Acute in vitro production of acylation stimulating protein in differentiated human adipocytes

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Abstract We have previously shown that in normolipidemic healthy adults, plasma acylation stimulating protein (ASP) increases postprandially and is produced in vitro by cultured differentiated human adipocytes. The present studies were undertaken to examine the influence of specific plasma components on endogenous ASP production in cultured human adipocytes. The results demonstrate that neither glucose nor fatty acids (over a wide range of concentrations) had any substantial effect on ASP production. Insulin increased ASP production up to 2-fold (208% ± 18%, $P < 0.01$). However, the most profound increase in ASP was generated by the addition of chylomicrons to the cell culture medium. Chylomicrons (CHYLO) obtained from postprandial plasma increased ASP production in a time- and concentration-dependent manner, producing up to a 150-fold increase in ASP at the highest concentration of CHYLO tested (500 μg triacylglycerol/mL medium ($P < 0.001$)). By contrast, very low (VLDL), high (HDL), and low density lipoproteins (LDI) had only marginal effects. The effects on ASP parallelled the changes in adipocyte C3 secretion (the precursor protein of ASP). As with ASP, glucose, oleate, insulin, and hepatic lipoproteins (VLDL, LDL, and HDL) had little or no effect on C3 secretion. In contrast, CHYLO had an even greater effect on C3 secretion than on ASP generation. Finally, the effects of CHYLO on generation of ASP and C3 were not dependent on lipolysis of CHYLO by lipoprotein lipase (LPL). These results are consistent with the changes in plasma ASP seen postprandially, and suggests a role of ASP as a positive feedback regulator of triacylglycerol synthesis in adipose tissue.—**Maslowska, M., T. Scantlebury, R. Germinario, and K. Cianflone.** Acute in vitro production of acylation stimulating protein in differentiated human adipocytes. *J. Lipid Res.* 1997. **38:** 1–11.

Supplementary key words triacylglycerol • postprandial metabolism • adipose tissue • complement C3

The role of adipose tissue in the storage and release of energy is well known. Fatty acids from adipose tissue are mobilized in response to specific stimuli through the action of hormone-sensitive lipase. The activity and regulation of this enzyme have been well characterized (1). Classically, interaction of a lipolytic hormone with the cell surface of the adipocyte results in activation of adenylate cyclase, increasing the production of cAMP which activates protein kinase A. This enzyme, in turn, phosphorylates hormone-sensitive lipase, thus activating it. To oppose this action, insulin stimulates phosphodiesterase which catabolizes cAMP, preventing activation of hormone sensitive lipase.

In contrast to lipolysis, although the enzymatic sequence for triacylglycerol synthesis is well known, none of the enzymes have been purified, nor is their regulation well characterized (2). Triacylglycerol molecules are formed through sequential enzymatic reactions in which fatty acid molecules are esterified to a glycerol-3-phosphate backbone. Both phosphatidate phosphohydrolase (which catalyzes the dephosphorylation of phosphatidate to form diacylglycerol) and diacylglycerol acyltransferase (which catalyzes the final esterification reaction to form triacylglycerol from diacylglycerol) have been implicated as the rate-limiting step (3, 4). To date, insulin has been considered to be the main factor responsible for stimulation of triacylglycerol synthesis in adipose tissue. In fact, insulin does have a profound effect on glucose transport and on inhibition of lipolysis mediated by hormone-sensitive lipase, but has little effect on the fatty acid esterification process itself which ultimately produces triacylglycerol (5).

Although triacylglycerol storage has usually been considered to be the main function of adipose tissue, more recently, it has been recognized as a secretory organ (6). In addition to lipoprotein lipase (LPL), adipocytes are also a source of cholesteryl ester transfer pro-

Abbreviations: ASP, acylation stimulating protein; BSA, bovine serum albumin; C3, complement C3, CHYLO, chylomicrons; DMEM/F12, Dulbecco's, minimum essential medium/Ham's F12; FBS, fetal bovine serum; HDL, high density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; MEM, minimum essential medium; PBS, phosphate-buffered saline; RIA, radioimmunoassay; VLDL, very low density lipoprotein.

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tein (7), apoE (8), estrogen (9), angiotensinogen (10), and tumor necrosis factor (TNF) (11), as well as the widely publicized discovery of leptin, the obese gene product responsible for the mutation in ob/ob mice (12, 13).

The discovery that adipose tissue also produces and secretes discrete proteins of the alternate complement pathway evolved from the work of White et al. (14) and Choy, Rosen, and Spiegelman (15) on murine 3T3-L1 clonal adipocyte differentiation. They demonstrated that murine cultured adipocytes synthesize and secrete a novel protein, in a differentiation-dependent manner, that was named adipsin (14). Cloning from human adipocytes indicated that adipsin is homologous to human plasma factor D and is one of the proteins involved in the alternate complement pathway (15). Similarly, complement C3 and factor B are also expressed and secreted in a differentiation-dependent manner in cultured adipocytes and mouse adipose tissue (15). These observations did not, however, define any specific function for the system, but interestingly, coincided with the molecular identification of acylation stimulating protein (ASP) by our group (16).

Upon purification, ASP, a human plasma protein, was found to be identical to C3adesArg (16), a cleavage product generated through the specific action of factor B and adipsin (factor D) on the C3 protein (15). ASP is produced in differentiated human adipocytes to a much greater extent than in human preadipocytes (17, 18). The increase in ASP production is differentiation-dependent and is associated with increased mRNA of the three factors needed: C3, adipsin, and factor B, and precedes the profound increase in triacylglycerol synthetic capacity seen in these cells (18). The production of ASP is correlated with the size of the adipocytes (17) and is dependent both on the secretion of all three proteins (C3, factor B, and adipsin) from adipocytes and on the appropriate activation of the enzymatic cleavage.

ASP was initially discovered based on its functional activity (16, 19). ASP actively stimulates triacylglycerol synthesis in human skin fibroblasts and to a much greater extent in human adipocytes (16). ASP also stimulates glucose transport in fibroblasts (20), adipocytes (21), and muscle cells (22) and does so through translocation of the glucose transporters (GLUT 1, GLUT 4, and GLUT 3) from intracellular pools to the plasma membrane. ASP action is achieved through interaction with the cell membrane, which results in stimulation of the second messenger diacylglycerol (23). Diacylglycerol then mediates stimulation and translocation of protein kinase C producing a downstream stimulation of triacylglycerol synthesis (23). Differentiated adipocytes are more responsive to ASP stimulation than are preadipocytes (17).

These findings suggest that the storage of triacylglycerols in adipocytes may, in fact, be regulated at the cellular level by the tissue itself and therefore provide a positive feedback stimulus. We have previously shown that plasma ASP is modulated through dietary intake. Prolonged fasting in obese subjects results in profound decreases in plasma ASP (24). Postprandially, ASP increases after a fatload (25), and the present studies were undertaken to examine the influence of specific plasma components on endogenous ASP production in cultured human adipocytes.

MATERIALS AND METHODS

Oleic acid (sodium salt), bovine serum albumin, essentially fatty acid-free (BSA), collagenase Type II, and all other tissue culture grade compounds were from Sigma (St. Louis, MO). General chemicals were from Fisher Scientific (Nepean, Canada). All tissue culture medium, Dulbecco's phosphate-buffered saline (D-PBS), fetal bovine serum (FBS), and all other tissue culture supplies were from Gibco (Gaithersburg, MD) or Flow Laboratories (Mississauga, Ontario).

Culture of human differentiating adipocytes

Human adipose tissue was obtained with informed consent from patients undergoing reduction mammoplasty and then processed as previously reported (17). Briefly, adipose tissue was cleaned of connective tissue and small blood vessels, then minced and treated with collagenase. The cell suspension was centrifuged to pellet the stromal-vascular cells (containing the preadipocytes) and was subsequently treated with the lysing buffer for 10 min to lyse the red blood cells. After filtration through a 50-μm filter and gentle centrifugation, the cell pellet was resuspended in minimum essential medium (MEM) containing 10% FBS. Preadipocytes were plated out on 24-well culture plates (10 g of cleaned tissue per 24-well plate). After 24 h, cells were changed to serum-free Dulbecco's minimum essential medium/Ham's F12 medium (DMEM/F12) supplemented with 7.5 mg/L insulin, 1 μM dexamethasone, 33 μM biotin, 17 μM pantothenate, and 0.2 nM triiodothyronine (18). Differentiating adipocytes were maintained in a 37°C incubator with 5% CO_2 and the medium was changed on the cells twice a week for a total of 3 weeks at which time the cells exhibited, by microscopy, clear adipocyte morphology with multiple fat droplets.

Experimental incubation

On the 21st day of culture, differentiated adipocytes were changed to serum-free, supplement-free DMEM/

F12 overnight prior to initiation of experiments. The next day, cells were exposed to the medium supplemented with different plasma components as indicated. Basal ASP production was linear for up to 8 h (data not shown); hence, the incubation time chosen for the experiments was 6 h. For the oleate experiments, oleic acid complexed to BSA in a 5:1 molar ratio as described by Van Harken, Dixon, and Heimberg (26) was added to the cells up to a final concentration of 0.8 mM. For the chylomicron (CHYLO) time course experiments, medium was changed anew to serum- and supplement-free medium. To this medium, CHYLO were then added at a concentration of 50 μg triacylglycerol/mL medium for the last 0, 2, 4, 6, 8, and 24 h of the 24-h incubation period.

In all experiments, after incubation 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 the cells and cell proteins were measured by the method of Bradford (27) using a commercial kit (Bio-Rad, Hercules, CA).

Medium ASP determination

ASP was measured in the medium of cultured human differentiating adipocytes after incubation of the cells in serum-and supplement-free medium with various additions using a radioimmunoassay (RIA) kit specific for C3adesArg (Amersham, Oakville, Canada) with values expressed as nmol ASP/mg cell protein. The use of the commercial RIA for C3adesArg for ASP determination has been validated in detail previously (17, 18).

Medium C3 determination

Medium C3 was determined by sandwich ELISA immunoassay. Murine monoclonal antibody to the C3d fragment of C3 (Quidel, San Rafael, CA) was coated at 1 μg/mL in PBS (100 μL per well) overnight at 4°C and blocked with 1.5% BSA 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 to 10 ng/mL) of C3 (Calbiochem, San Diego, CA), as well as test samples (conditioned culture media diluted appropriately) and in-house control samples were added at 100 μL per well. The plate was incubated for 1 h at 37°C, followed by incubation for 1 h at 37°C with 100 μL goat polyclonal anti-C3 (Quidel), diluted appropriately (1:5000) in PBS–0.05% Tween 20. The plate was then incubated for 30 min at 37°C with 100 μL rabbit anti-goat IgG conjugated to horseradish peroxidase (diluted 1:1250, Sigma, St. Louis, MO) diluted in PBS–0.05% Tween 20. After the final wash, the color reaction was initiated with 100 μL *o*-phenylamine dihydrochloride (1 mg/mL) in 100 mM Na citrate, 0.05% Tween 20. After visual development the reaction was stopped with 50 μL of 4 N H_2SO_4 and absorbance was read at 490 nm.

Lipoprotein isolation

Blood was obtained from healthy subjects with normal lipoprotein profiles and collected on ice into Vacutainer Tubes containing EDTA as anticoagulant. Plasma was immediately isolated by low-speed centrifugation at 4°C and the lipoproteins were subsequently separated by discontinuous preparative ultracentrifugation according to the procedure of Havel, Eder, and Bragdon (28). In summary, fresh plasma was initially layered under a salt solution of density 1.006 g/mL. CHYLO were isolated after centrifugation for 30 min at 30,000 rpm at 11°C. The infranate from the initial step was again overlaid with 1.006 g/mL solution and centrifuged for 18 h at 40,000 rpm (100,000 *g*) at 11°C. VLDL was recovered in the top 2-mL fraction. LDL was isolated from the infranate obtained in the second step by increasing solution density from 1.006 g/mL to 1.063 g/mL. The supernatant fraction was removed after centrifugation at 40,000 rpm (100,000 *g*) for 20 h. Finally, the remaining infranate was used for HDL isolation after increasing the solvent density to 1.21 g/mL and centrifuging for 48 h at 40,000 rpm (100,000 *g*) at 11°C. LDL and HDL fractions were dialyzed overnight in PBS at 4°C. Triacylglycerol and cholesterol concentrations of the lipoprotein fractions were measured using commercially available assays from Boehringer Mannheim (Laval, Quebec).

Statistics

Values are reported as means of experiments (with all determinations for each point in each experiment performed in triplicate) ± standard error of the mean (SEM). Statistical significance was set at $P = 0.05$ and was determined by computer-assisted analysis (SigmaStat Software, Jandel Scientific, San Rafael, CA) using either one-way ANOVA or paired Student's *t*-test as indicated in the results or figure legends where P = NS indicates not significant.

RESULTS

We have previously demonstrated that ASP is produced by cultured human differentiated adipocytes and that its production increases proportional to differentiation of adipocytes (17, 18). The aim of the present study was to determine whether ASP production could be influenced and to identify potential physiological stimulatory factors for this.

Human preadipocytes were obtained from adipose tissue and differentiated into adipocytes in culture over the period of 3 weeks as described in Materials and

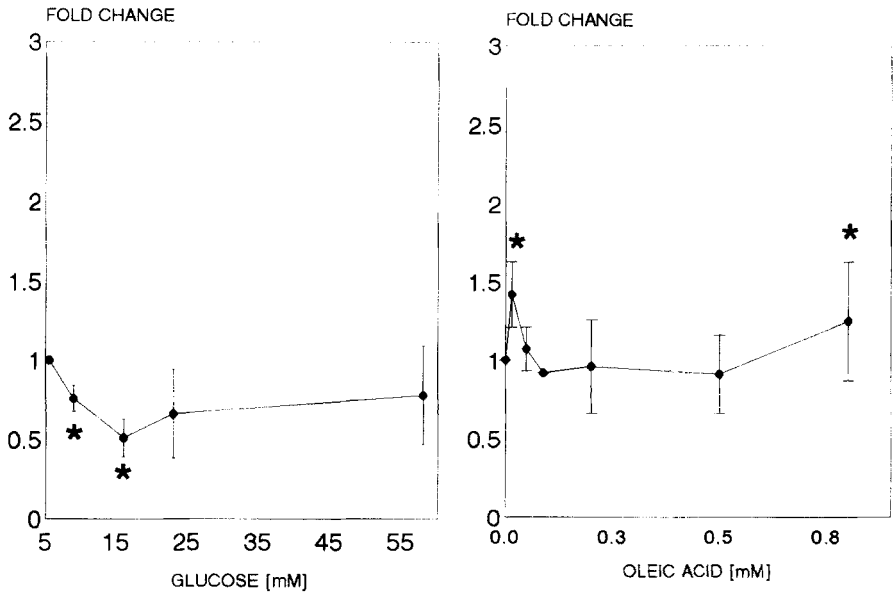

Fig. 1. Concentration-dependent effect of glucose and oleate on ASP generation by human differentiated adipocytes. Human preadipocytes were differentiated in serum-free supplemented medium for 3 weeks. Cells were then exposed to increasing concentrations of glucose (left panel) or oleic acid complexed to BSA (right panel) over a period of 6 h. Medium ASP was measured by RIA (pmol/mg cell protein) at each of the concentrations. The results are expressed as fold change in ASP ± SEM as compared to the control, where control values are defined as 1.0; $*P < 0.05$ by one-way ANOVA for n = 3 experiments from three different subjects each assayed in triplicate at each concentration (n = 9). Basal ASP concentration was 35.2 ± 2.1 and 31.1 ± 10.4 pmol/mg cell protein for glucose and oleate experiments, respectively.

Methods. At this point, the adipocytes were changed to fresh serum- and supplement-free medium for the indicated times and the amount of ASP produced by control cells was then measured. ASP production was found to be linear up to 8 h and for all incubations, unless otherwise indicated, medium ASP was measured after a 6-h incubation period.

As fatty acids and glucose provide essential building blocks for triacylglycerols and ASP is very effective in stimulating both triacylglycerol synthesis and glucose transport in human adipocytes, we first examined the effects of these two substrates on ASP production by cultured human differentiated adipocytes. As shown in **Fig. 1,** ASP generation in culture medium was measured by RIA in the presence of increasing concentrations of oleic acid and glucose. These concentrations represent physiological values in humans ranging from fasting to postprandial levels. Figure 1 (left panel) represents data obtained from cells that were exposed for 6 h to increasing concentrations of glucose (5.0 mM to 55.0 mM) where basal ASP = 35.2 ± 2.1 pmol/mg cell protein. The amount of ASP generated by the differentiated adipose cells in the medium under different glucose concentrations did not differ overall from the control values at any glucose concentration as determined by ANOVA. There was a slight decrease (−49% ± 12) at a glucose concentration of 16 mM ($P < 0.05$). The results from experiments in which increasing concentrations of oleic acid complexed to BSA (up to 0.8 mM) were added to the culture medium are also shown in Fig. 1 (right panel) where basal ASP = 31.1 ± 10.4 pmol/mg cell protein. Again, the amount of ASP generated by the cells differed little from the baseline other than the slight, but significant increase at a very low concentration. At 0.025 mM the increase was 42% ($P < 0.05$) and at 0.8 mM it was 25% ($P < 0.05$) as determined by ANOVA.

The capacity of cultured human adipocytes to generate ASP was also examined under different concentrations of insulin ranging from fasting to postprandial to pharmacological (up to 100 mU/mL) concentrations (**Fig. 2**). Insulin has a well-documented effect on triacylglycerol synthesis through its action on glucose transport and inhibition of hormone-sensitive lipolysis (5). It is also an essential component necessary for differentiation of both mouse and human adipocytes (29). With increasing medium insulin concentrations, there was a slow but steady increase in ASP in the medium where basal ASP = 11.3 ± 3.9 pmol/mg cell protein. The ASP increased a maximum of 2.1 ± 0.2-fold (208 ± 18%) at a concentration of 50 mU/mL of insulin, $P < 0.01$ as determined by ANOVA.

We have previously shown that plasma ASP increases after an oral fatload in normolipidemic subjects con-

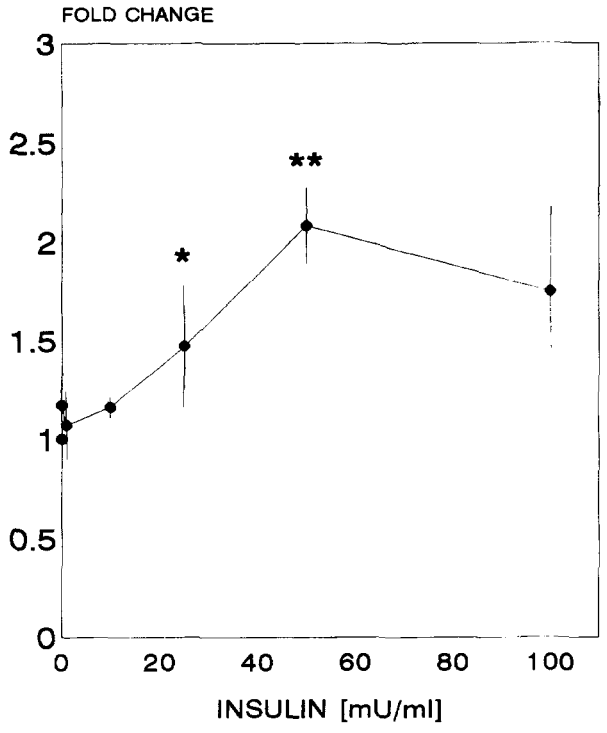

Fig. 2. Dose-dependent effect of insulin on ASP generation by human differentiated adipocytes. Human preadipocytes were differentiated in serum-free supplemented medium over a period of 3 weeks. Differentiated adipocytes were then exposed to increasing insulin concentrations for 6 h. Medium ASP was measured by RIA as described in Methods and the results are expressed as fold change in medium ASP ± SEM as compared to the control, where control values are given as 1.0; n = 3 experiments from three different subjects each assayed in triplicate (n = 9), $*P < 0.05$ and $**P < 0.01$. Basal ASP concentration was 11.3 ± 3.9 pmol/mg cell protein.

TABLE 1. Effect of lipoproteins on ASP generation by human differentiated adipocytes

Addition	ASP	*P*
	pmol/mg cell protein	
PBS	38.1 ± 2.1	
LPL	38.6 ± 1.8	ns
CHYLO	474.0 ± 94.0	<0.025
VLDL	51.6 ± 6.9	ns
LDL	47.8 ± 10.0	ns
HDL	38.0 ± 6.7	ns

Human preadipocytes were differentiated in serum-free hormone-supplemented medium over a period of 3 weeks. Differentiated adipocytes were then exposed to CHYLO or VLDL (at 50 μg lipoprotein triacylglycerol/mL medium), LDL, or HDL (at 25 μg lipoprotein cholesterol/mL medium) for 6 h. LPL was added to all lipoprotein fractions at 0.25 U/mL. Medium ASP was measured by RIA. The results are shown as pmol/mg cell protein ± SEM for an average of three different subjects from three experiments each assayed in triplicate (n = 9). Statistical significance was calculated using two mean *t*-test where *P* ns = not significant.

comitant with an increase in plasma triacylglycerol (25). We therefore tested the ability of various lipoprotein fractions to stimulate ASP production. Lipoprotein particles were isolated by sequential discontinuous ultracentrifugation from normolipidemic plasma as described in the Materials and Methods section. Each lipoprotein fraction was assayed for the concentrations of triacylglycerol and cholesterol. CHYLO and VLDL were added to the cultured cells at a concentration of 50 μg of lipoprotein triacylglycerol/mL and LDL and HDL were added at a concentration of 25 μg of lipoprotein cholesterol/mL. These concentrations represent low plasma levels. **Table 1** shows the changes in the medium ASP levels after a 6-h incubation with the indicated concentrations of CHYLO, VLDL, LDL, and HDL. As compared to control values there was on average a 12-fold increase ($P < 0.025$) in the amount of ASP generated by the cells after their exposure to CHYLO whereas all other lipoprotein fractions had no significant effect on the cells as compared to CHYLO. Nonetheless, the effects of VLDL, LDL, and HDL on ASP generation were greater than the effects of fatty acids and comparable to those of insulin. It should be pointed out that there is plasma ASP associated with the CHYLO lipoprotein fraction, but background ASP was subtracted from the total medium ASP. In all cases, the amount of plasma ASP associated with the CHYLO fraction was not greater than 10–20% of the total medium ASP generated by cultured adipocytes. There was no plasma ASP associated with the other lipoprotein fractions.

This profound stimulatory effect of CHYLO on ASP production was further examined. The effects of varying concentrations of CHYLO on cultured human adipocytes are shown in **Fig. 3.** The data demonstrate that increasing concentrations of CHYLO increase the amount of ASP that is generated in the medium of human adipocytes. Concentrations up to 500 μg/mL are shown in the graph, lower concentrations (up to 50 μg/mL) are shown in the inset. At both low and high concentrations, the increase in ASP is proportional to the amount of CHYLO reaching 150-fold at the highest concentration of 500 μg lipoprotein triacylglycerol/mL ($P < 0.001$). We did not observe a plateau at the CHYLO concentrations tested. However, one must keep in mind that the concentrations chosen were still well within the range of physiological postprandial levels; 500 μg/mL is equivalent to 50 mg/dL plasma triacylglycerol and postprandial increases can be substantially larger than that. It should also be noted that in each experiment, cells were derived from a different subject. Therefore, there is a certain amount of variability in the extent to which the cells differentiate, and thus in the basal amount of ASP produced (17, 18). Similarly, the responses to CHYLO stimulation vary from 4-fold to 20-

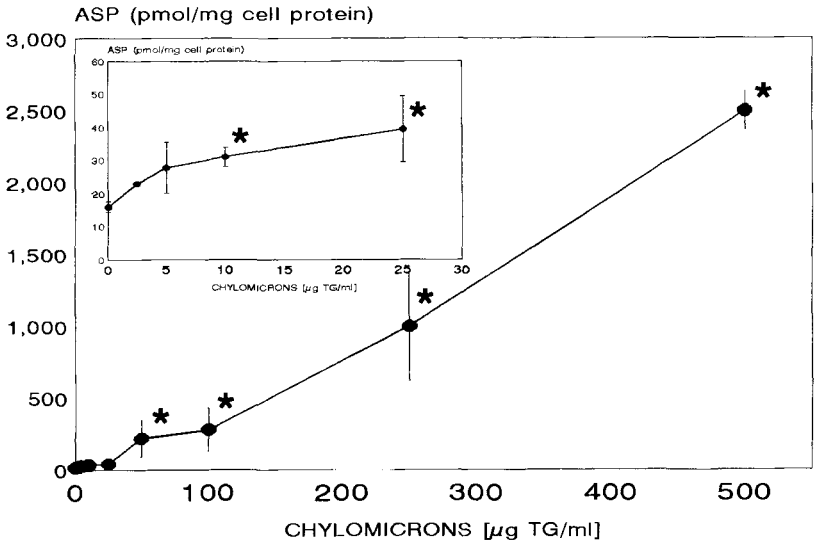

Fig. 3. Dose-dependent effect of chylomicrons on ASP generation by human differentiated adipocytes. Human preadipocytes were differentiated in serum-free supplemented medium over a period of 3 weeks. Differentiated adipocytes were then exposed to increasing concentrations of CHYLO (in μg lipoprotein triacylglycerol/mL medium) for 6 h and ASP generated was measured in the cell medium by RIA. LPL was added to all CHYLO concentrations at 0.25 U/mL. The results are shown as pmol ASP/mg cell protein ± SEM for an average of three different subjects from three experiments each assayed in triplicate (n = 9). Statistical significance was calculated by one-way ANOVA. Significance for the individual CHYLO concentrations was calculated by paired *t*-test where $*P < 0.05$.

fold, although they are significantly increased in all cases.

Next, to investigate the response rate to the CHYLO stimuli, the adipocytes were exposed to CHYLO at a selected concentration of 50 μg triacylglycerol/mL for varying incubation periods. In this experiment the cells were changed to fresh serum-free and supplement-free medium 24 h before the medium was collected for ASP determination. The CHYLO were then added during the final 0, 2, 4, 6, 8, and 24 h of the incubation period. Therefore, the 0 time point represents the amount of ASP generated basally over the 24-h time period. As shown in **Fig. 4** the amount of ASP generated when CHYLO were present for the last 2 h of the 24-h time period was almost the same as the baseline or 0 time point. The amount of ASP present increased rapidly when CHYLO were present during the last 4 to 6 h incubation reaching a plateau thereafter which represents a 4-fold increase over baseline in these experiments ($P < 0.01$ as measured by ANOVA).

Effects on ASP accumulation in the medium could be the result of two actions: an increase in the cellular secretion of C3, the precursor molecule from which ASP is generated through enzymatic cleavage, or an increase in the proportion of C3 that is enzymatically converted to ASP. We therefore examined C3 production

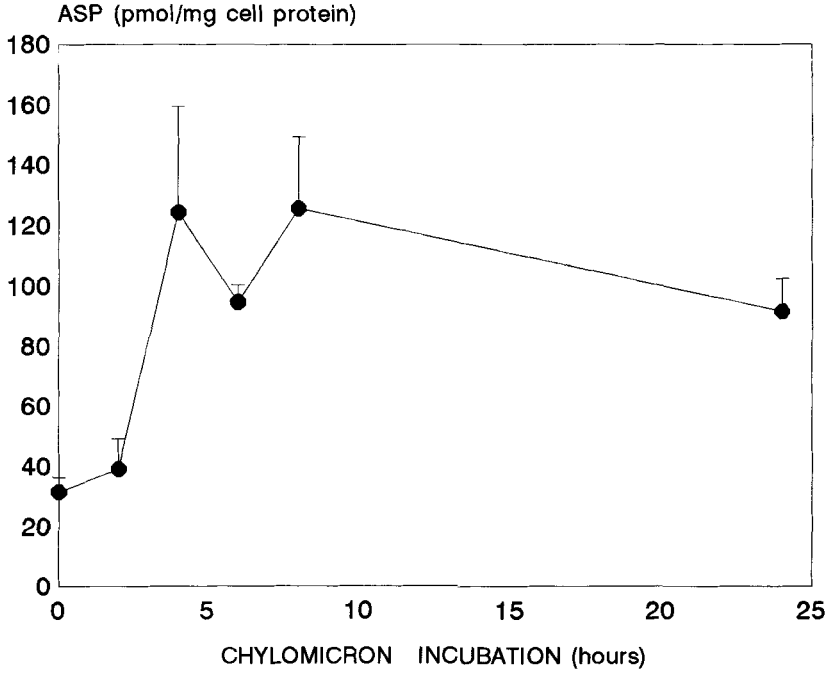

Fig. 4. Time course of the chylomicron effect on ASP generation by human differentiated adipocytes. Human preadipocytes were differentiated in serum-free supplemented medium over a period of 3 weeks. Differentiated adipocytes were then changed to serum-free medium and exposed to CHYLO at 50 μg lipoprotein triacylglycerol/mL medium for the indicated times. ASP levels were measured in the cell medium by RIA. The results are expressed as pmol/mg cell protein ± SEM for two experiments from two different subjects assayed in triplicate (n = 6); $*P < 0.01$ determined by ANOVA for all points except at 2 h.

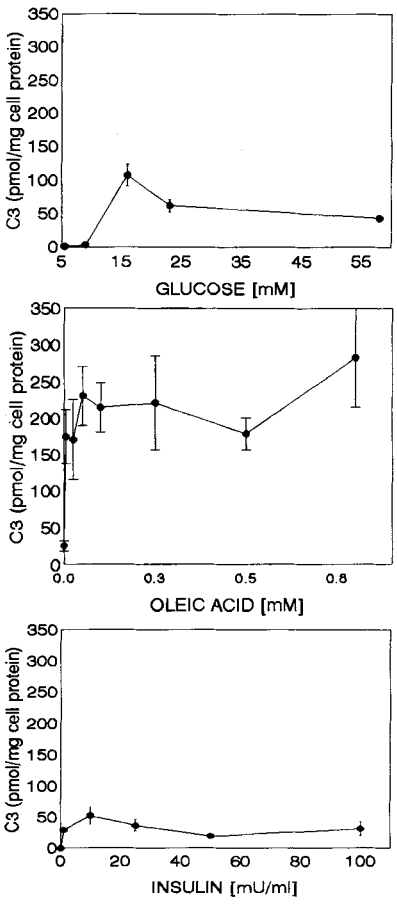

Fig. 5. Concentration-dependent effect of glucose, oleate, and insulin on C3 generation by human differentiated adipocytes. Human preadipocytes were differentiated in serum-free supplemented medium for 3 weeks. Cells were then exposed to increasing concentrations of glucose (top panel, n = 6), oleic acid complexed to BSA (middle panel, n = 6), or insulin (bottom panel, n = 9) over a period of 6 h from 2–3 different subjects. Medium C3 was measured by ELISA (pmol/mg cell protein) at each of the concentrations. The results are expressed average C3 ± SEM (pmol/mg cell protein); $*P < 0.01$ for all concentrations of glucose, oleic acid, and insulin (except 10 mM glucose, *P* ns).

in the adipocytes and the effects of various postprandial factors. As shown in **Fig. 5,** as with ASP, glucose, oleic acid, and insulin had only minimal but significant effects on C3 production in the adipocytes. The amount of C3 produced was, on average, much greater than the amount of ASP produced on a molar basis. Therefore, under these incubation conditions, only a portion of the C3 secreted from adipocytes is converted to ASP.

The effect of various lipoproteins on C3 production in adipocytes was tested as for ASP. As shown in **Table 2,** neither VLDL, LDL, nor HDL had any significant effect on C3 production. As with ASP increases, CHYLO had a profound stimulatory effect on C3 production ($P<0.0025$). There was a small amount of endogenous C3 associated only with the CHYLO, and this was subtracted from the total medium C3. Background CHYLO C3 levels did not exceed 10–20% of total medium C3.

TABLE 2. Effect of lipoproteins on C3 generation by human differentiated adipocytes

Addition	C3	*P*
	pmol/mg cell protein	
PBS	10 ± 3	
LPL	14 ± 4.3	ns
CHYLO	3009 ± 670	<0.0025
VLDL	26 ± 13	ns
LDL	18 ± 5.6	ns
HDL	0	—

Human preadipocytes were differentiated in serum-free supplemented medium over a period of 3 weeks. Differentiated adipocytes were then exposed to CHYLO or VLDL (at 50 μg lipoprotein triacylglycerol/mL medium), LDL, or HDL (at 25 μg lipoprotein cholesterol/mL medium) for 6 h. LPL was added to all lipoprotein fractions at 0.25 U/mL. Medium C3 was measured by ELISA. The results are shown as pmol/mg cell protein ± SEM for an average of three different subjects from three experiments each assayed in triplicate (n = 9). Statistical significance was calculated using two mean *t*-test where *P* ns = not significant.

We then tested the effects of a range of CHYLO concentrations on C3 production and these results are shown in **Fig. 6.** Again, as with ASP increases, CHYLO had a profound and significant stimulation on C3 production at all concentrations, $P < 0.0025$. The amount of C3 produced appeared to increase linearly up to 100 μg CHYLO TG/mL, and then began to level off at higher concentrations of CHYLO. Overall, the proportion of C3 that was converted to ASP was on average 12%.

LPL is made and secreted by adipose tissue and is necessary for hydrolysis of plasma lipoprotein triacylglycerol. In some cases, LPL was also added to the cultured adipocytes at a concentration of 0.25 U/mL. Addition of LPL alone had no effect on ASP generation by the cells (P = NS as determined by two mean *t*-test, results not shown). Addition of LPL to the incubations with the lipoproteins (CHYLO, VLDL, LDL, or HDL) also had no additional effect on generation of ASP. This is not surprising, as the differentiated adipocytes are capable of secreting active lipoprotein lipase. We then tested to see whether lipolysis was necessary for the CHYLO effect on ASP and C3 production. As shown in **Fig. 7,** the addition of BSA alone (2 mg/mL) to the culture medium had no effect on basal C3 or ASP production. As well, addition of BSA with CHYLO had no effect on the CHYLO induced increase in C3 and ASP. We also tested the effect of adding increasing concentrations of tetrahydrolipstatin (THL). THL is an effective lipoprotein lipase inhibitor that acts via binding to the active site (30). THL was added to the adipocytes at two different concentrations concurrently with the CHYLO, and both C3 and ASP production were assessed. As shown in Fig. 7, addition of THL did not prevent the CHYLO-induced increase in C3 and ASP, nor

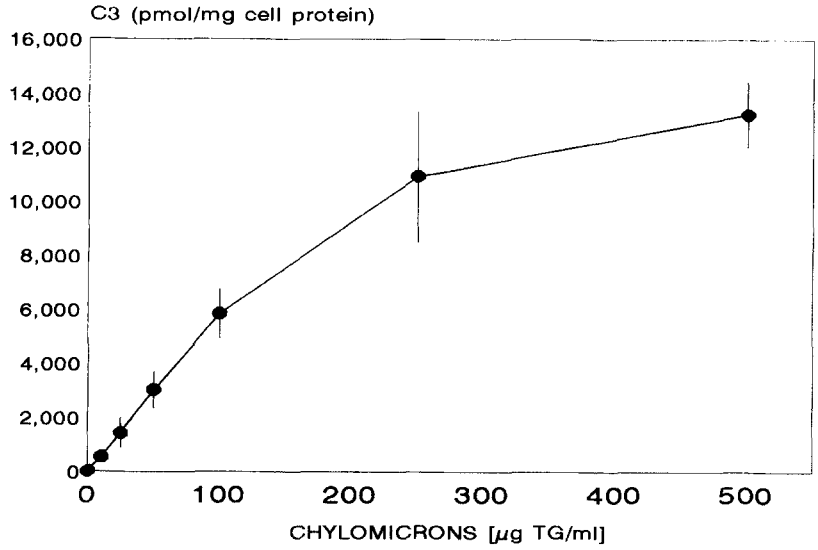

Fig. 6. Dose-dependent effect of chylomicrons on C3 generation by human differentiated adipocytes. Human preadipocytes were differentiated in serum-free supplemented medium over a period of three weeks. Differentiated adipocytes were then exposed to increasing concentrations of CHYLO (in μg lipoprotein triacylglycerol/mL medium) for 6 h and C3 generated was measured in the cell medium by ELISA. LPL was added to all CHYLO concentrations at 0.25 U/mL. The results are shown as pmol/mg cell protein ± SEM for an average of n = 6 from two different subjects. Significance for the individual CHYLO concentrations was calculated by *t*-test of CHYLO where $*P < 0.0025$ for all concentrations.

did THL have any effect on basal C3 and ASP production. This suggests that the release of C3 and production of ASP is not secondary to a detergent effect caused by the hydrolytic production of large amounts of fatty acid. Finally, we also examined the interaction of insulin and CHYLO on C3 and ASP production. Although the addition of insulin with CHYLO had no additive effect on ASP production, there was an additive effect with respect to C3 production as compared to CHYLO alone ($P < 0.01$).

DISCUSSION

The striking effects of ASP on triacylglycerol synthesis as well as on glucose transport in human fibroblasts and particularly in human adipocytes (16–22) have made it clear that the triacylglycerol synthetic pathway is regulated and that ASP is a key regulator of this pathway. This is particularly relevant as ASP not only has marked effects on adipocyte lipid metabolism, but is also produced by mature adipocytes (17, 18). Based on these observations, the goal of this study was to define the particular stimuli that modulate ASP production. The present results provide the first evidence that ASP generation from human adipocytes can be driven by specific postprandial plasma components.

Neither glucose nor oleate had significant effects on ASP generation in the culture medium. The fact that glucose and free fatty acids did not cause increases in medium ASP levels was initially surprising as both factors increase postprandially. However, it should be noted that fatty acids are also generated through the action of hormone-sensitive lipase in adipocytes for the purpose of releasing fatty acids for transport to other tissues (1). An increase in ASP and C3 generation at this point would indeed be paradoxical and would result in a futile cycle of lipolysis/reesterification. The results indicate that insulin does significantly increase ASP and C3 production. Increases in insulin may be one mechanism by which plasma ASP increases postprandially, particularly at the early stages (1–2 h) when insulin levels are elevated.

We have shown in fatload studies that plasma ASP levels rise concurrently with the rise of plasma triacylglycerols (22). We speculated, therefore, that the triggering signal might lie within the lipoprotein particles that carry dietary fat in the form of triacylglycerols. To test this hypothesis, experiments were performed in which different plasma lipoprotein fractions (CHYLO, VLDL, LDL, and HDL) were tested for their effect on ASP and C3 production. Addition of CHYLO to the cultured adipocytes caused dramatic elevations in both medium ASP and the precursor molecule to ASP, C3, and these effects were both time- and concentration-dependent. This suggests that the signalling mechanism is on the CHYLO particle itself. Certainly, the apolipoprotein composition of CHYLO is different from that of the other lipoproteins. One obvious difference is the presence of apoB-48 on CHYLO, whereas the other apoB-containing lipoproteins have only apoB-100 (31). There are many other differences as well, including differences in lipid composition and lipid-soluble factors such as retinol ester (32) and future experiments will focus on defining the key components in CHYLO responsible for the effects on ASP and C3 production. Based on the magnitude of the increase in ASP achieved with the addition of in vivo concentrations of CHYLO, this mechanism is most likely the major physiological source of the postprandial increase in ASP.

What, then, is the mechanism by which insulin and

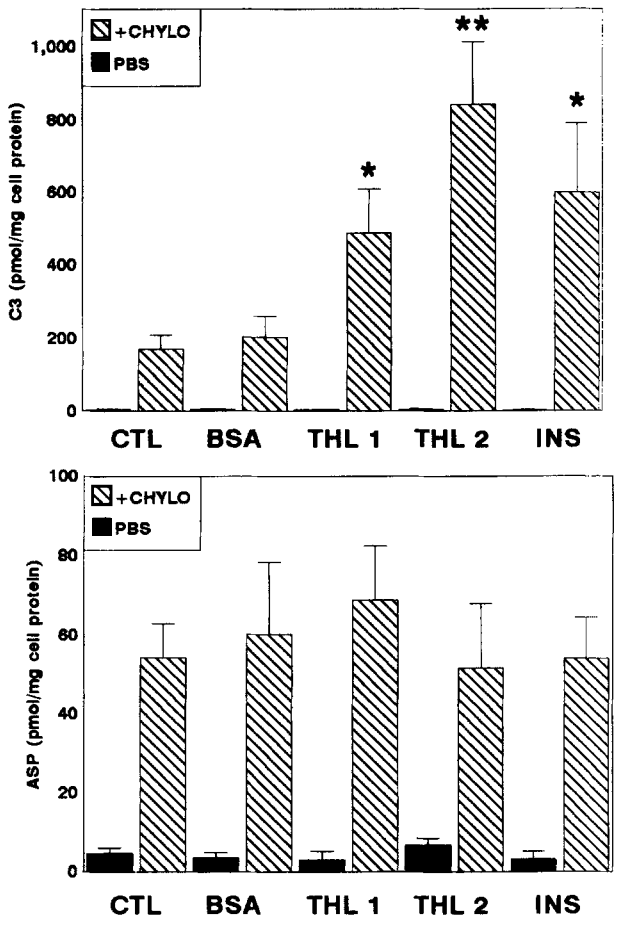

Fig. 7. Modulation of the CHYLO-mediated increase in medium C3 and ASP in human differentiated adipocytes. Human preadipocytes were differentiated in serum-free supplemented medium over a period of 3 weeks. Differentiated adipocytes were then exposed to the indicated conditions: insulin (50 mU/mL), BSA (2 mg/mL), or tetrahydrolipstatin (THL, 1 or 2 μM) with (hatched bars) or without (solid bars) CHYLO (50 μg lipoprotein triacylglycerol/mL medium) for 6 h. C3 (top panel) and ASP (bottom panel) were measured in the medium as indicated in Methods. The results are shown as pmol/mg cell protein ± SEM for an average of n = 7 from two different subjects from two experiments. All additions with CHYLO were significantly increased versus no CHYLO for each set of additions ($P < 0.005$) for both C3 and ASP. Significance for the individual CHYLO + additions versus CHYLO alone was calculated by paired *t*-test where $*P < 0.01$ and $**P < 0.005$.

CHYLO stimulate ASP production? Production of ASP is dependent on three protein factors (C3, factor B, and adipsin), two of which are consumed during the reaction (C3 and factor B) whereas adipsin, as a catalytic enzyme, is not. Therefore, the levels of bioactive ASP could be increased by increasing cellular synthesis and secretion of any one of these three proteins (substrates or enzyme), or by enhancing the catalytic cleavage reaction itself. In murine adipocytes, preformed adipsin is stored in intracellular secretory vesicles and translocation and secretion of adipsin is triggered through the action of insulin (33). In human adipocytes, insulin may affect not only the secretion of C3 (as shown here) but also the acute secretion of adipsin and factor B, although an effect on catalytic activity cannot be ruled out. Insulin appeared to have only minimal effects when added alone to cells (maximum 2-fold increase in ASP), although it markedly enhanced the CHYLO effect on C3 production.

CHYLO, also, may be acting via an effect on either catalytic conversion of C3 to ASP, or on the secretion of C3/B/adipsin through effects at the mRNA or protein level. The present study clearly documents a profound stimulatory effect of CHYLO on C3 production by adipocytes. However, we cannot rule out an additional effect on catalytic conversion of C3 to ASP. Certainly, catalytic conversion cannot occur in the absence of an "activated" C3-B complex and it has been suggested that this complex can only be formed through specific cell or membrane interaction (34); C3, B, and adipsin mixed together in solution will not produce ASP without artificial activation (15, 17, 35). Thus CHYLO may also provide the specific surface interaction that is required for activation. In addition, however, a key component of CHYLO may interact directly with adipocytes and stimulate increased secretion not only of C3, but also of B or adipsin. Clearly, all these potential mechanisms must be investigated in future experiments.

These in vitro experiments are not without physiological relevance. Postprandial generation of ASP will result in activation of adipose tissue and increase triacylglycerol synthesis as well as glucose transport (16, 17, 20–22). In this way, through autoregulation, the adipose tissue is primed to sequester the excess plasma glucose and dietary fatty acids much more efficiently via the ASP action on triacylglycerol synthesis. If this adipocyte–ASP positive feedback loop was ineffective, the resulting fatty acids would not be removed as quickly from plasma and the build-up of fatty acids in the microenvironment would result in inhibition of LPL action (36) and detachment of the LPL from the cell surface (37). Consequently, an increased flux of fatty acids or partially hydrolyzed lipoprotein particles containing detached LPL could flood the liver causing increased hepatic lipoprotein production (38, 39).

Modulations of plasma ASP in response to dietary fat intake as well as the metabolic state of the individual both in vitro and in vivo, suggest that ASP may play a role in positive feedback regulation of adipose tissue fat mass. The present data, therefore, provide new and important insights into how the ASP pathway is integrated into the complex process of energy storage in adipocytes.

This work was supported by a grant from the Medical Research Council of Canada #MA12462 to Dr. Katherine Cianflone who is a recipient of a scholarship from the Heart and

Stroke Foundation of Canada and les Fonds de la Recherche en Santé du Québec.

Manuscript received 22 March 1996 and in revised form 14 October 1996.

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