Coordinated release of acylation stimulating protein (ASP) and triacylglycerol clearance by human adipose tissue in vivo in the postprandial period

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Abstract The objective of this study was to determine whether Acylation Stimulating Protein (ASP) is generated in vivo by human adipose tissue during the postprandial period. After a fat meal, samples from 12 subjects were obtained (up to 6 h) from an arterialized hand vein and an anterior abdominal wall vein that drains adipose tissue. Veno-arterial (V-A) gradients across the subcutaneous adipose tissue bed were calculated. The data demonstrate that ASP is produced in vivo (positive V-A gradient) with maximal production at 3–5 h postprandially. The plasma triacylglycerol (TAG) clearance was evidenced by a negative V-A gradient. It increased substantially after 3 h and remained prominant until the final time point. There was, therefore, a close temporal coordination between ASP generation and TAG clearance. In contrast, plasma insulin and non-esterified fatty acid (NEFA) had an early (1–2 h) postprandial change. Fatty acid incorporation into adipose tissue (FIAT) was calculated from V-A glycerol and non-esterified fatty acid (NEFA) differences postprandially. FIAT was negative during the first hour, implying net fat mobilization. FIAT then became increasingly positive, implying net fat deposition, and overall followed the same time course as ASP and TAG clearance. There was a direct positive correlation between total ASP production and total FIAT ($r = 0.566$, $P < 0.05$). These data demonstrate that ASP is generated in vivo by human adipocytes and that this process is accentuated postprandially, supporting the concept that ASP plays an important role in clearance of TAG from plasma and fatty acid storage in adipose tissue.**—Saleh, J., L. K. M. Summers, K. Cianflone, B. A. Fielding, A. D. Sniderman, and K. N. Frayn.** Coordinated release of acylation stimulating protein (ASP) and triacylglycerol clearance by human adipose tissue in vivo in the postprandial period. *J. Lipid Res.* 1998. **39:** 884–891.

Supplementary key words complement 3A • adipose tissue • human • postprandial lipemia

The normal clearance of dietary triacylglycerol (TAG) from plasma demands close coordination of metabolic events within the capillary and subendothelial spaces. In the case of adipose tissue, the fatty acids that are abruptly and massively released from chylomicrons (CHYLO) by the action of lipoprotein lipase (LPL) either enter the venous plasma or are taken up and converted intracellularly to TAG by adipocytes (1). The critical roles of insulin and LPL in energy storage have long been recognized. Only recently, however, has it been appreciated that an increase in intracellular adipocyte TAG synthesis may also be an integral step in efficient energy storage (2).

In vitro studies have shown that acylation stimulating protein (ASP), the product of the ASP pathway, is a major determinant of the rate of adipocyte TAG synthesis (3, 4). ASP is a 76 amino acid fragment of the third component of complement (C3) and is produced by the interaction of C3, factor B, and adipsin (3). These three proteins are synthesized and secreted by both murine (5) and human adipocytes (4, 6) in a differentiation-dependent manner (7). The carboxy terminal arginine of the initial product, C3a, is cleaved by carboxypeptidase to generate C3adesArg. C3adesArg has no known function in immunological pathways but is identical to ASP (3). ASP increases the rate of TAG synthesis (8) by *i*) stimulating diacylglycerol acyltransferase (9), the enzyme that regulates the last step in TAG synthesis (10), and by *ii*) increasing specific membrane transport of glucose via translocation of intracellular glucose transporters to the plasma membrane sur-

Abbreviations: ASP, acylation stimulating protein; BMI, body mass index; CHYLO, chylomicron; ELISA, enzyme-linked immunosorbent assay; FIAT, fatty acid incorporated into adipose tissue; GLY, glycerol; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; NEFA, non-esterified fatty acids; PEG, polyethylene glycol; PBS, phosphate-buffered saline; TAG, triacylglycerol; V-A, veno-arterial.

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face. This last effect has been documented in human skin fibroblasts (8), human adipocytes (11), and L6 myotubes (12). ASP affects TAG synthetic capacity consequent to interaction with a specific cell membrane receptor (13) which then results in activation of a protein kinase C signal transduction pathway (14).

However, only limited in vivo data are yet available with respect to the role of ASP in plasma TAG clearance. In our initial study in normal subjects, plasma ASP appeared to increase after an oral fat load (15). By contrast, plasma ASP levels are unchanged after an oral glucose load (15). These observations do not establish whether ASP is, in fact, released from human adipocytes in the postprandial period, nor do they define the temporal relations of ASP generation to changes in the fatty acid uptake by adipocytes. Frayn, Coppack, and Humphreys (16) have established a method to sample the venous drainage directly from adipose tissue. Simultaneous sampling from an arterialized hand vein allows for the measurement of arterial concentrations. Hence differences between venous and arterial concentrations across the adipose tissue bed can be quantified. The present study was designed, therefore, to examine in vivo veno-arterial (V-A) changes for ASP across human subcutaneous adipose tissue of the anterior abdominal wall in the postprandial period.

METHODS

Subjects

Twelve subjects were studied, ranging in age from 18 to 70 years, with body mass indices (BMI) from 19.5 to 52.8 kg/m^2. Their ages, BMI (body mass (kg)/height^2(m^2)) and fasting lipid levels are shown in **Table 1**. All subjects fasted and drank only water for at least 12 h before the study. All studies were undertaken in a temperature-controlled room (23°C) and none of the subjects was taking any medication known to affect lipoprotein metabolism. All studies were approved by the Central Oxford Research Ethics Committee and the Research Institute Review Ethics Board of the Royal Victoria Hospital.

Experimental design

Arteriovenous studies were conducted as previously described (16). A cannula was inserted retrogradely into a hand vein and the hand was warmed in a box at 60–70°C so that arterialized blood samples could be obtained. A 10-cm, 22-gauge catheter was then introduced over a guide wire into one of the superficial veins on the anterior abdominal wall and threaded towards the groin, so that its tip lay just superior to the inguinal ligament. Samples from this cannula represent the venous effluent from the subcutaneous abdominal adipose tissue, uncontaminated by muscle drainage and with only a minor contribution from skin (16). Both catheters were kept patent with saline and heparin was not administered.

The subjects rested for at least 30 min after installation of the catheters and before any samples were taken. They then ate a mixed meal containing 60 g fat, 85 g carbohydrate, and 13 g protein and arterial and venous blood samples were taken simultaneously at times 0, 30, 60, and 90 min as well as 2, 3, 4, 5, and 6 h after eating. The samples were centrifuged and plasma stored at −70°C.

TABLE 1. Fasting plasma values on all subjects

Patients	BMI	Age	Sex	ASP	INS	apoB	CHOL	TAG	NEFA	GLY	HDL
	kg/m^2	*yr*		*n*m	*mU/L*	*mg/dL*	*m*m	*m*m	*m*m	μm	*m*m
1	19.5	18	F	85	5.8	39	4.3	1.17	0.39	53	1.4
2	21.6	70	F	63	4.4	141	6.3	1.64	1.17	143	1.3
3	22.0	29	F	121	4.8	61	4.1	0.83	0.69	56	1.2
4	22.2	40	F	41	4.1	73	3.3	0.73	0.51	48	0.9
5	22.4	55	F	37	3.9	65	4.3	0.85	0.67	60	1.0
6	24.2	26	F	32	4.2	68	4.0	0.53	0.82	80	1.3
7	25.0	59	F	33	4.3	70	4.4	0.58	0.83	71	1.3
8	26.3	25	M	76	5.6	83	6.0	1.52	0.42	48	0.7
9	30.0	54	M	13	10.8	118	4.4	3.28	0.74	65	N/A
10	37.9	68	F	29	11.5	93	5.6	1.40	0.78	83	0.8
11	46.6	49	F	77	103.1	93	5.1	1.80	0.86	137	0.8
12	52.8	43	F	57	38.6	116	5.0	2.46	0.91	129	0.5
Mean	29.2	44.7	2 males	55.4	16.8	85.0	4.7	1.40	0.73	81	1.0
SEM	3.0	4.8	10 females	8.4	8.0	7.9	0.24	0.23	0.06	10	0.1

BMI (body mass index) is measured as weight (kg)/height^2(m^2). Values for fasting arterial plasma are given for ASP (acylation stimulating protein); INS (insulin); apoB (total apolipoprotein B); CHOL (total cholesterol); TAG (total triacylglycerol), NEFA (total non-esterified fatty acid), and HDL (high density lipoprotein cholesterol). Values for GLY (glycerol) were measured in fasting arterial whole blood. N/A, not available. Results are expressed as mean ± standard error of the mean (SEM).

Analyses

Plasma TAG was measured enzymatically on an IL Monarch centrifugal analyzer (Instrumentation Laboratory, Warrington, Cheshire, UK) with correction for free glycerol (GLY) (17). Plasma total cholesterol was measured using a commercial enzymatic colorimetric method (cholesterol 50 kit; Sigma, Poole, UK). Chylomicrons were isolated by preparative ultracentrifugation at 60,000 *g* for 20 min based on the method of Cheng et al. (18). Plasma non-esterified fatty acid (NEFA) was measured by an enzymatic method (WAKO NEFA kit, Alpha Laboratories, Eastleigh, UK). Total apoB was measured by competitive ELISA assay using rabbit anti-human apoB antibody (in house), a commercial standard (#837237, Boehringer Mannheim, Laval, Quebec, Canada) and controls (Precipath (#1285874 and Precinorm #781827, Boehringer Mannheim) as previously described (19). Plasma insulin was measured by a double antibody radioimmunoassay kit (Pharmacia Ltd, Milton Kajnes, UK). GLY was measured in whole blood (20).

Sandwich ELISA for ASP determination

Human ASP was purified as previously described (14). Purity was confirmed by ion spray mass spectrometry, amino acid sequencing, and amino acid composition. Polyclonal antibody to human ASP was raised in rabbits by multiple injections of soluble ASP. Injection of denaturated reduced ASP contained in a polyacrylamide gel did not produce an antigenic response against native ASP. An 8 amino acid peptide representing the carboxy terminal tail of ASP (amino acid sequence: RASHLGLA) was conjugated to keyhole limpet hemocyanin. Mice were injected with this conjugate, and the spleen of immunoresponsive mice was used to produce monoclonal antibodies as previously described for C3a (21). The monoclonal antibody (Mab 4H3) used for this assay does not recognize plasma C3, but does recognize both ASP and C3a. C3a is rapidly converted to C3adesArg (ASP) by carboxypeptidases. Only ASP (C3adesArg) is found in plasma as confirmed by ion spray mass spectrometry.

The human plasma samples, controls, and standard ASP were pretreated with PEG 8000 according to van de Graaf et al. (22) to precipitate C3 to prevent any artefactual generation of ASP. Hamster serum (which does not cross react with Mab 4H3) was diluted 1:1 with phosphate-buffered saline (PBS) and added to the purified ASP standard prior to PEG precipitation to parallel the human plasma test and control samples. For PEG precipitation, 200 μL plasma was added to 200 μL PEG (20% (w/v) in borate buffer (0.1 m sodium borate and 0.01 m EDTA, pH 8.3)) and the samples were incubated on ice for 90 min. The samples were centrifuged (2000 g, 20 min), the supernatant was removed, and ASP was assayed immediately or frozen at −80°C. Two plasma pools were routinely measured for each assay as internal controls. Within assay variation of these controls was 4%; interassay variation was 8%.

Monoclonal antibody 4H3 was coated on 96 well Nunc-Immuno Module MaxiSorp F8 plates (#469949 Intermed, Nunc, Gibco, Burlington, Ontario, Canada) at 7 μg/mL in PBS (100 μL per well) overnight at 4°C and blocked with 1.5% BSA for 2 h at room temperature. The plate was washed 3 times with wash solution (0.05% Tween 20 in 0.9% NaCl) between each step of the assay. PEG precipitated plasma samples were routinely diluted 1:40. The ASP (PEG precipitated) standard was diluted to give final concentrations of 27.5, 16.5, 9.9, 5.9, 3.6, 2.1, and 1.3 ng/mL. PBS was used as the blank. The ASP standard, as well as test samples and in-house control samples (all diluted in PBS appropriately) were added at 100 μL per well. The plate was incubated for 90 min at 37°C and washed, followed by incubation for 90 min at 37°C with 100 μL rabbit polyclonal anti-ASP (diluted 1:2000 in PBS–0.05% Tween 20). The plate was then incubated for 30 min at 37°C with 100 μL goat anti-rabbit IgG conjugated to horseradish peroxidase (A4914, Sigma Chemicals, St. Louis, MO) diluted 1:3000 in PBS–0.05% Tween 20. After the final wash, the color reaction was initiated with 100 μL *o*-phenylenediamine dihydrochloride, (P-8287, Sigma Chemicals, St. Louis, MO) at 0.5 mg/mL in 100 mm Na citrate, pH 5.0, containing 0.05% (v/v) Tween 20. After visual development the reaction was stopped with 50 μL of 2 m H_2SO_4 and absorbance was read at 490 nm. The linear range was from 1.3 ng/ml to 27.46 ng/ml. Although, the results obtained for the sandwich ELISA were comparable to those obtained using a commercial radioimmunoassay kit for plasma C3adesArg (Amersham, Oakville, Canada) (4), it was more stable, practical, and cost effective.

Calculations for LPL, HSL, and FIAT activity

Indices of LPL activity and HSL activity were calculated using the V-A gradients for substrate and products (TAG, NEFA, and GLY) as described previously (23). Fatty acid incorporation into adipose tissue (FIAT) was calculated as described below. These calculations are based on the following lipolysis reaction: TAG→ 3NEFA+ 1 GLY where both LPL and HSL can hydrolyze TAG producing both NEFA and GLY. The NEFA can be taken up for re-esterification by the tissue but the GLY cannot be re-used by adipose tissue. V-A represents the veno-arterial difference. NEFA and TAG concentrations are converted to whole blood values using the hematocrit value such that blood NEFA = (plasma

NEFA)(1−hematocrit). All indices are expressed in units of mM NEFA in whole blood.

LPL = 3 (V-A TAG) *Eq. 1*)

HSL = [(total lipolysis) − (LPL)] where total lipolysis = 3 (V-A GLY) and LPL is given in equation (1) above. *Eq. 2*)

FIAT = [(total NEFA produced)−(net NEFA exiting tissue bed)]=3 (V-A GLY)− (V-A FFA). *Eq. 3*)

Statistics

All results are presented as mean ± standard error of the mean (SEM). Statistical differences for postprandial versus basal or venous versus arterial were calculated by repeated measures ANOVA. V-A differences were calculated for each subject at each time point individually and the values were averaged. Correlations were analyzed by Pearson Product Moment correlation by computer using the Sigma Stat program (Jandel Scientific, San Rafael, CA).

RESULTS

The individual and mean values for the subject group for age, BMI, fasting lipid, apolipoprotein B, and GLY, as well as fasting ASP and insulin, are given in Table 1. Subjects ranged from 18 to 70 years and from normal BMI (18 to 28) to morbidly obese (BMI greater than 35). Four of the twelve subjects had ASP values greater than the 95th percentile (67 nm) (24).

Figure 1 shows the temporal profiles taken after the meal for venous and arterial concentrations of ASP, total TAG, and CHYLO TAG. The plasma concentration of ASP increased markedly in the venous plasma samples that drain directly from the subcutaneous adipose tissue reaching a peak at 3 to 5 h (180 to 300 min) and decreasing to baseline by 6 h (360 min) (Fig. 1, left panel). At all time points, however, the venous ASP concentration was greater than the arterial ASP concentration, showing direct production of ASP from the adipose tissue bed. At the same time, plasma TAG increased in both the venous and arterial plasma samples reaching a peak at 3–5 h, decreasing thereafter although values at 6 h were still substantially higher than baseline (Fig. 1, center panel). In contrast to the ASP values, plasma TAG concentrations were consistently lower in the venous samples as compared to the arterial samples, suggesting hydrolysis of TAG during passage within the adipose tissue bed. This loss of TAG can also be seen clearly within the CHYLO lipoprotein density fraction that demonstrated a profile similar to that of the plasma TAG (Fig. 1, right panel). Again there was a consistent decrease in the venous as compared to the

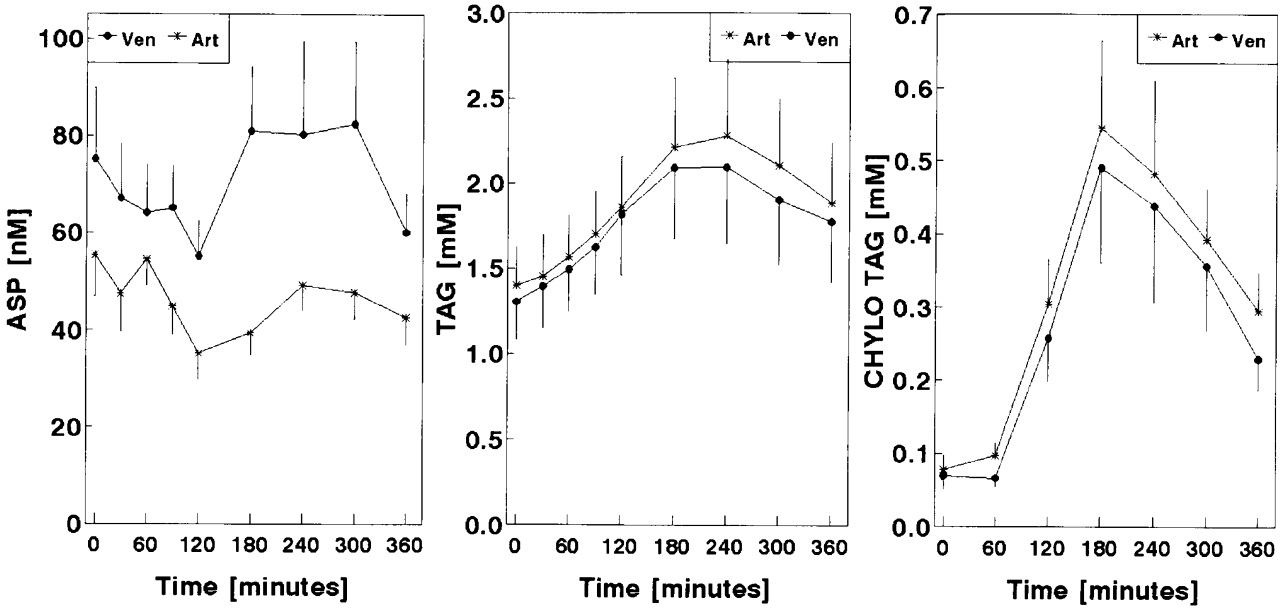

Fig. 1. Postprandial profile of arterial and venous plasma ASP, total plasma triacylglycerol, and chylomicron triacylglycerol. Blood samples were taken at the indicated times from an arterialized hand vein (∗) and subcutaneous adipose tissue venous drainage (●) and analyzed for plasma ASP (left panel), plasma total triacylglycerol (TAG) (center panel), and chylomicron (CHYLO) TAG (right panel). Results represent the mean of n = 12 subjects ± standard error of the mean.

arterial samples reaching a maximum at 3 h and decreasing thereafter.

The absolute V-A gradients are shown in **Fig. 2**. The basal V-A ASP gradient was about 20 nm which increased up to 2-fold ($P < 0.05$) from 3 to 5 h (180–300 min) at maximal production (Fig. 2, top panel). The V-A ASP gradient was consistently positive, whereas the V-A TAG gradient is consistently negative (Fig. 2, bottom panel). The temporal profiles of both were very similar, with both processes becoming more pronounced in the second half of the postprandial period. This contrasts with the temporal profiles of insulin and NEFA (V-A NEFA) that are shown in **Fig. 3**. Insulin increased postprandially, reaching a maximum at 1–2 h (60–120 min) and decreasing thereafter to baseline levels (Fig. 3, top panel). Similarly, total venous and arterial NEFA decreased initially over the first 1–2 h (60–120 min) and increased thereafter, with venous NEFA consistently greater than arterial NEFA (not shown). The V-A NEFA gradient is shown in Fig. 3, bottom panel. The NEFA gradient (that is, the amount of NEFA produced during passage through the adipose tissue bed), was lowest at the 1–2 h point (60–120 min) decreasing to 20% of basal values). This is consistent with the well-recognized role of insulin in inhibiting HSL and decreasing adipose intracellular TAG hydrolysis and release of NEFA.

We then examined the contributions of HSL, LPL,

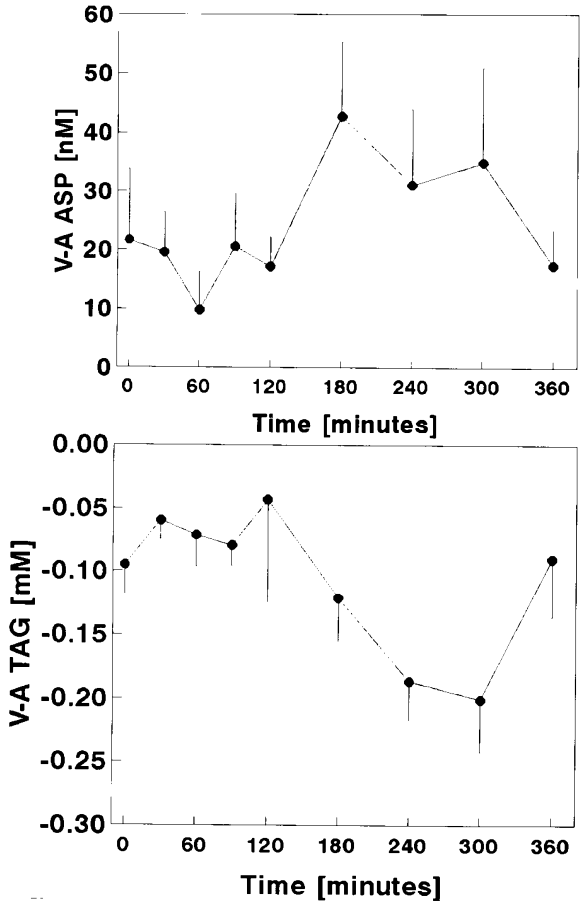

Fig. 2. Postprandial profile of veno-arterial gradient for plasma ASP and total plasma triacylglycerol. Veno-arterial gradient was calculated as venous-arterial concentration for each subject individually at each time point for plasma ASP (top panel) and plasma total triacylglycerol (TAG) (bottom panel). Results represent the mean of n = 12 subjects ± standard error of the mean. Significance was calculated by repeated measures ANOVA where $P < 0.05$ for both ASP and TAG curves.

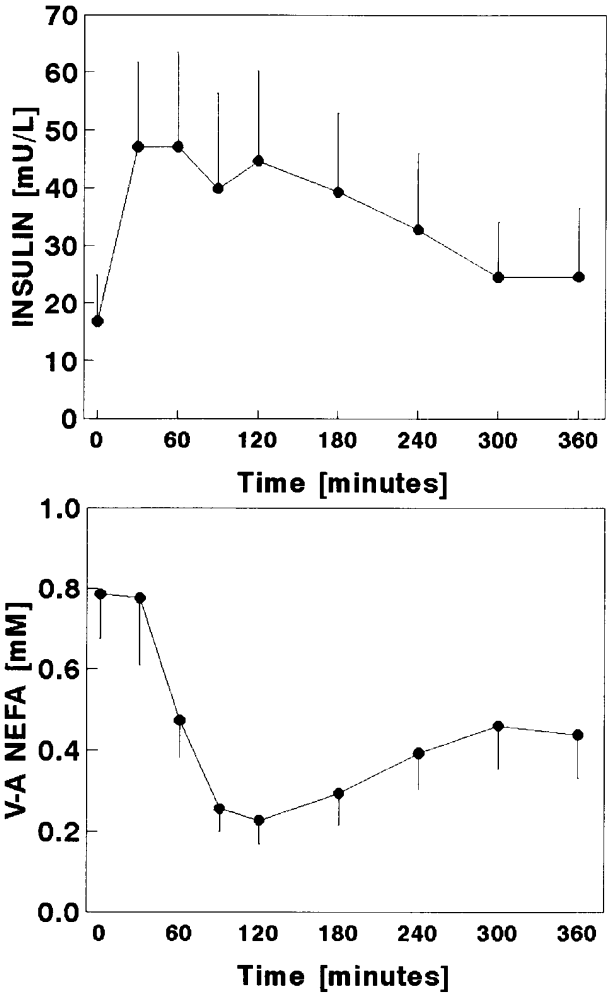

Fig. 3. Postprandial profile of plasma arterial insulin and veno-arterial gradient for plasma non-esterified fatty acid. Plasma arterial insulin values were measured at each time point (top panel). Veno-arterial non esterified fatty acid gradient (V-A NEFA) was calculated as venous-arterial concentration for each subject individually at each time point (bottom panel). Results represent the mean of n = 12 subjects ± standard error of the mean. Significance was calculated by repeated measures ANOVA where $P < 0.0001$ for both insulin and NEFA curves.

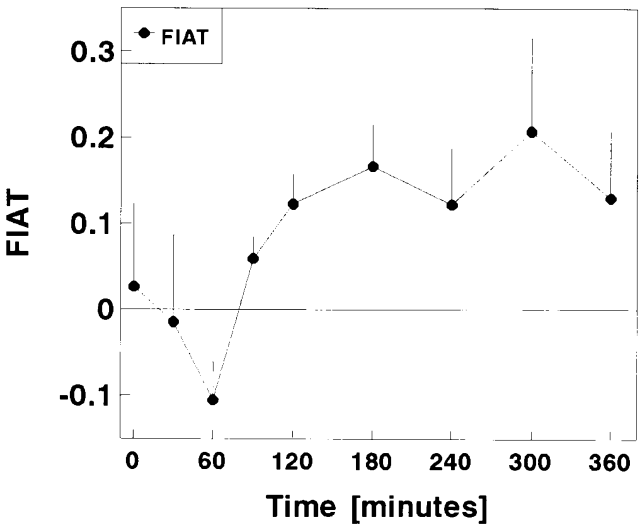

Fig. 4. Calculated postprandial fatty acid incorporation into adipose tissue. Fatty acid incorporation into adipose tissue (FIAT) was calculated for each subject at each time point as described in Materials and Methods. Results represent the mean of n = 12 subjects ± standard error of the mean and are expressed as [mm] NEFA incorporated into adipose tissue (FIAT activity). Significance was calculated by repeated measures ANOVA where $P < 0.01$.

and FIAT to the overall V-A gradients of substrates (TAG) and products (NEFA and GLY). Indices of the relative activities over the 6-h time course were calculated as described in Methods. The results for tissue uptake of fatty acids (FIAT) are shown in **Fig. 4**. In the early postprandial period, the tissue uptake of NEFA was negative, indicating efflux of NEFA from the adipose tissue. During the later postprandial period, from 3 to 6 hours (180–360 min), the overall flux became positive, pointing to net fatty acid storage in the tissue bed. To examine the relationship between ASP production and adipose tissue postprandial metabolism, the sum of the V-A gradients was calculated for each parameter for each of the 12 subjects including the sum V-A ASP. The postprandial production of ASP (sum V-A ASP) correlated with the fasting venous ASP: $r = 0.793$, $P < 0.002$. Of interest is the finding of a significant relation between the sum V-A ASP and sum FIAT ($r = 0.566$, $P < 0.05$) linking, therefore, generation of ASP and net fatty acid uptake by adipocytes. By contrast, there was no correlation between sum V-A ASP and indices of LPL or HSL activity (not shown).

DISCUSSION

A complex series of events occurs in the adipose tissue microenvironment in the postprandial period. The release of energy, which is essential when fasting, must be diminished because energy must now be stored, not released. The present study illustrates that these two vital, but opposed, processes occur sequentially. Plasma levels of insulin rise early after an oral fat load resulting in marked decreases in NEFA release from adipocytes due to inhibition of HSL (25). By contrast, energy storage, as evidenced by triglyceride clearance, begins in the early postprandial period, but only becomes maximal midway through the process.

The specific focus of this study was on the relationship between ASP and fatty acid storage in adipocytes. In vitro studies (3, 4) have shown ASP to be a potent stimulant of triglyceride synthesis in adipose tissue. As well, ASP is produced in vitro by adipocytes (4, 7). Until now, little was known about the generation and release of ASP by adipose tissue in vivo. The positive V-A gradient for ASP demonstrates that ASP is released into the circulation from adipocytes during both the fasting and postprandial periods. This does not rule out the fact that ASP may be produced elsewhere, but it does establish that ASP is generated in humans within the adipose tissue microenvironment. These observations are in accord with previous in vitro data that both human and murine adipocytes can synthesize and secrete the three precursor proteins necessary to generate ASP as well as produce the final product, ASP (5, 7).

A marked increase in the generation and release of ASP into the systemic circulation did not begin until midway in the postprandial period just preceding the increase in triglyceride clearance and uptake of fatty acids within the adipose tissue microenvironment. There is, therefore, close temporal synchrony between ASP generation, TAG removal, and net fatty acid uptake within the adipose tissue bed. In this paper we have chosen for simplicity to present measured concentrations. In interpreting the V-A gradients it should be borne in mind that adipose tissue blood flow increases after a meal like the one used here (26). Therefore, the absolute rates of TAG removal and ASP release will actually have been greater during the postprandial period compared with the fasting than would appear from the concentrations presented. Nevertheless, the relationships between TAG extraction and ASP production would not be affected.

The present in vivo data correspond to data obtained from in vitro studies of the regulation of ASP generation by differentiated human adipocytes (27). These in vitro experiments demonstrated that ASP generation was not influenced by either the concentration of NEFA or glucose in the medium. As well, very low density lipoproteins, low density lipoproteins, and high density lipoproteins had minimal effects. Insulin produced a 2- to 3-fold increase in ASP generation. CHYLO,

however, caused differentiated adipocytes to produce 10- to 20-fold more ASP. Not only was ASP generated in large amounts in the medium after addition of CHYLO, but secretion by the adipocyte of its parent molecule, C3, increased in parallel (27). Insulin substantially amplified the effects of CHYLO on C3, markedly increasing its production pointing to potential metabolic synergy in vivo as well. It should be noted that this in vitro profile closely parallels the results in the present in vivo study: that is, a marked increase in ASP production in close temporal association with the postprandial increase in CHYLO concentration and CHYLO TAG clearance. The greater fold increase (10- to 20-fold) seen in vitro is likely due to a local increase in ASP production in the tissue culture microenvironment. Results in vivo (2-fold) however, are influenced by the dilution effect of systemic plasma. Thus, in vivo as well as in vitro, the profound effect of influx of dietary CHYLO particles and insulin may trigger an increase in local adipose tissue ASP concentration stimulating efficient fatty acid uptake and storage.

In summary, the present data establish *1*) that ASP is generated in subcutaneous adipose tissue in the postprandial period; *2*) that maximal generation of ASP precedes and accompanies maximal removal of TAG; and *3*) that there is a significant relationship between ASP generation and net fatty acid uptake by adipocytes in the postprandial period. Moreover, the generation of ASP appears to be accelerated both in vivo and in vitro by CHYLO. All these data, therefore, are consistent with the hypothesis that the ASP pathway plays a critical physiologic role in the normal clearance of dietary TAG from plasma.

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