Metabolic response of Acylation Stimulating Protein to an oral fat load

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Abstract Acylation Stimulating Protein (ASP) is a small (mol wt 14,000), basic (pI 9.0) protein present in human plasma. When examined in vitro with normal human cultured skin fibroblasts and adipocytes, ASP appears to be the most potent stimulant of triglyceride synthesis yet described. In this study, a competitive ELISA assay for ASP has been developed using immunospecific polyclonal antibodies, and ASP levels have been measured in seven normal subjects. Following an oral fat load, a sustained significant increase in ASP occurs, whereas after an oral glucose load, ASP levels do not change significantly. These responses are entirely opposite to those of insulin, which rises sharply but transiently after an oral glucose load but is unchanged after an oral fat load. Both the fasting and peak ASP levels were significantly related to the postprandial lipemia. These data provide the first in vivo evidence that Acylation Stimulating Protein may play an important physiological role in the normal response to an oral fat load. —**Cianflone, K., H. Vu, M. Walsh, A. Baldo, and A. Sniderman.** Metabolic response of Acylation Stimulating Protein to an oral fat load. *J. Lipid Res.* 1989. **30:** 1727–1733.

Supplementary key words triglyceride clearance • postprandial lipemia

The role of chylomicrons in the plasma transport of dietary triglycerides is well known. Most of the triglycerides within these particles are hydrolyzed by lipoprotein lipase and the fatty acids and monoglycerides so released are taken up by various tissues. They may enter, for example, skeletal or cardiac muscle in which they may undergo oxidation to provide metabolic energy, or alternatively, they may enter adipose tissue where they are reesterified to store energy for future use. The rate at which fatty acids are liberated from chylomicrons is, in the first instance, a function of the activity of the enzyme, lipoprotein lipase, and its essential activator, apoC-II (1). To date, no other factors have been shown to be integral in the normal physiological response to an oral fat load.

We have purified from human plasma a small (mol wt 14,000), basic (pI 9.0) protein which we have named Acylation Stimulating Protein (ASP) (2). When ASP was examined in vitro using either cultured human skin fibroblasts or human adipocytes, it markedly stimulated fatty acid incorporation into triglycerides within these cells without affecting triglyceride hydrolysis (3). These observations suggested that ASP might play a role in the normal physiologic response to an oral fat load. Accordingly, the purpose of the present study was to measure the fasting levels of ASP in human plasma and then to determine the effects of both an oral fat load and an oral glucose load on ASP levels.

MATERIALS AND METHODS

Subjects

The response of plasma ASP to an oral fat load was studied in seven normal subjects. Five of these were also examined after an oral glucose load. All were in good health and none were taking any medication known to affect plasma lipids or lipoproteins.

Oral fat load

An oral fat load was administered following an overnight fast (12 h) (3). The volume administered was standardized for body surface area (BSA) calculated as weight (kg) × height (cm). The lipid meal consisted of 350 ml of 35% cream mixed with one tablespoon of granulated sugar and one tablespoon of instant dry nonfat milk per 2.5 m^2 BSA. This provided 1362 kcal of which, by weight, 5.1% was protein, 25.6% carbohydrate, and 69.3% fat (130 g). The total amount of cholesterol was 480 mg and the polyunsaturated to saturated fatty acid ratio was 0.059. All subjects were ambulatory but were not allowed food or liquid, except for water, during the study. The fat meal was ingested within 5 min and was well tolerated. Blood samples were collected fasting and at 2-h intervals following the fat load for 8 h.

Abbreviations: ASP, acylation stimulating protein; ELISA, enzyme-linked immunosorbent assay; PBS-T, 0.05% Tween 20 in phosphate-buffered saline; MBWI, mean body weight index; LDL, low density lipoproteins; HDL, high density lipoproteins.

Oral glucose load

The glucose load consisted of a standard 75 g oral glucose load. Blood samples were taken fasting and at 30 min, 1, 1.5, 2, 3, and 5 h. For both the fat load and the glucose load, blood was collected into Vacutainer tubes containing 1 mg/ml of EDTA and centrifuged at 2000 rpm for 15 min at 4°C, followed by isolation of the plasma.

Lipid and apolipoprotein assays

Plasma cholesterol, HDL cholesterol, LDL cholesterol, plasma apoB, and plasma apoA-I were determined in the fasting samples. Plasma triglycerides, glucose, insulin, and ASP levels were measured sequentially in all of the samples following the fat load and glucose load. All samples were assayed within 5 days (4°C) or frozen at −70°C and assayed within 1 month.

Plasma cholesterol and plasma triglyceride were measured by commercial colorimetric enzymatic determination (Boehringer, Mannheim #237574 and #701904, respectively). Plasma was treated with heparin-Mn^{2+} to precipitate apoB-containing lipoprotein (4) and the HDL cholesterol in the supernatant was measured by colorimetric enzymatic determination, as above. LDL cholesterol was calculated according to the method of Friedewald, Levy, and Fredrickson (5). Plasma apoB and plasma apoA-1 were measured using immunonephelometry kits (Beckman Instruments, CA). Glucose was measured by the glucose oxidase method in an Astra 8 analyzer (Beckman, CA) and insulin was analyzed by radioimmunoassay.

ASP purification

ASP was purified by a modification of the original method (2). Frozen human plasma was thawed at 4°C overnight. The plasma was centrifuged at 2000 rpm for 20 min and the supernatant was filtered to remove the precipitate. Plasma was then applied to a Fast-Flow-S-Sepharose column (Pharmacia, Sweden) (14 ml/ml gel) and washed with seven column-volumes of buffer A (0.02 M phosphate, pH 7.1, with 0.02% sodium azide). The bound protein fraction was eluted with seven column-volumes of 3 M NaCl in buffer A and absorbance was monitored at 280 nm. The fractions were pooled and concentrated by dialysis against 50% polyethylene glycol in buffer A.

This fraction was then separated by gel filtration chromatography on Sephadex G-75 (Pharmacia, Sweden) applied in a sample volume of 4–5% of the column volume (column volume = 1.7 ml/ml starting plasma). The column was eluted with buffer A and the fraction of interest (the second peak) emerged at a V_e/V_o of 3.4. The fractions were pooled and dialyzed against buffer A to remove any NaCl, then applied to a second Fast-Flow-S-Sepharose column (20 ml column volume/l starting plasma). The bound protein peak was again eluted, collected and concentrated as described above using buffer A without sodium azide. The ASP was dialyzed overnight, centrifuged to remove the precipitate, and the protein concentration in the supernate was measured by the method of Bradford using bovine serum albumin as a standard (6). Purification was monitored by both SDS-PAGE electrophoresis (see below), and biological activity as estimated by capacity to increase triglyceride synthesis in normal human cultured skin fibroblasts (2).

ASP ELISA assay

Antibody to ASP was prepared as follows. ASP purity was assessed by SDS-15% polyacrylamide electrophoresis (PAGE) according to the method of Laemmli (7). The purified preparation was also homogeneous by native basic gel electrophoresis (8). The gel was stained with Coomassie Blue and the appropriate band, as determined by reference to molecular weight standards (Sigma, St. Louis, MO), was excised and homogenized in buffer A without azide. The suspension was emulsified with Freund's adjuvant and injected subcutaneously into a rabbit (New Zealand White). Antibody was present following four injections at 2-week intervals, as determined by enzyme-linked immunosorbent assay (ELISA) screening assay. The antibody was shown by Western blotting (9) to be monospecific against both purified ASP and plasma.

A competitive ELISA assay was used to measure ASP in human plasma. ASP (100 μl, 2μg/ml) in phosphate-buffered saline (PBS) was coated to Immulon 2 (Dynatech, Chantilly, VA) 96-well microtiter plates overnight at 4°C. The plates were washed in PBS with 0.05% Tween 20 (PBS-T) and blocked with 1.5% bovine serum albumin in PBS-T for 30 min. Following six washes, 100 μl of PBS-T with 0.01% thimerosal was added to the plates for storage at 4°C and the plates were used within a month.

For the assay, plasma samples diluted 50- to 200-fold in PBS-T were incubated with an equal volume of ASP antibody diluted appropriately (usually 1:5000); 100-μl aliquots were added to the microtiter plates and incubated for 1 h. The plates were washed and incubated with a second antibody of goat anti-rabbit IgG complexed to peroxidase (Sigma, St. Louis, MO) at a dilution of 1:1000 in 4% polyethylene glycol in PBS-T at 37°C for 30 min in the dark. The plates were washed as described above and the substrate was added (1 mg/ml O-phenylenediamine in 100 μM sodium citrate, 0.05% Tween 20, pH 5.0) for 30 min. The colorimetric reaction was stopped with 50 μl of 4 N H_2SO_4 and the absorbance was read at a wavelength of 492 nm using Dynatech manual ELISA plate reader (Dynatech, Chantilly, VA).

ASP, purified to homogeneity as described above, was used as the standard for the assay. Homogeneity was verified by SDS-PAGE. The protein content of the primary standard was verified by modified Lowry protein assay (10) and Bradford protein assay (6) using bovine serum albumin as a standard. Purified ASP and plasma gave parallel titration curves in the ELISA assay, and two control samples of plasma were used to verify the reproducibility of each assay.

TABLE 1. Plasma lipid and lipoprotein values

Subject	Age	Sex	MBWI	Chol	TG	LDL Chol	HDL Chol	ApoB	ApoA-I	Glu	Ins	ASP
	yr						*mg/dl*			*mM/l*	*mU/l*	*mg/dl*
1	31	F	19.4	213	94	75	119	59	291	5.2	6.7	7.6
2	47	M	23.5	194	65	121	60	82	120	5.7	7.3	9.8
3	29	M	25.2	154	32	74	74	50	142	5.5	2.5	7.8
4	31	M	21.7	180	46	92	79	64	164	5.5	11.2	10.4
5	42	M	23.7	135	30	31	98	35	184	5.3	8.7	4.1
6	26	F	23.4	143	51	58	75	46	170	5.2	7.8	4.3
7	39	F	19.5	215	84	111	87	67	217	5.5	7.2	2.3
Average	35		22	176	57	80	85	58	184	5.4	7.3	6.6
SD	7		2	30	23	29	18	14	52	0.2	2.4	2.8

Abbreviations: MBWI, mean body weight index; Chol, plasma cholesterol; TG, plasma triglyceride; Glu, plasma glucose; Ins, plasma insulin; ASP, plasma ASP.

A standard curve of serial dilutions of 0.625 to 20 μg/ml ASP in PBS-T was constructed; the diluted samples were mixed with an equal volume of appropriately diluted antibody solution and assayed as described above. For calculation ln [concentration ASP] was plotted against ln [absorbance] and a least squares linear regression analysis was performed.

Statistics

All averages are expressed as mean ± standard deviation (SD). Following the oral fat load or the oral glucose load, the nonfasting plasma levels were compared to fasting levels by paired *t*-test. Area under the curve was calculated by area of the polygon using the fasting value as the baseline value. Least squares linear regression analysis and Pearson's correlation coefficient were used to analyze the relationships between individual values of two variables. Mean body weight index (MBWI) was calculated as weight (kg) divided by height (meters) squared.

RESULTS

Seven normal subjects were studied for their response to a fat load; five of these subjects were studied for their response to a glucose load. Their average age was 35 ± 8 years; four were males, three females. All were in good health and none were taking any medications. Their fasting plasma and lipoprotein lipid levels as well as plasma apoB, A-I, glucose, and insulin levels are given in **Table 1**.

The changes in plasma triglycerides following both the oral fat and glucose loads are shown in **Figs. 1A and 1B**.

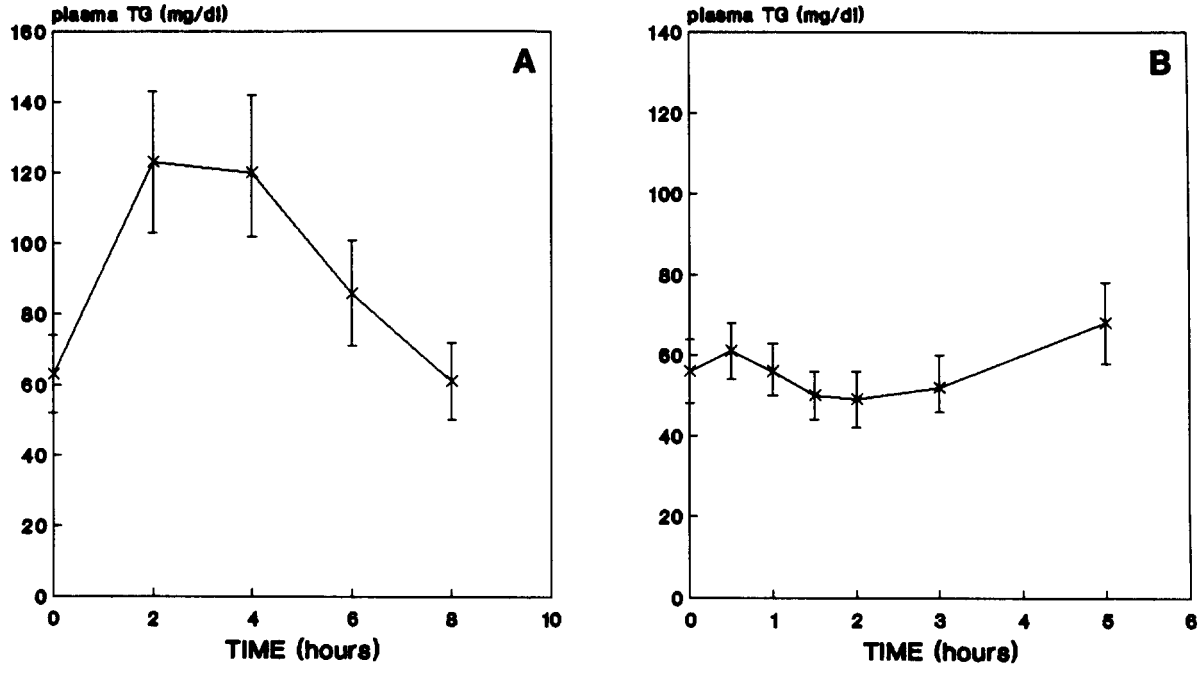

Fig. 1. Plasma triglyceride concentrations over time following an oral fat load (A) or an oral glucose load (B). Results are in mg triglyceride per dl plasma ± SD.

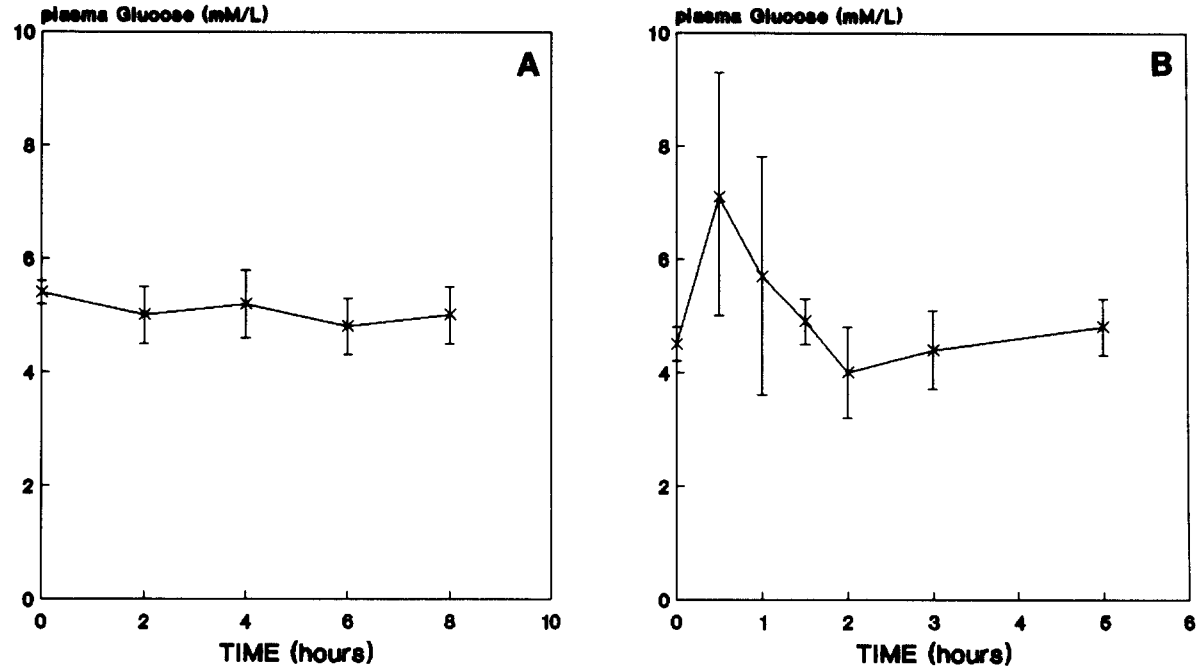

Fig. 2. Plasma glucose concentrations following an oral fat load (A) or an oral glucose load (B). Results are in mM plasma glucose ± SD.

As expected, following the ingestion of fat (Fig. 1A) the plasma triglyceride levels increased to reach a maximum between 2 and 4 h (peak: 136 ± 65 mg/dl, $P < 0.005$), and then declined quickly, returning to levels indistinguishable from fasting levels by 8 h (61 ± 29 mg/dl, NS). Following an oral glucose load (Fig. 1B), as expected, there was no significant change in plasma triglyceride levels.

The plasma glucose levels after the two oral challenges are shown in **Figs. 2A and 2B**. With the oral glucose load (Fig. 2B) there was a rapid increase in plasma glucose levels (peak: 7.1 ± 2.3 mM, $P < 0.0125$), which returned to baseline level by 1.5 h (4.9 ± 0.4 mM, NS) and changed little thereafter. After an oral fat load, glucose levels do not change significantly (Fig. 2A).

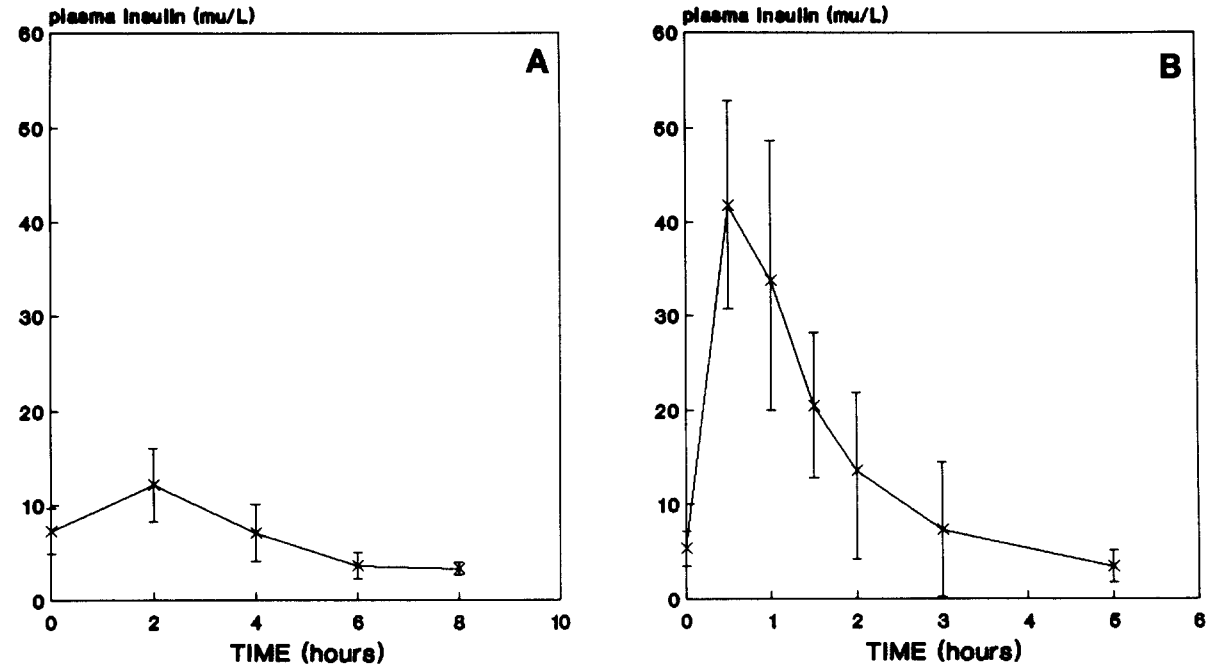

Fig. 3. Plasma insulin concentrations following an oral fat load (A) or an oral glucose load (B). Results are in mU per liter plasma ± SD.

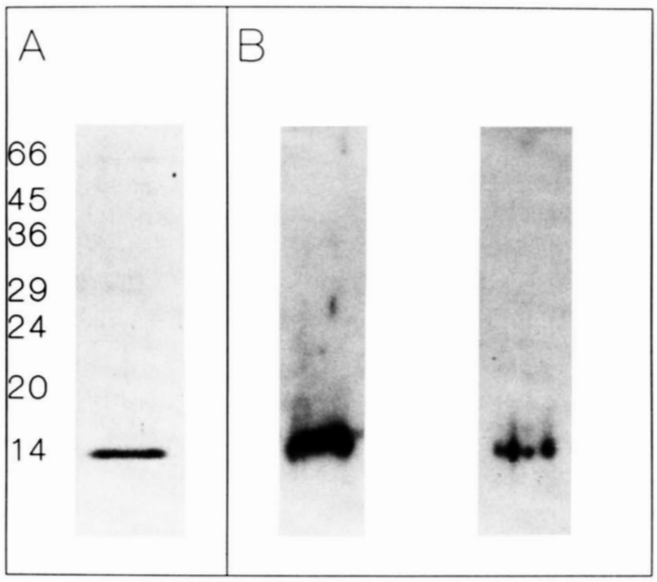

Fig. 4. ASP and Western blot analysis. Panel A: ASP (2 μg) was electrophoresed on SDS-PAGE as described in Materials and Methods and the gels were stained with Coomassie Blue R250. Panel B: ASP (2 μg, left) and human plasma (300 μg, right) were electrophoresed on SDS-15% PAGE, blotted onto nitrocellulose, and probed with rabbit anti-human ASP. Bands were visualized by autoradiography.

Changes in plasma insulin are shown in **Figs. 3A and 3B**. After an oral glucose load (Fig. 3B), insulin levels increased abruptly, approximately sevenfold within 30 min (37.8 ± 20.5 mU/l, $P < 0.0025$), and then began to decline almost immediately, returning to baseline by 2 h. After an oral fat load, insulin levels did not change significantly (Fig. 3A).

Plasma ASP was measured by a competitive ELISA assay using purified ASP as a standard (**Fig. 4A**). Polyclonal antiserum was shown to be monospecific to both purified ASP and human plasma by Western blotting (9) (Fig. 4B). The within-assay variability was 3.4% and the between-assay variability was 5.0%. The limit of sensitivity was 0.05 mg/dl of ASP in plasma. Fresh plasma assayed within 5 days and plasma frozen at −70°C and thawed were found to vary less than 5.0%. Recovery of assayable purified ASP added to human plasma was 95%.

Sequential plasma ASP levels are shown in **Figs. 5A and 5B** for the fat and glucose loads, respectively. The average level of ASP in fasting plasma for the seven subjects in which an oral fat load was conducted was 6.6 ± 2.9 mg/dl. After the oral fat load (Fig. 5A) this increased progressively, reaching peak levels between 4 and 6 h later. However, even by 2 h, the increase in ASP concentration in plasma was significant (9.2 ± 4.7 mg/dl, $P < 0.025$). The average ASP level at 4 h was higher still (11.1 ± 5.4 mg/dl, $P < 0.01$), but there was no further change from 4 to 6 h (11 ± 5.3 mg/dl, $P < 0.005$). By 8 h, the ASP level was declining but still significantly above baseline (9.7 ± 2.4 mg/dl, $P < 0.005$). The change from fasting to peak ASP levels for each individual is shown in **Fig. 6**. The average peak ASP level was 12.6 ± 4.9 mg/dl, $P < 0.005$, an increase of more than 100% in each individual. By contrast, ASP levels did not change following a glucose load (Fig. 5B).

Following both the glucose load and the fat load the plasma insulin levels paralleled the plasma glucose levels (Fig.

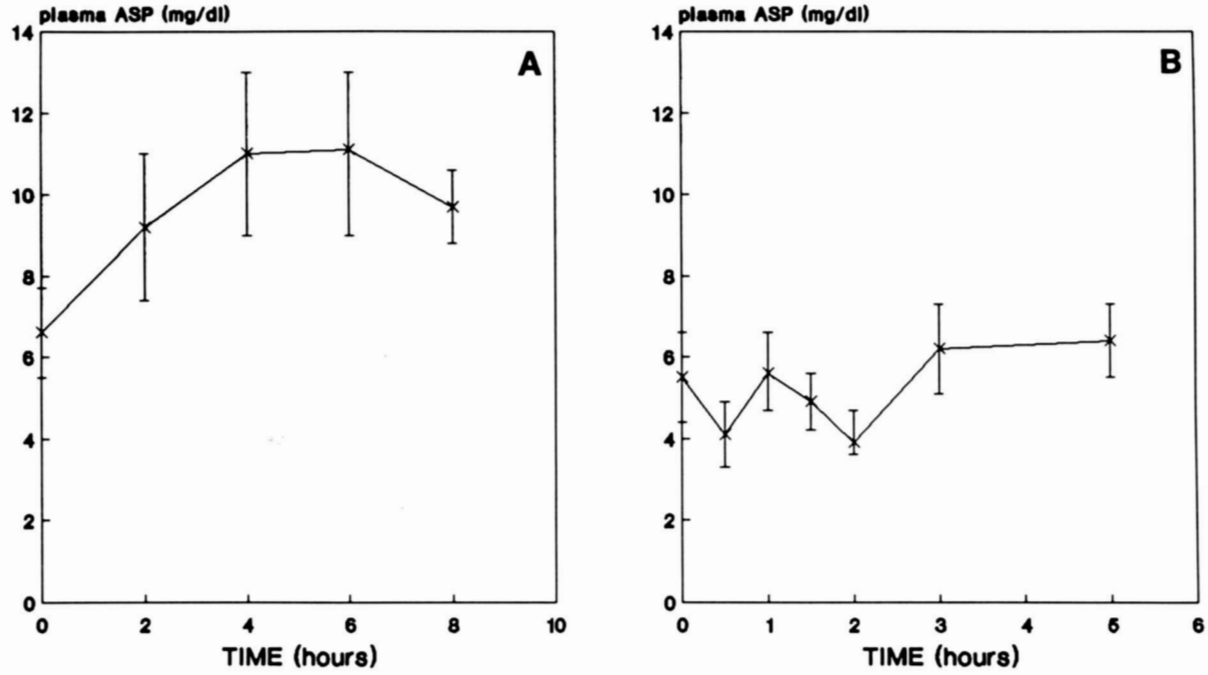

Fig. 5. Plasma ASP concentrations following an oral fat load (A) or an oral glucose load (B). Results are expressed in mg/dl plasma ± SD.

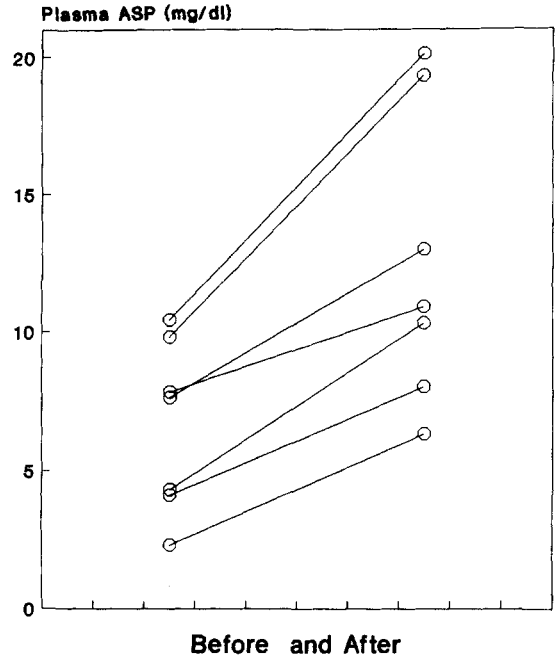

Fig. 6. Fasting ASP levels (before) and peak ASP levels (after) the fat-load for each individual.

2B and Fig. 3B) and the plasma ASP levels paralleled the plasma triglyceride levels as seen in Figs. 1A and 5B. The increase in insulin, therefore, to an oral glucose load was marked but short-lived, the change in area being 46.8 ± 20.3 mU/l-h. By contrast, the increase in ASP level, while substantial, was less marked and slower, but more sustained, the total change in area over baseline being 25.9 ± 6.0 mg/dl-h.

To compare directly changes in insulin levels during the glucose load against changes in ASP levels during the fat load, the plasma values were expressed as percentage of the baseline value, and the area under the curve was calculated for each subject. Percentage change in insulin levels over time was 959 ± 517 % · h following a glucose load and the percent change in ASP levels was 462 ± 120 % · h following a fat load.

The relationships between fasting ASP level and postprandial triglyceride response and between peak ASP level and postprandial triglyceride response are shown in **Figs. 7A and 7B**, respectively. In both instances there was a significant inverse relationship such that the higher the level of either fasting ASP or peak ASP (after the fat load) the less marked the postprandial lipemia ($P < 0.0125$ and $P < 0.05$, respectively).

DISCUSSION

This is the first report describing the concentration of ASP in normal human plasma, as well as the first evidence that ASP may play a role in the normal physiologic response to an oral fat load. The data indicate that, following an oral fat load, there was a prompt, significant, and sustained increase in plasma ASP levels in all subjects studied. By contrast, following an oral glucose load, there was no change in

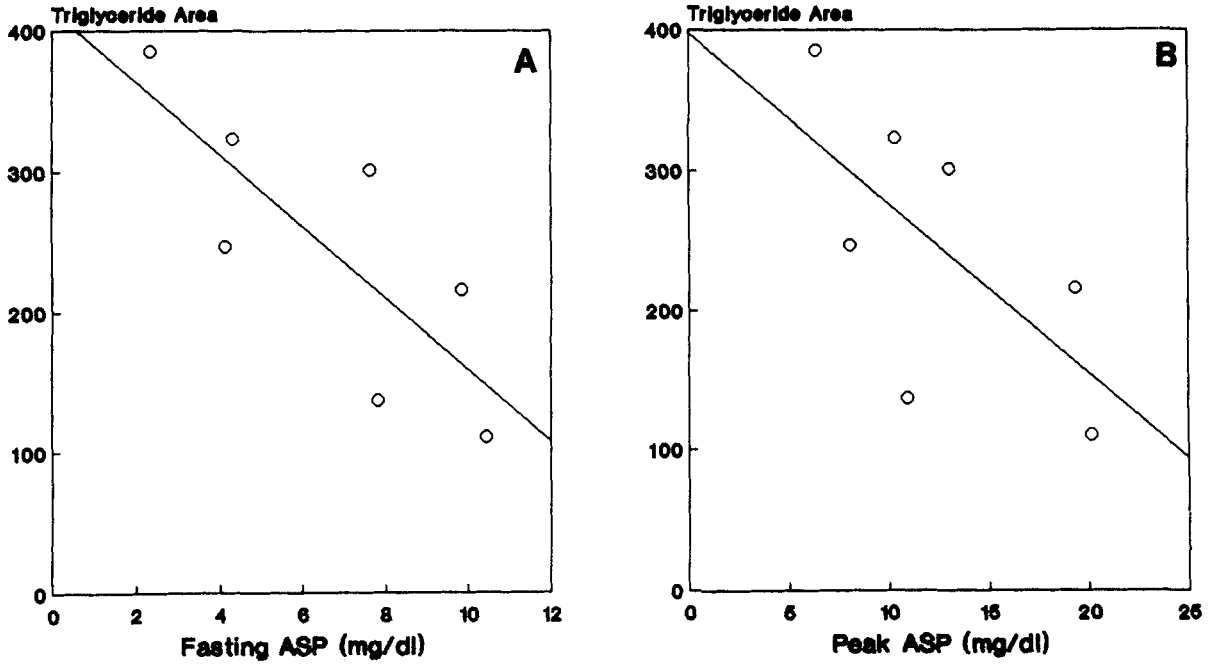

Fig. 7. Fasting ASP levels (A) and peak ASP levels (B) versus triglyceride clearance. Triglyceride clearance was measured as the area under the triglyceride time curve, using the fasting triglyceride level as the baseline value. Panel A: Linear regression correlation coefficient $r = 0.794$, $P < 0.0125$. Panel B: Linear regression correlation coefficient $r = 0.649$, $P < 0.05$.

the concentration of ASP in plasma. Similar results were seen with the plasma triglyceride profiles in both the oral fat load and the glucose load. Furthermore, there was a significant inverse relation between the level of ASP, both fasting and peak, and postprandial lipemia. That is to say, the higher the level of ASP, the more rapid the apparent clearance of triglyceride from plasma. By contrast, there was no relation between plasma concentration and changes in plasma glucose following an oral glucose load.

That such a plasma factor might exist was first recognized serendipitously in experiments examining the effect of human serum on triglyceride synthesis in human skin fibroblasts cultured from normal subjects and patients with hyperapobetalipoproteinemia (11). These studies demonstrated that normal human serum contained a factor that markedly stimulated triglyceride synthesis in the normal but not the abnormal cells. This factor was purified to homogeneity and named Acylation Stimulating Protein or ASP (2).

The exact mechanism of action of ASP remains to be elucidated but the initial observations are of interest in that ASP appears to be the most potent in vitro stimulant of triglyceride synthesis in intact cells yet described (2). The evidence to date has established that ASP is internalized by the cell after binding to a specific high-affinity receptor (12). Consistent with this are the observations that the metabolic effect of ASP on fibroblasts is directly proportional to the amount of specifically bound ASP. Moreover, ASP does not stimulate fatty acid synthesis de novo and does not appear to have any effect on triglyceride hydrolysis within the cell or the handling of glucose by the cell (11).

The process by which fatty acids enter cells begins with their release from chylomicrons. It is this step which, to date, has commanded attention. Unquestionably, the enzyme responsible for triglyceride hydrolysis, lipoprotein lipase, and its co-factor, apoC-II, are essential to the normal process. However, much remains to be learned about how fatty acids enter cells, and once such access has been gained, how they travel through the cytoplasm to reach their primary target organelles, the mitochondria and the smooth endoplasmic reticulum. It also remains to be determined exactly how ASP is involved in this sequence. Nevertheless, however ASP achieves its effect, increasing the rate of triglyceride synthesis intracellularly should result in an increased rate of fatty acid uptake by the tissue. This, in turn, may allow more effective action of lipoprotein lipase and so facilitate the clearance of triglyceride from plasma. If so, this would explain the marked inverse relationships observed in the present study between fasting or peak plasma ASP level and postprandial triglyceride response in plasma.

Much remains to be done to test this hypothesis. Nevertheless, the present data to establish that ASP is present in plasma in quantities sufficient to be biologically active. The data also demonstrate that following an oral fat load ASP levels rise significantly in every subject studied, whereas they do not change after an oral glucose load. Moreover, the higher the fasting ASP levels and the higher the peak level, the less marked the postprandial lipemia. Taken together with evidence provided by our in vitro studies, which have shown ASP to be far more potent than insulin in stimulating triglyceride synthesis in human adipocytes (2), the present data are consistent with the hypothesis that ASP may play a physiological role in the disposition of an oral fat load in humans. On the other hand, the present data are essentially correlative, and therefore further experimental evidence is still required before our hypothesis can be accepted.

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