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The Effect of ASP on Triglyceride Synthesis in the Human Adipocyte

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Abstract

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This thesis examines the isolation of the Acylation Stimulating Protein (ASP), a small molecular weight, basic protein from human plasma, as well as this protein's effect on triglyceride synthesis in the human adipocyte. ASP stimulates the incorporation of the fatty acid, oleic acid, into both diglyceride and triglyceride in adipocytes. This stimulatory activity is greater than that seen by insulin alone. The protein also stimulates triglyceride synthesis in the microsomal fraction of the adipocyte, but has no effect on beta oxidation of palmitoyl-Coenzyme A by adipose mitochondria. Its stimulatory effect on glyceride synthesis is the same for adipocytes isolated from normal weight and from morbidly obese individuals. However, the level of ASP in the plasma of morbidly obese females is elevated compared to the levels found in age matched normal weight females.

This work points to a physiological role for ASP in triglyceride synthesis as well as a possible role in the development of obesity.

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List of Abbreviations

ABS	Absorbance
ASP	Acylation Stimulating Protein
ATP	Adenosine Triphosphate
BMI	Body Mass Index
BSA	Bovine serum Albumin
cAMP	cyclic Adenosine 3',5' Monophosphate
CE	Cholesterol Ester
Chol	Cholesterol
CM	Chylomicron
CoA	Coenzyme A
CR	Chylomicron Remnant
DG	Diglyceride
DGAT	Diglyceride Acyl Transferase
FACS	Fatty Acyl-CoA Synthetase
FFA	Free Fatty Acid
GPAT	Glycerol Phosphate Acyl Transferase
G3P	Glycerol-3-Phosphate
HDL	High Density Lipoprotein
HSA	Human Serum Albumin
IDL	Intermediate Density Lipoprotein
Kd	Kilodalton
LCAT	Lecithin Cholesterol Acyl Transferase
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase

MES	2-(N-Morpholino) Ethanesulfonic Acid
Na_2EDTA	Disodium Ethylenediamine Tetraacetate
NADH	Nicotinamide Adenine Dinucleotide
PAGE	Polyacrylamide Gel Electrophoresis
Pi	Inorganic Phosphate
PIA	Phenylisopropyl Adenosine
PL	Phospholipid
POFFS	Plasma On Fast Flow S-Sepharose
PPH	Phosphatidate Phosphohydrolase
SEM	Standard Error of the Mean
TCA	Trichloroacetic Acid
TG	Triglyceride
TRIS	Tris(Hydroxymethyl)aminomethane
VLDL	Very Low Density Lipoprotein

Introduction

The regulation and control of lipid metabolism in the fat cell or adipocyte occurs intracellularly through the triglyceride synthetic and hydrolytic enzymes at the site of lipid synthesis as well as extracellularly in blood, where the lipoproteins are found. A brief review of these regulatory processes will be made, along with a discussion of morbid obesity and how these processes relate to it.

Lipoprotein Metabolism

Lipoproteins are a heterogenous mixture of macromolecular particles composed of lipid and protein. There are basically five major types of lipoproteins, Chylomicrons (CM), Very Low Density Lipoproteins (VLDL), Intermediate Density Lipoproteins (IDL), Low Density Lipoproteins (LDL) and High Density Lipoproteins (HDL). The initial classification of these lipoproteins was determined physically by their flotation rates on salt density gradient centrifugation (1). Each type, however, can also be differentiated from one another chemically by their lipid and protein content (2). The combination of proteins, also known as the apoproteins, contained in these particles will be unique to the particular lipoprotein type. As well, the lipid composition and the size of the lipoprotein particle will be unique for each lipoprotein type. Table 1 contains information on the percent

composition by weight of the lipid and apoprotein components of the different lipoproteins, and Table 2, a list of the apoprotein components of each lipoprotein.

The major function of the lipoprotein is to act as a transporter in plasma of lipid that is either absorbed through the gastrointestinal tract after eating or that is synthesized by the liver.

The chylomicrons are the triglyceride rich particles that are synthesized in the enterocytes of the small intestine after a meal. These lipoproteins are then secreted into the lymph from which they can enter the bloodstream (3). In the circulation they are acted on by the enzyme Lipoprotein Lipase (LPL). This enzyme requires apo CII as a cofactor, an apoprotein found in both chylomicrons and VLDL. United with its cofactor, LPL can then hydrolyze the triglyceride in the chylomicron to form glycerol and fatty acids, leaving behind a chylomicron remnant (CR) that contains cholesteryl-ester, cholesterol and phospholipid. This remnant is then taken up by the liver through a receptor mediated process (4). The free fatty acids and glycerol that are released upon hydrolysis of the chylomicron can then be used in a variety of tissue types to make either triglyceride for storage, as in the case of the adipocyte and the hepatocyte, or to make phospholipids to be used in membrane synthesis or to be used as a source of energy through the process of mitochondrial beta-oxidation, as is seen primarily in skeletal and cardiac muscle cells (5).

TABLE 1: PLASMA LIPOPROTEINS
PERCENT COMPOSITION BY WEIGHT

LIPOPROTEIN	TG	CHOL	CE	PL	PROTEIN
CHYLOMICRONS	85	1	3	9	2
VLDL	50	7	12	18	10
LDL	10	8	37	20	23
HDL	4	20	15	24	55

TG = TRIGLYCERIDE CE = CHOLESTERYL ESTER
CHOL = CHOLESTEROL PL = PHOSPHOLIPID

TABLE 2: PLASMA LIPOPROTEINS
APOLIPOPROTEIN COMPOSITION

LIPOPROTEINS	APOLIPOPROTEINS
CHYLOMICRONS	AI, AIV, B-48, CI, CII, CIII, E
VLDL	B-100, CI, CII, CIII, E
LDL	B-100
HDL	AI, AII, CI, CII, CIII, E

cholesterol and fatty acids derived from chylomicron remnants, LDL particles and circulating fatty acids that are taken up by the liver, as well as endogenously synthesized cholesterol and fatty acid of the liver. The triglyceride of the VLDL is also hydrolyzed by LPL to generate fatty acids, glycerol and IDL. The IDL is further hydrolyzed to form LDL, a cholesterol rich particle which can then be taken up into the liver or peripheral tissues through the LDL receptor. The fatty acids and glycerol are used by the various cells as previously discussed, while the cholesterol of the LDL particle is used in the synthesis of plasma membranes, cholesterol-ester and bile (6,7).

The HDL particles pick up cholesterol from cells as well as the surface remnants of both protein and lipid of VLDL and chylomicrons and then, through the action of Lecithin Cholesterol Acyl Transferase (LCAT), esterify the cholesterol to a fatty acyl chain (8). This process results in the formation of a larger and less dense HDL particle. The cholesterol-ester of this particle can then be exchanged with the triglyceride of other lipoproteins such as VLDL and chylomicron remnants by a cholesterol ester transport protein. As well, HDL can pick up protein, including apo CII after the hydrolysis of chylomicrons and VLDL by lipoprotein lipase (9).

The lipoproteins of interest in terms of fatty acid uptake and triglyceride synthesis in adipocytes are the

triglyceride rich particles, VLDL and chylomicrons. This is because they provide the substrate, ie. the fatty acids, that will be esterified to a glycerol-3-phosphate (G3P) backbone once they enter the adipocyte. Another source of fatty acids is de novo synthesis from glucose in the adipocyte as well as the hepatocyte by the fatty acid synthetase enzyme complex (10). There is also a circulating basal level of fatty acid that is bound to albumin and is available to the adipocyte for triglyceride synthesis. The processes that affect the extracellular concentration of fatty acid, ie. the level of circulating fatty acids, as well as the intracellular levels of fatty acid, will also effect the synthesis of triglyceride from these precursors.

Triglyceride Synthesis in the Adipocyte

The adipocyte can be thought of as being primarily a large triglyceride deposit, comprising 80% of the cell's weight, with a nucleus, some mitochondria and an endoplasmic reticulum surrounded by a plasma membrane (11). Its main function within the body is to act as a storage site for triglyceride. The level of the triglyceride storage pool is under the control of two processes. One is catabolic and involves the hydrolysis of triglyceride to release fatty acids by Hormone Sensitive Lipase (HSL). This enzyme is activated by the catecholamine epinephrine through its binding to an adrenergic receptor and activation of the adenylate cyclase

complex with the subsequent generation of cAMP (12,13). The fatty acid that is released can then be used by the mitochondria of the adipocyte, or it can enter the bloodstream and be taken up by other tissues such as skeletal and cardiac muscle and be used to produce energy (5,14). The second process, which is also the one of interest in terms of this work, is the synthesis of triglyceride from fatty acids and glycerol. This process is dependent upon the supply of substrate, glycerol-3-phosphate and fatty acid, as well as on the activity of the enzymes in the triglyceride synthetic pathway.

As was stated previously, fatty acids can be made either de novo from glucose, or they can be supplied from the diet in the form of triglyceride. In the adipocyte, the former process does not play a major role in providing fatty acids for esterification (15,16). Fatty acids from the blood stream are the major source of substrate, and the uptake of this substrate is dependent in part upon the activity of LPL as well as the transport of fatty acids into the adipocyte.

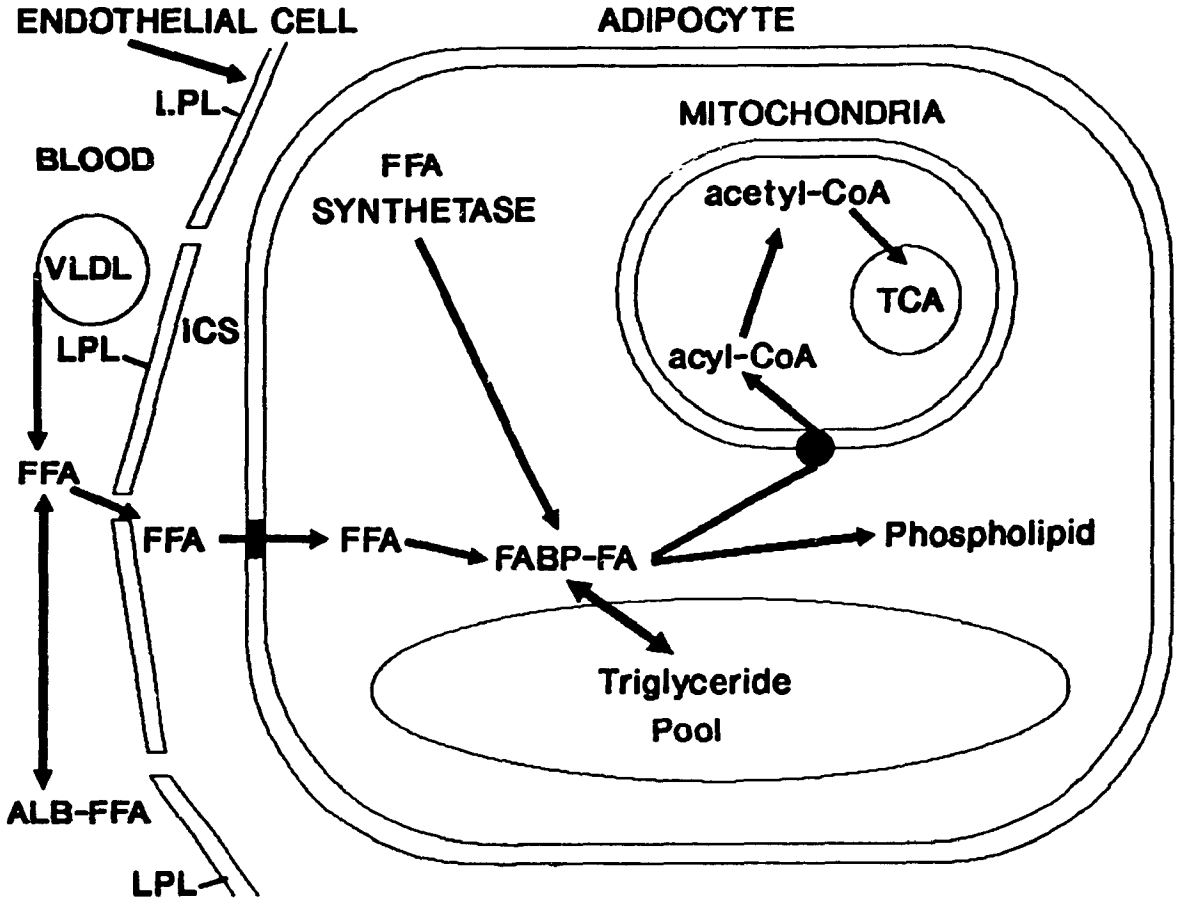
The lipolytic enzyme LPL is synthesized and secreted by the adipocyte from where it is transported to the luminal surface of the capillary endothelium (17). This process is increased in the presence of insulin and is inhibited by various lipolytic agents, such as glucagon and adrenaline (18,19). Once on the surface of the capillary endothelium, the chylomicrons and VLDL can interact with it, and the apo CII

in these particles will activate the LPL which in turn will result in the hydrolysis of triglyceride to form fatty acid and glycerol. From this point, the fatty acids can be either bound by albumin, or can be taken up by the adipocyte directly. A summary of this is shown in Figure 1.

The majority of the fatty acid that is released into serum from the hydrolysis of triglyceride rich lipoproteins by LPL or from the hydrolysis of triglyceride stores within the adipocyte by the action of HSL, will be bound by albumin. Albumin has 6 binding sites for fatty acids, three with high affinity and three with low affinity (20). It binds greater than 99.98% of the fatty acid in blood, and transports them throughout the body (21). This is necessary as high concentrations of free fatty acid, either intracellularly or extracellularly are toxic. Therefore, although the available free fatty acid within the adipocyte to incorporate into triglyceride is small, however this pool is constantly replenished.

Fatty acid uptake into the adipocyte is rapid and may be mediated through a 40 Kd plasma membrane fatty acid binding protein (22). The evidence to date suggests that albumin is not involved in this process. There is no albumin specific receptor on the plasma membrane to which it can bind and thus deliver fatty acids to the cell. Instead, it is the small extracellular pool of free fatty acid that binds to the membrane protein and enters the adipocyte.

FIGURE 1: FATTY ACID METABOLISM IN THE ADIPOCYTE



FABP = FATTY ACID BINDING PROTEIN; FFA = FREE FATTY ACID
ICS = INTRACELLULAR SPACE; ALB = SERUM ALBUMIN
TCA = TRICARBOXYLIC ACID CYCLE

Once in the adipocyte, the free fatty acids can be bound by cytosolic fatty acid binding protein (FABP), which belongs to a family of lipid binding proteins whose functions are not yet completely understood (23). These binding proteins prevent the accumulation of toxic levels of free fatty acids, as well as transporting them to the appropriate site for their metabolism.

Fatty acids are esterified to a glycerol-3-phosphate backbone, the other precursor of triglyceride synthesis. Its production within the cell is stimulated by insulin and glucose (19). Both glucose and insulin increase post-prandially and therefore will result in increased G3P production. The triglyceride levels in blood, and therefore the fatty acid levels will also increase post-prandially. The result of this is that both substrates for triglyceride synthesis will be increased and will therefore drive triglyceride synthesis.

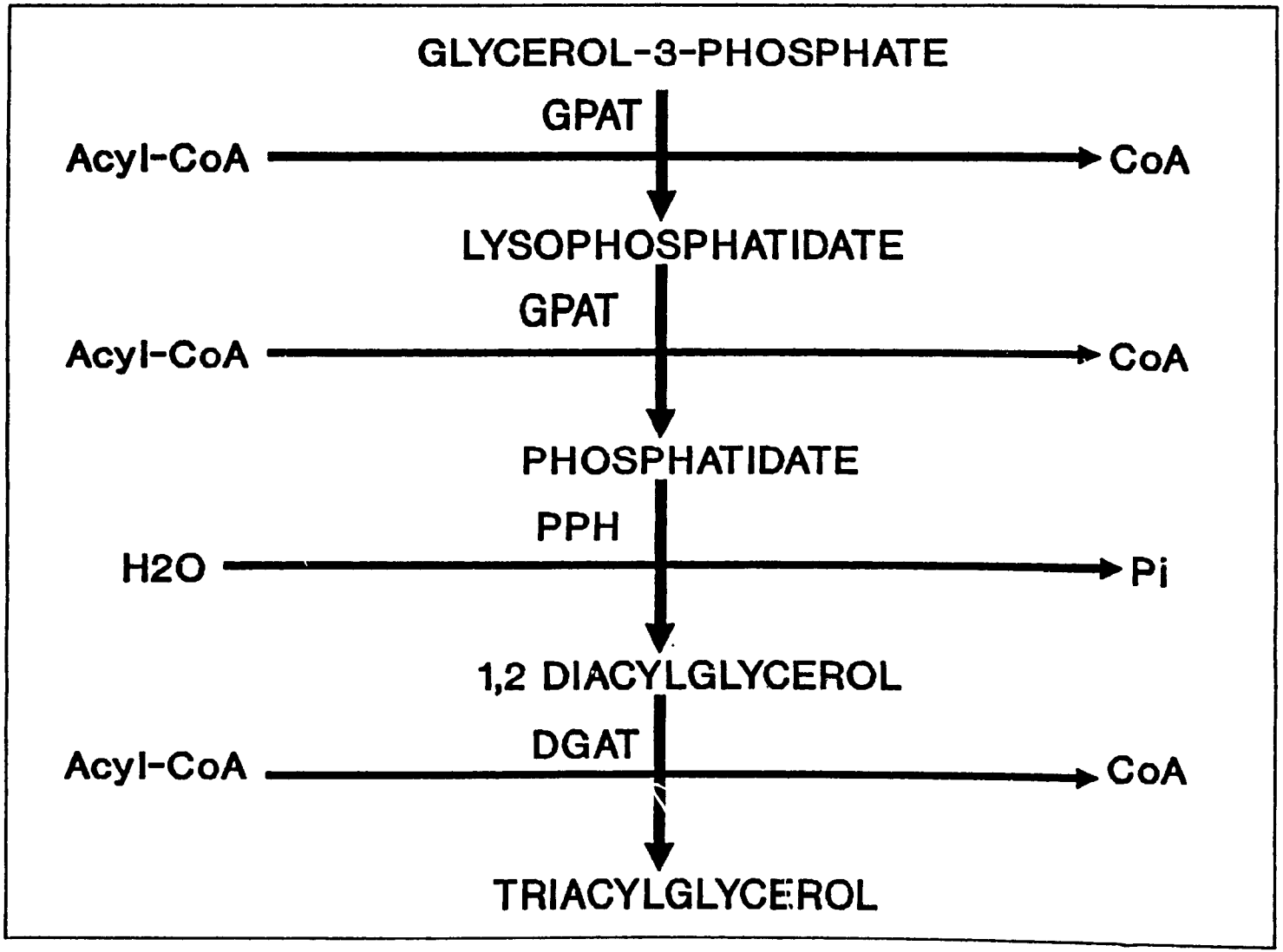
The process of triglyceride synthesis is initiated once the fatty acid enters the cell and is esterified through a thiol ester to Coenzyme A (CoA) by the enzyme Fatty Acyl-CoA Synthetase (FACS) to form acyl-CoA (13). This reaction requires energy in the form of Adenosine Triphosphate (ATP) as well as Mg^{++} ion. This activated form of a fatty acid is the primary substrate of the enzymes involved in triglyceride synthesis. It is esterified to glycerol-3-phosphate to form lysophosphatidate by the enzyme Glycerol Phosphate

Acyltransferase (GPAT). A second acyl-CoA is then esterified to the lysophosphatidate by GPAT to form phosphatidate. The phosphatidate is then dephosphorylated to form diglyceride (DG) and inorganic phosphate (Pi) by the enzyme Phosphatidate Phosphohydrolase (PPH). Finally, a third acyl-CoA molecule is esterified to the DG to form triglyceride (TG) by the enzyme Diglyceride Acyltransferase (DGAT) (11).

The enzymes and pathway involved in triglyceride synthesis are shown in Figure 2. All the enzymes are membrane bound and are situated in the endoplasmic reticulum. The one exception is PPH, which has a membrane bound form as well as a cytosolic form, although only the membrane bound form appears to be active in triglyceride synthesis (24). The regulation of these enzymes is under hormonal control. Under conditions of lipolysis, where triglyceride is being hydrolyzed to produce fatty acids, the activities of the triglyceride synthetic enzymes are decreased. This mechanism is thought to occur through a phosphorylation mechanism, whereby the enzymes are phosphorylated by a cAMP-dependent kinase that itself is activated upon hormonal stimulation of the adenylate cyclase complex (19).

Insulin is the best known stimulant of triglyceride synthesis to date. Insulin can do so by stimulating glucose transport, inhibiting inactivation of the triglyceride synthetic enzymes induced by catecholamines, stimulation of FACS and of de novo fatty acid synthesis, as well as

FIGURE 2: TRIGLYCERIDE SYNTHESIS IN THE ADIPOCYTE



stimulation of LPL activity (10,19,25).

Morbid Obesity

It has been estimated that from 25% to 50% of the population of affluent society is overweight (26,27). Of this segment of the population, most are only moderately obese, while another smaller group are considered to be morbidly obese. Clinically, obesity is divided into three categories, Grade I, Grade II and Grade III. Grade I obesity is defined as a Body-Mass Index (BMI) between 25-29.9, Grade II obesity as being between 30-40, while Grade III or morbid obesity is diagnosed in individuals having a BMI greater than 40 (28). The BMI of an individual is calculated by dividing their weight in kilograms by their height in meters squared. Morbid obesity is characterized by a massive accumulation of adipose tissue, and as triglyceride accounts for 80% of the the weight of adipose tissue, it is clear that factors controlling the level of intracellular triglyceride stores may well play a role in the etiology of this disorder.

The literature available on obesity and its causes is both voluminous and contradictory. Recently however, more precise and elegant studies in the area of the physiology and biochemistry of obesity have helped to gain an understanding in the various factors that may be controlling the development of obesity (27,29,30,31).

The level of triglyceride within a cell, and therefore

the amount of adipose tissue within a body is determined by a balance between the synthesis and the breakdown of triglyceride. Adipose tissue constantly releases fatty acids from triglyceride. This is done to supply fatty acid to tissue that will use it in preference to glucose to produce energy. In a stressed state, such as in starvation or exercise, the fatty acids are released and will be used by tissues once the glycogen stores of these tissues are depleted. On the other hand, as food is taken in, it will be stored as triglyceride in the adipose tissue, as previously discussed. So it would appear that obesity is simply an imbalance between energy expenditure and energy storage. If one takes in more energy than one uses, it will be stored. If, on the other hand, one uses more energy than one is taking in, then any energy reserves will have to be used in order to compensate for this deficit.

Recent evidence in two different populations, one of adults and one of infants, has shown that individuals from each group who became overweight also had a reduced energy expenditure (29). These findings point to a catabolic defect in lipid metabolism whereby the obese individuals are more efficient in their energy expenditure, and therefore will have more energy to store as compared to lean individuals. There is also considerable evidence to show that the adipocytes of individuals who have lost weight are metabolically quite different from the adipocytes of individuals who have always

been lean, as well as the adipocytes of individuals who are obese (27,30,31). The adipose tissue LPL activity in response to insulin and as well as to a meal was increased in obese individuals who had lost weight as compared to the same obese individuals before their weight loss. Also, the fasting adipose tissue LPL level was 3.5 times higher in obese individuals as compared to lean individuals. The increased LPL activity in response to a fed state, as well as the higher fasting levels of LPL suggest that in obese individuals as well as in obese individuals who have lost weight, the mechanisms by which triglycerides are stored are more active. In another study on obese individuals who had lost weight and maintained the weight loss, the intracellular ratio of fatty acid to glycerol was measured in adipocytes, and it was found to be significantly elevated above the ratio present in both normal weight and obese individuals.This finding indicates that the adipocyte is behaving as if it were starving, which it clearly was not. This condition could in turn result in an increasing efficency of energy utilization as well as a signal to take in more energy, or in other words, to eat more.

In another series of experiments, hybrid clones of human preadipocytes and murine renal adrenalcarcinoma cells were made (32). The hybrids composed of preadipocytes from obese individuals showed more frequent and prominent differentiation into adipocyte like hybrids than did the hybrids formed from adipocytes isolated from lean individuals. This suggests that

adipocytes isolated from lean individuals. This suggests that there may be some factor that enables preadipocytes from obese individuals to more readily become adipocytes. This is supported by other findings that adipocyte hypertrophy, an increase in cell size, and hyperplasia, an increase in cell number, may be major contributors to the development of obesity (31,33).

Obese individuals have also been found to be hyperinsulinemic, and as was discussed previously, insulin has been shown to stimulate triglyceride synthesis in a number of ways. In other cases of obesity, hypothyroidism leading to a decreased metabolic rate is responsible (34). In summary, there is evidence for the development of obesity being due to either an anabolic or catabolic defect. It is likely that in certain cases it may be due to one or the other, and in some cases, perhaps both. These defects may be genetic in origin, or may reflect a change in the state of the adipocyte after obesity.

Acylation Stimulating Protein

In 1988 and 1989, Cianflone and coworkers reported the purification and isolation of a novel protein present in human serum that was small, having a molecular weight of 14000 da, and basic, having an isoelectric point of 9.0 (46,47). This protein, named ASP, an acronym for Acylation Stimulating Protein, was found to markedly stimulate triglyceride syn-

thesis in cultured human skin fibroblasts. However, in vivo, the fibroblast is not the cell of importance in terms of triglyceride synthesis; the adipocyte is. It is obvious that the adipocyte must be implicated in some way in the development of morbid obesity. Therefore, a protein that is known to stimulate triglyceride synthesis is clearly of interest in terms of this action on adipocytes.

The major focus of the work reported in this thesis will be studies on triglyceride synthesis in morbidly obese individuals, with particular reference to the role of the ASP in this process. This protein may be playing a role in some cases of morbid obesity through its stimulatory effect on triglyceride synthesis. This is a novel explanation of morbid obesity in that it suggests a defect in the fundamental synthetic metabolic pathway of the adipocyte.

Materials and Methods

Isolation of ASP

Frozen, outdated plasma was obtained from the Canadian Red Cross, and was thawed overnight and then centrifuged at 600 x g for 15 minutes to remove any precipitated fibrin. A volume of this plasma (14 ml/ml of gel) was then loaded onto a Fast-Flow-S-Sepharose column (Pharmacia). This column is referred to as POFFS for Plasma On Fast-Flow-S-Sepharose. Once loaded, the column was washed with 7 column volumes of Buffer A (0.02 M Na_2HPO_4, 0.02 % sodium azide, pH 7.1) and the first peak, P0 was eluted. The second peak, P1, was then eluted in 7 column volumes of Buffer B (Buffer A + 3 M NaCl). The elution profile of each column was followed by measuring the aborbance at 280 nm of each fraction. The P1 peak contained the triglyceride synthetic stimulatory activity.

The fractions making up peak P1 were concentrated in 50% polyethylene glycol solution in Buffer A to a final volume of 3%-5% of a G-75 Sephadex gel filtration column (1.7 ml/ml of starting plasma). The column was eluted with Buffer A and two peaks were obtained. The second, lower molecular weight peak contained the triglyceride synthetic stimulatory activity. The fractions of this peak, SII, were pooled and then dialyzed against Buffer A to remove any NaCl from the first column that would have been present. The SII peak was retarded in its movement through the column, and eluted at a Ve/Vo of 3.4,

which is after the exclusion volume of the column as determined with molecular weight standards.

This fraction was then loaded onto a second POFFS column (20 ml/ ml of gel) and washed with 6 column volumes of Buffer A. The bound peak was then eluted in 6 column volumes of Buffer B. The fractions of this peak, PO1, were then combined and concentrated against 50% polyethylene glycol in Buffer A. Once concentrated to a volume of 8-10 mls, the peak was dialyzed overnight against Buffer A with no sodium azide and centrifuged for 10 minutes at 600 x g. Again, this final fraction contained the triglyceride stimulatory activity and is referred to as ASP (Acylation Stimulating Protein).

Purity and Quantitation of ASP

Protein in each peak eluted from the columns was quantitated using the Bradford method with Bovine Serum Albumin (BSA) as the standard (35). The purity of the ASP was checked on both 12% and 17% Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis run under reducing conditions using the method of Laemmli (36).

Adipocyte Isolation Procedure

Subcutaneous abdominal adipose tissue was obtained from both morbidly obese and normal weight subjects at the time of surgery. Individuals with a body-mass index (BMI) greater than 40 Kg/m^2 are considered to be morbidly obese, while normal

weight subjects have a BMI of less than 25 Kg/m2 (28).

The adipose tissue was rinsed twice in Krebs-bicarbonate buffer, and blood and connective tissue removed. The tissue was then finely minced with a scalpel and incubated for 45 minutes at 37°C in a volume 5X the tissue weight of Krebs-bicarbonate buffer, containing 2% fatty-acid free bovine serum albumin (BSA), 2mM glucose and 2 mg/ml of collagenase. Adipocytes were then isolated by filtration through a fine plastic mesh, followed by centrifugation at 600 x g for 2 minutes. The cells were then washed twice in the Krebs-bicarbonate glucose buffer to remove any collagenase and BSA, and resuspended in a volume of buffer that contained no collagenase nor BSA and that was 3X the volume of the packed cells (20,37).

Time Incubation Procedure

To measure acylglycerol synthesis, 50 ul suspension of adipocytes were incubated at 37oC in a final volume of 1 ml of Krebs-Bicarbonate buffer containing 2 mM glucose, 10 uM ^{3}H-oleate (20 Ci/mole), 1 nM 2-Phenylisopropyl Adenosine and 450 nM Insulin. The cells were incubated with and without ASP which was present at a concentration of 30 ug/ml. Incubation times were from 15 minutes up to a maximum of 3 hours. Incubations were stopped by the addition of 3 ml of 1:1 isopropanol:heptane.

An aliquot of the incubation buffer was counted in a

scintillation counter in order to calculate the specific radioactivity of the oleate marker. This value was them used to calculate the pmoles of oleate uptake into the acylglycerol fractions after extraction. As well, a 50 ul aliquot of cell suspension was added to 100 ul of 1 N NaOH and 850 ul of water, vortexed, and then spun for 5 minutes at 600 x g. A 500 ul aliquot was then analyzed for protein using the Bradford assay (36).

Incubation Procedures

The incubation protocol used in the experiments comparing insulin effects to ASP and ASP plus insulin was essentially the same method as used in the time experiments. The only difference is that all these experiments were carried out with a 2 hour incubation period. In these experiments, the insulin concentration was either varied (the insulin curve), or was 450 nM.

Extraction and Quantitation of Lipids

Following the incubation, the lipids were extracted from the cell by adding 3 mls of 1:1 isopropanol:heptane followed by vortexing and centrifugation to separate the aqueous and organic phases (38,39). The upper organic phase was then removed and placed in a new test tube where it was washed with 1 ml of 0.05% KOH and 1 ml of 4:1 isopropanol:heptane in order to remove any contaminating fatty acid from the media. The

tube was then vortexed and centrifuged to separate the two phases. The lower aqueous phase was removed and the organic phase was washed two more times using the same procedure. Finally, the organic phase was removed and dried under a stream of nitrogen. The sample was then resuspended in 50 ul of chloroform and 10 ul of this was applied to a thin-layer chromatography (TLC) plate to separate the lipids.

All TLC was carried out at 25°C using 20cm. x 20cm. silica gel-G plates. To each lane, either 10 ul of the 50 ul chloroform lipid mixture or standards were applied. The plate was run in a saturated atmosphere of 75:25:1 parts (by volume) hexane:ether:glacial acetic acid (39). The lipids were then visualized in iodine vapor and the appropriate area scraped. Ten ml of Scintiverse I scintillation cocktail (Fisher Scientific Co.) was added to the to the scraped peaks and they were then counted in a scintillation counter (Beckman Instruments). The disintegrations per minute were then used to calculate the incorporation of ^{3}H- or ^{14}C-oleate into the appropriate lipid. All plates were run with the following standards, cholesteryl-oleate, triolein, 1,2-dipalmitoyl-sn-glycerol, oleic acid and phosphatidic acid.

Subcellular Fractionation

Human adipose tissue was obtained at the time of gastroplasty from morbidly obese females and rat liver tissue was obtained after decapitation from 350 g male Sprague-Dawley

rats. The tissue was kept on ice at all times in Krebs-bicarbonate buffer, pH 7.4. Connective tissue and blood were then removed. All subsequent manipulations were carried out at 4°C using a standard subcellular fractionation method (40).

Tissue was homogenized using a glass homogenizer in a buffer (5 ml/ g of tissue) containing 250 mM sucrose, 10 mM Tris-HCL (Tris(hydroxy-methyl)amino methane Hydro Chloride), pH 7.4, 1 mM Na_2EDTA and 1 mM dithiothreitol (Buffer A). The homogenate was then collected in 50 ml conical, polyethylene centrifuge tubes and spun at 600 x g, resulting in a lipid rich cake that floats up above the supernatant, an aqueous supernatant and a pellet containing cellular debris. The supernatant is then filtered through glass wool and transferred to 10 ml centrifuge tubes and spun at 16,000 x g for 15 minutes.

In adipose tissue , the 16,000 x g pellet was resuspended in 10 ml of Buffer B (Buffer A without 1 mM Na_2EDTA), and centrifuged at 16,000 x g a second time. This pellet was then resuspended in Buffer B and is the crude mitochondrial fraction. In liver, the first 16000 x g pellet was resuspended in 10 ml of Buffer B and 3 ml of it was loaded on top of 6 mls of 1.45 M sucrose, and then spun at 59,000 x g for 45 minutes in a swinging bucket centrifuge. The pellet was resuspended in Buffer B and is the mitochondrial fraction.

In both tissue types, the 16000 x g supernatant was centrifuged at 106,000 x g for 70 minutes. The 106,000 x g

supernatant is the cytosolic fraction. The pellet was resuspended in 8 mls of Buffer B and centrifuged a second time at 106,000 x g for 70 minutes. This pellet was then resuspended once again in Buffer B and is the microsomal fraction.

Quantitation of Subcellular Fractions

All subcellular fractions were assayed for protein content using the Bradford assay, and were stored at -90°C and used within one week.

Cross contamination of the fractions with other subcellular fractions was determined by assaying each fraction for enzyme specific activity. Plasma membrane content was determined by assaying for 5'Nucleotidase activity, microsomal content was determined by assaying for Glucose-3-phosphatase activity and mitochondrial content was determined by assaying for Monoamine oxidase activity (41,42).

5'- Nucleotidase Assay

5'- Nucleotidase is an enzyme found on the plasma membrane that catalyzes the hydrolysis of adenosine-5'-monophosphate (AMP) to adenosine and inorganic phosphate (Pi).

Subcellular fractions were incubated at 30°C for 20 minutes at 5 different protein concentrations ranging from 0.5 ug/ml to 200 ug/ml of subcellular protein. The fractions were in a final assay volume of 1 ml containing 10 mM Tris-acetate,

10 mM magnesium acetate and 10 mM AMP. The incubations were stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA), and placed in an ice bath for 15 minutes. This solution was then centrifuged at 3000 rpm for 10 minutes, and 1 ml of the resulting supernatant was assayed for inorganic phosphate content as described below.

Glucose-6-phosphatase Assay

Glucose-6-phosphatase is an enzyme found only on the endoplasmic reticulum, which is in the microsomal portion of the subcellular fractions. It catalyzes the hydrolysis of glucose-3-phosphate to glucose and inorganic phosphate.

Subcellular fractions were incubated at 30°C for 20 minutes at 5 different protein concentrations ranging from 0.5 ug/ml to 200 ug/ml of subcellular protein. The incubations were carried out in a volume of 1 ml containing 25 mM MES (2-(N-morpholino)ethane sulfonic acid), pH 6.6 and 10 mM glucose-6-phosphate. The incubations were stopped by the addition of 1 ml of 10% TCA and were then placed on an ice bath for 15 minutes. The solution was then centrifuged at 3000 rpm for 10 minutes and the supernatant analyzed for inorganic phosphate content as described below.

Monoamine Oxidase Assay

Monoamine oxidase is a mitochondrial specific enzyme that catalyzes the conversion of the artificial substrate

kynuramine to a transient aldehyde which then undergoes non-enzymatic ring closure to become 4-hydroxyquinoline. The kynuramine absorbs at 360 nm, and its absorbance will decrease as it is converted to 4-hydroxyquinoline. The decrease in absorbance was measured at a single protein concentration for each subcellular fraction, ranging from 100 ug/ml to 500 ug/ml of protein.

The assay was carried out at 25°C in a final volume of 3 ml containing 50mM Na_2HPO_4, pH 7.4, 0.2% Triton X-100 and 33 ug/ml kynuramine. Readings at 360 nm were taken every 20 seconds over a 20 minute time period, after the addition of substrate. Specific activity is calculated as picomoles of kynuramine converted to 4-hydroxyquinoline/ 10 minutes/ ug of cell fraction. Total activity in the fraction was determined by multiplying the specific activity by the total protein in each fraction.

Determination of Inorganic Phosphate

Inorganic phosphate was quantitated by measuring spectrophotometrically at 660nm the absorbance of an inorganic phosphate - molybdate complex. A 1 ml volume of the 3000g supernatant was made up to 4 mls with a final concentration of 10 mg/ml ascorbic acid, 2.5 mg/ml ammonium molybdate and 0.6 N H_2SO_4. The incubations were carried out at 45°C for 20 minutes. The tubes were then transferred to an ice bath for 20 minutes, and then read at 660nm on a spectrophotometer. A

standard curve was plotted using known concentrations of KH_2PO_4 (43).

Mitochondrial Activity Assay

Beta-oxidation in mitochondria was measured by monitoring the generation of acetyl-CoA from palmitoyl-CoA oxidation in the presence of increasing concentrations of ASP in 20 mM Na_2HPO_4 buffer (44). Mitochondria, 25 ug, were incubated in 1.5 ml eppendorf tubes (Fisher Scientific Co.) at 37°C for 11 minutes in a volume of 600 ul of buffer containing increasing concentrations of ASP along with 10 uM ^{14}Palmitoyl-Coenzyme A, 100 mM Coenzyme A, 200 uM Nicotinamide Adenine Dinucleotide, 10 uM Flavin Adenine Dinucleotide, 100 uM Dithiothreitol, 1 mM Carnitine, 75 ug/ml Bovine Serum Albumin, 250 mM sucrose and 50 mM Tris-HCL, pH 8.0. The final volume of phosphate buffer with or without ASP was the same in each tube. The incubation was stopped by the addition of 500 ul of ice cold 6% perchloric acid. This mixture was then centrifuged for 10 minutes at 13000 rpm in a bench top micro centrifuge. A volume of 500 ul of the supernatant which contains the acid soluble ^{14}C-Acetyl-CoA produced by the beta-oxidation of the ^{14}C-Palmitoyl-CoA was then added to 10 ml of Scintiverse 1 scintillation cocktail (Fisher Scientific Co.) and counted in a scintillation counter (Beckman Instruments).

Rat liver mitochondria were used in each experiment to insure that the assay was working. As well, a concentration

curve of adipose mitochondria was run in order to determine the optimal concentration of mitochondria to use.

Microsomal Lipid Synthesis Activity Assay

Radioactive oleate incorporation into triglyceride in human adipose microsomes was determined using the following method (45). Microsomes, 12.5 ug, were incubated at 37°C for 30 minutes in a buffer containing 2.5 mM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 500 mM Coenzyme A, 100 mM Tris-HCL, pH 7.4, and 20 uM ^3H- or ^{14}C-oleate. The incubation was carried out in a volume of 500 ul, with increasing concentrations of ASP in 20 mM Na_2HPO_4. The total volume of phosphate buffer with or without ASP added to each tube was constant.

The incubation was stopped, and the triglycerides separated from the other lipids using the same method as that used in the adipocyte experiments. Quantitation of oleate incorporation into triglyceride was determined in the same way as for the adipocytes.

Blood Collection

Blood was obtained from female, human subjects after a 12 hour overnight fast. A volume of 7 ml was collected into vacutainer tubes containing an anticoagulant, Na_2EDTA, at a final concentration of 1 mg/ml. The blood was then spun at 600 x g for 15 minutes and the plasma was removed. This plasma was then immediately assayed or frozen and stored at -90°C.

Enzyme Linked Immunosorbent Assay (ELISA)

Plasma ASP levels were determined using an indirect competitive ELISA method with an anti-human rabbit polyclonal antibody against ASP (personal communication, Dr.K.Cianflone and Mrs.H.Vu) (53). A standard curve of anti-ASP and purified ASP was used and the plasma ASP levels were determined from this.

Results

Modification of the Isolation of ASP

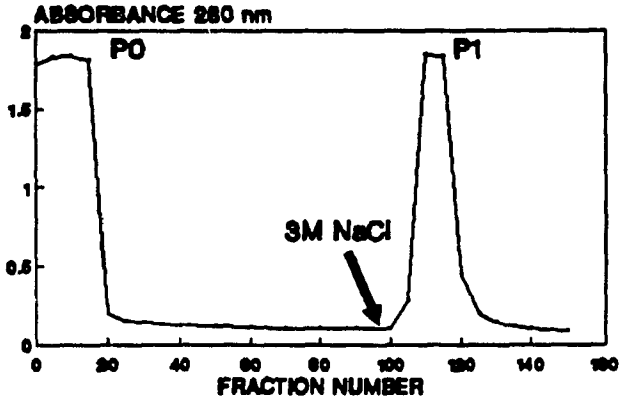
ASP was initially isolated from human plasma by a three step chromatographic procedure using the affinity matrix Affigel Blue (Biorad Co.), G-75 Sephadex and Carboxymethyl Sepharose (Pharmacia Co.) (46,47). This method was modified in order to achieve a higher yield of ASP, as well as to develop a simpler and more rapid isolation procedure.

Figure 3a shows the results of the binding and elution of human plasma from the first column used in the modified isolation method, a Fast-Flow-S-Sepharose column. This column is referred to as POFFS, an acronym for Plasma On Fast-Flow-S-Sepharose. This matrix was chosen for the first step of the purification because it is a strong cation exchanger, and ASP is a basic protein with a pI of 9.0 and will therefore have an overall positive charge at pH 7.0 and can thus bind to the negatively charged POFFS column (48). The absorbance of the fractions at 280nm is plotted against the fraction number. The first peak on the column, P0, consists of the unbound plasma proteins that elute with the loading buffer, Buffer A, which is 20 mM Na_2HPO_4, pH 7.1. The second peak, P1, is eluted with Buffer B, which is 3 M NaCl, 20 mM Na_2HPO_4, pH 7.1.

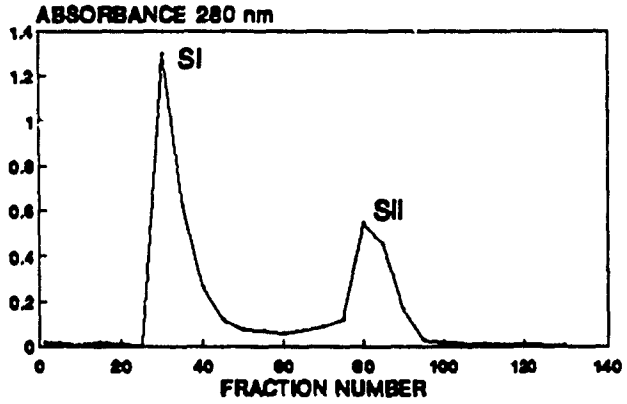
Peak P1 was then concentrated against a 50% polyethylene glycol solution of Buffer A, and loaded onto a G-75 Sephadex gel filtration column that had been equilibrated with Buffer

FIGURE 3: ELUTION PROFILE

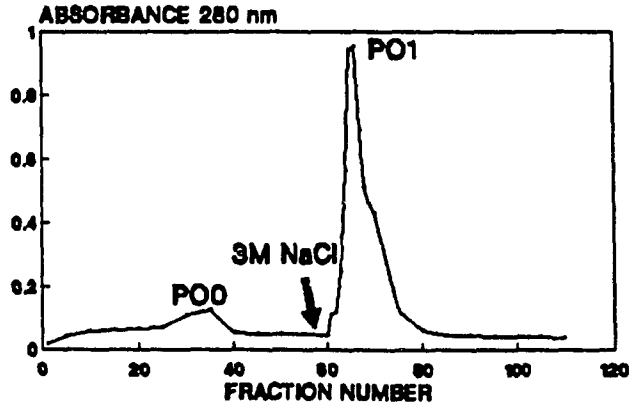
3a: PLASMA ON FAST-FLOW S-SEPHAROSE
pH 7.1, ELUTION WITH 3M NaCl



3b: PEAK P1 ON G-75 SEPHADEX,
0.02 M PHOSPHATE, pH 7.1



3c: SII ON FAST-FLOW SEPHAROSE
pH 7.1, ELUTION WITH 3M NaCl



A. Figure 3b is a representative column profile. The absorbance of each fraction at 280nm is plotted against fraction number. Two peaks elute on this column, SI and SII. Peak SII was collected and dialyzed against Buffer A to remove any salt from the first column which would elute at the exclusion volume.

Each of these protein fractions was assayed for triglyceride stimulatory activity on human adipocytes.The activities of these peaks in terms of stimulation above baseline of oleate incorporation into triglyceride over 2 hours in the presence of 15 ug/ml of protein in adipocytes from 2 morbidly obese individuals are shown in Figure 4 and Table 3a. Peaks P0 and P1 stimulate triglyceride synthesis 40% and 77% respectively. The 40% stimulation seen with P0 is likely due to the effect of Insulin (pI 6.0) and Albumin (pI 5.85) that are both contained within this peak and that have been shown to stimulate triglyceride synthesis in human adipocytes (37,49). Peak P1 shows an almost 2 fold stimulation of triglyceride synthesis as compared to P0. This peak contains neither insulin nor albumin as determined by antibodies to these proteins. When the P1 peak was further fractionated on the G-75 Sephadex gel filtration column, two peaks were obtained. The first peak, S1, shows almost no stimulation above baseline, while the second peak, S11, shows a 98% increase in triglyceride synthesis.

Although the S11 fraction contains the triglyceride

TABLE 3a: STIMULATION OF OLEATE INCORPORATION INTO TRIGLYCERIDE IN HUMAN ADIPOCYTES

PROTEIN FRACTIONS	pMOLES FFA INC/ ug CELL PROTEIN	% STIMULATION OVER BASELINE	+/- SEM % STIMULATION
NONE	3.8	0	
P0	6.6	40	10
P1	6.2	77	2
S1	4.0	6	2
S11	7.7	98	6

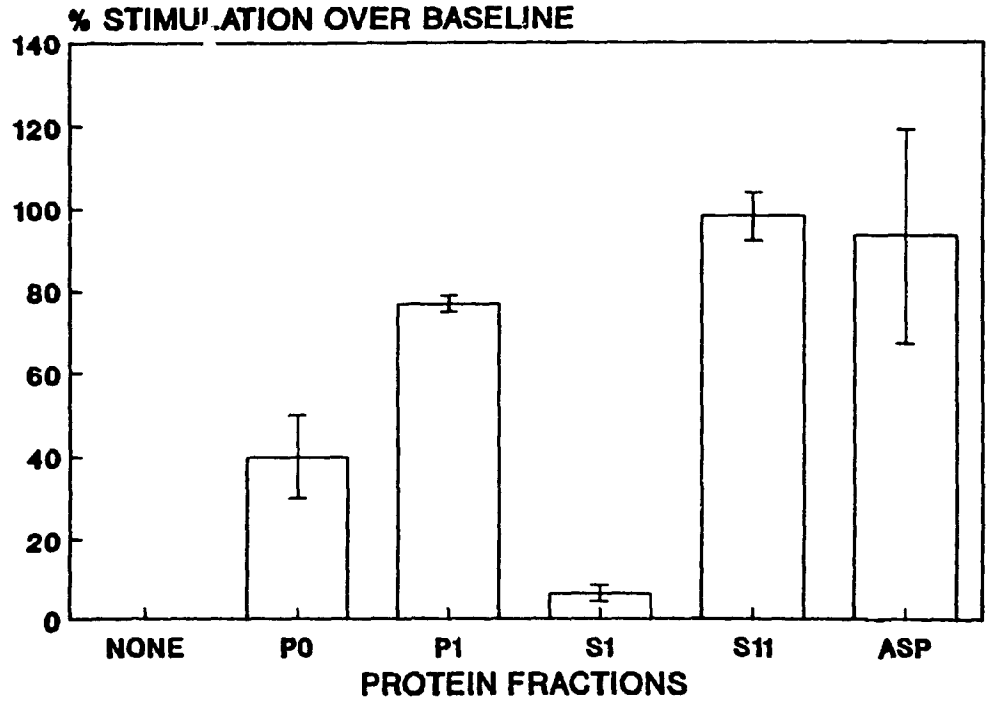
Adipocytes (n=2) were incubated for 2 hours at 37°C in 2 mM glucose, 10 uM 3H-oleate, 450 nM insulin, 1 nM PIA and 15 ug/ml of the appropriate protein fractions.

TABLE 3b: STIMULATION OF OLEATE INCORPORATION INTO TRIGLYCERIDE IN HUMAN ADIPOCYTES

PROTEIN FRACTIONS	pMOLES FFA INC/ ug CELL PROTEIN	% STIMULATION OVER BASELINE	+/- SEM % STIMULATION
NONE	7.8	0	
ASP (P01)	16.2	93	26

Adipocytes (n=2) were incubated for 2 hours at 37°C in 2 mM glucose, 10 uM 3H-oleate, 450 nM insulin, 1 nM PIA and 25 ug/ml of ASP.

FIGURE 4: PROTEIN FRACTIONS' EFFECTS ON TRIGLYCERIDE SYNTHESIS IN THE HUMAN ADIPOCYTE



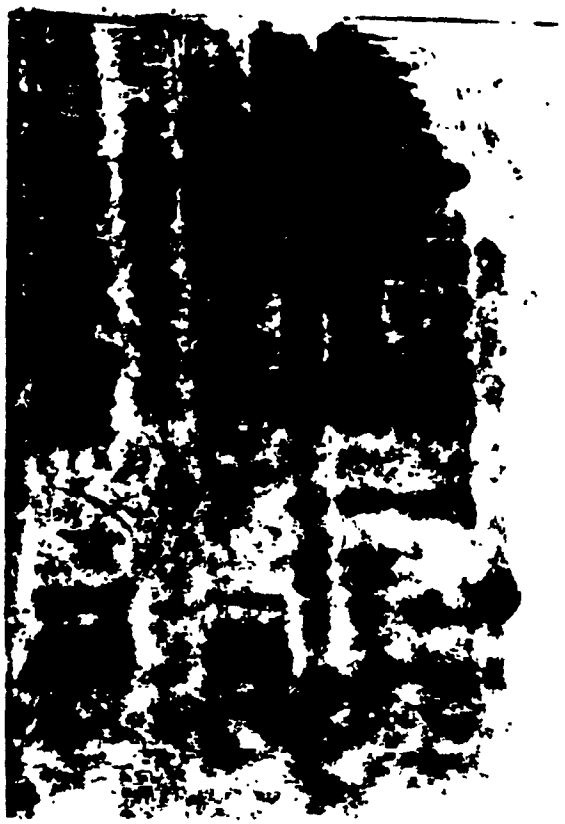
Adipocytes (n=2) were incubated for 2 hours at 37 C in a Krebs-bicarbonate buffer, pH 7.4, containing 2 mM glucose, 10 uM 3H-oleate, 450 nM insulin, 1 nM PIA and 15 ug/ml of P0, P1, S1, S11 and 25 ug/ml of ASP. Results are given in % stimulation of oleate incorporation into triglyceride over baseline (adipocytes alone).

stimulatory activity, the peak is not purified ASP. Figure 5 is a picture of a 12% polyacrylamide gel of protein fractions run under reducing conditions (SDS-PAGE). In lane 1 is seen 27 ug of S11 run on this gel. There is a lower molecular weight band at an Rf of 0.79 that corresponds to ASP. As well, there are a number of higher molecular weight contaminants that must be removed in order to further purify the protein. In lane 2 is seen 12 ug of S1. This demonstrates that the G-75 Sephadex gel filtration column effectively removes a number of contaminants from the ASP band without removing the ASP band itself. Lane 3, which contains 25 ug of P1, shows that the first POFFS column removes all the ASP from plasma, as no ASP band is present in lane 4, which contains PO, which is the wash from the first POFFS column, while there is an ASP band in lane 5, which is plasma.

In order to remove the remaining contaminants, the S11 was loaded onto a second POFFS column equilibrated with Buffer A. This column was used a second time since it was so effective at binding ASP and removing it from the majority of the other plasma proteins. Two peaks were again eluted from this column, POO and PO1. POO was eluted with the loading buffer, while PO1 eluted with Buffer B. This peak was then concentrated to a volume of 8-10 mLs in 50% polyethylene glycol in Buffer A, and then dialyzed overnight in 1 liter of Buffer A, and centrifuged for 10 minutes at 600 X g to remove any precipitated protein in the pellet, PO1 precipitate (PO1ppt).

FIGURE 5: PROTEIN ISOLATION COLUMN FRACTIONS P0, P1, SI, SII AND PLASMA ON A POLYACRYLAMIDE GEL RUN UNDER REDUCING CONDITIONS

1 2 3 4 5 6 7



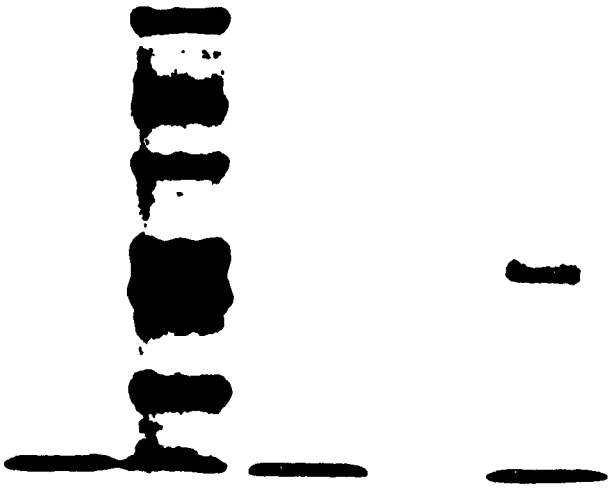
The results of a 12 % SDS polyacrylamide gel. Lane 1 contains 27 ug of S11, lane 2 contains 12 ug of S1, lane 3 contains 25 ug of P1, lane 4 contains 100 ug of P0, and lane 5 contains 200 ug of plasma. Lane 6 contains 6.6 ug of S7 standards and lane 7 contains 3.3 ug of lysozyme. For molecular weight markers and gel running conditions see Figure 6. For column fractions, see Figure 3.

Figure 3c shows the profile of this column, with the absorbance of the fractions plotted against the fraction number. Figure 6 is a 12% SDS-PAGE of protein fractions run under reducing conditions. Lane 3 shows that the PO1 peak is more than 95% pure, by scanning densitometry, and has an approximate molecular weight of 12500 da, as determined by reference against the molecular weight standards Bovine Serum Albumin (66000 da), Egg Albumin (45000 da), Glycerol-3-Phosphate Dehydrogenase (36000), Carbonic Anhydrase (29000), Trypsinogen (24000), Trypsin Inhibitor (20100) and Alpha-Lactalbumin (14200 da) (Sigma Co.). The purified ASP (PO1) was then assayed for activity on human adipocytes from 2 morbidly obese individuals. Table 3b contains the results of incubation of these cells for 2 hours in the presence of 25 ug/mL of ASP. The effect of ASP on the stimulation of oleate incorporation into triglyceride per ug of cell protein is approximately 2 fold over baseline. POO and PO1ppt do not show any stimulatory activity. PO1ppt does have some ASP present in it along with the higher molecular weight contaminants seen in S11, however it does not stimulate triglyceride synthesis.

Table 4 contains the results of 5 isolations of ASP from human plasma with an average yield of 1.46 mg/mL of ASP for each isolation. In the previous method of isolation of ASP, it was reported that 1.6 g of serum protein in a volume of 50 ml yielded 0.5 mg of ASP (47). The procedure used in this work isolated 1.46 mg of ASP from 48 g of plasma protein in 11 of

FIGURE 6: ASP ON A POLYACRYLAMIDE GEL RUN UNDER REDUCING CONDITIONS

1 2 3 4 5



The results of a 6 X 10 cm. 12 % SDS polyacrylamide gel are shown. The gel was run for 45 minutes at 100 V and 30 mA. Lane 1 contains 3.3 ug of cytochrome C (12.5 Kd). Lane 2 contains 6.6 ug of S7, a mixture of bovine serum albumin (66 Kd), egg albumin (45 Kd), G3P dehydrogenase (36 Kd), carbonicanhydrase (29 Kd), trypsinogen (24 Kd), trypsin inhibitor (20 Kd) and alpha lactalbumin (14.2 Kd). Lane 3 contains 1.6 ug of ASP, lane 4 is empty and lane 5 contains 3.3 ug of lysozyme (14 Kd).

For column fractions see Figure 3.

TABLE 4: AVERAGE PROTEIN PEAK YIELD IN 5 ISOLATIONS

PROTEIN FRACTION	MASS PER FRACTION	S.E.M.	% TOTAL PROTEIN
PLASMA	47.9 g	2.5	100
P0	47.8 g	2.8	99.8
P1	204 mg	54	0.42
S1	93 mg	60	0.20
S11	9.8 mg	2.2	0.02
PO0	1.25 mg	0.25	.003
PO1	1.46 mg	0.21	.003
PO1ppt	0.87 mg	0.40	.002

The results of 5 isolations of ASP(PO1) starting from 1 liter of human plasma. The yield of ASP is 0.003 % of the total starting protein. For column fractions , see Figure 3.

plasma. The second method appears to be less efficent in isolating ASP from human plasma. However, in the first method, the plasma was defibrinated by the addition of thrombin in order to make serum. This results in a decrease in the protein concentration of the starting material, as fibrinogen makes up from 5 - 6 % by weight of the plasma protein (50). So, the % yield of ASP in the first method is in fact an over estimate. The second method is more rapid, taking only 3 days as opposed to 5-6 days for the first method. It is also simpler in that it does not use a chromatofocusing step and therefore avoids contamination of ASP with polybuffer which interferes both with polyacrlyamide gel electrophoresis and amino acid analysis.

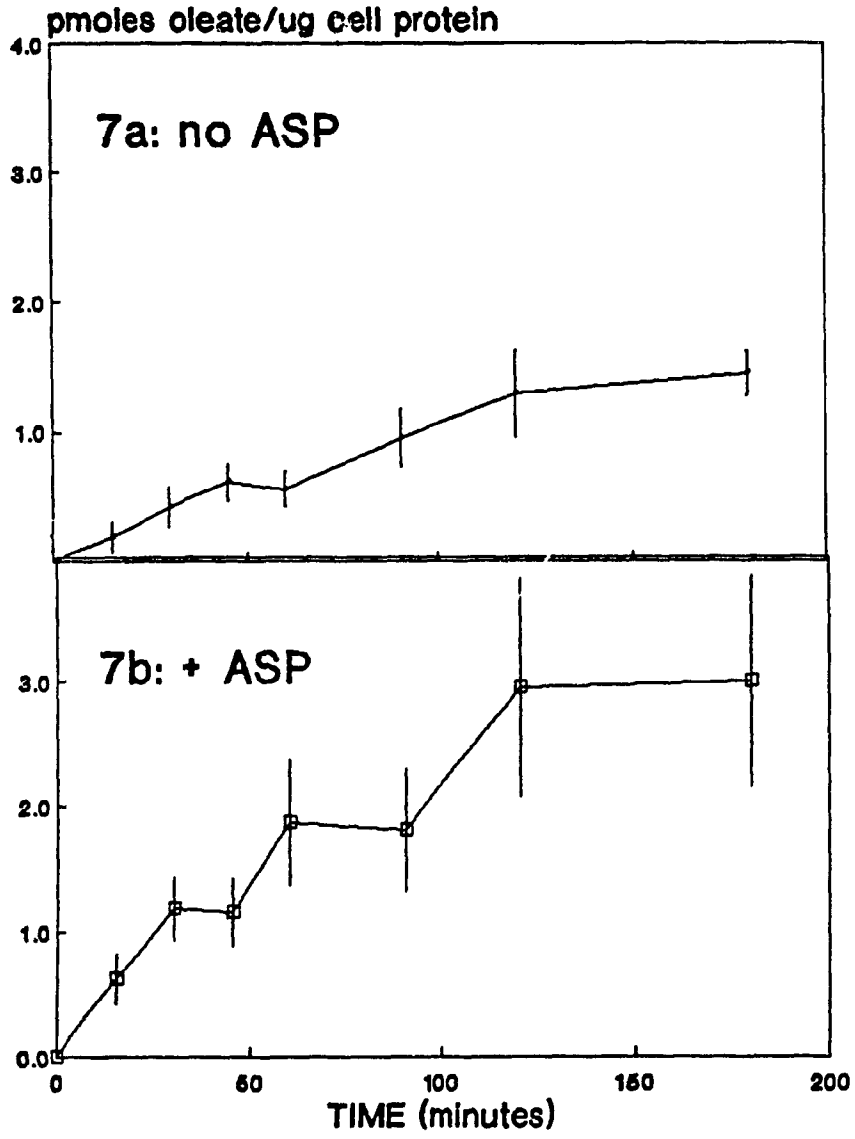
Time Course Studies

It has been previously shown that ASP stimulates triglyceride synthesis in cultured human skin fibroblasts(47). These experiments were carried out over time periods ranging from 2 to 24 hours and were done on cells that serve as a model of peripheral tissue. In vivo, however, these cells do not play a major role in triglyceride synthesis and storage. The major site of triglyceride storage in vivo is the adipocyte. The following set of experiments was done in order to determine the time course of the stimulatory effect of ASP in the major cell type involved in triglyceride synthesis, the adipocyte.

Adipose tissue was obtained from 3 morbidly obese patients, all females, age = 43 +/- 5.1 years, all of whom were undergoing gastroplasty surgery. An aliquot of 50 ul of adipocytes isolated from this tissue was incubated in the presence of ^{3}H-oleate and 30 ug/mL of ASP for time periods ranging from 0 to 3 hours. Figures 7 and 8 are plots of pmoles of ^{3}H-oleate incorporated into diglyceride per ug of cell protein with respect to time. The values are expressed an average of the 3 experiments, +/- sem. Figure 7a represents the basal activity of the cell and Figure 7b, the activity in the presence of ASP. Figure 8 is a plot of the two curves. The incorporation of oleate into diglyceride is stimulated in the presence of ASP and the effect occurs within fifteen minutes and continues for at least 3 hours. The increase in stimulation of oleate incorporation into diglyceride synthesis is significant at all time points over baseline using a paired t-test, $p<0.05$. The % stimulation over baseline ranges from 150% at 2 and 3 hours to 250% at 15 minutes. These results clearly demonstrate that ASP's stimulation of diglyceride synthesis is potent, rapid and prolonged.

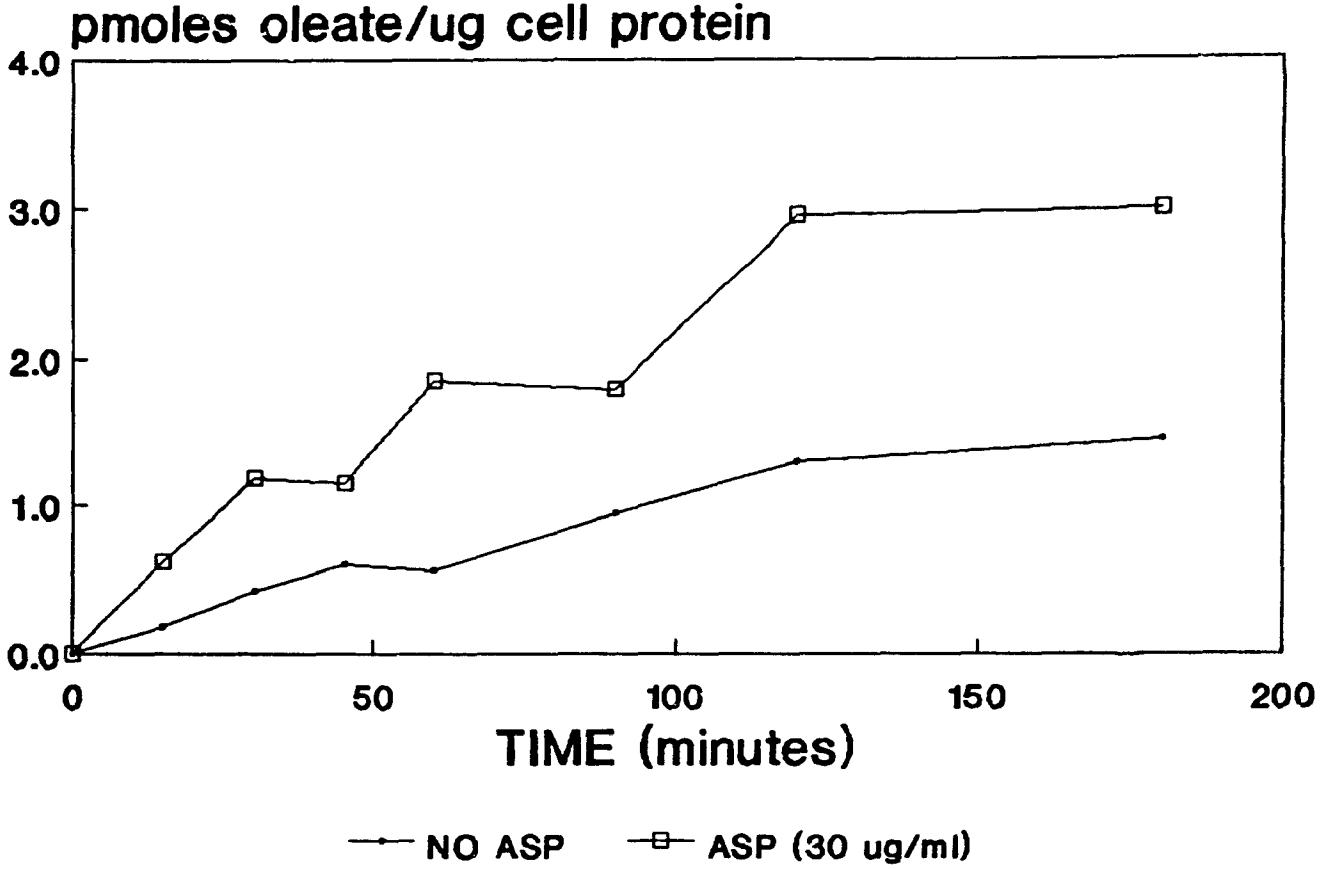
Figures 9 and 10 are results from a set of five experiments. Adipose tissue was obtained at the time of gastroplasty from five morbidly obese individuals, 1 male and 4 females, with an average age of 46 years, +/- 4.8. ^{3}H-oleate incorporation into triglyceride per ug of cell protein is plotted against the time of incubation. Figures 9a and 9b show oleate

FIGURE 7: 3H-OLEATE INCORPORATION INTO DIGLYCERIDE IN HUMAN ADIPOCYTES



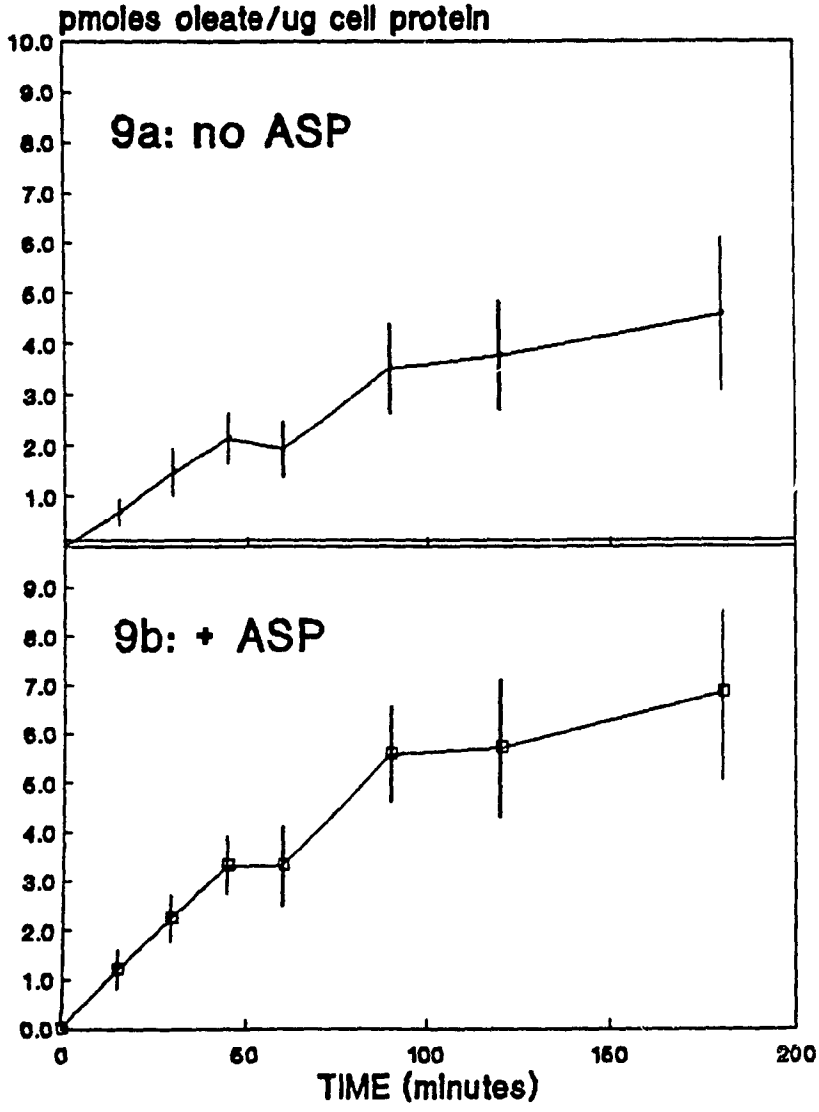
Adipocytes (n=3) were incubated at increasing time intervals at 37 C in a Krebs-bicarbonate buffer, pH 7.4. containing 2 mM glucose, 10 uM 3H-oleate, 450 nM insulin, 1 nM PIA and 30 ug/ml ASP (lower graph). Results +/- SEM are expressed as pmoles of oleate incorporated into diglyceride per ug of cell protein.

FIGURE 8: 3H-OLEATE INCORPORATION INTO DIGLYCERIDE IN HUMAN ADIPOCYTES



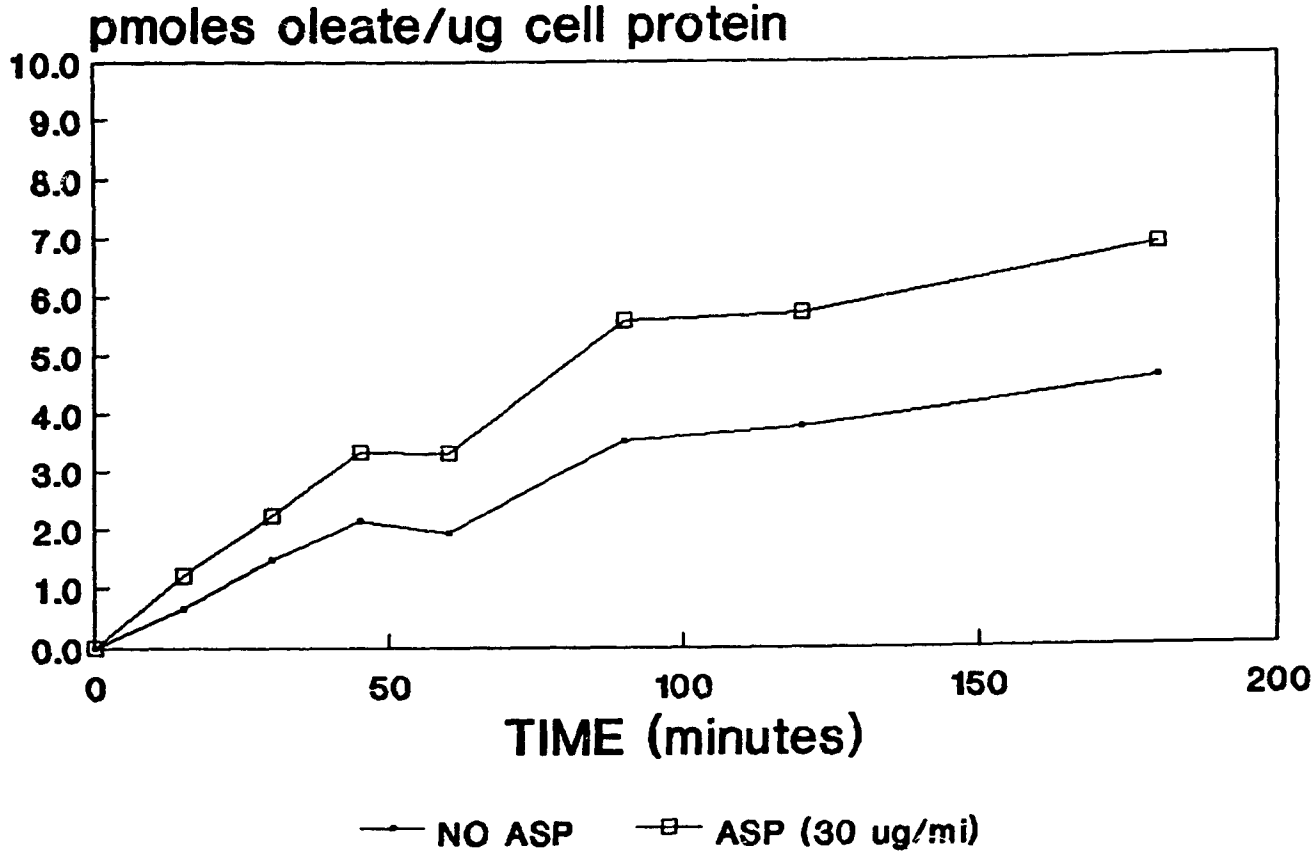
For incubation conditions see Figure 7. $p < 0.05$ for all time points.

FIGURE 9: 3H-OLEATE INCORPORATION INTO TRIGLYCERIDE IN HUMAN ADIPOCYTES



Adipocytes (n=5) were incubated under the same conditions as Figure 7. Results +/- SEM are expressed as pmoles oleate incorporated into triglyceride per ug of cell protein.

FIGURE 10: 3H-OLEATE INCORPORATION INTO TRIGLYCERIDE IN HUMAN ADIPOCYTES



For incubation conditions see Figure 9. $p < 0.05$ at all time points except for 15 minutes.

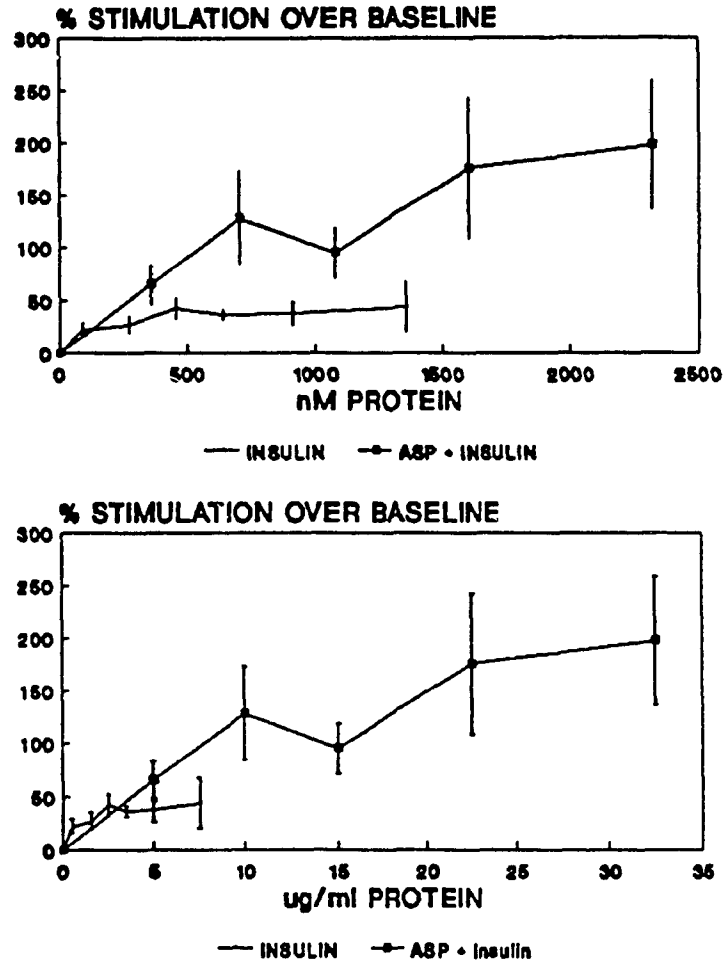
incorporation into triglyceride in the absence and presence of ASP. The values are expressed +/- the sem for the 5 experiments. Figure 10 is the combination the two previous plots. The effect of ASP on the stimulation of oleate incorporation into triglyceride is significantly different at all time points except at 15 minutes by a paired t-test, $p<0.05$. The % stimulation of triglyceride synthesis does not vary with time of incubation, remaining at a level of 50% above baseline.

These results demonstrate that ASP stimulates both the production of triglyceride as well as its precursor, diglyceride.

Comparison of the ASP and Insulin Effect on Triglyceride Synthesis

To date, insulin is the most potent stimulant of triglyceride synthesis known. The effect of insulin and insulin plus ASP was studied in adipocytes from morbidly obese individuals undergoing gastroplasty. The only difference in the two groups is that the insulin group is all female, while the ASP plus insulin group is comprised of both males and females. This however should not effect the results, as insulin has been shown to act equally in both groups. In Figure 11 are plots of % stimulation over baseline of ^{3}H-oleate incorporation into triglyceride in adipocytes against increasing concentrations of protein. The two different plots

FIGURE 11: ASP EFFECT AS COMPARED TO INSULIN



Adipocytes were incubated in the presence of increasing insulin concentrations (n=9) or increasing ASP concentrations (n=12) at a fixed insulin concentration of 450 nM. Incubations were at 37 C for 2 hours in a Krebs-bicarbonate buffer, pH 7.4, with 2mM glucose and 1nM PIA. Results +/- SEM are expressed as % stimulation of oleate incorporation into triglyceride in 50 ul of cells. In the upper graph the X axis is plotted in nM protein, while in the lower graph the X axis is in ug/ml of protein.

of protein values, the first in nanomolar concentration and the second in ug/ml is done simply to accentuate the difference in effects. The lowest insulin concentration that is used in these experiments is 90nM, which is pharmacological rather than physiological. The plasma level of insulin in humans ranges from 1 to 9 nM (6). Therefore, the lowest concentration used in these experiments is 10 fold higher than the physiological level of insulin in humans. At this and higher concentrations, the maximal stimulation achieved is 50%. However, in the presence of insulin plus ASP, the maximal stimulation reaches 200% at a concentration of ASP that is not outside the physiological range found in human plasma (53).

ASP Stimulation of Triglyceride Synthesis in Normal and Obese Adipocytes

The data described so far is based on ASP's effect on adipocytes from morbidly obese individuals, since large amounts of adipose tissue were readily available from these patients. A question of interest is whether or not there is any difference in ASP responsiveness between adipocytes obtained from morbidly obese individuals compared with adipocytes obtained from normal weight individuals.

Adipose tissue was obtained from obese individuals at the time of gastroplasty, while adipose tissue from normal weight individuals was obtained at the time of elective abdominal surgery. The differences in the size of the two groups, obese

n=12 and normal n=4 is due to the fact that sufficent abdominal adipose tissue is readily available from obese individuals, but not easily obtained from normal weight individuals.

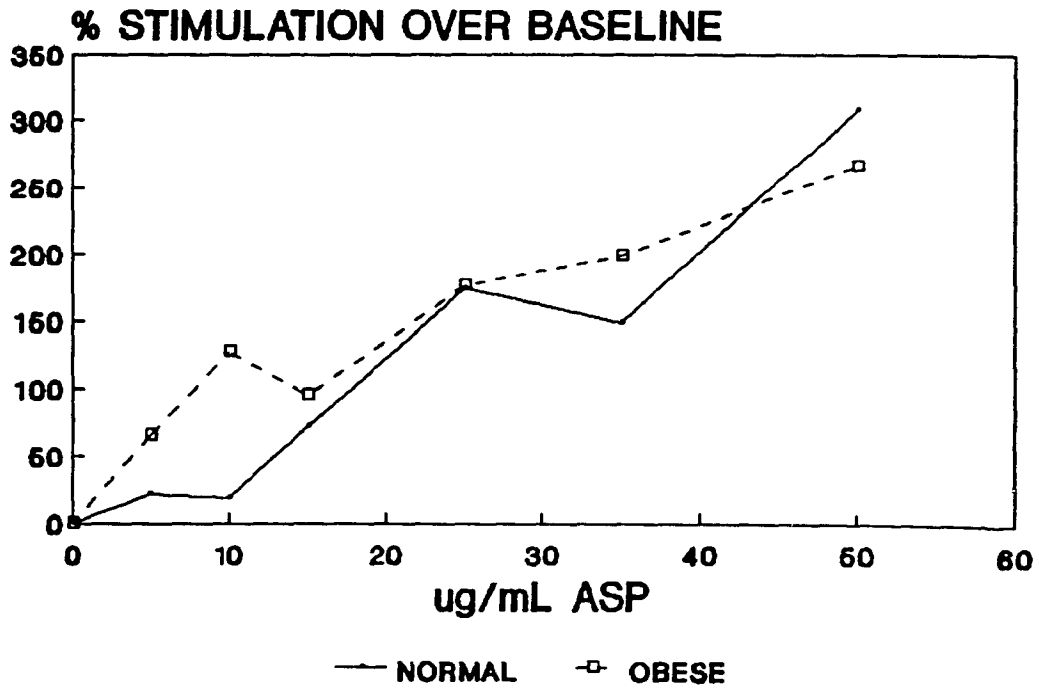
Figure 12 is a plot of the % stimulation above baseline of ^{3}H-oleate incorporation into triglyceride with increasing concentrations of ASP. A comparison of the % stimulation in the two groups by a 2 group t-test shows that there is no significant difference between them at any ASP concentration. From this graph, it is clear that the % stimulation increases with increasing concentrations of ASP and that it does not yet reach saturation at 50 ug/mL of protein in either group of subjects.

Plasma ASP Levels

The previous results were all obtained from experiments done using isolated adipocytes. The comparison of the stimulatory effect of ASP on triglyceride synthesis in normal weight and morbidly obese subjects shows that there is no significant difference between the two groups in terms of their response to ASP. The question now is, does ASP play any role at all in the etiology of morbid obesity?

In order to answer this question, ASP plasma levels were measured in two groups of females using an Enzyme Linked Immunosorbent Assay (ELISA) with a rabbit anti-human ASP polyclonal antibody. Fasting plasma was obtained from a normal

FIGURE 12: ASP STIMULATION OF 3H-OLEATE INCORPORATION INTO TRIGLYCERIDE IN NORMAL AND MORBIDLY OBESE HUMAN ADIPOCYTES



Normal adipocytes (n=4) and morbidly obese adipocytes (n=12) were incubated at 37 C for 2 hours in Krebs-bicarbonate buffer, pH 7.4, with 2 mM glucose, 10 uM 3H-oleate, 450 nM insulin, 1 nM PIA and increasing amounts of ASP. Results are expressed as % stimulation over baseline (no ASP) of oleate incorporation into triglyceride per 50 ul of cells.

weight group, n=8, BMI = 20.1 +/- 0.43 kg/m^2, age = 30.4 +/- 1.7 years, and a morbidly obese group, n=9, BMI = 48.3 +/- 2.1 kg/m^2, age = 33.7 +/- 2.4 years. Figure 13 shows the distribution of fasting plasma ASP values in mg/dL of each individual in the two groups. There is considerable overlap between the two populations, but there is a subgroup within the morbidly obese population with levels of ASP in plasma that are well outside the range of the normal weight group. Also, the mean ASP value of the two groups as well as their BMI's are significantly different by a 2 sample t-test, while their mean ages are not. The ASP levels are 10.45 +/- 1.2 mg/dL and 18.3 +/- 2.3 mg/dL for the normal weight and morbidly obese group respectively.

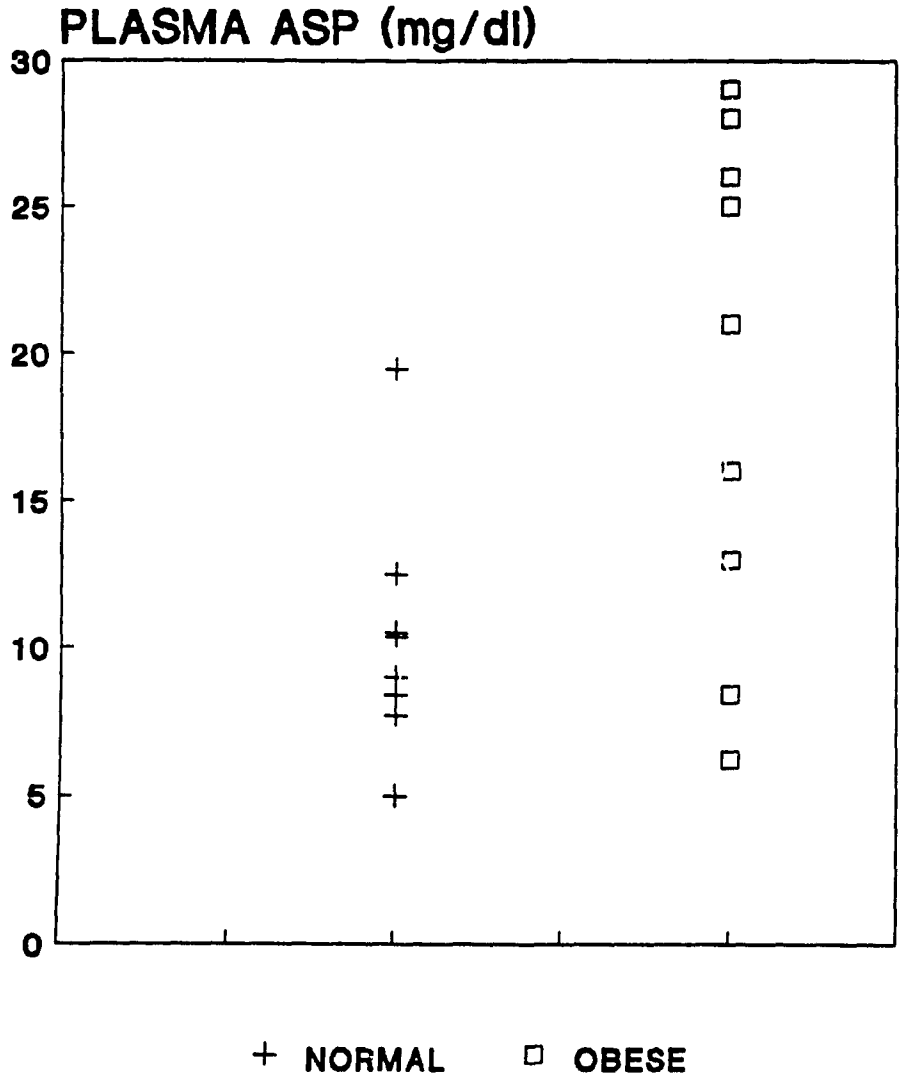
The results show that on average, the fasting plasma ASP level is elevated in morbidly obese females as compared to normal weight individuals. It also suggests that ASP may playing a role in at least some cases of morbid obesity.

Studies on the Effect of ASP on Intracellular Organelles

Within the adipocyte, triglyceride synthesis occurs at the smooth endoplasmic reticulum, the organelle on which the enzymes of the triglyceride synthetic pathway are found (13,24). It is at this intracellular site that fatty acids are activated and then esterified to the glycerol backbone to become lysophosphitidate, diglyceride and triglyceride.

Experiments done using human skin fibroblasts indicate

FIGURE 13: FASTING PLASMA ASP LEVELS IN HUMAN FEMALES



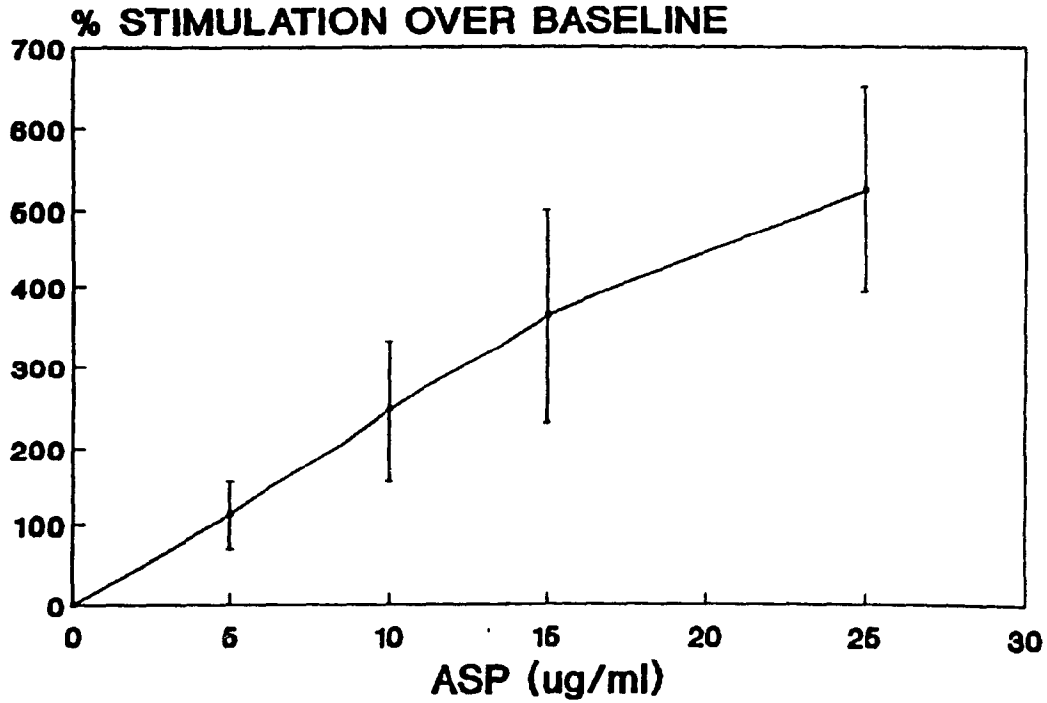
MEAN NORMALS = 10.45 +/- 1.2 mg/dl (n=8)

MEAN OBESE = 18.3 +/- 2.3 mg/dl (n=8)

that ASP is acting through an endocytotic mechanism. This would involve the ASP binding to the plasma membrane of the cell and then entering the cell in order to have its effect. The two most likely sites for ASP to act within the cell would be the mitochondria and/or the endoplasmic reticulum as these two organelles both use fatty acids. If ASP is acting simply by binding fatty acids and then delivering them to the site at which they will be further metabolized, then both organelles should be stimulated by ASP in terms of their action on fatty acids.

The microsome is the subcellular fraction of the cell containing the endoplasmic reticulum. This is obtained by homogenization of cells followed by ultracentrifugation. Figure 14 is a plot of the percent stimulation of incorporation of ^{14}C-oleate into triglyceride per ug of microsomal protein over 30 minutes against an increasing concentration of ASP in 5 experiments. Microsomes were isolated from 5 female patients undergoing gastroplasty procedures, age = 36 +/- 3.2 years and BMI = 47 +/- 1.7 kg/m^2. ASP stimulates triglyceride synthesis up to 600% above the basal level. The microsomal fraction was not completely free of contamination by plasma membrane. The method used to fractionate the cell results in a 30% plasma membrane content in the microsomal fraction, as assayed by 5'nucleotidase activity (41). Our preparations give a more pure preparation with only 20% contamination by the plasma membrane. However, even at this

FIGURE 14: 3H-OLEATE INCORPORATION INTO TRIGLYCERIDE IN HUMAN ADIPOCYTE MICROSOMES



Adipocyte microsomes, 25 ug/ml, (n=5), were incubated at 37°C for 30 minutes in buffer containing 20 uM 3H-oleate and increasing concentrations of ASP. Results are expressed as % stimulation over baseline (no ASP present), of pmoles of oleate incorporated per ug of microsomal protein.

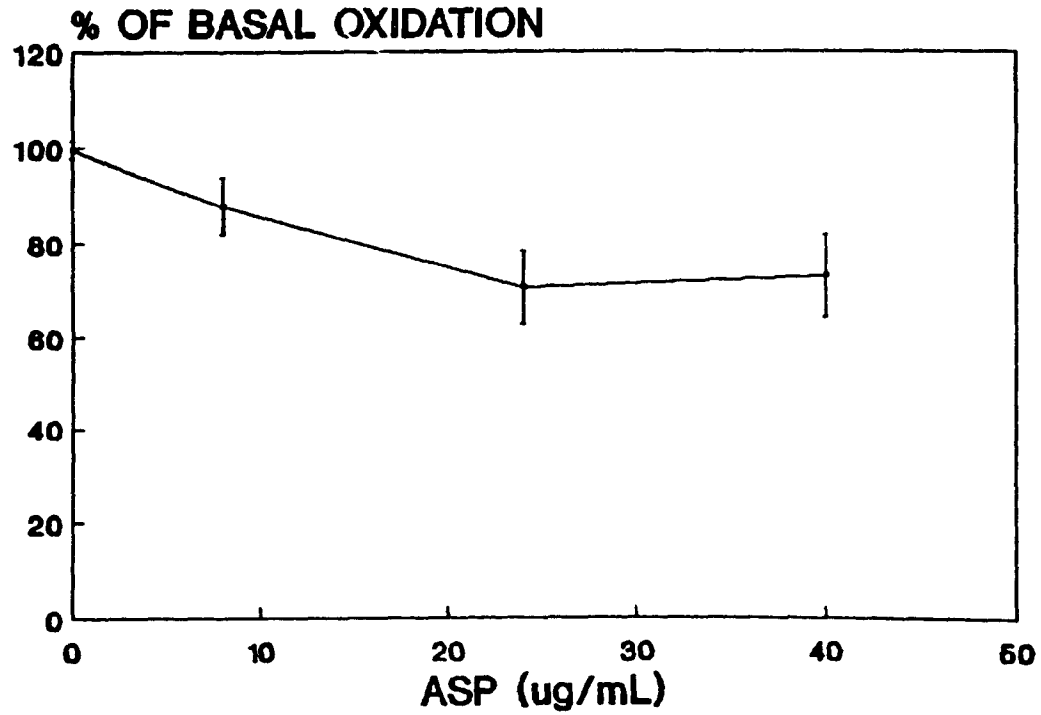
low level of contamination, a receptor mediated effect due to the presence of plasma membrane containing the ASP receptor in the microsomal fraction cannot be ruled out.

Another intracellular organelle that uses fatty acids as substrate is the mitochondrion, which oxidizes them to produce NADH and acetyl-CoA. In this organelle, the use of fatty acid is catabolic, while in the endoplasmic reticulum the process is an anabolic one.

Figure 15 shows the results of increasing ASP concentrations on the basal level of oxidation of fatty acyl-CoA by human adipose mitochondria obtained from 3 female patients undergoing gastroplasty, age = 40 +/- 3.1 years and BMI = 46 +/- 4.3 kg/m^2. 100% basal oxidation is defined as the picomoles of ^{14}C-acetyl-CoA formed per minute per ug of mitochondrial protein in the absence of ASP. The results of the experiments are shown +/- the SEM. The graph shows that as ASP concentration increases, the oxidation of ^{14}C-palmitoyl-CoA does not significantly increase or decrease, as confirmed by a paired t-test against basal activity. Therefore, ASP is not stimulating the oxidation of fatty acids by mitochondria.

The microsome results, when considered along with those obtained from the mitochondria indicate that ASP has some specificity within the cell. It is not acting by indiscriminately transporting fatty acids throughout the cell, rather it is acting by specifically stimulating their incorporation into triglyceride at the site of triglyceride synthesis.

FIGURE 15: ASP EFFECT ON THE OXIDATION OF PALMITOYL-CoA IN HUMAN ADIPOCYTE MITOCHONDRIA



Adipocyte mitochondria, 25 ug/ml, (n=3) were incubated for 11 minutes at 37^{o}C Results +/- SEM are expressed as the % change in the level of beta-oxidation which is the pmoles of 14C-acetyl-CoA produced per ug of mitochondrial protein in the absence of ASP.

Conclusion

ASP is a small, basic protein that has been isolated from human plasma. The capacity of ASP to stimulate triglyceride synthesis in human skin fibroblasts, and its potential role in the pathogenesis of hyperapobetalipoproteinemia, have already been demonstrated (46,47). The tissue of interest in terms of triglyceride metabolism however is not the fibroblast, but the adipocyte. The subject of this thesis has dealt with ASP's effect on triglyceride synthesis in the adipocyte (51).

ASP was isolated from human plasma using a three step chromatographic procedure. This method was used rather than the original method in order to increase the amount of ASP obtained per isolation as well as to decrease the isolation time, since ASP has a tendency to precipitate out of solution upon standing at 4°C. The amount of starting material, which is plasma, is larger using this method, since the capacity of the S-Sepharose resin is far greater than that of the Affi-Gel Blue resin, and therefore more ASP is obtained in each isolation even though the % yield is in fact lower as compared to the previous method.

The majority of the experiments reported in this thesis were carried out on adipocytes isolated from morbidly obese individuals. The results of these experiments show that ASP stimulates both digyceride and triglyceride formation. The

stimulatory effect is immediate, occuring at fifteen minutes after the addition of ASP, as well as long lasting, continuing for at least three hours. Also, ASP's action is far more potent than insulin, which has been the most documented stimulant of triglyceride synthesis prior to the discovery of ASP.

There are some limitations in terms of the interpretation of ASP's seemingly non-differential activity on triglyceride synthesis in normal weight and morbidly obese adipocytes. The first such limitation is that different preparations of ASP were used in each experiment, and since there is as yet no standardized activity assay for ASP, it is likely that the various ASP preparations could differ in their activity. A second limitation is that in the isolation of adipocytes, a collagenase digestion is used and this treatment may inactivate the putative ASP receptor on the cell surface as well as disturb the cell membrane. Another problem is that since the system used is a primary cell culture system in which the isolated adipocytes are used immediately, then the cells from the different individuals may be in a different metabolic state and therfore may not all respond to ASP to the same degree. Keeping these limitations in mind, ASP does stimulate triglyceride synthesis to the same level in both the normal weight and morbidly obese groups, however it should be noted that this does not rule out the possibility that ASP may show a differential binding to the cell surface of these two

groups of adipocytes. It is well known that adipocytes from the morbidly obese are larger than those obtained from normal weight individuals. Therefore, in an aliquot of cells, there will be more of the smaller normal cells as opposed to the larger morbidly obese cells. This suggests that we are underestimating the response of the morbidly obese cells to ASP , since there should be fewer of these cells present when compared to the normal group. One must also consider that since the morbidly obese cell is larger, then the surface area ot the cell will be larger, and receptor density per area of cell surface will be smaller. This in turn will mean that in a given volume of cells, there will be fewer ASP receptors present for the obese group versus the normal weight group. Therefore, if the obese cell in the presence of ASP is synthesizing triglyceride to the same extent as the normal cell in the presence of ASP, and if it has fewer ASP receptors, then the obese cell is a hyper-responsive to ASP.

The plasma levels of ASP show that ASP levels on average are elevated in the morbidly obese group of females. Within both the normal weight and morbidly obese groups, there are some individuals with dramatically elevated levels of ASP, but there are more of these individuals in the morbidly obese group. This finding clearly demonstrates that the morbidly obese population is a heterogenous group in terms of plasma ASP levels, and that therefore an elevated plasma ASP level is unlikely to be the only cause of morbid obesity.

In the cases where the ASP level is elevated, there are two possible explanations for this phenomenon. The first explanation is that the elevated levels of ASP are secondary to the development of morbid obesity. Once the metabolic disorder that leads to the development of morbid obesity is operating, then there is an elevation of ASP production in response to this condition. On the other hand, ASP overproduction resulting in elevated plasma levels may be the primary defect resulting in the development of obesity. This could be explained by the fact that ASP overproduction would result in increased triglyceride synthesis and thus, hypertrophic adipocytes.

An extremely exciting, and potentially important finding of this thesis is that ASP may be acting directly at the site of triglyceride synthesis within the cell. To date, no one has reported the existence of a protein which binds to the cell surface, is internalized, and then acts directly at an intracellular site. Rather, proteins that bind to cells either initiate a second messenger cascade, or they are endocytosed and then degraded or recycled (6,52). The evidence that ASP acts at the endoplasmic reticulum is shown by in vitro incubations of ASP with microsomes, resulting in increased triglyceride synthesis with increasing ASP concentrations. The other organelle that uses fatty acids is the mitochondrion, and ASP has no effect on oxidation of fatty acids by this organelle. A limitation of these findings and the hypothesis

developed from them is that the microsome is not completely free of plasma membrane, and therefore plasma membrane may be mediating the effect of ASP on the microsome. Also, all the evidence to date on ASP endocytosis is based on work done on fibroblasts rather than adipocytes.

In summary, ASP is the most potent stimulant of triglyceride synthesis reported to date. Its action is far more pronounced than that of insulin. ASP may be playing a role in some cases of morbid obesity, and its stimulatory activity may be through a direct action at the intracellular site of triglyceride synthesis, the endoplasmic reticulum.

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