^{2+}, 0.25 M NaCl				10.0 ± 0.6)

[a]The values are means ± SEM of triplicate determinations in either phosphate-buffered (7.3 mM, pH 7.4) saline (0.14 M) containing 10 mg/ml bovine albumin for the calcium studies or minimal essential media supplemented with NaCl to the indicated concentrations for the experiments with differing concentrations of NaCl.

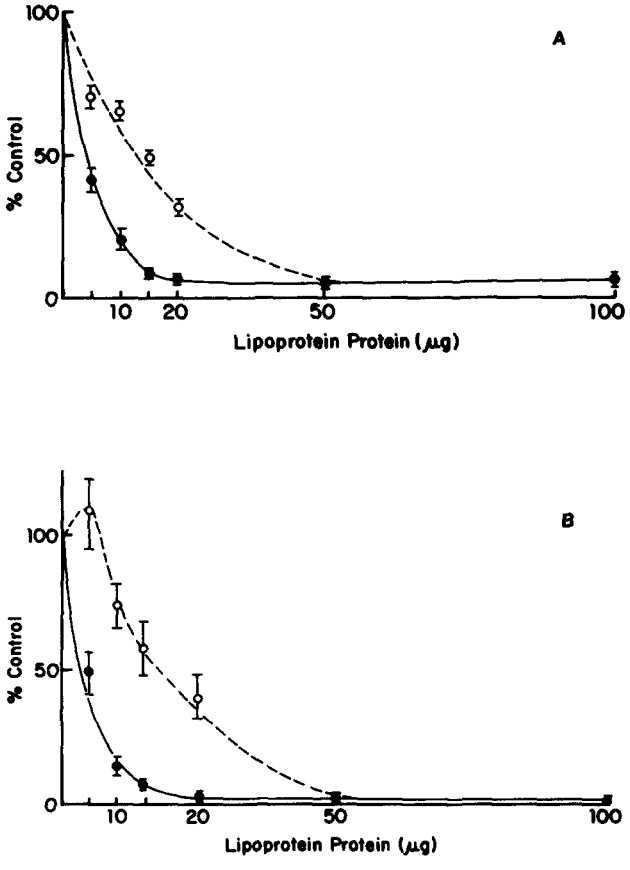

Fig. 6. The inhibition of [^{14}C]triolein recovery (given as a percent of baseline) after 1-hr incubations at 37°C with apoE-enriched triolein emulsion (25 μg of triolein/7.5 μg of apoE) containing increments of rat LDL (●) and rat HDL (○) in hepatocytes (A) and hepatoma cells (B).

apoE-enriched substrate. When the radiolabel was in the triglyceride fatty acid, a greater recovery was noted at 24 hr than when the tracer was in the glycerol fraction of the molecule, possibly reflecting reesterification. The recovery of tritium in the aqueous fraction of hepatocytes and media after a 12-hr incubation was 1.6 ± 0.2% and 10.7 ± 0.6% of the administered tracer for the mean ± SEM of four incubations each of control and apoE-supplemented substrate, respectively ($P < 0.001$).

The enhanced cellular recovery of triolein produced by addition of apoE was reduced by incubations of hepatoma cells in media at low calcium concentrations **(Table 5).** The addition of EDTA completely suppressed the apoE stimulation and produced no change in cell morphology or lactic dehydrogenase release. Sodium chloride concentrations at 0.12 M permitted apoE-enhanced cell lipid recovery, but 0.2 M and above inhibited this effect again with no apparent effect on the cells. Pronase exposure (1 μg/ml) also removed the apoE-mediated stimulation of hepatocyte lipid association.

Additions of rat LDL (d 1.019–1.063 g/ml) at low concentrations appreciably decreased the rat hepatocyte recovery of apoE-enriched triolein emulsions **(Fig. 6A).** The delipidated rat LDL protein was predominantly apoB with apoE accounting for 13% of the protein mass by scanning densitometry of Coomassie blue-stained SDS polyacrylamide gels. Rat HDL (d 1.10–1.21 g/ml) contained 5% of the total protein as apoE by the same assay. Rat HDL also decreased the recovery of apoE-rich hepatocyte [^{14}C]triolein but required about three times the concentration of LDL protein to produce a 50% reduction in recovery. Similar rat LDL and HDL inhibitions were observed for the hepatoma G2 cells (Fig. 6B). Human HDL inhibited the apoE-induced cell lipid recovery in both hepatocytes and hepatoma cells at much higher concentrations than the rat HDL **(Fig. 7).** Neither hu-

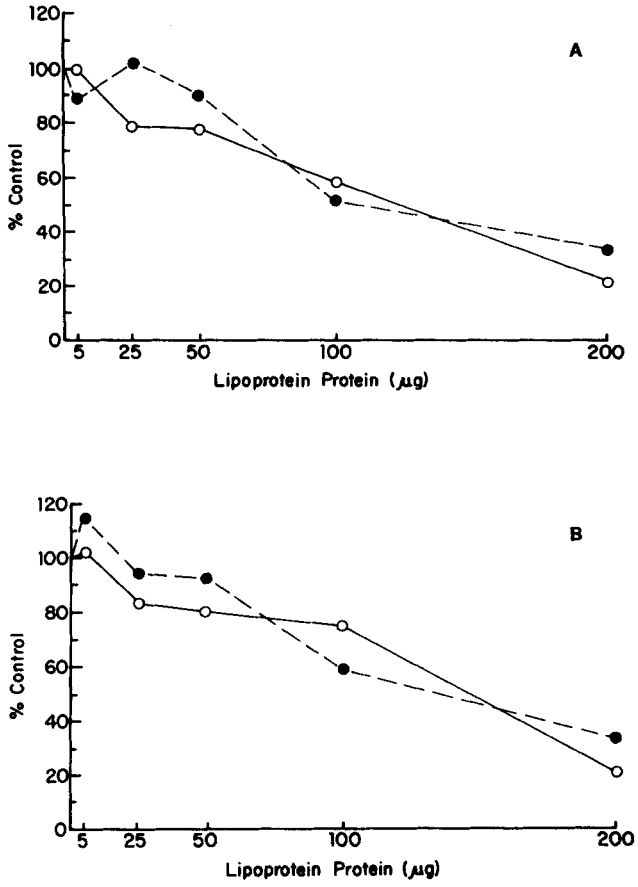

Fig. 7. The inhibition of [^{14}C]triolein recovery (given as a percent of baseline) after 1-hr incubations at 37°C with apoE-enriched triolein emulsion (25 μg of triolein/7.5 μg of apoE) containing added human HDL (●) and human LDL (○) in hepatocytes (A) and hepatoma cells (B).

man HDL nor human LDL contained detectable apoE by Coomassie staining of heavily loaded SDS polyacrylamide gels, but HDL contained appreciable amounts of apoC. The HDL inhibitory potency of four human HDLs was associated with the apoC content of the lipoprotein. The inhibitory potential of the HDL could be substantially diminished by exposing the lipoprotein to a phospholipid-stabilized triglyceride emulsion **(Fig. 8)** which effectively removed the apoC protein from the HDL.

Both hepatoma G2 cells and rat hepatocyte apoE-induced triglyceride recovery were inhibited by the presence of C apoproteins **(Table 6).** ApoC-III produced the greatest inhibition at lower concentrations. At high concentrations, apoC-I and C-III were equivalent, both demonstrating greater inhibition than apoC-II. It was observed that the molar ratio of fatty acid present on the media bovine albumin also influenced the apoE-induced hepatocyte triglyceride uptake. An inverse correlation was observed **(Table 7)** between the fatty acid/albumin molar ratio and the apoE-induced hepatocyte triolein recovery.

DISCUSSION

The interactions of apoE-rich lipoproteins with hepatocytes have properties that differ depending on the species evaluated and the technique of study. The canine (1), swine, and human (2) hepatic membranes appear to have an apoE receptor that is pronase-sensitive and calcium-dependent but less sensitive to ionic strength or metabolic perturbations than the receptor for LDL. The binding of the apoE-rich HDL_c to the canine membranes was not inhibited by either canine HDL or LDL. These properties differ from data (21, 22) on rat apoE-rich chylomicron remnant interaction with rat hepatocyte suspensions or monolayer cultures. The binding in this system was not calcium-sensitive and was only partially influenced by pronase treatment. The canine membrane data also differ from the earlier study (23) of rat hepatocyte membranes where a substantial inhibition of chylomicron remnant binding was observed with human HDL. None of these studies of apoE-rich lipoprotein binding to hepatocyte membranes or hepatocytes relate binding to subsequent lipoprotein catabolism. Therefore, the metabolic significance of the binding properties cannot be assessed. Although different proteins have been isolated from canine (3) and rat (4) hepatic membranes that specifically interact with apoE, it is not clear at this time whether any of these proteins mediate cellular uptake and metabolism of apoE rich lipoproteins.

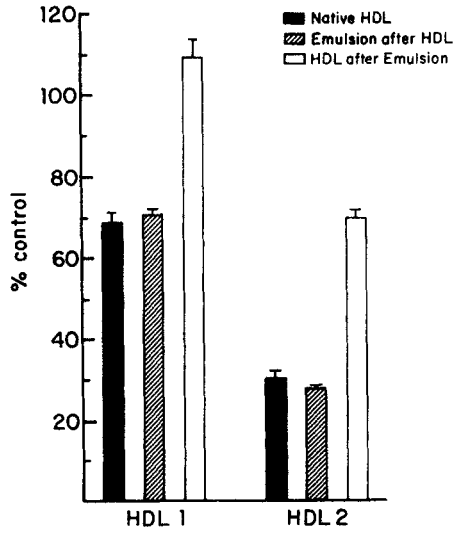

Fig. 8. The inhibition of [^{14}C]triolein recovery after 1-hr incubations at 37°C with apoE-enriched triolein emulsion (25 μg of triolein/7.5 of apoE) by human HDL from two individuals (HDL1, apoC-poor; HDL2, apoC-rich) prior to and after exposure to an emulsion (as described in the text) at a concentration of 25 μg and 100 μg.

TABLE 6. Effect of C apoproteins on apoE-induced hepatocyte triglyceride uptake[a]

Substrate	C Protein	Triolein Uptake
		nmol/mg cell protein
Without apoE	None	0.425 ± 0.03
With apoE	None	4.13 ± 0.6[b]
With apoE	25 μg apoC-I	0.983 ± 0.1[b]
With apoE	50 μg apoC-I	0.495 ± 0.03
With apoE	25 μg apoC-II	1.06 ± 0.06[c]
With apoE	50 μg apoC-II	1.04 ± 0.04[c]
With apoE	25 μg apoC-III	0.565 ± 0.07
With apoE	50 μg apoC-III	0.403 ± 0.02

[a]Values are means ± SEM of triplicate incubations with conditions described in Table 1. The values are significantly different from the substrate without apoprotein at [b]$P < 0.01$, [c]$P < 0.001$.

The emulsions employed in these studies previously demonstrated hepatic uptake and degradation similar to rat chylomicrons when supplemented with apoE in an isolated perfused rat liver (24). The addition of apoE to the triolein substrate significantly enhanced uptake and metabolism in this hepatocyte monolayer system. Differing from the previous data (1, 2, 21, 22), these results correlate binding properties with subsequent metabolism. The properties of apoE emulsion interaction with rat hepatocytes and a human hepatoma described here differ somewhat from previous studies. The interaction appears to be calcium-dependent as are the apoE and apoB,E receptors and is sensitive to changes in the ionic strength of the media similar to that observed for the B,E receptor. The interaction is completely inhibited by low concentrations of rat LDL and somewhat higher concentrations of rat HDL. This inhibition is directly related to the apoE content of both lipoproteins similar to the inhibition of remnant uptake in the isolated perfused canine liver by apoE-containing lipoproteins (25). These properties are similar to those described for canine hepatocyte membrane apoE binding (1). The inhibition of the hepatocyte and hepatoma apoE-mediated cellular [^{14}C]triolein recovery by 200 mM NaCl and by higher concentrations of human LDL and human HDL is different from the membrane data of Hui, Innerarity, and Mahley (1). Neither the human LDL nor the human HDL demonstrated any apoE on the heavily loaded SDS gels by either Coomassie blue or silver staining. The loss of human HDL inhibition after incubation of the lipoprotein with emulsions that effectively removed only the apoC proteins indicate that this inhibition was apoC-mediated. The observation that the HDL from four human subjects with different contents of apoC protein appeared to inhibit with potencies directly related to the amount of apoC also emphasized the importance of these apoproteins in HDL inhibition.

The lack of an increment in cellular [^{14}C]triolein recovery as a consequence of apoE addition in the hepatocyte suspensions differs from the data (21) on chylomicron remnant uptake by hepatocyte suspensions. Since the E protein is the major apoprotein of these remnants (22) and is suggested to be the mediator of the rat hepatocyte remnant uptake (22), the chylomicron and emulsion may behave differently in this system. We have utilized rat chylomicrons in the place of the emulsion (data not shown) and have observed them to be interchangeable with respect to the apoE effect. The calcium-independent binding of rat chylomicron remnants (21) and estradiol-treated rat chylomicrons (20) to hepatocyte suspensions, observed previously, suggest a mediator of this interaction other than apoE.

The hepatocyte suspension and the early (<24 hr) monolayer culture demonstrated a significantly greater recovery of unsupplemented emulsion lipid than the recovery at later times. It was consistently observed that, at a time when the recovery of control emulsion triglyceride decreased, the cells demonstrated apoE responsiveness. This transition usually occurred between 24 and 36 hr but was occasionally later. The initial interpretation of this finding was that the collagenase perfusion destroyed the receptor and the plating interval was necessary to resynthesize receptor. The inability to destroy apoE stimulation in hepatoma cells with exposure of the cells to greater collagenase concentrations than in the perfusate make this unlikely. The lack of an effect of sufficient colla-

TABLE 7. Albumin fatty acid molar ratio influence on apoE-induced triolein uptake in hepatocytes

Substrate	Molar Ratio	Triolein Recovery/Dish
		nmol/hr
11.5 nmol of Triolein + 0.11 nmol of apoE	0.05	0.88 ± 0.06
11.5 nmol of Triolein + 0.11 nmol of apoE	0.5	0.70 ± 0.03[b]
11.5 nmol of Triolein + 0.11 nmol of apoE	1.0	0.64 ± 0.02[c]
11.5 nmol of Triolein + 0.11 nmol of apoE	2.0	0.47 ± 0.06[d]
28.5 nmol of Triolein + 0.28 nmol of apoE	0.6	1.67 ± 0.12
28.5 nmol of Triolein + 0.28 nmol of apoE	3.0	0.74 ± 0.12[e]

[a]Values are means ± SEM of quadruplicate determinations of [^{3}H]glycerol-labeled triolein at incubation conditions described in Table 1. Significantly different from 0.05 molar ratio in the 11.4 nmol triolein study at [b]$P < 0.05$, [c]$P < 0.02$, [d]$P < 0.01$. Significantly different from the 0.6 molar ratio in the 28.5 nmol triolein study at [e]$P < 0.01$.

genase to detach hepatocytes also makes this improbable. The quick return to apoE responsiveness after collagenase treatment of these monolayers makes it likely that the 24-hr interval before apoE responsiveness is seen, after the initial plating, is on a basis other than collagenase destruction of the receptor. The observation of preserved apoE responsiveness, shortly after the replating of collagenase-treated hepatomas and hepatocytes, indicates that the plating process had nothing to do with the delay in recovery after the initial perfusion. One appreciable difference between the rat hepatocytes initially plated and the replated cells was in cellular morphology. The post-perfusion hepatocytes were nearly spherical and underwent a well-recognized (18) flattening during the first day in culture. The collagenase-treated monolayers were flattened at the time of enzyme addition and remained in this configuration with replating. It is possible that the cytoskeletal configuration changes are the major reasons for the delay in rat hepatocyte apoE responsiveness. The time delay, for apoE catabolic effectiveness, was not observed for LDL-specific binding to the hepatocyte.

The ability of rat hepatocytes to interact with asialoglycoproteins deteriorates (26) during time in monolayer culture and shows no restoration of activity similar to apoE responsiveness. A number of plasma membrane enzymes show an appreciable increment in activities (27) at the same time interval as the appearance of apoE responsiveness. The restoration of receptor activity for insulin and other peptide hormones (28), as well as cell surface enzyme markers, has been noted at a similar time for rat hepatocytes in culture. Aside from the possible cytoskeletal change, which has been suggested as a reason for the lag in apoE responsiveness, other equally speculative reasons exist. It is possible that this appearance of apoE responsiveness is an indication of the fetalization (18) of the hepatocyte in culture. The apoB,E receptor (1) activity is present in neonatal tissue and this activity has many properties similar to the apoB,E receptor. However, the observation of specific human LDL binding to the hepatocyte at times of no apoE responsiveness, and the poor inhibition characteristics of apoE binding with human LDL, does not favor this argument.

Although apoE appreciably enhances emulsion triglyceride recovery in both hepatocytes and hepatoma G2 cells similar to in vitro perfused livers, the subsequent metabolism of the lipid differs for the rat hepatocyte monolayer and the perfused rat liver. More rapid lipolysis and re-esterification was observed for the chylomicron or emulsion triglyceride in the perfused liver than in the monolayer culture. It is possible that hepatic lipase is virtually only on the sinusoidal endothelial cells (29), which are absent from these monolayers. We have been unable to recover this enzyme by heparin washes of the surface of hepatoma and hepatocyte monolayers. The apparent lack of this enzyme may leave solely the hepatic lysosomal lipase (30) to effect lipolysis, possibly a much slower process.

The rat hepatocyte monolayer and the hepatoma G2 cells were quite similar in terms of the properties of apoE-induced cell lipid uptake. Both cells demonstrated saturable high affinity binding with a K_d which, when expressed in the molarity of the emulsion particle, would be in the picomolar range. The lipoprotein inhibition of the apoE-induced lipid recovery was also quite similar. It has been proposed (31) that hepatomas in vivo lack the capacity for remnant binding and it is this defect that accounts for their lack of feedback inhibition when exposed to cholesterol. The hepatoma G2 cell line, in culture, does not appear to be defective in terms of apoE responsiveness. We have examined a number of rat hepatomas in culture with regard to this property and have noted a spectrum of apoE responsiveness. However, it is possible that apoE (32) is not the most important mediator of hepatocyte remnant recognition and the data presented may have little bearing on in vivo hepatoma or hepatocyte remnant retrieval.

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