

# Chylomicron-specific Enhancement of Acylation Stimulating Protein and Precursor Protein C3 Production in Differentiated Human Adipocytes\*

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Acylation stimulating protein (ASP) is a potent stimulator of adipocyte triacylglycerol storage. *In vivo* studies have shown that ASP production by adipocytes increases locally after a fat meal. Initial *in vitro* studies demonstrated increased production of ASP in the presence of chylomicrons (CHYLO). The present aim was to define the CHYLO component responsible. None of the apoproteins tested (AI, AII, AIV, CI, CII, CIII, and E) were capable of stimulating C3 (the precursor protein) or ASP production. Rather, the active component is a nonlipid, loosely associated, trypsin-sensitive molecule. High pressure liquid chromatography fractionation of the CHYLO infranate proteins identified the critical protein as transthyretin (TTR), which binds retinoid-binding protein and complexes thyroxine and retinol. Addition of TTR alone, with lipid emulsion, or with respun CHYLO to human differentiated adipocytes had little effect on C3 and ASP production. By contrast, when transthyretin was added to CHYLO, C3 and ASP production were substantially enhanced up to 75- and 7.5-fold respectively, compared with the effect of native CHYLO alone. Finally, a polyclonal antibody against TTR could inhibit stimulation of C3 and ASP production by CHYLO (by 98 and 100%, respectively) and by CHYLO infranate proteins (by 99 and 94%, respectively). We hypothesize that TTR mediates the transfer of the active components from CHYLO to adipocytes, which then stimulates increased C3 and ASP production. Thus the CHYLO provides the physiologic trigger of the ASP pathway.

Adipose tissue is recognized as an active organ releasing hormones, enzymes, and proteins such as lipoprotein lipase (1), cholesteryl ester transfer protein (2), apoprotein E (3), angiotensinogen (4), estrogen (5), tumor necrosis factor (6), and leptin (7). Both human and murine adipose tissue have been shown to synthesize and secrete complement C3, factor B, and adipsin, proteins involved in the alternate complement pathway (8–12). Furthermore, complement C3, factor B, and adipsin have been shown to be synthesized and secreted in a differentiation-dependent manner by adipocytes (10–12). The

interaction of these three proteins results in the cleavage of complement C3 generating the bioactive protein acylation stimulating protein (ASP/C3adesArg)<sup>1</sup> (9–11), which was initially recognized as a small basic protein present in human plasma (13). ASP can actively stimulate triacylglycerol synthesis in human adipocytes through a coordinated effect on translocation of the glucose transporters (GLUT1, GLUT3, and GLUT4) (14–16) and increases in the activity of the enzyme diacylglycerol acyltransferase. These effects of ASP are mediated through the diacylglycerol protein kinase C pathway (17) *via* specific interaction with the cell surface. The most responsive target to the action of ASP is adipose tissue, which is also known as the primary tissue for storage and release of energy. Within the cell, free fatty acids are enzymatically esterified to a glycerol-3-phosphate backbone to form triacylglycerol, the main storage fuel for the body (18). The hormone-sensitive lipase pathway then allows the release of energy in the form of free fatty acids in response to appropriate stimuli. Thus, the physiological role of ASP is to regulate the synthesis and storage of triacylglycerol in adipose tissue (19).

Triacylglycerol-rich lipoproteins play a major role in the transportation of fatty acids to the tissues. Chylomicrons (CHYLO) are intestinally derived postprandial lipoprotein particles that carry dietary fat in the form of triacylglycerols (20). In humans, venous and arterial measurements of ASP, triacylglycerol, and CHYLO triacylglycerol across an adipose tissue bed at fasting and after a meal suggest that the postprandial production of ASP is coordinated with the increase of triacylglycerol clearance (20). By what mechanism is this ASP pathway activated? *In vitro* studies have identified CHYLO as a plasma component that greatly augments the production of complement C3 and ASP in adipocytes (8). The other lipoproteins such as very low density lipoprotein, low density lipoprotein, and high density lipoprotein had no significant effect on C3 and ASP production, whereas insulin had a 2-fold effect on ASP, although this is small compared with the increases of both C3 and ASP by CHYLO (8). These *in vivo* and *in vitro* data associating ASP production to CHYLO clearance lend importance to identifying the active component of the CHYLO, which is responsible for the increase in both C3 (the precursor protein) and ASP.

## MATERIALS AND METHODS

Essentially fatty acid-free bovine serum albumin, collagenase type II, lipid substrate, cholesterol, and human transthyretin (TTR) were from Sigma. All tissue culture medium, Dulbecco's phosphate-buffered saline

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<sup>1</sup> The abbreviations used are: ASP, acylation stimulating protein; CHYLO, chylomicron(s); PBS, phosphate-buffered saline; TTR, transthyretin; HPLC, high pressure liquid chromatography; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay.

(PBS), fetal bovine serum, and tissue culture supplies were from Life Technologies, Inc. or Flow Laboratories (Mississauga, ON, Canada). Antiserum to human TTR was from Cedarlane Laboratories (Hornby, ON, Canada). Apoprotein AII, apoprotein CII, and apoprotein CIII were purchased from Calbiochem (La Jolla, CA). Apoprotein CI was obtained from PerImmune (Rockville, MD). Apoprotein AI and apoprotein E were a gift from Dr. J. Westerlund. Apoprotein AIV was a gift from Dr. J. C. Fruchart (Pasteur Institute, Lille, France).

**Culture of Human Differentiating Adipocytes**—Human adipose tissue was obtained with informed consent from patients undergoing reduction mammoplasty and then processed as previously reported (8). Briefly, adipose tissue was cleaned of connective tissue and small blood vessels, then minced, and treated with 0.1% collagenase. The cell suspension was centrifuged to pellet the stromal-vascular cells (containing the preadipocytes), and the resuspended pellet was subsequently treated with buffer for 10 min to lyse the red blood cells. After filtration through a 50- $\mu$ m filter and gentle centrifugation, the cell pellet was resuspended in minimum essential medium containing 10% fetal bovine serum. Preadipocytes were plated out on 24-well culture plates (cells from 10 g of cleaned tissue/24-well plate) at a concentration of  $3 \times 10^4$  cells/cm<sup>2</sup>. After 24 h, cells were changed to serum-free Dulbecco's minimum essential medium/Ham's F-12 medium supplemented with 7.5 mg/liter insulin, 1  $\mu$ M dexamethasone, 33  $\mu$ M biotin, 17  $\mu$ M pantothenate, and 0.2 nM triiodothyronine (21). Differentiating adipocytes were maintained in a 37 °C incubator with 5% CO<sub>2</sub>, and the medium was changed twice a week for a total of 2–3 weeks, at which time the cells exhibited adipocyte-like morphology with multiple fat droplets.

**Experimental Incubation**—On the 21st day of culture, differentiated adipocytes were changed to serum-free, supplement-free Dulbecco's minimum essential medium/Ham's F-12 medium overnight prior to initiation of experiments. The next day, the medium was changed to fresh medium supplemented with components as indicated. All additions were diluted with PBS to a final volume of 125  $\mu$ l and added to the cells with 375  $\mu$ l of Dulbecco's minimum essential medium/Ham's F-12 medium. Lipoprotein lipase (Sigma) was added to all CHYLO-containing samples at a concentration of 0.25 IU/ml. Apoproteins, TTR, and polyclonal antibody to TTR were added to CHYLO and incubated (1 h, 37 °C) prior to addition to cells. Following incubation (6 h, unless otherwise indicated) the medium was removed and frozen immediately at -70 °C for later analysis of medium ASP and C3 levels. The cells were washed twice with ice-cold PBS, 0.5 ml of 0.1 N NaOH was added to dissolve cells, and cell proteins were measured by the method of Bradford (22) using a commercial kit (Bio-Rad).

**Medium ASP Determination**—ASP was measured *via* a sandwich ELISA immunoassay. A murine (in house) monoclonal antibody raised to the last eight amino acids of the carboxyl-terminal of ASP was used as capture antibody (as described previously) (20). The monoclonal antibody was coated at 7  $\mu$ g/ml in PBS (100  $\mu$ l/well in a 96-well plate) overnight at 4 °C and blocked with 1.5% bovine serum albumin for 2 h. The plate was washed three times with wash solution between every step (0.05% Tween 20 in 0.9% NaCl). Standard solutions (0–2.13 ng/ml) of ASP, purified as described previously (20), as well as samples (conditioned culture medium diluted appropriately) and control plasma samples (precipitated and diluted as described for plasma ASP assays (20)) were added at 100  $\mu$ l/well. The plate was incubated for 1 h at 37 °C and washed, followed by an incubation for 1 h at 37 °C with 100  $\mu$ l of rabbit antiserum to human ASP (raised against the holoprotein), diluted appropriately (1:2000) in PBS with 0.05% Tween 20. The plate was then incubated for 30 min at 37 °C with 100  $\mu$ l of goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000, Sigma) diluted in PBS with 0.05% Tween 20. Following the final wash, the color reaction was initiated with 100  $\mu$ l of *O*-phenylenediamine dihydrochloride (1 mg/ml) in 100 mM sodium citrate, 0.05% Tween 20. After visual development the reaction was stopped with 50  $\mu$ l of 4 N H<sub>2</sub>SO<sub>4</sub>, and absorbance was read at 490 nm. ASP concentration *versus* absorbance was graphed and calculated by linear regression.

**Medium C3 Determination**—Medium C3 was also determined by sandwich ELISA as described previously (8). Murine monoclonal antibody to the C3d fragment of C3 (Quidel, San Rafael, CA) was coated at 1  $\mu$ g/ml in PBS (100  $\mu$ l/well) overnight at 4 °C and blocked with 1.5% bovine serum albumin for 2 h. The plate was washed three times with wash solution (0.05% Tween 20 in 0.9% NaCl) between every step. A standard solution (0–10 ng/ml) of C3 (Calbiochem) as well as test samples (conditioned culture medium diluted appropriately) and in house control plasma samples (diluted 1:10<sup>5</sup>) were added at 100  $\mu$ l/well. Subsequent steps were identical to those found in the ASP sandwich ELISA. Note that goat polyclonal anti-C3 (1:5000, Quidel) and anti-serum IgG conjugated to horseradish peroxidase (1:1250, Sigma) were

used for the C3 sandwich ELISA. Linear C3 concentration was plotted against absorbance, and sample C3 concentration was calculated by linear regression.

**Chylomicron Preparation and Fractionation**—Blood was obtained from healthy subjects with normal lipoprotein profiles 3 h following a fat meal and collected on ice into Vacutainer tubes containing EDTA as anticoagulant. Plasma was immediately isolated by low speed centrifugation at 4 °C, and the CHYLO were isolated by discontinuous preparative ultracentrifugation according to the procedure of Havel *et al.* (23). Plasma was layered under a salt solution of density 1.006 g/ml, and CHYLO were isolated after centrifugation for 30 min at 30,000 rpm (40,000  $\times$  *g*) at 11 °C. Where indicated, CHYLO were again overlaid with 1.006 g/ml solution and recentrifuged for 18 h at 40,000 rpm (100,000  $\times$  *g*) at 11 °C. The respun CHYLO were recovered in the top 2-ml fraction. The loosely associated CHYLO proteins in the infranate (lower layer) were collected and fractionated by hydrophobic interaction HPLC on a C4 column (Vydac C4, Scientific Products and Equipment Ltd, ON, Canada) from 0 to 80% acetonitrile in 0.1% fluoroacetic acid. SDS-polyacrylamide gel electrophoresis (4.8%/10%/16.5% discontinuous gradient gel) was performed on the isolated fractions according to Schagger and von Jagow (24). Triacylglycerol and cholesterol concentrations of the CHYLO were measured using commercially available colorimetric enzyme assays (Boehringer Mannheim, Laval, PQ, Canada). The protein concentrations were measured by the Lowry method (25).

**Chylomicron Modifications**—CHYLO were gently delipidated with 25 volumes of ethanol/ether (3:1 v/v) added to a known concentration of CHYLO. After incubation on ice for 2 h, the CHYLO were centrifuged at 1200 rpm (500  $\times$  *g*) for 15 min at 4 °C. The CHYLO pellet was reconstituted to its original volume using the concentration of lipid substrate (Sigma) or lipid substrate/cholesterol (50  $\mu$ g cholesterol/ml lipid substrate) equal to that of the original CHYLO triacylglycerol concentration or with a comparable volume of PBS and incubated for 1 h (37 °C) prior to addition to cells. For heat inactivation of CHYLO, a known concentration of CHYLO was incubated at 70 °C for 30 min and then quenched on ice. CHYLO were trypsinized with an equal concentration of trypsin incubated at 37 °C for 1 h, after which soybean trypsin inhibitor was added in 4-fold excess concentration.

**Statistics**—Values are reported as means of experiments (with all determinations for each point in each experiment performed in triplicate)  $\pm$  S.E. Statistical significance was set at *p* = 0.05 and was determined using either Student's *t* test or one-way ANOVA (with multiple comparisons by Dunnett's method) as indicated; where *p* = NS indicates not significant.

## RESULTS

The secretion of C3 in human differentiated adipocytes was first examined. Medium C3 was measured by sandwich ELISA. We have shown previously that ASP production was linear up to 8 h (8) and was stimulated by CHYLO. In Fig. 1 (*top panel*), C3 production in cultured medium was measured in the presence and absence of CHYLO up to a 24-h incubation period. Cells were changed to fresh serum-free and supplement-free medium 24 h before the medium was collected for C3 measurements. The CHYLO were then added during the final 0, 2, 4, 6, 8, and 24 h of the incubation period. Hence, the zero time represents the amount of C3 generated basally over the 24-h time period. In the absence of CHYLO, relatively little C3 was produced over the time period assessed (up to 24 h). In the presence of CHYLO, C3 production increased markedly and reached a maximum between the last 4–6 h of incubation, which parallels that of ASP production (Fig. 1, *bottom panel*) as shown previously (8). Thus, the CHYLO effect on C3 production, as with ASP (8), is time-dependent. Also, as shown previously, the CHYLO effect on C3 and ASP production was concentration-dependent (8). Based on these data, the remaining experiments utilized a 6-h incubation period containing 50  $\mu$ g/ml CHYLO triacylglycerol following which medium C3 and ASP were measured. Overall, under these conditions, the average stimulation of C3 was 43-fold, and the effect on ASP was 6-fold where the basal levels of C3 and ASP were  $3.40 \pm 1.09$  and  $2.40 \pm 0.90$  pmol/mg cell protein, respectively.

To characterize the component of the CHYLO that caused this effect on ASP and C3, the CHYLO were manipulated in

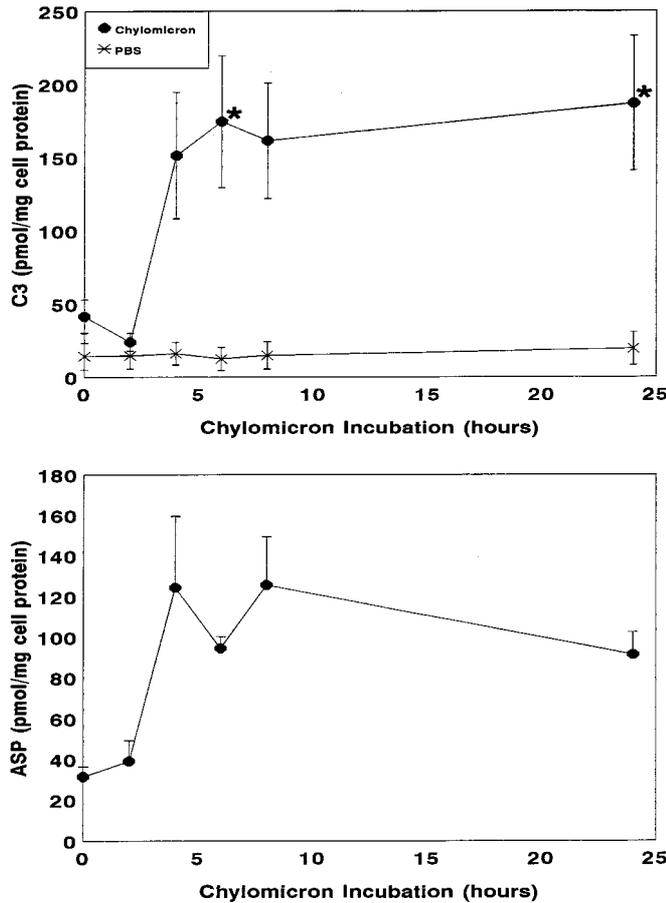


FIG. 1. Chylomicron stimulation of C3 and ASP production by human differentiated adipocytes. Differentiated human adipocytes were exposed to a constant concentration of chylomicrons (50  $\mu$ g of lipoprotein triacylglycerol/ml of medium) for the indicated times (PBS was added for control incubations). Cells were changed to fresh serum-free and supplement-free medium 24 h before the medium was collected for C3 (top panel) and ASP (bottom panel) measurements. The CHYLO were then added during the final 0, 2, 4, 6, 8, and 24 h of the incubation period. C3 and ASP levels were measured in medium by a sandwich ELISA immunoassay. The results are expressed as pmol/mg cell protein  $\pm$  S.E. for four experiments assayed in quadruplicate. CHYLO,  $p < 0.003$ ; PBS,  $p = \text{NS}$  by ANOVA; \*,  $p < 0.05$  versus zero time point for multiple comparison analysis.

several ways. As shown in Fig. 2 (top panel), delipidation of CHYLO diminished the stimulation of C3 by CHYLO. The results for ASP (bottom panel) were similar to those for C3. The stimulation of both C3 and ASP production were reinstated when the triacylglycerol-phospholipid (lipid substrate) cholesterol emulsion plus cholesterol was added back to the delipidated CHYLO, although the lipid emulsion alone had no effect on C3 or ASP production.

As shown in Table I, mild heat treatment of the CHYLO resulted in a slight decrease in C3 production and a more pronounced decrease in ASP production as compared with the positive (CHYLO) controls. Protein digestion with trypsinization of CHYLO eliminated the CHYLO-induced stimulation of both C3 and ASP production by 92% ( $p < 0.003$ ) and 100% ( $p < 0.002$ ), respectively. This would suggest that the protein component of the CHYLO is essential for C3 and ASP production; the lipid component may play a role in maintaining the proteins in the appropriate conformation for the effect.

Many apoproteins are associated with CHYLO particles (18). As shown in Table II, addition of individual apoproteins alone to the tissue culture medium had no effect on C3 production. When the apoproteins were preincubated with CHYLO and

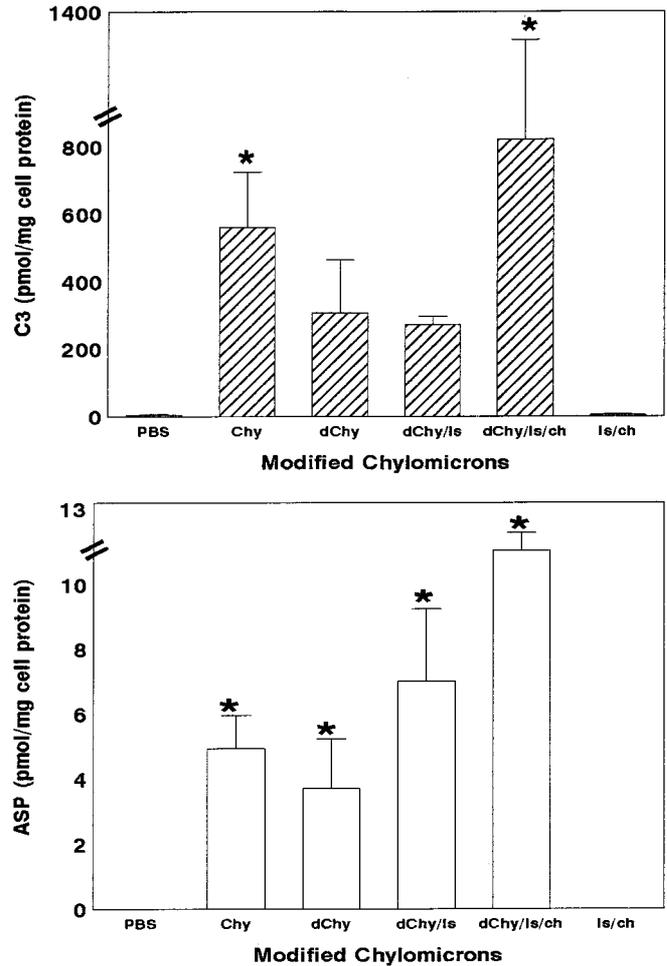


FIG. 2. The effect of lipid modified chylomicrons on C3 and ASP production by human differentiated adipocytes. Differentiated human adipocytes were exposed to a concentration (50  $\mu$ g of triacylglycerol/ml of medium) of chylomicrons (Chy), delipidated chylomicrons (dChy), delipidated chylomicrons with lipid substrate (dChy/lis), or delipidated chylomicrons with lipid substrate and cholesterol (dChy/lis/ch) for a period of 6 h. PBS or lipid substrate plus cholesterol (lis/ch) were used for the control incubations. C3 (top panel) and ASP (bottom panel) levels were measured in cell medium, and the results are expressed as pmol/mg cell protein  $\pm$  S.E. ( $n = 3$ ), where  $p < 0.0001$  for C3 and ASP by ANOVA and  $p < 0.05$  versus PBS for multiple comparison analysis (asterisk).

TABLE I

The effects of modified chylomicrons on C3 and ASP production in human differentiated adipocytes

Differentiated adipocytes were exposed to a concentration (50  $\mu$ g of triacylglycerol/ml medium) of CHYLO, heat-treated CHYLO, or trypsinized CHYLO for a period of 6 h. The modifications are described under "Materials and Methods." C3 and ASP levels were measured in cell medium, and the results are expressed as pmol/mg cell protein  $\pm$  S.E. ( $n = 7-9$ ). One-way ANOVA was performed versus PBS control, and  $p$  values are indicated accordingly.

Addition	C3	ASP
	<i>pmol/mg cell protein</i>	
PBS	3.4 $\pm$ 0.9	0.14 $\pm$ 0.12
CHYLO	371.7 $\pm$ 92.9	3.39 $\pm$ 1.15
CHYLO + heat treatment	229.3 $\pm$ 156.0	0.41 $\pm$ 0.28
CHYLO + trypsinization	7.4 $\pm$ 4.2	0.0 $\pm$ 0.0
$p$ value (ANOVA)	$p < 0.003$	$p < 0.002$

then added to the cells, again there was no significant increase in C3 production over and above that of the CHYLO positive control. Only apoprotein AIV had a small but significant effect

on C3 production. Similarly, with the same treatments, there was no significant increase in ASP production following incubation alone or in combination with CHYLO (Table II). Thus, the apoproteins tested do not appear to be the active protein component of the CHYLO responsible for the increase in C3 and ASP production.

To further investigate the protein component of the CHYLO, an additional ultracentrifugation step was performed to re-isolate CHYLO (density < 1.006) and remove loosely associated proteins (100,000 × g for 18 h). Following re-isolation of CHYLO, there was a loss of C3 and ASP stimulation as com-

pared with the CHYLO alone as shown in Table III (84% loss for C3,  $p < 0.0001$ ; 83% loss for ASP,  $p < 0.0001$ ). By contrast, the loosely associated proteins now present in the infranate (lower layer) following ultracentrifugation maintained partial capacity to stimulate the production of both C3 and ASP as compared with CHYLO (45% C3,  $p < 0.0001$ ; 59% ASP,  $p < 0.0001$ ). Therefore, it appears that the loosely associated protein(s) initially associated with the CHYLO are required for the stimulation of C3 and ASP production.

The CHYLO infranate proteins were fractionated using hydrophobic interaction C4 HPLC (five fractions on a gradient from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid). Fractions were collected, lyophilized and reconstituted in PBS. SDS-polyacrylamide gel electrophoresis demonstrated distinct protein bands in each fraction isolated. The activity of the fractions was tested in the same manner as with the infranate proteins (above). An equivalent concentration of each fraction was added to the adipocytes for a period of 6 h, after which the medium was removed and medium levels of ASP and C3 were measured by ELISA. One fraction (55–59% acetonitrile on HPLC) demonstrated activity (283%,  $p < 0.05$ ) with a later fraction (69–73% acetonitrile on HPLC) having lesser activity (184%,  $p = \text{NS}$ ) on C3 production. Of note, the activity of the CHYLO infranate (Table III) could not be fully reconstituted by the addition of the HPLC fraction alone. Therefore, it appears that this active fraction containing TTR requires the presence of other protein components to have an effect. However, no other fractions tested possessed significant activity, and the fraction with the most pronounced effect was further analyzed. This fraction contained two distinct bands on SDS-polyacrylamide gel electrophoresis under reducing conditions. An 83-kDa band was present in several fractions and co-migrated with the albumin standard. We have shown previously that albumin had no stimulatory effect on C3 and ASP production alone or in the presence of CHYLO (8). The second band had an apparent molecular mass of 20 kDa. The 20-kDa band was transferred to polyvinylidene difluoride, sequenced, and identified as TTR based on the amino-terminal 19 amino acids.

TTR is a plasma protein that complexes to retinol-binding protein and transports both thyroxine and retinol (26) in plasma. The TTR used in the following experiments is the purified uncomplexed protein (95% purified human protein, Sigma). The addition of purified TTR alone or with triacylglycerol-phospholipid (lipid substrate) cholesterol emulsion to the tissue culture medium did not result in any significant effect on C3 and ASP stimulation as compared with the basal level of C3 and ASP production (Table IV), although at higher concentrations there was some effect (Fig. 3). As well, addition of TTR back to respun CHYLO had little effect on C3 and ASP production. However, when an increasing concentration of TTR was added to a constant amount of CHYLO, there was an enhancement (up to 75-fold) of the CHYLO effect on C3 stimulation where the CHYLO effect alone was already 16-fold (Fig. 3). The

TABLE II

The effect of apoproteins on C3 and ASP production by human differentiated adipocytes

Differentiated adipocytes were exposed to a constant concentration of the selected apoprotein (10 µg/ml medium) or a mixture of all apoproteins (10 µg/ml medium) with or without CHYLO (50 µg lipoprotein triacylglycerol/ml medium) for a period of 6 h. C3 and ASP levels were measured in cell medium, and the results are expressed as pmol/mg cell protein ± S.E. ( $n = 4-8$ ). Significance was determined by one-way ANOVA (as indicated) versus the PBS control. NS, not significant.

Addition	C3		ASP	
	-CHYLO	+CHYLO	-CHYLO	+CHYLO
	<i>pmol/mg cell protein</i>			
PBS	6.8 ± 2.5	407 ± 143	2.26 ± 0.81	11.2 ± 3.6
AI	2.3 ± 1.7	763 ± 357	0.24 ± 0.21	8.9 ± 4.7
AII	0.5 ± 0.3	918 ± 289	0.0 ± 0.0	8.9 ± 4.5
AIV	16.4 ± 4.2 <sup>a</sup>	892 ± 205		
CI	3.0 ± 1.8	520 ± 224	1.14 ± 0.35	7.2 ± 2.6
CII	5.8 ± 1.3	855 ± 420	1.56 ± 0.79	17.0 ± 7.7
CIII	6.5 ± 2.1	523 ± 174	1.30 ± 0.47	8.4 ± 2.9
E	5.8 ± 1.6	910 ± 374	0.0 ± 0.0	6.7 ± 5.0
All apoproteins	8.0 ± 3.2	423 ± 15	1.10 ± 0.35	8.5 ± 4.2
<i>p</i> value (ANOVA)	$p < 0.01$	NS	NS	NS

<sup>a</sup>  $p < 0.05$  by multiple comparison analysis versus PBS control.

TABLE III

Effect of chylomicron components on C3 and ASP production in differentiated human adipocytes

Differentiated adipocytes were exposed to a constant concentration of chylomicrons (50 µg of lipoprotein triacylglycerol/ml medium), recentrifuged CHYLO (CHYLO respun, 50 µg of lipoprotein triacylglycerol/ml medium), or CHYLO protein infranate (CHYLO infranate, 2.5 µg of protein/ml medium) for a period of 6 h. C3 and ASP levels were measured in cell medium, and the results are expressed as pmol/mg cell protein ± S.E. from eleven and nine different subjects for C3 and ASP, respectively ( $n = 31-66$ ). Significance was calculated by one-way ANOVA (as indicated) versus PBS control.

Treatment	C3	ASP
	<i>pmol/mg cell protein</i>	
PBS	2.24 ± 0.34	1.45 ± 0.47
CHYLO	90.79 ± 13.18 <sup>a</sup>	11.98 ± 1.88 <sup>a</sup>
CHYLO respun	14.34 ± 4.17	2.87 ± 1.13
CHYLO infranate	41.10 ± 10.11 <sup>a</sup>	7.05 ± 2.54 <sup>a</sup>
<i>p</i> value (ANOVA)	$p < 0.0001$	$p < 0.0001$

<sup>a</sup>  $p < 0.05$  versus PBS control by multiple comparison analysis.

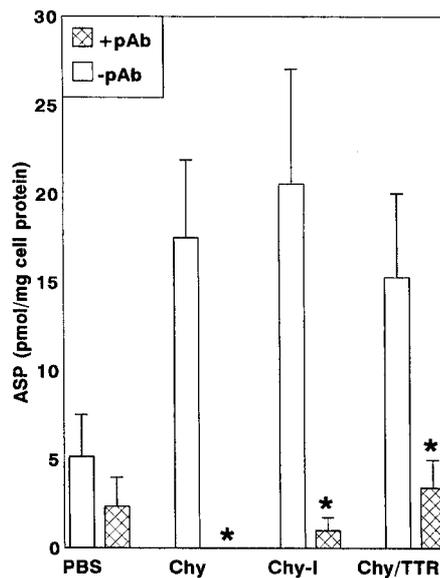
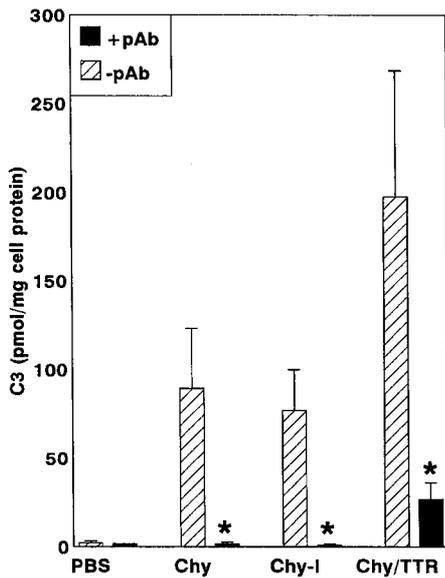
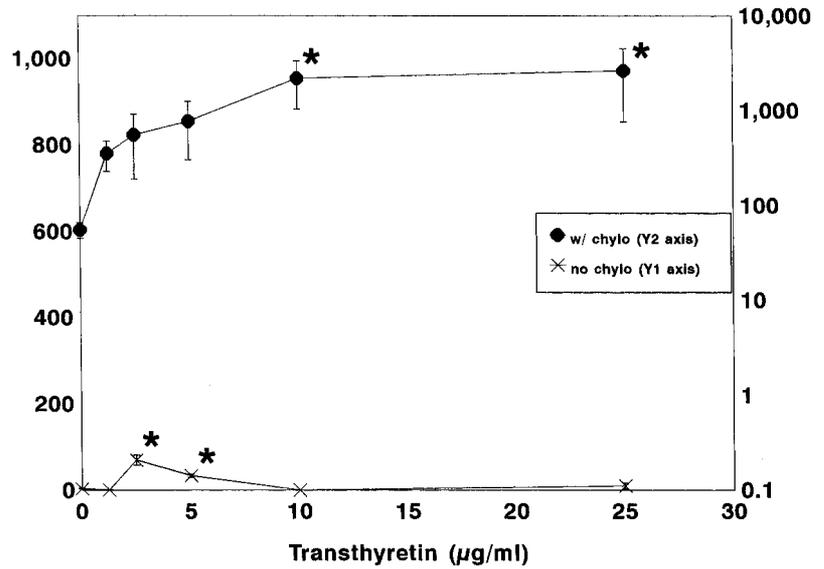
TABLE IV

The effect of transthyretin on C3 and ASP production by differentiated human adipocytes

Differentiated human adipocytes were exposed to a constant concentration of CHYLO, CHYLO respun (50 µg of lipoprotein triacylglycerol/ml medium), PBS, or lipid substrate, with or without TTR (1.25 µg/ml medium) for a period of 6 h. The results are expressed as pmol/mg cell protein ± S.E. C3 and ASP levels were measured in cell medium for three experiments assayed in quadruplicate ( $n = 12$ ). Multiple comparison analysis (ANOVA) was analyzed comparing in the presence of TTR versus absence of TTR. NS, not significant.

Addition	C3			ASP		
	-TTR	+TTR	<i>p</i> value	-TTR	+TTR	<i>p</i> value
	<i>pmol/mg cell protein</i>			<i>pmol/mg cell protein</i>		
PBS	3.48 ± 1.35	1.02 ± 0.34	NS	1.96 ± 0.55	2.53 ± 1.70	NS
Lipid substrate + cholesterol	1.17 ± 0.70	0.98 ± 0.38	NS	1.65 ± 0.73	1.30 ± 0.64	NS
CHYLO respun	2.25 ± 0.44	3.23 ± 0.72	NS	1.31 ± 0.58	3.68 ± 1.65	NS
CHYLO	55.33 ± 10.53	352.15 ± 123.19	$p < 0.0001$	13.07 ± 1.69	18.01 ± 2.90	$p < 0.0002$

**FIG. 3. Concentration-dependent enhancement of C3 production by transthyretin in the presence of chylomicrons in differentiated human adipocytes.** Differentiated human adipocytes were exposed to increasing amounts of TTR in the presence of a constant concentration of chylomicrons (50  $\mu\text{g}$  of lipoprotein triacylglycerol/ml of medium) and in the absence of chylomicrons over a period of 6 h. C3 levels were measured in cell medium, and the results are expressed as pmol/mg cell protein  $\pm$  S.E. The TTR concentration curve without chylomicrons (linear Y1 axis) was performed in duplicate ( $n = 2$ ). The TTR concentration curve with chylomicrons (logarithmic Y2 axis) was performed in triplicate in two experiments ( $n = 6$ ). One-way ANOVA was performed. \*,  $p < 0.0001$  versus no TTR additions where CHYLO stimulation (no TTR) is  $15 \pm 0.2$ -fold increase versus PBS alone.



**FIG. 4. Immunospecific blocking of transthyretin enhancement of chylomicron stimulation of C3 and ASP production.** Differentiated human adipocytes were exposed to a constant concentration of PBS, chylomicrons (Chy, 50  $\mu\text{g}$  of triacylglycerol/ml of medium), chylomicron infranate proteins (Chy-I, 2.5  $\mu\text{g/ml}$  of medium), or chylomicrons plus transthyretin (Chy/TTR, 1.25  $\mu\text{g}$  of transthyretin/ml of medium). Antiserum to TTR was added as indicated (+pAb, 2.5  $\mu\text{g/ml}$  of medium). C3 (left panel) and ASP (right panel) levels were measured in cell medium, and the results are expressed as pmol/mg cell protein  $\pm$  S.E. ( $n = 8$  in three separate experiments). Multiple comparison analysis was performed comparing results in the presence of antiserum versus the absence of antiserum. \*,  $p < 0.001$ .

results of ASP (not shown) parallel those of C3. These data suggest that although TTR plays a role in stimulating C3 and ASP production, it does not function alone.

To further examine the role of TTR in stimulating C3 and ASP production, a polyclonal antibody to TTR was tested. As shown in Fig. 4, the addition of a TTR antiserum to the culture medium (+pAb) resulted in blockage of the CHYLO-induced stimulation of both C3 and ASP production by 98% for C3 (left panel,  $p < 0.001$ ) and 100% for ASP (right panel,  $p < 0.001$ ). Similarly, addition of the TTR antiserum also blocked the increases in both C3 and ASP production induced by the CHYLO infranate proteins to the same extent, 99% for C3 ( $p < 0.001$ ) and 94% for ASP ( $p < 0.001$ ). Finally, addition of TTR to CHYLO, which enhanced the CHYLO effect, could also be blocked by addition of the TTR antiserum (86% decrease for C3,  $p < 0.05$ ; 80% decrease for ASP,  $p < 0.05$ ). A nonimmune preparation had no inhibitory effect on the TTR-CHYLO or CHYLO infranate protein stimulation (data not shown). Thus the data indicate that a loosely associated protein, TTR, found associated with CHYLO is critical to the overall mediation of the CHYLO-induced stimulation of both C3 and ASP production.

DISCUSSION

In our species, as in most others, energy intake is intermittent, and therefore the rate at which energy must be stored in adipose tissue varies markedly. Storage of dietary fatty acids in adipocytes is a two-step process: first, there must be release of fatty acids from CHYLO triacylglycerol by lipoprotein lipase, and second, there must be uptake of fatty acids and incorporation into triacylglycerol by adipocytes (18). The first takes place in the capillary space; the second takes place in the subendothelial space. However, unless the fatty acids liberated from CHYLO triacylglycerol are rapidly taken up by the adipocytes, lipoprotein lipase activity will be inhibited and triacylglycerol clearance from plasma will be reduced (27). By increasing the rate at which fatty acids are incorporated into adipose tissue triacylglycerol, the ASP pathway allows the sudden influx of the dietary fatty acids from CHYLO to be transferred rapidly and efficiently from the capillary space into adipocytes that lie in the subendothelial space (19). Thus, the rate of adipocyte triacylglycerol synthesis *in vivo* appears to govern the rate of CHYLO lipolysis by lipoprotein lipase.

The present data add powerfully to the model of the ASP pathway. *In vivo* studies in humans have shown that ASP was

released into the systemic circulation by subcutaneous adipocytes of the anterior abdominal wall (20). This release was steady during fasting and during the first 3 h after an oral fat load. However, the production of ASP increased markedly thereafter. CHYLO triacylglycerol clearance by subcutaneous adipocytes also markedly increased in the second half of the postprandial period, as did fatty acid storage in adipocytes. These data suggested that CHYLO stimulated adipocytes to increase their production of ASP. This hypothesis is in accord with previous *in vitro* studies demonstrating that CHYLO had a profound stimulatory effect on the production of ASP, as well as its precursor protein C3, from human adipocytes (8). This effect was both concentration- and time-dependent and was distinctive in that other lipoproteins (very low density lipoprotein, low density lipoprotein, and high density lipoprotein) had minimal effect. Other postprandial components such as fatty acids or glucose had little effect, and insulin had only a moderate effect (8). Based on these results, our aim was to identify the CHYLO component that was responsible for the acute stimulatory effect on C3 and ASP production.

The results of the present study indicate, first of all, that the effect of CHYLO is not only on ASP, the bioactive protein, but is also manifested through a marked increase in the precursor protein, C3. Under all experimental conditions, parallel trends in both C3 and ASP were evident. The few instances where ASP does not fully parallel C3 production may result from changes in the activation of the enzymes (adipsin and C3 convertase) involved in the conversion of C3 to ASP. The CHYLO component responsible for the increase in ASP and C3 is a protein, loosely associated with the lipoprotein particle. Although the lipid-free mixture of proteins possesses most of the stimulatory capacity, reassociation with a lipid substrate increases its potential, suggesting that a change in conformation with lipid association may enhance its capacity to stimulate. The active protein component was demonstrated to be TTR: (i) by the identification of TTR as the active protein in the CHYLO infranate protein mixture, (ii) by the demonstration that the addition of TTR to CHYLO increases the stimulatory capacity, and (iii) by the ability of a polyclonal antiserum to TTR to obliterate the stimulatory capacity of both native CHYLO and the active loosely associated protein complex. Thus, TTR appears to be necessary but not sufficient, because addition of TTR alone (purified uncomplexed protein) in the presence of lipid substrate or respun CHYLO does not produce any increase in either medium ASP or C3. Thus an additional substance that remains associated with TTR during the additional ultracentrifugation or remains with the native CHYLO particle appears to be required.

How then can TTR play a role in mediating increases in C3 and ASP production? TTR is a plasma protein that is found associated in a complex with retinol-binding protein (26). This TTR-retinol-binding protein complex binds both thyroxine and retinol and transports them through plasma to the sites of action of the active forms: T3 and retinoic acid. Both T3 and retinoic acid are regulators of gene transcription (28, 29). Both have been implicated in adipocyte differentiation (30) and acute gene regulation of adipsin and phosphoenolpyruvate carboxykinase (31–33). Cross-talk has been demonstrated between thyroid hormone, peroxisome proliferator-activated receptors, and retinoid X receptors (34, 35). Retinol is an exogenously derived dietary compound. It is a fat-soluble vitamin (vitamin A) and is absorbed and delivered to tissues through incorporation into the lipid core of the dietary triacylglycerol-rich CHYLO in intestinal villus cells (36). We propose that the TTR associated with the CHYLO may serve as the vehicle to shuttle the hormones to the adipocyte. Preliminary

studies suggest that retinoic acid associated with TTR plays a role in the stimulatory effect of TTR on C3 and ASP production.<sup>2</sup> As the CHYLO docks with lipoprotein lipase on the endothelial surface, TTR may disassociate from the CHYLO and mediate transport of the hormones to the adipocytes. This results in initiation of increased secretion of C3, which is converted to ASP. ASP will then activate triacylglycerol synthesis within the adipocyte by coordinately stimulating both glucose transport and esterification to generate storage triacylglycerol.

By this means CHYLO, once they bind to lipoprotein lipase on the adipose tissue capillary endothelium, would activate the ASP pathway. ASP, by increasing the rate of fatty acid storage in adipocytes, would allow rapid hydrolysis of CHYLO triacylglycerol to continue. Chylomicron triacylglycerol hydrolysis is then coupled to adipocyte triacylglycerol synthesis, and the ASP pathway would link events in the capillary space to events in the subendothelial space. It appears, therefore, that the ASP pathway constitutes a novel model of microenvironmental metabolic regulation that allows effective and rapid storage of energy in adipose tissue.

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