The Role of Acylation Stimulating Protein in Lipid Metabolism in a Human Adipocyte Cell Model

Magdalena Hanna Maslowska

A Thesis
in
The Department
of
Biology

Presented in Partial Fulfillment of the Requirements for the Degree of Masters of Science at Concordia University Montreal, Quebec, Canada

April 1996

© Magdalena Hanna Maslowska. 1996
The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
ABSTRACT

The Role of Acylation Stimulating Protein in Lipid Metabolism in a Human Adipocyte Cell Model

Magdalena Hanna Maslowska

This thesis examines the effects of Acylation Stimulating Protein (ASP) on triacylglycerol synthesis and glucose transport in human adipose cells. In addition, it also looks into possible plasma components necessary for regulation of ASP levels in this cell system.

Adipose tissue is continuously involved in the storage of energy in the form of triacylglycerols during the fed state and their hydrolysis in the fasting state. ASP is a small basic protein isolated from human plasma which stimulates triacylglycerol synthesis and glucose transport in human skin fibroblasts. A cultured human adipose cell model was developed to test the effects of ASP. It was found that ASP 1) greatly stimulates triacylglycerol synthesis in adipocytes and 2) stimulates glucose transport to levels comparable to that of insulin.

Adipose tissue has also been shown to be a secretory organ. Among other proteins, it secretes Factor B, adipin, and complement C3 (which is the precursor of ASP). Cultured adipocytes produce ASP and the amount produced is positively correlated to the adipocyte triacylglycerol mass. In our human adipose cell model, ASP levels were shown to be regulated by specific plasma components. Among many tested, chylomicrons had the greatest stimulatory effect. Thus the incoming dietary fat has a way by which it can prompt adipose
tissue to enhance efficient storage of energy in the form of triacylglycerols.

This work provides evidence for a positive feedback loop in human adipocytes. Imbalances in this process may be involved in such pathological states as obesity, cardiovascular disease and diabetes.
ACKNOWLEDGEMENTS

Human adipose tissue was generously provided by the plastic surgery department at the Royal Victoria Hospital

Plasma for lipoprotein isolation was kindly provided by Mr. David Kalant

I would like especially to express my deep appreciation and gratitude to Dr. Katherine Cianflone for her guidance, help and continuous stimulation. Without her, this thesis would not exist.

I would like to thank Dr. Ralph Germinario for his advice and support during the course of this work.

I wish to also express special thanks to Dr. Allan Sniderman for his helpful discussions and encouragement and Dr. Allain Baldo for all the constructive criticism.

Finally, I would like to thank Enrique, Natasha and Jonathan for their patience and love.
To my Mother and my Father who have helped me in so many ways over the years

Mojej Matce i Ojcu za ich pomoc i bezgraniczna miłość
# TABLE OF CONTENTS

List of Figures .................................................................................................................. ix  
List of Tables .................................................................................................................... x  
Abbreviations .................................................................................................................... xi  

1. Introduction .................................................................................................................... 1  
   1.1 Lipoprotein Metabolism ............................................................................................. 1  
   1.2 Adipose Tissue Metabolism ..................................................................................... 5  
   1.3 Acylation Stimulating Protein (ASP) ........................................................................ 9  

2. Materials and Methods .................................................................................................. 13  
   2.1 Materials .................................................................................................................. 13  
   2.2 Instrumentation ........................................................................................................ 14  
   2.3 Experimental Methodology ...................................................................................... 14  
      2.3.1 Isolation of Human Preadipocytes ..................................................................... 14  
      2.3.2 Preadipocyte and adipocyte cell culture ............................................................. 15  
      2.3.3 Experimental Procedure for Triacylglycerol Synthesis ...................................... 16  
      2.3.4 Isolation and Measurement of Intracellular Triacylglycerol ............................... 16  
      2.3.5 Measurement of Intracellular Triacylglycerol Mass ........................................... 17  
      2.3.6 Experimental Procedure for Glucose Transport ................................................. 17  
      2.3.7 Measurement of Glucose Transport .................................................................... 18  

vii
2.3.8 Isolation of Human Plasma Lipoprotein Fractions

2.3.9 Experimental Procedure for ASP Generation

2.3.10 Measurement of Media ASP

2.4 Statistical Analysis of Data

3 Results

3.1 Cultured Adipose Cell System

3.2 ASP Effect on Triacylglycerol Synthesis

3.3 ASP Effect on Glucose Transport

3.4 Regulation of ASP Generation

4 Discussion

5 References
LIST OF FIGURES

Figure 1  Lipid metabolism in adipose tissue  
Figure 2  Alternate complement pathway  
Figure 3  Morphology of cultured human adipose cells  
Figure 4  Triacylglycerol mass in human preadipocytes and adipocytes  
Figure 5  Concentration-dependent effect of ASP on triacylglycerol 
   synthesis in differentiated and non-differentiated adipocytes  
Figure 6  Concentration-dependent effect of ASP on glucose transport 
   in cultured preadipocytes and adipocytes  
Figure 7  Effect of time exposure to ASP on glucose transport in 
   cultured human preadipocytes and adipocytes  
Figure 8  Comparison of glucose transport at different times of 
   differentiation of human adipocytes in cell culture  
Figure 9  The effects of ASP and insulin on glucose transport in 
   cultured human preadipocytes  
Figure 10 The effects of ASP and insulin on glucose transport in 
   cultured human adipocytes  
Figure 11 Basal ASP production in cultured human adipocytes  
Figure 12 Concentration-dependent effect of glucose on ASP generation 
   by human adipocytes
Figure 13  Concentration-dependent effect of oleate on ASP generation by human adipocytes

Figure 14  Concentration-dependent effect of insulin on ASP generation by human adipocytes

Figure 15  Effect of lipoproteins on ASP generation by human adipocytes

Figure 16  Dose-dependent effect of chylomicrons on ASP generation by human adipocytes

Figure 17  Time course of chylomicron effect on ASP generation by human adipocytes

Figure 18  Proposed model for ASP generation

LIST OF TABLES

Table I  Lipoprotein lipid and apolipoprotein percent composition

Table II  Lipoprotein apolipoprotein composition
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td>Acylation Stimulating Protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C3adesArg</td>
<td>Fragment of the third component of plasma complement without terminal argenine</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3', 5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CH</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>DGAT</td>
<td>Diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco's minimum essential medium/ Ham's F12</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G3P</td>
<td>Glycerol 3-phosphate</td>
</tr>
<tr>
<td>GPAT</td>
<td>Glycerolphosphate acyltransferase</td>
</tr>
<tr>
<td>Glut</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PPH</td>
<td>Phosphatidate phosphohydrolase</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 13-myristate 12-acetate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Obesity is a well recognized health problem faced by many in our affluent society. It is also one of the important risk factors for cardiovascular disease and diabetes (1, 2). Understanding the different aspects of the pathology of obesity is a key to its eventual cure or control. The imbalance between energy intake and energy expenditure results in excessive storage of energy in the form of triglycerides in adipose tissue, which in turn, will result in an increased fat mass accumulation and obesity. Every day, there is additional information obtained regarding the regulation of established biochemical processes along with the discoveries of new functions and new proteins. This thesis will review fatty acid absorption and transport to adipose tissue, as well as the processes pertinent to the physiology of adipose tissue with emphasis on the involvement of Acylation Stimulating Protein (ASP) in adipose tissue metabolism.

1.1 LIPOPROTEIN METABOLISM

Water-insoluble lipids such as cholesterol and triglycerides are absorbed from the diet each time we eat, and are transported in the blood circulation by the means of macromolecular complexes known as lipoprotein particles. This specialized carrier system is composed of four main classes of particles: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Each lipoprotein class has a distinct lipid and apolipoprotein composition which is shown in Table 1 (3). The outer monolayer of
<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Triglyceride</th>
<th>Cholesterol &amp; cholesterol ester</th>
<th>Phospholipid</th>
<th>Apolipoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron</td>
<td>90%</td>
<td>4%</td>
<td>4%</td>
<td>2%</td>
</tr>
<tr>
<td>VLDL</td>
<td>60%</td>
<td>15%</td>
<td>15%</td>
<td>10%</td>
</tr>
<tr>
<td>LDL</td>
<td>10%</td>
<td>45%</td>
<td>20%</td>
<td>25%</td>
</tr>
<tr>
<td>HDL</td>
<td>3%</td>
<td>20%</td>
<td>27%</td>
<td>50%</td>
</tr>
</tbody>
</table>
each lipoprotein particle is made of phospholipids, cholesterol and apolipoproteins. This combination of molecules provides an interface between the aqueous phase of plasma and core neutral lipids. Apolipoproteins stabilize the particle in plasma and provide specific recognition sites necessary for receptor-mediated uptake or for enzymatic action on the lipids (4, 5). The core of the lipoproteins is composed mainly of neutral lipids like cholesterol ester and triacylglycerols. The core lipid composition varies from one lipoprotein class to another giving rise to triacylglycerol-rich particles (chylomicrons and VLDL) or cholesterol ester-rich particles (LDL and HDL). The apolipoprotein composition also differs for each of the different classes of lipoproteins and is given in Table II (3). The function of some of the apolipoproteins has yet to be defined.

Chylomicrons, the most triacylglycerol-rich particles, are synthesized in the intestine after ingestion of a meal (6). They are subsequently released into the lymphatic circulation and later into blood circulation. These triacylglycerol-rich particles function mainly to carry fatty acids in the form of triacylglycerols to the peripheral tissues (mostly adipose tissue and muscle) for their usage in various metabolic processes. As chylomicrons pass through the circulation they are acted on by lipoprotein lipase (LPL) (7). LPL is an enzyme which is bound to the endothelial cells lining the lumen of the capillaries. LPL is activated by apolipoprotein CII, a protein present on the surface of the chylomicron particle itself (8). In an elegant series of experiments, Scow and co-workers have shown, by electron microscopy, that the chylomicron particle is partially enveloped by the endothelial cell during its association (9). As a consequence of triacylglycerol
TABLE II: LIPOPROTEINS APOLIPROTEIN COMPOSITION

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Apolipoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron</td>
<td>A1, AIV, B-48, CI, CII, CIII, E</td>
</tr>
<tr>
<td>VLDL</td>
<td>B-100, CI ,CII, CIII, E</td>
</tr>
<tr>
<td>LDL</td>
<td>B-100</td>
</tr>
<tr>
<td>HDL</td>
<td>A1, AII, AIV, CI, CII, E</td>
</tr>
</tbody>
</table>

hydrolysis, free fatty acids are liberated, which can then be taken up for storage by adipose tissue. Fatty acids that are not stored are carried, complexed to albumin, throughout the circulation and are taken up by the liver. Excessive influx of fatty acids into the liver will give rise to another lipoprotein particle - VLDL as well as LDL, which are then secreted by the liver into the circulation (10).

VLDL is a triacylglycerol-rich particle of hepatic origin, which is also hydrolysed by LPL located on the endothelial surface. Fatty acids are liberated generating an intermediate density lipoprotein (IDL) particle some of which is taken up by the liver and the rest is eventually converted to an LDL particle (10). Fatty acids are, again, used by the cells in the manner described above and the circulating LDL particle (now rich in cholesterol ester) can be taken up by the tissues through receptor-mediated endocytosis (11) and used for the synthesis of plasma membrane, steroid hormones, cholesterol ester or eliminated from the body in the form of bile acids (12). Defects in the uptake of the LDL particles, however, will result in the accumulation of a large number of these particles in the circulation, predisposing individuals to coronary artery disease (13). The main function of HDL lipoprotein may be the clearance of the tissue cholesterol (14). HDL cholesterol is then degraded in the liver, used by steroid hormone producing tissues or removed in the form of bile acids (12). HDL apolipoproteins can be recycled.

The lipoproteins which are of interest in the context of obesity are chylomicrons and VLDL since they provide fatty acids which are the building blocks of triacylglycerols.
1.2 ADIPOSE TISSUE METABOLISM

The main function of white adipose tissue is its involvement in energy metabolism and for many years research has focused on different aspects of lipogenesis and lipolysis. It has only recently been appreciated that adipose tissue is a metabolically active tissue, constantly involved not only in the storage and release of energy but also in secretion of specific peptides. Figure 1 illustrates metabolic processes relevant to adipose tissue metabolism. These processes are under the control of various hormonal, neural and other local components (15).

Adipocytes store energy surplus in the form of triacylglycerol fat droplets. Triacylglycerol molecules are formed through sequential enzymatic reactions in which three fatty acid molecules are esterified to a glycerol-3-phosphate (G3P) backbone. The enzymes involved in this esterification process are glycerolphosphate acytransferase (GPAT), phosphatidate phosphohydrolase (PPH) and diacylglycerol acyltransferase (DGAT) and are located on the membrane of the endoplasmic reticulum. Free fatty acids used for triacylglycerol synthesis in esterification reactions are either supplied by chylomicrons or VLDL from the diet or made de novo from available glucose. In adipose tissue, however, de novo fatty acid synthesis is low, when the supply of exogenous fatty acids is satisfactory (16). G3P, on the other hand, is obtained from the metabolism of glucose in the process known as glycolysis. In mammalian cells, glucose enters the cell via a membrane carrier protein. Until now, the translocation of these specialized glucose transporters from intracellular compartments to the plasma membrane was believed to be mainly responsible for the rapid uptake of glucose (17). Recently, however, this view has
Figure 1. Lipid metabolism in adipose tissue. LPL - lipoprotein lipase. HSL - hormone sensitive lipase. G3P - glycerol-3-phosphate. GPAT - glycerolphosphate acyltransferase. PPH - phosphatidate phosphorylase. DGAT - diacylglycerol acyltransferase. FFA - free fatty acids. TG - triacylglycerol. Chylo - chylomicron.
been challenged and it has been demonstrated that "intrinsic" activity of the glucose transporter itself can be, in fact, affected. The catalytic activity of glucose transporters can be either suppressed (18) or stimulated (19).

There is now considerable evidence that the esterification process is under hormonal control (20). Insulin can increase the rate of triacylglycerol synthesis not only through its effects on glucose uptake and fatty acid synthesis but also by its inhibitory action on hormone-sensitive lipase (21). In addition, both PPH and DGAT have been implicated as the rate-limiting steps of triacylglycerol synthesis. These observations are based on circumstantial evidence in subcellular fractionation enzyme assays. Either enzyme has as yet to be purified to homogeneity and fully characterized (22, 23). Overall, our current knowledge of the mechanisms regulating adipose tissue-specific processes is limited.

In the fasting state triacylglycerols from adipose tissue are mobilized in response to specific hormonal stimuli (24). Following hormonal binding (e.g., noradrenaline) to β-adrenergic receptors, adenylate cyclase is activated and cAMP is produced intracellularly. This cAMP, in turn, activates protein kinase A (PKA) which phosphorylates and activates hormone-sensitive lipase (HSL). HSL, in turn, catalyses the rate-limiting step in the lipolysis pathway (25). As a result, the triacylglycerols are hydrolysed into glycerol and free fatty acids and are subsequently released from the cells to provide the needed energy to the peripheral tissues.

Although triglyceride storage and hydrolysis have always been considered to be the main function of adipose tissue, more recently, adipose tissue has also been
recognized as a secretory organ. Initially, lipoprotein lipase was known to be synthesized and secreted by adipocytes (26), a process which is hormonally regulated (27). The requirement of lipoprotein lipase for triacylglycerol hydrolysis of chylomicrons and VLDL has been well documented (7). More recent data reveal that in addition to lipoprotein lipase, adipocytes are also a source of cholesterol ester transfer protein (28), angiotensin (29), apolipoprotein E (30) and leptin (31).

Furthermore, the discovery that adipose tissue also produces and secretes proteins of the alternate complement pathway has evolved from the work of Spiegelman and colleagues (32). In their work they demonstrated that cultured murine adipocytes synthesize and secrete a novel protein, in a differentiation-dependent manner, which they called adipsin. Further analysis and cloning from human adipocytes recognized this protein as homologous to factor D, a protein involved in alternate complement pathway (33). Additional research revealed that two other complement proteins are secreted by murine adipocytes: complement C3 and factor B (34). Moreover, the mRNA levels of these three complement proteins were shown to increase with differentiation in cultured murine adipocytes as well as to be elevated in adipose tissue in mice (34). These observations did not, however, point to any specific function for the system, but interestingly, coincided with the identification and characterization of Acylation Stimulating Protein (ASP) in our laboratory. Cianflone et al have shown that mRNA levels for C3, factor B and adipsin are increased in mature human fat cells (35). Moreover, it was demonstrated that not only mRNA levels are higher in adipocytes as compared to fibroblasts, but human adipocytes produce increased levels of ASP (36).
1.3 ACYLFATION STIMULATING PROTEIN (ASP)

ASP is a human plasma protein discovered based on its functional activity. ASP has been shown to markedly stimulate triacylglycerol synthesis in human skin fibroblasts and mature fat cells and it is the most potent stimulator described yet (37, 38). Upon purification based on its functional activity, ASP was found to be identical to C3adesArg, a cleavage product generated through the action of factor B and adipsin (factor D) on the third component of complement - C3 (39).

Figure 2 illustrates the intricate interactions of the three proteins involved in the generation of ASP. This process initiates with the immobilization of C3. Factor B interacts with the C3b fragment of C3 resulting in a C3bB complex. This complex is then susceptible to the action of a catalytic enzyme - adipsin. As a result a C3bBb macromolecule is generated which in turn has enzymatic properties and acts on C3, cleaving it into C3b and C3a fragments. In this process C3 and factor B are constantly being used up but not adipsin. In plasma, terminal arginine (Arg) is removed through the action of carboxypeptidase B and C3a is rapidly converted to ASP (C3adesArg).

In addition to the stimulatory action of ASP on triacylglycerol synthesis, ASP also increases specific glucose transport. In terms of glucose transport, the effects of ASP, whether in human skin fibroblasts or L6 muscle cells are comparable in magnitude to and additive to, those of insulin (40, 41). Germinario et al. have shown that the effects on triacylglycerol synthesis are not solely due to the effects on glucose transport. When glucose in the media was substituted for pyruvate the effect on triacylglycerol synthesis was only slightly diminished, indicating that the
Figure 2.  Alternate complement pathway.
presence of glucose potentiates, but is not required for ASP stimulation of triacylglycerol synthesis (40). The ASP effect on glucose transport is mediated by translocation of specific glucose transporters (Glut1 in fibroblasts and Glut1, Glut3 and Glut4 in L6 muscle cells) to the surface of the cell (40, 41). In addition, the ASP effect on triacylglycerol synthesis has been shown to be achieved by stimulating DGAT activity - a rate limiting enzyme for triacylglycerol synthetic pathway (42) and not by increasing the specific transport of fatty acids across plasma membrane (41).

Preliminary experiments demonstrate the existence of a potential specific receptor for ASP (43). ASP binding is greater in cultured human adipocytes as compared to preadipocytes or skin fibroblasts (43). At this point the ASP receptor has not been fully characterized, however, initial studies indicate that the effects of ASP are exerted through activation of the protein kinase C (PKC) pathway (44). The stimulatory effects of phorbol 12-myristate 13-acetate (PMA), a known activator of PKC pathway, and ASP on triacylglycerol synthesis are non-additive suggesting that they act through the same mechanism. In addition, competitive inhibitors of PKC inhibit both ASP and PMA stimulatory effects (44).

These in vivo data are not without physiological relevance. In fat-load studies in normal adult human subjects, it was observed that plasma ASP levels rise concurrently with the rise of plasma triacylglycerols (45, 46). Moreover, the rate at which triacylglycerols were cleared from plasma was proportional to the plasma ASP. In addition, plasma ASP levels have been found to be 2-3 times higher in obese people than in normal weight individuals (47). These modulations of plasma ASP in response to dietary fat intake as well as to the individual metabolic state,
suggest that ASP may play a key physiological role in the regulation of adipose tissue fat mass.

The aim of this thesis was to develop a human adipocyte cell model in which the pertinence of the ASP involvement in lipid metabolism could be tested. Using this cell model, the specific goals were 1) to examine the effects of ASP on triacylglycerol synthesis and glucose transport and 2) to define the influence of specific plasma components on the endogenous ASP production in cultured human adipocytes.
MATERIALS AND METHODS

2.1 MATERIALS

All chemicals used were of reagent grade quality and were purchased from Fisher Scientific (Montreal, Canada). Tissue culture media and supplies were from Gibco (Burlington, Ontario) or Flow Laboratories (Mississauga, Ontario). [3H] 2-deoxy D-glucose (specific activity 50 dpm/pmol) was purchased from ICN Biochemicals Canada (Mississauga, Ontario) and [9,10-3H(N)] oleic acid (specific activity 100 dpm/pmol) from DuPont-New England Nuclear (Mississauga, Ontario). Insulin, oleic acid (sodium salt), bovine serum albumin essentially fatty acid free, collagenase Type II, lipoprotein lipase and all other tissue culture grade compounds were from Sigma Chemicals (St. Louis, MO). Commercial protein assay kit as well as bovine serum albumin used for standard curves were obtained from Bio-Rad Laboratories (Mississauga, Ontario). Kits used for measurements of triglyceride and total cholesterol content of plasma lipoproteins were purchased from Boehringer Mannheim (Laval, Quebec). Thin layer chromatography (TLC) plates were from Mandel Scientific (Rockwood, Ontario), TLC solvents from Fisher Scientific and TLC lipid standards from Sigma Chemicals. Scintillation fluid was purchased from ICN Biochemicals Canada and scintillation vials from Fisher Scientific. Acylation Stimulating Protein was isolated and purified from frozen human plasma according to the published method (48). The radioimmunoassay kit specific for C3adesArg used for ASP measurements was obtained from Amersham (Oakville, Canada).
2.2 INSTRUMENTATION

Lipid samples were dried using a Jouan vacuum centrifuge (Canberra-Packard, Montreal, Quebec). [9,10-³H(N)] oleic acid (³H-oleate) incorporation into triacylglycerol as well as ³H-2-deoxy-glucose (³H-2-DG) uptake by the cells were measured using scintillation counter (Beckman Instruments, Mississauga, Ontario). Results from ASP radioimmunoassay were measured using RiaGamma counter (LKB Wallac, Wallac, Finland). All spectrophotometric readings were performed using a microtiter plate reader (Molecular Devices, Melno Park, CA). Lipoprotein isolation was accomplished by sequential ultracentrifugation using a Ti50 type rotor (Beckman Instruments, Mississauga, Ontario) on a Beckman ultracentrifuge.

2.3 EXPERIMENTAL METHODOLOGY

2.3.1 Isolation of human preadipocytes. The isolation procedure for stromal vascular cells was modified from that of Hauner et al (49). Briefly, human adipose tissue was obtained with informed consent from normal weight females, ages 16-45, undergoing mammoplasty reduction. Fat lobules were excised under sterile conditions immediately after breast tissue was removed from the patient and transferred to the laboratory. The adipose tissue was then cleaned of any connective and glandular material as well as visible blood vessels. Remaining fat was finely minced with scalpels and digested for 30 minutes at 37°C in Hank's Buffered Salt Solution containing 1 mg/ml Type II collagenase and 0.5% fatty acid free bovine serum albumin (BSA). Stromal-vascular cells containing preadipocytes were separated from mature adipocytes by centrifugation at 2,500 rpm for 10
minutes. The fat cake and the supernatant were discarded and the remaining pellet was resuspended in a lysis buffer consisting of 0.154 M NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA for 10 minutes to lyse contaminating red blood cells. To further remove remaining undigested connective tissue that could interfere with cell adherence, the cell suspension was filtered through a 53μm filter (Spectrum, Huston, Texas) and centrifuged at 2,500 rpm for 10 minutes to pellet down cells of interest. The sedimented preadipocytes were resuspended in Minimum Essential Medium (MEM) medium supplemented with 10% fetal bovine serum (FBS). Cells to be differentiated were plated out on 24 well plates at a high density (3x10⁴ cells/cm² or 10 gm of cleaned tissue per one 24 well plate; where one 24 well plate = 1.7 cm² surface area/well). Cells not to be differentiated were plated out at 1/10 of the cell density.

2.3.2 Preadipocyte and adipocyte cell culture. Plated cells remained in MEM medium containing 10% FBS for a period of 24 hours. Preadipocytes were grown in 10% FBS containing MEM. Cells destined for differentiation to adipocytes were changed to serum-free Dulbecco’s minimum essential medium/Ham’s F12 (DMEM/F12) medium supplemented with 1.25 μM bovine insulin, 1 μM dexamethasone, 0.2 nM triiodothyronine, 33 μM biotin, 17 μM pantothenate, 15 mM NaHCO₃, and 15 mM Hepes (49). Preadipocytes and differentiating adipocytes were maintained in a 37°C humid incubator with 5% CO₂ with media changes twice a week. By the 21st day preadipocytes formed a confluent cell layer and the differentiating cells exhibited a typical adipocyte morphology of rounded cells.
containing multilocular droplets (49). At this time cells were used for experiments.

2.3.3 Experimental procedure for triacylglycerol synthesis. On the 21st day differentiated and nondifferentiated preadipocytes were changed to serum- and supplement-free DMEM/F12 overnight prior to initiation of experiments. The following day, the cells were incubated with fresh DMEM/F12 supplemented with $^3$H-oleate complexed to BSA in a 5.1 molar ratio as described by Van Harken (50) and added to the cells at a final concentration of 100 $\mu$M (average specific activity 100 dpm/pm). In addition, the following concentrations of ASP (0, 0.3, 0.6, 1.8, 2.4, 2.8, and 5.5 $\mu$M) were added. The effect of ASP on triacylglycerol synthesis was measured over the period of 6 hours as $^3$H-oleate incorporation into triacylglycerol. This was on the linear part of the incorporation curve (0 hours to 24 hours) (35).

2.3.4 Isolation and measurement of intracellular triacylglycerol. Following incubation, at 6 hours the media was discarded and the cells were washed 3 times with 1 ml of ice-cold phosphate buffered saline (PBS). Lipids were then extracted with 1 ml of heptane:isopropanol (3:2 v/v) for 30 minutes at room temperature. Cells were extracted with an additional 1 ml of the same organic solvent which was then added to the first fraction and the total lipid extract was dried in a vacuum-centrifuge. Dried samples were reconstituted in 100 $\mu$l of chloroform:methanol (2.1 v/v) to be separated by thin-layer chromatography (TLC). An aliquot (25 $\mu$l) of each sample was spotted onto prescored Silica thin 150A TLC plates. Lanes in each TLC plate were spotted with triolein as reference lipid. The plates were developed in
hexane:ethyl ether:acetic acid (75:25:1 v/v/v). The lipids were visualized by exposure of plates to iodine vapor and the triacylglycerol spots corresponding to triolein were scraped into scintillation vials. Scintillation fluid (5 ml) was added to each vial and the radioactivity was determined using a scintillation counter. Cell proteins were dissolved by addition of 1 ml of 0.1 N NaOH and quantitated by the method of Bradford (51) using a commercial Bio-Rad protein assay.

2.3.5 Measurement of intracellular triacylglycerol mass. Triacylglycerol mass was measured on the remaining lipid extract isolated by TLC extraction by the method of Neri and Frings (52). In summary, triacylglycerol extracts were saponified with alcoholic KOH to fatty acids and glycerol, followed by oxidation of the latter with sodium metaperiodate to form glycolaldehyde and formaldehyde. The formaldehyde was then reacted with acetylacetone and ammonium acetate to produce a colored product 3,5-diacetyl-1,4-dihydrolutidine. This colorimetric reaction was then measured spectrophotometrically at 405 nm. Triacylglycerol mass results are expressed as nmol triacylglycerol per mg cell protein.

2.3.6 Experimental procedure for glucose transport. Cells were grown and differentiated as described above in 35 mm diameter Petri dishes. On the 18th day of culture the medium on differentiating adipocytes was changed to serum-free, hormone-supplemented differentiation medium which did not contain insulin. This was done to minimize the effects of prolonged exposure to insulin on the downregulation of insulin receptors in differentiating adipocytes in order to allow
testing of insulin effects. On the 21st day the medium on all the cells was changed to serum- and supplement-free DMEM/F12 medium for a period of 18 hours prior to experiment. The next day, cells were stimulated with ASP, insulin or both at specific concentrations for one hour at 37°C. After exactly one hour, glucose transport was assessed by measuring the cellular uptake of $^3$H-2-DG.

2.3.7 Measurement of glucose transport. Conditioned media was removed and the cells were washed once with 2 ml of PBS at 37°C. Next, 0.8 ml of PBS containing $^3$H-2-DG (0.05 mM; average specific activity 50 dpm/pmol) was added to each dish and incubated at 37°C in a water bath for exactly 10 minutes (53). In all the experiments zero-time controls were performed to account for background binding of $^3$H-2-DG. At exactly 10 minutes, the radioactive solution was aspirated and the cells were washed twice with 2 ml of ice-cold PBS. Finally, the cell monolayer was dissolved in 0.5 ml of 0.1 N NaOH. Aliquots of 0.2 ml were then transferred into a scintillation vial containing scintillation fluid and counted with a scintillation counter. Protein concentration was determined using the Bio-Rad protein assay as described above.

2.3.8 Isolation of human plasma lipoprotein fractions. Blood was obtained from healthy subject 4 hours postprandially and collected in Vacutainer Tubes containing anticoagulant (0.15% EDTA) and placed on ice. Plasma was immediately isolated by low-speed centrifugation at 4°C and the lipoproteins were subsequently separated by discontinuous preparative ultracentrifugation according to the
procedure of Havel et al (54). In summary, plasma was initially layered under a salt solution of density 1.006 g/ml. Chylomicrons were separated from the top layer after centrifugation for 30 minutes at 30,000 rpm (40,000 g) at 11°C. The infranate from the initial step was again overlaid with the 1.006 g/ml solution and centrifuged for 18 hours at 40,000 rpm (68,000 g) at 11°C. Very low density lipoprotein (VLDL) was recovered in the top fraction. Low density lipoprotein (LDL) was isolated from the infranate obtained in the second step by increasing its solvent density from 1.006 g/ml to 1.063 g/ml. The supernatant fraction was removed after centrifugation at 40,000 rpm (100,000 g) for 20 hours. Finally, the remaining infranate was used for high density lipoprotein (HDL) isolation after increasing the solvent density to 1.21 g/ml and centrifuging for 48 hours at 40,000 rpm (100,000 g) at 11°C. LDL and HDL fractions were subsequently dialyzed in large volume of PBS for 24 hours at 4°C to remove excess salt. Chylomicrons and VLDL were sterilized by filtering through a 0.45 µm pore syringe filter, LDL and HDL were sterilized through a 0.22 µm pore syringe filter. The triacylglycerol and cholesterol concentration of each fraction was measured using commercially available assays from Boehringer Mannheim.

2.3.9 Experimental procedure for ASP generation experiments. On the 21st day of culture, differentiated adipocytes were changed to serum- and supplement-free DMEM/F12 overnight prior to initiation of experiments. The next day, cells were incubated with selected plasma components added at different concentration ranges to the medium. Basal ASP production was linear for up to 4 hours with only a slight decrease at 8 hours, hence the incubation time chosen for most of the experiments
was 6 hours. For the oleate experiments, oleic acid complexed to BSA in a 5:1 molar ratio as described by Van Harken (50) was added to the cells up to a final concentration of 0.8 mM. For the chylomicron time course experiments, cells had their media changed again to serum- and supplement-free media for an additional 24 hours. To this medium chylomicrons were added to a concentration of 50 μg triacylglycerol/ml for the last 0, 2, 4, 6, 8 and 24 hours of the 24 hour incubation period. In all experiments, following incubation, the medium was removed and frozen immediately at -70°C for later ASP quantification. The cells were washed twice with 1 ml ice-cold PBS, 0.5 ml of 0.1 N NaOH was added to the cells and cell proteins were measured by the method of Bradford (51) using a commercial kit.

2.3.10 Measurement of media ASP. ASP was measured in the medium of cultured human differentiating adipocytes following a 6 hour incubation of the cells in serum- and supplement-free medium with selected plasma components using a modified version of a radioimmunoassay kit specific for C3adesArg. The use of the commercial radioimmunoassay for C3adesArg for ASP determination has been validated in detail previously (35). Briefly, in the first step, complement factor C3 (the precursor protein of ASP) was acid-precipitated from the media by the addition of an equal volume of a precipitating reagent supplied in the kit. At this point the medium was supplemented with BSA to a final concentration of 1.0 mg/ml to act as a carrier protein. The precipitation was carried out at room temperature for 30 minutes. The samples were then centrifuged at 2,500 rpm for 15 minutes at 4°C to remove the protein precipitate, including C3 molecules, which otherwise would react with the
anti-C3adesArg antibodies. The cell medium was then mixed with a fixed volume of 
\(^{125}\)I-C3adesArg and an equal, fixed volume of primary rabbit polyclonal anti-
C3adesArg antibody and incubated at room temperature for 30 minutes. Secondary 
(goat anti-rabbit) antibody was then added to that solution mixture and incubated for 
an additional 30 minutes. Following this step, 1 ml of saline was added and the 
samples were centrifuged at 2,000 rpm for 10 minutes at 4° C. The supernatant 
containing unbound ASP and free \(^{125}\)I-C3adesArg was then removed by aspiration 
and the remaining pellet was counted in a Gamma counter. The basis of the assay 
is the competition between unlabelled ASP (in the media) and a constant amount of 
\(^{125}\)I-C3adesArg for the primary anti-C3adesArg antibody. Because the amounts of 
radioactive ligand and antibody are limiting, the quantity of the \(^{125}\)I-ligand bound and 
precipitated will be inversely proportional to the concentration of the added non-
radioactive ligand in the sample. Measurements of the radioactivity in the pellet 
allows the calculation of the labelled C3adesArg in the bound fraction. The 
concentration of unlabelled ASP (C3adesArg) in each sample is then determined by 
interpolation from the standard curve.

2.4 Statistical Analysis of Data.

For all experiments, values are reported as means of experiments with all 
determinations performed in triplicate (or duplicate where indicated) in each 
experiment ± standard error of the mean (SEM). Statistical significance was set at 
p=0.05 and was determined using either one-way ANOVA or paired t-test as 
indicated in the Figure legends and results.
3. RESULTS

3.1 CULTURED ADIPOSE CELL SYSTEM

To study the effects of ASP in human adipose tissue as well as to determine the potential regulators of ASP secretion from that tissue, it was important to develop an *in vivo* system that could represent human adipose tissue physiologically. The initial step was to grow and differentiate human adipocytes in tissue culture. Human adipose tissue obtained at the time of elective surgery was dissected and preadipocytes (precursors of adipocytes) were isolated. These cells were then differentiated in tissue culture over the period of 21 days according to the procedure described in Materials and Methods. Figure 3 demonstrates the changes that take place during this differentiation process. Figure 3A shows the adipocyte precursors grown in culture. However, when given the right and specific differentiating conditions the morphology of these cells changes and they become fat loaded adipocytes which is shown in Figure 3B.

One of the criteria used to evaluate whether the differentiation process is taking place and to decide if the cells could be used for experiments, is the visual examination of the morphology of the cells. However, triglyceride mass accumulation in preadipocytes and adipocytes can also be used as an index of differentiation, and this was done using a biochemical assay specific for triglyceride (as described in Materials and Methods). Since each cell preparation comes from different individual there is a wide range of triglyceride mass accumulated from one cell line to the next. Figure 4 shows the amount of triglyceride accumulated in preadipocytes as
Figure 3. Phase-contrast micrograph of cultured human adipose cells: Human preadipocytes were either grown in tissue culture in MEM medium containing 10% FBS (A) or differentiated, to become adipocytes, in DMEM/F12 serum-free medium supplemented with 1.25 μM insulin, 1.0 μM dexamethasone and 0.2 nM triiodothyronine (B) for 21 days as described in Materials and Methods, (100x magnification for A and B).
Figure 4. Triacylglycerol mass in human preadipocytes and differentiated adipocytes: Preadipocytes obtained from eight different individuals were either grown in MEM medium containing 10% FBS or were differentiated to become adipocytes for 21 days. Intracellular triacylglycerol (TG) mass accumulation was measured on the 21st day of culture for each cell line. The results are expressed as nmol TG produced per mg cell protein. Average TG mass for preadipocytes was 698.93 ± 467.54 nmol/mg cell protein and 4970.38 ± 2267.68 nmol/mg cell protein for adipocytes, (p<0.05, paired t-test).
compared to adipocytes from eight different individuals during the course of 21 days of differentiation in culture. Preadipocytes have relatively little triglyceride mass (range 78 to 1485 nmol/mg cell protein) which increases substantially during differentiation (range 1941 to 9132 nmol/mg cell protein) by an average of 10.3 fold, p<0.05

3.2 ASP EFFECT ON TRIGLYCERIDE SYNTHESIS

Until recently it was believed that the process of adipose tissue triglyceride storage was passive. Only insulin was thought to regulate lipogenesis through its inhibitory action on hormone sensitive lipase (21). However, in recent years, Cianflone and co-workers have shown that plasma Acylation Stimulating Protein (ASP) is a potent stimulator of triacylglycerol synthesis in cultured human skin fibroblasts (40).

In this set of experiments the effects of ASP on triacylglycerol synthesis were examined in differentiated adipocytes and compared to the effects on non-differentiated preadipocytes. Figure 5 is a representative experiment showing the concentration-dependent effect of ASP on triacylglycerol synthesis in differentiated and non-differentiated adipocytes. Increasing concentrations of ASP were added to the cells in the presence of 100μM 3H-oleate and triacylglycerol synthesis was measured over the period of 6 hours as described in Materials and Methods. Based on the previous studies done in cultured human skin fibroblasts the 6 hour time period was chosen since it was on the linear part of the incorporation curve (0 to 24 hours) (35). The results indicate that ASP causes a concentration-dependent
Figure 5. Concentration-dependent effect of ASP on triacylglycerol synthesis in differentiated and non-differentiated adipocytes: Both human non-differentiated (PREAD) and differentiated (AD) adipocytes were grown in culture for 21 days. Cells were then exposed to serum-free medium supplemented with 100 μM oleate and increasing concentrations of ASP for a period of 6 hours. Conditioned media was removed and triacylglycerol (TG) synthesis was measured as $^3$H-oleate incorporation into TG. The results are expressed as nmol of oleate/mg cell protein/6 hours. Data points depict a representative experiment assayed in triplicate.
increase in triacylglycerol synthesis in differentiating as well as in non-differentiating adipocytes (577 to 1640 nmol/mg cell protein vs. 252 to 393 nmol/mg cell protein, respectively). Although, the basal levels of triacylglycerol synthesis are already much higher in adipocytes (577 nmol/mg cell protein) than in preadipocytes (252 nmol/mg cell protein), the maximal percent increase above the basal in the presence of ASP is greater in differentiated adipocytes (285%) than in preadipocytes (156%), p<0.05

3.3 ASP EFFECT ON GlUCOSE TRANSPORT

To date insulin has been considered the most potent hormone affecting glucose transport in muscle and adipose tissue (55). It has been shown that ASP exerts similar effects to that of insulin in cultured skin fibroblasts (40) and more recently, in muscle L6 cells (41). Here (Figure 6), cultured human preadipocytes and adipocytes were exposed for 1 hour to increasing concentrations of ASP. Basal levels of glucose transport were 5.19 ± 0.84 nmol/mg cell protein for preadipocytes and 2.44 ± 0.39 nmol/mg cell protein for adipocytes. In adipocytes, glucose uptake was slightly stimulated at a concentration as low as 0.3 μM of ASP with 118% ± 12% stimulation and reached its maximum of 206% ± 13% (p<0.05) stimulation (2.44 ± 0.39 nmol/mg cell protein basal vs. 6.54 ± 0.28 nmol/mg cell protein stimulated) at the highest ASP concentration tested of 5.5 μM. The graph also shows that cultured adipocytes are more responsive at the lower ASP concentrations (0.3 μM to 2.5 μM); however, the difference in response between preadipocytes and adipocytes disappears at the higher ASP concentrations (3.0 μM and 5.5 μM). Thus the half-
Figure 6. Concentration-dependent effect of ASP on glucose transport in cultured human preadipocytes and adipocytes. Preadipocytes (PREAD) and adipocytes (AD) were incubated at 37°C for 1 hour with 0.3, 0.6, 1.8, 2.4, 2.8 and 5.5 μM concentrations of ASP. Glucose uptake was then measured for 10 minutes with 0.05 mM of 3H-2-DG. The results are expressed as % stimulation ± %SEM above basal glucose transport. Basal (no ASP, 100%) levels for preadipocytes were 5.19 ± 0.84 nmol/mg cell protein and 2.44 ± 0.39 nmol/mg cell protein for adipocytes. The graph shows an average of 4 experiments with duplicate determinations in each experiment. *p < 0.05 at all concentration points vs. basal glucose transport.
maximal dose for preadipocytes is 2.8 μM, whereas it is 0.6 μM for adipocytes, which are 4.7 fold more sensitive. Overall the effect of ASP on glucose transport was significant over the whole concentration range for adipocytes, p< 0.05 and at very high ASP concentrations (greater than 2.8 μM) for preadipocytes, p< 0.05. In order to determine the kinetics and stability of the preadipocyte and adipocyte response to ASP, the response of exposure to a high concentration of ASP (5.5 μM) was tested for different periods of time. Figure 7 shows glucose uptake at different time points. Basal levels were 5.04 ± 0.49 nmol/mg cell protein for preadipocytes and 5.5 ± 1.68 nmol/mg cell protein for adipocytes. When ASP was added to the cells the effect on glucose uptake was observed as early as 15 minutes (8.05 ± 2.04 nmol/mg cell protein for preadipocytes vs. 6.14 ± 1.57 nmol/mg cell protein for adipocytes) and reached its maximum at approximately 4 hours with the values 13.69 ± 3.1 nmol/mg cell protein for preadipocytes and 8.6 ± 2.6 nmol/mg cell protein for adipocytes. In this particular set of experiments the response to ASP was higher in preadipocytes than in adipocytes, a result which is in contrast to what was seen in all the other experiments. One possible explanation could be that the cells were derived from different subjects and direct comparison of preadipocytes and adipocytes for the same subject was not possible. The data indicate, nevertheless, that the effect of ASP is prolonged (up to 4 hours) with subsequent, possible down-regulation at 24 hours. This is consistent with the studies done in human skin fibroblasts (40) but contrasts with the studies done in L6 muscle cells (41).

Most of our studies have been performed using cells that have been differentiated for 3 weeks. At that time the cells exhibit adipocyte morphology. After
Figure 7. **Effect of time exposure to ASP on glucose transport in cultured human preadipocytes and adipocytes.** Preadipocytes (PREAD) and adipocytes (AD) were incubated with 5.5 μM ASP at 37°C for 15 min, 30 min, 1, 2, 4, 8, and 24 hours. Glucose uptake was then measured over 10 minutes with 0.05 mM of 2-DG. The results are expressed as nmols of ^3^H-2-DG taken up by the cell per mg of total cell protein. The graph shows an average of 3 experiments with duplicate determinations in each experiment. *p< 0.05 for *.
longer differentiation times, fat loaded cells become more fragile and break up or detach and float up. In this set of experiments the response of the cells to ASP was examined at different stages during the time of differentiation. As shown in Figure 8, basal (no ASP) glucose transport in cultured human differentiating cells increases as the cells progress and become more and more differentiated. Their basal glucose transport increased from $4.27 \pm 0.54$ nmol/mg cell protein at week 1 to $6.54 \pm 1.93$ nmol/mg cell protein at week 2 to $9.76 \pm 2.55$ nmol/mg cell protein at week 3. Overall, this is an average increase of 136%, which is statistically significant ($p < 0.05$). In addition, ASP-stimulated glucose transport (5.5 µM ASP) is significantly increased by $142\% \pm 22.3\%$ above basal levels in the second week ($p < 0.05$) and by $124\% \pm 4.8\%$ SEM in the third week ($p < 0.05$). In the first week of differentiation little effect was observed.

In order to gain some understanding of the mechanisms by which ASP induces glucose uptake in human adipocytes, the effects of ASP were compared to those of insulin. Figure 9 represents data from the experiments in which the effects of ASP were tested in addition to those of insulin. When preadipocytes were exposed to 1 mU/ml of insulin (observed to exert its maximal effect in our cell system, data not shown) together with an increasing concentration of ASP, plasma membrane glucose transport was increased above that observed with ASP alone. This effect was only statistically significant at the highest ASP concentration of 5.5 µM ($p < 0.05$). At the lowest concentration of ASP (0.3 µM) glucose transport was $4.8 \pm 0.51$ nmol/mg cell protein for ASP alone and $5.8 \pm 0.11$ nmol/mg cell protein for ASP and insulin and reached its maximum of $9.7 \pm 1.47$ nmol/mg cell protein and
Figure 8. **Comparison of glucose transport at different times of differentiation of human adipocytes in cell culture.** Freshly isolated preadipocytes were differentiated into adipocytes for a period of 1, 2 and 3 weeks. Glucose uptake was measured over 10 minutes with 0.05 mM of 2-DG following 1 hour incubation with and without 5.5 μM of ASP. The results are expressed as nmols of ^3H-2-DG taken up by the cells per mg of total cell protein. The graph shows an average of 3 experiments with duplicate determinations in each experiment. p<0.05 at the indicated points (*) and **) vs. basal.
Figure 9. The effects of ASP and insulin on glucose transport in cultured human preadipocytes. Cultured preadipocytes were exposed to ASP and ASP + insulin at 37°C for 1 hour with ASP concentrations of 0.3, 0.6, 1.8, 2.4, 2.8 and 5.5 μM and constant insulin concentration of 1 mU/mL. Glucose uptake was then measured over 10 minutes with 0.05 mM of ³H-2-DG. The results are expressed as % stimulation ± % SEM above basal glucose transport where basal glucose transport equals 100%. Basal levels (no additions) for preadipocytes were 4.37 ± 0.31 nmol/mg cell protein. The graph shows an average of 4-5 experiments with duplicate determinations in each experiment, p< 0.05 at the highest ASP concentration for cells treated with ASP and insulin vs. ASP alone.
11.9 ± 1.71 nmol/mg cell protein, respectively, at the highest concentration of ASP tested. Therefore, the effect on glucose transport appeared to be additive only at the highest ASP concentration tested. In contrast, in cultured differentiated adipocytes, even though the cells were responsive to ASP-induced glucose transport the additional stimulation with 1 mU/ml of insulin was not observed (Figure 10).

3.4 REGULATION OF ASP GENERATION

Adipocytes, when compared to preadipocytes or skin fibroblasts, have been shown to express high levels of mRNA for the three proteins involved in the generation of ASP, namely C3 - the precursor molecule, Factor B and adipin - the enzyme (35). When the culture media was analyzed for the presence of ASP, the average ASP levels were much higher in the media from differentiated adipocytes than from nondifferentiating preadipocytes (35). Previous results have demonstrated that plasma ASP levels rise in response to oral fat load (45). Moreover, very recent data from transcavillarly sampling experiments shows that adipocytes are the source of postprandial plasma rise in ASP (Sniderman, A.D., unpublished observation, work in progress). Based on these observations experiments were performed to establish which plasma component may be a potential regulator of ASP production from adipocytes in the postprandial state.

As portrayed on the graph in Figure 11, in cultured adipocytes basal ASP levels increase up to 4 hours at which time they reach a maximum of 297 ± 68 ng/mg cell protein (166%), slightly decrease by 8 hours and even more by 24 hours.
Figure 10. The effect of ASP and insulin on glucose transport in cultured human adipocytes. Culture differentiated adipocytes were exposed to ASP and ASP + insulin at 37°C for 1 hour with ASP concentrations of 0.3, 0.6, 1.8, 2.4, 2.8 and 5.5 μM and single insulin concentration of 1 mU/mL. Glucose uptake was then measured over 10 minutes with 0.05 mM of ^3H-2-DG. The results are expressed as % stimulation ± % SEM above basal glucose transport were basal glucose transport equals 100%. Basal levels (no addition) for adipocytes were 3.6 ± 0.41 nmol/mg cell protein. The graph shows an average of 4-5 experiments with duplicate determinations in each experiment, p=NS (not significant) at all concentrations for cells treated with ASP + insulin vs. ASP alone.
Figure 11. Basal ASP production in cultured human adipocytes. Human preadipocytes were differentiated in culture as described in Materials and Methods for 21 days. Preadipocytes and adipocytes were incubated in serum- and hormone-free media for 2, 4, 8 and 24 hours. Basal ASP content in the media was then measured by radioimmunoassay at each of the time points. The results are expressed as ng of ASP generated per mg cell protein ± SEM. Shown are the results from 3 experiments assayed in triplicates.
Therefore, to study ASP generation in cultured human adipocytes a six hour time point was chosen for all the incubations for most of the experiments since at this point the ASP production was observed to be within maximal range.

Since glucose and fatty acids provide essential building blocks for triacylglycerols and ASP is effective in stimulating triacylglycerol synthesis in human adipocytes from these substrates, we examined the effects of these two substrates on ASP production by human adipocytes differentiated in culture. ASP generation in culture media was measured by radioimmunoassay in the presence of increasing concentrations of glucose and oleic acid. The substrate concentrations used represent physiological values in humans ranging from fasting to postprandial levels for glucose and for fatty acids. Figure 12 shows data obtained from cells that were exposed for 6 hours to increasing concentrations of glucose (5.0 mM to 75.0 mM) The amount of ASP generated in the media bathing differentiated adipocytes did not increase above the baseline (315.01 ± 19.42 ng/mg cell protein) with varying glucose concentrations (p<NS, one-way ANOVA). The results from experiments in which increasing concentrations of oleate complexed to bovine serum albumin (up to 0.8 mM) were added to the culture media are shown in Figure 13. Again, the amount of ASP generated by the cells differed little from the baseline other than the small increase at the very low and very high oleate concentrations. At 0.015 mM the increase was 1.42 fold (p<NS) and at 0.8 mM it was 1.25 fold (p<NS, one-way ANOVA).

The capacity of cultured human adipocytes to generate ASP was also examined under different concentrations of insulin ranging from 0.01 mU/ml (fasting)
Figure 12. Concentration dependent effect of glucose on ASP generation by human adipocytes: Human preadipocytes were differentiated in serum free medium for 21 days. Cells were then exposed to increasing concentrations of glucose over a period of 6 hours. Medium ASP was measured (ng/ml/mg cell protein) at each of the concentrations by radioimmunoassay. Basal (5.0 mM glucose) levels are 315.01 ± 19.42 ng/mg cell protein. The results are expressed as fold change in ASP generated above the basal ± SEM, where basal values are given as 1.0 and n=3 experiments from 3 different subjects assayed in triplicate. p=NS (not significant) by one-way ANOVA.
Figure 13. **Concentration-dependent effect of oleate on ASP generation by human adipocytes:** Human preadipocytes were differentiated in serum-free medium for 21 days. Cells were then exposed to increasing concentrations of oleic acid complexed to BSA over a period of 6 hours. Medium ASP was measured at each of the oleate concentrations by radioimmunoassay. Basal levels (no oleate) are 278.67 ± 92.68 ng/mg cell protein. The results are expressed as fold change in ASP generated above the basal ± SEM, where basal values are given as 1.0 and n=3 experiments from 3 different subjects assayed in triplicate. p=NS (not significant) by one-way ANOVA.
to 100 mU/ml (pharmacological) (56). Insulin has a well documented effect on triacylglycerol synthesis through its action on glucose transport and hormone sensitive lipase (21). It is also an essential component for differentiation of mouse and human adipocytes (49). Figure 14 demonstrates that with increasing insulin concentrations there was a slow but steady rise of ASP in the media. The increase of medium ASP reached a maximum of 208% ± 19% (p<0.05. ANOVA) at a concentration of 50 mU/ml of insulin and reached a plateau thereafter.

Previous studies have shown that plasma ASP levels increase following an oral fat load in normolipidemic patients concomitant with an increase in plasma triacylglycerols (45). Lipoprotein particles are the plasma components that carry triacylglycerol and cholesterol in the circulation. Lipoprotein particles were, therefore, isolated by sequential ultracentrifugation from a normolipidemic individual, 4 hours postprandially as described in Materials and Methods section. Each lipoprotein fraction was assayed for triacylglycerol (TG) and cholesterol (CH) concentration. Chylomicrons and VLDL were added to the cultured cells at a concentration of 50 µg of TG/ml and LDL and HDL were added at a concentration of 25 µg of CH/ml. These concentrations were chosen because they represent the lower end of the physiological postprandial range (56).

Figure 15 depicts the changes in the media ASP levels 6 hours following incubations with either CHYLO, VLDL, LDL or HDL. As compared to basal values (no addition) there was a 171 ± 45 fold increase in the amount of ASP generated by the cells after exposure to chylomicrons, whereas all the other lipoprotein fractions had little effect on the cells as compared to CHYLO, p<0.05 for CHYLO.
Figure 14. Concentration-dependent effect of insulin on ASP generation by human adipocytes: Human preadipocytes were differentiated in serum free medium over a period of 21 days. Differentiated adipocytes were then exposed to increasing insulin concentrations for 6 hours. Medium ASP was measured by radioimmunoassay. Basal (no insulin) levels were 101.0 ± 35.1 ng/mg cell protein. The results are expressed as fold change in medium ASP generated above the basal ± SEM, where basal values are given as 1.0 and n=3 experiments from three different subjects assayed in triplicate. * p<0.05 by one-way ANOVA.
Figure 15. **Effect of lipoproteins on ASP generation by differentiated human adipocytes:** Human preadipocytes were differentiated in serum free medium over a period of 21 days. Differentiated adipocytes were then exposed to CHYLO, VLDL (50 µg TG/ml) or LDL and HDL (25 µg CH/ml) for 6 hours. LPL was added to all lipoprotein fractions at 10 mU/ml. Medium ASP was measured by radioimmunoassay. Basal levels (no additions) were 315.00 ± 130.61 ng/mg cell protein. The results are shown as fold change in medium ASP generated above the basal ± SEM, where basal values are given as 1.0 and n=3 experiments from three different subjects assayed in triplicate. Statistical significance was calculated using paired t-test, * p<0.05.
and p<NS for VLDL, LDL and HDL vs. basal as determined by paired t-test. Nonetheless, the effects of VLDL, LDL and HDL on ASP generation were still greater than the effects of fatty acids and insulin (note the difference in scale between figure 8, 9 and 10).

LPL is an enzyme made and secreted by adipose tissue and is necessary for the hydrolysis of plasma lipoprotein triacylglycerol (57). This enzyme was added to the cultured adipocytes at a concentration of 10 mU/ml to all incubations with lipoproteins. Addition of LPL alone or addition of LPL to lipoproteins had no effect on ASP generation by the cells (p=NS, paired t-test). Similarly, addition of BSA, which is a carrier protein for fatty acids, alone at the concentration of 1.0 mg/ml had no effect on medium ASP levels.

It should be pointed out that although there is some ASP associated with the chylomicron lipoprotein fraction, this amount of background ASP was subtracted from the total medium ASP. In all cases, the amount of ASP associated with the chylomicron fraction was not greater than 27% ± 6.8% of the total medium ASP generated by cultured adipocytes.

The effects of varying concentrations of chylomicrons on cultured human adipocytes are shown in Figure 16. The data demonstrate that increasing concentrations of chylomicrons result in an increase in ASP levels detected in the media of human adipocytes. This increase in ASP reaches 142.0 ± 64.9 fold at the highest concentration of 500 μg TG/ml of chylomicrons added. There was no plateau observed at the chylomicron concentrations tested; however, it should be noted that the concentrations chosen were still well within the range of physiological
Figure 16. **Dose-dependent effect of chylomicrons on ASP generation by human adipocytes:** Human preadipocytes were differentiated in serum-free medium over a period of 21 days. Differentiated adipocytes were then exposed to increasing concentrations of chylomicrons for 6 hours and ASP generated was measured in the cell media by radioimmunoassay. Basal (no additions) levels were 151.5 ± 27.2 ng/mg cell protein. LPL was added to all chylomicron concentrations at 10 mU/ml. The ASP concentration present in chylomicrons (background) was subtracted from total medium ASP. Background levels of ASP did not exceed 27% ± 6.8% of total medium ASP. The results are shown as fold change in medium ASP generated above basal ± SEM, where basal values are given as 1.0 and n=3 experiments from three different subjects assayed in triplicate, * p<0.05 determined by one-way ANOVA.
postprandial levels (56).

Finally, to investigate the response rate to the chylomicron stimuli, the adipocytes were exposed to chylomicrons at a selected concentration of 50 μg TG/ml for varying periods of time. In this set of experiments the cells were switched to serum- and supplement-free media 24 hours before medium ASP levels were measured. The chylomicrons were then added for the final 0, 2, 4, 6, 8 and 24 hours of the incubation period. As shown in Figure 17 the amount of ASP generated after 2 hours exposure to chylomicrons was almost the same as the baseline or at time zero with no exposure to chylomicrons (241.03 ± 17.10 ng/mg cell protein vs. 292.32 ± 16.22 ng/mg cell protein respectively). This level increased rapidly at 4-6 hours chylomicron exposure reaching a plateau of 821 ± 115 ng/mg cell protein (p<0.05, one-way ANOVA).
Figure 17. Time course of the chylomicron effect on ASP generation by human adipocytes: Human preadipocytes were differentiated in serum free medium over a period of 21 days. Differentiated adipocytes were then exposed to chylomicrons at 50 μg TG/ml medium for different lengths of time. ASP levels were measured in the cell media by radioimmunoassay. The results are expressed as ng/mg cell protein ± SEM for 2 experiments from 2 different subjects assayed in triplicate; * p< 0.05 determined by one-way ANOVA.
4. DISCUSSION

Initial interest in ASP was based on its function of stimulating triacylglycerol synthesis, and over several years, some aspects of ASP such as its definitive characterization, together with its mode of action, have been unraveled. ASP is a small (8932.5 Dalton), basic protein (pl 9.0) which was initially shown to stimulate triacylglycerol synthesis in human skin fibroblasts and freshly isolated mature fat cells (35, 38). Later, it was also observed that ASP had a stimulatory effect on glucose transport (40). However, the association between ASP and adipocytes extends beyond the effects of ASP on adipocytes. ASP is in fact produced by adipocytes (36).

The human adipocyte cell model was developed by modifying the initial procedure of Hauner and his colleagues (49). This cell model was of particular interest for a number of reasons. First, ASP is a protein which was isolated from human plasma and therefore a human cell model was specifically chosen instead of, for example, the murine 3T3 preadipocyte cell system, which has been used extensively (58). Second, the effects of ASP had previously been studied mainly in human skin fibroblasts but the tissue that is actively involved in synthesizing and storing triacylglycerols is, in fact, adipose tissue. It was previously shown that mature adipocytes are more responsive to ASP than fibroblasts (59). Third, the effects of ASP on differentiated human adipocytes could be studied and compared to the effects on preadipocytes - the adipocyte precursor cells. Fourth, although freshly isolated mature fat cells can be used
experimentally, the interpretations of studies done in that system must then take into account the influence of the environmental metabolic milieu acting on the tissue at the time of harvesting. Fifth, isolation of mature fat cells is done through collagenase digestion. This particular treatment has been shown not only to break up very large fat cells (which might be important) and damage plasma receptor proteins in the remaining cells but also to decrease secretion of certain proteins such as lipoprotein lipase (60). Finally, in recent years adipose tissue has been shown to be not only a storage site, but a metabolically active secretory organ for proteins and non-protein factors involved in lipid metabolism (61). Lipoprotein lipase, cholesterol ester transfer protein, apolipoprotein E and recently adipisin and complement C3 are only a few molecules shown to be made and secreted by adipose tissue. For that reason, the human adipocyte cell model offers a fully controlled system to study not only ASP action but also ASP generation in the basal and stimulated states.

Using the human adipocyte cell model, I first examined the effects of ASP on triacylglycerol synthesis and glucose transport. In the second part of this thesis, attention focused on acute regulation of ASP generation. One shortcoming of this model is the fact that human adipocytes cannot be propagated in culture for long periods of time and so only primary cultures can be used. Each experiment, therefore, must be done on cells obtained from different subject and thus a wide range of responses from one cell line to another is seen. The experiments are validated by including numerous internal controls which take into account this natural variation.
The results of the experiments show that ASP stimulates triacylglycerol synthesis to a much greater extent in adipocytes than in preadipocytes indicating that as the preadipocytes enter the differentiation pathway they become much more responsive to ASP than their precursors. Moreover, the stimulatory effects of ASP seen here are greater than shown previously in human skin fibroblasts but are similar to those observed in mature fat cells (59). This observation supports the use of the human adipocyte cell model for studying the role of ASP in lipid metabolism.

The response of adipocytes to an ASP challenge was observed at low ASP concentrations which raises the possibility that adipocytes may have an increased number of ASP receptors. This hypothesis was confirmed in very recent preliminary experiments in our laboratory (43). For this reason one might suggest that adipose tissue is not only the primary site of ASP action but also that any changes affecting either the production of ASP and/or of its receptor might result in dysfunctional storage of fat in adipose tissue.

Furthermore, in the present work, the stimulatory effect of ASP on glucose transport in adipocytes has been demonstrated for the first time. It was observed that ASP stimulates glucose transport in adipocytes to a comparable extent to that seen in human fibroblasts and in L6 muscle cells (40, 41). In addition, the maximum effect is accomplished at very low ASP concentrations. As with cultured fibroblasts and L6 muscle cells, the maximal stimulation with ASP is comparable to that of insulin.
There is now strong evidence that ASP affects the translocation of not only Glut 1, but also Glut 4 and Glut 3 glucose transporters from intracellular pools to plasma membrane in specific cells. In addition, preliminary observations suggest that ASP may also affect the intrinsic activity of the Glut 1 transporter. However, it is the Glut 4 glucose transporter that is specific to muscle and adipose tissue and the changes in the levels of expression of this transporter in adipose tissue have been implicated in the development of obesity (62). It is also known that translocation of Glut 4 is the means by which insulin stimulates glucose transport in fat and muscle (63). It would, of course, be of great interest to see which glucose transporter is affected by ASP in adipocytes and preadipocytes. However, due to limited availability of human adipose tissue and, therefore, the number of cells necessary for differentiation, these experiments were not feasible.

As preadipocytes differentiate into adipocytes the responsiveness to ASP becomes much more rapid and the maximum effects are achieved at physiological concentrations of ASP. This is seen whether we monitor triacylglycerol synthesis or glucose transport. This coordinate response of adipocytes at levels of ASP found in the circulation further supports a physiological role for ASP.

It was particularly interesting to see, in the case of glucose transport, that at much higher ASP concentrations preadipocytes become as responsive as adipocytes. Since preadipocytes are in some aspects similar to adipocytes it cannot be excluded that, under specific conditions, the ASP effects on this
particular group of cells may facilitate the entry of these cells into the differentiation pathway. In fact, preliminary experiments give an indication that ASP does facilitate the differentiation process of preadipocytes (I. Murray, M. Maslowska, K. Cianfione, unpublished observations). The fact that the effects of ASP are sustained may be of importance as well. In particular, this may suggest that as the adipose tissue responds to the nutritional challenge, adipocytes are the first cells that become rapidly recruited to take up the incoming glucose and the liberated free fatty acids for storage. In some situations, however, when the challenge becomes overwhelming, preadipocytes must be recruited as well. In this way the tissue could increase not only the size of the existing adipocytes but also the number of cells that will be "the rapid responders". Indeed, this may be the pathophysiologic process which ultimately leads to obesity.

An important question to address at this point was the interaction between the effects of ASP and those of insulin. Are these two powerful agents acting independently? Results presented in this thesis show that the stimulatory effects of ASP on glucose transport are most probably independent of the stimulatory effects of insulin in preadipocytes. However, this was not the case in differentiated adipocytes. There is one possible explanation for this phenomenon. Stimulated glucose transport is achieved by the means of recruiting specific glucose transporters. In adipose tissue, there are two different glucose transporters involved, the Glut1 and the Glut4 transporters. Their levels of expression differ not only during the differentiation course of adipose cells. It
is possible that the degree of translocation, as well as the intrinsic activity of these transporters might be affected differently in preadipocytes and in adipocytes. Regrettably, this particular enigma could not be answered in this thesis leaving room only for speculations that in preadipocytes, ASP and insulin effects are via two different mechanisms but in adipocytes, most probably, both act via the same mechanism.

The extent of ASP's role in the regulation of lipid metabolism is becoming increasingly relevant and at the same time very challenging. The striking effects of ASP on both triacylglycerol synthesis as well as on glucose transport in cultured human fibroblasts (40) and now in cultured human adipocytes, have not only substantiated the view that the triacylglycerol synthetic pathway may be regulated but also motivated further studies towards understanding the regulation of ASP itself. ASP not only has marked effects on adipocyte metabolism, but is also produced by differentiated adipocytes (36). Based on these observations, the second part of this thesis examined which particular stimuli modulates ASP production in cultured cells. The results of these experiments provide the first evidence that ASP generation from human adipocytes can be modulated by specific plasma components.

Neither glucose nor oleate had significant effects on ASP generation in the media. The fact that glucose and free fatty acids did not cause increases in media ASP levels was initially surprising since both factors increase postprandially and we have previously demonstrated that ASP levels also rise during that time (45). However, it should be noted that fatty acids can also be generated through the
action of hormone sensitive lipase from adipocytes for export to other tissues (24). An increase in ASP production at this point would indeed be paradoxical and would result in a futile cycle of lipolysis/lipogenesis.

The experimental results indicate that, at high concentrations, insulin significantly increases ASP production. Although a direct effect on the conversion of C3 to ASP cannot be ruled out, insulin may be affecting the secretion of adipsin (the catalytic enzyme), as shown previously in murine clonal adipocytes (64) as well as (hypothetically) C3 and factor B.

It was shown in fat-load studies that plasma ASP levels rise concurrently with the rise of plasma triacylglycerols (45). It was speculated, therefore, that the triggering signal might lie within the lipoprotein particles themselves. To test this hypothesis, experiments were performed in which ASP production was assessed following the exposure of the cells with different plasma lipoprotein fractions (chylomicrons, VLDL, LDL and HDL). Addition of chylomicrons to cultured adipocytes caused dramatic elevations in media ASP, whereas other lipoproteins had relatively little effect. This effect was both time and concentration dependent. This suggests that the signalling mechanism is on the chylomicron particle itself. The lack of effect by other lipoprotein particles may provide a clue.

Certainly the apolipoprotein composition of chylomicrons is different from the other lipoproteins. For example, chylomicrons are the only lipoprotein particle that contain apolipoprotein B-48, the remaining apolipoprotein B containing lipoproteins (VLDL and LDL) contain a hepatic form of apolipoprotein B, apolipoprotein B-100. In addition to differences in apolipoproteins, chylomicrons also contain lipid
components not present in the other lipoproteins, such as retinol ester. Thus there are several potential candidates for the active factor. Further studies will focus on defining the active component in chylomicrons and the mechanism by which it affects increases in ASP production. Although postprandial increases in insulin may be one mechanism by which plasma ASP levels increase, based on the magnitude of the increase in ASP achieved with the addition of \textit{in vivo} concentrations of chylomicrons (which far exceeds the insulin effect) this mechanism is most likely the physiological source of the postprandial increase in ASP levels.

What is the mechanism by which postprandial factors (chylomicrons and insulin) increase media ASP concentration? Production of ASP is dependent on three protein factors (C3, factor B and adipsin), two of which are consumed during the reaction (C3 and factor B) whereas adipsin, as a catalytic enzyme is not. It is, therefore, possible to stimulate ASP production by increasing cellular secretion of any one of these three proteins, or by enhancing the catalytic conversion reaction itself. With respect to the first mechanism, C3 and factor B secretion can be regulated under specific stimuli in a number of different cells. In addition, human adipocytes have also been shown to express increased levels of mRNA for C3 and factor B (36) and recently shown to secrete these proteins (T. Scantlenbury, unpublished observation). In the case of adipsin, however, expression in humans is primarily confined to adipose tissue (33). This is particularly relevant since the generation of ASP is dependent on both the presence of the catalytic enzyme adipsin and on C3 and factor B.
Based on the observations presented in this thesis, Figure 18 illustrates a proposed mechanism of ASP generation. Adipocytes are known to make and secrete lipoprotein lipase (LPL) - an enzyme which is translocated from adipose tissue onto the luminal surface of endothelial cells (65). It is possible that, as adipocytes secrete complement C3, factor B, and adipsin, these proteins are also translocated to the vascular space through a mechanism similar to that responsible for LPL translocation. When dietary fat enters the circulation in the form of a chylomicron particle, the particle associates with the endothelial cell surface lining the blood vessel. The lipoprotein particle binds the endothelial surface through interaction with LPL located on the endothelial cell surface and apolipoprotein CII. Apolipoprotein CII is the cofactor for LPL and is located on the surface of the chylomicron particle, resulting in TG hydrolysis. It is possible that during the same time another chylomicron protein or lipid component facilitates attachment of C3, an event which could conceivably cause the activation of the proximal portion of the alternate complement pathway. The activation of this pathway, which involves C3, factor B and adipsin, would thus account for the postprandial generation of ASP. In addition, a particular component of chylomicrons may also enhance adipocyte secretion of the ASP precursor protein C3 as well as the two factors necessary for C3 cleavage - factor B and adipsin.

The scenario outlined above would result in the generation of ASP which in turn will act back on the adipose tissue itself to increase triacylglycerol synthesis and glucose transport. In this way the adipose tissue is primed to efficiently sequester any excess dietary plasma fatty acids and glucose for storage.
Figure 18. Proposed model for ASP generation.
One can then see how excessive efficiency of this system might be associated with obesity. And indeed, plasma ASP levels are 2-3 times higher in obese people than in age-matched, normal weight individuals (47). Fasting of obese individuals results in substantial decreases of plasma ASP to within the normal range (47). This is seen even in the absence of large decreases in adipose fat mass indicating that the amount of ASP produced per fat mass, has clearly been down-regulated. Similarly, small but significant variations in plasma adipsin levels are also found in human obesity (increased adipsin) and eating disorders such as anorexia nervosa (decreased adipsin) (66).

In summary, ASP is a potent stimulator of triacylglycerol synthesis and glucose transport in adipocytes. Moreover, levels of ASP can in fact be regulated in vivo with a chylomicron challenge. These data suggests that modulations of plasma ASP levels in response to dietary fat intake may play a physiological role in positive feedback regulation of adipose tissue fat mass.
6. REFERENCES


24. Belfrage, P., Fredrikson, G., Olsson, H., Stralfors, P., Regulation of adipose tissue lipolysis through reversible phosphorylation of hormone-sensitive


33. White, R T., Damm, D., Hancock, N., Rosen, B.L., Lowell, B.B., et al, Human adipsin is identical to complement factor D and is expressed at high levels of adipose tissue., J. Biol. Chem., 267: 9210-9213, 1992


62. Shepherd, P.R., Gnudi, L., Tozzo, E., Yang, H., Leach, F., Kahn, B., Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue., J. Biol. Chem.

