Adipsin/Acylation Stimulating Protein System in Human Adipocytes: Regulation of Triacylglycerol Synthesis†

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ABSTRACT: Through their capacity to store fatty acids as triacylglycerol molecules, adipocytes serve a vital physiologic role. This study presents further evidence that this process can be modulated in human adipocytes by the adipsin/acylation stimulating protein (ASP) pathway and suggests a novel function for the product of this system—ASP. The data demonstrate the following: (1) ASP stimulates triacylglycerol synthesis within adipocytes, and this occurs to a greater extent in differentiating than undifferentiated cells (242% ± 32% vs 168% ± 11%, $p < 0.01$, respectively, at an ASP concentration of 88 ng/mL; (2) ASP does not affect the K_m for triacylglycerol synthesis but does substantially increase V_{max}; (3) when ASP is generated *in vitro* through incubation of its precursor proteins under appropriate conditions, triacylglycerol synthesis increases to the same extent as when plasma-purified ASP is added to the medium; (4) human adipocytes contain mRNA for the specific serine protease adipsin and the two precursor proteins C3 and factor B required to interact for the production of ASP; and (5) the extent to which cultured differentiating adipocytes produce ASP is proportional to the degree to which they have accumulated triacylglycerol mass during differentiation ($r^2 = 0.7523$, $p < 0.0005$). These findings provide the first evidence for the existence of the adipsin/ASP pathway in human adipocytes, and this may markedly enhance our understanding of the processes which regulate triacylglycerol clearance from plasma.

Because fatty acids can be stored as triacylglycerols in adipose tissue, we are able to move about freely, since without this reservoir of energy our musculoskeletal system would soon fail due to lack of fuel. In 1989, we reported that a basic protein present in human plasma markedly stimulated triacylglycerol synthesis in cultured human skin fibroblasts and human adipocytes. On the basis of its physiologic effect, this protein was named acylation stimulating protein (ASP) (Cianflone et al., 1989a). When its identity was determined, it was apparent that ASP purified from plasma was identical to the biologic fragment of the third component of plasma complement known as C3adesArg (Baldo et al., 1993). At first, this result appeared to be physiologically implausible given that its precursor C3a is widely believed to be an anaphylatoxin generated during complement activation (Hugli, 1975).

However, a series of studies by Spiegelman and his colleagues persuaded us to consider otherwise (Cook et al., 1985, 1987; Flier et al., 1987; White et al., 1992). They have shown that rodent adipocytes synthesize and secrete the three proteins necessary to generate C3a: adipsin (or complement factor D), factor B, and C3 (Choy et al., 1992). Since adipsin mRNA as well as protein production and secretion was markedly reduced in several murine obesity models (Flier et al., 1987), they suggested that this system modulated the rate of triacylglycerol hydrolysis within adipocytes. Importantly, in none of their studies, however, was the functional activity of the adipsin-initiated pathway tested directly. By contrast, our data indicate that ASP, which is the result of B, C3, and adipsin interaction, causes triacylglycerol synthesis to increase markedly in fibroblasts and, as set out below, in cultured human adipocytes as well. On this basis, we suggest the adipsin/ASP pathway may be a major determinant of triacylglycerol synthesis in human adipocytes.

MATERIALS AND METHODS

[9,10-^{3}H(N)]Oleic acid (10.0 Ci/mmol) and D-[1-^{3}H(N)]-glucose (15 Ci/mmol) were obtained from DuPont—New England Nuclear (Mississauga, Canada). Oleic acid (sodium salt) and bovine serum albumin, essentially fatty acid free (BSA), were from Sigma (St. Louis, MO). General chemicals and solvents were from Fisher Scientific (Nepean, Canada). Minimum essential medium (MEM) and Dulbecco's minimum essential medium/F12 (D-MEM/F12), Dulbecco's phosphate-buffered saline (D-PBS), and all other tissue culture supplies were from Gibco (Gaithersburg, MD). The following reagents for reverse transcriptase/PCR were obtained from the listed suppliers: Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD); hexanucleotides (Pharmacia LKB Biotechnology Products, Baie d'Urfe, Quebec, Canada); dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Laval, Quebec, Canada), Taq polymerase (ProMega/Fisher Scientific, Nepean, Ontario, Canada).

Culture of Human Skin Fibroblasts and Human Preadipocytes. Fibroblasts were obtained from forearm skin biopsies of normolipidemic subjects. Primary cultures were established from explants and maintained in MEM with 10% fetal calf serum supplemented with penicillin/streptomycin (100 IU/mL). Fibroblasts were subcultured every 7–10 days with a

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Table 1: PCR Primer Pairs

RNA	message	primers	product	size (bp)	ref
GAP	Sn	GGTGAAGGTCGGAGTCAACGGATTTGG	69–95	978	Arcari et al. (1984)
	Asn	GGCCATGAGGTCCACCACCCTGTT	1047–1024		
LPL	Sn	GAGATTTCTCTGTATGGCACC		277	Perkin Elmer Cetus (Norwalk, CT)
	Asn	CTGCAAATGAGACACTTTCTC			
adipsin[a]	Sn	GTCACCCAAGCAACAAAGTCC	765–785	268	White et al. (1992)
	Asn	TCCTGCGTTCAAGTCATCC	1014–1032		
factor C3	Sn	GCTGCTCCTGCTACTAACCCA	87–107	444	Botlo et al. (1990)
	Asn	TAGCAGCTTGTGGTTGAC	531–514		
factor B[a]	Sn	GTTGAAGTCAGGGACTAACACC	8–29	548	Campbell et al. (1983)
	Asn	CCACAGTGAAACAATGTGC	537–555		

[a] Primer design based on the computer-assisted program from cDNA from the indicated references (Kandel et al., 1991). Sn = sense; Asn = antisense; product indicates the location of the primer sequences in the published cDNA sequence (indicated as ref). Size indicates the amplified PCR fragment size in bp (base pairs).

split ratio of 1:2 following a 10 min incubation with 0.25% trypsin in Mg^{2+}- and Ca^{2+}-free D-PBS to detach the cells from the flask. Cells were used for experiments between passages 5 and 15 and plated out at a concentration of 1×10^4 cells/cm^2 in 24-well dishes in 0.5 mL of medium. At or near confluency, the day prior to the experiment, cells were changed to serum-free D-MEM/F12.

Human adipose tissue was obtained with informed consent at time of elective laparotomy. The adipose tissue was minced and treated with collagenase, and the stromal fraction was prepared as described by Hauner et al. (1989). Preadipocytes were plated out in 24-well culture dishes and cultured in 10% fetal calf serum (preadipocytes) or in differentiation medium (adipocytes) containing 7.5 mg/L insulin, 1 μM dexamethasone, 33 μM biotin, 17 μM pantothenate, and 0.2 nM triiodothyronine for an average of 28 days (Hauner et al., 1989). Cells were changed to serum-free DMEM/F12 the day prior to initiation of the experiment.

Measurement of Intracellular Triacylglycerol Synthesis and Mass. For experiments, cells were incubated in DMEM/F12 supplemented with D-[1-^{3}H(N)]glucose (specific activity 2.4 dpm/pmol) or [9,10-^{3}H(N)]oleic acid complexed to BSA in a 5:1 molar ratio as described by Van Harken (Van Harken et al., 1969) and added to the cells at a final concentration of 100 μM (average specific activity, 100 dpm/pmol). Triacylglycerol synthesis was measured over a 4 h period as [^{3}H]oleate incorporation into triacylglycerol. Following incubation, the cells were washed 3 times with 1 mL of ice-cold PBS and extracted with two 1 mL volumes of heptane/2-propanol (3:2). Lipid samples were dried in a centrifuge-evaporator (Canberra-Packard, Canada), reconstituted in 100 μL of chloroform/methanol (2:1), and resolved by thin-layer chromatography (silica gel 150A; Whatman, England). Plates were developed in hexane/ether/acetic acid (75:25:1) with reference lipids run concurrently. The lipids were visualized in iodine vapor, and the spots corresponding to triolein were scraped into scintillation vials. The radioactivity was counted in 5 mL of scintillation fluid (Cytoscint-ES; ICN, Costa Mesa, CA) and counted by scintillation (Beckman model counter). Triacylglycerol mass was measured as described by Neri and Frings (1973) and expressed as micrograms of triacylglycerol per milligram of soluble cell protein. Cell proteins were solubilized on the dishes by addition of 0.1 N NaOH and measured by the method of Bradford (1976) using a commercial kit (Bio-Rad, Richmond, CA). Data are reported as means of experiments (with all determinations performed in triplicate) ± standard deviation. Statistical significance was set at $p = 0.05$ and was determined using a paired or two-mean Student's t-test as indicated in the figure legends and under Results.

Isolation and Measurement of Plasma and Medium ASP. Acylation stimulating protein was partially purified from human plasma in three sequential chromatographic steps that were performed in the following order: cation exchange on S-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden); gel filtration on Sephadex G-75 (Pharmacia); and finally reverse phase on a semipreparative Vydac Protein C4 (1.0 × 25 cm) column (Separations Group, Hesperia, CA). For each chromatographic step, the activity of the column fractions was tested for their ability to stimulate triacylglycerol synthesis in cultured human skin fibroblasts. Overall recovery of activity from plasma averaged 60%. The fractionation method has been described in detail previously (Baldo et al., 1993). For validation of the radioimmunoassay, homogeneous ASP was prepared in five sequential chromatographic steps, and purity was assessed by amino acid composition, amino acid sequencing, and ion spray mass spectrometry as previously described (Baldo et al., 1993). Complement factors B, C3, and D (Calbiochem, San Diego, CA) were incubated as described to generate C3a/ASP *in vitro* (O'Keefe, 1988). ASP was measured in the medium of cultured fibroblasts, cultured human preadipocytes (undifferentiated), and adipocytes (differentiating) following a 24 h incubation of the cells in DMEM/F12 serum-free medium. In all cases, ASP was measured in the medium by a radioimmunoassay kit specific for C3adesArg (Amersham, Oakville, Canada). For Western analysis, ASP purified from plasma to homogeneity and C3adesArg (courtesy of Dr. T. Hugli, Scripps Institute, La Jolla, CA) were electrophoresed on 15% SDS discontinuous polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose paper. Western analysis with rabbit polyclonal anti-human C3adesArg from the radioimmunoassay kit (Amersham) was performed with a commercial colorimetric kit using alkaline phosphatase conjugated secondary antibody (Bio-Rad). Ouchterlony was performed over 18 h in a 1% agarose gel using the same antibody.

mRNA Isolation and RT–PCR Amplification. Confluent human skin fibroblasts in T150 flasks were extracted with 3 mL of guanidinium thiocyanate solution. Mature fat cells released from the collagenase digestion of adipose tissue during the preparation of the stromal adipocyte precursor cells were also extracted in an equal volume of guanidinium thiocyanate solution. RNA was isolated as described by Chomczynski and Sacchi (1987) and quantitated by fluorometry. RNA (1 μg) was reverse-transcribed (Kandel et al., 1991), and 8% of this product was amplified by polymerase chain reaction. The primers used as described in Table 1. In-house primers were designed using a computer-assisted program (Lowe et al., 1990) and were obtained through HSC/Pharmacia Biotechnology Service Centre, Department of Clinical Biochemistry, University of Toronto, Toronto, Ontario. The final reaction contained 0.5 unit of Taq polymerase, 0.2 mM dNTPs, and

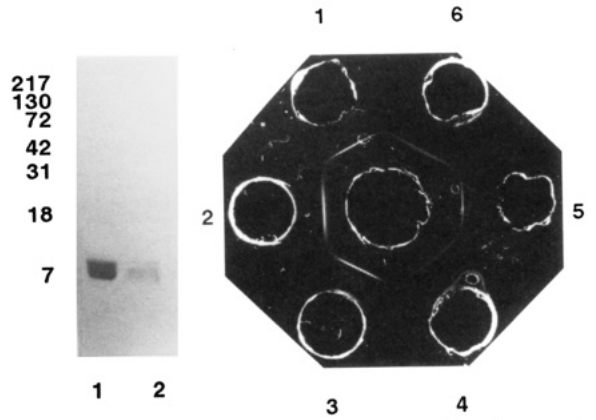

FIGURE 1: Western and Ouchterlony of ASP and C3adesArg. Left panel: C3adesArg (10 μg) and ASP purified from plasma (2 μg) were electrophoresed on 15% SDS discontinuous polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose paper. Western analysis was performed with rabbit polyclonal anti-human C3adesArg (Amersham) using a commercial colorimetric kit with alkaline phosphatase conjugated secondary antibody as detection (Bio-Rad). Both C3adesArg (lane 1) and ASP (lane 2) react with the specific antibody. Right panel: Ouchterlony was performed in a 1% agarose gel. ASP (10, 5, and 2.5 μg in wells 1, 3, and 5) and C3adesArg (10, 5, and 2.5 μg in wells 2, 4, and 6) were immunoreacted against the same rabbit polyclonal anti-human C3adesArg as above for 18 h. A line of identity is obtained between ASP and C3adesArg at all concentrations applied.

1 μM of each primer in a final volume of 20 μL amplified on a Perkin Elmer Cetus DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). For each of the primer products, cycling controls were run to establish the assay conditions. One cycle consisted of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. The product signal for each message was linear up to 37 cycles in mature fat cell RNA where there was greater abundance of the mRNA of interest. GAP was linear up to 29 cycles in mature fat cell RNA and fibroblast RNA. Therefore, a standard cycle number was chosen within the linear range to assay all of the samples. Individual conditions were as follows: glyceraldehyde-3-phosphate dehydrogenase (10^{-5} M TMAC, 2.0 mM $MgCl_2$, 25 cycles), lipoprotein lipase (2.0 mM $MgCl_2$, 35 cycles), adipsin (2.5 mM $MgCl_2$, 35 cycles), factor B (10^{-5} M TMAC, 2.0 mM $MgCl_2$, 35 cycles), and complement C3 (10^{-5} M TMAC, 2.0 mM $MgCl_2$, 35 cycles) where TMAC is tetramethylammonium chloride. Following PCR amplification, samples were separated on a 9% polyacrylamide gel (Laemmli, 1970) using 0.8% piperazinediacrylamide as the cross-linker with a 100 base pair ladder as reference (Pharmacia LKB Biotechnology Products). The gel was silver-stained (Bio-Rad silver stain kit) and scanned by densitometry on a computer-assisted LKB Ultroscan XL laser densitometer (Pharmacia LKB Biotechnology Products). Each sample was measured in separate experiments at least 2–3 times, and the results were averaged.

RESULTS

ASP/C3a was purified from human plasma to homogeneity as determined by amino acid sequencing, amino acid composition, and ion spray mass spectrometry as described previously (Baldo et al., 1993), and ASP concentration was determined by radioimmunoassay. As shown in Figure 1, left panel, the specific polyclonal antibody supplied in the commercial radioimmunoassay (Amersham) recognizes homogeneous ASP and human C3adesArg (courtesy of Dr. Tony Hugli, Scripps Institute). Similar results were obtained with a commercially available monoclonal antibody to C3adesArg (Quidel, San Diego, CA; results not shown). In addition, an

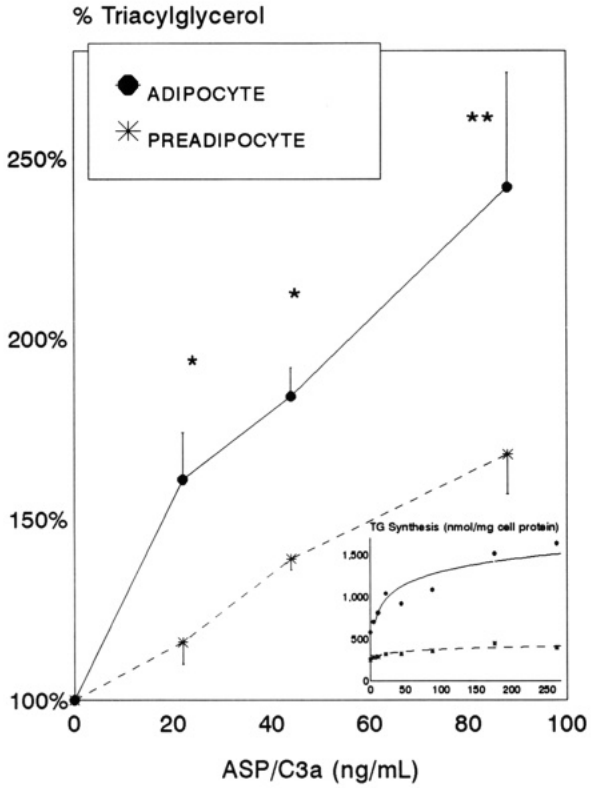

FIGURE 2: ASP stimulation of cultured human differentiating adipocytes. Human preadipocytes were isolated, and both preadipocytes and differentiating adipocytes were cultured from each tissue sample. ASP concentration was measured in a partially purified fraction of plasma ASP by radioimmunoassay. After 24 h in serum-free DMEM/F12, differentiating adipocytes and preadipocytes were changed to serum-free DMEM/F12 supplemented with 100 μM [^{3}H]-oleate complexed to bovine serum albumin and ASP at the indicated concentrations. Triacylglycerol synthesis was measured over a 4 h period as [^{3}H]oleate incorporation (nmol/mg of cell protein ± standard deviation, n = 7 experiments). In the differentiating adipocytes, triacylglycerol synthesis was linear for at least 24 h, and 100 μM oleate was within the linear portion of the fatty acid concentration curve (data not shown). Basal triacylglycerol synthetic rates (shown as 100% on the figure) were 12.8 ± 2.4 and 65.6 ± 9.3 nmol of triacylglycerol (mg of soluble cell protein)$^{-1}$ (4 h)$^{-1}$ ± standard deviation in preadipocytes and differentiating adipocytes, respectively. One asterisk, $p < 0.05$; two asterisks, $p < 0.01$ for percent TG in differentiating adipocytes vs preadipocytes by the two-mean t-test.

Ouchterlony using the same polyclonal antibody with different dilutions of homogeneous ASP and human C3adesArg indicates immunochemical identity of ASP and C3adesArg at all concentrations tested (Figure 1, right panel). Therefore, the commercial radioimmunoassay antibody is valid for ASP determination.

Human adipose tissue obtained at the time of laparotomy was digested, and the cell pellet yielded stromal cells that were cultured to yield differentiating adipocytes. The first question examined was the effect of ASP on triacylglycerol synthesis in preadipocytes and differentiating adipocytes. ASP was partially purified through a three-step chromatographic procedure as described under Materials and Methods and the concentration of ASP determined by radioimmunoassay. ASP was then added to the cell culture medium in the presence of 100 μM [^{3}H]oleate, and these results are shown in Figure 2. Triacylglycerol synthesis was measured over a 4 h period as [^{3}H]oleate incorporation into triacylglycerol. For each of the two cell types, basal triacylglycerol synthesis was taken as 100%, and triacylglycerol synthesis was determined over a concentration range of plasma-purified ASP which was added to the medium. The results indicate that ASP induced a

concentration-dependent increase in triacylglycerol synthesis in both differentiating adipocytes and preadipocytes. Of interest, the percent increase above basal produced by addition of ASP to the medium was significantly greater in the differentiating adipocytes than in the preadipocytes ($p < 0.05$ for each concentration of ASP by the two-mean t-test; $p < 0.01$ for the highest concentration of ASP). Such an analysis, however, minimizes the absolute effects on triacylglycerol synthesis caused by ASP. For example, the basal triacylglycerol synthetic rate in preadipocytes was 12.8 ± 2.4 nmol of triacylglycerol (mg of soluble cell protein)$^{-1}$ (4 h)$^{-1}$ (mean ± standard deviation), whereas it was 65.6 ± 9.3 nmol of triacylglycerol (mg of soluble cell protein)$^{-1}$ (4 h)$^{-1}$ for the differentiating cells. With addition of 88 ng/mL ASP to the medium, the two absolute rates of synthesis were 20.3 ± 3.0 and 174.0 ± 50.1 nmol of triacylglycerol (mg of cell protein)$^{-1}$ (4 h)$^{-1}$, respectively (168% ± 11%, $p < 0.0005$, and 242% ± 32%, $p < 0.025$, respectively). With higher concentrations of ASP and longer incubation times (24 h), the ASP effect saturated in both preadipocytes and differentiating adipocytes although the absolute triacylglycerol synthetic rate in differentiating adipocytes was still much greater (Figure 2, inset). It is apparent, therefore, that while ASP acts on both undifferentiated and differentiating adipocytes, it is far more potent in the latter and this may be important in terms of ASP function *in vivo*.

The effect of ASP on the apparent K_m and V_{max} for triacylglycerol synthesis was measured at saturating concentrations of ASP (88 ng/mL), and a representative experiment is shown in Figure 3. In both the preadipocytes (top panel) and the differentiating adipocytes (lower panel), there is a clear effect of ASP at all of the concentrations of oleate tested. However, both the net increases (note the different y-axis scales) and the percentage increase are much greater in the differentiating adipocytes than the preadipocytes (351% ± 72% vs 230% ± 66%, $p < 0.005$, respectively). Double-reciprocal linear regression analysis indicates no change in the K_m for either the preadipocytes or the differentiating adipocytes (15.0 μmol/L basal vs 15.8 μmol/L with ASP for preadipocytes and 4.1 μmol/L basal vs 2.6 μmol/L with ASP for differentiating adipocytes). However, there is a large change in V_{max}, particularly in the differentiating adipocytes: 10.0 nmol/mg of cell protein basal vs 20.2 nmol/mg of cell protein with ASP in preadipocytes and 52.0 nmol/mg of cell protein basal vs 181.5 nmol/mg of cell protein with ASP in differentiating adipocytes. Over three experiments, the average change in V_{max} in differentiating adipocytes was 260% ± 64%, $p < 0.05$, and in preadipocytes was 219% ± 76%, $p < 0.05$, with no significant change in K_m (69% ± 6% in differentiating adipocytes and 122% ± 49% in preadipocytes). These effects of ASP on the cellular V_{max}, which suggest enhanced triacylglycerol synthesis capacity, are consistent with our previous results of the ASP effect on human skin fibroblast triacylglycerol synthesis (Germinario, 1993) although the results in differentiating adipocytes are far more pronounced.

We have shown previously that ASP can be generated by incubation of factors B, C3, and D (adipsin) (Baldo et al., 1993). Accordingly, we examined whether ASP generated *in vitro* would be competent to stimulate triacylglycerol synthesis in human adipocytes. ASP was generated *in vitro* through the interaction of factors B, C3, and adipsin. The amount of ASP generated was measured by radioimmunoassay, and these measured values are given on the x-axis as ASP/C3a (ng/mL) (Figure 4). The total amount of protein present in the mixture is actually much greater and consists of intact C3, B, and adipsin and the byproducts of ASP/C3a generation

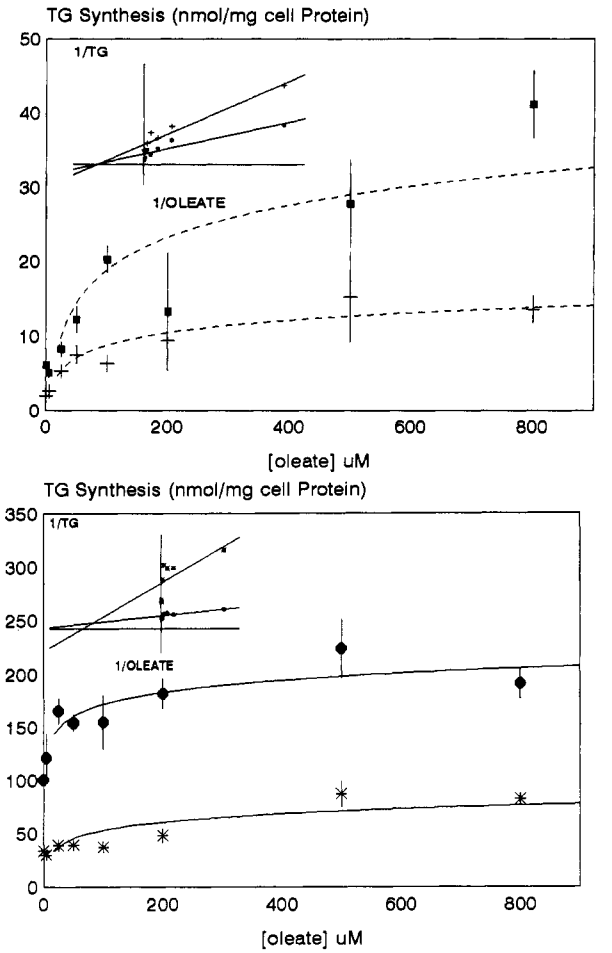

FIGURE 3: Kinetic analysis of the ASP effect on triacylglycerol synthesis in cultured human preadipocytes and differentiating adipocytes. Human preadipocytes and differentiating adipocytes were cultured as described under Materials and Methods. ASP concentration was measured in a partially purified fraction of plasma ASP by radioimmunoassay. After 24 h in serum-free DMEM/F12, differentiating adipocytes and preadipocytes were changed to serum-free DMEM/F12 supplemented with ASP (88 ng/mL), [^{3}H]glucose (specific activity 2.42 dpm/pmol), and the indicated concentrations of oleate complexed to bovine serum albumin. Triacylglycerol synthesis was measured over a 6 h period as [^{3}H]glucose incorporation into triacylglycerol (nmol/mg of cell protein). Upper panel: preadipocytes with (■) and without (+) ASP. Lower panel: differentiating adipocytes with (●) and without (*) ASP. Reciprocal analysis linear regression was used to calculate K_m and V_{max} as shown in the inset panels.

including C3A′, Ba, Bb, and C3b as shown previously (Baldo et al., 1993). Similarly, although the plasma-purified ASP is not completely homogeneous, the amount of immunoreactive ASP was determined by radioimmunoassay, and this value is indicated on the x-axis. As shown in Figure 4, when the mixture of precursor proteins and specific serine protease is added to cells, it stimulates triacylglycerol synthesis to a degree comparable to that achieved by adding an equivalent amount of plasma-purified ASP (p not significant for plasma ASP vs *in vitro* generated ASP from B/C3/D, but $p < 0.025$ for all points vs basal). Therefore, the specific activities of the plasma-purified ASP and the *in vitro* generated ASP are equivalent since an equal amount of immunoreactive ASP in both cases increases triacylglycerol synthesis to the same extent. The much greater specific activity of ASP in these experiments as compared to our previous results (Cianflone et al., 1989a) can be attributed to a number of factors: first, the initial purification procedures used chromatofocusing under denaturing conditions in the presence of urea and high

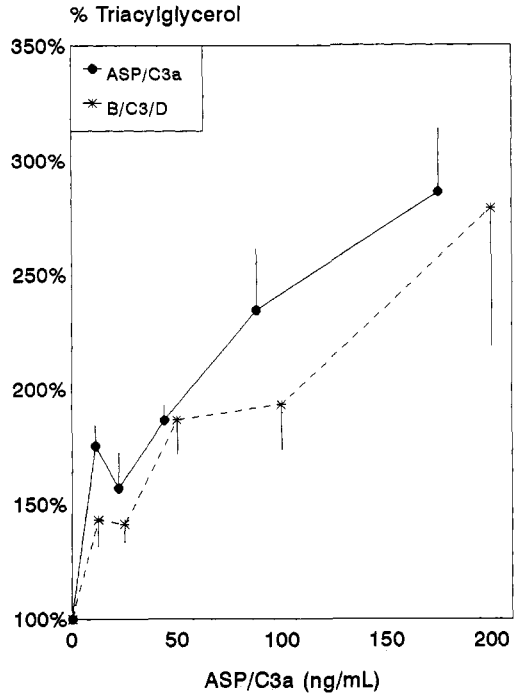

FIGURE 4: Generation of ASP by factors B, C3, and D. Human differentiating adipocytes were cultured as described under Materials and Methods. Complement factors B, C3, and D (Calbiochem) were incubated to generate ASP (C3a) *in vitro*, and the concentration was measured by radioimmunoassay. ASP concentration was measured in a partially purified fraction of plasma ASP by radioimmunoassay and added to the adipocytes at the indicated concentrations in serum-free DMEM/F12 supplemented with 100 μM [^{3}H]oleate complexed to bovine serum albumin for 4 h. Triacylglycerol synthesis was measured as described and expressed as nanomoles per milligram of cell protein per 4 h ± standard deviation for an average of six experiments: p not significant for plasma ASP stimulation vs *in vitro* generated ASP stimulation from B/C3/D by the two-mean t-test, but $p < 0.0025$ for all points vs basal by the paired t-test.

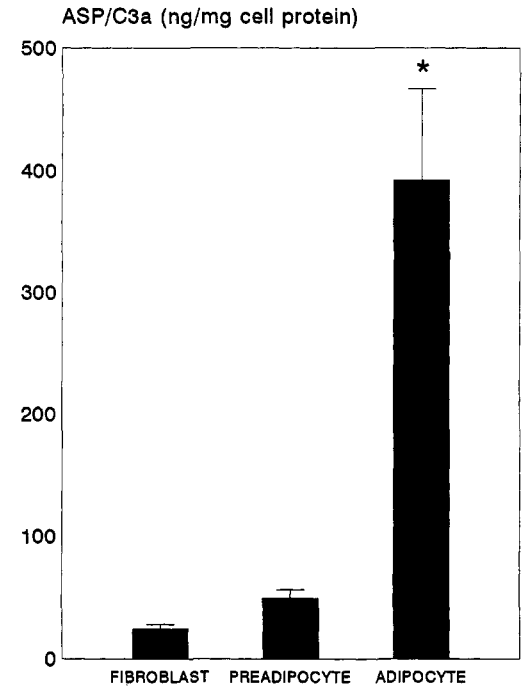

FIGURE 5: ASP secretion by cultured human skin fibroblast, preadipocytes, and differentiating adipocytes. The preadipocyte stromal fraction of collagenase-digested human adipose tissue was cultured in 10% fetal calf serum in minimal essential medium or in differentiation medium for an average of 28 days prior to experimentation as described under Materials and Methods. Human skin fibroblasts were cultured as previously described (Cianflone, 1989a) and were incubated in 10% fetal calf serum. ASP was measured in the medium of cultured human preadipocytes (undifferentiated, n = 30 subjects), adipocytes (differentiating, n = 30 subjects), and human skin fibroblasts (n = 4) following a 24 h incubation in DMEM/F12 serum-free medium. Medium ASP was measured by a radioimmunoassay kit specific for C3a (Amersham) and expressed as nanograms per milligram of soluble cell protein ± standard deviation. One asterisk, $p < 0.0005$ compared to preadipocytes (two-mean t-test).

pH (pH > 9.0), which may have caused inactivation of a portion of ASP and reduced the activity yield; second, the purified material at this time yielded a single band on SDS–polyacrylamide gel electrophoresis which later proved to be misleading when analyzed by amino acid sequencing and ion spray mass spectrometry, and the fraction was not, in fact, homogeneous; finally, differentiating adipocytes respond to ASP to a greater extent than preadipocytes or fibroblasts (Baldo et al., 1993), and this may also account for increases in the specific activity of ASP.

We then examined ASP secretion in these cells, and the results are shown in Figure 5. Three types of cells were studied: human skin fibroblasts, human preadipocytes, and human differentiating adipocytes. Both human skin fibroblasts and undifferentiated preadipocytes produce small but detectable amounts of ASP in the medium over a 24 h period. Incubation in differentiation medium did not affect the production of ASP by human skin fibroblasts. Differentiating adipocytes, by contrast, secrete 8-fold more ASP into the medium over the same time period. The differences in ASP secretion between preadipocytes and adipocytes are also clear at shorter incubation times (4 and 18 h, unpublished observations).

Since ASP is generated through the combined action of adipsin, a serine protease, and factors B and C3, we examined whether human mature fat cells possess the mRNA message for these three proteins and compared the findings to human skin fibroblasts which do not produce substantial amounts of ASP in conditioned culture medium. Primers were prepared for adipsin (factor D), factor B, and C3 as described under Materials and Methods. Human mature fat cells, isolated from the floating layer of the digested human adipose tissue, were extracted and the message levels examined. Care was taken that the cycling number used was within the linear range (i.e., signal to PCR cycle number) even in mature fat cells where there is greater cellular abundance of the particular mRNA of interest. Average results of 8 human skin fibroblast cell lines and mature fat cell preparations from 13 subjects are shown in Figure 6. There was no difference in glyceraldehyde-3-phosphate dehydrogenase (GAP) between human skin fibroblasts and mature fat cells. As previously reported, there was a very strong signal for the message levels of lipoprotein lipase (LPL) in mature fat cells, with little signal apparent in human skin fibroblasts (Dani et al., 1990). As well, mature fat cells were also shown to have much higher message levels present for adipsin and C3 ($p < 0.0005$ and $p < 0.005$, respectively) with a trend toward higher factor B message levels as compared to fibroblasts. This is consistent with the increased levels of ASP secreted by cultured differentiating adipocytes shown in Figure 5.

The relation between adipose differentiation, triacylglycerol synthesis, and ASP production was then examined in more detail. For this purpose, the mass of triacylglycerol per cell protein was taken as an index of differentiation (Hauner et al., 1987). As shown in Figure 7, there is a strong positive correlation between changes in triacylglycerol cell mass, which increases as the cell differentiates, and changes in triacylglycerol synthetic rates tested at two different fatty acid

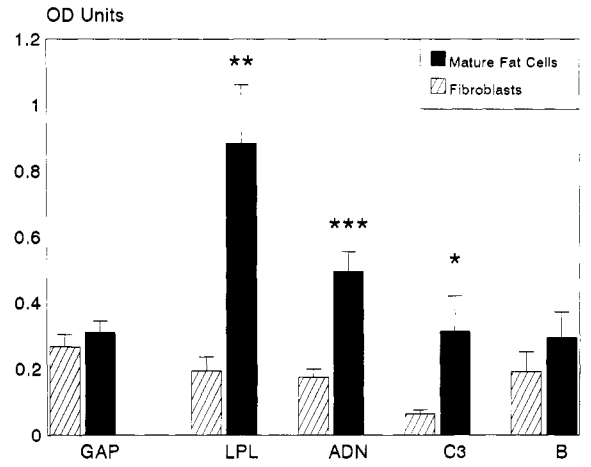

FIGURE 6: PCR amplification of cultured human skin fibroblast and mature fat cell RNA. RNA was isolated from one T150 flask of human skin fibroblasts or from mature fat cells isolated by collagenase digestion of adipose tissue. One microgram of RNA was reverse-transcribed and amplified by PCR. The following messages were amplified by PCR: GAP (glyceraldehyde-3-phosphate dehydrogenase, 978 bp); LPL (lipoprotein lipase, 277 bp); ADN (adipsin, 268 bp); C3 (complement C3, 444 bp); and B (factor B, 548 bp). PCR products were separated by gel electrophoresis and silver-stained. Gels were scanned, and results are expressed in arbitrary densitometric units as average ± standard deviation. Results are the average of 8 human skin fibroblast cell lines and mature fat cell preparations from 13 subjects where one asterisk is $p < 0.05$, two asterisks are $p < 0.005$, and three asterisks are $p < 0.0005$ by the two-mean t-test.

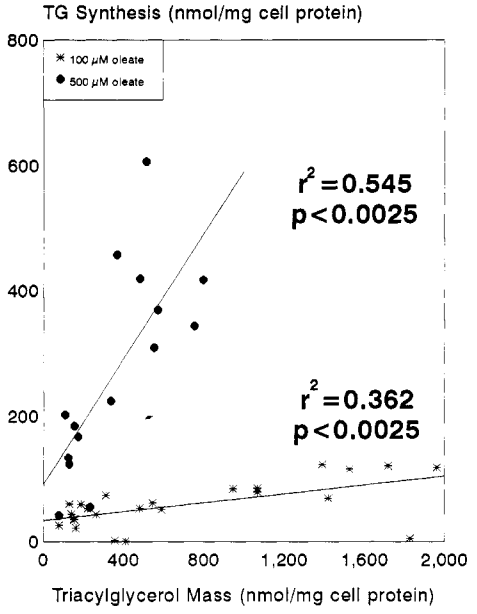

FIGURE 7: Correlation of triacylglycerol synthesis to adipocyte differentiation. Cells were cultured as described under Materials and Methods. Following a 24 h incubation in serum-free medium, labeled [^{3}H]oleate complexed to bovine serum albumin was added at a concentration of 100 μM (*) or 500 μM (●) for the last 4 h of the 24 h incubation time. The cells were extracted, and triacylglycerol mass was measured and expressed as nanomoles of triacylglycerol per milligram of soluble cell protein. [^{3}H]Oleate incorporation into triacylglycerol is expressed as nanomoles per milligram of soluble cell protein (TG synthesis). Linear regression correlation: for 100 μM oleate, $y = 0.0349x + 34.5$, $r^2 = 0.3621$, and $p < 0.0025$; for 500 μM oleate, $y = 0.499x + 91.7$, $r^2 = 0.545$, and $p < 0.0025$.

concentrations. As the adipose cell differentiates, it becomes larger and triacylglycerol-enriched. The triacylglycerol synthetic capacity increases concurrently (100 μM oleate: $r^2 = 0.362$, $p < 0.0025$; 500 μM oleate: $r^2 = 0.545$, $p < 0.0025$). With time, as cells differentiate, adipocytes also acquire an increased capacity to secrete ASP. The data in Figure 8

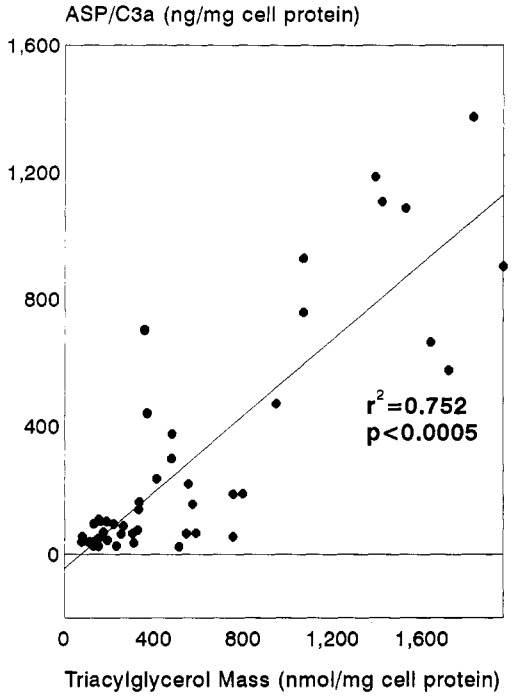

FIGURE 8: Correlation of medium ASP to adipocyte differentiation. Preadipocytes and differentiating adipocytes were cultured as described under Materials and Methods. Following a 24 h incubation, medium ASP was measured by radioimmunoassay and expressed as nanograms per milligram of cell protein. The cells were extracted, and triacylglycerol mass was measured and expressed as nanomoles of triacylglycerol per milligram of soluble cell protein. Linear regression correlation: $y = 0.6295x - 44.2$, $r^2 = 0.752$, and $p < 0.0005$.

establish that ASP secretion is proportional to their level of differentiation ($r^2 = 0.752$, $p < 0.0005$). Thus, as adipocytes mature, they become increasingly competent to synthesize triacylglycerols and also to secrete ASP.

DISCUSSION

This study presents the first evidence of the production of ASP and, more importantly, of the potential function of ASP in human adipocytes, where ASP or C3a is the final effector molecule produced by the interaction of factor B, adipsin (or factor D), and complement C3 (Hugli, 1975). Once generated, ASP facilitates membrane transport of glucose and increases the activity of the final enzyme involved in triacylglycerol synthesis, diacylglycerol acyltransferase, in human fibroblasts and human adipocytes (Germinario et al., 1993; Yasruel et al., 1991). A single protein, therefore, is able to stimulate two critical processes involved in the construction of a triacylglycerol molecule.

In this study, we have shown that ASP induces a concentration-dependent increase in triacylglycerol synthesis in both preadipocytes and differentiating adipocytes, the absolute effect, though, being more pronounced in the latter. ASP does not significantly affect the K_m for triacylglycerol synthesis but does substantially increase V_{max}. Not only are differentiating adipocytes more responsive to ASP, but they also secrete more ASP into the medium than do preadipocytes. Moreover, message levels for the three proteins necessary to generate ASP were shown to increase during differentiation, and, finally, strong relations were demonstrated between the rate of triacylglycerol synthesis, ASP accumulation in the medium, and the extent of adipocyte differentiation.

Such a system might be of considerable physiologic importance. For example, there is now evidence that the rate

of triacylglycerol clearance from plasma is not a simple function of the mass of lipoprotein lipase present on the capillary endothelium (Olivecrona et al., 1990). Rather, lipoprotein lipase activity seems to be determined by the ambient fatty acid concentration. This, in turn, may be governed by the rate at which triacylglycerol synthesis occurs in adipocytes, thus ensuring that fatty acid concentrations never rise to deleterious levels in the microenvironment. According to this model, the action of ASP generated through the adipsin/ASP system may allow up-regulation of intracellular triacylglycerol synthesis and thus enhancement of triacylglycerol clearance from plasma (Cianflone et al., 1989b; Sniderman et al., 1992).

On the other hand, dysfunction of this system may be key to the pathogenesis of HyperapoB, a common dyslipoproteinemia in patients with premature coronary artery disease (Sniderman et al., 1980). Adipocytes from patients with this disorder synthesize triacylglycerols less rapidly than adipocytes from normals (Teng et al., 1988), and studies in cultured skin fibroblasts from such patients indicate they respond less well to ASP than do cells from normal subjects (Cianflone et al., 1990a). A decreased rate of peripheral fatty acid uptake might result in diversion of these fatty acids to the liver, leading, in turn, to an increased rate of secretion of hepatic apoB100 particles (Cianflone et al., 1990b). The accumulation of these particles in plasma can then lead to accelerated atherogenesis.

Finally, there is the issue of obesity. Because adipsin message and plasma serum levels were reduced in many murine models of obesity, Spiegelman and his colleagues concluded that down-regulation of the system might be a metabolic cause of the obese state (Flier et al., 1987). In humans, in both moderate and severe obesity, plasma ASP levels are elevated (Sniderman et al., 1991) as are plasma adipsin levels (Napolitano, 1991). Whether the source is adipocytes or some other tissue as well remains to be determined, but the observation raises the possibility that the diminished adipsin expression observed by Spiegelman and colleagues (Flier et al., 1987) was an adaptive rather than a causal event.

Clearly, much further work is required to test these hypotheses. They are raised to indicate that the present findings may be of broad physiological as well as clinical relevance. For the moment though, the issue of interest is that human adipocytes appear able to up-regulate their capacity to synthesize triacylglycerols by virtue of a complex but nonetheless effective mechanism—the adipsin/ASP pathway. If valid, this model will provide, at a minimum, a new framework to understand the processes which regulate triacylglycerol clearance from plasma and disorders which may reflect dysfunction of this pathway such as HyperapoB and obesity.

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