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Acylation Stimulating Protein (ASP) Structure & Function Studies: *in vitro* and *in vivo* in mouse models

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctorate of Philosophy

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Occams Razor; Proposes that the fewest possible assumptions are to be made in explaining a thing.

William of Occam

"Nature goes her own way and all that to us seems an exception is really according to order."

Johann Wolfgang von Goethe

Abstract

Acylation stimulating protein (ASP or C3a desArg) is a complement derived product produced by adipocytes and is able to alter their metabolism, stimulating both triglyceride synthesis and glucose transport. This stimulatory activity has been shown to be due to ASP as both plasma derived protein and recombinant protein stimulate glucose transport and triglyceride synthesis. Furthermore, we demonstrated that ASP is functionally distinct from C3a. A two site model for ligand interaction with the receptor is proposed with the carboxy-terminal involved in receptor binding and the disulphide core in stimulating triglyceride synthesis. Functionality of ASP *in vivo* supported the hypothesis that ASP is involved in plasma triglyceride and glucose clearance postprandially after a fat load. The following data were obtained: 1. Administration of ASP and ASP functional knockouts displayed increased and delayed triglyceride clearance respectively. 2. Administered ASP decreased plasma glucose levels, which was independent from its effects on plasma triglycerides. 3. In ASP functional knockout mice gender differences of greater postprandial lipemia in males and more pronounced reductions of adipose tissue in females were observed. In conclusion the function of ASP was determined as well as structural regions involved. Furthermore, the *In vivo* physiology of ASP has been determined, with an effect on postprandial metabolism, regulation of adiposity and gender dependent penetrance of the ASP functional knockout phenotype.

Abrégé

Acylation Stimulating Protein (ASP ou C3a desArg) est une protéine dérivée du système complément qui est produite par les adipocytes, et qui est capable d'influencer leur métabolisme en stimulant la synthèse de triglycérides et le transport du glucose. L'activité stimulatoire sur la synthèse de triglycérides due à l'ASP isolée à partir du plasma, est pareil à l'effect de l'ASP recombinante, et les deux ont un effet identique sur le transport du glucose et la synthèse de triglycérides. En plus, nous avons démontré que l'ASP est fonctionnellement distincte de C3a. Nous avons proposé un modèle d'interaction à deux sites entre le ligand et le recepteur: le terminal-carboxylique est impliqué dans la liaison avec le récepteur, et la structure interne disulphure impliquée dans la synthèse de triglycérides. Le fonctionnement d'ASP *in vivo* supporte l'hypothèse que l'ASP est impliquée dans le processus postprandial d'élimination, dans le plasma, du glucose et triglycérides, suite à un repas riche en graisses. Les données suivantes ont été obtenues: 1. Les souris injectées avec de l'ASP exogène démontrent un taux d'élimination des triglycérides augmenté, tandis que les souris "knockouts" ont un taux d'élimination retardé. 2. Les niveaux de glucose dans le plasma sont inférieurs chez les souris injectées d'ASP, indépendamment des triglycérides. 3. Chez les souris ASP "knockouts" fonctionnelles, il y a une différence de genre: les mâles ayant une lipémie postprandiale supérieure, et les femelles ayant une réduction beaucoup plus prononcée du tissu adipeux. En conclusion, la fonction de l'ASP a été déterminée, ainsi que les régions structurelles impliqués. En plus, la physiologie *in vivo* de l'ASP a été déterminée, avec un effet sur le métabolisme postprandial, sur la régulation de la masse adipeuse, et un effet spécifique de genre sur les souris de phénotype ASP "knockouts".

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Index of Abbreviations

(-/-)	denotes knockout mouse
(+/+)	denotes wildtype mouse
(129SvxC57Bl/6)	hybrid mouse, strains in brackets
129Sv	mouse strain
2-DG	2 deoxyglucose
3T3	murine embryonic fibroblast cell line
A/J	mouse strain
ACTH	adrenocorticotropic hormone
ASP	acylation stimulating protein
Asp-N	endoproteinase Asp-N
ANOVA	analysis of variance
AUC	area under the curve
B1, BK1	bradykinin 1
B2, BK2	bradykinin 2
BSA	bovine serum albumin
C57Bl/6	mouse strain
C/EBP	CCAAT enhancer binding protein
C3	complement C3
C3a	complement C3a
C3aR	c3a receptor
cAMP	adenosine 3',5'-cyclic monophosphate
cASP	*in vitro* complement derived ASP
CD	circular dichroism
CNBr	cyanogen bromide
db/db	diabetic mouse mutation, natural
DEPC	diethyl pyrocarbonate
DGAT	diacylglycerol acyltansferase
DMEM/F12	dubelco's minimal esential medium F12
Fn	filial generation, n=number
FATP	fatty acid transport protein
FABP	fatty acid binding protein
FFA	free fatty acids
Glut	glucose transporter
HDL	high-density lipoprotein
HF	high fat diet
HPLC	high pressure liquid chromatography
HSL	hormone sensitive lipase
IC_{50}	50% inhibitory concentration
IgG	immunoglobulin G
k_d	dissociation constant
LDL	low-density lipoprotein
LF	low fat diet
LPL	lipoprotein lipase
MRG	membranoproliferative glomerulonephritis

MW	molecular weight
NEFA	non-esterified fatty acids
Neo	neomycin
NMR	nuclear magnetic resonance
ob/ob	obese mouse mutation, natural
OD	optical density
PAGE	polyacrylamide gel electrophoresis
pASP	plasma derived ASP
PBS	phosphate buffered saline
pI	isoelectric point
PKC	protein kinase C
PLD	partial lipodystrophy
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
rASP	recombinant ASP
RIA	radioimmunoassay
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosus
SREBP	steroid response element binding protein
TCA	trichloroacetic acid
TG	triglyceride
TGS	triglyceride synthesis
TGSS	triglyceride synthesis stimulation
TNF	tumour necrosis factor
UCP	uncoupling protein
U937	macrophage type cell line
V_{max}	maximum rate
VLDL	very-low density lipoprotein
WAT	white adipose tissue

Acknowledgements

I would like to express my greatest gratitude to both my supervisors, Dr Allan.D. Sniderman and Dr Katherine Cianflone. They have been instrumental in my development during my graduate education, and they have had always generously provided not only explanations, discussions and technical help but also critical insights into the processes at hand.

I would also like to thank the people with whom I shared a room for many years, performing research and contributing to the discussions and generation of concepts Magdalena Maslowska and David Kalant. Magda has helped with posters, presentations and cell culture while David has aided with logical and theoretical aspects.

As a general acknowledgment, I must thank Magdalena Maslowska and Thea Scantlebury for their technical assistance in provision of cells and cell culture, David Kalant for the radiolabelled ASP and initial setup of the competition binding protocol, Steve Phelis for the purification and measurement of human ASP.

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Dr. A. Bell for his discussions in protein modifications, Dr Bernie Gibbs for his igniting my interest in protein modeling, Drs W. Chazin and M. Kalnik for the provision of the Complement C3a solution co-ordinates, Dr J. Turnbull for aid in protein modification and circular dichroism, Dr A. English for Mass Spectrophotometer analysis, Dr J, Westerlund who was an external advisor from Merck Frosst. And finally our collaborators Dr J. Kohl for provision of modified peptides, Drs R. A. Parker and T.G. Kirchgessner for the recombinant ASP bacteria.

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Personnel of the Royal Victoria Animal Centre and McGill Animal Resources Centre: Mrs D Greinke, Dr. Scriber's laboratory , Dr R. Sladeck and Ms M. McAuliffe for consultations and advice regarding animal experiments. I would like to thank my collaborators: and Drs R. Wetsel, H. Colten for the transgenic mice, Dr Lan-Ying Chen for research into tissue distribution of lipids in knockout mice and finally Dr P. Havel for measurement of murine leptin levels.

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Preface I

The faculty of Graduate studies and research of McGill University requires that the following excerpt be cited in full in the introductory section of a thesis containing original publications as part of the text:

As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers that have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)
2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.
3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

   The thesis must include the following:

   (a) a table of contents;

   (b) an abstract in English and French;

   (c) an introduction which clearly states the rational and objectives of the research;

   (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper);

   (e) a final conclusion and summary;
4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in

sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

6. When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

7. Irrespective of the internal and external examiners reports, if the oral defense committee feels that the thesis has major omissions with regard to the above guidelines, the candidate may be required to resubmit an amended version of the thesis. See the "Guidelines for Doctoral Oral Examinations," which can be obtained from the web (http://www.mcgill.ca/fgsr), Graduate Secretaries of departments or from the Thesis Office, Dawson Hall, Room 409, 398-3997.

8. In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis.

The following papers, published, in press or in preparation are presented as part of the text of this thesis. Chapter 2 is published, chapters 3, 4 and 5 are in press and chapter 6 is a manuscript in preparation.

Chapter 2: Murray I, Parker, R.A., Kirchgessner T.G., Tran J., Zhang Z.J., Westerlund J. and Cianflone K. Functional bioactive recombinant acylation stimulating protein is distinct from C3a anaphylatoxin. J Lipid Res. 1997 Dec;38(12):2492-501.

Chapter 3: Murray I, Kohl, J, Cianflone K. Acylation Stimulating Protein (ASP): Structure:Function determinants of cell surface binding and triglyceride synthetic activity. Biochem. J. 1999 342 (Pt 1): 41-48

Chapter 4: Murray I., Sniderman A.D., Cianflone K. Enhanced triglyceride clearance with intraperitoneal Acylation Stimulating Protein (ASP) in C57Bl/6 mice. Am. J. Physiology (Endo & Metab) 1999 277(3): E474-E480.

Chapter 5: Murray I, Sniderman A.D., Cianflone K. Mice lacking Acylation Stimulating Protein (ASP) have delayed postprandial triglyceride clearance. 1999 J. Lipid Res. 40: 1671-1676.

Chapter 6: Murray I., Sniderman A.D., Cianflone K. et al Female Mice lacking Acylation Stimulating Protein (ASP) have reduced body weights and leptin levels. 1999 Submitted Endocrinology.

Chapter 1 is an introduction providing a comprehensive overview as well as the objectives and rational for the research described in the dissertation. The conclusion and summary is a cohesive overview of the findings, the physiological role of ASP and future directions. The general references for the introduction and summary are presented at the end of the thesis.

Contribution of Authors

The data presented here are the results of my five years of laboratory research at Mike Rosenbloom's Laboratory for Cardiovascular Research.

Chapter 2 (Functional bioactive recombinant acylation stimulating protein is distinct from C3a anaphylatoxin) is the result of a collaborative effort between the members of our laboratory and at Bristol-Myers Squibb. I have carried out the majority of the work, determining the protocols for purification of the recombinant and performing the assays of triglyceride synthesis and optimizing conditions for competitive binding. It is necessary to acknowledge Drs. Parker and Kirschgessner for their role in producing the construct and transfection of the bacteria, the intracellular calcium and glucose transport assays, Dr Zhang for performing the immunoprecipitation experiments, Dr J Westerlund for his role as an external advisor from Merck Frosst, Ms J Tran a summer student who aided in recombinant ASP purification and Dr Cianflone for her initial studies in stability and recovery of ASP.

Chapter 3 (Acylation Stimulating Protein (ASP): Structure:Function determinants of cell surface binding and triglyceride synthetic activity). I undertook all of the chemical and enzymatic modifications and purifications of ASP described in the paper. Dr Alex Bell and Dr Turnbull were instrumental in discussions as to the modifications of the protein, with Dr Turnbull training and allowing us to use her Circular Dichroism spectrophotometer. Dr Anne English and her students performed the mass spectrophotometry, while both Dr Bell and I analyzed the results. Dr J $\bar{r}$g Kohl kindly provided the C-terminal peptides for the experiments.

Chapter 4 (Enhanced triglyceride clearance with intraperitoneal Acylation Stimulating Protein (ASP) in C57Bl/6 mice). All experiments and assays were performed by myself, wit hthe exception of measuring human plasma ASP. I would like to thank Steve Phelis for the ASP measurement. Furthermore, I must acknowldge McGill University Animal Resources, Rob Sladeck, Annie Mattheson both of Dr Gigueres Lab., Malia McAuliffe of Merck Frosst and Dr Schrieber's Laboratory at Montreal Children's Hospital for assiatnce in setting up animal protocols.

Chapter 5, 6 were experiments performed on mice bred from stock kindly provided by Dr Rick Wetsel and Dr Harvey Colten. All experiments and measurements (except leptin)

were performed and setup by myself. Dr Katherine Cianflone was of great assistance in dissection and assistance in the preparation of the manuscripts, Thea Scantelbury, Jumana Saleh, along with summer students, aided in food and animal weighing. Rob Sladeck provided protocols for the glucose tolerance test and Dr Scriber's lab demonstrated the tail bleeding technique. Lastly but not least, Dr Sniderman who provided insightful interpretation and experimental input.

Work performed during the course of my training has appeared in the following co-authored abstracts and publications:

Abstracts

1. Murray I, Sniderman, A.D.S, Phelis S, Wetsel, R, Colten H, Cianflone K (1998). Postprandial triglyceride metabolism in a functional Acylation Stimulating Protein (ASP) knockout mouse. O120 Intl J Obesity, 22 (Suppl 3): O120 pg S33
2. Cianflone K, Murray I, Kalant D, Zhang Z, Sniderman AD (1996) Structure-function analysis of human and recombinant acylation stimulating protein. Circulation 94(8): I-347
3. Murray I, Maslowska M., Sniderman AD, Cianflone K (1995) Acylation stimulating protein (ASP) and preadipocyte differentiation. Clin. Invest. Med. 18 (Suppl. B): B43
4. Cianflone K, Maslowska M, Murray I, Sniderman AD (1995) The ASP pathway: Evidence for a positive feedback loop in triglyceride syntheis in human adipocytes. Int'l . J. Obesity 19 (Suppl 2): 69
5. Maslowska M, Sniderman AD, Murray I, Cianflone K (1995) The ASP pathway: Evidence for a positive feedback loop in human adipocytes. Circulation Suppl. 1: 92(8):I-427.

Publications

1. Murray I, et al (1999) Mice lacking Acylation Stimulating Protein (ASP) have delayed postprandial triglyceride clearance. J. Lipid Res. 40: 1671-76.
2. Murray I, et al (1999) Acylation Stimulating Protein (ASP): Structure:Function determinants of cell surface binding and triglyceride synthetic activity. Biochem. J. 342(Pt 1): 41-48
3. Murray I, et al (1998) Enhanced triglyceride clearance with intraperitoneal Acylation Stimulating Protein (ASP) in C57Bl/6 mice. Am. J. Physiology (Endo & Metab) 277(3): E474-E480.
4. Murray I, et al (1997) Functional bioactivity of recombinant Acylation Stimulating Protein is distinct from that of C3a. J. Lipid Res. 38(12): 2492-2501.

Introduction

The main focus of the present introductory chapter is of energy storage, in particular its relationship to the effects of acylation stimulating protein (ASP) on *in vitro* stimulation of triglyceride synthesis and *in vivo* postprandial triglyceride, free fatty acids and glucose clearance from the plasma compartment and obesity.

The major energy storage forms are proteins, glycogen and fat, with the latter two playing a much more prominent role in energy metabolism under normal conditions. When energy is in excess of that required metabolically, it is shunted into storage. Glycogen is the primary short-term energy storage site unit and its levels are closely regulated as it can readily be converted back to glucose. Fats are the most economic storage unit, due to their relative inertness, low hydration, density and thus high-energy yield per weight. Although the storage capacity of fats is regulated, the expansion of adipose tissue is remarkable and in extreme cases can reach 57% of the body weight (1).

Part 1 Postprandial Metabolism

1.1 Dietary Input

The primary source of energy in mammals is derived from the diet. The ingested carbohydrates, fats and proteins are catabolised in the gastrointestinal tract. Carbohydrates are converted to saccharides and oligosaccharides, while peptides are digested to short peptides and amino acids. The fats are emulsified with bile salts, increasing their surface area for interaction with pancreatic and intestinal lipases to form glycerol, mono and diglycerides and various chain length free fatty acids (FFA). The liver plays a central role in regulating the concentrations of substrates taken up directly *via* the hepatic portal system, such as medium and short chain free fatty acids, peptides and sugars, exiting the liver into the general blood circulation. However the long-chain FFA are re-esterified into triglycerides within the lacteals in the intestinal villi and bypass the liver.

1.2 Triglyceride Rich Plasma Lipoproteins

The re-esterified long chain FFA form lipoproteins (chylomicron) particles consisting of a monolayer amphipathic phospholipids and cholesterol with a core of cholesterol (4%) and triglycerides (90%) (2). These chylomicrons, dietary lipoproteins, have apoproteins associated with their surface including apoprotein B_{48}, A-IV, E, C-I and C-III (2), (3). The chylomicrons bypass the liver completely and are transported by the lymphatic system, to be released into the venous circulation. Here they acquire the apoprotein C-II *via* exchange with HDL (4). Upon passage through capillaries of tissues such as adipose and muscle that express the enzyme lipoprotein lipase (LPL), the LPL co-factor (apoC-II) activates LPL resulting in hydrolysis of the triglyceride of the chylomicron particle. In humans approximately 50% of the FFA released by LPL are taken up in the periphery with the remainder entering the circulation bound to albumin (5), while the liver removes the delipidated chylomicron remnants. One plausible reason for the chylomicron particles to bypass the liver is to prevent uptake and re-secretion of lipoproteins, a futile cycle. This has been demonstrated experimentally with liver specific expression of LPL in a transgenic lacking LPL. The consequence of LPL expression in the liver, where expression is normally absent, is liver specific chylomicron clearance and increased secretion of liver lipoproteins (6).

The liver also secretes lipoproteins, smaller in size than the chylomicron, termed very low-density lipoproteins (VLDL). The structure is similar to that of chylomicrons and contains similar structural and targeting surface apoproteins. Chylomicrons however, are present only after a meal, are relatively short-lived whereas VLDL are constitutively secreted, contain less core TG and a longer splice variant of apoprotein B_{100} (apoB) rather than $apoB_{48}$ (intestinal). The clearance of VLDL in the periphery is also mediated by the LPL specific pathway and competes with chylomicrons for clearance (7). Reduction of the triglyceride content of the VLDL results in smaller particles with less triglyceride and a greater relative enrichment in cholesterol and cholesterol ester particles (namely intermediate density lipoprotein IDL). IDL can be cleared by the liver or further hydrolyzed to form low-density lipoprotein (LDL) which has a long plasma-half life.

Competition for clearance between lipoprotein particles, particularly chylomicrons and very low-density lipoprotein (VLDL), also exists. VLDL is less efficiently

hydrolyzed than chylomicrons, accumulating in plasma after injection, and this has been proposed to be due to competition for sites on the enzyme lipoprotein lipase (LPL) (8). This raises the question as to how is chylomicron hydrolysis more rapid than VLDL, although the VLDL numbers are increased. This differential hydrolysis may be a function of differences in binding affinity for LPL, with chylomicrons possessing greater binding affinity than VLDL (9).

1.3 Lipoprotein lipase

Hydrolysis of the triglyceride within these triglyceride-rich lipoprotein particles (chylomicrons and VLDL), mediated by the enzyme LPL results in free fatty acids and glycerol release. Complete absence of LPL in a knockout mouse results in severe hypertriglyceridemia and early neonatal death (10). LPL is secreted from predominantly adipose and muscle, tissues with the greatest demands for FFA (11). The LPL is then translocated to the luminal surface of the endothelial cells, where it forms an active homodimer attached via charge interaction with glycosaminoglycans (negatively charged heparan sulphate). It has been proposed that active LPL is sequestered in vesicles and thereafter released upon stimulation by insulin (12).

The hydrolysis of lipoprotein triglycerides by the rate-limiting enzyme LPL is controlled in several ways. Firstly, LPL can be inhibited by its end-product, fatty acids. In the presence of excess fatty acids, LPL is released from the endothelial cell surface and inactivated (13). FFA have also been shown to interfere with the activation of LPL by the co-activator apolipoprotein C-II (14). This results in feedback regulation of lipoprotein hydrolysis and could regulate the entry of FFA into tissues. Free fatty acids can reduce secretion of LPL and interfere with its subsequent translocation (15). The levels of expression of LPL also govern the site at which hydrolysis can occur. LPL is reciprocally expressed in adipose and muscle, with increased expression in the adipose tissue postprandially while there is increased expression in muscle during fasting (review (16). While adipose has traditionally been thought of as the major sink for fatty acids from lipoprotein hydrolyzed triglycerides, muscle has been shown to play an active role as well. In studies by Eckel, muscle was shown to be the primary site of free fatty acid uptake in the early postprandial period in normal weight male rats (17), with

redistribution to adipose later. In other studies, muscle specific LPL expression, onto a LPL knockout mouse, was able to reduce lipoprotein levels to normal (18). Presumably this normal hierarchy is to satisfy energy requirements of the muscle before the excesses are stored in adipose tissue. Finally, in addition to acute postprandial FFA and tissue regulation of LPL, its activity is also enhanced by insulin. The mass is increased with a high fat diet and activity is reduced by catecholamines. Recently it has been shown, *in vivo*, that inactive LPL is able to bridge between lipoprotein particles and proteoglycans or lipoprotein receptors, enhancing lipoprotein clearance (19). This places LPL in a central role for distribution of fats (review (16) and energy storage.

However LPL levels and activity have not been found to correlate with the triglyceride clearance from the plasma. It has been shown that post-heparin LPL levels are in excess of that required for rapid triglyceride clearance (20) and that there was only a weak correlation of triglyceride clearance and LPL levels (21). If LPL is present in excess for triglyceride clearance, perhaps the displaced LPL levels (pre-heparin) would correlate more closely with lipolysis in the tissue beds as shown by Eckel (22). Indeed pre-heparin LPL mass or activity has been found to correlate with triglyceride rich lipoproteins (23), (24), free fatty acids (25) and also for essential FFA (26). It is also possible that other lipases contribute to postprandial lipoprotein clearance, such as hepatic lipase (7) especially in mice where the murine hepatic lipase is present in plasma in contrast to human hepatic lipase (27). Hepatic lipase may not contribute significantly to lipoprotein hydrolysis however, as its levels in humans are not thought to change significantly postprandially (28), (26). Thus LPL plays a significant role in regulation of tissue site and rate of hydrolysis, and the microenvironmental control of this hydrolysis, as evidenced by pre-heparin LPL studies, can regulate substrate supply.

Plasma triglyceride clearance depends upon LPL activity. Overloading of LPL such as in insulin resistance, with defective insulin suppression of VLDL secretion, LPL deficiency or postprandial overloading of fat can occur (29). Patients displaying heterozygosity for a genetic LPL deficiency have hypertriglyceridemia (30), (31) and delayed triglyceride clearance (32). This indicates that when LPL levels are reduced, triglyceride clearance is reduced. This underlies the multi-step clearance of plasma triglycerides involving initial hydrolysis followed by clearance of the FFA generated.

This multi-step triglyceride clearance is seen in a defect in the ASP pathway. It has been poatulated that due to a defect in the ASP pathway, reduced fatty acid trapping would feedback to inhibit LPL, resulting in not only delayed triglyceride clearance but also elevated FFA levels (33).

1.4 FFA

Once the FFA are released from the triglyceride rich particle, they enter the cell or bind to albumin in plasma. The FFA in the cell can either be utilized to produce energy or converted into triglycerides. Considerable debate remains as to whether cellular uptake of FFA is mediated by passive diffusion or active transport. Specific saturable and temperature sensitive cellular uptake processes have been shown to exist for fatty acids (34). Fatty acid transporters have been found in animal cells, with increased expression upon differentiation of preadipocytes to adipocytes and high expression levels also found in skeletal muscle and heart (35). It has been proposed that perhaps both processes occur with specific transport being low capacity/ high affinity and diffusion being high capacity / low affinity (34). A defect in a fatty acid transporter isolated by Abumrad (36) CD36 or fatty acid translocase (FAT) accounted for the majority of the fatty acid lipolytic release defect, free fatty acid elevation in plasma and a portion of the insulin resistance in rats (37). Other membrane fatty acid transport proteins, such as plasma membrane fatty acid binding protein (FABPpm) and fatty acid transporter protein (FATP) have also been identified. The homology of fatty acid transporters and evolutionary conservation (38), points to a specific and important role of these transporters.

FFA bind to several cytoplasmic proteins once they enter the cell. Fatty acid binding proteins (FABP) have been found in tissues with increased requirements for FFA uptake such as the liver, intestine, muscle, adipose and brain. It has been proposed, for the muscle FABP, that their content is related to fatty acid utilization (39). The aP2 fatty acid binding protein was first identified by Speigelman and colleagues as a protein which increased upon adipocyte differentiation (40). In aP2 knockout mice, there was a distinct separation between obesity and insulin resistance (41) which was determined to be due to a downregulation of hormone sensitive lipase (Scheja IJO conference,1998 presentation,

(42)). In conclusion, the intracellular regulation of FFA is important and FFA are now taking on a more important role.

1.5 Glucose

Triglyceride storage requires not only FFA but also glucose for production of the triglyceride glycerol backbone. Glucose entry into the cells is controlled via specific transporters and is prevented from leaving the cell by introduction of a charged phosphate by phosphorylation to produce glucose-6-phosphate. The glucose transporters fall into two categories, active transport coupled to the sodium electrochemical gradient as in the intestine and kidney and facilitated diffusion via glucose transporters. The glucose transporters are Glut 1 (ubiquitous), Glut 2 (liver, kidney, intestine), Glut 3 (brain), Glut 4 (insulin responsive cells such as adipose and muscle) and Glut 5 found in the small intestine. Glucose plasma levels are closely regulated by glucagon and insulin, released from the α and β cells in the pancreas respectively, modulating liver gluconeogenesis and peripheral tissue uptake.

Glucose uptake is regulated primarily in insulin sensitive tissues such as muscle and adipose tissue. Upon insulin stimulation, the glucose transport is increased as the Glut 4 transporter is translocated from intracellular stores to the cell surface. Muscle is thought to play a major role in peripheral glucose uptake, however recent experiments implicate other tissues (43) such as adipose tissue in a greater role in peripheral glucose uptake. It has been proposed that alterations in glucose transporter levels in adipocytes may contribute to impaired glucose disposal. This was seen in impaired glucose disposal with decreases in Glut 4 in rats (44) or improved glucose control with adipose specific overexpression of Glut 4 in transgenic mice (45).

Glucose secretion occurs primarily from the liver *via* gluconeogenesis or glycogenolysis. The islets of Langerhans cells in the pancreas monitor the glucose concentration in the blood. Decreases of glucose result in secretion of the hormone glucagon from the α cells of the islets of Langerhans. The main targets of glucagon are adipose tissue and the liver. In hepatocytes, glucagon stimulates adenylate cyclase production of cAMP resulting in glycogenolysis, gluconeogenesis, prevention of conversion of free fatty acids to ketone bodies and blockage of glycolysis (46). In

adipocytes, glucagon results in lipolysis although to a lesser extent in the presence of insulin. Insulin released by the β cells in the islets of Langerhans, acts upon many tissues with the major effect on muscle adipose and liver. Insulin secretion is simulated by increased glucose and amino acid levels and glucagon and is inhibited by somatostatin. Insulin stimulates glycogen storage (liver), increases glucose and amino acid uptake (muscles). It also increases glycerol-phosphate formation and fatty acid supply (via increased LPL activity) for triglyceride synthesis (adipocytes) (46).

Glucose levels change chronically with environmental conditions leading to decreases during fasting or increases in diabetes, obesity or a high fat diet. Several factors have been implicated in the cause of insulin resistance defined as a reduced response to insulin with elevated blood glucose and insulin levels. A high fat diet has been implicated in the development of insulin resistance in humans. Elevated fatty acids increase hepatic glucose production and compete for utilization with glucose in muscle, resulting in free fatty acid oxidation in preference to glucose (Randle cycle). Fatty acids also stimulate insulin secretion and reduce insulin clearance by the liver (reviewed (47), (48)). Besides FFA, TNFα is another candidate implicated in or insulin resistance and inhibition of insulin receptor signaling (49). In mice, a high fat diet and obesity also increased glucose levels and reduce Glut 4 expression in adipose with no change in skeletal muscle levels in normal mice (50), suggesting changes in tissue specific expression in glycemic control.

Part 2 Intracelllular Processes: Lipogenesis and Lipolysis

2.1 Lipogenesis

Triglyceride synthesis requires free fatty acids and glycerol. Free fatty acids can enter the cell subsequent to lipolysis or be synthesized *de novo*. In humans, the majority of the FFA are from external sources as *de novo* synthesis is negligible. However this may be a consequence of the high fat content of our diet resulting in reduced endogenous fat synthesis (51). In humans with LPL deficiency, the adipose tissue mass is normal (52), (53) and this may be due to the activity of hepatic lipase, *de novo* FFA synthesis or an adipose tissue lipase (53). In mice FFA *de novo* synthesis was also noted in the LPL

knockout mice as the adipose tissue were normal and found to be maintained *via de novo* synthesis (54) or possibly increased activity of the ASP pathway. The glycerol-3-phosphate in adipose tissue is synthesized from glucose while in the liver glycerol can be converted directly to glycerol 3-phosphate by the liver specific enzyme glycerol kinase. Triglyceride synthesis occurs by sequential addition of activated free fatty acids (acyl CoA fatty acids) to the glycerol phosphate backbone via the enzymes glycerol 3-phosphate acyltransferase (GPAT), phosphatidate phosphohydrolase (PPH) and diacylglycerol acyltransferase (DGAT).

2.2 Lipolysis

The rate-limiting enzyme for lipolysis of intracellular triglycerides is hormone sensitive lipase (HSL). This concept is supported by the observation that overexpression of HSL in adipocytes prevented an accumulation of intracellular triglyceride storage (55) and is supported by experiments by Langin and colleagues (56). Diacylglycerols and monoacylglycerols are the result of HSL lipolysis of triglycerides. The monoacylglycerols are then catabolised to glycerol and FFA by the enzyme monacylglycerol lipase (57). Furthermore HSL activity is activated by β adrenergic receptor stimulation. This activation of the G-protein coupled receptor releases the $G_{s\alpha}$ subunit leading to the cascade of adenylate cyclase activation, cAMP release, stimulation of protein kinase A (PKA) and finally HSL activation (58). Phosphodiesterase activation by insulin or β adrenergic receptor agonists, converts cAMP to 5' AMP and reduces HSL activity (59). ASP is also able to reduce the activity of HSL *via* phosphodiesterase activity (60).

The activity of HSL and LPL are, under normal conditions, reciprocal to one another (cited (61)). However in transgenic mice parallel changes in activity have been observed, without a change in obesity as seen in LPL knockout mice with adipose specific LPL overexpression (62). Thus HSL and LPL are able to regulate substrate utilization or storage by control of cellular substrate influx and efflux.

Part 3 Adipose Tissue Physiology

3.1 High Fat Diets

Flatt proposed that fatty acids utilization is not coupled to fat intake and that a high fat diet would lead to obesity until the relative utilization of fatty acids increase (63). This increase in obesity with a high fat diet could be attributed to hyperphagia, since the high fat diet leads to passive over-consumption due to its increased energy density, potent oral stimulation of ingestion and slower satiety signals as compared to carbohydrate (64). As such there have been several studies to determine the effect of high fat or high fat / high sucrose diets on adipose tissue physiology.

However, hyperphagia is not the only cause of obesity on a high fat diet. A sedentary lifestyle and reduced exercise in affluent societies contributes greatly to obesity because while daily caloric intake has decreased by 10% obesity has increased two fold in the U.S (65). Reduction of sympathetic nervous system activity and the concomitant lipolysis have also been implicated.

With a high fat diet, many species have shown weight increases (66), with some mice strains, such as the A/J, demonstrating resistance to obesity (67). Triglyceride levels in mice placed on a high fat diet at times display no change or decreases (68), (69) or increases in other studies (70). In the murine models, increased levels of insulin and glucose have accompanied obesity and this has been proposed to occur before obesity (71) or due to weight increase (72). This increase in both insulin and glucose levels (insulin resistance) occurs in several in mice models on a high fat diet (68), (73), (70).

3.2 Insulin Resistance

Insulin resistance is a syndrome where there is reduced response of target tissues to a given dose of insulin. However insulin resistance has been inferred from glucose tolerance tests with concomitant measurement of insulin as well as increased fasting levels of glucose and insulin. It has been proposed that insulin resistance arises due to elevated FFA and these metabolic disturbances are most commonly seen in humans, predominantly males, with visceral obesity. The model proposed for this insulin increase is that the elevated FFA reduces insulin degradation in the liver and the substrate competition between FFA and glucose utilization at the muscle leads to glucose increases

begetting more insulin secretion. It has been proposed that there is a cycle put into effect by increased glucose, insulin and FFA levels in obesity that worsens insulin resistance (47). In brief, elevated FFA and thus long chain fatty Acyl CoA result in increased FFA oxidation and reduced glucose utilization (Randle or glucose-fatty acid cycle). The increased insulin levels promote glucose utilization resulting in an inhibition of FFA oxidation. This reduced FFA oxidation by increased insulin levels then feeds back to result in increased long chain Co-A further reducing glucose utilization. Thus there is a cycle competition for glucose and fatty acid utilization each resulting in exacerbation of the insulin resistance. Insulin resistance is proposed as a mechanism of reducing weight increase (74). The experimental data for this glucose-fatty acid cycle has mainly been obtained in humans or rats.

This theory of insulin resistance necessitates the elevation of FFA. Although a high fat diet induces elevation in both plasma glucose and insulin in mice, there is no accompanying increase in free fatty acids in mice (68), (69) indeed lowered in one study (75), as compared to humans (review (48)). Furthermore free fatty acid levels were not elevated in genetically obese mice (76), (77). However an elevation of free fatty acids may be diet or strain specific as elevated fatty acids were noted in mice on a high fat diet (78), (79), (70). Diabetes occurred in transgenic murine models lacking white adipose tissue. In these mice, increased FFA were seen with the dominant negative protein inhibition of C/EBP function (80) and no increases in FFA, in a SREBP-1c adipose overexpressor (81). Thus plasma FFA do not accurately reflect the etiology of insulin resistance or their fluxes in the microenvironment. Mice may be able to adapt to reduce elevations in FFA *via* upregulation of UCP, as seen in obesity resistant mice strains (73), (82). Alternatively, it could be that not one factor could be singled out as a cause for insulin resistance. However, it is undisputed that insulin resistance occurs with obesity and high fat diets in murine models.

3.2 Obesity

That adipose tissue is not a passive acceptor of free fatty acids but can regulate the uptake of substrates such as glucose and FFA is a novel idea and physiologically a potentially critically important idea.

Obesity is prevalent in the Western Society where energy consumption, especially in the percentage of fat, generally exceeds that of energy expenditure. Many factors are likely to contribute to the pathophysiological condition of obesity such as behavioral, psychological, environmental and genetic factors. Recent research in humans and animal models has suggested a genetic component to obesity (83).

Obesity is no longer regarded as a homogenous phenotype and has been proposed by Bouchard (84) to be able to be divided into 4 different morphological types:

1. excess total body fat
2. excess subcutaneous trunkal obesity or android obesity
3. excess abdominal visceral obesity
4. gluteofemoral obesity.

The above distinctions are not mutually exclusive and are not based on clinical manifestations but on anthropomorphic or morphological differences. Visceral obesity has been recognized to be associated with syndrome X (hyperlipidemia, obesity, insulin resistance and atherosclerosis). The metabolic mechanisms proposed for this are similar to those outlined for insulin resistance; increased TNF α or free fatty acids. The main focus has been on the free fatty acid theory. Visceral adipose exhibits increased lipolysis (85), (86) and decreased triglyceride synthesis (87),(88). It is theorized that the increased FFA flux results in more substrate returning to the liver resulting in increased VLDL secretion, glucose concentrations (via the Randle or glucose-fatty acid cycle), insulin secretion, and reduced liver insulin clearance (reviewed (48), (47)).

These morphological divisions of obesity can be combined into two metabolic classes, adipose tissue that is efficient or inefficient at trapping FFA (89). In efficient trapping there is no or little aberrations in plasma lipoproteins, insulin or glucose. It appears that the adipose tissues are able to both rapidly and efficiently act as a buffer preventing excessive fluxes in FFA and lipoproteins. This is indeed seen in the femoral-gluteal obesity, where the degree of adiposity is closely related to fertility. It is also conceivable that loss of adipose tissue below that of the set point would result in an increased efficiency of adipose tissue. Indeed weight loss resulted in an increased energy efficiency as compared to patients who were never obese (90).

With inefficient trapping, in contrast, there are elevations of extracellular FFA. This leads to the syndrome X or visceral fat syndrome a clinical condition manifested by both insulin resistance and dyslipidemia. Here free fatty acids utilization is increased and there is a decrease in glucose transporters, LPL activity and mass increase and there is insulin resistance. This has been suggested as an adaptation to maintain weight (74) and is associated with lipoprotein disorders. Flatt proposed that fatty acids are allowed to increase to a certain mass and then begin to be oxidized, and that glycogen levels control or regulate the lipid pool (63).

In the obese state several changes occur such as decreased glucose transporters (50), increased lipoprotein lipase levels and activity in the muscle (16), reduced adipocyte LPL lipolysis (16) and increased UCP in brown adipose tissue (73). These changes would enable adipose tissue to modify its activity in order to alter body weight or attempt to reduce obesity.

3.3 Sexual Dimorphism

Vague first noted the morphological distribution of adiposity is sexually dimorphic, with males tending to have upper body or android obesity while women have lower body or gluteal femoral obesity. This difference is not only due to the sex hormones but also to the effect of corticosteroids, insulin and growth hormone (91). The link between adipose tissue distribution and gender is still under investigation, however different depots of adipose tissue have distinct characteristics. The ability of visceral adipose tissue to store triglycerides is less than those of subcutaneous adipose tissue (87), (88) and visceral adipose cells in men had increased sensitivity to catecholamine induced lipolyis (92). Finally, subcutaneous adipose tissue lipolysis is reduced in upper regions as compared to lower body obesity, where lipolysis was measured as free fatty acids release *in situ* (86).

Plasma parameters are also different between the sexes. Females have lower fasting insulin, cholesterol and triglycerides. Furthermore the postprandial lipid metabolism changes with menopause. Postprandial triglyceride clearance has been shown to be faster in females in both human and animal studies (93), (94). In general this leads to a protective effect against cardiovascular disease. It is currently under investigation as

whether this difference is gender or sex hormone specific. Hormone therapy for sex changes also causes redistribution of adipose tissue with hormone therapy in postmenopausal women preventing redistribution of adipose tissue to upper body (95), (96). The protective effect against cardiovascular disease is also lost in post-menopausal women (Framingham study (97). Several murine studies in adipose tissue noted sex specific (female) decreases in adiposity, such as in the protein kinase A subunit knockout (98) and aP2 adipose specific diptheria toxin expression (99). Other studies have noted increased adiposity in females as compared to males with examples such as the neuropeptide Y receptor knockout mouse (100), β3 adrenergic knockout mouse (101), LPL muscle specific overexpression (18). To my knowledge there are no natural mutations resulting in reduced or an absence of adipose tissue. There are transgenic models in which adipose tissue mass is reduced in both sexes (102). However in many cases differentiation is not made between the sexes for adipose tissue loss or reduced growth curves (81), (80), (103), (99) or comparisons to females are not reported (104), (105), (106). In one study gonadal fat weight was decreased to a greater extent than that of males however they experienced less weight loss than males (107).

It has been suggested that females may control or protect their body fat more than males (18), in order to stay within a certain adiposity range for reproduction (108) (cited (109)). Certainly, females (in both human and mice) tend to have greater percent body fat (fat mass as assessed by skinfold in humans) (65), (110). Indeed, sexual maturation of humans females has been linked to attainment of a "critical mass" or body fat content (109). Therefore the metabolic differences in the adipose depots may be a reflection this evolved physiology in reproduction.

3.4 Adipose Hormones

There are also several other genes altered with obesity or with a high fat diet such as increases in uncoupling protein in brown adipose tissue- generating heat through uncoupling energy storage in the energy transport chain in the mitochondrion. With increased fat mass, there is the release of several signaling molecules such as TNF α and leptin. Elevated levels of TNFα are postulated to cause insulin resistance and adipocyte

de-differentiation. Leptin levels correlate with body weight and are higher in females than males irrespective of body weight.

Glucose levels may play a role in leptin secretion as *in vitro*, non-metabolizable 2-deoxyglucose reduces leptin secretion (111). Leptin is now thought to play a role in reproductive status in the female and long term weight regulation rather than acute effects.

Part 4 Acylation Stimulating Protein (ASP)

4.1 ASP formation

One of the adipocyte molecules is complement C3a des Arg or Acylation Stimulating Protein (ASP), a non-glycosylated protein. Spiegelman and colleagues along with Cianflone, Sniderman and colleagues, working from different perspectives discovered the role of the alternate complement pathway in adipose tissue in tandem. Cianflone identified ASP (C3a desArg) as having triglyceride synthetic stimulation activity (112) while Spiegelman identified adipsin (or complement factor D) as having a role in murine obese models (113) and the complement system in adipose tissue (114).

Complement C3a is part of the proximal alternate complement pathway. It is formed by interaction of complement C3, factor D (adipsin) and factor B producing C3a, presumably in the extracellular space. The C-terminal arginine is removed by carboxypeptidase B forming C3a desArg or acylation stimulating protein (ASP). Under normal circumstances, there is very little C3a in plasma and it is rapidly converted to (C3adesArg) ASP (115). The complement system is highly regulated and has many regulatory factors including H, I, and Crry. Furthermore adipsin, the catalyst for ASP formation, has a self-regulatory loop that blocks the active site which is specifically activated by the binding of cleaved factor B, Bb (116). The latter portion of the complement system, which causes complement mediated cell lysis, is absent in adipose tissue (117) and there is continual low level production of ASP via C3 hydrolysis (118).

The evolution of ASP is highly conserved, with complement C3 being found in many species (119) including primitive vertebrates (120), (121) and the lytic portion of the complement system appearing later in evolution (122).

The role of complement in lipid metabolism is perhaps an adaptive function demonstrating a close linkage between immune function and lipid metabolism. The lymphatic system is the main transporter of dietary lipid from the intestine to the general circulation and contains many lymph nodes. The gastrointestinal tract plays an important role in absorption of and subsequent packaging of dietary lipid for release within the bloodstream. It also has several lymphatic nodes, Peyers patches, which are important in immune function. During infection hypertriglyceridemia is observed (123) and is associated with increased bacterial clearance and reduced susceptibility to infection (124). Furthermore, obesity such as in the *ob/ob* mouse results in impaired immune function (125). Several factors are secreted by both adipocytes and macrophages such as adipsin/factor D, C3 and TNFα which have known immunological function. Finally, preadipocytes have been shown to posses the macrophage-like properties of phagocytosis (126).

4.2 ASP Structure

Before detailing the function of ASP, the structure will be examined. ASP is a highly charged molecule, with an isoelectric point of 9.1 and is thought to contain a hydrophobic core. The structure of the molecule is shown in stereo pair in figure 1.1. The molecule appears as a ball and chain with three α-helices held together in a globular core by 3 disulfide bonds. The third helix extends out of the core and terminates in a flexible C terminal tail. The center of the core is thought to be hydrophobic or inaccessible, based on differential iodination of Tyr 15 *vs.* Tyr 59 (cited in review (115)). Complement C3a and ASP have identical three-dimensional structures (127) however due to lack of resolution at the terminals there may be differences of structure within the C-terminal end.

4.3 ASP Function *In vitro*

The function of ASP *in vitro* is to increase the rate of triglyceride synthetic stimulation via diacylglycerol acyltansferase DGAT (recently cloned Dr Farese (128)) and glucose transport. DGAT is highly expressed in several tissues most notably the small intestine and colon and, surprisingly there are low mRNA levels in the liver (128). ASP also plays a role in modulating the activity of hormone sensitive lipase (60). ASP mediates these effects by binding to a cell surface receptor and activating intracellular

pathways, including that of protein kinase C (review (129). ASP has many effects, with greater effects on differentiated cells.

4.3.1 Glucose Transport

ASP has been shown to increase the translocation of glucose transporters from intracellular stores to the cell surface (130). Glucose uptake rates using 2-deoxyglucose, a form of glucose unable to be metabolized, indicated that ASP did not affect the rate of transport and its action was additive to that of insulin at maximal effective doses (review (129)). This suggests different signaling pathways.

4.3.2 Triglyceride Synthesis

ASP is able to stimulate triglyceride synthesis in a variety of human cell types such as fibroblasts, cultured adipocytes and mature primary adipocytes (review (129)) and from several species such as mouse, rat, primate and human (119). This triglyceride synthesis stimulation is dependent upon the C-terminal tail and core region of the molecule (131) with the C-terminal tail implicated in cell surface binding and the core involved in activation (131). Recombinant ASP and *in vitro* produced ASP (from complement C3, B and adipsin incubated under appropriate conditions) had equivalent stimulation of triglyceride synthesis as plasma ASP (119). Furthermore, immunoprecipitation with anti-ASP antibodies, of recombinant, plasma and *in vitro* generated ASP abolished triglyceride synthetic stimulation of the supernatant (119).

4.3.3 Receptor Binding

ASP has been shown to bind specifically and saturably to a specific cell surface receptor on human fibroblasts (132) and is competitively displaced by unlabelled ASP (131) with a K_d in the 10^{-8} M range (review (129). Cytochrome C, a molecule of similar size (133) and pI (134) to ASP was unable to compete out this specific binding. This specific binding was inhibited by heparin and protamine sulphate indicating structural and/or charge interactions of ASP with its receptor. Although ASP is identical in structure to the precursor molecule C3a, (other than the C-terminal arginine) it is unable to bind to the recently isolated complement C3a receptor (135), (unpublished observations).

Fibroblasts from patients with HyperapoB (hyperlipidemia) displayed reduced receptor binding by approximately two-fold and reduced ASP stimulated triglyceride synthesis (136). This matching of both reduced receptor binding and reduced triglyceride synthesis indicates that there is a specific binding of ASP to a cell surface receptor.

4.4 Postprandial ASP production

4.4.1 *In vitro*

Adipocytes *in vitro* increase ASP production when stimulated with factors such as insulin and dietary chylomicrons. The chylomicron stimulation was markedly greater (10 fold) than that of insulin (2 fold) (137) and the active component of the chylomicron was identified to be transthyretin (138). Thus this implicates postprandial metabolism in increasing ASP production, a novel signaling system that would increase both triglyceride synthetic stimulation and glucose transport at the time of dietary storage.

4.4.2 *In vivo*

In human studies no postprandial change in ASP was observed in total plasma (139), (unpublished observations) suggesting that ASP, and its effects occur within the adipose tissue microenvironment. Indeed, in a recent study in humans, ASP is produced postprandially from the adipose tissue bed and its appearance in plasma was concordant with triglyceride clearance, where triglyceride and ASP were both measured as arterio-venous difference across subcutaneous adipose tissue (140).

In vivo data, with intraperitoneal injection of ASP into mice resulted in marked decreases in glucose and triglyceride levels in the *db/db* strain (manuscript in preparation).

Part 5 Aims:

Previously insulin was the only molecule known to have effects on both glucose and triglyceride synthesis. Now ASP has been shown to posses these functions as well. Thus studies were undertaken to determine the "active sites" of the molecule.

5.1 In Vitro

1. To determine if recombinant ASP is identical in activity, glucose transport and stimulation of triglyceride synthesis to plasma ASP
2. To identify which regions of ASP are required for function (stimulation of triglyceride synthesis and glucose transport) and competition binding

5.2 In Vivo

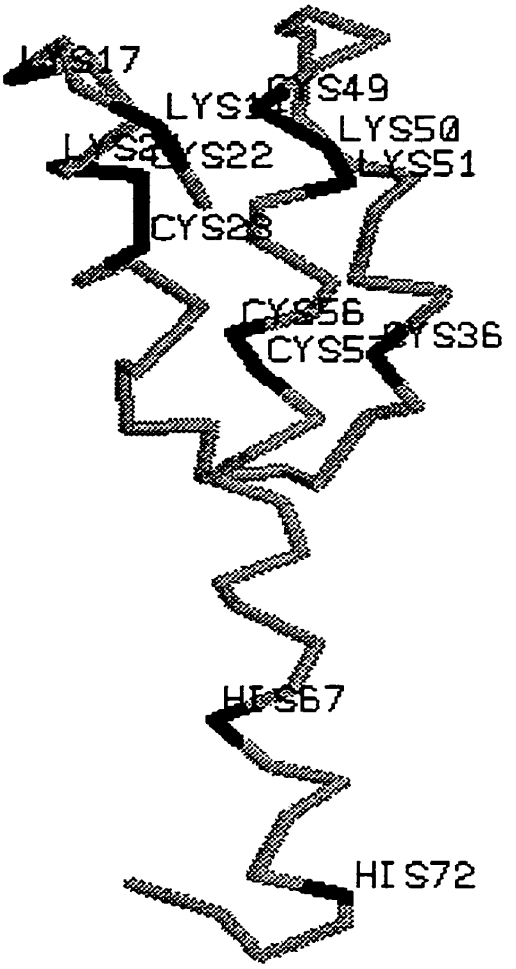
ASP has been implicated in triglyceride clearance in *in vitro* and *in vivo* studies. Thus to directly determine the physiological role of ASP, experimental models-murine models and knockouts were used.

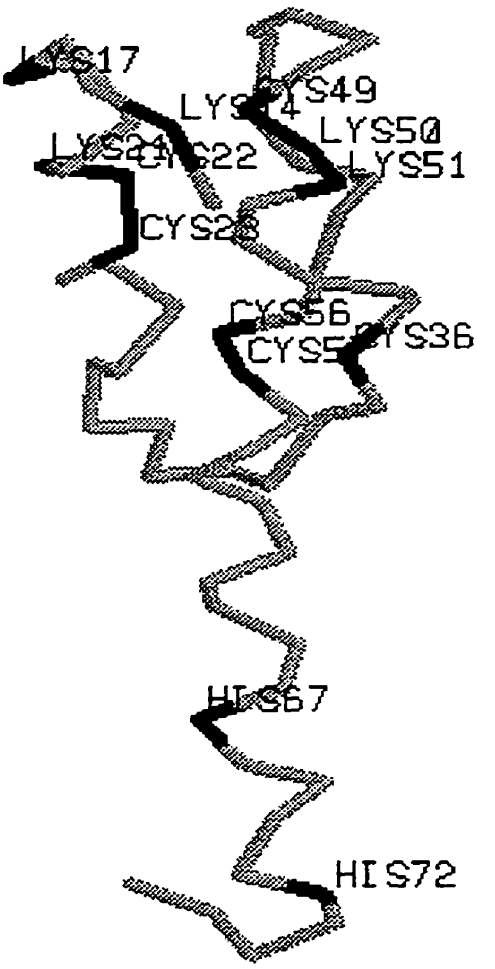
1. To determine if exogenous ASP is able to decrease plasma glucose and triglyceride levels after oral fat load in C57Bl/6 mice.
2. To determine if knockout mice have delayed triglyceride, glucose and free fatty acid clearance and to characterize fasting plasma characteristics with increases in age and high/ low fat diet in a longitudinal study (4 months) as compared to wild type mice.
3. To determine if there are decreases in adipose, muscle, liver or brown adipose tissue and body weight and increased food intake in a 4 month longitudinal study as compared to wild types.

Figure 1.1 3D-steroscopic image of acylation stimulating protein (ASP).

Solution co-ordinates for ASP courteously provided by Dr W. Chazin. Steroscopic image and labeling generated with the Rasmol (created by Dr R Sayle, homepage www.umass.edu/microbio/rasmol/index.html). The residues targeted for amino acid modification are labeled with the amino acid code and sequence position.

Plastic hand held print viewers provided by Reel 3D Enterprises Inc, C.A: stereoscopy.com/reel3d e-mail Reel3D@aol.com. The focal length is 24.7cm. One 3D image should be observed using these viewers.


LYS17
LYS50
LYS51
CYS28
CYS56
CYS36
HIS67
HIS72


LYS50
LYS51
CYS28
CYS36
HIS67
HIS72

Prelude I

ASP (C3a desArg) is a novel molecule, isolated from human plasma, which is able to stimulate triglyceride synthesis and glucose transport. Recombinant ASP was purified and assayed to determine if it possessed the same qualities as native plasma ASP. Furthermore, the activity of ASP was contrasted with that of the similar molecule C3a.

(Formatted for the Journal of Lipid Research)

Chapter 2
Functional Bioactive Recombinant Acylation Stimulating Protein is Distinct from C3a Anaphylatoxin

2.1 Abstract

Acylation Stimulating Protein (ASP) acts upon adipose tissue to stimulate triglyceride synthesis and glucose transport. The aim of the present study was to produce recombinant ASP and to measure its bioactivity. The cDNA region of the parent complement C3 sequence coding for ASP (C3adesArg) was cloned and expressed in *E.coli*. Bioactivity of the purified recombinant material was tested by determining its effect on triglyceride synthesis, glucose transport and competition binding assays. In standard assays, concentrations of 5.5 μM recombinant ASP (rASP) stimulated triglyceride synthesis comparably to plasma ASP (pASP): 228% vs 237% respectively in 3T3 preadipocytes and 568% vs 440% in human differentiated adipocytes. rASP also increased glucose transport in L6 myocytes (163% at 10 μM rASP) and in human differentiated adipocytes (334% rASP vs 329% pASP at 5 μM). rASP competitively displaced radiolabelled plasma ASP from high affinity association with the cell surface in both human differentiated adipocytes and 3T3 preadipocyte fibroblasts. Furthermore, immunoprecipitation of rASP and pASP with a specific monoclonal antibody abolished stimulation of cellular triglyceride synthesis. Lastly, we contrasted the structure:function activities of the arginated (C3a) and desarginated (ASP) proteins. The lipogenic activity and the anaphylatoxic activity result from distinct structural domains of the polypeptides. Thus rASP retains full biologic ASP activity and may provide a tool to study structure-function relationships in this physiologic system.

2.1 Introduction

Acylation Stimulating Protein (ASP) is a 76 amino acid, basic protein (pI 9.0) which stimulates triglyceride synthesis and glucose transport (1-3). ASP was initially isolated as a serum component that stimulated triglyceride synthesis in normal human fibroblasts (1). This effect on adipocytes, though, was much more pronounced than on fibroblasts (2). This serum fraction was purified to homogeneity and was found to be identical to C3adesArg (molecular weight 8932.5 Da), a biologic fragment of complement C3 produced through the alternate complement pathway (1). ASP is a non-glycosylated product of the N-terminal cleavage of the α-chain of complement C3, mediated through the interaction of complement factors B and adipsin (complement factor D), followed by C-terminal arginine cleavage by serum carboxypeptidase N (4). Studies by Speigelman demonstrated that mRNA of complement factors B, C3 and adipsin were produced in murine adipose tissue (5,6). We have shown, in human adipocytes, that C3, B and adipsin increase with adipocyte differentiation and that the C3 cleavage product, ASP, also increases in the medium of differentiating cells (2,7). The increased production of ASP correlates with the increased basal triglyceride synthetic capacity of human adipocytes as well as an increased response to ASP (2,7). Furthermore, triglyceride synthesis, using *in vitro* complement-derived ASP (cdASP) (from factors B, C3 and adipsin) produced triglyceride synthetic activity comparable to that of plasma ASP (pASP) in human fibroblasts (2). ASP is thus the bioactive component of the adipsin pathway described by Speigelman in adipose tissue.

It should be noted that although C3a has potent anaphylatoxic activity in immunologically relevant cells such as U937 human monocytes (8) and other cells (review 9), this activity is entirely dependent on the presence of the carboxy terminal arginine (8,9). C3adesArg (ASP) has no known immunologic function and does not appear to bind to the recently identified C3a receptor (10,11).

In vitro expression of eukaryotic proteins has refined the process of structure - function analysis. In the present paper we report the production and purification of rASP and assess its biological activity. Similar to pASP, rASP was able (i) to stimulate triglyceride synthesis (ii) to increase glucose transport and (iii) to competitively displace

radiolabelled plasma ASP from association with apparent cell surface receptors. Immunoprecipitation abolished cellular triglyceride stimulation activity. Lastly, ASP activity was shown to be sensitive to solubilization conditions in contrast to C3a anaphylatoxic activity. Thus rASP has full biological activity and may be useful to study structure-function relationships of ASP in future research.

2.3 Materials and Methods

All human tissue was obtained with informed consent and all experimental protocols were approved by the ethics committee of the Royal Victoria Hospital.

2.3.1 Recombinant ASP (rASP) and plasma (pASP) Production: The cDNA sequence encoding human ASP (C3adesArg) was assembled in the pET22b expression vector (Novagen, Inc., Madison, WI) using synthetic oligonucleotides in which mammalian codons were modified to reflect *E.coli.* codon bias as described previously for C3a (12). To our knowledge, this has never been done for ASP (C3adesArg).

Recombinant ASP (rASP) protein was purified as follows from BL21 (DE3) cells transformed with the above expression vector. Cells from the IPTG induced cultures were pelleted and then lysed by sonication in 20 mM Tris-HCL pH7.4, 0.1 M NaCl, 1.0 mM EDTA and 0.2 mM PMSF. Inclusion bodies, which contained rASP, were sedimented by centrifugation (2500 x g, 4°C for 30 minutes). The inclusion bodies were resuspended in 50 mL of 1.0 N HCl, sonicated, neutralized with 10 N NaOH to pH7.0 and the debris removed by centrifugation. The rASP present in the supernatant was isolated by C-18 SepPak, followed by S-Sepharose chromatography, and lastly by reverse phase HPLC on a C-4 column as described in detail for plasma ASP (13). The yield of rASP was 0.5 to 1.0 mg per litre of starting culture. Human pASP was prepared as previously described (13). Protein concentrations were determined by a modification (14) of the Lowry protein assay (15) using a commercial albumin standard (BioRad, Mississauga, Ontario, Canada). The pASP and rASP were stored at -80°C in siliconized glass vials.

2.3.2 Preparation of *in vitro* complement-derived ASP (cdASP): 500 µg of human factor C3 and B (Calbiochem, LaJolla, CA) in 50 mM magnesium chloride were incubated in a glass test tube for 3 minutes at 37°C to activate C3. To this, 50 µg of human factor D (Calbiochem) was added and the mixture was incubated for a further 15 minutes at 37°C. From this mixture 25 µg (15 µg/mL) of cdASP was obtained, based on 100% conversion as assessed by SDS-PAGE (1,2). Preparations of C3a were treated with carboxypeptidase B (1 unit/mL for 5 minutes at 37° C) to generate *in vitro* ASP.

2.2.3 Cell Culture: Human fibroblasts were obtained from forearm biopsies of normolipidemic subjects and cultured as previously described (1). 3T3-L1 preadipocytes (obtained from ATCC) were cultured in the same fashion as the human fibroblasts. Cells were subcultured, plated out at 3 x 10^3 cells/cm^2 and grown in 10% fetal calf serum in DMEM/F12 medium. Cells were used for experiments at 80% confluency. For experiments with 3T3 adipocytes, cells were differentiated as previously described (2). Human adipose tissue was obtained with informed consent at the time of elective laparotomy. Following collagenase digestion and centrifugation, stromal vascular preadipocytes were isolated from the cell pellet and primary adipocytes from the floating layer. Human preadipocytes were differentiated to adipocytes in hormone supplemented DMEM/F12 medium for 18 days as described previously (2,7). U937 cells were obtained from ATCC and cultured as described by Klos *et al* (8).

2.3.4 Triglyceride Synthesis Assay: Cultured cells in 24 well dishes (1.7 cm^2/well) were preincubated in serum-free DMEM/F12 medium overnight (18 hours) prior to the experiments. Triglyceride synthesis was measured as incorporation of ^3H oleate (specific activity 10.0 Ci/mmol, Du Pont-New England Nuclear, Mississauga, Ont, CA) incorporation into triglyceride. Cells were incubated for 4-18 hours (as indicated) in 100 μM oleate complexed to BSA (5:1 molar ratio, average final specific activity = 100 dpm/pmol) in serum free DMEM/F12 as previously described (13). Results are expressed as nmol ^3H oleate incorporated into triglyceride per mg of cell protein.

2.3.5 Glucose Transport Assay: Cells were cultured, as described above, in 35 mm tissue culture dishes and preincubated in serum-free DMEM/F12 medium 18 hours prior to the experiments. Cells were stimulated with rASP or pASP for one hour at 37°C and glucose transport was assessed by measurement of cellular uptake of ^3H 2-deoxy-glucose (^3H 2-DG, specific activity 25-50 Ci/mmol, Dupont-New England Nuclear) as previously described (3). Results were corrected for background binding assessed as zero time counts. The results are expressed as nmol of ^3H 2-DG uptake per mg of cell protein.

2.3.6 Cellular Ca^{2+} flux: Ca^{2+} uptake into U937 cells was assayed by the Fura-2 method as described by Klos et al (8).

2.3.7 Radiolabelled I^{125} ASP Competition Assay: pASP was radiolabelled using Iodogen (Pierce Chemicals, Rockford, IL) and specific activity measured as dpm per μg of trichloroacetic acid (10% TCA) precipitable protein (average specific activity = 20 dpm/fmol). Competitive binding was performed on cells cultured in 96 well plates. Cells were preincubated with serum-free DMEM/F12 medium overnight. Cells were prechilled on ice for 15 minutes followed by incubation for 1 hour with 50 or 500 nM ^{125}I ASP in 100 μL of 1% bovine serum albumin (BSA) in PBS and increasing concentrations of unlabelled ASP. After a 1 hour incubation, 5 μL aliquots (in triplicate) of medium were counted for calculation of free ASP. Cells were washed three times with ice cold PBS and soluble cell protein dissolved in 100 μl of 0.1 N NaOH. Aliquots were taken for counting of bound ASP and cell protein determination by the method of Bradford (16) using a commercial assay (BioRad, Mississauga, Ontario, Canada). The results are expressed as nmol of ASP bound per mg soluble protein. Calculation of competition (IC_{50}) was performed by iterative four parameter logistic function analysis (Sigma Plot, Jandel Scientific, San Rafael, CA).

2.3.8 ASP Immunoprecipitation Assay: Protein G agarose (Calbiochem) was prewashed three times with 20 volumes of PBS, with centrifugation (100 x g) between the washes to allow for removal of the supernatant. For immunoprecipitation, 15 μg pASP, rASP or cdASP was added to 316 μg of monoclonal anti-human C3a IgG (Quidel, San Diego, CA) or the same amount of non-immune mouse IgG (Sigma, St. Louis, MO) (molar ratio of IgG to ASP was 1:1). Following incubation at 20° C for 20 minutes and then 4 ° C for 14 hours, the entire mixture was then added to the protein G agarose pellet and incubated at 20 ° C for 20 minutes, followed by 4 ° C for 2 hours, with gentle mixing. The sample was centrifuged (100 x g), to pellet the protein G agarose-antibody-ASP complex and the supernatant collected and tested for the ability to stimulate triglyceride synthesis.

2.3.9 SDS Polyacrylamide Gels: Proteins were separated on discontinuous 15% SDS-PAGE under denaturing conditions according to the method of Laemmli (17). Proteins were dissolved in 0.125 M Tris, glycerol (0.125 g/mL), 4% w/v SDS and 0.11 g/mL dithiothreitol and heated (100 ° C, 5 minutes). Gels were stained with Coomassie blue R-250.

2.3.10 Western Blot Analysis: Proteins were separated on SDS polyacrylamide gels as described above. Proteins were transferred by electroblotting to a PVDF membrane. Western analysis was performed using a monoclonal murine anti-human C3a antibody (Quidel) as primary antibody and detected with an alkaline phosphatase conjugated rabbit anti-mouse IgG secondary antibody using a commercial colorimetric kit (BioRad).

2.4 Results

The purification of rASP produced in *E.coli.* is shown in Figure 2.1 (top left). With IPTG induction, there is an increase in rASP as compared to the non-stimulated cells (lane 4 vs lane 3) which is present in inclusion bodies (lane 5). Following sonication of the inclusion bodies in 1 N HCL, the majority of the large globular proteins precipitate. However, ASP remains in the soluble fraction. The fractions from the SepPak and S-Sepharose purification steps are shown in lanes 7 and 8. Finally, the proteins were fractionated by C-4 HPLC and a typical profile obtained during the purification is shown in Figure 2.1B. Following HPLC fractionation of rASP, each of the protein fractions (numbered in Figure 2.1 top right) was tested for triglyceride synthetic capacity on human fibroblasts (Figure 2.1 bottom right). pASP was fractionated in parallel and the isolated fractions also tested for activity (Figure 2.1 bottom right). The pre-HPLC samples had activities of 233%±30 for pASP and 224%±32 for rASP where basal triglyceride synthesis was defined as 100%. The pASP elutes in a narrow band and rASP followed a similar profile. The fractions eluting at 55% to 65% acetonitrile (fraction #3) caused the greatest increase in triglyceride synthesis for both pASP (343%±48) and rASP (376%±39). This yielded a protein of identical molecular weight and immunogenicity as pASP as shown by SDS-PAGE and Western blot (Figure 1 bottom left). This process yielded 0.5 to 1 mg of rASP per litre of starting culture. Both the rASP cDNA and recombinant protein sequences were verified by DNA and amino acid sequencing.

During the course of purification of the recombinant ASP, it became clear that the yield and activity were sensitive to isolation conditions. Equal amounts of ASP were aliquotted and treated as described in Figure 2.2. As shown, losses could be kept to a minimum through the use of siliconized plastic tubes and by adding a small amount of BSA to dilute preparations (condition F in figure 2.2). On the other hand, there were losses (both mass and activity) when ASP was concentrated by centrifugation (C and D) or lyophilized in non-siliconized tubes ("A and B"). Sticking to plastic and glass is characteristic of basic proteins.

The functional activity of the rASP was first assessed for stimulation of triglyceride synthetic activity. As shown in Figure 2.3 (left panel) the triglyceride synthetic activity of 3T3-L1 preadipocytes was stimulated equivalently by both rASP and

pASP to a maximum of 237%±1% pASP vs 228%±20% at the highest ASP concentration (5.6 μM). The response of cultured human differentiated human adipocytes is shown in Figure 3, right panel. It should be noted that the responses in the differentiated adipocytes were much higher, with a proportional increase to 568%±34% at 5.6 μM with rASP in human cultured adipocytes.

We have previously shown that pASP increases 2-deoxy glucose (2-DG) transport in rat L6 myotubes, adipocytes and fibroblasts (3,18,19). Thus rASP was tested for stimulation of 2-DG transport in human adipocytes and L6 myotubes. rASP stimulated glucose transport in L6 differentiated myotubes (160%±20 at 10 μM ASP), an effect comparable to insulin as shown in Figure 2.4, left panel. In human differentiated adipocytes, rASP and pASP also stimulated glucose transport equivalently (Figure 2.4, right panel): 329% (pASP) vs 344% (rASP) at 5.6 μM (where basal glucose transport is shown as 100%). The percent response in adipocytes was greater than in L6 myotubes.

Further evidence of the functionality of rASP was obtained by assaying the ability of rASP to displace radiolabelled ^{125}I pASP (500 nM) binding to cell surface receptor binidng sites in cultured human skin fibroblasts and human differentiated adipocytes (Figure 2.5). In both cell types, rASP was as effective as pASP in displacement of receptor binding. The IC_{50} (500 nM ASP) for rASP ($2.7x10^{-6}M$ and $4.2x10^{-7}M$) was comparable to pASP ($3.9x10^{-6}M$ and $8.8x10^{-7}M$) for fibroblasts and adipocytes respectively.

The effect of ASP across several species was also examined. As shown in Table 2.I, ASP was as potent as insulin in stimulating triglyceride synthesis at equimolar amounts (0.7 μM) in primary adipocytes from other primates (cynomolgus and African green monkey). In contrast, ASP did not have as great an effect on glucose transport stimulation as did insulin in murine cells (Table 2.I) although the effects of ASP and insulin on glucose transport are comparable in human differentiated adipocytes (18) and in L6 myotubes (19). Nonetheless, ASP was effective at stimulating adipocytes from all species tested regardless of the activity index used (triglyceride synthesis or glucose transport).

To demonstrate the specificity of the effect on triglyceride synthesis, rASP, pASP and cdASP were immunoprecipitated and the remaining supernatant was tested for the capacity to stimulate triglyceride synthesis in human skin fibroblasts (Figure 2.6). Basal triglyceride synthesis (control) did not change significantly with the addition of either non-immune mouse IgG or monoclonal anti-C3a. The triglyceride stimulatory capacity of the positive controls for pASP (189%±23), cdASP (190%±14) and rASP (167%±18) were not significantly different from each other, and all stimulated triglyceride synthesis effectively. When non-immune mouse IgG was added, there was also no significant change in triglyceride synthesis stimulation of pASP (176%±18), cdASP (159%±1) or rASP (181%±19). Non-immune IgG did not immunoprecipitate ASP and all of the ASP remained in the supernatant as assessed by SDS-PAGE (not shown). However, triglyceride synthesis was significantly reduced by 126%±5, (pASP), 78%±14%, (cdASP) and 120%±7% (rASP) when the monoclonal anti-C3a antibody was used for immunoprecipitation. Thus triglyceride synthesis stimulation is abolished when ASP is immunoprecipitated. SDS-PAGE analysis indicates that 88% of the ASP was present in the IgG-Protein G agarose immunoprecipitate and no ASP was detected in the supernatant (not shown). Thus the triglyceride synthetic stimulatory activity was attributable specifically to bioactive ASP derived from either pASP, cdASP or produced by recombinant *E.coli* (rASP).

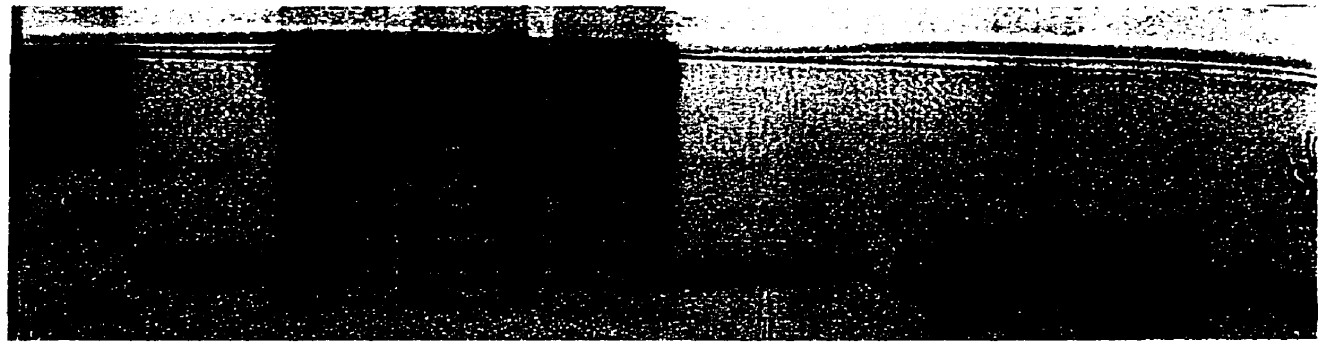
Functional bioactivity of the rASP appeared to be sensitive to solubilization conditions. It has previously been shown that treatment of C3a with urea under reducing conditions has no effect on its anaphylatoxic bioactivity (9,20). However, solubilization of the inclusion bodies containing rASP with either urea or guanidium isothiocyanate plus dithiothreitol, followed by purification, resulted in isolation of an inactive protein. This structure:function effect was investigated in more detail. The effect of various solubilization conditions used in purification of recombinant proteins was assessed. ASP was treated for 1 hour as indicated, and then ASP was repurified by HPLC and TGS activity measured. As shown in Table 2.II, high salt (1 M NaCl) or acid (1 M HCl) did not affect the ASP activity. As well, urea alone had little negative effect. In contrast, the combination of urea and β-mercaptoethanol caused a loss of 75% of the activity of ASP. This treatment of ASP resulted in a marked change in the charge/shape of the molecule as

demonstrated by reduced migration on native gel electrophoresis (data not shown). This loss of bioactivity was parallelled by a decrease in the affinity of the modified ASP for the receptor as shown in competition binding studies (Figure 2.7, 50 nM ASP) where the IC_{50} concentrations were $4.1x10^{-8}$ M (unmodified ASP), $1.7x10^{-7}$ M (urea treated ASP) and $3.39x10^{-7}$ M (urea+ β-mercaptoethanol treated ASP, $p<0.0001$ by ANOVA).

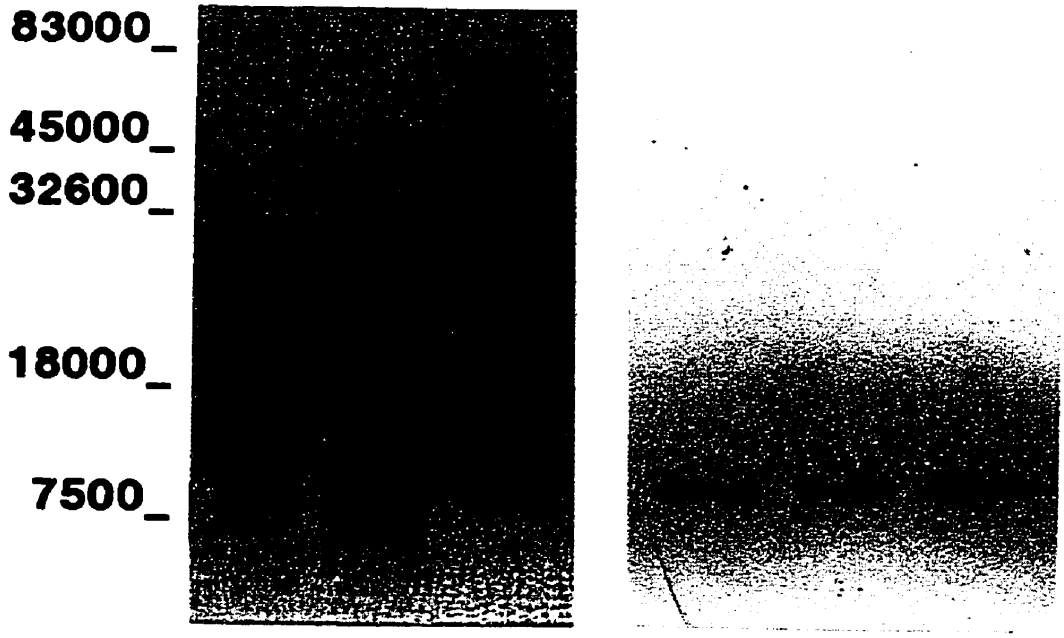
This contrast in structural stability between ASP and C3a was further investigated. C3a was prepared by *in vitro* cleavage of C3, B and adipsin and treated with carboxypeptidase B to produce *in vitro* generated ASP. The activity of these two proteins, as well as carboxy terminal peptides was tested. Hugli and colleagues have previously demonstrated that the 21 carboxy terminal peptide (C57-77) is sufficient for C3a anaphylatoxic activity (21,22). As shown in Figure 2.8 (top panel), C3a increased Ca^{2+} flux into U937 cells by 160%. The 21 residue peptide (C57-77) also increased Ca^{2+} flux to the same extent (Figure 8, bottom panel), 200%. Relative activities are shown in Table 2.III. Neither ASP (C3adesArg) nor the 20 amino acid residue desArg peptide (C57-76) had any effect on Ca^{2+} flux (Figure 2.8). Amino terminal peptides (N 1-19, N 1-30 and N 10-29) used as negative controls, also had no effect on Ca^{2+} flux. By contrast, although ASP and C3a had comparable effects on triglyceride synthesis assayed in parallel in fibroblasts, neither the 21 or the 20 residue carboxy peptides had any effect on triglyceride synthesis even at concentrations 50 times higher (100 μM) than the holo-proteins (Table 2.III).

Figure 2.1. Purification and activity of recombinant ASP

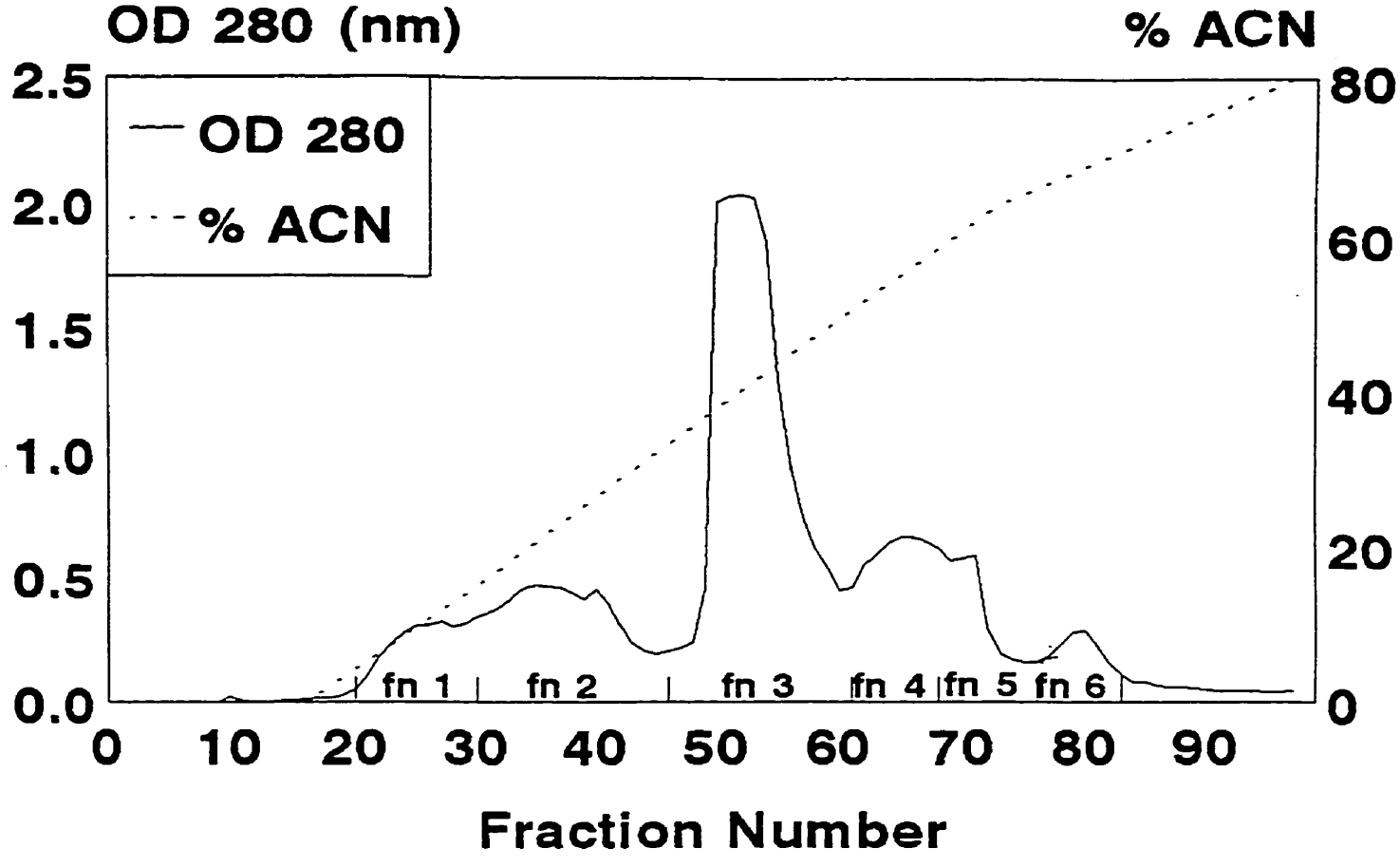
The left and right panels are shown on separate pages, pages 34 and 35 respectively
Top Left: The fractions obtained during purification of rASP were separated on 15% SDS-PAGE and stained with 0.12% Coomassie R250 blue (10 μg protein/lane). Lane 1: molecular weight markers, lanes 2,6,9: control plasma ASP, lane 3: uninduced *E.coli* bacterial pellet, lane 4:4 hour induction with IPTG, lane 5: inclusion body pellet, lane 7: C-18 SepPak eluant, lane 8: S-Sepharose eluant. **Bottom Left:** 15% SDS-PAGE stained with Coomassie blue (lanes 1-3) and Western blot using monoclonal anti-ASP (lanes 4-6) of the final C-4 HPLC purified rASP from two different recombinant preparations (lanes 2,3,5,6) and pASP (lanes 1 and 3) with 2 μg protein loaded per lane.**Top Right:** rASP was purified from *E.coli* exclusion bodies by hydrophobic (C-18 SepPak), ion exchange (S-Sepharose) and C-4 HPLC separation (a sample profile is shown). **Bottom Right:** Triglyceride synthetic capacity of rASP and pASP fractions from C-4 HPLC separation. Triglyceride synthesis was measured as ^{3}H-oleate incorporation into triglyceride in human skin fibroblasts over 6 hours. The results are expressed as percent change (average±std) where basal triglyceride synthetic activity was 7.1 nmol/mg cell protein/6 hours and is shown as 100% (n=3).



1 2 3 4 5 6 7 8 9



1 2 3 4 5 6


OD 280 (nm)
% ACN
OD 280
% ACN
2.5
2.0
1.5
1.0
0.5
0.0
80
60
40
20
0
fn 1
fn 2
fn 3
fn 4
fn 5
fn 6
0 10 20 30 40 50 60 70 80 90
Fraction Number

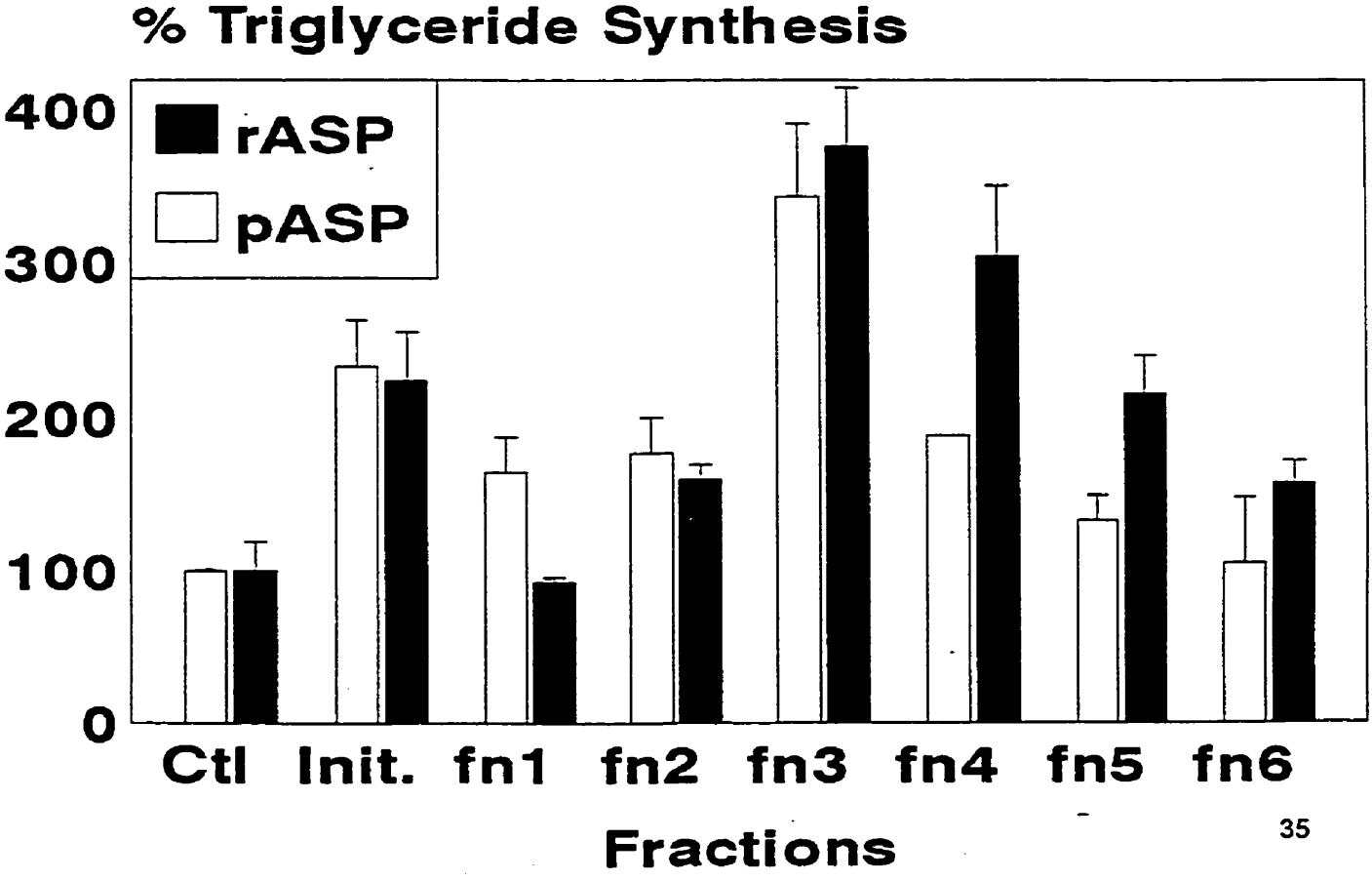

% Triglyceride Synthesis
rASP
pASP
400
300
200
100
0
Ctl
Init.
fn1
fn2
fn3
fn4
fn5
fn6
Fractions

Figure 2.2. Effect of isolation conditions on triglyceride synthetic activity of ASP

Cells were preincubated overnight in serum-free DMEM/F12 and then changed to serum-free DMEM/F12 supplemented with 100 μM ^{3}H oleate complexed to BSA and 125 μL of ASP. Equivalent aliquots of ASP were prepared under the following conditions: (A) lyophilized, (B) lyophilized + 1 mg/mL BSA, (C) concentrated by membrane centrifugation, (D) as in C plus 1 mg/mL BSA, (E) lyophilized in plastic siliconized vials or (F) as in E plus 1 mg/mL BSA. Triglyceride (TG) synthetic capacity of equivalent aliquots was measured in human skin fibroblasts overnight as ^{3}H oleate incorporation into triglyceride. Results are expressed as percent change (average±std, [■] where basal triglyceride synthesis (shown as 100%) was 27.3±4.7 nmol/mg cell protein/18 hours (n=3). Corresponding ASP concentration is indicated in the hatched bars [//].

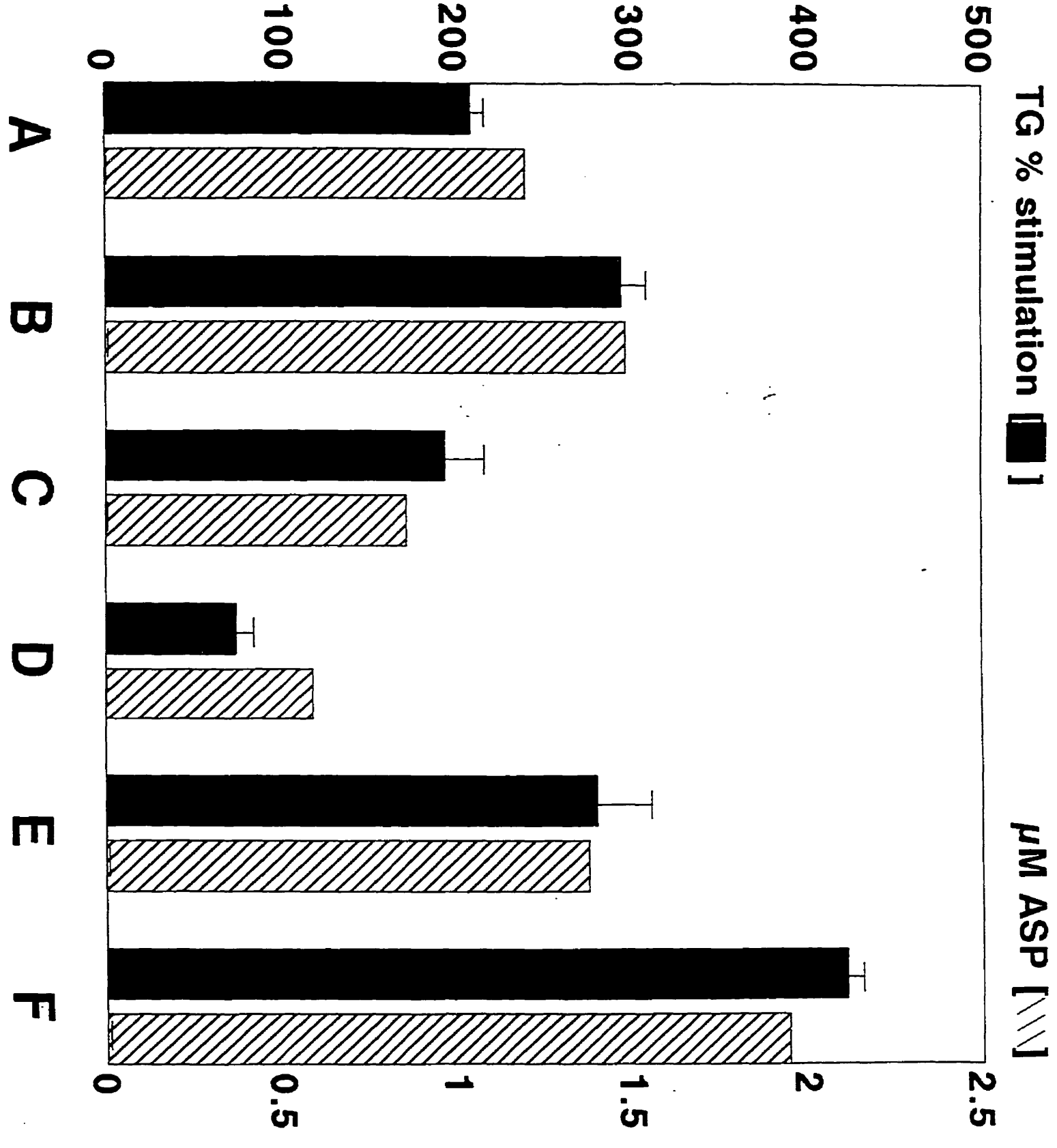
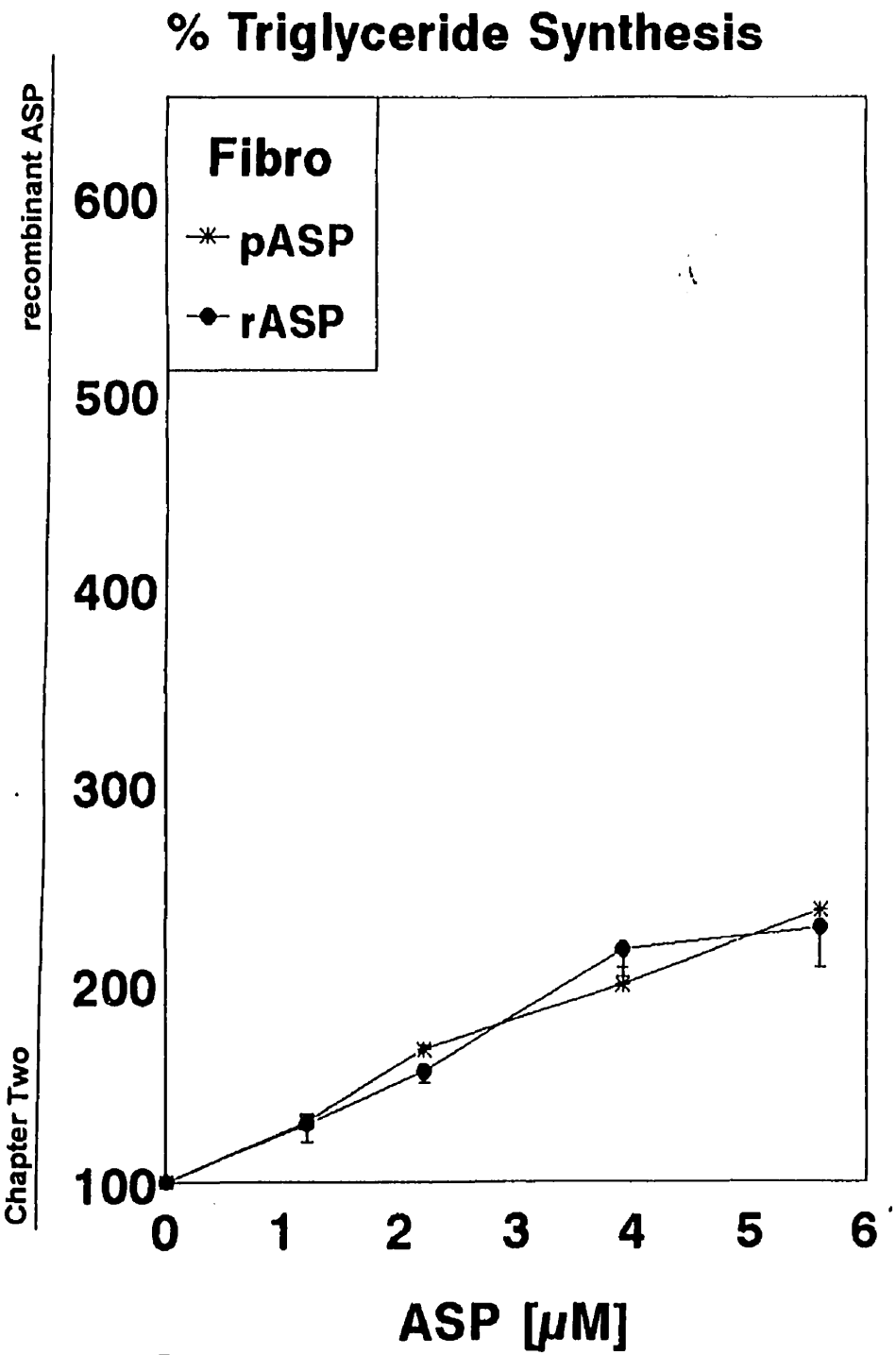

0
100
200
300
400
500
TG % stimulation [ ]
A
B
C
D
E
F
μM ASP [ ]
0
0.5
1
1.5
2
2.5

Figure 2.3. Triglyceride synthetic capacity of purified rASP and pASP

Cells were preincubated overnight in serum-free DMEM/F12 and then changed to serum-free DMEM/F12 supplemented with 100 μM ^{3}H oleate complexed to BSA and increasing concentrations of rASP or pASP. Triglyceride synthetic capacity was measured in 3T3 fibroblasts for 24 hours (left panel) and in cultured human differentiated adipocytes for 2 hours (right panel) as ^{3}H oleate incorporation into triglyceride. Results are expressed as percent change (average±std) where basal triglyceride synthesis (shown as 100%) was 54.9±1 nmol/mg cell protein/24 hours for 3T3 fibroblasts (n=3) and 11.9±1.3 nmol/mg cell protein/2 hours for human differentiated adipocytes (n=3).


% Triglyceride Synthesis
Fibro
pASP
rASP
600
500
400
300
200
100
0
1
2
3
4
5
6
ASP [μM]

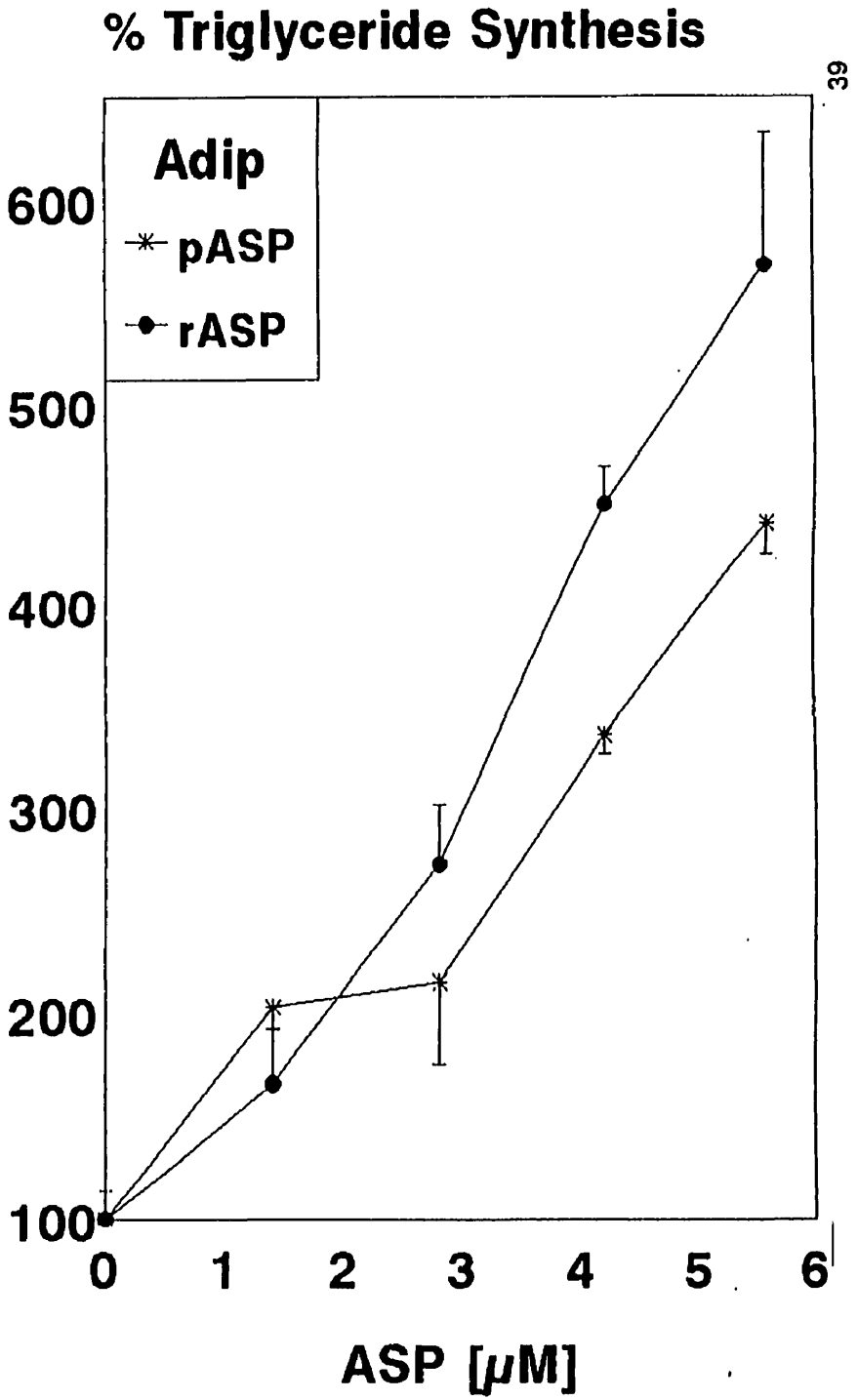
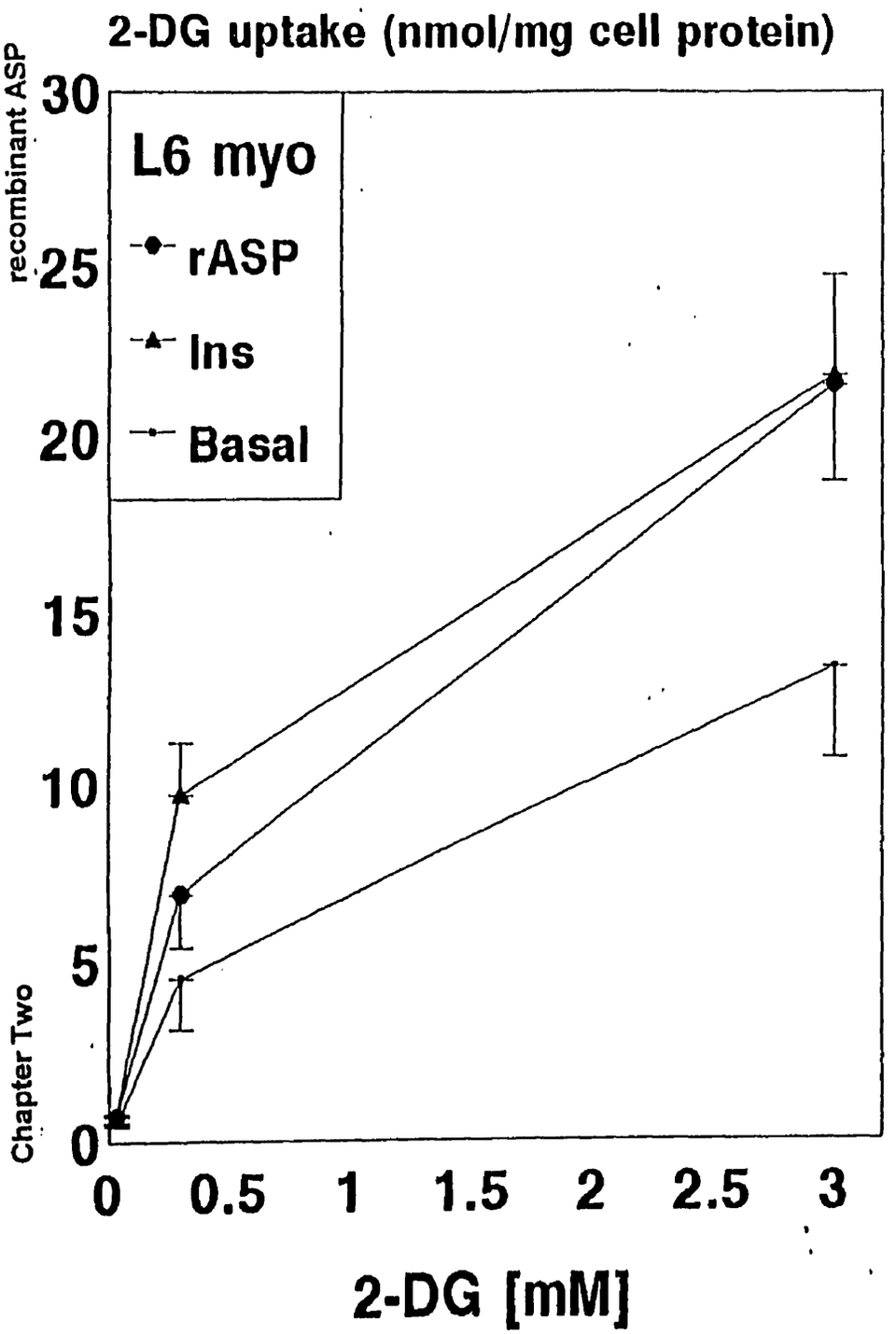

% Triglyceride Synthesis
Adip
pASP
rASP
600
500
400
300
200
100
0
1
2
3
4
5
6
ASP [μM]

Figure 2.4. Effect of rASP and pASP on glucose transport

Cells were preincubated in serum-free DMEM/F12 media and then stimulated with rASP, pASP or insulin for one hour. Glucose transport was assessed as ^{3}H 2-deoxyglucose (2-DG) uptake over 10 minutes in differentiated L6 myotubes (rASP = 10 μM, insulin = 1 nM) at 3 concentrations of 2-DG (left panel, n=4) and in differentiated human adipocytes with 0.05 mM ^{3}H 2-DG (right panel, n=3). Results are expressed as average±std nmol 2-DG per mg cell protein.


2-DG uptake (nmol/mg cell protein)
recombinant ASP
L6 myo
rASP
Ins
Basal
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
2-DG [mM]

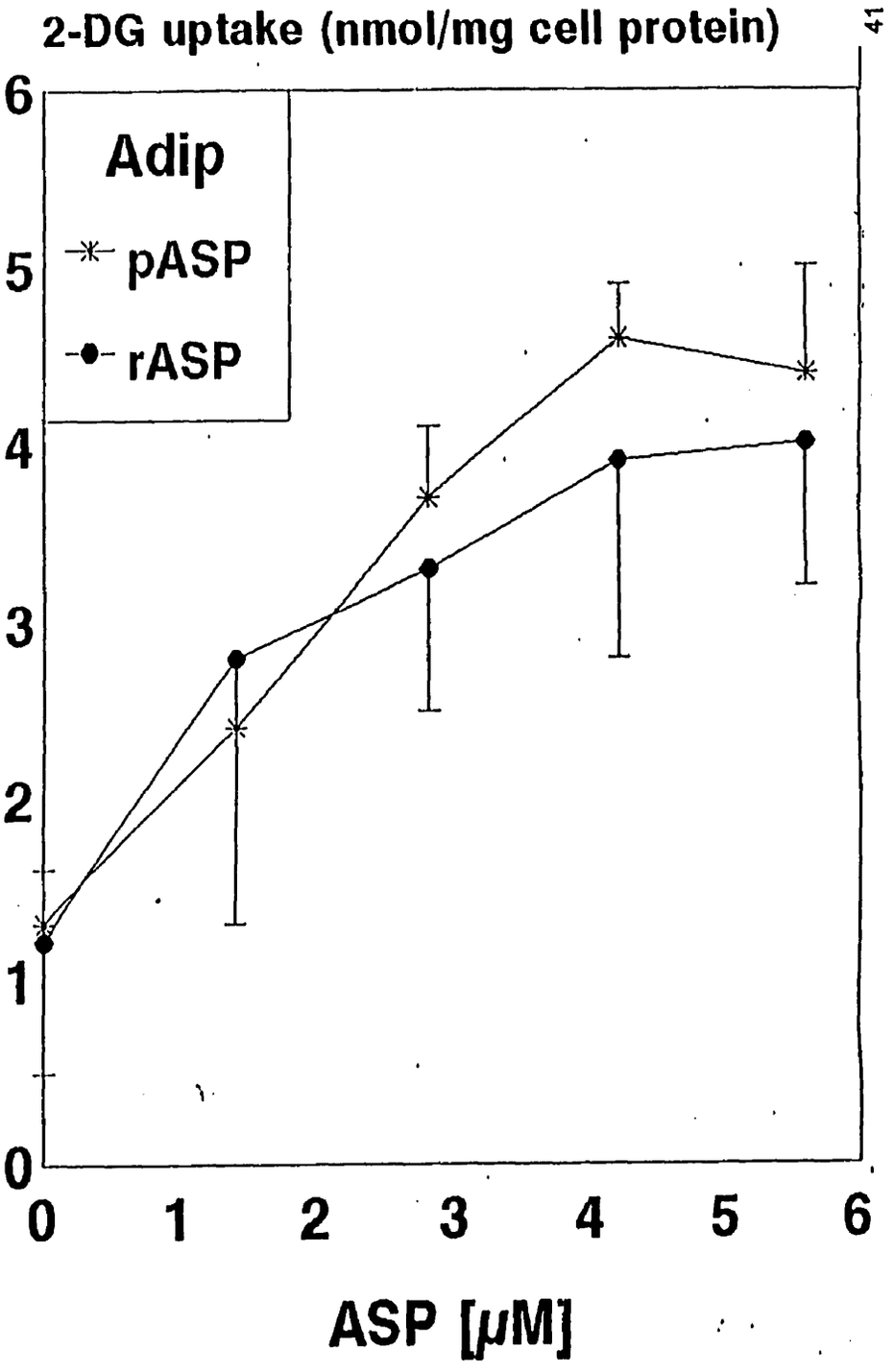
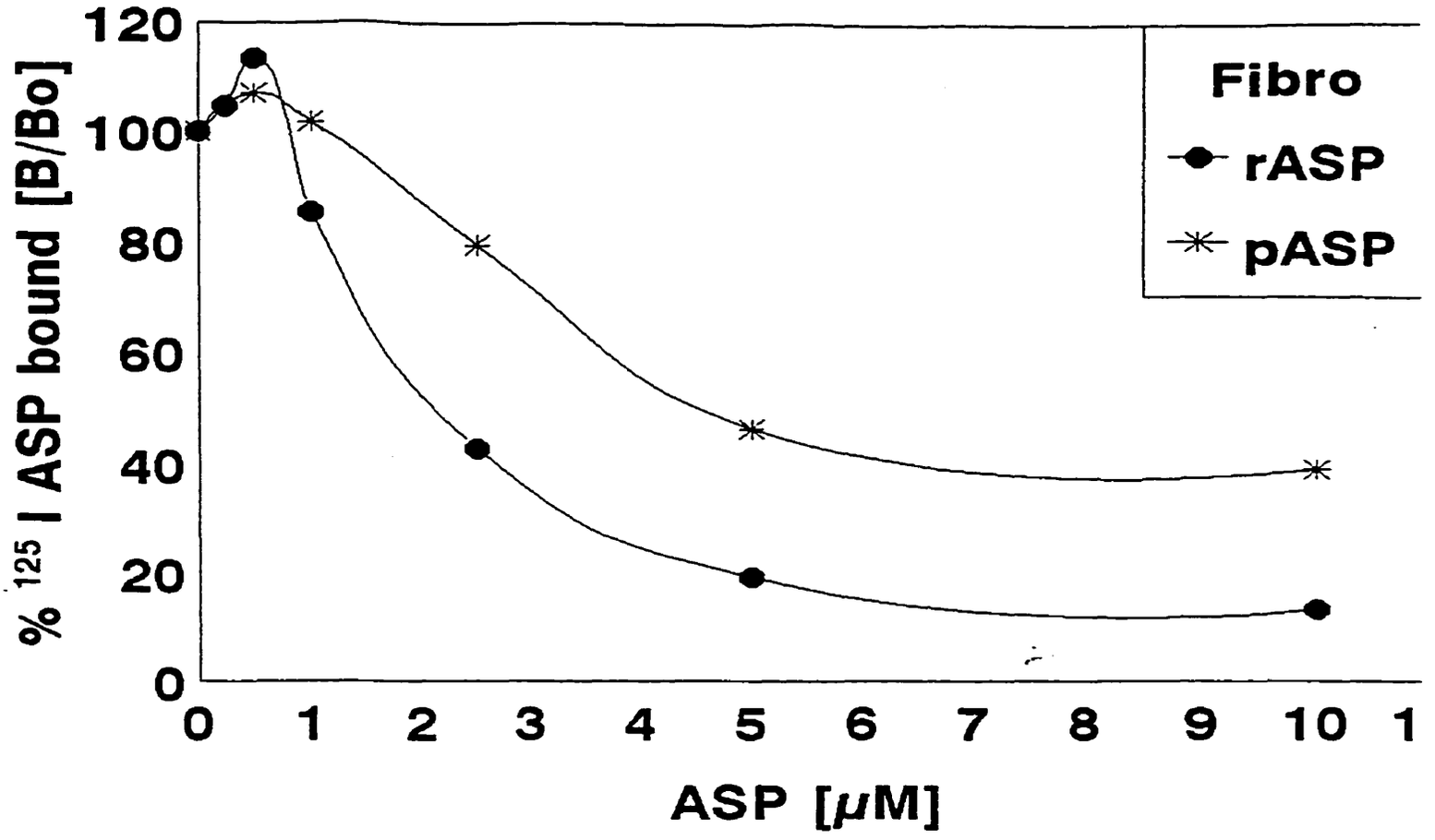

2-DG uptake (nmol/mg cell protein)
Adip
pASP
rASP
6
5
4
3
2
1
0
0
1
2
3
4
5
6
ASP [μM]

Figure 2.5. Competition binding of rASP and pASP

3T3 fibroblasts (top panel) and human differentiated adipocytes (bottom panel) were preincubated in serum-free DMEM/F12 overnight. ^{125}I ASP (500 nM, specific activity = 20 dpm/fmol) in 1% BSA in PBS was added with increasing concentrations of unlabelled ASP (up to 10 μM) and incubated for one hour on ice. Aliquots of solubilized cells were taken for counting and protein determination. Results are expressed as percent ASP bound (average n=2 experiments, triplicate wells in each experiment) where 100% is the amount of ^{125}I ASP bound in the absence of competitor (0.290±0.045 and 0.488±0.066 nmol/mg cell protein for 3T3 fibroblasts and human adipocytes respectively).


Fibro
rASP
pASP
% 125 I ASP bound [B/Bo]
ASP [μM]
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8 9 10 1

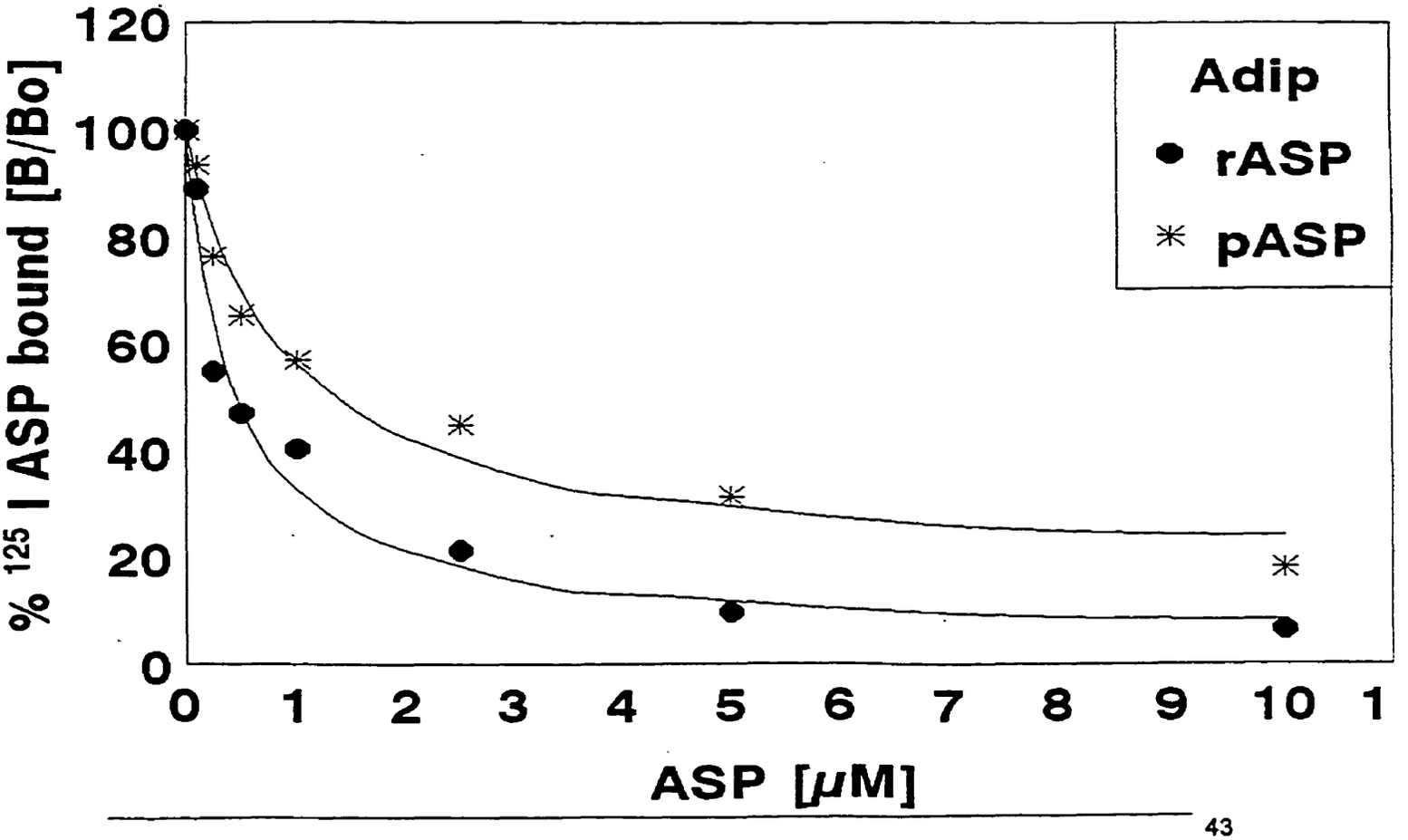

Adip
rASP
pASP
% 125 I ASP bound [B/Bo]
ASP [μM]
0
20
40
60
80
100
120
0 1 2 3 4 5 6 7 8 9 10 1

Figure 2.6. Immunoprecipitation of rASP, pASP and cdASP

Basal triglyceride synthetic activity is shown as 100% (control, open bars) measured as ^{3}H oleate incorporation into triglyceride over 4 hours (19.9±6.5 nmol/mg cell protein). The stimulatory effect of pASP, cdASP, and rASP (1.1 μM) is shown in the open bars. Comparable amounts of rASP, pASP and cdASP were immunoprecipitated with monoclonal anti-C3a/ASP antibody (solid bars) or non-immune mouse IgG (hatched bars) and the supernatant was then tested for triglyceride synthetic capacity in human fibroblasts. Results are shown as average ± std for n=4 experiments with duplicate dishes in each experiment.

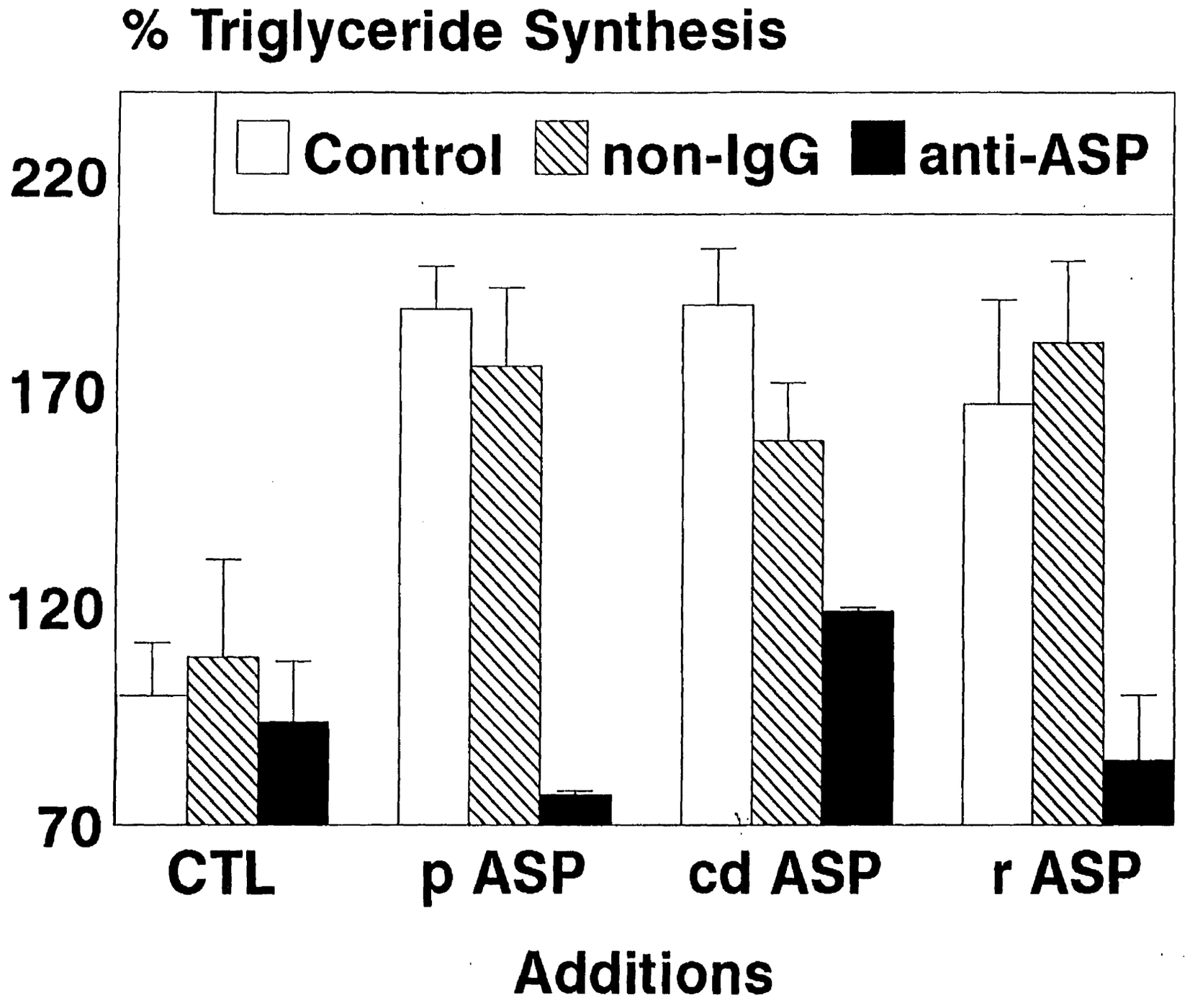

% Triglyceride Synthesis
Control
non-IgG
anti-ASP
220
170
120
70
CTL
p ASP
cd ASP
r ASP
Additions

Figure 2.7. Competition binding of modified ASP

3T3 fibroblasts (top panel) were preincubated in serum-free DMEM/F12 overnight. ^{125}I ASP (50 nM, specific activity = 20 dpm/fmol) in 1% BSA in PBS was added with increasing concentrations of unlabelled native ASP (dotted line) or modified ASP (top panel: urea-treated ASP (solid line), bottom panel: urea + β-mercaptoethanol treated ASP (solid line) and incubated for one hour on ice. Aliquots of solubilized cells were taken for counting and protein determination. Results are expressed as percent ASP bound (average n=2) where 100% is the amount of ^{125} I ASP bound in the absence of competitor.

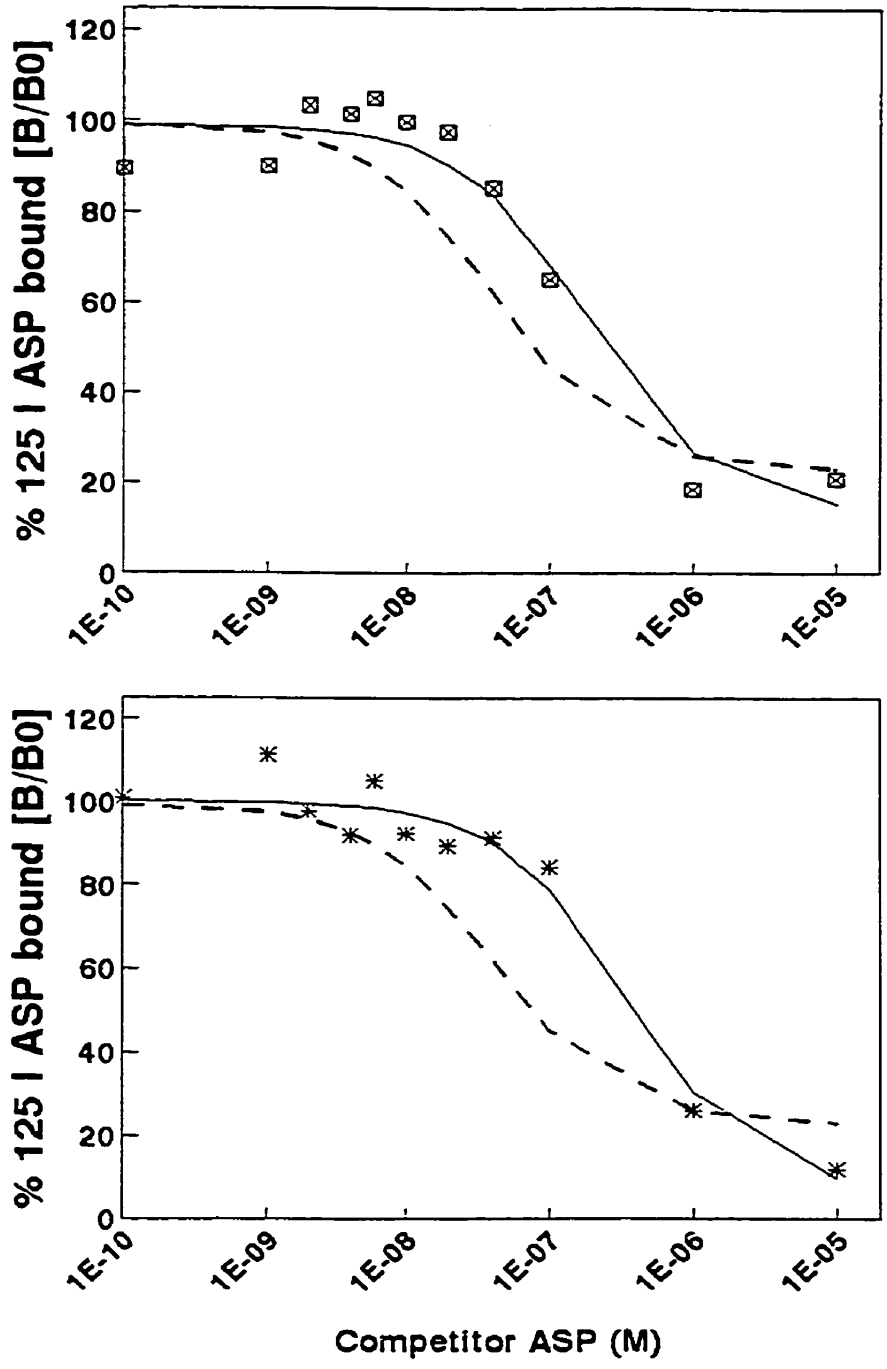
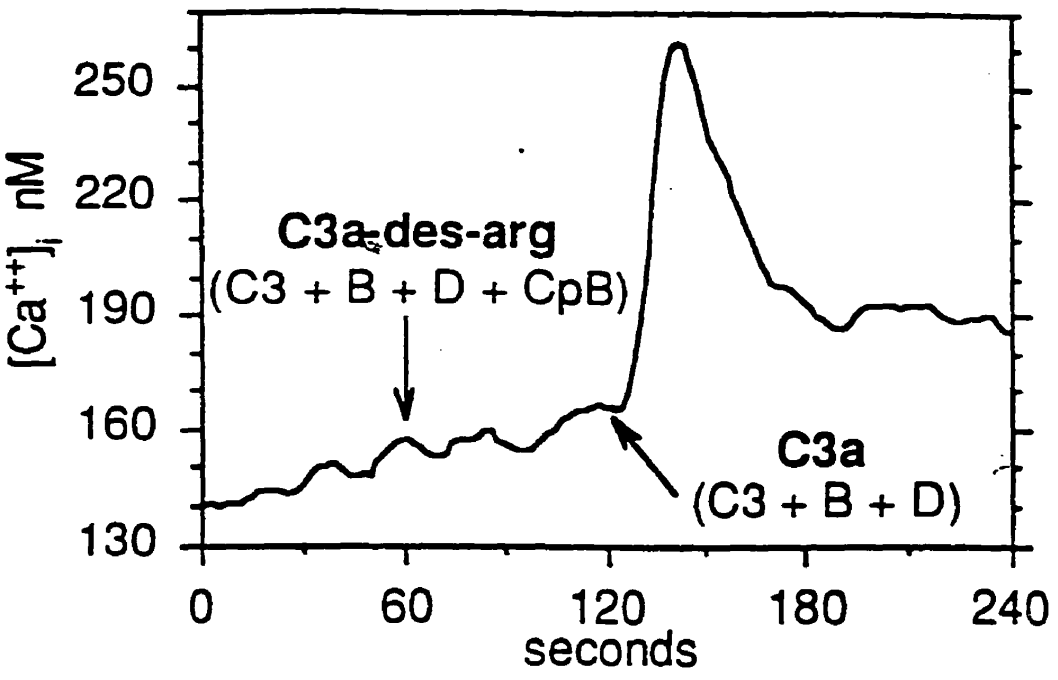

% 125 I ASP bound [B/B0]
120
100
80
60
40
20
0
1E-10
1E-09
1E-08
1E-07
1E-06
1E-05
% 125 I ASP bound [B/B0]
120
100
80
60
40
20
0
1E-10
1E-09
1E-08
1E-07
1E-06
1E-05
Competitor ASP (M)

Figure 2.8. Activity of ASP, C3a and carboxy terminal peptides on Ca^{2+} flux and TGS

Ca^{2+} flux was measured in human macrophage U937 cells by the Fura-2 method for C3a and ASP (C3adesArg) in the top panel, and for the 21(solid line, C57-77) and 20 (C57-76) carboxy terminal amino acid peptides as well as several amino terminal peptides (N1-19, N1-30 and N10-29) in the bottom panel. Relative activities are given in Table 2.III.


250
220
190
160
130
$[Ca^{++}]_i$ nM
C3a-des-arg
(C3 + B + D + CpB)
C3a
(C3 + B + D)
0
60
120
180
240
seconds

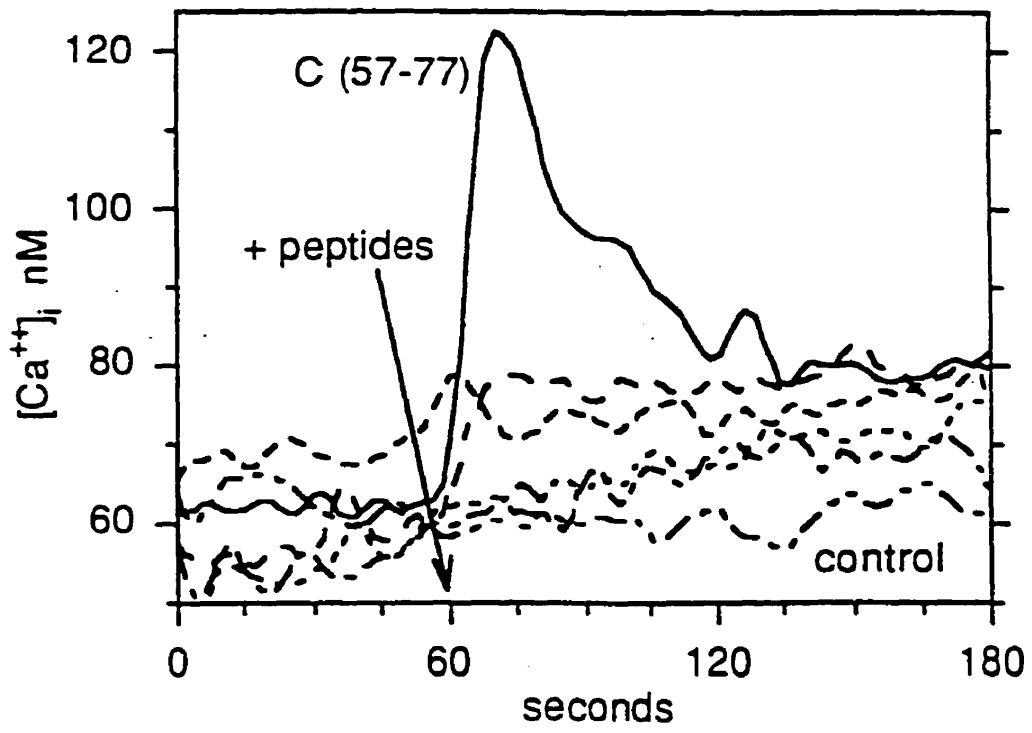

120
100
80
60
$[Ca^{++}]_i$ nM
C (57-77)
+ peptides
control
0
60
120
180
seconds

Table 2.I: Effect of ASP and Insulin on Adipocytes

		Triglyceride Synthesis		Glucose Uptake	
Adipocytes	n	% of Basal	P	% of Basal	P
Cynomologous omental primary	6				
ASP, 0.7 μM		190±16	<0.005		
Insulin, 0.7 μM		221±16	0.0005		
African green, omental primary	6				
ASP, 0.7 μM		194±44	<0.05		
Insulin, 0.7 μM		155±38	=0.065		
3T3 differentiated cultured	6				
ASP, 10μM				168±19	<0.005
Insulin, 2 nM				347±156	<0.001

Primary isolated adipocytes from primate omental adipose tissue were prepared by collagenase digestion. Cells were then incubated for 2.5 hours with 100 μM ^{3}H oleate and the indicated concentrations of ASP or insulin to measure triglyceride synthesis (TGS). 3T3 murine cells were differentiated to adipocytes for 5 days. Cells were preincubated with ASP or insulin for 1 hour, then glucose transport (glucose uptake) was measured as ^{3}H 2-deoxy-glucose transport (0.3 mM) uptake over 10 minutes. Results are expressed as percent change compared to basal levels of triglyceride synthesis or glucose transport (average ± std).

Table 2.II: Effect of Solubilization conditions on ASP Activity

Treatment	Basal	+ ASP	% Stimulation	+ Treated ASP	% Inhibition	P
NaCl, 1 M	83.8±5.3 (8)	251±7.3 (12)	300+9	239+11 (12)	8	ns
HCl, 1 M	90±5.4 (11)	243±8.2 (15)	279±13	223±12.6 (15)	13	ns
Urea, 4 M	109±22 (10)	232±32 (14)	234±12	257±32 (8)	0	ns
Urea, 4 M + β-Me, 8.8 mM	164±28 (5)	373±22 (6)	234±27	215±34 (6)	75	<0.001

ASP was treated with the indicated conditions for 1 hour, then repurified by reverse phase HPLC. Cells were preincubated overnight in serum-free DMEM/F12 and then changed to serum-free DMEM/F12 supplemented with 100 μM ^{3}H oleate complexed to BSA and 5.6 μM ASP. Triglyceride synthetic capacity was measured in human skin fibroblasts overnight as ^{3}H oleate incorporation into triglyceride over 18 hours. Results are expressed as nmol triglyceride (TG)/mg cell protein (average±std) with the number of assays indicated in parentheses, where ns=not significantly different.

Table 2.III: Comparison of lipogenic and anaphylatoxic activities of C3a, ASP and carboxy terminal peptides in human skin fibroblasts and differentiated U937 cells.

Addition	Concentration	TGS	Ca^{2+} Flux
	μM	*(% of control)*	
C3a holo form	1.7	181±8	160
C57-77 peptide	100	110±10	200
ASP (C3adesArg) holo from	1.7	158±11	nc
C57-76 peptide	100	105±15	nc

Human skin fibroblasts were preincubated overnight in serum-free DMEM/F12 and then changed to serum-free DMEM/F12 supplemented with 100 M ^3H oleate complexed to BSA and the indicated concentrations of ASP, C3a or peptides. Triglyceride synthetic capacity (TGS) was measured for 6 hours as ^3H oleate incorporation into triglyceride. Results are expressed as percent change (average±std) for n=4. U937 cells were differentiated with dibutryl cAMP for 3 days and Ca^{2+} flux was measured by the Fura - 2 method for C3a, ASP and the 21 and 20 carboxy terminal amino acid peptides as shown in Figure 2.8, where nc= no change from basal values.

2.5 Discussion

To our knowledge, this is the first report detailing functional expresssion of bioactive ASP (C3a desArg). Although previous expression of partially bioactive C3a has been described (12), it is important to note that these two polypeptides (ASP and C3a) appear to be functionally distinct. The results demonstrate that rASP is functionally active, whether assessed by triglyceride synthesis or glucose transport. As well, in competitive binding studies, the recombinant ASP was able to inhibit the binding of radiolabelled ASP to the same extent as native plasma ASP. Finally, immmunoprecipitation removed the rASP bioactivity as well as plasma ASP. The activity of rASP confirms that ASP (C3adesArg) is indeed the bioactive plasma component, which acts in an autocrine/paracrine manner to stimulate TG synthesis in adipocytes *in vivo*.

Clearly, although ASP (C3a desArg) is bioactive for triglyceride synthesis, it is not competent to stimulate anaphylatoxic activity in U937 cells and other cells (8,9) whereas C3a (with the carboxy terminal arginine intact) is a potent activator of this pathway. As well, C3a activity is not sensitive to denaturing conditions that change the structural conformation of the protein (9,20), while ASP certainly is. Thus the function of ASP ASP may be more sensitive to refolding conditions than is the functionality of C3a, a finding which would not be unexpected considering the presence of 6 cysteines (3 disulphide bridges) within a 36 amino acid region. On the other hand, C3a bioactivity is clearly dependent on the presence of the carboxy terminal arginine as shown here and in many other studies (8,9,20-22). By contrast, the ASP triglyceride stimulatory capacity is obviously not dependent on the presence of the terminal arginine. Thus, the lipogenic activity and the anaphylatoxic activity appear to reside in specific and distinct structural domains of the polypeptide. It is important to note that purification techniques that maintain the anaphylatoxic activity of C3a may abrogate the lipogenic activity of ASP as shown in the present study. The present study also demonstrates that human ASP can stimulate TGS not only in human cells, but across several species (primates, mice, rats). It is interesting to note that, although the primary amino acid sequence can vary from species to species, there appears to be phylogenetic conservation of both the carboxy

latter structural domain is important in ASP lipogenic activity, although not for C3a anaphylatoxic activity.

Why do both ASP and C3a stimulate triglyceride synthesis in adipocytes, yet only C3a is bioactive in macrophage and other immunologic cells? The differences may be due to distinct cell surface receptors, or to differences in cell signalling and links to downstream cell specific pathways triggering alternate events: lipogenesis or immunologic response. A human C3a receptor has been cloned and characterized (10,11) although the reports did not examine whether it is present in human adipocytes. In the literature there are examples of specific receptors which can differentiate between the desArginated form *versus* arginated polypeptides. For example, bradykinin and bradykinin desArg bind specifically and with different affinities to two distinct receptors (BK1 and BK2) which can be present in the same cell type to generate differential responses (for review, see 23-25). This may also be true of ASP (C3a desArg) and C3a. Future studies using site-directed mutagenesis and deletion analysis to investigate the structure-function relationships of ASP in a recombinant model, as well as receptor interactions will allow these possibilities to be explored.

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Prelude II

We wished to determine which regions of the ASP molecule were responsible for triglyceride synthesis and cell surface binding. In C3a, a molecule differing only by the presence of a carboxy-terminal Arginine, activity was found to reside predominantly in the C-terminal octapeptide and little of disulphide linked core. Extending on these studies for ASP, the contributions of the carboxy-terminal, amino-terminal and amino acids such as the disulphide core were tested for cell surface binding and stimulation of triglyceride synthesis.

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Chapter 3
Acylation Stimulating Protein (ASP): Structure:Function Determinants of Cell Surface Binding and Triacylglycerol Synthetic Activity

3.1 Synopsis

Acylation Stimulating Protein (ASP or C3adesArg) is a potent lipogenic factor in human and murine adipocytes and fibroblasts. The arginated form of ASP, i.e. C3a, stimulates immunological responses in human granulocytes, mast cells, guinea pig platelets and guinea pig macrophages, however, C3adesArg (ASP) is inactive in stimulating these responses. Thus both ASP and C3a are bioactive across species but are not functionally interchangeable. Tertiary structure of both proteins by X-ray crystallography and NMR predicts a tightly linked core region consisting of 3 alpha helices linked *via* three disulphide bonds with one of the alpha helices extending out from the core, and terminating in a flexible irregular conformation carboxyl tail region. The present studies were undertaken in order to define the functionally active domains of ASP distinctive from those of C3a using chemical modifications, enzymatic cleavage and synthetic peptide fragments. The results indicate that: (i) the amino terminal region (<10 amino acids) plays little role in ASP receptor binding and triacylglycerol synthesis stimulation (TGSS); (ii) the native carboxyl terminal region had no activity, but modifications which increased hydrophobicity increased receptor binding and led to some activation of TGSS and (iii) an intact disulphide linked core region is essential for TGSS activity but not receptor interaction. Finally, basic charges in the carboxyl region (His) are essential for ASP TGSS but not receptor binding whereas both functions are abrogated by Lys modification in the disulphide-linked core region. The present results suggest that there are two functional domains in ASP, one of which is responsible for the initial binding to the cell surface receptor. A second domain activates and increases TGSS. This contrasts markedly with the structure-function studies of C3a where both binding competency and function were dependent on the carboxyl terminal Arg. Thus ASP demonstrates distinct bioactivity.

3. 2 Introduction

Acylation Stimulating Protein (ASP) has a unique function in lipid metabolism. ASP stimulates triacylglycerol synthesis in primary human adipocytes and to a lesser degree in preadipocytes and fibroblasts (1). The effect of ASP is mediated through a coordinate effect on fatty acid esterification by increasing the activity of diacylglycerol acyltransferase, the final enzyme in triacylglycerol synthesis (2), and stimulation of glucose transport. In adipocytes, smooth muscle cells, preadipocytes and fibroblasts, glucose transport increases due to translocation of the glucose transporters (Glut 1, Glut 3 and Glut 4) from intracellular sites to the plasma membrane surface (3,4). These dual effects are mediated through specific cell surface binding (5) resulting in activation of a signalling pathway which includes activation of protein kinase C (6).

Sequence analysis of ASP indicated that it was identical to the desarginated form of C3a, that is C3adesArg (1). C3a is generated when the complement system is activated *via* either the classical or alternative pathway. In adipose tissue, alternative pathway activation involving C3 (the precursor protein), factor B and adipsin (factor D) has been demonstrated (7). The terminal arginine of C3a is cleaved by carboxypeptidase N to generate ASP (C3adesArg), and in normal human plasma, only the ASP form of the protein is present (8). C3a stimulates proinflammatory responses in different immunocompetent cells such as histamine release from IL-3 stimulated basophils (9), chemotaxis of eosinophils (10) and mast cells (11), release of reactive oxygen metabolites from polymorphonuclear leukocytes (12) and aggregation and degranulation of guinea pig platelets (13). These bioactive effects are mediated by specific interaction with the C3a receptor, which belongs to the large group of guanine-nucleotide-binding protein (G-protein) -coupled receptors (14,15). The desarginated form of the protein (C3adesArg/ASP) has no effect on the above mentioned cell populations and does not bind to the cloned C3a receptor (16,17). These data are in contrast to its potent bioactivity in adipocyte lipogenesis (5). Human ASP has been shown to stimulate triacylglycerol synthesis and glucose transport in human adipocytes (1,4,5), murine 3T3 cells (5), primate cynomologous and African Green monkey adipocytes (5), rat L6 myotubes (18) and human fibroblasts (1). Thus these two similar polypeptides have distinct metabolic properties.

The tertiary structures of both C3a and ASP (C3adesArg) have been analyzed using X-ray crystallography and solution NMR (19,20). No structural changes induced by the presence of the carboxyl terminal arginine could be demonstrated (20) but due to the flexible irregular conformation of the carboxyl tail region, this might be difficult. Nevertheless, the presence of this terminal arginine appears crucial for C3a bioactivity (16). The physical analyses indicate that ASP/C3adesArg is composed of a tightly linked core region consisting of 3 alpha helices (residues 17-27, 35-41 and 47-73) held intact through three disulphide bonds with one of the alpha helices extending out, terminating in a flexible irregular conformation carboxyl tail region of 5-6 amino acids that is free from interactions with the rest of the molecule (21). The N-terminal segment does not remain docked to the cysteine stabilized core of the molecule but is in dynamic motion and is helical from residues 5-15.

It is striking that both ASP and C3a are bioactive across species but ASP is not functionally interchangeable with C3a. Extensive functional studies have been performed on C3a (review (21)). Initial studies indicated that the disulphide linked core region was not essential for C3a bioactivity as unfolding, reduction and refolding of the molecule did not affect activity (21). However, it did appear that the native carboxyl terminal region was sufficient for bioactivity to a greater (22) or lesser (23) degree. A number of C3a analogues were developed based on this information (24) and indeed such a bioactive peptide was instrumental in cloning and characterizing the C3a receptor (14,15). However, since ASP and C3a share few biologic effects, any modification to C3a may not be relevant to ASP. Although desargination of C3a destroys proinflammatory responses, both the desArg form of the protein (ASP) and the arginated form (C3a) are equally competent for triglyceride synthesis stimulation (5). Preliminary studies indicated that the native carboxyl terminal peptide of ASP had no triacylglycerol synthetic stimulatory bioactivity (5), although it has been reported to have from 10% to full (100%) C3a activity (22,25). The aims of the present paper were twofold: (i) to determine if those modifications or peptides which have been previously shown to conserve C3a stimulatory activity (see above) also maintain ASP stimulatory activity and (ii) to determine if other modifications, which have not been examined with respect to C3a but have been shown to

be important for a structurally similar molecule (C5a), might be important for functional activity of ASP.

3.3 Materials and Methods

3.3.1 ASP Purification and Peptides: Human plasma ASP was prepared as described previously (6). Purity and molecular mass were verified by ion spray mass spectrometry. The following carboxy terminal native and modified peptides were used; RASHLGLA (native ASP-(69-76)), LRRQHARASHLGLA (native ASP - (63-76)), LRRQAWRASALGLAR (P117) and Fmoc-YAAALGLAR (P32). Peptides P32 and P117 were prepared by solid phase synthesis and analyzed by thin layer chromatography, HPLC and amino acid analysis as described (26). The modified residues of P32 and P117 are underlined.

3.3.2 ASP Digestion: ASP (500 μg) was digested with endoproteinase Asp-N (Boehringer Mannheim, Laval, Quebec, Canada) 1:333 w/w for 60 minutes at 37°C. Chemical cleavage of methionines was performed using cyanogen bromide (27). A crystal of cyanogen bromide (0.292 g) was added to 1 mL of 88% formic acid (final reaction concentration = 70%) (v/v) followed by addition of 500 μg ASP (molar ratio of CNBr : total methionines was 300:1). The reaction was carried out in a fumehood for 4 hours at room temperature in darkness. Cyanogen bromide was removed under a stream of nitrogen in a fumehood and the ASP lyophilized and reconstituted in 500 μL of PBS. The fragments were separated on HPLC and the molecular weight of the isolated fragment determined by ion spray mass spectrometry. Protein concentration was measured by Lowry assay (28).

3.3.3 ASP Chemical Modifications: The disulphides were reduced and blocked to prevent dimerization of cysteines (29). Purifed ASP (500 μg) in PBS was incubated at 37°C for 1 hour with an equal volume of 8 M urea in 0.29 M Tris solution (pH 8.6) with 0.4% 2-mercaptoethanol and 5.25 mM EDTA in a 2 mL siliconized tube and flushed with N_2 to prevent oxidation. The cysteines were then blocked with the addition of iodoacetic acid (1.44 M) in 1N NaOH (room temperature) or iodoacetamide (0.724 M) in PBS (4°C) for 30 minutes in the dark. The reaction was terminated by re-purifying ASP by HPLC.

The basic lysine residues of ASP were modified as described by Turk and Macek (30). Pyridoxal-5-phosphate (66 mM final concentration) was added to plasma ASP (500 μg) in 0.1 M phosphate buffer (pH 7.1) and incubated at 4°C for 60 minutes in the dark.

Ice cold sodium borohydride was added (final concentration 250 nM) to reduce the imine bond and a drop of octanol was added (to reduce foaming) and incubated at 4°C (50 minutes) then at room temperature (10 minutes). The sample was degassed under vacuum then re-purified by HPLC.

Histidine residues were modified with diethyl pyrocarbonate (DEPC) (30). DEPC concentration is determined in the solution prior to use (31). To 500 μg of ASP in 0.1 M phosphate buffer (pH 6.0), DEPC (1% in anhydrous ethanol, 4°C) is added to a final concentration of 1.4 mM and incubated for 60 minutes at room temperature. To prevent protein denaturation the alcohol concentration did not exceed 2%.

As additional controls, for all of the chemical modifications, reagents were added to ASP and injected immediately on HPLC for re-purification. Protein concentration of modified ASP was determined by Lowry assay (28). Changes in the overall charge of the molecule were assessed by native gel electrophoresis (16.7% polyacrylamide) as described by Reisfield for basic proteins (32) as well as by circular dichroism (see below).

3.3.4 Circular Dichroism of ASP: Circular dichroism (CD) spectra of ASP preparations in PBS, pH 7.4 were measured in the far UV range (200-250 nm) using a Jasco 710 spectro- polarimeter. Spectra represent the accumulation of 10 scans per sample at a scan speed of 10 nm per minute, bandwidth 1.0 nm, resolution 0.2 nm at a sensitivity setting of 100 millidegrees. Dynode voltage did not exceed 600 volts and sample concentration was from 0.3 to 1.1 mg/mL in cuvettes of 0.1 cm path length. Protein concentrations of all preparations were determined by Lowry assay (28) and the spectra adjusted accordingly by computer assisted analysis (J-700 for Windows Standard Analysis, Ver 1.10.00, Jasco Corp) and expressed as molar mean residue ellipticity.

3.3.5 Lipid Synthesis in Cultured Cells: Human fibroblasts were obtained from forearm biopsies of normolipidemic subjects and cultured as previously described (1). 3T3-L1 preadipocytes (obtained from ATCC Rockville, Maryland) were cultured in the same fashion as the human fibroblasts. ASP stimulated both types of cells to the same extent and the cell type used is indicated in the figure legends (5). Cells were subcultured, plated out at 3×10^3 cells/cm^2 and grown in 10% fetal calf serum in DMEM/F12 medium. Cells were used for experiments at 80% confluency. Cultured cells in 24 well dishes (1.7

cm^2/well) were preincubated in serum-free DMEM/F12 medium overnight (18 hours) prior to the experiments. Triacylglycerol synthesis was measured as incorporation of (^3H) oleate (specific activity 10.0 Ci/mmol, Du Pont-New England Nuclear, Mississauga, Ont, CA) into triacylglycerol. Cells were incubated for 18 hours in 100 μM oleate complexed to BSA (5:1 molar ratio, average final specific activity = 100 dpm/pmol) in serum free DMEM/F12 as previously described (5). Results are expressed as nmol (^3H) oleate incorporated into triacylglycerol per mg of cell protein.

3.3.6 Radiolabelled Competition Binding Assay: ASP was radiolabelled using Iodogen (Pierce Chemicals, Rockford, IL) and specific activity was measured as dpm per μg of trichloroacetic acid (10% TCA) precipitable protein (average specific activity = 20 dpm/fmol). Competitive binding was performed on human skin fibroblasts cultured in 96 well plates. Cells were preincubated with serum-free DMEM/F12 medium overnight. Cells were prechilled on ice for 15 minutes followed by incubation for 1 hour with 1 or 50 nM (^{125}I) ASP in 100 μL of 1% (w/v) bovine serum albumin (BSA) in PBS and increasing concentrations of unlabelled ASP. After a 1 hour incubation, 5 μL aliquots (in triplicate) of medium were counted for calculation of free ASP. Cells were washed three times with ice cold PBS and soluble cell protein dissolved in 100 μl of 0.1 N NaOH. Aliquots were taken for counting of bound ASP and cell protein determination by the method of Bradford (33) using a commercial assay (BioRad, Mississauga, Ontario, CA). The results are calculated as nmol of ASP bound per mg soluble cell protein and expressed as % competition where 100% = amount of (^{125}I) ASP bound in the absence of competitor. Calculation of competition was performed by iterative four parameter logistic function analysis (Sigma Plot, Jandel Scientific, San Rafael, CA) in order to calculate specific binding, non-specific binding, and IC_{50} (50% inhibitory concentration).

3.3.3.7 Statistics: All results are expressed as average ± standard error of the mean (sem). Significant differences are calculated by 1 way or 2 way ANOVA or t-test (as indicated) where pNS=not significant.

3.4 Results

Phylogenetic comparison of the primary sequence of ASP from a number of species with human (Accession number M65080) (34), rat (Accession number P01026) (35), mouse (Accession number P01027) (36), guinea pig (Accession number P12387) (37), chicken (Accession number V16848) (38), cobra (Accession number L02365) (39), trout (Accession number P98093) (40), lamprey (Accession number Q00685) (41), hagfish (Accession number P98094) (41) and pig (Accession number P01025) (42) was performed by computer assisted analyses. Overall there is 67% to 72% primary sequence homology between the mammals. The carboxyl terminal region (LGLA) is highly conserved with 100% identity between mammals for the last 4 amino acids. Similarly, the amino terminal region (amino acids 1-15) is 73% to 80% identical within the mammals. For all sequences, there were 6 cysteine residues, with three disulphide bonds in the motif $CC(X)_{12}$-$C(X)_{12}$-$C(X)_6$-CC. This is not only characteristic of ASP/C3adesArg sequences but also of C5a, C4a, fibulin and sex linked protein (43). Finally, human ASP is a very basic molecule with a pI of 9.1 (1). A basic pI (9.05-9.99) is also predicted from the other mammalian sequences. The positively charged amino acids in human ASP are primarily Arg (n=10), Lys (n=7) and His (n=2) with the Lys and His located primarily in the amino terminal core "head" region and carboxyl terminal "tail" regions respectively. Conserved sequences, which are common across species, may represent important functional sites. Structure:function modifications were targeted to the following aspects of the molecule: the disulphide knot core region, basic charges, and the carboxyl- and amino- termini. The modifications used were (i) chemical modifications of specific amino acids, (ii) enzymatic and chemical cleavage of specific regions of the protein and (iii) use of synthetic peptide fragments, which have been previously documented to retain C3a activity (15,22,24). In each case, the modifications were tested in two ways: first, for triacylglycerol synthesis stimulatory capacity determined as the increase in (^{3}H) oleate incorporation into storage triacylglycerol in human fibroblasts or murine 3T3 cells, and second, for the capacity to competitively inhibit binding of native radiolabelled ASP (^{125}I ASP) to cell surface receptor binding sites.

Since native and modified carboxyl peptides of C3a have previously been shown to possess C3a biological activity (22,23,44), we first examined the effects of native as

well as modified carboxyl terminal peptides on triacylglycerol synthesis. The results from the effects on triacylglycerol synthesis are shown in Figure 3.1, top panels. The native 8 carboxyl terminal peptide of ASP (P8aa) (top right) did not stimulate triacylglycerol synthesis even at molar concentrations 3 times higher than that of ASP, which at 11.2 μM, stimulated triacylglycerol synthesis in 3T3 cells by 205% *vs* basal ($p<0.002$ by ANOVA). The native 8 amino acid peptide was also ineffective at inhibiting (^{125}I) ASP binding to cell surface receptors as assessed in a competition binding assay. Only at extremely high concentrations was the peptide capable of inhibiting ASP binding (IC_{50}:1.2 mM, Figure 3.1, bottom panel).

This peptide sequence was modified as described by Gerardy-Schahn *et al* to increase the hydrophobicity of the peptide through addition of a fluorenylmethyloxycarbonyl (Fmoc) group which they proposed would enhance interaction with biomembranes and alter the association with the receptor from a 3-dimensional to 2-dimensional interaction to increase the rate of association (23). This peptide (P32) was fully bioactive for C3a activity (23). This modification increased the capacity of the peptide (P32) to inhibit native ASP binding although the concentration of peptide required was still greater as compared to ASP (IC_{50}: 5.6 μM *vs* IC_{50}: 44 nM for native ASP $p<0.05$). However, this modified peptide P32 demonstrated no stimulation of triacylglycerol synthesis event at high molar concentrations (top left panel).

Longer peptides were also used. A 15-mer native peptide was incapable of stimulating triacylglycerol synthesis or inhibiting binding of ASP (data not shown). A modified 15-mer peptide (P117), which has been shown to possess full C3a activity (15) was also tested for triacylglycerol synthetic stimulatory activity. The modifications of the peptide from the native sequence were designed to increase its hydrophobicity and enhance membrane interaction as with P32, as described by Crass *et al* (15). P117 was competent to inhibit ASP binding at higher concentrations (IC_{50}: 120 nM not significantly different *vs* native ASP), and had a consistent effect on stimulation of triacylglycerol synthesis in both human skin fibroblasts (HSF, top left, $p<0.001$ by ANOVA *vs* basal) and 3T3 cells (top right, $p<0.001$ by ANOVA *vs* basal), although at higher concentrations than ASP (166% HSF and 159% 3T3 at 25 μM) (Figure 3.1). Thus, the modified carboxyl

terminal region is not sufficient for complete ASP bioactivity but does appear to mimic partial receptor binding.

We then tested the involvement of the amino terminal region in binding and triacylglycerol synthesis stimulation. ASP was treated with CNBr or with Asp-N endoproteinase in order to remove the N-terminal region and the remaining ASP was re-isolated and tested. Mass spectrometry analysis indicated that with Asp-N endoproteinase treatment, the amino terminal peptide (1-9) was removed leaving a protein of 7860 daltons. In the cyanogen bromide (CNBr) treated ASP both the amino terminal peptide (1-9) and the peptide fragment (28-32) which forms part of a loop region in the core were removed (remaining protein = 7203 daltons). In both cases, as shown in Table 3.1, the remaining ASP fragment was capable of stimulating triacylglycerol synthesis in both HSF and 3T3 (199% ± 2% Asp-N, $p<0.05$ and 222% ± 25% CNBr, $p<0.05$ *vs* basal for HSF and 165% ± 2% ASP -N, $p<0.05$ and 231% ± 11% CNBr $p<0.05$ *vs* basal for 3T3). As well, both proteins were capable of inhibiting ASP binding to the same extent as native, intact ASP (IC_{50}: 123 nM Asp-N and 113 nM CNBr, pNS by ANOVA *vs* ASP). Thus, this small amino terminal segment in the flexible amino region (less than 10 amino acids) appears to play little role in ASP bioactivity.

The disulfide-linked core region structure was modified to alter the secondary structure of ASP. The protein was unfolded with urea, and the disulfides cleaved and blocked with either iodoacetamide (Cys-NH), which does not alter the overall protein charge, or iodoacetic acid (Cys-COOH), which adds one new acidic group/cysteine to the protein and renders the protein less basic. As a result, the migration on native basic gel electrophoresis is substantially retarded for Cys-C00H, but less so for Cys-NH (data not shown). The conformation of these modifications was analyzed by circular dichroism (CD) in the far UV region as shown in Figure 3.2 (left panel). Native ASP demonstrated a characteristic "w" profile indicating high alpha helix content. Denaturation of ASP (100°C + β-mercaptoethanol for 5 minutes) reduced the molar ellipticity dramatically (a similar profile was obtained with ASP boiled 100°C for 3 hours, data not shown). These results are comparable to those previously reported for C3a (45). Treatment of ASP with urea alone (without reduction of the Cys bonds) followed by removal of urea has been shown to have no inhibitory effect on triacylglycerol stimulating activity or competition

binding (5) and as shown here, had no effect on molar ellipticity analyzed by CD (Figure 3.2, left panel). In both cases, treatment to block Cys residues resulted in an increase in the IC_{50} from 3 to 14 times compared to native ASP (IC_{50}: 37 nM native ASP *vs* 120 nM Cys-NH and 510 nM Cys-COOH, $p<0.001$ by ANOVA) (Figure 3.3). By contrast, the triacylglycerol stimulating activity was lost (85% ± 6% inhibition Cys-NH, $p<0.05$; 60% ± 2% inhibition Cys-COOH, $p<0.05$ *vs* ASP treatment, $p<0.003$ ANOVA (Table 3.2). As might be expected, there was a marked change in the structural conformation of the treated ASP, and for both Cys-NH ASP and Cys-COOH ASP, CD analysis demonstrated a marked reduction in the alpha-helicity of both molecules (Figure 3.2, left panel). Thus an intact core region appears to be essential for activity, although not necessarily for receptor interaction.

ASP is a very basic polypeptide (pI 9.1) (1). These basic charges are due principally to His, Lys and Arg residues. The Arg residues (10) are distributed along the "backbone" of the protein, while the His residues (2) are found in the carboxyl tail region. Of the 7 Lys residues, 5 are found in the amino terminal region (between residues 7 to 21) and two at residues 50 and 51. These residues cluster as a "crown" in the "head" region of the alpha-helix/disulfide linked core. We targeted Lys and His for chemical modification. In each case, modification resulted in changes in mobility as assessed by native basic gel electrophoresis (data not shown). The results for competition binding are shown in Figure 3.3 (right panel). His modification reduced the capacity to inhibit specific binding (IC_{50}=707 nM *vs* 44 nM for native ASP), whereas with the Lys modification, there was a complete loss of competitive binding. With respect to CD analysis of the chemically modified ASP, His-ASP demonstrated a "w" profile comparable to native ASP, whereas the molar ellipticity for Lys-ASP was substantially diminished suggesting major changes in the structure of the molecule. In each case, the capacity to stimulate triacylglycerol synthesis was markedly reduced: 85% ± 11% inhibition His-ASP, $p<0.05$ and 100% ± 8% inhibition Lys-ASP, $p<0.001$ *vs* positive ASP control which stimulated up to 2 fold (Table 3.2). Thus modifications which interfere with the receptor binding capacity also result in a loss of activity and the present results indicate that the basic residues appear to be important for both receptor interaction and subsequent activity.

Table 3.1:Effect of Amino Terminal Cleavage of ASP on Triacylglycerol Synthesis Stimulating Activity and Competition Binding:

TGSS	CNBr-ASP		AspN-ASP	
	HSF	3T3	HSF	3T3
Basal TGS (100%) (nmol/mg cell protein)	127 ± 14	56 ± 1	144 ± 18	56 ± 1
ASP (native)	228% ± 17%*	185% ± 2%*	175% ± 28%*	138% ± 3%*
ASP (modified)	222% ± 25%*	231% ± 11%*	199% ± 2%*	165% ± 2%*
ANOVA	p<0.006	p<0.001	p<0.001	p<0.001
IC_{50}	113 nM	nd	123 nM	nd

Human skin fibroblasts (HSF) and 3T3-L1 fibroblasts (3T3) were cultured as described in methods. ASP was treated with CNBr or Asp-N endoproteinase and re-purified on HPLC. Triacylglycerol synthetic stimulation (TGSS) is measured as (^{3}H) oleate incorporation into triacylglycerol for n= 5 to 7 (HSF) and n= 2 (3T3) experiments. Basal triacylglycerol synthesis (TGS) is expressed as nmol ^{3}H oleate incorporated into triacylglycerol/mg cell protein (=100%) where * $p<0.05$ *vs* basal (results from Bonferroni text, ANOVA). For competition experiments, the effectiveness of the competitor (for IC_{50}) was tested over 15 different concentrations (duplicate points at each concentration). The degree of inhibition (top - bottom plateau) was compared to native ASP where binding at 1nM ^{125}I ASP = 173 ± 14 fmoles (^{125}I) ASP bound/mg cell protein (n=11). The IC_{50} was calculated by iterative four parameter logistic function analysis where IC_{50} native ASP = 35 ± 5 nM and nd= not determined

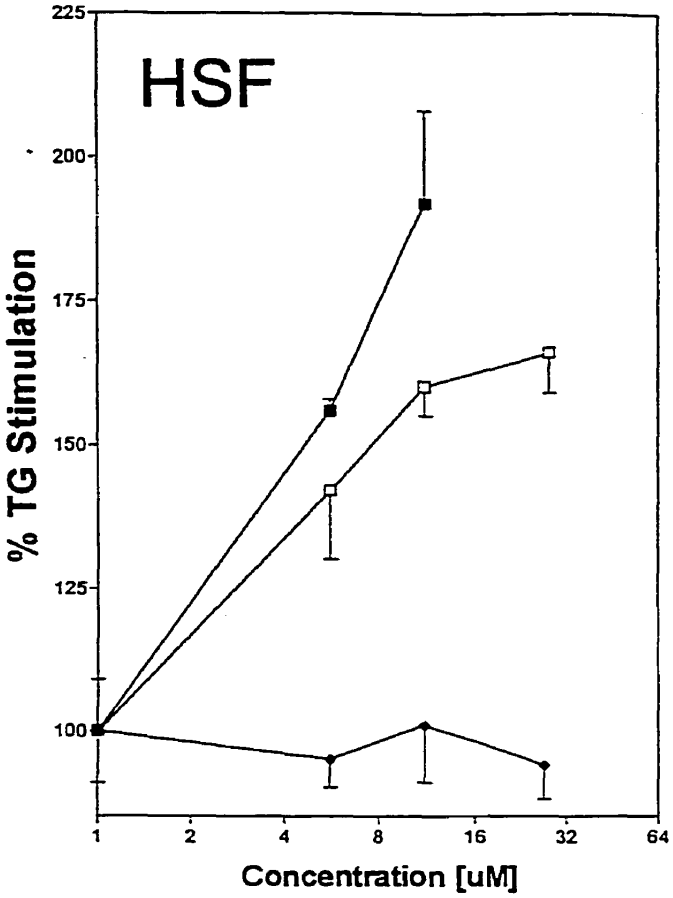
Table 3.2: Triacyglycerol Synthetic Activity of Modified ASP

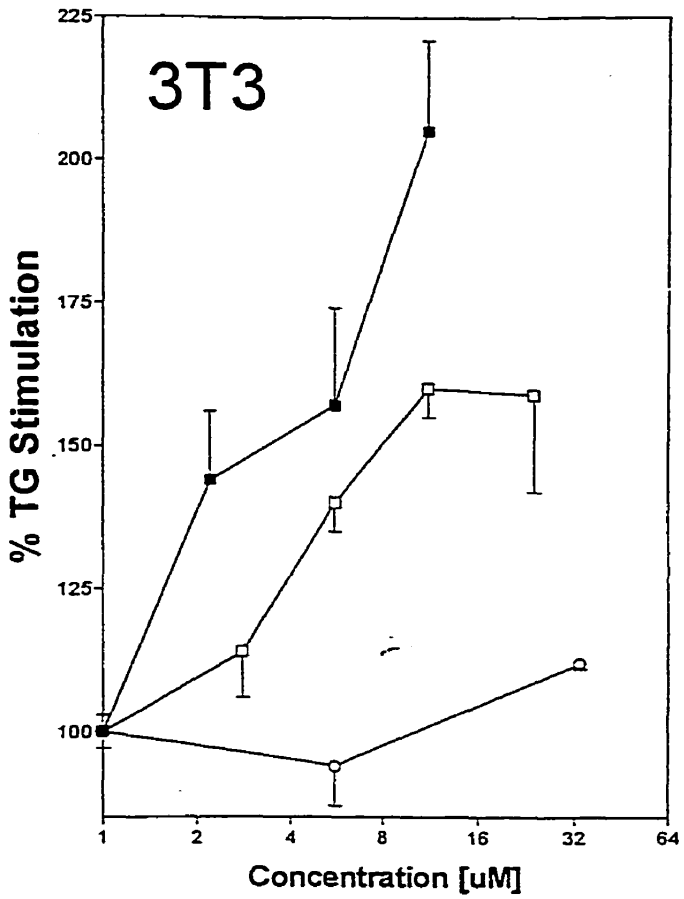
Modification	Basal TGS	Native ASP (%)	Modified ASP (%)	% Inhibition
Cys-NH ASP (n = 8)	109 ± 22	232 ± 34 (213%)	128 ± 23* (117%)	85%
Cys-COOH ASP (n = 7)	109 ± 22	232 ± 34 (213%)	158 ± 30* (145%)	60%
His-ASP (n = 4)	94 ± 23	165 ± 56 (175%)	104 ± 41* (111%)	85%
Lys-ASP (n = 9)	80 ± 12	177 ± 27 (221%)	76 ± 15** (95%)	100%

Cells (3T3-L1 fibroblasts) were prepared as described in methods. ASP was modified by treatment with urea and iodoacetamide (Cys-NH), urea and iodoacetic acid (Cys-COOH ASP), DEPC (His-ASP), or pyridoxal-5-phosphate (Lys-ASP) and re-purified on HPLC following treatment. Activity is measured as (^{3}H) oleate incorporation into triacylglycerol (nmol/mg cell protein) (see Legend to Figure 1), where * $p<0.05$ and ** $p<0.001$ for modified *vs* native (untreated) ASP.

Figure 3.1: The effect of native and modified peptides on triacylglycerol synthesis stimulation and ASP competition binding:

Human skin fibroblasts and 3T3-L1 fibroblasts (as indicated) were cultured as described in Materials and Methods. **Top Panel:** For triacylglycerol synthesis stimulation, the indicated concentrations of ASP or peptides were added to serum free medium containing 100 μM (^{3}H) oleate complexed to BSA. The cells were incubated for a further 6 hours. Triacylglycerol synthesis stimulation is measured as (^{3}H) oleate incorporation into triacylglycerol. Results are expressed as % TG stimulation where basal triglyceride synthesis (nmol triacylglycerol/mg cell protein) for HSF = 71.9 ± 7.0 (n= 2 experiments) and 3T3 = 78.3 ± 10.3 (n= 4 experiments). **Bottom Panel:** For competition binding studies, HSF were incubated at 4°C for 1 hour with 1 nM ^{125}I ASP and increasing concentrations of unlabelled ASP or peptides as competitor (15 concentrations in duplicate). Results are expressed as percent competition, each competitor was tested in 2-3 separate experiments, where ASP binding at 100% (no competitor) = 140 ± 22 fmoles (^{125} I) ASP/mg cell protein (n = 13). Both the degree of inhibition (top - bottom plateau) as well as the IC_{50} were calculated by iterative four parameter logistic function analysis (IC_{50}: 44 nM native ASP, 120 nM P117, 5.6 μM P32, 1.2 mM P8aa; $p<0.01$ by ANOVA, $p<0.05$ for P32 and P8aa *vs* ASP, pNS for P117 *vs* native ASP).


HSF
% TG Stimulation
225
200
175
150
125
100
1
2
4
8
16
32
64
Concentration [uM]


3T3
% TG Stimulation
225
200
175
150
125
100
1
2
4
8
16
32
64
Concentration [uM]

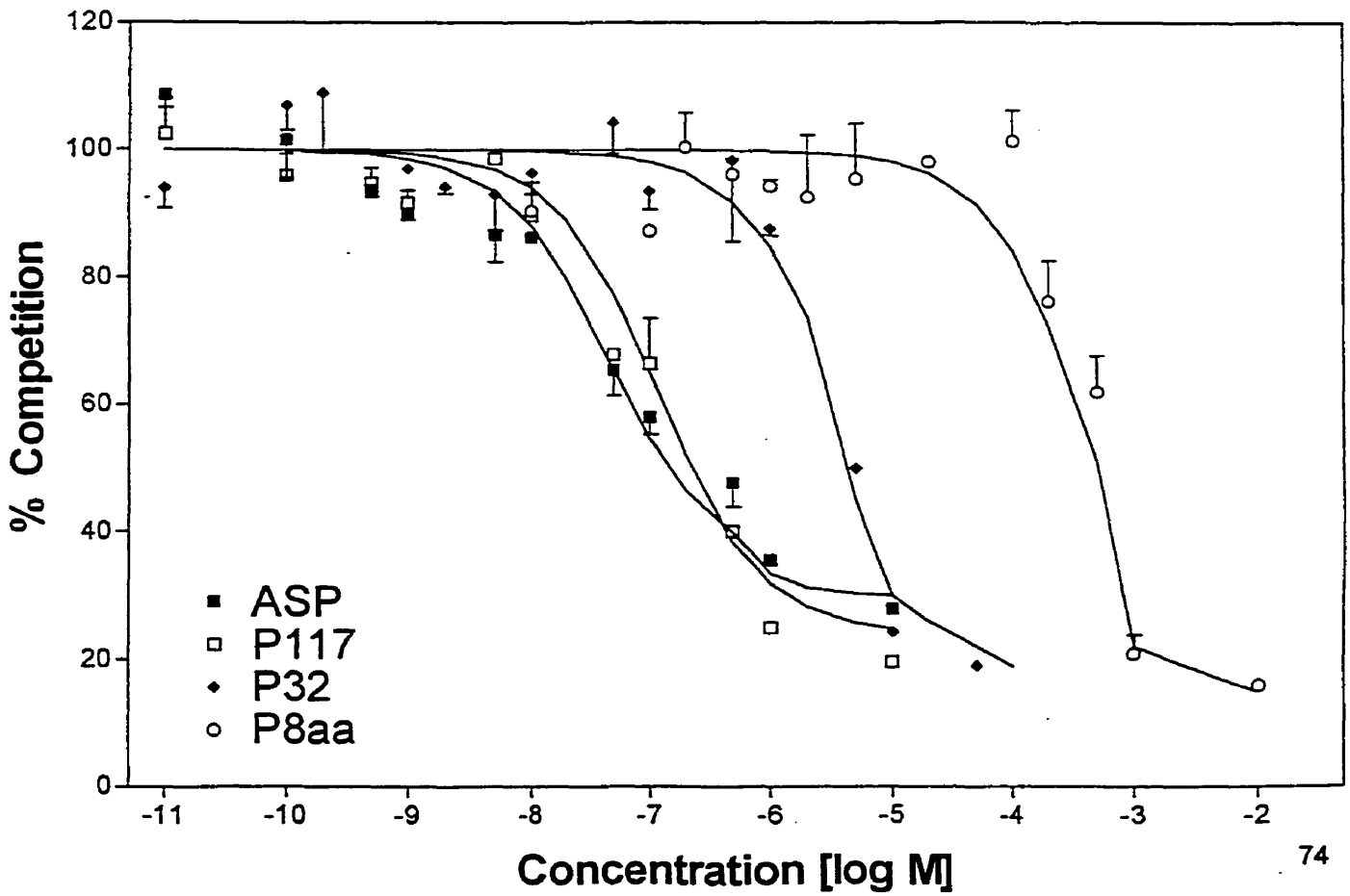
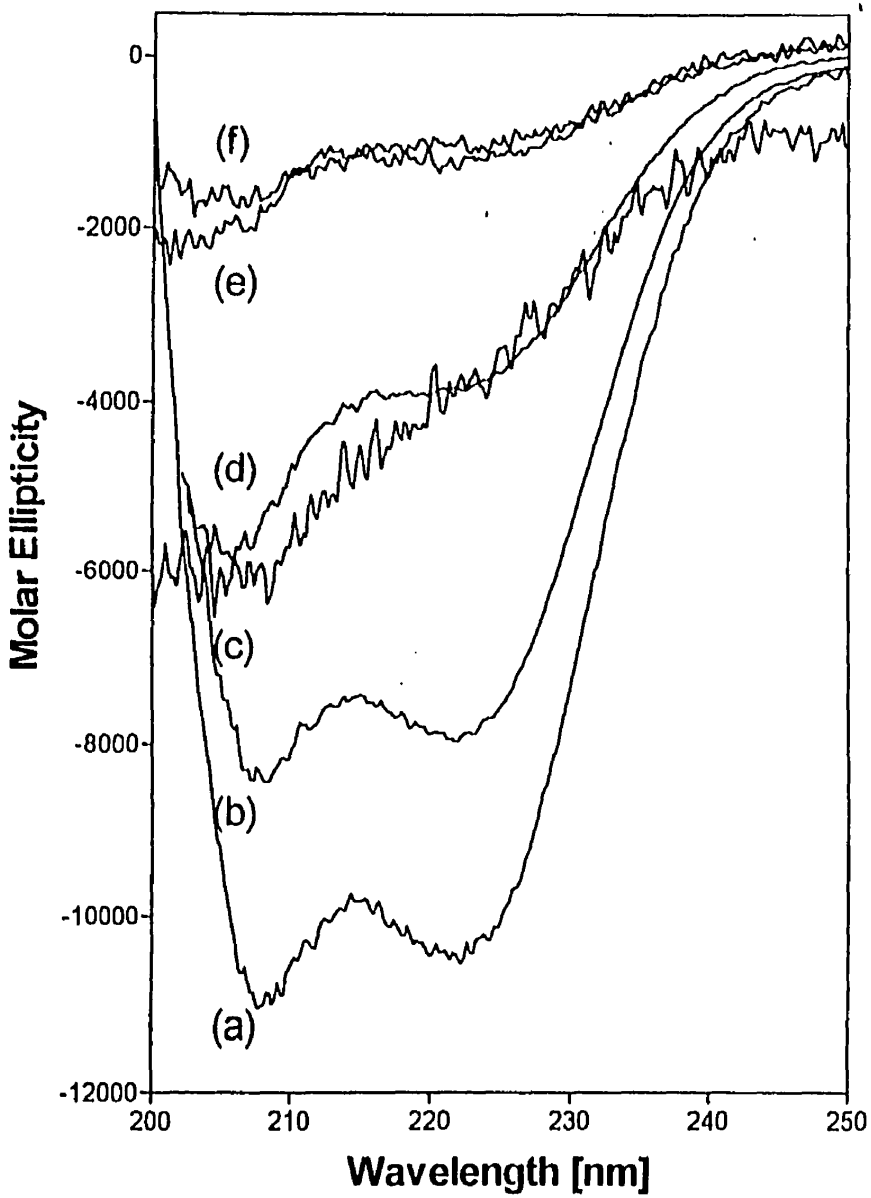

% Competition
120
100
80
60
40
20
0
ASP
P117
P32
P8aa
-11
-10
-9
-8
-7
-6
-5
-4
-3
-2
Concentration [log M]

Figure 3.2: Effect of cysteine modified ASP on circular dichroism profile and competition binding.

Right Panel: Human skin fibroblasts were cultured as described in methods. ASP was modified following treatment with urea and iodoacetamide (Cys-NH) or iodoacetic acid (Cys-CooH) and was re-purified on HPLC following treatment. Results are expressed as percent competition where each competitor was tested in 2-3 separate experiments where ASP binding at 1 nM (^{125}I) ASP (100% no competitor)=202 ± 25 fmoles (^{125} I) ASP/mg cell protein (n = 9). Both the degree of inhibition (top-bottom plateau) as well as the IC_{50} were calculated by iterative four parameter logistic function analysis (IC_{50}:37 nM native ASP, 120 nM Cys-NH and 510 nM Cys-COOH; $p<0.001$ by ANOVA, $p<0.05$ for Cys-NH and Cys-COOH *vs* native ASP. **Left Panel:** Circular dichroism of native and modified ASP in PBS where a = native ASP, b = urea-treated ASP, c = boiled + β-mercaptoethanol treated ASP, d = ASP treated with urea and β-mercaptoethanol, e = Cys-NH ASP, and f = Cys-COOH ASP. In all cases, ASP was repurified by HPLC, then tested for bioactivity and circular dichroism analysis.


Molar Ellipticity
0
-2000
-4000
-6000
-8000
-10000
-12000
(f)
(e)
(d)
(c)
(b)
(a)
200
210
220
230
240
250
Wavelength [nm]

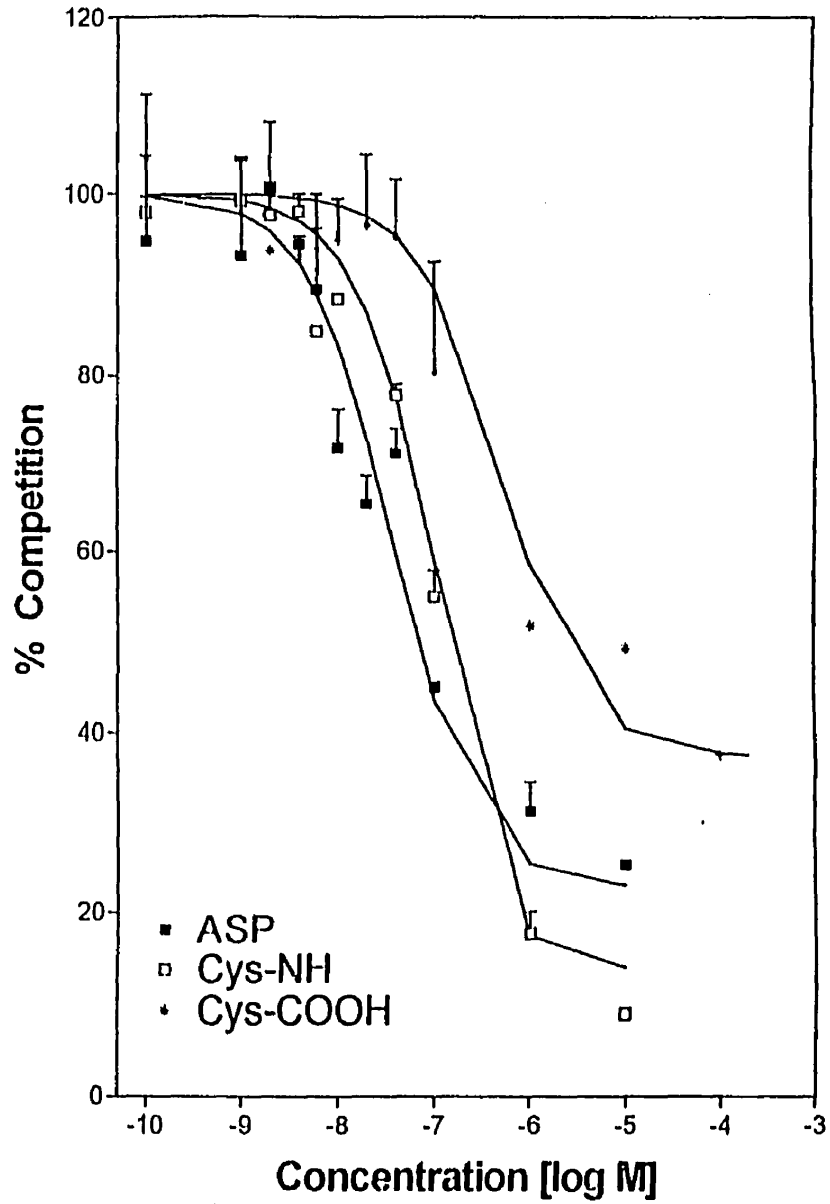
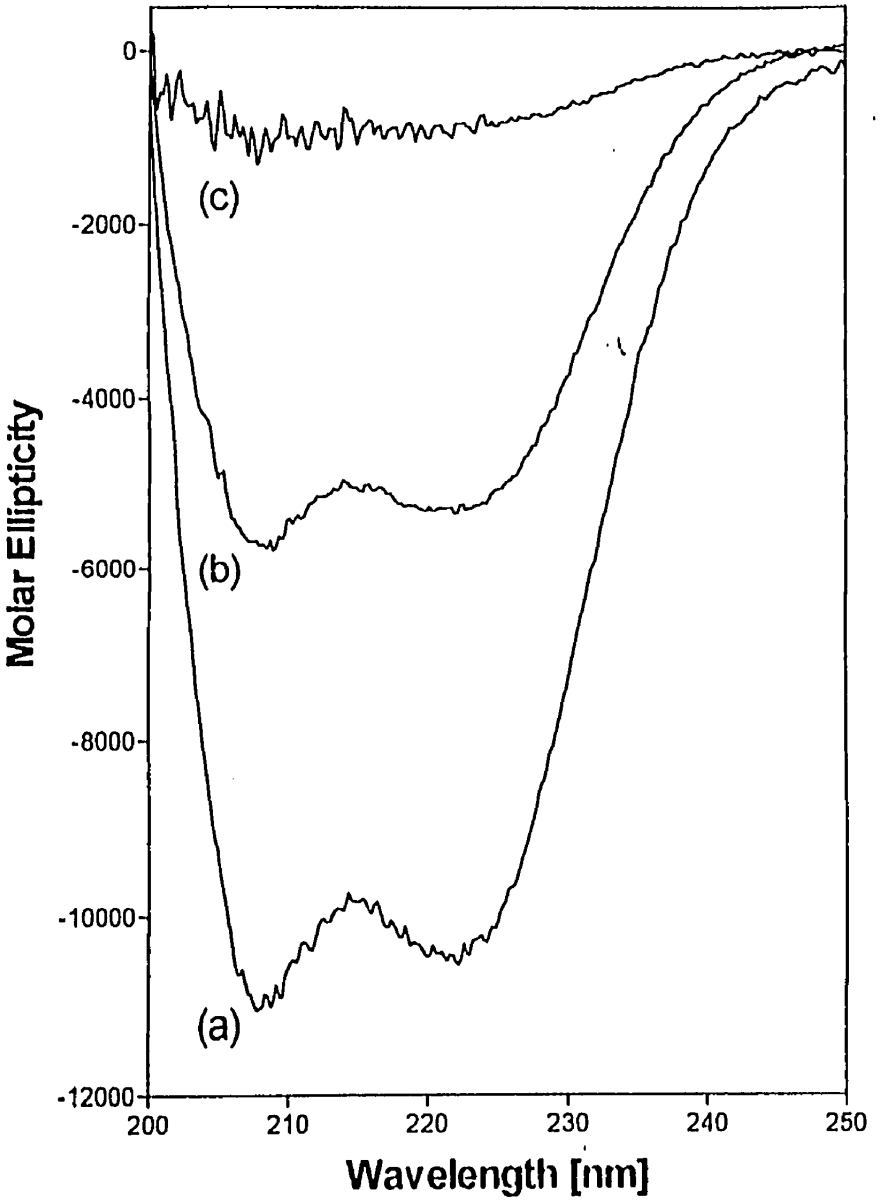
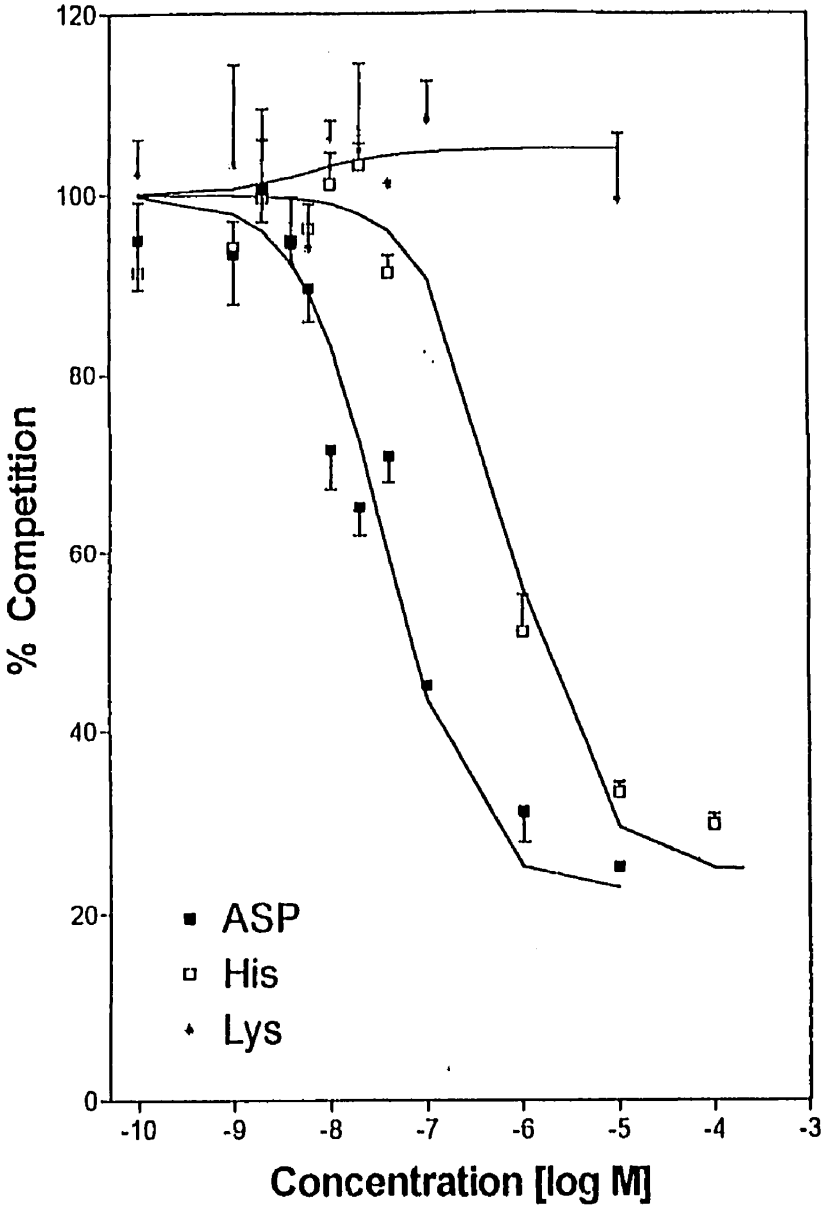

% Competition
120
100
80
60
40
20
0
ASP
Cys-NH
Cys-COOH
-10
-9
-8
-7
-6
-5
-4
-3
Concentration [log M]

Figure 3.3: Effect of modification of basic residues of ASP on competition binding

Human skin fibroblasts were cultured as described in methods. ASP was modified following treatment with DEPC (for His modification), or pyridoxal-5-phosphate (for Lys modification) and re-purified on HPLC following treatment. **Right Panel:** Results are expressed as percent competition for the curve calculated from duplicate points at each of 15 concentrations; each competitor was tested in 2-3 separate experiments where ASP binding at 100% (no competitor) = 142 ± 84 fmoles (^{125} I) ASP/mg cell protein (n = 12). Both the degree of inhibition (top - bottom plateau) as well as the IC_{50} were calculated by iterative four parameter logistic function analysis (IC_{50}:44 nM native ASP, 707 nM His-ASP and cannot be calculated for Lys-ASP; $p<0.003$ by ANOVA, $p<0.05$ for His-ASP and Lys-ASP vs native ASP). **Left Panel:** Circular dichroism of native ASP (a), His-ASP(b), and Lys-ASP(c). Conditions are described in Figure 3.2.


Molar Ellipticity
0
-2000
-4000
-6000
-8000
-10000
-12000
(c)
(b)
(a)
200
210
220
230
240
250
Wavelength [nm]


% Competition
120
100
80
60
40
20
0
ASP
His
Lys
-10
-9
-8
-7
-6
-5
-4
-3
Concentration [log M]

3.5 Discussion

The present results suggest that ASP function is mediated through initial binding to a cell surface receptor, and then cellular activation resulting in increased triacylglycerol synthesis. Binding can occur without stimulation of triacylglycerol synthesis but triacylglycerol synthesis stimulation cannot occur in the absence of ASP binding. Modifications which altered the cysteine bound core region had little effect on specific binding. Similarly, removal of the amino terminal 9 amino acid portion of ASP had no effect on binding. By contrast, modification of the Lys residues, 5 of which are present in the amino terminal region of the core (between residues 7 to 21) did affect binding greatly. In addition, the carboxyl terminal region appeared to be involved directly in receptor binding since alteration of the two C-terminal His residues decreased binding affinity substantially. As well, although the native 8 or 15 carboxyl terminal amino acid peptide could not inhibit ASP binding, modifications which resulted in increased hydrophobicity (and presumably increased cell surface contact) did possess partial inhibitory capabilities, suggesting that this region participates but is not sufficient for complete receptor binding competency.

Triacylglycerol synthesis stimulatory activity of ASP was dependent on binding. In the absence of binding to the cell surface there was no triacylglycerol stimulatory activity remaining. This was clearly demonstrated by the modifications which targeted the basic residues (His and Lys) which were poor competitive inhibitors and had no significant triacylglycerol stimulatory activity. However, the distinct nature of a postulated "binding domain" *vs* an "active domain" could be seen in some modifications where the molecule was clearly competent to bind, but had little bioactivity. For example, the carboxyl terminal peptide P117 had an IC_{50} that was three-fold native ASP, yet could only partially stimulate triacylglycerol synthesis. The P32 peptide was even less efficient at inhibiting radiolabelled ASP binding, and stimulating triacylglycerol synthesis. It has been suggested that hydrophobic elements enhance the structural conformation of the peptide to stabilize receptor interaction and increase activation (44) or that the local concentration of the peptides is increased by hydrophobic interaction of the Fmoc-moiety and/or the modified amino acid residues with the cell membrane (46). An even more striking example is the two treatments to modify the Cys residues within the globular

core: in both cases, the polypeptides were effective binding inhibitors, but had no significant residual triacylglycerol stimulatory activity. This also points to the importance of structures in the tightly folded core region in activation of triacylglycerol synthesis.

The results in the present study on ASP binding and activity contrast markedly with the structure-function studies on C3a as shown by several groups (22,23,47). In those studies, it was clearly demonstrated that both the binding competency and the functional domain as assessed in differentiated U937 cells as well as other immunocompetent cells were dependent on the carboxyl terminal arginine (which is not present in ASP). Thus a native carboxyl terminal peptide was sufficient to demonstrate activity, and modified carboxyl terminal peptides of 8 to15 residues (22,23) retained complete binding capacity and degranulation activity although peptides lacking the terminal arginine as well as ASP (C3adesArg) did not demonstrate any degranulation activity (5,22,23,47). These same modified peptides only contained partial inhibitory effects on ASP binding and some triacylglycerol stimulatory activity highlighting the contrasts in the two different biological systems.

Other examples of peptides which exist in both the desArginated as well as the arginated form have been documented. For example C5a, which has a structure very similar to that of ASP with a tightly folded cysteine linked core region and a flexible carboxyl terminal tail region, also exists in a desArg form. In this case, the two forms of C5a differ in their binding affinities by a factor of 10-30 (48,49) while their activity level is either decreased from nM to μM (as demonstrated for smooth muscle cells) or not affected at all (enzyme release from differentiated U937 cells) (49). Structure: function analysis through site-directed mutagenesis suggests that domains other than the carboxyl terminal arginine, such as basic residues located in the core region, are responsible not only for C5a activity, but also for binding and these studies were used as the basis for the ASP modifications examined in the present paper. A two-site model is proposed for the C5a receptor accomodating a "binding region" of the anaphylatoxin composed of the amino terminal region knotted by disulfides which presents an "activating domain" containing the C-terminal positions 67-74 (50) and we hypothesize that this may also be so for ASP.

Bradykinin and bradykinin desArg exhibit characteristics similar to those of C3a *vs* ASP. Bradykinin (BK) is a 9 residue peptide which has potent dilatory and motility effects through various second messenger systems including effects on intracellular calcium, cAMP and cGMP, in aortic mesangial and endothelial cells, to name but a few. Bradykinin desArg has similar effects (for reviews see (51,52)). The effects of these two peptides, however, are mediated through interaction with two distinct receptors (B1 and B2) derived from two different genes. At the protein level, these receptors are similar in secondary structure (both are G protein linked receptors) but share only 54% sequence identity in humans. Thus B2 binds BK with high affinity, whereas B1 binds BKdesArg to a similar extent although different affinity forms of the receptor also exist. The differences in structure between the two peptides is sufficient that agonists/antagonists have been developed which can selectively block the activity of one peptide without affecting the activity of the other. Some cells express only B1 receptors (rabbit aorta) or only B2 receptors (rabbit jugular vein), whereas other cells express both (HUVEC, fibroblast).

Thus we hypothesize that the situation with respect to ASP/C3a would appear to be similar to that of BKdesArg/BK. In human adipocytes and fibroblasts, both ASP and C3a are able to stimulate triacylglycerol synthesis (5), whereas in human granulocytes, mast cells and differentiated U937 cells, only C3a produces a degranulation response (5,9-12,16). Differential competitive binding characteristics are also demonstrated: both ASP and C3a can bind with high affinity to adipocytes and fibroblasts (manuscript in preparation), but only C3a can bind to basophils, polymorphonuclear leukocytes, monocytes, mast cells, differentiated U937 cells or guinea pig platelets (16,53-57). These results suggest that there may be two distinct receptors, one which binds ASP and C3a, and a second one which only binds C3a and is dependent on the presence of the terminal arginine of C3a. A C3a receptor has been cloned from a U937 expression library (15) and from a neutrophil cDNA library using expression tag analysis (14) and this receptor does not bind ASP (16,17). An ASP receptor remains yet to be identified.

We have demonstrated that ASP plays a role in regulating adipose tissue metabolism both *in vitro* and *in vivo* (8,58). A number of disorders such as obesity, diabetes and cardiovascular disease have been associated with abnormal adipose tissue

metabolism and understanding adipose tissue metabolism and regulation has become a major research area in recent years (59). ASP dysfunction is present in specific metabolic disorders (58) with increased ASP levels in obesity (60), and ASP dysfunction in a subgroup of HyperapoB patients (61-63). Thus, one of the implications of defining regions that are important in ASP activity, but have no effect on C3a bioactivity, is that this may permit the identification of peptides or other organic compounds that may be used as selective ASP agonists or antagonists. These would be valuable tools in elucidating further the physiological role of ASP, or defining future pharmacological treatments for use in modifying adipose tissue function.

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Prelude III

The function of ASP was determined to be due to the whole molecule: the carboxy-terminal and core. *In vivo* and *in vitro* studies implicated a role of ASP in postprandial clearance of plasma triglyceride and glucose. Thus in order to determine the *in vivo* postprandial role of ASP, it was injected intrapeitoneally into mice during an oral fat load.

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Chapter 4
Enhanced Triglyceride Clearance with Intraperitoneal Human Acylation Stimulating Protein (ASP) in C57BL/6 Mice

4.1 Abstract

Acylation Stimulating Protein (ASP), a novel adipocyte derived autocrine protein, stimulates triglyceride synthesis and glucose transport *in vitro* in human and murine adipocytes. *In vitro,* chylomicrons increase ASP and precursor complement C3 production in adipocytes. Furthermore, *in vivo*, ASP production from human adipose tissue correlates positively with triglyceride clearance postprandially. The aim of the present study was to determine if intraperitoneally injected ASP accelerated triglyceride clearance *in vivo* following a fat load in C57Bl/6 mice.

ASP increased the triglyceride clearance with a reduction of the triglyceride area under the curve over 6 hours (AUC_{0-6}) from 102.6±30.0 to 61.0±14.5 mg/dL*hr ($p<0.05$) especially in the latter postprandial period (AUC_{3-6}, 56.2±18.0 *vs* 24.9±8.9, $p<0.025$). ASP also reduced plasma glucose both in the mice with accelerated plasma triglyceride clearance and in those with relatively delayed triglyceride clearance ($p<0.025$). Therefore, ASP alters both postprandial triglyceride and glucose metabolism.

4.2 Introduction

Clearance and storage of postprandial dietary triglycerides is an essential part of energy homeostasis. Triglyceride (TG) rich, dietary lipoproteins (chylomicrons) are released into the peripheral blood circulation and a large proportion of dietary TG within them is cleared by adipose tissue and muscle (5,12). Chylomicron clearance is effected in a two step pathway. First, in the interstitial space, lipoprotein lipase (LPL) hydrolyses the chylomicron TG producing free fatty acids (FFA). Next, these FFA are taken up by muscle (utilization) or by adipocytes, where they are activated to form acyl CoA and re-esterified to stored triglyceride in adipose tissue.

Adipose tissue is a metabolically active tissue which regulates its own metabolism *via* secretion of paracrine and autocrine factors and influences other tissues as well. Adipocytes produce many factors such as apolipoprotein E (41), leptin (25), tumour necrosis factor (TNF) (22), lipoprotein lipase (LPL) (2) as well as complement C3, the precursor of acylation stimulating protein (ASP) (8). Adipocytes secrete C3, factor B and adipsin (3,6). C3a is a cleavage product of the amino-terminal alpha chain of complement C3, and is formed through the enzymatic interaction of complement C3, factor B and adipsin (factor D) (18,23). C3a has a short half-life in plasma, rapid carboxypeptidase cleavage of the carboxyl-terminal arginine of C3a produces ASP (C3a des Arg) (15).

ASP is produced by adipocytes and acts back upon adipose tissue to stimulate both glucose transport and triglyceride synthesis. It therefore appears to act as an autocrine factor (7,33). ASP has been shown to increase triglyceride synthesis *in vitro* by increasing the V_{max} of diacylglycerol acyltransferase (DGAT) (38). ASP also increases glucose transport in adipocytes (21), myotubes (34) and fibroblasts (13). In the latter two cell types, Western blot analysis demonstrated that glucose transport increases consequent to increased translocation of glucose transporters (Glut 1, Glut 3, Glut 4) to the cell surface (13,34). Although long term (24 hour) incubation with ASP does not appear to increase total Glut 1, Glut 3, or Glut 4 transporter mass, ASP does appear to augment the insulin effect on increased Glut 3 and Glut 4 mass in muscle (L6 myotubes) (34). The effects of ASP are more pronounced in differentiated adipocytes and myotubes than in undifferentiated cells (21,34).

The importance of these effects is underscored by the conservation of function amongst species. Thus, ASP has been shown to affect both triglyceride synthesis and glucose transport not only in human adipocytes but also in primate and rodent adipocytes and myotubes (24,34). In an *in vivo* human study, both adipose tissue ASP production and dietary TG clearance increased within the adipose tissue bed in the latter half of the postprandial period (29). This coordinated change suggests a specific physiologic postprandial role of ASP since *in vitro,* ASP production from adipocytes is increased by specific chylomicron components. (20,31).

The use of murine models to elucidate *in vivo* metabolic pathways has increased over the past years. The murine strain C57Bl/6, especially the leptin (*ob/ob*) and leptin receptor (*db/db*) mutants, have commonly been used to determine lipid related parameters (1,14,16,36) as well as for oral fat loads (36). Since we have demonstrated that ASP increases TG formation *in vitro* in adipocytes and glucose transport in myotubes, we hypothesized that *in vivo* administration of ASP would accelerate TG clearance following a fat load in a murine model.

4.3 Methods

4.3.1 Animals The Royal Victoria Hospital Research Institute Animal Ethics Committee approved all procedures, which were in accord with the Canadian Animal Care Committee (CACC) guidelines. Twelve female C57Bl/6 NHsd mice were obtained from Harlan Sprague Dawley (Chicago,Il.) and were fed a Purina chow 5075 diet. Mice were housed in sterile barrier facilities with controlled humidity, temperature and light/dark cycles. Experiments were conducted on acclimatized mice at 14-16 weeks of age with two weeks between experiments to allow for restoration of blood volume.

4.3.2 Postprandial Fat load on Mice Fat loads were performed on mice, half were injected intraperitoneally with human ASP (acylation stimulating protein), the other half with vehicle (see below). Two weeks later, using a crossover design, the mice that received ASP at the time of the first fat load received vehicle, and *vice versa*. Following an overnight fast (16 hours), 400 µL of olive oil (followed by 100 µL air) was given by gastric gavage using a feeding tube (1½ cm curved ball tipped feeding needle #20) according to standard procedures as published previously (19, 32, 36, 39) and the ASP intraperitoneal injection was performed immediately afterwards. The mice were free running, with water *ad libitum*, but no food. The unanaesthetized mice were restrained in a plastic cylinder (modified 50 mL Falcon tube) for the duration of the bleed (2 minutes). The tail was cut with a scalpel for the first bleed only. For repeated bleeds, the scab was removed and blood was collected by gently stroking the tail (lubricated with Vaseline). Blood samples (40 µl) were collected with heparinized capillary tubes by tail bleeding at 0, 1, 2, 3, 4 and 6 hours into EDTA containing tubes on ice (maximum 10% blood volume of the mouse for the total fat load). The plasma was separated by centrifugation within 30 minutes of collection at 12,000 x g for 5 minutes and stored at -80°C. Triglycerides, glucose, free fatty acids and human ASP were measured in these samples as described below.

4.3.3 ASP injections ASP (8932.5 MW) was prepared as described previously (4) and its purity (99%) was ascertained by ion spray mass spectrophotometry (3). Each mouse received intraperitoneally either sterile ASP (500 µg) dissolved in phosphate buffered saline, pH 7.4 containing 1 mg/mL fatty acid free bovine serum albumin (Sigma, St Louis, MO) or sterile vehicle (same solution without ASP). Preliminary experiments

solution without ASP). Preliminary experiments demonstrated that injection of the vehicle solution (1 mg/mL BSA in PBS, 300 μL) had no effect on postprandial triglyceride clearance in the mice as compared to the same mice without vehicle. The two week interval permitted restoration of blood volume. Mice were injected intraperitoneally (300 μl), immediately after receiving the oral fat load.

4.3.4 Assays Triglycerides, free fatty acids (Boeringher Mannheim, Laval, Quebec, Canada) and glucose (Trinder Kit, Sigma,St Louis, MO) were measured using commercial enzymatic colorimetric kits. ASP was measured by a sandwich enzyme linked immunoassay (ELISA) as described previously (29). In all assays, each sample was measured in duplicate. The anti-human ASP murine monoclonal capture antibody does not crossreact with mouse ASP. Results at each time point during the postprandial period are expressed as the mean ± standard error of the mean (sem) of the difference from fasting levels. Individual time points were compared by t-test. As well, the area under the curve (AUC) was determined using linear trapezoidal equation (Sigma Stat, Jandel Scientific, San Rafael, CA) and the results in the two groups compared using one way ANOVA or two way repeated measures ANOVA. Significance was set at $p<0.05$.

4.4 Results

The role of ASP in postprandial triglyceride clearance *in vivo* was determined in 12 C57Bl/6 mice. Fat loads were performed on mice injected intraperitoneally with ASP or vehicle in a crossover design with a two week interval between the two fat loads. In Figure 4.1, the plasma triglyceride levels are shown as difference from basal (mg/dL) where average fasting plasma triglyceride level was 34.8±2.1 mg/dL (n=24). Triglycerides of control mice (vehicle only) increased rapidly within 1 hour reaching a maximum at 4 hours then returned towards baseline levels, similar to published studies (19,32,39). In separate studies we verified that the triglyceride profiles of the mice were not altered by the procedure of intraperitoneal injection (data not shown). At all time points postprandially, the plasma triglycerides were significantly elevated compared to fasting TG levels (Figure 4.1). With injection of ASP, there was no effect on the initial rise (first hour) in plasma triglycerides but the clearance from 2 hours on was markedly altered. At 1 hour, plasma TG was significantly elevated as compared to fasting triglyceride ($p<0.01$). However, at all time points past 1 hour, ASP effected a decrease in plasma TG such that the levels were no longer significantly different from fasting TG. The total triglyceride area under the curve from 0 to 6 hours (AUC_{0-6}) was reduced from 102.6±30.0 to 61.0±14.5 mg/dL*hr ($p<0.05$). Although the first half of the AUC_{0-3} (from 0 to 3 hours postprandially) was not significantly different with ASP (46.4 ±15.3 control *vs* 36.1±7.9 with ASP) there was a 2 fold reduction in the latter half of the postprandial period (AUC_{3-6}) 56.2±18.0 control *vs* 24.9±8.9 mg/dL*hr with ASP ($p<0.025$). The differences were due to the administration of ASP since basal lipemic response was the same whether the mice received vehicle first or second in the crossover design. Fat loads repeated in the same mouse differed by only 20% ± 2.6% for area under the curve (AUC) over 6 hours (n = 6). As well, when a sham fat load was performed (saline only given), plasma triglycerides, free fatty acids, and glucose have small changes compared to fasting plasma levels over a 3 hour time course (-6% ± 4%, 19% ± 3%, and -19% ± 3% change respectively, n = 6 mice).

ASP entered the plasma compartment following its intraperitoneal administration (Figure 4.2). There was no cross-reaction of the murine monoclonal anti-human ASP capture antibody with mouse ASP. ASP levels peaked rapidly at 1 hour to 771± 78 nM

and declined rapidly to baseline at 3 hours with an average total AUC_{0-6} of 1091± 86 nM*hr. The ASP peak preceded its effects on plasma triglyceride, with triglyceride changes beginning after 1 hour.

As dietary plasma triglycerides are hydrolyzed by lipoprotein lipase, free fatty acids (FFA) are generated. The plasma increase in FFA is shown in Figure 4.3, again as difference from basal where the fasting free fatty acid plasma concentration was 0.408 ± 0.036 mM (n=24). As with triglycerides, FFA increased rapidly by one hour corresponding to the rise in postprandial triglyceride. Throughout the postprandial period (up to 6 hours), the fasting free fatty acids remained significantly elevated above fasting in both the mice with and without ASP injections. At 6 hours, postprandial FFA was still significantly above baseline levels, with a significant difference between with ASP *vs* without at 6 hours ($p<0.05$).

ASP has been shown *in vitro* to increase glucose transport and in the present fat load studies we also measured plasma glucose. As shown in Figure 4.4, postprandial glucose levels increased slightly but significantly from baseline, up to a maximum of 9 % by 1 hour, with basal glucose levels being 111± 4 mg/dL (n=24). The glucose changes were similar to those of the triglyceride curves for both control and ASP treated groups. In the control group, glucose levels remained significantly elevated over the 6 hour time course. Compared to controls, ASP treatment significantly reduced plasma glucose levels to fasting levels within 2 hours and then below fasting such that, at all time points, there was no significant difference between postprandial glucose and fasting levels. The drop in glucose from 2 to 6 hours with ASP was significantly greater compared to mice without ASP (Figure 4.4). The overall ASP effect on plasma glucose was a reduction in postprandial AUC_{0-6} from 67.6 ± 25.9 to -19.5 ± 46.7 mg/dL*hr ($p<0.025$). As with TG clearance, the greatest changes were noted in the second half of the postprandial period AUC_{3-6} 31.3 ± 18.3 vs -26.9 ± 16.2 mg/dL*hr ($p<0.01$).

Overall, following the fat load in the control mice, plasma triglyceride AUC_{0-6} increased by 83% over basal fasting triglyceride. There was considerable inter-individual variation of AUC_{0-6} within the mice, ranging from no change in postprandial TG to a 352% increase in postprandial TG as compared to basal triglycerides. We therefore separated the animals into 2 groups: normal postprandial lipemia (NOR, average 159% ±

52 increase in TG AUC_{0-6}, n=6, range 44% to 352%) and those with accelerated dietary TG clearance resulting in less pronounced postprandial TG levels (ACC, average 8% ± 6 change in TG AUC_{0-6}, n=6, range 26% to -18%). The fasting parameters of the 2 groups are shown in Table 4.1. Both fasting glucose and FFA levels were significantly lower in the accelerated clearance (ACC) group than the normal NOR group and may be related to the specific breeding colony that the animals derived from. Postprandial FFA followed a similar trend with higher increases in the NOR group than in the ACC group although there was no effect of ASP (1.93 ± 0.50 *vs* 2.30 ± 0.15 NOR and 0.011 ± 0.16 *vs* 0.56 ± 0.16 ACC; without and with ASP respectively for AUC_{0-6}, pNS). There was, however, no difference in fasting TG between the two groups.

Triglyceride clearance of the NOR group *vs* the ACC group was examined. As shown in Figure 4.5 for the NOR group, plasma triglyceride levels were significantly increased at all time points for control mice (1 hour up to 6 hours). However, in the presence of ASP, postprandial triglycerides were only increased above baseline at 1 hour. At all other time points, the triglycerides were reduced so that there was no difference from fasting TG levels and there was a significant decrease compared to the same time point without ASP ($p<0.005$ by 2 way repeated measures ANOVA). On the other hand, in the ACC group, there was little postprandial change in TG with a significant increase over fasting only at 1 and 2 hours, and therefore ASP had little or no effect.

The ASP effect on glucose in both groups was also examined. The glucose profile in the absence of ASP was similar in both groups of mice (Figure 4.6). In the absence of ASP, in both NOR and ACC mice, postprandial glucose levels were consistently elevated throughout. Interestingly, in both groups, there was an equivalent effect of ASP on glucose lowering to below fasting levels such that with ASP there was no significant change from fasting glucose at any time point (Figure 6). In the ACC group, although there was little postprandial change in TG and thus little effect of exogenous ASP, nonetheless there was still an effect of ASP on plasma glucose levels, independent of an effect on TG clearance. In addition, there was a strong negative correlation between both total glucose AUC_{0-6} and ASP AUC_{0-6} ($r=0.71$, $p<0.01$).

Figure 4.1: Change in plasma triglyceride in C57Bl/6 mice upon injection with human ASP following an oral fat load.

An oral fat load was administered by gastric gavage as described in methods. Twelve C57Bl/6 mice were studied either with ASP or vehicle (1 mg/mL bovine serum albumin in PBS). Results at each time point during the postprandial period are expressed as the mean ± sem (mg/dL) of the difference in triglyceride (TG) levels from fasting (34.8±2.1 mg/dL, n=24) by subtracting the basal value (time zero) from each time point. Comparisons of time points *vs* fasting TG, *$p<0.01$ and **$p<0.005$, and between mice with ASP *vs* without ASP were performed, # $p<0.025$.

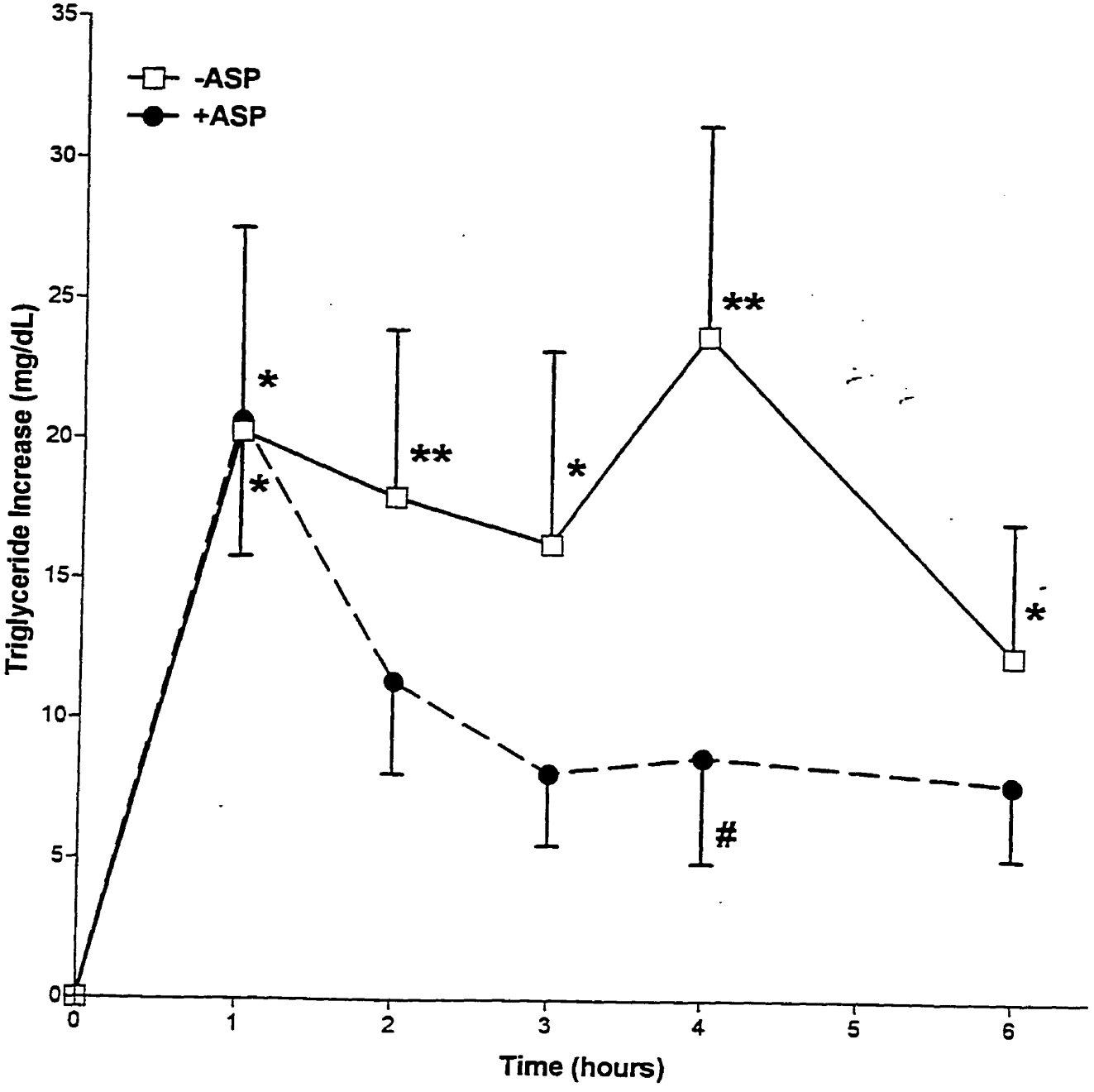

-ASP
+ASP
Triglyceride Increase (mg/dL)
Time (hours)
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
*
*
**
*
**
*
#

Figure 4.2: Changes in plasma levels of exogenous human ASP following an oral fat load.

Human acylation stimulating protein (ASP) or vehicle was injected intraperitoneally into C57Bl/6 mice as described in methods. Human ASP was measured by sandwich ELISA at each time point for each animal. The data here represents mean ± sem for all twelve mice.

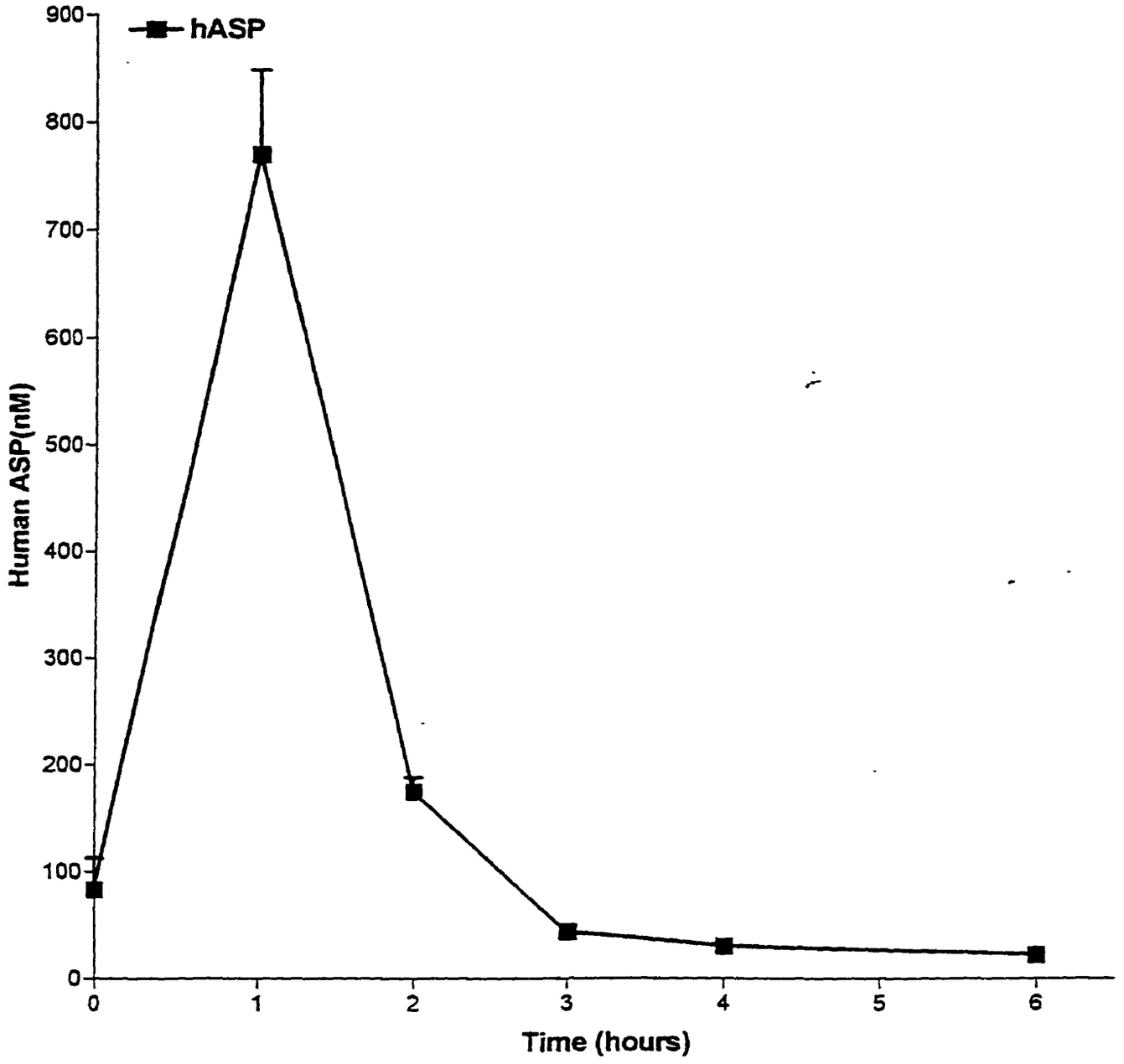

hASP
900
800
700
600
500
400
300
200
100
0
Human ASP(nM)
0
1
2
3
4
5
6
Time (hours)

Figure 4.3 Changes in plasma free fatty acids in C57Bl/6 mice upon injection with human ASP following an oral fat load.

The change in plasma free fatty acids (FFA) was determined at each time point following the fat load as described in Figure 4.1, where basal FFA = 0.408±0.036 (n=24) and *p<0.05, **p<0.01 *vs* fasting FFA. Data are shown here as mean ± sem and represent the difference from basal for n=12 mice with ASP and 12 without ASP.

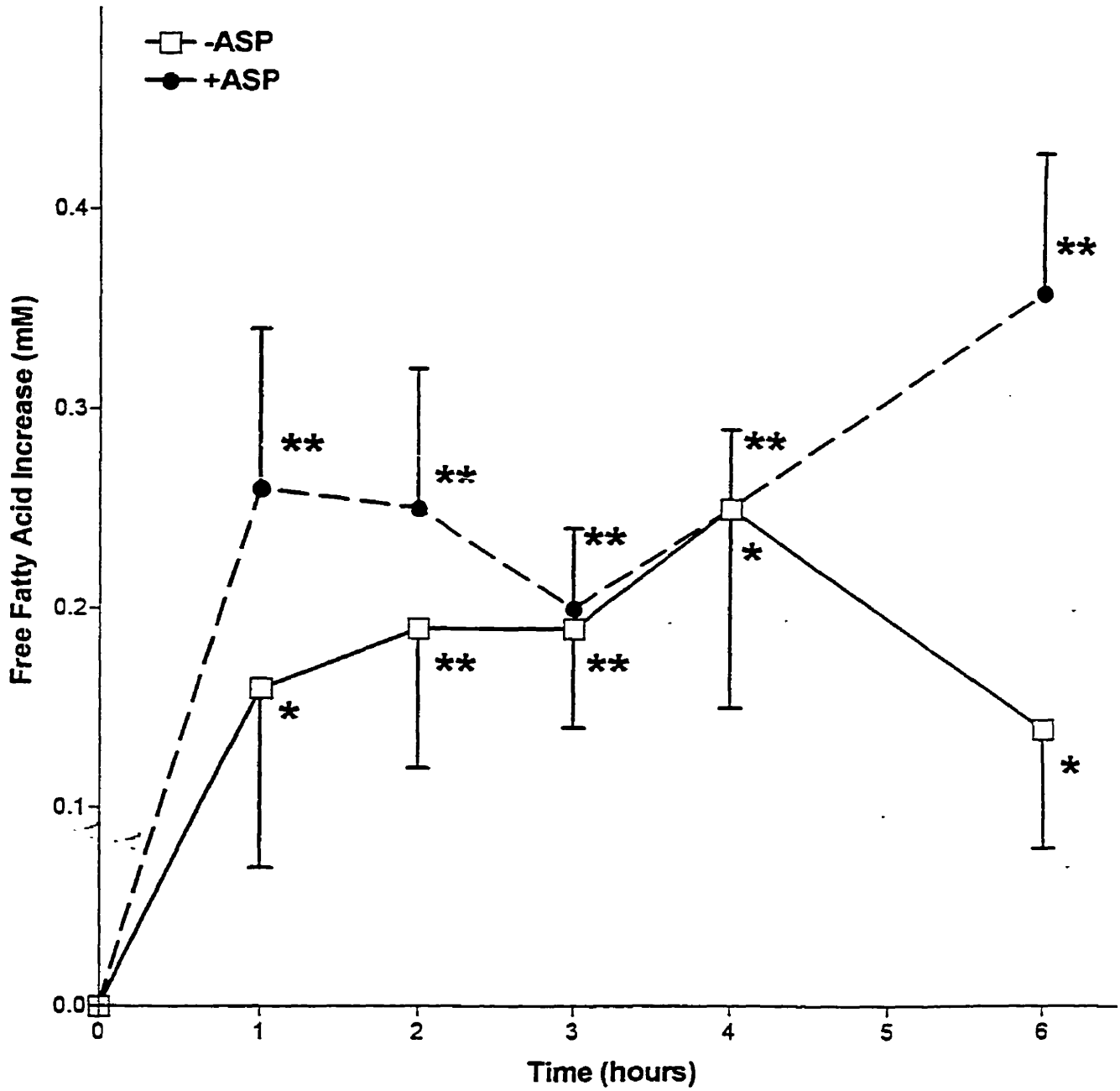

-ASP
+ASP
Free Fatty Acid Increase (mM)
0.0
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6
Time (hours)
**
**
**
**
**
*
*
**
**
*

Figure 4.4:Changes in plasma glucose levels in C57Bl/6 mice upon injection of human ASP following an oral fat load.

Plasma glucose was measured at each time point following the fat load as described in Figure 4.1. Data are shown here as mean ± sem of difference from basal (111±4 mg/dL n=24) where *p<0.05 and **p<0.025 *vs* fasting plasma glucose. Plasma glucose levels that decreased below basal fasting levels are depicted below the x-abcissa where #p<0.01 for mice with *vs* without ASP.

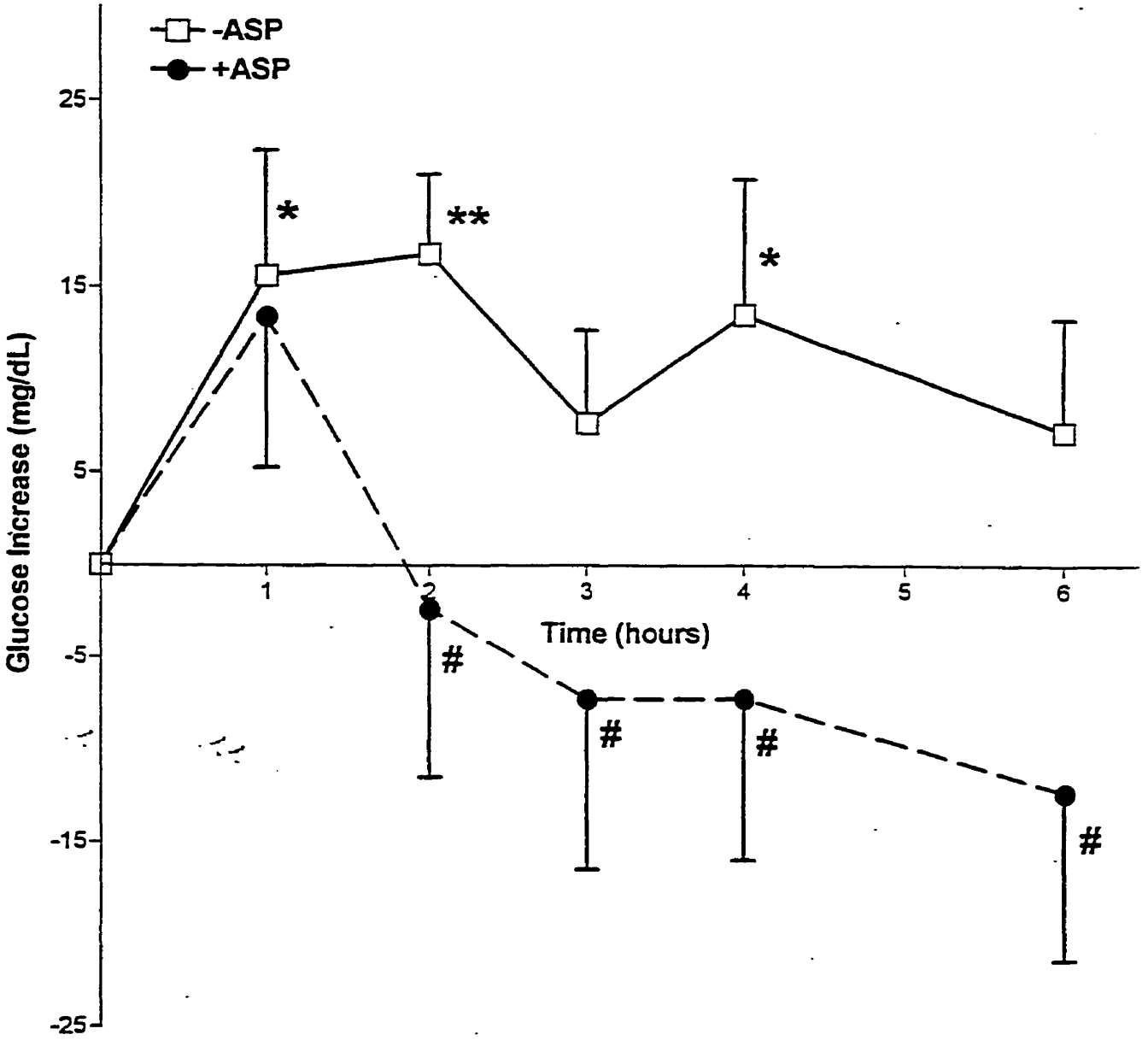

-ASP
+ASP
25
15
5
-5
-15
-25
Glucose Increase (mg/dL)
1
2
3
4
5
6
Time (hours)
*
**
*
#
#
#
#

Figure 4.5: Comparison of plasma triglyceride between normal postprandial lipemia and accelerated clearance groups of mice following an oral fat load.

Plasma triglycerides were measured for the two groups of C57Bl/6 mice: normal postprandial lipemia (NOR, left panel, n=6, basal TG = 32.8 ± 4.0 mg/dL) and accelerated clearance (ACC, right panel, n=6, basal TG = 36.7 ± 1.2 mg/dL) are represented as mean ± sem of difference from basal where for NOR, with ASP is significantly different from without ASP by 2-way repeated measures ANOVA ($p<0.005$) and *$p<0.05$, **$p<0.0125$ *vs* fasting TG and #$p<0.05$ and ##$p<0.005$ for mice with ASP *vs* without ASP. For ACC, there is no difference with *vs* without ASP.

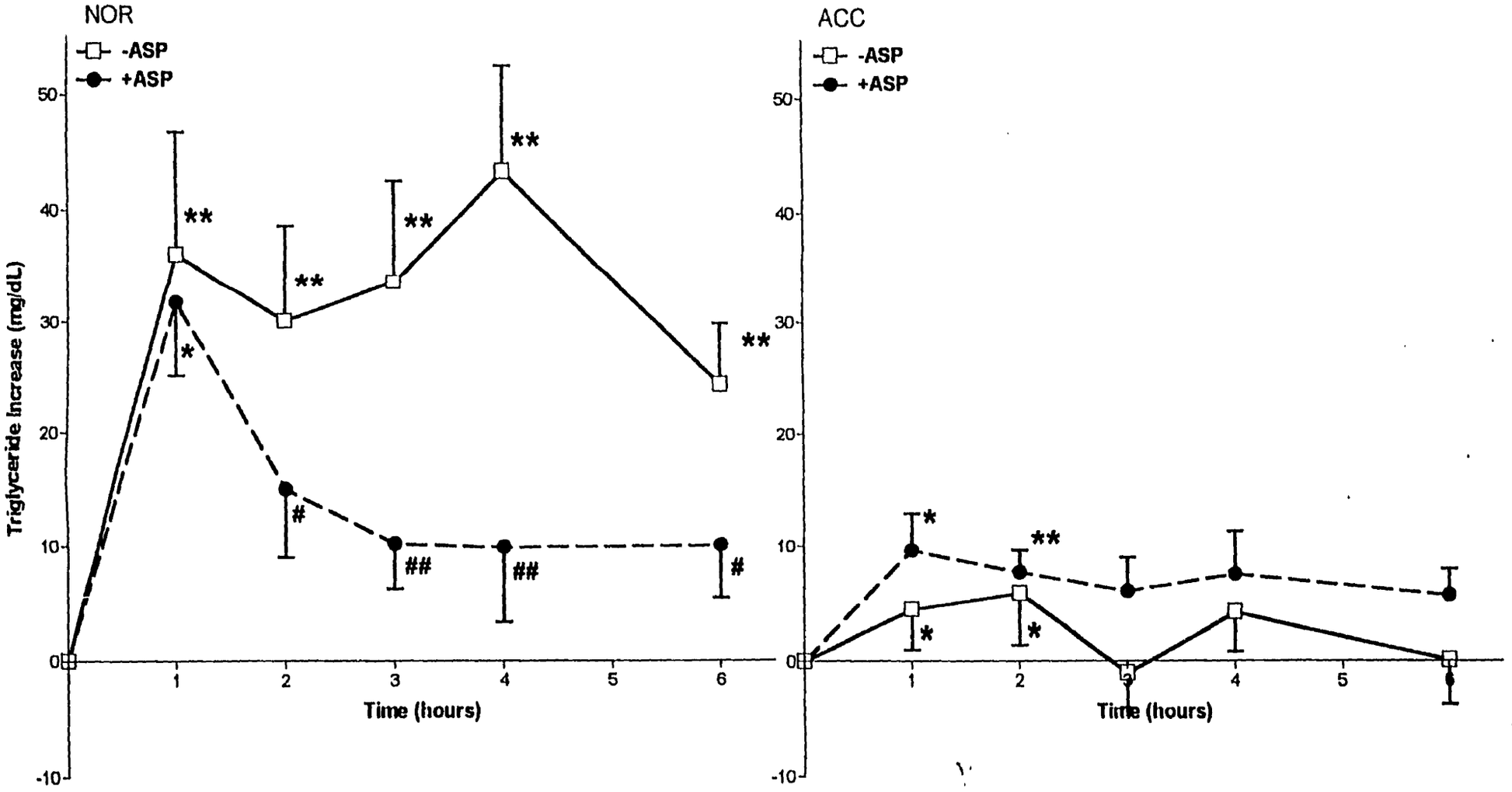
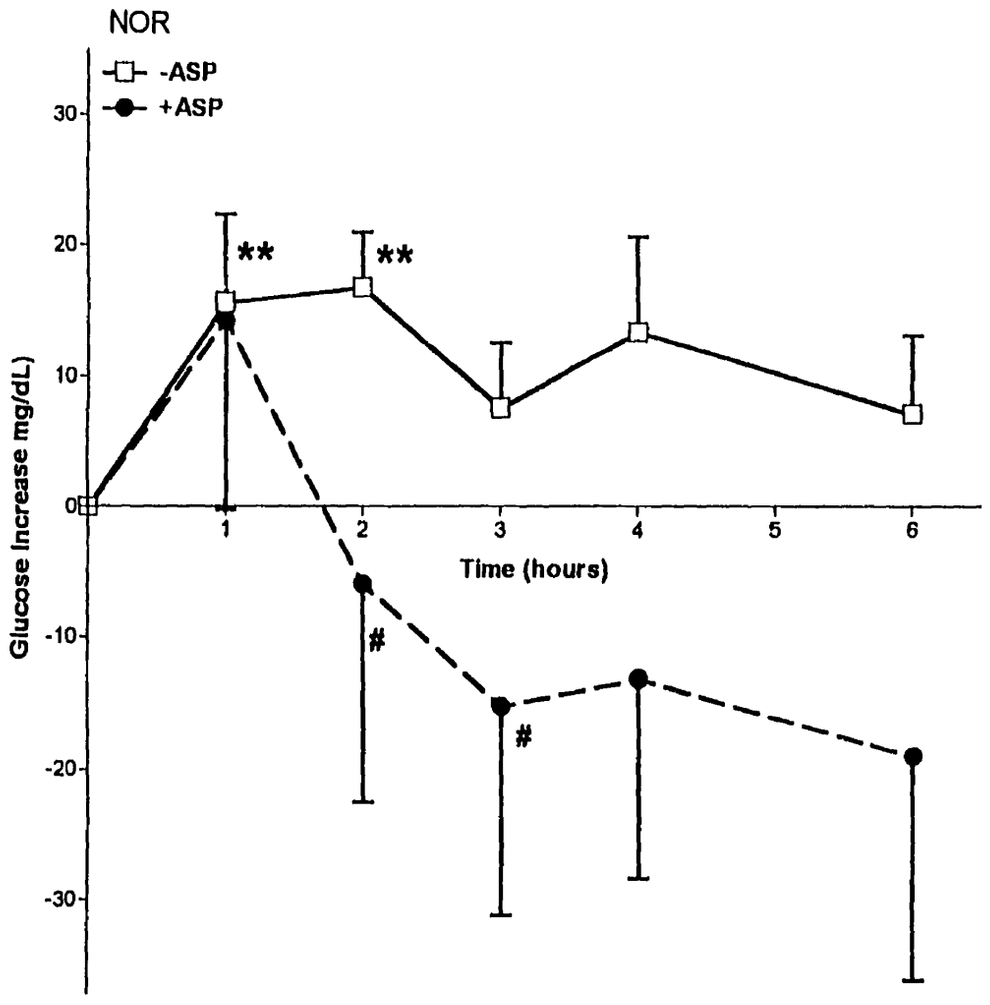

NOR
-ASP
+ASP
Triglyceride Increase (mg/dL)
50
40
30
20
10
0
-10
1
2
3
4
5
6
Time (hours)
**
**
**
**
**
*
#
##
##
#
ACC
-ASP
+ASP
50
40
30
20
10
0
-10
1
2
4
5
Time (hours)
*
**
*
*

Figure 4.6: Comparison of plasma glucose between normal postprandial lipemia and accelerated clearance groups of mice following an oral fat load.

Plasma glucose was measured for the two groups of C57Bl/6 mice: the normal postprandial lipemia (NOR, left panel, n=6, basal glucose = 119.8 ± 6.8) and accelerated clearance (ACC, right panel, n=6, basal glucose = 101.5 ± 5.3) as described in Figure 5 where *p<0.05 and **p<0.01 *vs* fasting TG and #p<0.05 and ##p<0.0125 with *vs* without ASP.


NOR
-ASP
+ASP
Glucose Increase mg/dL)
30
20
10
0
-10
-20
-30
1
2
3
4
5
6
Time (hours)
**
**
#
#

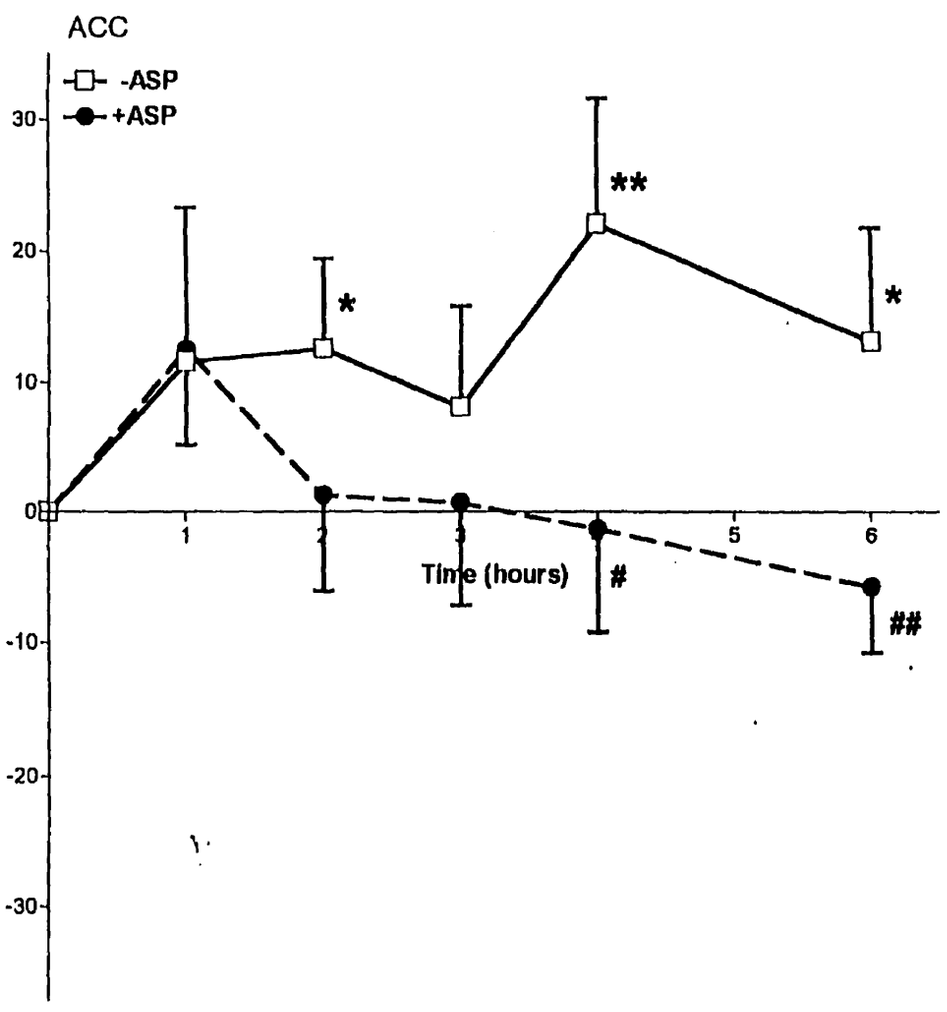

ACC
-ASP
+ASP
30
20
10
0
-10
-20
-30
1
2
3
5
6
Time (hours)
*
**
*
#
##

Table 4.1. Fasting basal parameters in normal postprandial lipemia mice and accelerated clearance mice.

	Group 1(NOR) Normal Postprandial Lipemia (n=6)	Group 2(ACC) Accelerated Clearance (n=6)
TG (mg/dL)	32.8±4.0	36.7±1.2
Glucose (mg/dL)	119.8±6.8	101.5±3.3 *
FFA (mM)	0.505±0.055	0.311±0.023 **

Average ± sem basal fasting values of triglyceride (TG), glucose and free fatty acid (FFA) measured twice for each mouse where * $p<0.025$, ** $p<0.001$

4.5 Discussion

The present study indicates clearly that exogenous administration of human ASP to normal wildtype C57Bl/6 mice altered postprandial metabolism. There were reductions in both postprandial triglycerides as well as glucose and these changes were greater in those animals where the postprandial lipemia was greater. The timing of the effects on triglyceride and glucose lowering was consistent with the time lag of appearance of human ASP in plasma where maximal ASP concentrations occurred at 1 hour and the lipid and glucose lowering effects occurred after this peak. This time course was consistent with the time delay in achieving ASP effects as shown in *in vitro* cellular assays, where it took 30 minutes (13,14,35) to 1 hour (21) to detect cellular changes in glucose transport or triglyceride synthesis.

ASP may achieve these *in vivo* effects on lipid and glucose metabolism through multiple pathways. In cellular studies we have shown that ASP increases fatty acid esterification in adipocytes to form storage triglycerides through a direct effect on the lipid synthetic enzymes in this pathway. Although ASP also increased glucose transport, the TG esterification effect was independent of the glucose transport effect (13). Thus we propose that by increasing the amount of fatty acid that is taken up by cells and esterified, the local micro-environmental concentration of fatty acids is decreased, allowing more efficient hydrolysis of dietary triglycerides by lipoprotein lipase, since lipoprotein lipase exhibits end product inhibition by high FFA (26,30). This hypothesis is consistent with the data obtained in cellular function assays. In fact, the increased plasma free fatty acid concentrations at 6 hours in those mice that received ASP, would suggest lipoprotein lipase exceeds the capacity of tissue fatty acid uptake resulting in a buildup in circulating fatty acids. The lack of exogenously administered ASP at this time point may account for a reduced fatty acid uptake. However, we cannot rule out a direct effect of ASP on lipoprotein lipase activity or production by the adipocytes as well during the fat load. As the body switches from a fasted to a fed state, lipoprotein lipase activity increases in adipose tissue and decreases in muscle (11,40) and FFA uptake changes (12). ASP may enhance this FFA partitioning to tissues.

The results also show an effect of ASP on glucose levels. Although the meal itself was a high fat meal with no carbohydrate, increases in plasma glucose may be due to fatty

acid stimulation of hepatic gluconeogenisis (17). The glucose lowering occurred over the same time frame as the triglyceride effects and this could be a direct or an indirect effect of ASP. ASP has been shown to stimulate glucose transport directly in adipocytes *in vitro* through translocation of Glut 1 and Glut 4 from intracellular stores to plasma membrane (13,21,34) and this could enhance glucose clearance from the plasma. ASP could also indirectly mediate glucose uptake through its effects on fatty acid uptake and storage in adipocytes, which then results in reduced fatty acid inhibition of glucose metabolism in muscle (27,28). However, even in the absence of an ASP effect on triglyceride clearance (as shown by the low lipemia in one group of mice) there was still an effect on glucose clearance, albeit not as marked. Thus, both of these explanations probably contribute to the ASP effects on plasma glucose.

We have focused on adipose tissue as the key target tissue for ASP. Certainly in *in vivo* cellular studies, the major effect of ASP on glucose transport and triglyceride synthesis was found in adipocytes (7). However, administration of ASP may exert actions that increase TG clearance independent of its known effects on adipose tissue determined *in vitro* thus far. However, both muscle and liver also play major roles in lipid and glucose metabolism *in vivo*. While the liver is capable of taking up and esterifying fatty acids efficiently, ASP appears to have little effect on increasing triglyceride synthesis in these cells. Only a 25% increase was obtained at high doses of ASP in human HepG2 cells (10) in contrast to the 2-10 fold increase in triglyceride synthesis obtained in mature fat cells or cultured differentiated adipocytes (9, 35 for review see 7). We have also found a consistent effect of ASP on stimulation of glucose transport in muscle (cultured differentiated myotubes) which was effected through translocation of Glut 1, Glut 3 and Glut 4 to the plasma membrane (34). Preliminary studies in humans suggest that there is no ASP production in muscle during fasting or postprandial states in arterio-venous studies (unpublished observations), in contrast to the adipose tissue ASP production found postprandially (29). As well, it is possible that the systemic administration of ASP in the present studies might induce responses not only in adipose tissue but also in muscle or other tissues.

In summary, these studies indicate that systemic administration of ASP reduced postprandial lipemia and postprandial glucose and these effects are consistent with the *in*

vitro cellular function data. Clearly, the exact mechanism and tissue specific impact need to be examined in much greater detail in future studies. However, the present data are the first *in vivo* data to demonstrate the potential physiological role that ASP may play in changing clearance of dietary fat.

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Prelude IV

The role of ASP was demonstrated *in vivo* utilizing excesses of ASP. The postprandial role of ASP after a fat load was thus determined in transgenic mice, mice with an absence of ASP. It was hypothesized that an absence of ASP should have opposite effects of an excess of ASP. In addition, these knockout mice were supplemented with exogenous ASP to see if the effects of a lack of ASP could be complemented.

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Chapter 5
Mice Lacking Acylation Stimulating Protein (ASP) have Delayed Postprandial Triglyceride Clearance

5.1 Abstract

Acylation Stimulating Protein (ASP) is a 76 amino acid fragment of the third component of complement (C3) which is generated by the interaction of adipsin and factor B with C3. In vitro studies have shown that ASP can markedly increase triglyceride synthesis in adipocytes. To test the ASP pathway in vivo, C3 deficient mice- and therefore ASP deficient mice- were generated and oral fat loads were conducted in wild type (C3+/+) and mutant (C3-/-) animals. The principal results were: 1) postprandial triglyceride clearance was significantly delayed in mutant compared to wild type mice; 2) this difference was more pronounced in males compared to females; 3) in both males and females, the differences were more pronounced in the second half of the postprandial period; 4) fasting and postprandial free fatty acid (FFA) were higher in C3(-/-) than in C3(+/+) males; and 5) intraperitoneal administration of ASP accelerated triglyceride clearance in C3(-/-) males. The data are consistent therefore, with the hypothesis that the ASP pathway is an important physiologic determinant of normal postprandial triglyceride clearance.

5.2 Introduction

Storage of energy when it is in excess and release of energy when it is needed are the critical biologic functions of adipose tissue. Lipoprotein lipase (LPL) and insulin are both widely recognized to play important roles in the regulation of lipogenesis. Recently, in vitro studies have demonstrated that the Acylation Stimulating Protein (ASP) pathway may also be a determinant of the rate at which adipocytes can store energy (1). Accordingly, the present study was designed to test this hypothesis *in vivo* using a mouse model in which synthesis of the obligate precursor of ASP, complement C3, was disrupted by targeted deletion.

ASP is generated by the interaction of factor B and adipsin with the third component of complement (C3), all three of which are synthesized and secreted by both murine (2) and human adipocytes (3-5). The product, C3a, is a non-glycosylated 77 amino acid N-terminal fragment of the chain of C3. The terminal arginine is then rapidly removed by carboxypeptidase N to produce ASP. ASP is identical to C3adesArg as established by amino terminal sequence analysis, ion spray ionization mass spectroscopy, and amino acid analysis (6). Subsequently, a recombinant ASP was produced and shown to be identical in bioactivity to native ASP (7). Both native (plasma) and recombinant ASP were bioactive in specific target tissues such as adipose tissue but not in other cells such as macrophages (7). ASP, therefore, differs in function as well as composition from C3a.

In vitro studies have shown that ASP increases triglyceride (TG) synthesis by increasing the activity of the last enzyme involved in the synthesis of a TG molecule, diacylglycerol acyltransferase (8) and by increasing specific membrane transport of glucose through translocation of GLUT 1 and GLUT 4 from intracellular vesicles to the plasma membrane (9,10). Of importance, ASP and insulin have independent and additive effects on TG metabolism (9,11). Interaction with a cell membrane receptor (7,12) and subsequent activation of a protein kinase C signal transduction pathway (13) appear to be critical to production of these coordinated effects on TG synthesis. The structure of ASP is broadly conserved phylogenetically (14) consistent with the demonstration in vitro of the bioactivity of human ASP in a variety of species including murine cells (7).

In vivo studies in humans have shown that ASP is produced in adipose tissue and that the production of ASP is markedly accelerated in the second half (i.e. 3-6 hours) of the

postprandial period (15). Furthermore, the increase in ASP production during this period correlates with maximal TG clearance and fatty acid uptake by adipocytes. Of interest also, obese subjects have higher plasma ASP than normal, but their plasma ASP diminishes with prolonged fasting and weight loss. Thus plasma ASP correlates with expansion and contraction of adipose tissue mass (16).

There is, therefore, considerable circumstantial *in vivo* and *in vitro* evidence in humans indicating that the ASP pathway plays a physiologically important role in postprandial TG clearance. However, a direct test of this hypothesis has not yet been made. To do so, C3 knockout (-/-) mice were engineered. In the absence of C3, the precursor to ASP, these mice are unable to produce ASP. Accordingly, TG clearance following an oral fat load was determined in C3(-/-) mice and compared to that observed in wild type C3(+/+) mice.

5.3 Methods

5.3.1 Targeted Disruption of the C3 Locus

C3(-/-) and (+/+) mice were kindly provided by Dr. H. Colton and Dr. R. Wetsel. Development of the C3 knockout mice has been described in detail elsewhere (17,18). The murine C3 locus was disrupted by replacing 2.4 Kb of the 5' flanking region and the first 105 bp of exon 1 of the C3 gene with the neomycin resistant gene oriented in the opposite direction from C3 transcription (1.14 Kb Neocassette from pMC1 NEO vector (Stratagene, LaJolla, CA)). RW4 and D3 ES (from 129 SVJ mice) were transfected with the targeting vector. Homologous recombinant RW4 ES cells were micro-injected into C57Bl/6 blastocysts. Chimeric founders were established (F_0) and germ line transmission into C57Bl/6 background was accomplished via mating to C57BI/6 females (F_1). C3 heterozygous (129SVJ x C57Bl/6 strain) F_1 were interbred with each other (brother/sister). Because these animals are not interbred strains, there is some genetic variation, and to control for this heterozygotes were intercrossed to produce heterozygotes (+/-), homozygotes (-/-) and wild type (+/+) (F_3 and F_4). Thus in all studies paired littermates (-/- and +/+) were used to control for, and randomize genetic variation.

5.3.2 Genotyping of Mice

Animal care was in accordance with Royal Victoria Hospital Animal Care Committee institutional guidelines. For genotyping analysis to identify hetrozygotes (+/-), homozygotes (-/-) and wild type (+/+) mice, tail DNA was extracted. PCR was performed using 800 nM each of the following primers: C3 sense: CTT AAC TGT CCC ACT GCC AAG AAA CCG TCC CAG ATC, C3 antisense: CTC TGG TCC CTC CCT GTT CCT GCA CCA GGG ACT GCC CAA AAT TTC GCA AC. Neomycin sense: ATC GCA TCG AGC GAG CAC GTA CTC GGA. Neomycin antisense: AGC TCT TCA GCA ATA TCA CGG CTA GCC. PCR conditions were: 30 cycles 94° C 1 minute, 67° C 2 minutes, and 72° C 3 minutes. Products were separated by electrophoresis on a 7% polyacrylamide gel and visualized with ethidium bromide staining.

5.3.3 Baseline Characterization of Mice

At 8 weeks of age, having been on a Purina chow 5075 diet from the time of weaning (3 weeks), the mice (littermates) were weighed and fasting lipid levels, glucose and insulin determined. Blood was obtained by tail bleeding after an overnight fast. Plasma FFA and TG

were measured using colorimetric enzymatic kits (Boehringer Mannheim, Laval, Quebec, Canada). Fasting insulin was measured using a rat insulin radioimmunoassay kit which fully crossreacts with mouse insulin (Linco Research Inc, St. Charles, MO). Fasting plasma glucose was measured using a Trinder glucose kit (Sigma, St. Louis, MO). HDL lipids were measured following precipitation of apoB lipoproteins with heparin Mn^{2+} and non-HDL (VLDL + LDL) cholesterol and triglyceride were calculated from the difference (total HDL).

5.3.4 Postprandial Fatload on Mice

An oral fat load was administered by intragastric feeding to C3(-/-) and wild type mice C3(+/+) (10-12 weeks old). The mice were hand held and were not anesthetized. Following an overnight fast, 400 μL of olive oil (followed by 100 μL air) were given as described (19-21). Blood samples (40 μL) were collected from each mouse at 0, 1, 2,3 ,4 and 6 hours into EDTA containing tubes and plasma isolated to measure TG and FFA. Twenty-four C3(-/-) mice were studied (9 males, 15 females) and 13 C3(+/+) wild type (7 males, 6 females). There was no evidence of fat malabsorption (loose stools, diarrhea). Results at each time point during the postprandial period are expressed as the mean ± sem of the difference in TG from fasting levels. Individual time points were compared. As well, the area under the TG curve was determined using a linear trapezoidal equation (Sigma Stat, Jandel Scientific, San Rafael, CA) and the results in the two groups compared.

The effect of ASP injection on postprandial lipemia was tested in separate studies (n=8 male C3(-/-) mice, 10-12 weeks old). Two hours following administration of the fatload (as described above), ASP (500 μg in 250 μl) was injected intraperitoneally with half of the animals receiving a sham injection (buffer only containing 1 mg/mL(bovine serum albumin in phosphate buffered saline pH7.2) Two weeks later, the same fatload was repeated with the ASP/sham injections reversed. ASP was purified and assessed for purity as previously described (13).

5.3.5 Statistical Analysis

Data was analyzed by two-way ANOVA for genotype (-/- vs +/+) and time effect (0 to 6 hours) and significance of individual points was determined by Bonferroni t-test.

5.4 Results:

At 8 weeks of age, the mice (-/- and +/+ littermates) were weighed and fasting cholesterol, triglyceride (TG), glucose and insulin levels were measured. These results are shown in Table 5.1. Both male C3(-/-) and wild type mice C3(+/+) weighed more than the females ($p<0.005$). The fasting TG and cholesterol values tended to be higher in the C3(-/-) males but these differences were not statistically significant. By contrast, fasting plasma FFA were significantly higher in the male C3(-/-) compared to the wild-type C3(+/+) mice ($p<0.05$).

The results of the changes in plasma TG during the oral fat load in the ASP C3(-/-) and the wild type C3(+/+) animals are illustrated in Figure 5.1 for both males and females separately. In both the male and female C3(+/+) mice, there was only a moderate increase in postprandial TG reaching a maximum of 54% increase over baseline (Figure 5.1). None of the individual time points were significantly increased above fasting TG although the overall curve in the male C3(+/+) was significantly different from fasting ($p<0.01$). By contrast, TG clearance from plasma after the oral fatload is significantly delayed in the C3(-/-) group with a significant postprandial increase in both male C3(-/-) ($p<0.01$) and in female C3(-/-) ($p<0.001$) mice. In the females, the 3 and 4 hour time points were significantly above fasting ($p<0.05$). The differences, however, were most marked in the male C3(-/-) mice in which plasma TG increased 112% above basal. At the 2, 3 and 4 hour time points, postprandial TG were significantly increased above basal TG (81% to 112%) and even at 6 hours were still significantly increased above basal TG (50%, $p<0.01$ by ANOVA for the postprandial curve).

The triglyceride curves from the C3(-/-) mice were also directly compared to the C3(+/+) mice. Both the 4 and 6 hour time points were both significantly higher in the C3(-/-) males compared to the C3(+/+) males (112±28 mg/dL vs 39±12 mg/dL, $p<0.025$ and 51±37 mg/dL vs -8±8 mg/dL, $p<0.05$, respectively). Similarly, the total TG area under the curve (AUC) was substantially greater in the male C3(-/-) mice compared to the male wild type group (+/+) (AUC = 558±120 mg/dL*h vs 264±45 mg/dL*h, $p<0.05$). A similar although less marked trend was present in the C3(-/-) females, (AUC = 357±63 mg/dL*h vs 247±64 mg/dL*h) (Figure 5.1, right panel). Importantly, since the fasting TG levels were not significantly different amongst the groups, the difference in postprandial TG clearance

cannot be a simple consequence of disproportionately elevated fasting TG levels in the C3(-/-) animals.

Because *in vivo* studies in humans have shown that maximal activation of the ASP pathway and TG clearance by adipocytes does not occur until the second half of the postprandial period (15). The areas under the TG (AUC) curve in the first and second halves of the postprandial periods in the C3(-/-) and C3(+/+) mice were calculated separately and these early and late phases were compared. These data are given in Table 5.II. In all cases, there was no significant difference between C3(-/-) and wild type C3(+/+) animals in the early portion of the TG curve. By contrast, the late TG (AUC) curve is significantly greater for the C3(-/-) mice vs the C3(+/+). The differences were particularly pronounced in the male C3(-/-) vs male wild typeC3 (+/+), with there being almost a threefold difference in the late postprandial area under the curve. While the difference between female C3(-/-) vs female wild type C3(+/+) was not statistically significant, the same trend is apparent in these animals as well.

As noted previously, the fasting plasma FFA concentration was significantly higher in the male C3(-/-) compared to the three other groups. As can be seen in Figure 5.2, postprandial FFA in the C3 (+/+) were only significantly increased postprandially at 3 and 4 hours ($p<0.05$), returning to basal levels by 6 hours. By contrast, although fasting FFA were already significantly increased in the C3(-/-) (Table 5.1), they continued to increase postprandially and at 4 and 6 hours were still significantly increased over basal ($p<0.05$). At 6 hours, when the C3 (+/+) had returned to fasting levels, C3(-/-) were reaching their highest point (67% above basal, $p<0.05$) and were significantly increased vs C3 (+/+) ($p<0.05$).

These data prompted more detailed studies in C3(-/-) and C3 (+/+) males. As shown in Table 5.III, there was a slight decrease (30%) in the glucose/insulin ratio. There was also a small, but not significant, decrease in fasting HDL cholesterol levels. By contrast there was a substantial (3-5 fold) increase in fasting non-HDL (VLDL + LDL) cholesterol and triglyceride (Table 5.III) ($p<0.005$ and $p<0.05$ respectively).

As an additional test of our hypothesis, since C3(-/-) mice have no functional ASP, we examined the effects of injecting human ASP intraperitoneally following administration of the fat load. As shown in Figure 5.3, in the absence of ASP, plasma TG increased 324% above basal (4 hours). Administration of ASP at 2 hours caused a rapid decrease in plasma

triglyceride towards basal and a substantial (42%) decrease in the area under the curve over 6 hours vs C3(-/-) mice that received a sham injection (669± 216 control vs 391 ± 77 with ASP, $p<0.05$).

Table 5.I: Characteristics of C3(-/-) and wild type (+/+) mice

	Male		Female	
	C3(-/-) (n=9)	C3(+/+) (n=7)	C3(-/-) (n=15)	C3(+/+) (n=6)
Wt (gm)	28.3± 0.8	27.7± 1.1	23. 0± 0.4	22.6± 0.9
TG (mg/dL)	100.6± 16.1	83.5± 12.9	70.1± 10.5	64.2± 3.1
Chol (mg/dL)	67.1± 4.3	63.0± 4.6	57.6± 4.0	56.6± 7.0
FFA (mM)	1.97± 0.52*	0.87± 0.18	1.54± 0.40	0.91± 0.23
Glu (mg/mL)	1.12±.08	1.12±.16	0.91±.03	1.06±.077
Ins (ng/mL)	0.295±.021	0.234±.033	0.200±.014	0.217±.016

Fasting blood was obtained in mice 8 weeks of age. The data are the mean±sem where Wt= weight; TG= plasma triglycerides; Chol= total plasma cholesterol; FFA= plasma free fatty acids; Glu= plasma glucose; Ins= plasma insulin, where *$p < 0.05$ C3(-/-) vs C3(+/+) mice and **$p<0.01$ C3(-/-) vs C3(+/+) by one-way ANOVA..

Table 5.II: Area under the plasma triglyceride curves during the early and late postprandial periods

Mice	n	Early	Late
All:			
C3 (-/-)	(n=24)	181± 30	252± 41
C3 (+/+)	(n=13)	132 ±23	124± 21*
Male:			
C3 (-/-)	(n=9)	226± 68	331± 63
C3 (+/+)	(n=7)	137± 34	127± 21**
Female:			
C3 (-/-)	(n=15)	153± 23	204± 50
C3 (+/+)	(n=6)	128± 30	119± 38

Plasma triglyceride was determined at each time point shown and the difference from the fasting value determined (Figure 5.1 and 5.2). The area was then determined under the plasma triglyceride curve for the first half (0 to 3 hours) and the second half (3 to 6 hours) of the postprandial period. The data are shown as mean ± sem mg/dL*h. The number of animals in each group is shown in brackets. Significance for C3(-/-) vs C3(+/+) is indicated, where *$p<0.025$ and ** $p<0.01$ for C3(-/-) vs C3(+/+).

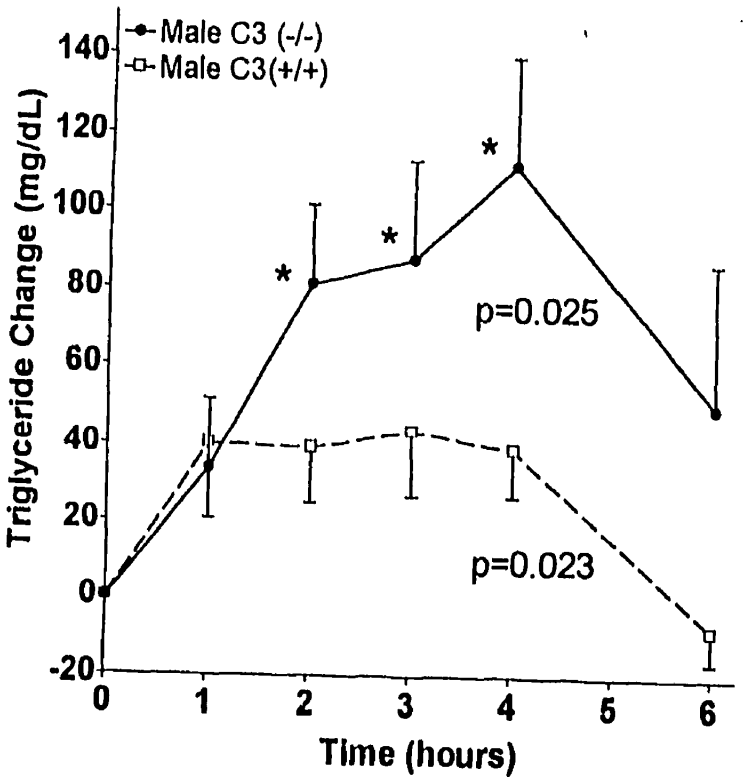
Table 5.III. Fasting Lipoproteins and Glucose/Insulin Ratio in Male C3 (-/-) Mice.

	C3 (+/+) (n = 5)	C3 (-/-) (n = 8)	% Change
HDL cholesterol	53.4 ± 3.1	45.8 ± 3.9	14%
VLDL + LDL cholesterol (non-HDL cholesterol)	5.6 ± 0.5	16.9 ± 1.9	302% $p<0.005$
VLDL + LDL triglyceride (non-HDL triglyceride)	7.7 ± 2.3	45.2 ± 11.2	587% $p<0.05$
Glucose/Insulin	5.00 ± 78	3.92 ± 33	22%

Cholesterol and triglyceride values are given as mg/dL and glucose/insulin as mg/ng

Figure 5.1: Changes in plasma triglyceride during the postprandial period in male and female C3(-/-) and wild type (+/+) mice.

The differences in plasma triglycerides (mean± sem) from fasting (shown in Table 5.I) were determined at each time point in each animal for males (left panel) and females (right panel) where *p<0.05 vs fasting TG (Bonferroni t-test). Triglyceride area under the curve was calculated in male C3(-/-) (n=9) and male C3(+/+) (n=7) (558±120 mg/dL*h C3(-/-) vs 264±45 mg/dL*h, C3(+/+), p<0.05). Female C3(-/-) (n=15) and C3(+/+) (n=6) mice were compared separately (357±63 mg/dL*h C3(-/-) vs 247±64 mg/dL*h C3(+/+). For each curve, the ANOVA results vs time are given in the figure where ns = not significant. By two-way ANOVA, there was a significant effect of genotype (-/- vs +/+) for males (p=0.0052) whereas the effect was not quite significant for females (p=0.07).


Male C3 (-/-)
Male C3(+/+)
Triglyceride Change (mg/dL)
140
120
100
80
60
40
20
0
-20
0
1
2
3
4
5
6
Time (hours)
*
*
*
p=0.025
p=0.023

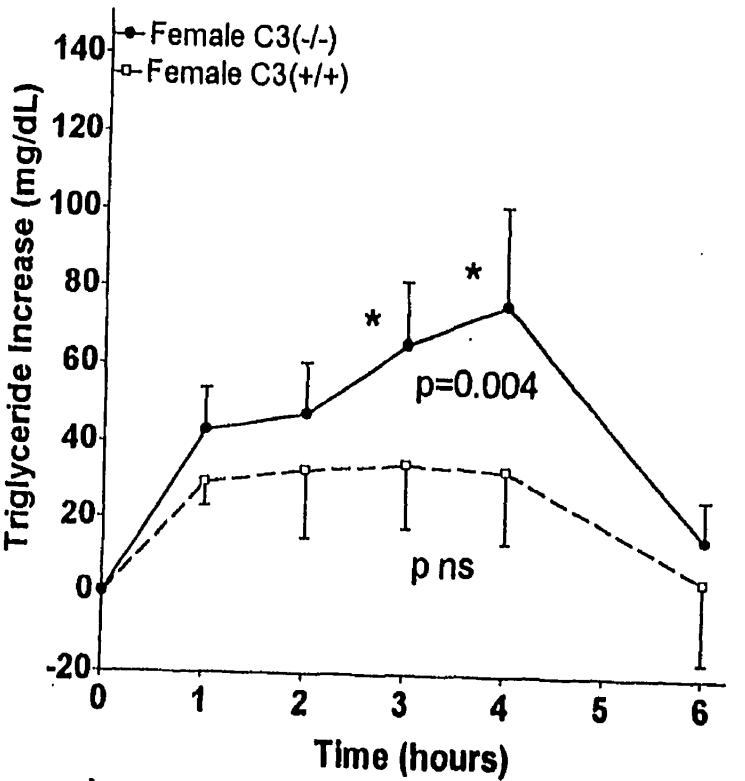

Female C3(-/-)
Female C3(+/+)
Triglyceride Increase (mg/dL)
140
120
100
80
60
40
20
0
-20
0
1
2
3
4
5
6
Time (hours)
*
*
p=0.004
p ns

Figure 5.2: Free fatty acid levels in male C3(-/-) and wild type mice during the postprandial period.

Plasma free fatty acids were determined at each time point in each animal following the fat load as described in Figure 5.1. Nine male C3(-/-) animals and 6 male wild type C3(+/+) animals were studied, where *$p<0.05$ vs fasting FFA (Bonferroni t-test), and the results for one-way ANOVA for each curve are given in the figure. By two-way ANOVA, there was a significant effect of genotype ($p=0.013$) for C3(-/-) vs C3(+/+) mice.

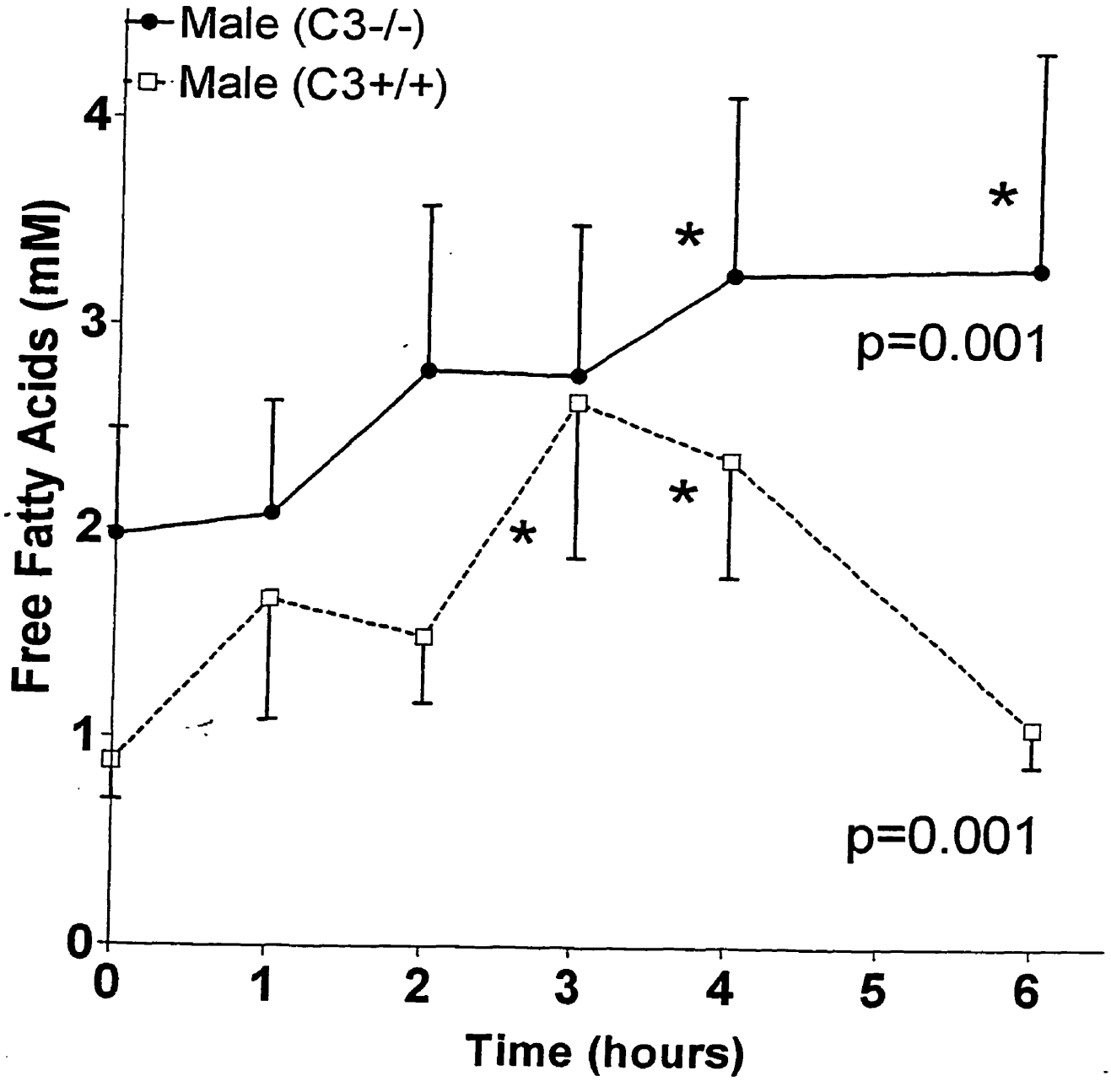
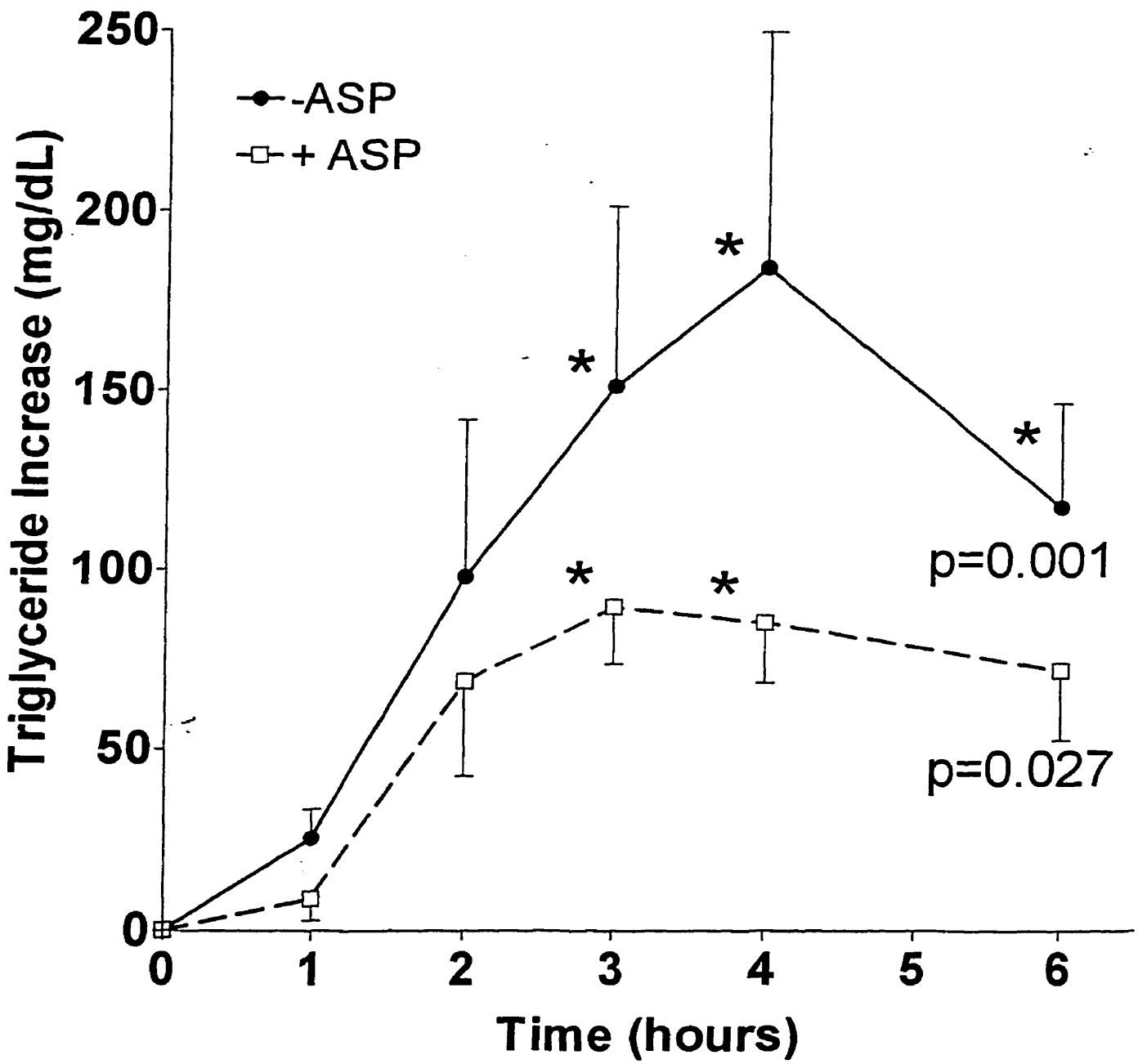

Male (C3-/-)
Male (C3+/+)
Free Fatty Acids (mM)
Time (hours)
p=0.001
p=0.001
*
*
*
*
0
1
2
3
4
5
6

Figure 5.3: Changes in plasma triglyceride during the postprandial period in male C3 (-/-) mice with injected ASP.

The differences in plasma triglycerides (mean ± sem) from fasting were determined at each time point in each mouse where fasting TG = 56 ± 6.0 mg/dL without ASP (n=8) and 59 ± 9.8 with ASP injection (n=8) where *$p<0.05$ vs fasting plasma triglyceride (Bonferroni t-test). The results for one-way ANOVA on each curve are given in the figure. By two-way ANOVA, there was a significant effect of ASP ($p=0.026$) for +ASP vs -ASP.


250
200
150
100
50
0
Triglyceride Increase (mg/dL)
-ASP
+ ASP
*
*
*
*
*
p=0.001
p=0.027
0
1
2
3
4
5
6
Time (hours)

5.5 Discussion

These findings represent direct evidence that the ASP pathway plays an important role in postprandial TG clearance from plasma. They constitute, therefore, an important step towards proof of concept that the ASP pathway, by increasing the rate of adipocyte TG synthesis, increases the rate of chylomicron TG clearance from plasma.

In vitro studies have shown that fatty acids themselves do not cause increased ASP generation by adipocytes (5). Insulin does produce a modest increase in ASP whereas chylomicrons cause a profound increase in ASP generation by adipocytes (5). As noted previously, *in vivo* studies in humans have demonstrated that production and release of ASP by adipocytes markedly increases in the second half of the postprandial period (15), a delay that would appear to reflect the time required to maximally activate the pathway by chylomicrons and insulin. The correspondence between these *in vivo* findings in humans and the present *in vivo* data in the C3(-/-) mice are of considerable interest. In the C3(-/-) mice injected intraperitoneally with ASP, the major differences also occur in the second half of the postprandial period.

Through activation of the ASP pathway by chylomicrons, the efficiency of postprandial TG clearance can be increased. If the substantial amounts of fatty acids released from chylomicrons were not quickly removed from the capillary space, then LPL action would be inhibited, and TG hydrolysis reduced (22). Thus, enhanced (21) or decreased (20) lipoprotein lipase activity can profoundly affect plasma triglyceride clearance as shown in over expressing or knockout mice models. As well, a decreased efficiency of triglyceride clearance may be reflected in our C3(-/-) mice by increased circulating free fatty acids not only postprandially, but also in the fasting state, as well as delayed postprandial triglyceride clearance.

The ASP pathway provides, therefore, a form of metabolic intergration. Chylomicrons stimulate the generation of ASP by adipocytes, and ASP, by increasing fatty acid uptake and esterification into adipocytes, allows continued action of LPL and therefore allows continued hydrolysis of chylomicron TG. There is independent evidence supporting this model of modulation of LPL activity by fatty acids. *In vitro* experiments indicate that LPL forms complexes with fatty acids resulting in decreased lipolysis and

detachment of both lipase and lipoproteins from their endothelial sites (22-24). *In vivo* support of this hypothesis has also been obtained (25).

Based on the present *in vivo* study, and our previous *in vitro* data, we propose that a major site of action of ASP *in vivo* is on adipose tissue. However, it is also possible that ASP may act at other tissues, such as muscle. *In vitro* data indicate that ASP stimulates glucose transport via translocation of Glut1, Glut3 and Glut4 to the plasma membrane in L6 myotubes (9-11).

Just as normal function of the ASP pathway appears integral to the normal deposition of dietary fatty acids into adipocytes during the postprandial period, dysfunction of the ASP pathway may be associated with lipoprotein abnormalities that are linked to the pathogenesis of coronary artery disease. If fatty acid trapping and storage by adipocytes are reduced in the postprandial period, an excessive proportion of dietary fatty acids will be diverted to the liver and hepatic secretion of apoB100 lipoproteins [VLDL and LDL] will increase (26,27). However in our C3(-/-) mice, although the fasting cholesterol and triglycerides were not elevated, there were indeed increases in [VLDL + LDL] cholesterol and triglycerides. The elevated plasma LDL particle number that results from the increased secretion of apoB100 lipoprotein particles by the liver increases the risk of coronary disease in humans. Unquestionably, further study will be required to define the role of the ASP as a determinant of the rate of retention and release of fatty acids from adipose tissue. The present data, however, indicate that for normal TG clearance following an oral fat load, the ASP pathway must be intact and thus the ASP pathway may plan an important role in energy storage.

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Prelude V

The acute effects of ASP on postprandial metabolism after a fat load were previously studied. A long-term effect of a lack of ASP in the female transgenic C3 knockout mice was determined. These mice were placed on different diets to determine the effects of obesity and age on lipid parameters, body weight, adiposity as well as plasma leptin levels.

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Chapter 6
Reduced body weight, adipose tissue and leptin levels in female mice lacking Acylation Stimulating Protein (ASP).

6.1 Abstract

ASP is a potent lipogenic protein produced by adipocytes. *In vitro* studies have shown that ASP increases triglyceride (TG) synthesis and glucose transport in both murine and human adipocytes. Our initial study indicated that complement C3 (-/-) deficient mice (and therefore ASP deficient) demonstrated altered dietary postprandial triglyceride clearance. In the present study, we have examined the phenotype of female mice longitudinally on different diets. Female C3(-/-) mice on both low and high fat diets displayed a reduction in weight of 10.4 ± 0.5% ($p<0.0001$) ANOVA as compared to the C3(+/+) littermates. Reductions in white adipose tissue mass accounted for most of this weight difference (50.3 % reduction $p<0.03$ high fat diet). In conjunction, leptin plasma levels were significantly reduced C3(-/-) on both high ($p<0.001$) and low fat diets ($p<0.01$). This reduction was significant even after adjusting for reduced body weight ($p<0.001$). Leptin reductions in the C3(-/-) were greater on the high fat diet (63%) and were associated with an increased food intake (14± 2.1% $p<0.001$). Furthermore, there was also a decrease in basal glucose levels and basal insulin levels (and 12.8% $p<0.05$ at 14 weeks and 41% $p<0.05$ at 26 weeks of age on both diets and low fat respectively). These *in vivo* experiments in female mice demonstrate that ASP plays a role in weight gain and adiposity, leptin and insulin plasma levels.

6.2 Introduction

Acylation Stimulating Protein (ASP) stimulates both adipocyte fatty acid esterification into triglyceride and glucose transport (1). This occurs via an increase in diacylglycerol acyltransferase activity (2) and translocation of glucose transporters from intracellular sites to the cell surface (3). These dual effects are mediated through specific cell surface binding (4), (5) resulting in activation of a signaling pathway which includes protein kinase C (6). In addition to stimulation of free fatty acid esterification, ASP has recently been shown to also inhibit hormone sensitive lipase in adipocytes, in concert with insulin (7).

ASP is identical to complement C3a des Arg. It is formed *via* the specific cleavage of complement C3 through the alternate complement pathway via the interaction of complement C3, factor B and adipsin, followed by rapid cleavage of the C-terminal arginine by carboxypeptidase N (8). Adipocytes are one of the few cells capable of producing all three of the factors (factor B, adipsin and C3) that are required for the production of complement C3a *via* the alternate pathway (9). ASP production increases consequent to adipocyte differentiation (10), (11) and also following chylomicron stimulation *in vitro*, in adipocytes (12), (13). *In vivo* arterial venous gradients across a subcutaneous adipose tissue bed demonstrated postprandial production of ASP (14) and plasma levels are elevated in obesity (15). The postprandial increase in ASP is adipose tissue specific and is not observed in the general circulation (1,) (unpublished observations) or in arterial venous differences across muscle tissue (unpublished observations).

The major site of action of ASP appears to be adipocytes as determined by competitive binding, stimulation of triglyceride synthesis and enhanced glucose transport and transporter translocation (reviewed (1)). There is also a differentiation specific increase in ASP binding and functionality in cells (1).

The correlation between *in vivo* postprandial adipose tissue ASP production, dietary triglyceride clearance and both *in vitro* functionality data and chylomicron stimulated ASP production suggested that ASP might play an important role in dietary fat clearance and storage deposition. Our initial studies in murine models with excess exogenous ASP confirm this. Administration of ASP to C57Bl/6 mice resulted in

decreased plasma triglyceride levels after a fat load of olive oil (6 hours duration) (16). This increase in triglyceride clearance was dependent on the degree of postprandial lipemia where greater decreases in triglyceride levels occurred in mice demonstrating greater postprandial lipemia (16). Studies were also performed in complement C3 knockout mice, which being deficient in C3, are unable to produce ASP. In young male mice (129Sv x C57Bl/6 strain), although fasting triglycerides were not different, elevated triglyceride levels were observed postprandially after an oral fat load suggesting delayed triglyceride clearance in the absence of ASP (17). Furthermore, administration of exogenous ASP to these functional ASP knockout male mice was able to reduce this postprandial lipemia and decrease plasma triglyceride levels (17). The female mice displayed similar but less striking differences in postprandial lipid metabolism (17). Longitudinal experiments then were undertaken to study the effects of age and diet on energy intake and adipose storage and related parameters such as insulin and leptin in the female ASP functional knockout mice.

6.3 Methods and Materials:

6.3.1 Ethics: All experimental protocols were approved by the Royal Victoria Hospital Animal Ethics committee and were in accordance with the guidelines set out by the Canadian Committee on Animal Care.

6.3.2 Mice: Dr H Colten and Dr R.H. Wetsel kindly provided the knockout mice. Development of the complement C3 knockout has been described elsewhere in detail (18), (19). The mice were of (129Sv x C57Bl/6) strain and heterozygous mating produced the littermates (wildtype C3(+/+) and knockout C3(-/-)) used for the present experiments. Mice were housed in sterile barrier facilities with equal day/night periods. In all cases, littermates were used to randomize genetic variation.

6.3.3 Genotyping: For genotyping, tail DNA was extracted and PCR was performed. PCR was performed using 800 nM each of the following primers: C3 sense: CTT AAC TGT CCC ACT GCC AAG AAA CCG TCC CAG ATC, C3 antisense: CTC TGG TCC CTC CCT GTT CCT GCA CCA GGG ACT GCC CAA AAT TTC GCA AC. Neomycin sense: ATC GCA TCG AGC GAG CAC GTA CTC GGA. Neomycin antisense: AGC TCT TCA GCA ATA TCA CGG CTA GCC. PCR conditions were: 30 cycles 94°C 1 minute, 67°C 2 minutes, and 72°C 3 minutes. Products were separated by electrophoresis on a 7% polyacrylamide gel and visualized with ethidium bromide staining.

6.3.4 Diet, feeding and weighing: C3(+/+) and C3(-/-) female mice were weighed once weekly from weaning at 4 weeks of age. At 8 weeks, the mice were housed individually and allowed to acclimatize for 2 weeks. The mice were placed on pelleted low fat diet (LF) consisting of, 19.3% protein, 67.3% carbohydrates and 4.3% fat (w/w) and high fat diet (HF) of 22.9% protein, 45.8% carbohydrate and 20.3% fat w/w modified from VanHeek et al (20) and obtained from Research Diets Inc (New Brunswick, NJ, Diets D12477 and D12478 respectively). The diets contained 10% Kcal (LF) and 40% (HF) Kcal energy from fat, with a 1:1:1 ratio of saturated: monounsaturated: polyunsaturated fats and were stored at 4°C. Carbohydrate was in the form of cornstarch instead of sucrose (70% LF and 40% HF Kcal). The vitamin and mineral content conformed with the AIN guidelines. The food was weighed 3 times weekly over a period of 16 weeks and food intake determined over the time period of 10 to 26 weeks of age.

6.3.5 Plasma Assays: Blood was collected into EDTA containing tubes by tail bleeding as previously described (16) from mice fasted overnight (16 hours), with water *ad libitum*, at 10, 14, 26, 32 and 48 weeks of age. Blood was separated by centrifugation and stored at -80°C. Leptin was measured using a mouse leptin RIA assay (Linco Inc. St Charles, MO). Fasting insulin was measured using a rat insulin RIA kit which has 100% cross-reactivity to mouse insulin (as described by the manufacturer Linco Inc). Glucose was measured using a Trinder glucose kit (Sigma St Louis, MO). Plasma free fatty acids (FFA), cholesterol and triglyceride were measured using colorimetric enzymatic kits (Roche, Laval, Quebec, Canada).

6.3.6 Tissues: Mice were anaesthetized (0.01 ml/10 g body weight intramuscularly) with a cocktail composed of 5 ml Ketamine (100 mg/ml), 2.5 ml xylazine (20 mg/ml), 1 ml acepromazine (10 mg/ml) and 1.5 ml sterile saline. Blood was drawn (0.5 mL) and the mice sacrificed by cervical dislocation. Tissues were dissected out, weighed and frozen in liquid nitrogen. Four adipose tissues depots were collected: inguinal, pectoral and suprascapular together, gonadal fat up to the apex of the ovary and perirenal adipose tissue with the adrenal gland removed. Additional tissues collected were heart, liver, intrascapular brown adipose tissue, both kidneys and quadriceps muscles with all visible fat removed.

6.3.7 Fat load: Following an overnight fast (16 hours), 400 µL of olive oil (followed by 100 µL air above the oil) was given by gastric gavage using a feeding tube (12 cm curved ball tipped feeding needle #20) according to standard procedures as previously described (16), (17) and similar to previously published methods (21), (22), (23), (24). There was a 2-week interval between the fasting blood sample and the fat load. Blood (40uL) was collected at 0, 1, 2, 3, 4 and 6 hours.

6.3.7 Glucose load: For glucose tolerance tests, mice were fasted overnight for 16 hours with water *ad libitum*. Basal blood was taken (80 µL) and mice injected intraperitoneally with a sterile D-glucose solution in saline, 2 mg/gm body weight from a stock solution of 200 mg/ml (0.010 ml/gm body weight). Blood (20 µL) was collected at 15, 30, 60, 90 and 120 minutes, with 80 µL for insulin time points. Insulin was measured at 0, 30, 60 and 120 minutes.

6.3.8 Fecal Analysis: The fat content of the feces was analyzed as described previously with modifications (25). The animals were placed in a chamber with a metabolic screen and feces were collected after 24 hours on normal chow and/or collected for 24 hours after a fat load, with 6 hours fasting, followed by food *ad libitum*. The stool was weighed and dried to constant weight and the fat extracted similar to Schwartz (25).

6.3.9 Statistical Analyses: Results are presented as the mean ± standard error of the mean (sem). The two groups were compared by repeated measures two way ANOVA followed by Bonferroni post test (where indicated), t-test or Pearson correlation using computer assisted analysis (Sigma Stat, Jandel Scientific, San Rafael, CA and Prism, GraphPad San Diego, CA).

6.4 Results

In this study of female C3(+/+) and C3(-/-) mice, growth curves were measured from 4 weeks of age until 26 weeks. Specific diets were fed from the age of 10 weeks on: low fat (LF) (C3(-/-), n=12; C3(+/+), n=8) and high fat diets (HF) (C3(-/-), n=11 ; C3(+/+), n= 6). On the low fat diet (Figure 6.1 left panel), the C3(-/-) mice weighed 10.9±0.5% less ($p<0.0001$ by ANOVA) and this occurred primarily after 9 weeks of age (following puberty) and maturation of the fat pad (6 weeks cited Shimomura (26). The results for the HF diet are shown in figure 6.1 (right panel). Average weight gain in the knockout and wild type mice (4 weeks to 25 weeks) was similar, 3.43±0.60 C3(-/-) and 3.692±0.62 grams/week (12.4% increase C3(+/+) and 12.47% increase C3(-/-) on the high fat vs low fat diet). However there was still a significant decrease in the growth curve in the C3(-/-) *vs* the C3(+/+) of 9.3±0.4% ($p<0.0001$ by ANOVA), which also occurred over the same time period.

In order to determine the cause of this reduced weight, following the 4 months on specific diets, tissues were collected from a subset of both genotypes and diets at 32 and 48 weeks of age. As shown in Figure 6.2, at 32 weeks there were marked changes in the amount of white adipose tissue in specific depots with a 67.7% decrease in LF (left panel) and 50.3% in the HF group (right panel). There were no significant changes seen in liver, kidney, brown adipose tissue or heart tissue weights. This difference in adipose tissue was also observed at 48 weeks on the low fat diet, although to a lesser extent (35.3% $p<0.04$, data not shown). By 48 weeks on the HF diet, the differences between the knockout and wild type appeared to be neutralized.

When the total adipose tissue weight (sum of all 4 adipose depots, excluding brown adipose tissue) is expressed per body weight, the C3(+/+) mice always have significantly greater adiposity on both diets (24.4±0.8% C3(+/+) *vs* 15.4±0.7% C3(-/-) $p<0.002$ on HF diet). Again, at 48 weeks of age the percent adiposity differences were less marked between wild type and knockout.

Leptin levels are highly correlated with adipose tissue mass and were thus measured in these animals at 10, 14 and 26 weeks on the corresponding diets (Figure 6.3). Leptin levels increased with age and even more so with fat content of the diet (as previously published elsewhere (20), (27) and (28)), accurately reflecting increases in

body weight and adipose tissue mass. The leptin levels were significantly reduced in the knockout on both diets (LF, $p<0.01$ ANOVA and HF, $p< 0.0001$ by ANOVA). Leptin levels were also measured at 32 and 48 weeks of age on a subset of mice at the time of dissection. At 32 weeks there were significant differences in leptin in the knockout *vs* the wild type mice on LF diet (11.8±0 *vs* 21.9±6.1 ng/mL) and on HF diet (14.6±2.8 *vs* 38.6±2.0 ng/mL, $p<0.005$) which corresponded with the reduced adipose tissue mass (Figure 6.2). By 48 weeks of age, although the differences were maintained on a LF diet, on the HF diet the differences between C3(+/+) and C3(-/-) were no longer evident, again consistent with the adipose tissue weights. The relationship between body weight and leptin levels (which is an indicator of adiposity) in both the C3(+/+) and C3(-/-) for all mice from 10 weeks to 48 weeks of age was also significantly different. The data shows that although there is a linear relationship between body weight and leptin in both sets of animals ($r^2= 0.85$, slope= 1.08±0.123, n=36 C3(+/+); $r^2 = 0.70$, slope= 1.36±0.095 and n=38 C3(-/-)), for any given body weight, leptin levels are significantly lower in C3(-/-) mice ($p<0.0001$).

Food intake was also measured and food efficiency calculated as caloric intake/g body weight over the 16 week diet period. There was no increase in food intake noted for the C3(-/-) mice on the low fat diet (96.8±1.1 C3(+/+) *vs* 97.9±1.4 C3(-/-) average calories/week) in spite of the decreased body weight in the knockout. On the high fat diet, food intake of the knockout increased markedly, compared to the wild type, after 14 weeks of age and this difference was maintained for the duration of the study (figure 4, left panel). The average caloric intake was 107.4±1.8 calories/ week C3(+/+), *vs* a caloric intake of 125.9±2.3 calories/week in the C3(-/-), $p<0.0001$, an average increase of 17.5±2.1%. When food efficiency was calculated as caloric intake per body weight, this was substantially greater in the knockouts than the wild types on the HF diet (Figure 6.4, right panel) (30.1%±2.2% increase calories/g body weight C3(-/-) *vs* C3(+/+), $p<0.0001$).

To determine if this phenotype could be attributed to a lack of intestinal absorption, fecal fat content was analyzed on a subset of mice on a chow diet equal in fat content to the LF diet. There was no change in the fecal fat weight (5.7±0.5 *vs* 6.1±1.0 on chow diet and 11.3±1.1 *vs* 10.0±0.2 after fat load C3(-/-) *vs* C3(+/+) respectively) or fecal lipid profile on thin layer chromatography (data not shown).

Fasting plasma parameters were measured for to determine if changes were associated with the differences in adipose tissue weights as shown in Table 6.1. There was no significant difference in triglyceride, cholesterol or free fatty acid (FFA) between C3(+/+) and C3(-/-) although in both there was an age dependent increase in cholesterol and decrease in free fatty acids(as noted previously (29), (30). Interestingly, glucose and insulin were significantly decreased in the C3(-/-), as well as the glucose insulin product, compared to the wild types and these differences were more marked in the HF diet. Correlation between insulin and glucose (all ages and diets) indicated that for any given concentration of insulin, glucose levels were always lower in the C3(-/-) $p<0.002$).

The glucose levels were investigated further with a glucose tolerance test (GGT) in a subset of mice at 30 weeks of age (following 4 months on LF of HF diet). The glucose area under the curve increased slightly following 4 months on a high fat diet, but again, there was no significant difference in between the C3(+/+) and C3(-/-) (Table 6.2). What was striking, however, was that the serum insulin in the C3(-/-) mice was markedly lower both fasting (40% HF) (Table 6.1) and following the glucose tolerance test as shown by the insulin area under the curve (Table 6.2).

In the early postprandial period hormone sensitive lipase is inhibited. Both insulin and ASP have been shown to inhibit hormone sensitive lipase (7). We therefore examined the mice for changes in FFA (the early (1hour drop) in plasma FFA and also for other net postprandial increases in FFA) and triglyceride changes postprandially after a fat load of 400 μL of olive oil. In mice on the LF diet there was a significant decrease in the C3(-/-) total triglyceride area under the curve compared to wild type (C3+/+) at 26 weeks of age, although there was no difference at 14 weeks old (Table 6.3). With regards to the FFA, there was no significant difference in fasting FFA. However there was a significant increase in FFA AUC in the C3(-/-) upon transition from a LF to a HF diet (Figure 6.5 left panel) and there was always an increase in postprandial FFA at 14 and 26 weeks of age (C3(-/-) compared to C3(+/+)). As shown in figure 6.5, right panel, there as a consistent drop in FFA at 1 hour in the C3(+/+) mice on both LF and HF diet (-24.0±8.5 %LF and -32.3±11.3% HF) at 4 months on the diet (26 weeks of age). This drop was absent in the C3(-/-) mice (average 1 hour change of 1.2±7.2% LF and 2.4±8.6 HF).

Thus in the female mice significant decreases were observed in body weight, adipose tissue, leptin levels and food efficiency as well as moderate changes in insulin, glucose and fatty acid metabolism.

Table 6.1: Fasting parameters measured in plasma

Diet		Basal Chow	Low Fat	Hi Fat	Low Fat	Hi Fat
Time		10wks	14wks	14wks	26 wks	26wks
N		(6/10)	(6/10)	(10/11)	(6/11)	(9/11)
TG	(+/+)	59.9±4.17	46.8±5.3	44.2±6.1	44.0±3.6	50.0±6.1
	(-/-)	69.6±10.6	41.4±2.7	50.8±5.6	37.5±2.7	45.0±3.1
Chol	(+/+)	56.6±7.0	64.3±7.1	84.8±17.3	79.5±7.3	99.9±11.1
	(-/-)	57.6±3.9	68.8±6.3	65.1±4.3	83.4±6.8	97.3±15.0
FFA	(+/+)	0.91±0.23	0.43±0.06	0.43±0.06	0.37±0.05	0.31±0.06
	(-/-)	1.54±0.41	0.54±0.06	0.37±0.04	0.29±0.03	0.29±0.03
Gluc	(+/+)	105.6±7.3	100.5±8.1	102.5±6.3	100.9±10.0	105.2±7.9
	(-/-)	90.8±3.2*	89.4±6.8	89.4±2.9*	96.1±3.5	102.2±5.8
Ins	(+/+)	0.19±0.03	0.19±0.02	0.19±0.03	0.30±0.04	0.51±0.05
	(-/-)	0.20±0.01	0.21±0.02	0.23±0.02	0.22±0.02*	0.28±0.03*
Glu*Ins	(+/+)	22.9±2.4	19.2±1.7	21.0±3.3	29.2±3.8	54.1±7.9
	(-/-)	18.2±1.6*	18.7±3.2	20.3±1.8	21.7±2.2*	30.7±4.4*

Fasting plasma parameters measured in knockout C3(-/-) and wild type C3(+/+) mice at 10,14 and 26 weeks of age on low fat (LF, 10% Kcal) and high fat (HF,40%Kcal) diets. Data is represented as mean±sem, with significance * at $p<0.05$, comparing between genotypes within the age groups. The abbreviations are TG= triglyceride (mg/dL), Chol= cholesterol (mg/dL), Glu= glucose (mg/dL), Ins= insulin (ng/dL), Gluc*Ins= the product of glucose and insulin. The sample size are displayed at the top of the columns for wild type C3(+/+) and C3(-/-) respectively.

Table 6.2: Postprandial glucose area under the curve (AUC) after a glucose tolerance test

Glucose Tolerance Test	Low Fat		High Fat	
	C3(+/+) (6)	C3(-/-) (1)	C3(+/+) (3)	C3(-/-) (4)
Glucose AUC (mg/dL*hours)	229±8.3	221	252±57	309±29
Insulin AUC (mg/dL*hours)	1.628±0.189	0.769	1.636±0.137	0.781±0.023**

Postprandial glucose area under the curve (AUC) and insulin area under the curve (AUC) after a glucose tolerance test. The data shown is for knockout C3(-/-) and wild type C3(+/+) mice at 30 weeks of age (4 months on diet) for the glucose tolerance test. The diets are low fat (LF, 10% Kcal) and high fat (HF, 40%Kcal). Data is represented as mean±sem, with significance ** $p<0.005$ comparing LF data betwee the mice genotypes. The sample sizes are displayed at the top of the columns for wild type C3(+/+) and C3(-/-) respectively.

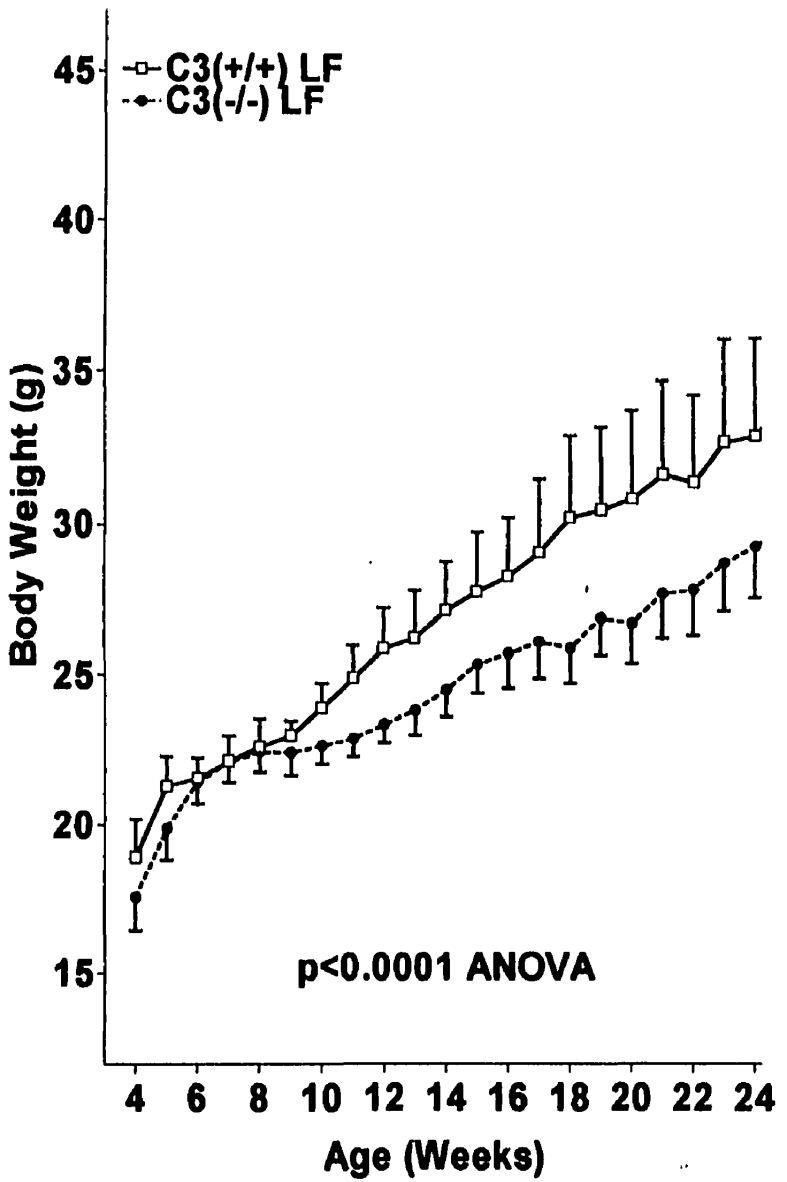
Table 6.3: Postprandial triglyceride area under the curve (AUC) after an oral fat load.

Oral Fat Load	Low Fat		High Fat	
	C3(+/+) (6)	C3(-/-) (10)	C3(+/+) (10)	C3(-/-) (10)
14 weeks old	441.4±62.5	418.8±91.1	476.8±78.3	564.5±105.3
26 weeks old	563.1±76.8	330.2±22.0**	652.2±113.8	553.6±56.1***

Postprandial triglyceride area under the curve (AUC) after an oral fat load of 400 μL olive oil described in Methods. The data shown is for knockout C3(-/-) and wild type C3(+/+) mice at 28 weeks of age (4 months on diet). The diets are low fat (LF, 10% Kcal) and high fat (HF, 40%Kcal). Data is represented as mean±sem, with a significant difference between mice comparing C3(+/+) and C3(-/-) mice on low fat and within C3(-/-) upon transition from low to high fat diet shown ** as $p<0.002$ and *** $p<0.001$. The sample sizes are displayed at the top of the columns for wild type C3(+/+) and C3(-/-) respectively.

Figure 6.1. Reduced growth curves in female knockout mice on both diets

Knockout C3(-/-)mice (filled circles, dotted line) and wild type C3(+/+)mice (open squares, solid line). on low fat 10%Kcal (left panel, LF) and high fat 40% Kcal diet (right panel,HF) from 4 weeks till 26 weeks of age. The diets were started at 10 weeks of age and mice were weighed once weekly. The data is represented as mean±sem with C3(-/-) n=12 and n=11 and C3(+/+) n=8 and n=6.


C3(+/+) LF
C3(-/-) LF
Body Weight (g)
45
40
35
30
25
20
15
p<0.0001 ANOVA
4 6 8 10 12 14 16 18 20 22 24
Age (Weeks)

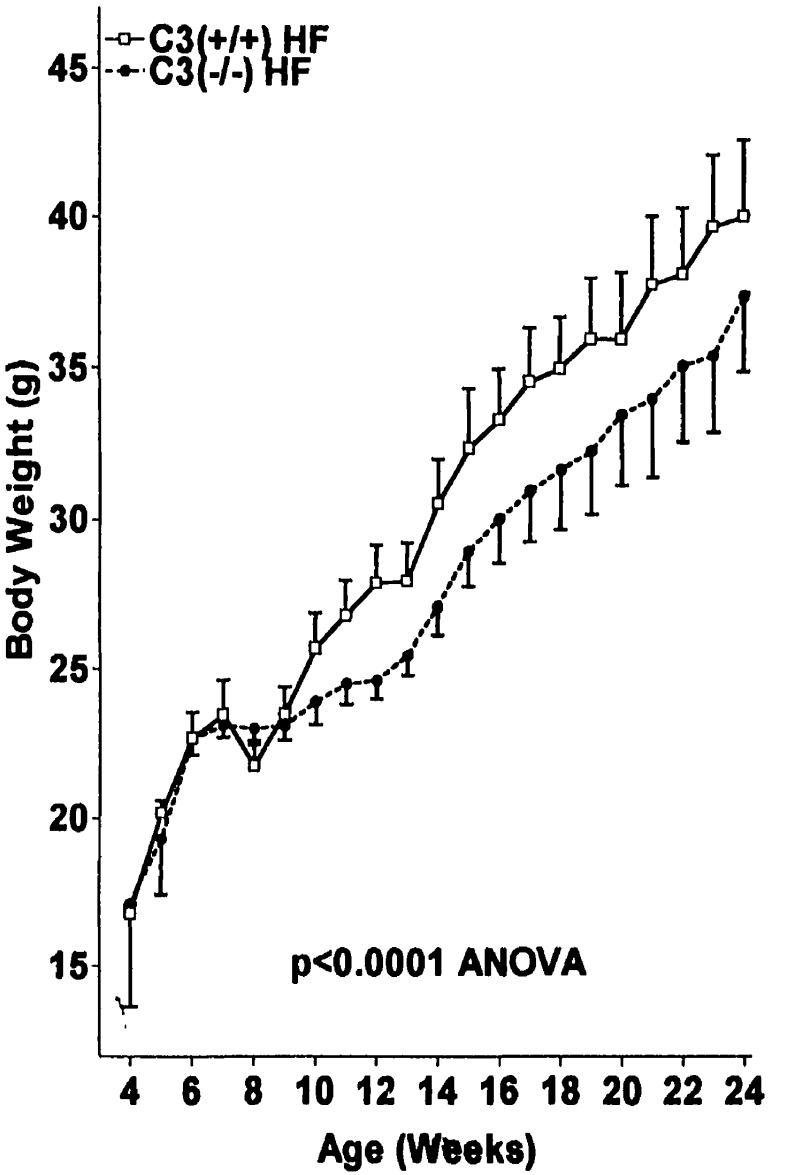
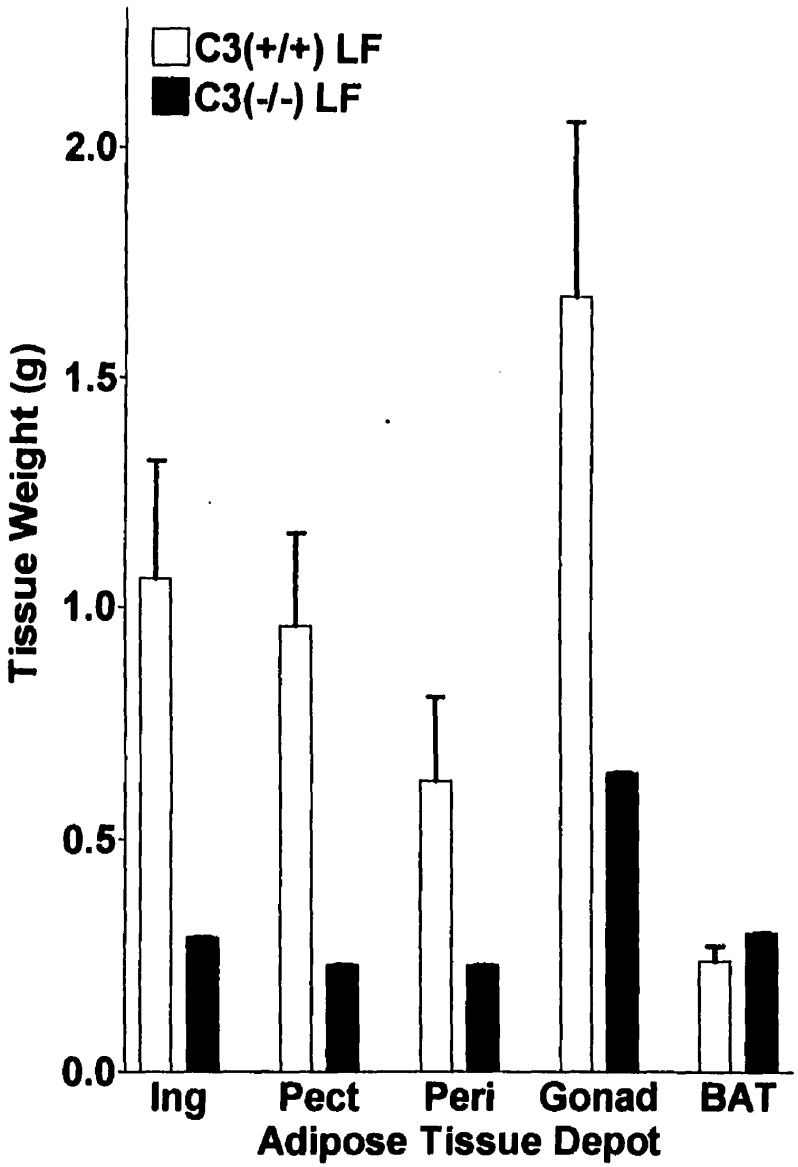

C3(+/+) HF
C3(-/-) HF
Body Weight (g)
45
40
35
30
25
20
15
p<0.0001 ANOVA
4 6 8 10 12 14 16 18 20 22 24
Age (Weeks)

Figure 6.2. Reduced adipose tissue weights in female knockouts on both diets

The adiopose tissue weights of female knockout C3(-/-) mice (filled bars) and wild type C3(+/+) mice (open bars) on a low fat 10% Kcal diet (left panel, LF, C3(-/-) n=1, C3(+/+) n=5) and a high fat 40% Kcal diet (right panel, HF, C3(-/-) n=4, C3(+/+) n=4). Adipose tissue was removed from mice at 32 weeks of age and the data is represented as mean±sem. The abbreviations for the adipose tissue depots are Ing= inguinal, Pect= pectoral, Peri= perirenal, Gonad= gonadal and BAT= brown adipose tissue. The symbols ** and * represent a significance level of $p<0.001$ and $p<0.05$ respectively.


C3(+/+) LF
C3(-/-) LF
Tissue Weight (g)
0.0
0.5
1.0
1.5
2.0
Ing
Pect
Peri
Gonad
BAT
Adipose Tissue Depot

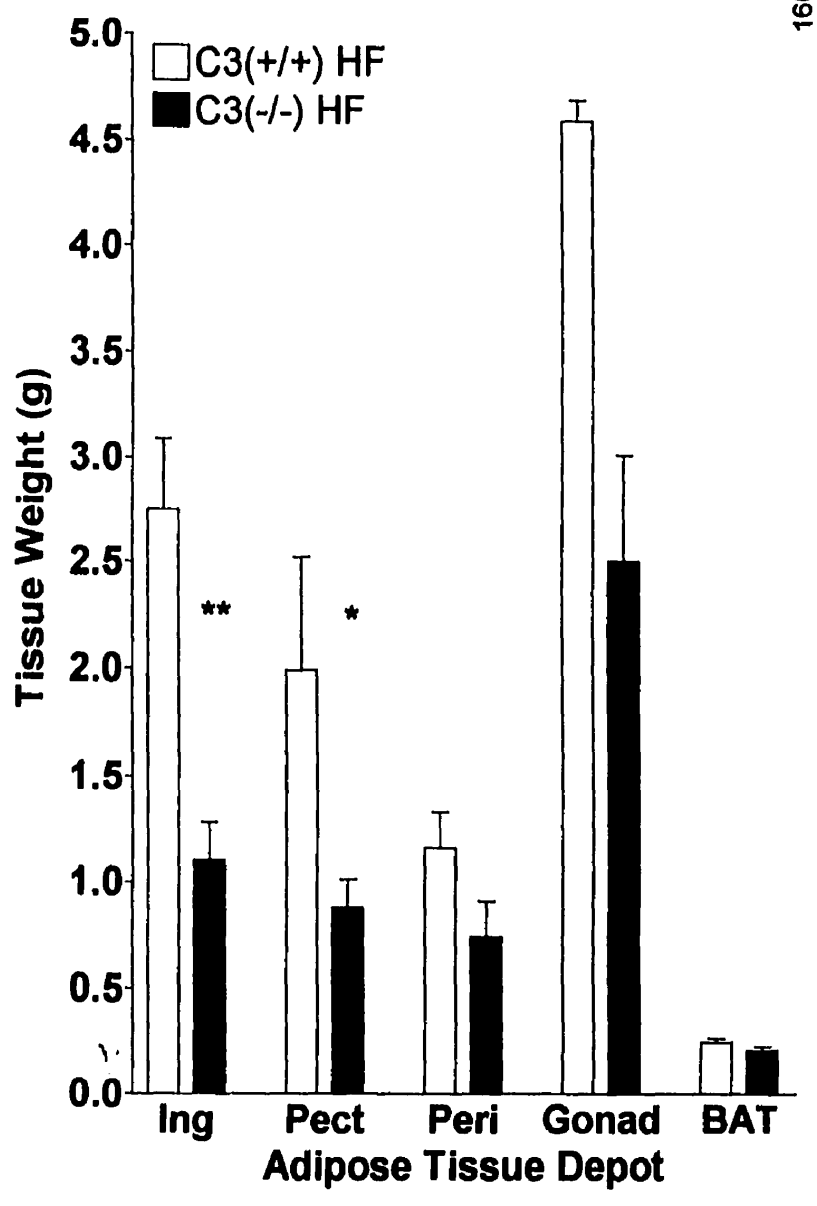
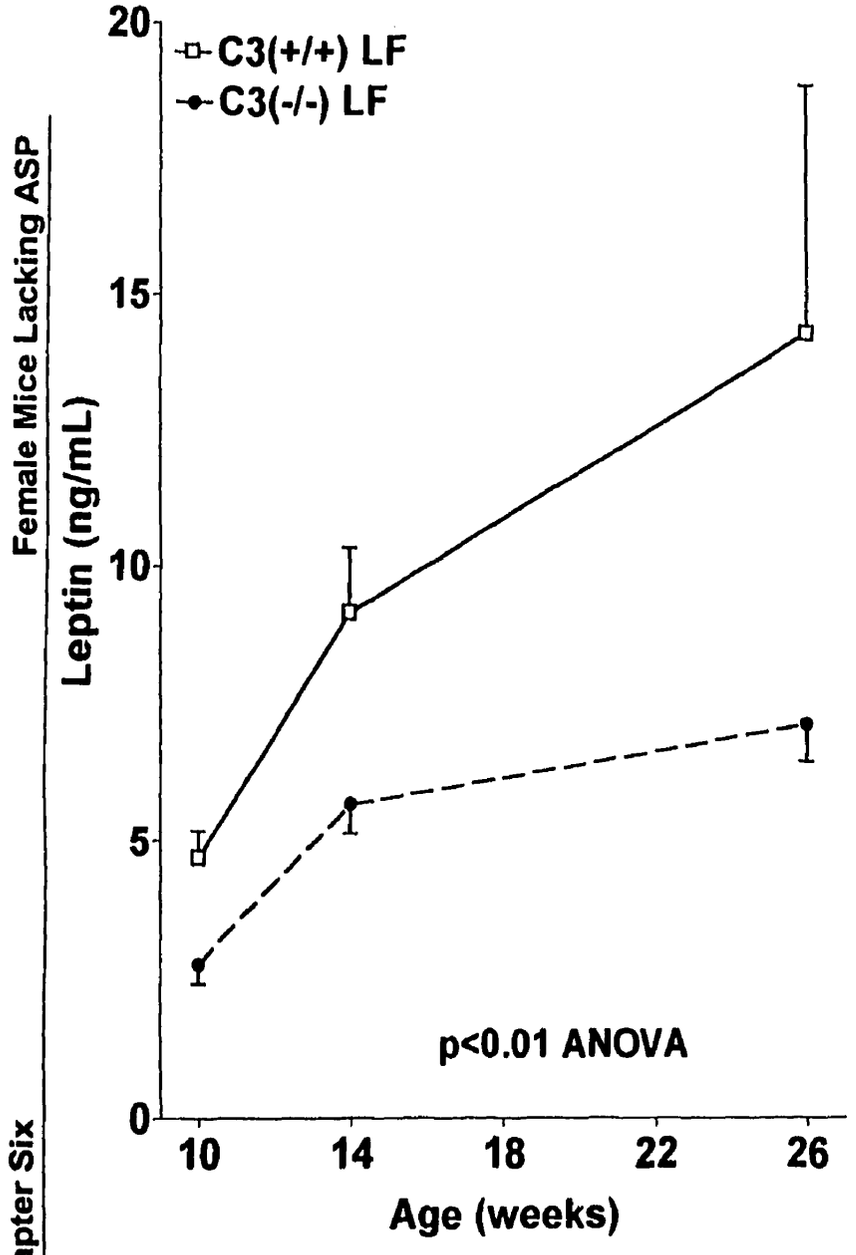

C3(+/+) HF
C3(-/-) HF
Tissue Weight (g)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
**
*
Ing
Pect
Peri
Gonad
BAT
Adipose Tissue Depot

Figure 6.3. Plasma leptin levels are lower in the female knockout on both diets

Plasma leptin levels at 10, 14 and 26 weeks for mice on the low fat (LF) and high fat (HF) diets are shown in the left and right panels respectively. The sample sizes are n= 5 and n=3 for the knockout C3(-/-) and n=4 and n=4 for the wild type C3(+/+) on the low and high fat diets respectively.


C3(+/+) LF
C3(-/-) LF
Leptin (ng/mL)
20
15
10
5
0
10
14
18
22
26
Age (weeks)
p<0.01 ANOVA

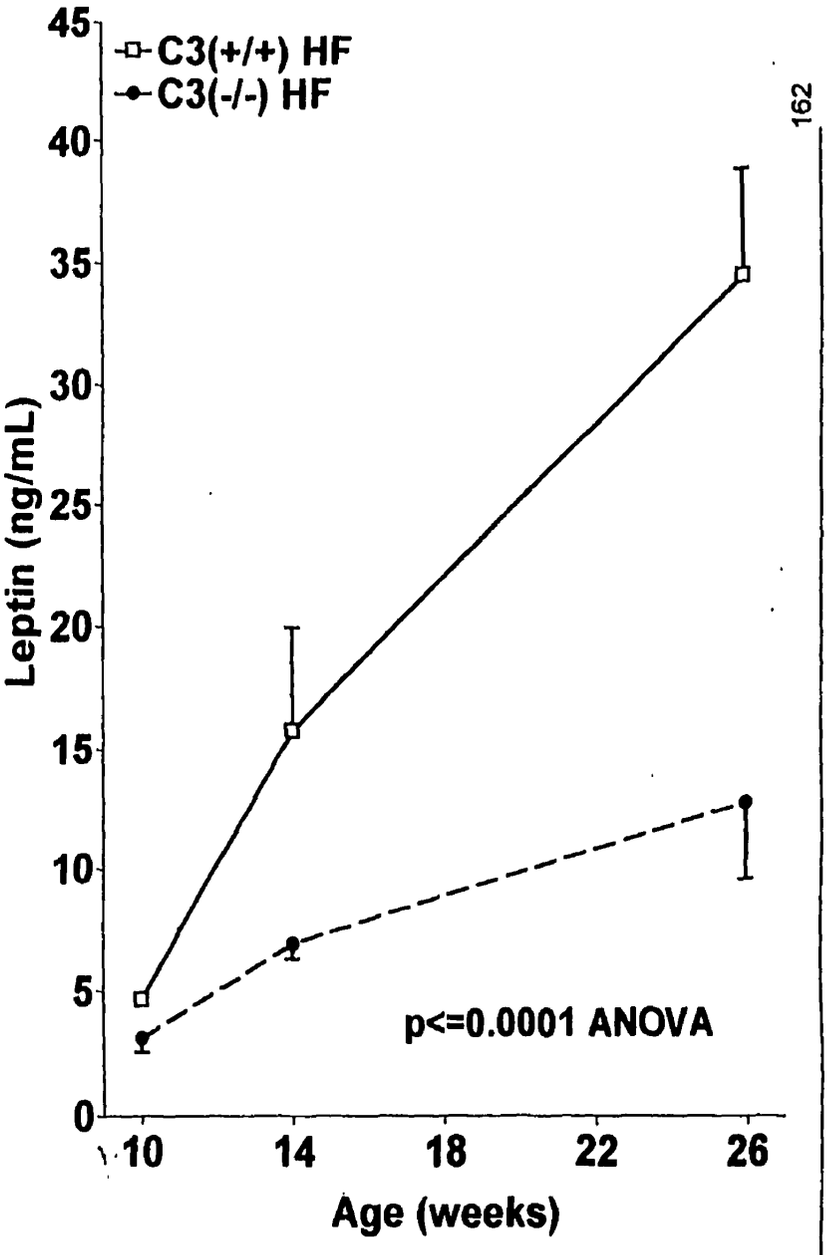
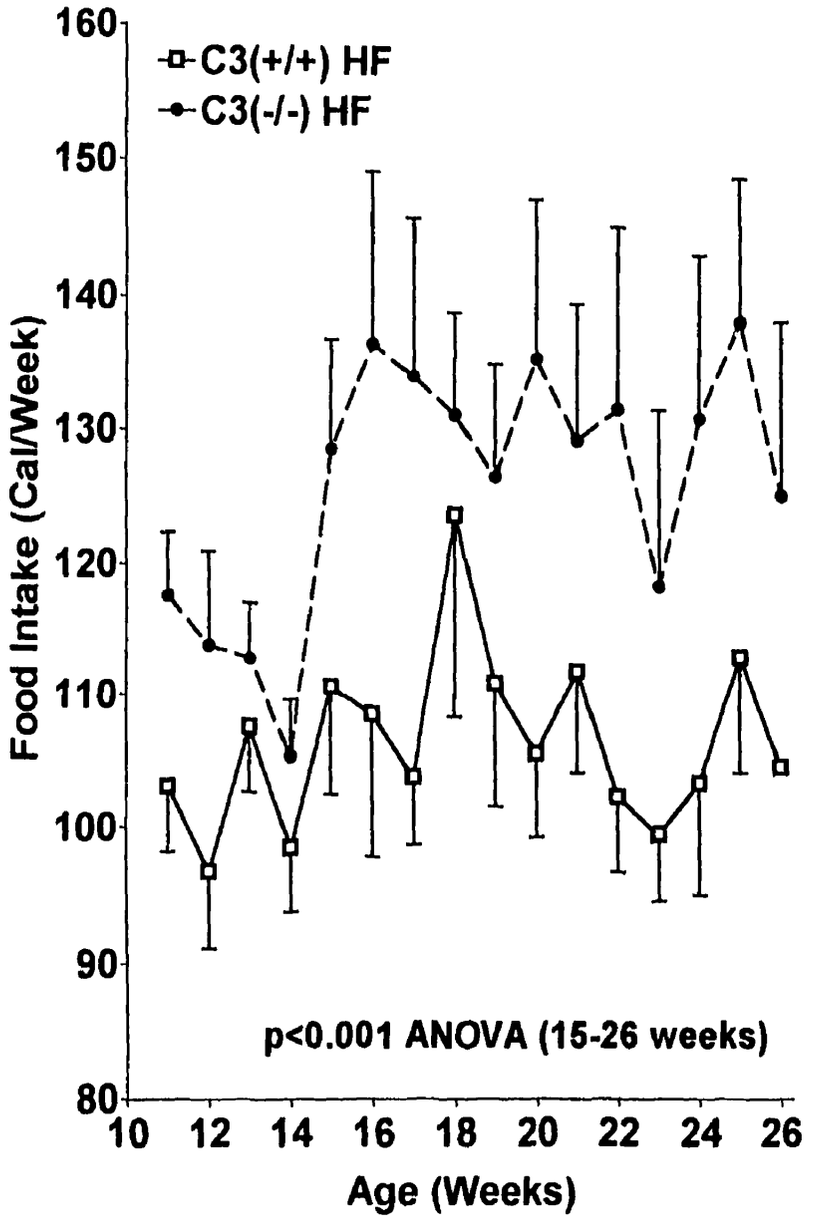

C3(+/+) HF
C3(-/-) HF
Leptin (ng/mL)
45
40
35
30
25
20
15
10
5
0
10
14
18
22
26
Age (weeks)
p<=0.0001 ANOVA

Figure 6. 4. Food intake and food efficiency for mice on a high fat diet

Food intake and food efficiency for the mice on high fat diet are displayed in the left and right panels respectively. The symbols and legends as described in figure 6. Food efficiency is calculated as calories food intake per week/body weight in grams.


C3(+/+) HF
C3(-/-) HF
Food Intake (Cal/Week)
160
150
140
130
120
110
100
90
80
p<0.001 ANOVA (15-26 weeks)
10 12 14 16 18 20 22 24 26
Age (Weeks)

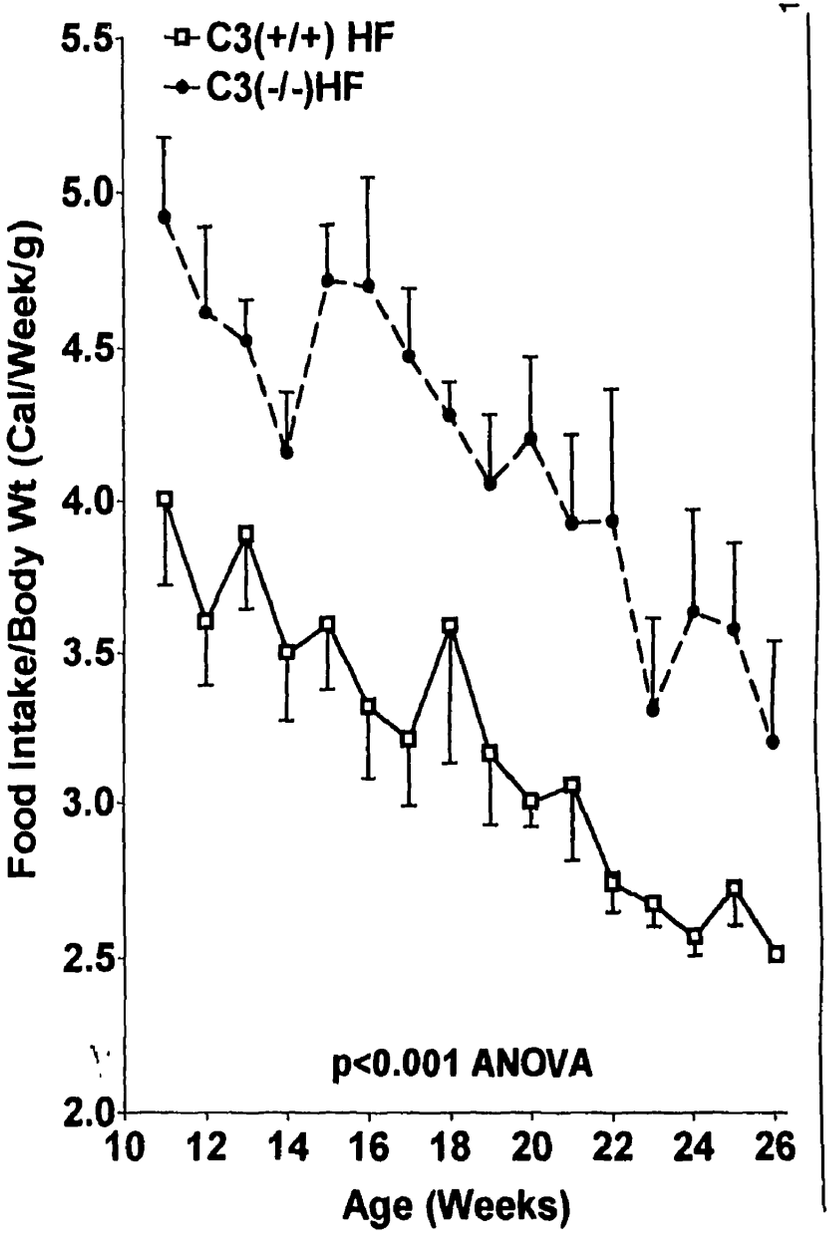
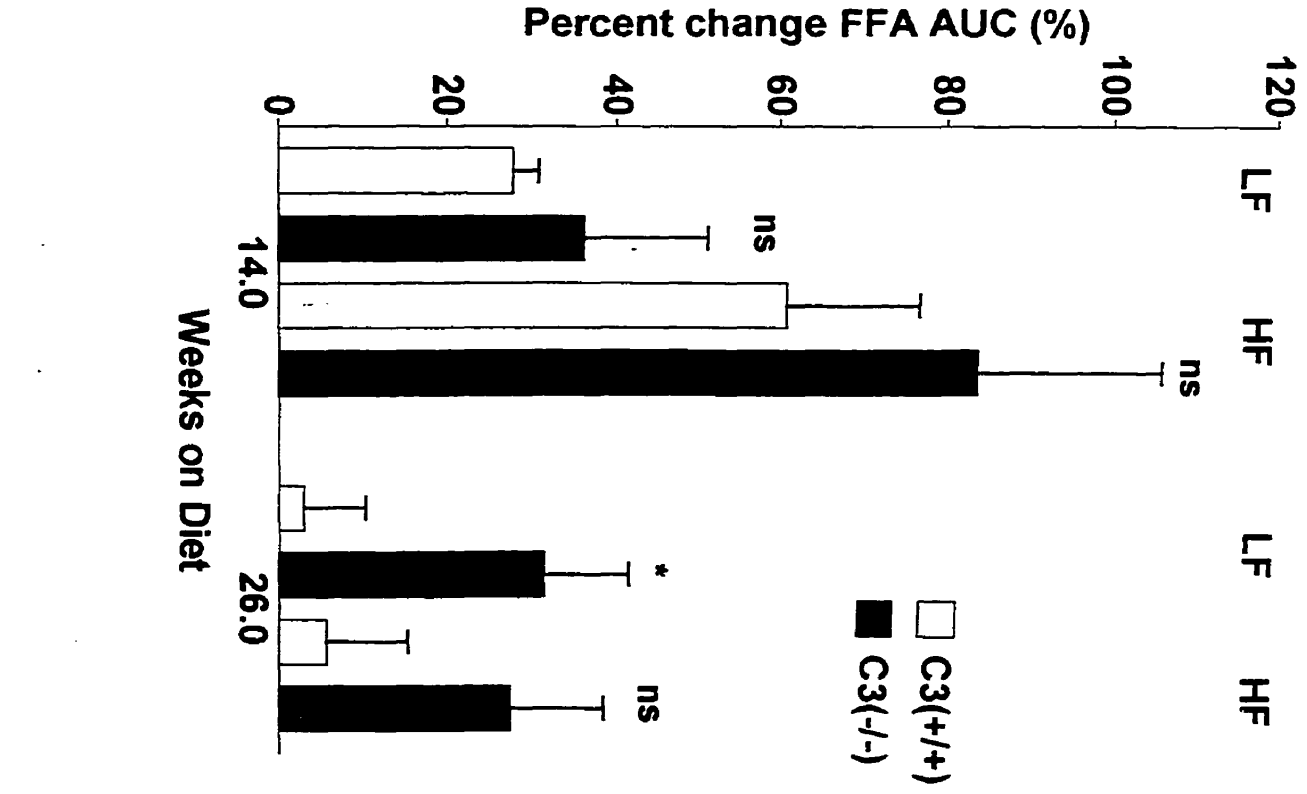
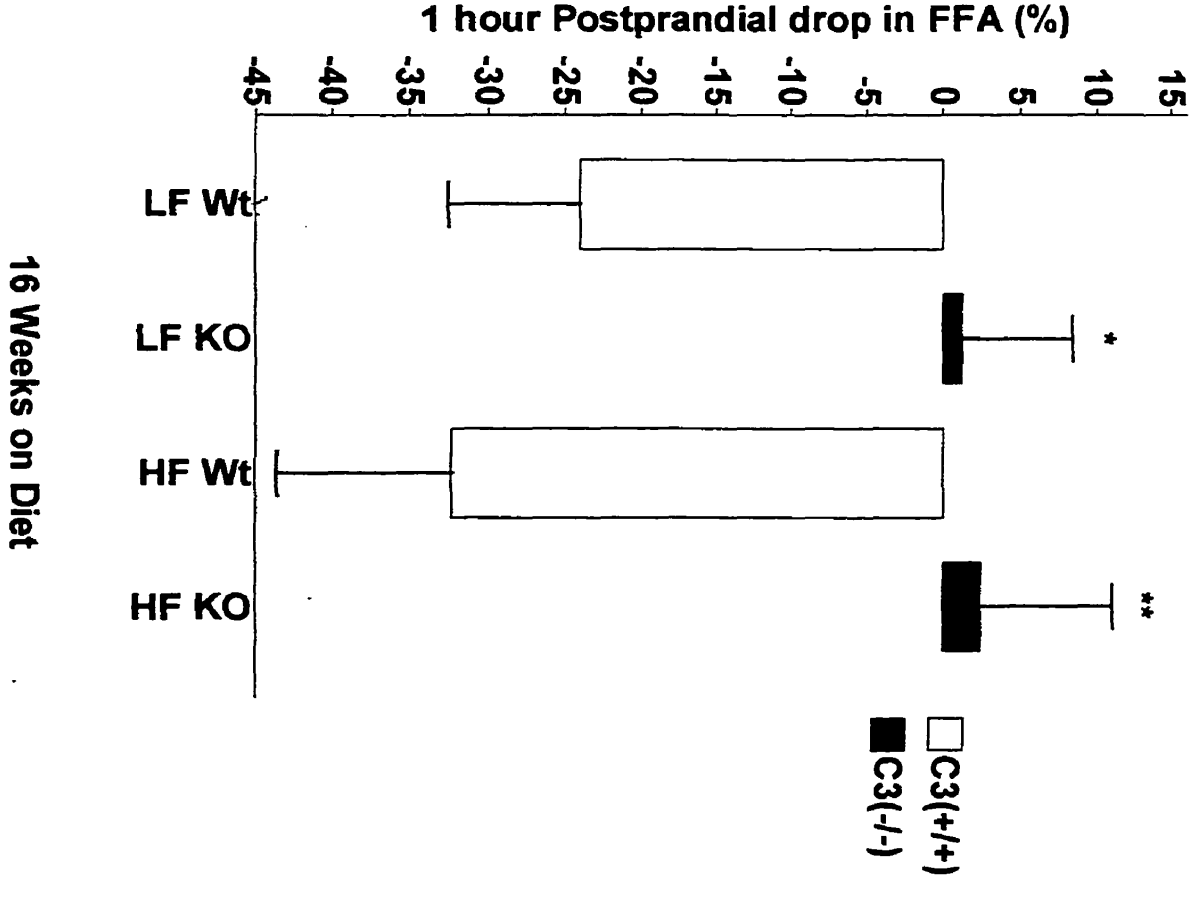

C3(+/+) HF
C3(-/-)HF
Food Intake/Body Wt (Cal/Week/g)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
p<0.001 ANOVA
10 12 14 16 18 20 22 24 26
Age (Weeks)

Figure 6.5.Increases in area under the curve (AUC) and 1 hour change postprandially after an oral fat load.

Percent increase in area under the curve (AUC) for free fatty acids after an oral fat load (left panel) or change at 1 hour postprandially (right panel). Data is shown in knockout (C3(-/-), solid bars) and wildtype (C3(+/+), open bars) mice on low fat (10%, LF) and high fat (40%, HF) diets. The number for mice is the same as reported for mice at 26 weeks of age in Table 6.1.


Percent change FFA AUC (%)
0
20
40
60
80
100
120
LF
HF
LF
HF
ns
ns
*
ns
14.0
26.0
Weeks on Diet
C3(+/+)
C3(-/-)


1 hour Postprandial drop in FFA (%)
-45
-40
-35
-30
-25
-20
-15
-10
-5
0
5
10
15
LF Wt
LF KO
HF Wt
HF KO
*
**
16 Weeks on Diet
C3(+/+)
C3(-/-)

6.5 Discussion

The size of adipose tissue is regulated by the efficiency of triglyceride synthesis and storage, as well as through inhibition of lipolysis *via* hormone sensitive lipase. ASP appears to affect both of these processes *in vitro*, and the present studies support a role for ASP in these functions *in vivo*. In these studies, the lack of ASP in female mice resulted in a decreased body weight and adipose tissue, implicating ASP in regulation of body and adipose tissue size. This decrease in adiposity could be due to a reduction in gastrointestinal absorption but malabsorption in this study was ruled out, as there were no changes in the lipid mass or species in the fecal analysis. Since the delivery of fat into the system is normal and postprandial lipemia is the same in C3(+/+) and C3(-/-), the reduction in adipose tissue size could be a consequence of reduced storage of dietary triglycerides (through effects on esterification enzymes) or enhanced lipolysis. The consequences of lack of ASP are consistent with changes in both, since ASP is able to affect each process.

There are similarities between male and female mice lacking ASP, the knockouts. In both there are increases in postprandial FFA and reduced glucose levels as compared to the wild type. This trend of increased postprandial FFA, we would hypothesis be due to reduced fatty acid trapping. The reduced fatty acid trapping may lead to general disturbances in the fatty acid metabolism. In one study, where the delivery of FFA to the peripheral tissues was disrupted with a targeted knockout of LPL, fasting glucose was lower and found in this extreme case to lead to neonatal death (31). It was proposed that reduced FFA tissue delivery and utilization resulted in the lower glucose levels (31). Thus reduced fasting glucose levels in our model may be due to a similar cause, reduced FFA utilization and tissue delivery.

We postulated that a lack of fatty acid trapping would result in FFA inhibition of LPL. However, it appears as though females are resistant to postprandial lipemia, where the knockout (lakcing ASP) and the wildtype mice do not display increased lipemia as seen in the male mice. In a study in rats it was found that this increased triglyceride clearance was due to increased mass and activity of LPL (32). This illustrates the multiple-step process of chylomicron clearance, with triglyceride hydrolysis followed by FFA uptake. If the LPL levels are in excess, as presumed in the females, then even in the

presence of elevated FFA and reduced fatty acid trapping, triglyceride hydrolysis is normal. The lack of postprandial lipemia in our study, and indeed an increase in efficiency of clearance in the knockout at 26 weeks of age, would indicate a mechanism leading to increased clearance.

In the females the loss of adipose tissue is more marked than that in the males while postprandial elevation of triglycerides. In the male knockout is more marked. There are gender related differences noted in insulin levels, with the males insulin levels being higher in both wild type and knockout males. This gender difference in insulin has also been noted previously in mice (33), (30). This increased insulin sensitivity in the females may result in enhanced activity of LPL and explain the absence of postprandial lipemia as compared to the males.

The reduction in both fasting insulin and glucose levels and the reduced insulin in the glucose tolerance test would suggest increased insulin sensitivity. This was especially pronounced on a high fat diet, where there was marked protection against increased insulin levels as compared to the C3(+/+). Hyperinsulinemia was absent in rats that failed to gain weight on a high fat diet (cited (34)) and it was suggested that an increased body weight rather than direct caloric intake might be responsible (34). The reduced body weight and adipose tissue in our animals, in tandem with reduced insulin levels in the C3(-/-) mice would fit with this hypothesis. These mice would appear to be resistant to high fat diet induced hyperinsulinemia. Further experimentation is required and is currently underway.

The lack of ASP also resulted in marked decreases in leptin levels. This decrease was at least partly due to decreases in adipose tissue, however the decreases were disproportionate to (and greater than) the decreases in body weight alone would appear to account for. Thus ASP may have a direct (unknown mechanism) or indirect role in leptin secretion, *via* changes in glucose or insulin, since both have been shown to have a positive effect on adipocyte leptin secretion (35), (36), (37) respectively. It is possible that the decreased leptin levels are an attempt to increase the adipose tissue mass. There was certainly a reduction in food efficiency (calories/g body weight) for a given food intake evidenced as decreased body weight on a low fat diet, and hyperphagia on a high fat diet. Females may control or protect their body fat more closely than males (38), in order to

stay within a certain adiposity range for reproduction (39), cited (40). They appear to have substantially more body fat that do males, even in wild type mice (12.7% female *vs* 10.53% males, unpublished data), (41).

There are other instances of weight loss in murine models exhibiting moderate (42), (43), (44) or dramatic losses in adipose tissue (45), (26), (46). In the mice with complete loss of adipose tissue they demonstrated increased levels of insulin, glucose and decreased leptin (46), (45). With complete lack of adipose tissue the fatty acids levels did not change (45) or increased (26). With moderate losses of adipose tissue (similar to our mice) there was little changes from normal in fasting plasma cholesterol, triglycerides, fatty acids, insulin and glucose noted (42). Interestingly, in the aP2/diptheria toxin white adipose tissue (WAT) ablation mouse (45), adipose tissue reduction only developed post maturation of the fat pads (after 8 weeks of age) similar to our C3(-/-) murine model, suggesting that it is at the point of puberty and adipose maturation that these differences become apparent. In the protein kinase A subunit knockout (42), females were predominantly affected as compared to males and displayed hyperphagia (42). Our mice demonstrated hyperphagia on a high fat diet, presumably due to reduced leptin levels, that was nonetheless unable to restore adiposity to the level of the wild type C3(+/+) mice. This was also observed in both the protein kinase A subunit knockouts (42) and WAT ablation mice (45).

Adipose tissue loss in humans can also produce metabolic consequences similar to those seen in the C3(-/-). In post-obese women there appears to be a drive to regain body weight and re-establish the set point of adiposity. Increased LPL activity was observed (44) and in some studies no changes in insulin sensitivity (47) or improved sensitivity were reported with weight loss (48). Lowered insulin levels (49), enhanced triglyceride clearance (unpublished observations), reduced glucose levels (50), reduced adipose tissue mass and reduced serum leptin (51), (52) have been observed. In many ways, the description is similar to our C3(-/-) mice.

Human genetic C3 deficiency is extremely rare with only 20 cases reported (53). Wetsel et al indicated that these "C3-deficient individuals do not appear to display increased predisposition to hyperlipidemia or coronary artery disease" (54). In the review cited (53), no lipid or lipoprotein data is cited. Unfortunately the majority of the studies of

any detailed lipid and/or lipoprotein analyses on patients with C3 deficiency. Complement C3 deficiencies are commonly associated with membranoproliferative glomerulonephritus (MRG), partial liposytrophy (PLD) and at times with systemic lupus erythematosus (SLE) symptoms (reviewed (55), (56), (57), (53). A human case of C3 deficiency has been reported with partial lipodystrophy (58) and reduced C3 levels have been frequently associated with partial lipodystrophy (PLD) (55).

The reduced adiposity in the female C3 knockout mice resembles the phenotype of acquired partial lipodystrophy seen in humans. Partial lipodystrophy may be classified as congenital or acquired. The acquired form affects both males and females. It is associated specifically with hypocomplementemia of complement C3 (55) with enhanced catabolism as well as reduced biosynthesis of C3 (59). This enhanced catabolism is the consequence of the presence of a C3 "nephritic factor" which acts as an auto-antibody, stabilizing the C3 convertase against spontaneous decay, enhancing C3 breakdown and clearance (60), cited (56), thus specifically affecting the proximal portion of the alternate complement pathway. In a review of 21cases with acquired partial lipodystrophy 80.9% demonstrated reduced complement C3 concentrations (55).

Our murine model demonstrates similarities to humans with acquired partial lipodystrophy as evidenced by depleted C3 levels and reduced adipose tissue. In our model we do not see the hyperlipidemia, hyperinsulinemia or abnormal glucose tolerance as reported for hereditary PLD (61), (62), (63)

In summary, ASP has been shown previously to play a role in postprandially in both humans (14) and mice (16). The present study demonstrates the effects of a lack of ASP on adipose metabolism in female mice. In the future, ASP may be a potential pharmacological target for reducing obesity in females.

6.6 References

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7.1 Summary

Acylation Stimulating Protein (ASP) regulates the entry and release of FFA into adipocytes *via* stimulation of triglyceride synthesis and inhibition of lipolysis. (60). Increased esterification of intracellular fatty acids into triglyceride results in an alteration of the FFA concentration gradient and increased influx of FFA. The effects of ASP on hormone sensitive lipase also reduce the efflux of FFA from the cell.

Adipocytes produce the three factors required for the production of the ASP (factor B, D/adipsin and Complement C3) (141). However adipocytes do not synthesize the proteins involved in the later part of alternate complement system (117) which would lead to cell lysis and death.

Although we have concentrated on adipocytes to date, the effects of ASP are not restricted to adipocytes since ASP is able to stimulate glucose transport and triglyceride synthesis in other cell lines such as fibroblasts and myotubes.

Utilizing recombinant ASP, we were able to rule out the possibility that the effects we have found with ASP purified from plasma were due to a contaminant. Furthermore, we have shown that the activity of ASP in stimulating triglyceride synthesis and glucose transport is due to specific cell surface binding.

We have proposed a two-site interaction of the ligand with the receptor based on studies which determined regions of the molecule responsible for function. They revealed that the carboxy-terminal is implicated in cell surface binding and the disulphide linked core in stimulating triglyceride synthesis. It was determined from the structure-function studies that entire molecule was required for functionality.

The effects of ASP on lipid synthesis contrast with its immunological inactivity in myeloid cells. By contrast complement C3a, with only a carboxy-terminal arginine difference, possesses immunological activity. We have confirmed the differences between ASP and C3a, where C3a is able, and ASP unable, to stimulate calcium release from a macrophage cell line. Subsequently it has been shown that ASP is unable to bind to the recently cloned C3a receptor

(unpublished observations), (135). It is possible that the receptors large second extracellular loop determines specificity of binding between C3a and C3a des Arg as 65% of the receptors loop can be removed before interfering with C3a binding (142).

Since the entire molecule would be required for bioactivity, the entire molecule was used for *in vivo* studies in mice. *In vivo* studies were performed to determine the effects of short-term administration of exogenous ASP to mice and the long-term effects of a lack of ASP in functional ASP knockout mice. The rational for such studies stemmed from the *in vitro* activity of ASP. The hypothesis was that ASP would cause an increase in dietary lipoprotein (chylomicron) clearance, reduced plasma glucose levels and increase body weight and adipose tissue mass. The mechanism for triglyceride clearance was that increased adipocyte fatty acid esterification would lead to local decreases in fatty acids, resulting in less FFA inhibition of LPL activity and increased triglyceride hydrolysis. Decreased glucose plasma levels was postulated to result from increased glucose transport. Long-term changes in adipose tissue mass would be due to the overall actions of ASP in increasing triglyceride synthesis and inhibiting lipolysis.

Administration of ASP into C57Bl/6 mice, during a fat load, allowed insight into the contribution of the molecule to postprandial metabolism of triglyceride as well as glucose. This data provides proof of concept that ASP is able to alter plasma triglyceride clearance and glucose levels in accordance with hypotheses generated based on its *in vitro* functionality. Furthermore the data indicate that ASP action on plasma glucose levels occur without a concurrent change in plasma triglyceride clearance.

We have postulated that ASP increases FFA clearance. This is supported by the knockout data, where a lack of ASP in these mice results in delayed postprandial FFA clearance. This action of ASP on FFA clearance is not apparent when Black 6 mice were injected with exogenous human ASP. The mice receiving ASP had accelerated TG clearance, but no reductions in FFA. The fact that this rapid hydrolysis of TG does not lead to elevated FA supports the

hypothesis that ASP increases FFA clearance. Thus the FFA plasma pool concentration does not accurately reflect what is occurring in the capillary microenvironment where FFA uptake or trapping is occurring. The microenvironment uptake of chylomicron derived FFA is sufficiently rapid to prevent equilibrium with the total FFA plasma pool. However, with a lack or reduction of ASP (knockouts and Black 6 mice at 3-6 hours postprandially), FFA is not as efficient, allowing for greater equilibrium with the FFA plasma pool. The result is elevated plasma FFA levels.

Studies in the knockout mice enabled us to determine the effects of a lack of ASP. The lack of ASP in male knockouts, resulted in elevated postprandial triglycerides whereas an excess of exogenous ASP reduced postprandial triglycerides in knockout males and C57Bl/6 females. This confirms the role of ASP in postprandial triglyceride clearance. Further evidence towards our hypothesis that ASP plays a role in fatty acid trapping, there was a trend to elevated postprandial FFA area under the curve (in the females) and significant elevation of postprandial FFA in preliminary studies in male knockout mice on high fat diets. The postprandial effect on triglyceride clearance is presumed to be secondary to ASP effects on FFA.

Although both males (preliminary studies) and females exhibited reduced adiposity and postprandial triglyceride clearance, sexual dimorphism was observed with regards to the degree of penetrance of the phenotype. Markedly reduced adiposity was seen in the females and decreased postprandial triglyceride clearance seen in the males. This initial difference, in wild types, may be due to the reduced total adiposity in the males as compared to the females, or decreased efficiency of adipose FFA uptake. In the absence of ASP this difference between males and females becomes more marked. The adipose tissue mass may also partially explain the increased efficiency of triglyceride clearance in the females as compared to the males. In wild type females, total adiposity is greater than that of males (unpublished observations) and the adipose tissue may function as a buffer to reduce plasma fluctuations in triglyceride and FFA levels. However, the knockout females have adiposity that is

comparable to the males and triglyceride clearance is still enhanced. This indicates that there is either increased efficiency of the adipose tissue or ancillary mechanisms present.

Insulin sensitivity, measured in the females and preliminary studies in the males, may be the answer. Fasting insulin levels are lower in female mice as compared to males and for glucose tolerance tests are markedly lower, even compared to the female wildtype. This indicates greater insulin sensitivity. Thus due to insulin's ability to increase triglyceride synthesis, glucose transport and reduce lipolysis, adipose tissue in females may be more efficient in triglyceride clearance.

We are left however with a paradox. Increased insulin sensitivity, while proposed to enhance triglyceride clearance between the sexes, should also prevent adipose tissue mass reductions as seen in the female knockouts. While the triglyceride clearance appears normal in female mice, deficiency of FFA uptake occurs, becoming more pronounced with age. Thus while insulin, in the absence of ASP, appears to compensate for triglyceride clearance (presumably *via* modifying LPL levels) it is unable to do so for intracellular triglyceride synthesis, resulting in reduced adipose tissue mass.

In addition to ASP playing a role in triglyceride clearance it also plays a role in glucose metabolism. From the *in vitro* studies, the effect of exogenous ASP on glucose uptake is presumed to be direct and independent of the ASP effect on triglyceride clearance (143). In the knockout mice, without ASP, glucose levels were reduced compared to the wild type, contrasting decreased glucose levels seen with exogenous ASP. In the knockout, lacking ASP, this may be due to an increased use of glucose because of reduced fatty acid trapping in tissues, compared to the direct effects of exogenous ASP on glucose uptake. The theory of lack of fatty acid uptake resulting in increased glucose utilization was proposed for LPL knockout mice, which have lower glucose levels and defective hydrolysis of triglyceride to FFA (6). Although the glucose tolerance tests did not display any differences between female knockout and wild type, the glucose levels observed

are a combination of both utilization and hepatic release. Further experiments to differentiate between the two are underway.

Although we have focused on the effects of ASP on triglyceride hydrolysis hypothesized to resulting from reduced inhibition of LPL with increased intracellular FFA trapping, other processes could be affected. The presence and clearance of chylomicron triglyceride is a multi-step process requiring the following:

-Chylomicron formation within the intestine
-Binding of chylomicrons to LPL and triglyceride hydrolysis
-FFA release, uptake and esterification into cells
-Chylomicron remnant removal by the liver

We presume that ASP does not affect chylomicron formation since the rate of chylomicron plasma appearance was not altered by ASP injection. At present, it is unknown if ASP has direct effects on LPL secretion and activity, similar to insulin, or effects on remnant removal.

In humans a downstream effect of an inefficient ASP pathway and reduced FFA trapping is hyperapoB (hyperlipidemia). In humans with visceral obesity, elevated FFA delivery to the liver leads to elevated secretion of VLDL from the liver, therefore resulting in hyperapoB.

Thus lack of ASP produce delays in FFA clearance and increases in FFA delivery to the liver are associated with increased hepatic lipoprotein production. There may be other causes of FFA disturbances such as alterations in fatty acid binding protein and enzymes involved in FFA storage. It should be noted that increased knowledge of regulation and control of the intracellular FFA pathway and FFA uptake would shed light on hyperapoB and ASP. For example FABP could alter the effective transmembrane FFA concentration and the cytosolic or metabolic distribution of FFA may counteract the effects of a lack of ASP. Presently the ASP affects on these transporters or FABP are unknown. Recent developments such as isolation of specific fatty acid transporters in adipocytes (36), fatty acid binding proteins (FABP) and the cloning of the triglyceride synthetic molecular DGAT (128) will certainly add to the field. Indeed DGAT

cloning would allow for utilization of molecular tools to determine the specific mechanisms of ASP action on DGAT, the intracellular FFA pathway and hyperapoB.

Although increased plasma FFA levels in humans leads to hyperapoB, this does not appear to be true in our mice. A lack of ASP, in the complement C3(-/-) knockout mice, does not lead to hypertriglyceridemia (144) or hyperapoB (145). Indeed in several studies, genetic obesity or high fat diets do not result in increased plasma triglycerides (76), (146), (69) nor changes in apoB (68), although changes in apoB may be mouse strain dependent (146). However, the theory that increased delivery of FFA to the liver results in increased VLDL secretion has been supported by recent evidence in mice. Mice expressing LPL only in the liver resulted in increased liver triglyceride clearance and increased VLDL production (6).

The lack of elevated fasting triglycerides in our mice or hyperapoB (145), as compared to humans, indicates that additional factors may be necessary to produce a hyperapoB phenotype in mice. Factors such as the environment or diversion of FFA to the liver may play a major role for this difference between mice and man. FFA regulation and tissue uptake in mice appears different than that in humans or the environmental factors leading to hyperapoB are absent, namely a high fat, sucrose and cholesterol diet. In regards to diet, hypertriglyceridemia has been observed in mice placed on a high sucrose diet (147) and perhaps sucrose is also required, in addition to a high fat/cholesterol diet for a hyperapoB phenotype in mice.

Fat partitioning between tissue may also result in increased FFA delivery to the liver in human than in the mice. Preliminary results during a fat load with radiolabelled oleate involving injection of ASP into male knockout mice resulted in greatest counts in the brown adipose tissue followed by the liver (unpublished observations). Less label was seen in the muscle and white adipose tissue (unpublished observations), the tissues supposedly important in postprandial triglyceride clearance (17). This would presumably lead to less FFA delivery to the liver and may explain the lack of hyperapoB seen in our model.

In conclusion, we have demonstrated that recombinant Acylation Stimulating Protein (ASP) is competent to stimulate triglyceride synthesis and glucose transport and confirmed previous reports of ASP's immunological inactivity. We have defined structural regions of the molecule responsible for the functionality of ASP. Finally we have demonstrated *in vivo* functionality of ASP in murine models with an excess and absence of ASP.

7.2 Future Work

In light of these discoveries and insights several future experiments can be envisioned *in vitro*, in *vivo* and in clinical studies. *In vitro* experiments into the stability and activity of ASP could enable molecules with enhanced activity to be produced, and even peptide mimetics could be synthesized.

The isolation and characterization of the receptor is presently an intense area of focus. This would allow for molecular tools to evaluate the levels of the ASP receptor in both human and animal models in diseases such as obesity, diabetes and hyperapoB patients. In diabetic *db/db* mice administration of ASP was able to reduce glucose and lipid levels (manuscript in preparation). This could be of benefit in human diabetics exhibiting insulin resistance and would add an extra hormone in the battle against this disease.

In animal models, especially the knockouts, various aspects of metabolism could be studied such as thermogenesis, insulin, glucose and lipoprotein release, effects of different diets and further radiolabelled tissue uptake studies can be performed. Complementary to these studies would be the *in vitro* analysis of the tissues to determine rates of triglyceride synthesis, lipolysis, glucose uptake, effects on LPL, leptin and various message RNA levels.

In conclusion, we have been able to test our hypotheses generated from *in vitro* data in animal murine models, leading to new insights into the physiological role of ASP. Clinical analysis of ASP in humans would be the eventual outcome of these studies.

7.3 Claims to Originality

The work performed using recombinant ASP and structure-function studies have not been previously performed or published. The administration of exogenous ASP *in vivo* also has not been previously published. The longitudinal studies of the functional ASP knockout mice, physiological determination of the effects of fat loads and glucose tolerance tests, body weight, food intake, leptin and other fasting parameters to my knowledge has not been previously published or performed.

7.4 General References

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