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**The Postprandial Stimulation of Acylation
Stimulating Protein (ASP) Production in Adipocytes**

Thea Scantlebury

**A Thesis in the
Department of Chemistry and Biochemistry**

**Presented in Partial Fulfillment of the Requirements for the Doctorate of
Philosophy at Concordia University
Montreal, Quebec, Canada**

September 2001

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After this, I can say that
I truly understand what
patience and
perseverance really
means!!!!

DEDICATION

In loving memory, I would like to dedicate my PhD thesis to my parents, Mytra and Michael Scantlebury. My Father first established the quest for knowledge and my Mother lovingly gave endless support and understanding throughout my life. I could not have made it this far with out her faith in me.

To my Brother, Michel Scantlebury, we may have had some rough times but I know that you have always tried to protect, love, and support me even though we may not have seen eye to eye all the time.

To my Husband, Roger Manning, you have lovingly supported me during the impossible times and have unknowingly given me the strength to continue. You have been extremely patient and understanding throughout and I could not have finished this without you. I can only thank God for bringing you into my life because you are an exceptional person. My Love, I truly appreciate the sacrifices you have made in order for me to accomplish my dream. Thank-you.

ABSTRACT

The Postprandial Stimulation of Acylation Stimulating Protein (ASP) Production in Adipocytes

Thea Scantlebury, Ph.D.
Concordia University, 2001

Acylation stimulating protein (ASP), a cleavage product of complement C3, is a potent stimulator of triacylglycerol synthesis in adipocytes. The three proteins necessary for ASP generation are complement C3, factor B and adipsin. *In vivo* studies have shown that there is an increased production of ASP in the micro-environment of adipose tissue after a meal and the level of ASP in the blood is substantially higher in obese individuals. Initial *in vitro* studies in our laboratory demonstrated that cultured differentiated adipocytes produce ASP and the postprandial component, chylomicrons (CHYLO), increased ASP production. Thus the present studies were undertaken firstly to identify the component of CHYLO responsible for the increase in ASP, and secondly to examine the CHYLO effects on the C3 (precursor), factor B and adipsin production. The results demonstrated that CHYLO stimulated ASP, C3, factor B and adipsin production in adipocytes. The stimulatory effects of CHYLO on ASP and C3 production were time- and concentration-dependant, with maximum levels at four to six hours of CHYLO stimulation. Transthyretin (TTR) was identified as a CHYLO component involved in the stimulatory effect. TTR and CHYLO transport

retinyl ester/retinol, and it was determined that the TTR mediated retinoic acid transfer from CHYLO was involved in stimulating C3, factor B, and subsequently ASP, but not adipsin production. This CHYLO effect includes both *de novo* protein synthesis and secretion. While retinoic acid partly accounts for the stimulatory effect of CHYLO on C3, factor B and ASP production, we believe there are other factors that are relevant in regulating C3, factor B, adipsin, and ASP production. Taken together, these studies demonstrated that CHYLO is a novel physiological trigger that stimulates ASP production and the necessary proteins (C3, factor B, adipsin) required for ASP generation. This in turn can result in the direct stimulation of triacylglycerol storage in adipose tissue after a meal thereby enhancing the overall body fat stores. With the increase in obesity prevalence over the last decade, we believe that understanding the ASP pathway will allow us to better ascertain the physiological development of obesity and possible new drug targets in controlling it.

(word count 350)

RESUMÉ

Stimulation Post-prandiale de la Production d'Acylation Stimulating Protein (ASP) dans les Adipocytes

Thea Scantlebury, Ph.D.

Université Concordia, 2001

La protéine stimulant l'acylation ou ASP (de l'anglais "acylation stimulating protein"), un produit de clivage de la protéine du complément C3, est un puissant stimulateur de la synthèse des triacylglycérols chez l'adipocyte. Les trois protéines nécessaires à la génération d'ASP sont la protéine du complément C3, le facteur B et l'adipsin. Des études *in vivo* ont démontré une augmentation de la production d'ASP dans le tissu adipeux après la prise d'un repas. Aussi, les niveaux sanguins d'ASP sont substantiellement augmentés chez les sujets obèses. Les études initiales *in vitro* réalisées dans notre laboratoire ont démontré qu'en culture, les adipocytes différenciés produisent de l'ASP et que les chylomicrons (CHYLO), un composé post-prandial, augmente la production d'ASP. La présente étude a donc été entreprise premièrement pour identifier la composante des CHYLO responsable de l'augmentation d'ASP et, deuxièmement, pour examiner les effets des CHYLO sur la production de C3 (précurseur), du facteur B et de l'adipsin. Les résultats ont démontré que les CHYLO stimulent la production d'ASP, de C3, du facteur B et de l'adipsin dans les adipocytes. L'effet stimulant des CHYLO sur la production d'ASP et de C3 est dépendante du temps et des doses utilisés, avec un effet maximal entre quatre et six heures. La transthyretin (TTR) a été identifiée comme étant un composé des CHYLO impliqué dans leur effet stimulant. La TTR et les CHYLO transportent l'ester de rétinyl et/ou rétinol. Il a subséquemment été démontré que le transfert de l'acide rétinoïque médié par la TTR des CHYLO vers les cellules

est impliqué dans la stimulation de la production de C3, du facteur B, et conséquemment de l'ASP, mais non de l'adipsin. Cet effet des CHYLO inclut autant la synthèse protéique et la sécrétion. Tandis que l'acide rétinoïque n'est impliqué que partiellement dans l'effet stimulant des CHYLO, nous croyons que d'autres facteurs sont nécessaires à la production de C3, du facteur B et de l'ASP. En résumé, ces études démontrent que les CHYLO représentent un nouvel élément déclencheur pour la production d'ASP et des protéines nécessaires à sa production (C3, facteur B, adipsin). Cela permet la stimulation directe de l'accumulation des triacylglycérols dans le tissu adipeux après un repas, augmentant alors les niveaux de réserve de gras corporels. Avec l'augmentation de la prévalence de l'obésité au cours des derniers dix ans, nous croyons qu'une meilleure compréhension du métabolisme de l'ASP permettra de mieux établir les causes physiologiques du développement de l'obésité et pourra conduire au développement de nouvelles cibles thérapeutiques pour son contrôle.

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I would like to express my greatest gratitude to Dr. Katherine Cianflone, and Dr. Allan A. Sniderman for guiding me through my development into an independent thinker and scientist. Thank you for giving me this amazing opportunity to be a part of your research team in the first place. I would like to express my gratitude to Dr. Judith Komblatt for her critical and insightful discussions during the course of my graduate education. They have all been instrumental in my development as a researcher, by providing explanations and discussions that allowed me to tackle many of my questions with a new and different light.

I would like to thank Magdalena Maslowska, Jumana Saleh, Ian Murray and May Faraj for their contribution to those discussions in the generation of concepts and in the preparation of posters and presentations.

I would also like to thank the Plastic Surgery Division at the Royal Victoria Hospital for aiding me in obtaining the adipose tissue needed for my studies.

I would like to thank Magdalena Maslowska in making and screening the monoclonal antibody to ASP, and training me in all tissue culture techniques. Steve Phelis purified the human ASP used as a standard in ASP ELISA.

David Kalant provided electro-competent bacteria, and was a constant resource in debugging the molecular biology techniques.

I would like thank Hai Vu for measuring adipsin, and Jumana Saleh for measuring factor B, purifying the monoclonal to ASP and generating the polyclonal antibody to ASP.

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TABLE OF CONTENTS

Abstract	iii
Resumé	v
General Acknowledgements	vii
Table of Contents	viii
List of Figures	xii
List of Tables	xiv
General Abbreviations	xv
Contribution of Authors	xvii

CHAPTER ONE INTRODUCTION

1.1: Obesity and Energy Balance	1
1.2: Lipid Storage and Metabolism in Adipose Tissue	3
1.2.1: Lipogenesis	5
1.2.2: Lipolysis	5
1.3: Adipose Tissue as a Dynamic Organ	8
1.4: Complement Proteins	11
1.5: ASP Generation	12
1.6: Regulation of TG Storage	14
1.7: Rational	17

CHAPTER TWO ACUTE IN VITRO PRODUCTION OF ACYLATION STIMULATING PROTEIN IN DIFFERENTIATED HUMAN ADIPOCYTES

2.1: ABSTRACT	20
2.2: Introduction	23
2.3: Materials and Methods	27
2.3.1: Culture of human differentiating adipocytes	27
2.3.2: Experimental Incubation	28
2.3.3: Medium ASP Determination	29
2.3.4: Medium C3 Determination	29
2.3.5: Lipoprotein Isolation	30
2.3.6: Statistics	31

2.4: Results	32
2.5: Discussion	39
2.6: Tables and Figures	43
Prelude One	59

CHAPTER THREE

CHYLOMICRON SPECIFIC ENHANCEMENT OF ACYLATION STIMULATING PROTEIN (ASP) AND PRECURSOR PROTEIN C3 PRODUCTION IN DIFFERENTIATED HUMAN ADIPOCYTES

3.1: ABSTRACT	63
3.2: Introduction	64
3.3: Materials and Methods	66
3.3.1: Culture of human differentiating adipocytes	66
3.3.2: Experimental Incubation	67
3.3.3: Medium ASP Determination	68
3.3.4: Medium C3 Determination	69
3.3.5: Chylomicron Preparation and Fractionation	69
3.3.6: Chylomicron Modifications	70
3.3.7: Statistics	71
3.4: Results	72
3.5: Discussion	78
3.6: Tables and Figures	82
Prelude Two	94

CHAPTER FOUR

RETINOIC ACID REGULATION OF ACYLATION STIMULATING PROTEIN (ASP) AND COMPLEMENT C3 IN HUMAN ADIPOCYTES

4.1: SUMMARY	98
4.2: Introduction	99
4.3: Materials and Methods	102
4.3.1: Culture of human differentiating adipocytes	102
4.3.2: Experimental Incubation for C3 and ASP Secretion	103
4.3.3: Medium ASP Determination	104

4.3.4: Medium C3 Determination	105
4.3.5: Chylomicron Preparation and Fractionation	106
4.3.6: mRNA Analysis via Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)	107
4.3.7: 3T3 Cell Culture and C3 Promoter Activity	108
4.3.8: Statistics	108
4.4: Results	109
4.4.1: Effect of Protein Synthesis and Secretion Inhibitors	109
4.4.2: Effect of RA on C3 and ASP Secretion	110
4.4.3: Effect of RA Antagonist on C3 and ASP Secretion	111
4.4.4: Effect on C3 mRNA Expression in Human Adipocytes	112
4.4.5: Effect on C3 Promoter-Luciferase Reporter Gene Expression in 3T3 Cells	113
4.5: Discussion	115
4.6: Tables and Figures	120
Prelude Three	129

CHAPTER FIVE
CHYLOMICRON-SPECIFIC ENHANCEMENT OF FACTOR B AND ADIPSIN
IN DIFFERENTIATED HUMAN ADIPOCYTES

5.1: Introduction	130
5.2: Methodology	134
5.2.1: Sandwich Elisa for Factor B	134
5.2.2: Sandwich ELISA assay for Adipsin	134
5.2.3: Factor B and Adipsin RT PCR	135
5.2.4: Statistics	136
5.3: Results	137
5.4: Discussion	140
5.5: Tables and Figures	144

CHAPTER SIX
GENERAL DISCUSSION

6.1: Summary of Research Presented	154
6.2: Novelty of the Results	155
6.3.1: Limitations of the <i>In vitro</i> Experimental Cell Model	158
6.3.2: Limitations of the Data	159
6.4: <i>In vivo</i> Postprandial ASP Metabolism	161
6.5: Mice Models of ASP Metabolism	162
6.6: Importance of ASP in Fat Metabolism	163

CHAPTER SEVEN
CONSTRUCTION OF ASP PSEUDOGENE USED TO GENERATE THE ASP
OVEREXPRESSOR TRANSGENIC MOUSE

7.1: Introduction	166
7.2: Strategy	168
7.3: Methods	170
7.3.1: ASP minigene	170
7.3.2: Insertion of SP-ASP into the pBk-CMV Phagemid (PBk-CMV-SP-ASP)	172
7.3.3: Removal of the Cytomegelo virus Promoter (PBk-SP-ASP)	173
7.3.4: Insertion of the Adipocyte P2 Promoter (PBk-aP2-SP-ASP)	174
7.3.5: Linearization of the ASP Pseudogene (aP2-SP-ASP)	175
7.3.6: Generation of Transgenic Mice	176
7.4: Future Experiments	177
7.4.1: <i>In vitro</i>	177
7.4.2: <i>In vivo</i>	178
General Bibliography	179

LIST OF FIGURES

Figures:	Page
1.1: Outline of Lipid Metabolism in Adipose Tissue	7
1.2: Overview of ASP Generation	13
1.3: Hormonal Regulation of TG storage	16
2.1: Concentration dependent effect of glucose and oleate on ASP generation by human differentiated adipocytes	46
2.2: Dose dependent effect of insulin on ASP generation by human differentiated adipocytes	48
2.3: Dose dependent effect of chylomicrons on ASP generation by human differentiated adipocytes	50
2.4: Time course of the chylomicron effect on ASP generation by human differentiated adipocytes	52
2.5: Concentration dependent effect of glucose, oleate and insulin on C3 generation by human differentiated adipocytes	54
2.6: Dose dependent effect of chylomicrons on C3 generation by human differentiated adipocytes	56
2.7: Modulation of the CHYLO mediated increase in medium C3 and ASP in human differentiated adipocytes	58
3.1: Chylomicron Stimulation of C3 and ASP Production by Human Differentiated Adipocytes	87
3.2: The Effect of Lipid Modified Chylomicrons on C3 and ASP Production by Human Differentiated Adipocytes	89
3.3: Concentration-Dependant Enhancement of C3 Production by Transthyretin in the Presence of Chylomicrons in Differentiated Human Adipocytes	91
3.4: Immunospecific Blocking of Transthyretin Enhancement of Chylomicron Stimulation of C3 and ASP Production	93

4.1: RA Effect on C3 Production in Differentiated Human Adipocytes	124
4.2: RA Receptor Antagonist Blocks the Stimulation by Chylomicrons and RA on C3 and ASP Production	126
4.3: RA Receptor Antagonist and TTR Antibody Blocks the Chylomicron Stimulation of C3 mRNA	128
5.1: Chylomicron concentration curve for Factor B	148
5.2: The Effect of CHYLO on C3, Factor B and Adipsin mRNA levels	150
5.3: Heparin Inhibits ASP Production but not C3 production	152
6.1: Postprandial Mechanism of ASP Production	157
7.1: Construction of ASP 'pseudogene'	169

LIST OF TABLES

Tables:	Page
1.1: Summary of factors that are synthesized or secreted by adipose tissue.	10
2.1: Effect of lipoproteins on ASP generation by human differentiated adipocytes	43
2.2. Effect of lipoproteins on C3 generation by human differentiated adipocytes	44
3.1: The Effects of Modified Chylomicrons on C3 and ASP Production in Human Differentiated Adipocytes	82
3.2: The Effect of Apoproteins on C3 and ASP Production by Human Differentiated Adipocytes	83
3.3: Effect of Chylomicron Components on C3 and ASP Production in Differentiated Human Adipocytes	84
3.4: The Effect of Transthyretin on C3 and ASP Production by Differentiated Human Adipocytes	85
4.1: The Effect of Protein Secretion Inhibitors on C3 and ASP Production in Human Differentiated Adipocytes	120
4.2: The Effect of Protein Synthesis inhibitors on C3 and ASP Production by Human Differentiated Adipocytes	121
4.3: RA Activation of the C3 promoter	122
5.1: Regulation of Complement Proteins by Various Proteins	144
5.2: Effects on C3, Factor B, Adipsin and ASP secretion	145
5.3: Effect of Transthyretin antibody and Retinoic Acid Receptor Antagonist on C3, Factor B and Adipsin mRNA	146
6.1: Changes in ASP, C3, Factor B and Adipsin levels with obesity and related pathophysiological conditions.	164

GENERAL ABBREVIATIONS

ACN	acetonitrile
AGPAT	1-acylglycerol phosphate acyltransferase
ASP	acylation stimulating protein
BSA	bovine serum albumin
C3	complement C3
CHYLO	chylomicrons
DGAT	diacylglycerol acyltransferase
DMEM/F12	Dulbecco's minimum essential medium / Ham's F12
FBS	fetal bovine serum
FFA	free fatty acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT4	glucose transporter 4
HDL	high density lipoprotein
HSL	hormone sensitive lipase
LDL	low density lipoprotein
LPL	lipoprotein lipase
MAGL	monoacylglycerol lipase
MEM	minimum essential medium
MGAT	monoacylglycerol acyltransferase
NEFA	non-esterified fatty
PBS	phosphate buffered saline

PPAR	peroxisome proliferator-activated receptor
PPH	phosphatidate phosphohydrolase
RA	retinoic acid
RIA	radioimmunoassay
sem	standard error of the mean
T4	thyroxine
TG	triacylglycerol
TMAC	tetramethylammonium chloride
TTR	transthyretin
VLDL	very low density lipoprotein

CONTRIBUTION OF AUTHORS

The data presented here are the results of Ph.D. laboratory research at the Mike Rosenbloom Laboratory for Cardiovascular Research

Chapter 2: Acute in vitro production of acylation stimulating protein in differentiated human adipocytes.

Magdalena Maslowska:

- Isolated chylomicrons, VLDL, LDL, and HDL used for experiments.
- Isolated, cultured and differentiated human preadipocytes for all experiments to measure ASP, except for the experiment with THL.
- Performed all the experiments from which ASP was measured, except for the experiment with THL.
- Performed all the ASP medium measurements using the RIA kit, and performed all the statistical analysis of the ASP data (except for the experiments with THL).
- Initiated the writing of the paper and wrote the methods, results and interpretation for the ASP data (except for the THL experiment).

Thea Scantlebury:

- Isolated chylomicrons and VLDL used for experiments.
- Isolated, cultured, and differentiated human preadipocytes for all the experiments where C3 was measured.
- Repeated all the experiments performed to initially measure ASP.
- Developed the sandwich ELISA used to measure C3 levels.
- Performed all C3 medium measurements and measured ASP levels on the experiment with THL.
- Performed the entire statistical analysis and interpretations for the C3 data and the THL data.
- Wrote the methods, results and interpretation for the C3 data and for the THL experiment (C3 and ASP).
- Intellectually contributed to the development of the paper and was involved in re-structuring and re-writing the body of work into the final version for publication.

Ralph Germinario:

- Provided helpful discussions in the overall project.

Katherine Cianflone:

- Supervised the overall project and provided financial support.

Chapter 3: Chylomicron-specific enhancement of acylation stimulating protein and precursor protein C3 production in differentiated human adipocytes.

All the tissue culture, experiments and measurements were performed and setup by myself, except for transthyretin sequencing. In order to demonstrate similar temporal regulation of ASP and C3 by chylomicrons, we decided to utilize the previously document chylomicron time curve for ASP generation that was performed by Magdalena Maslowska in addition to all the new ASP and C3 data used for the manuscript. Dr. Alex Bell of the Sheldon Polypeptide Biotechnology Center sequenced transthyretin from the band of interest. Aside from supervising the overall project, Dr. Katherine Cianflone was a great assistance in the preparation of the manuscript.

Chapter 4: Regulation by retinoic acid of acylation-stimulating protein and complement C3 in human adipocytes.

All the tissue culture, experiments and measurements were performed and set-up by myself. Dr. Allan D. Sniderman provided helpful discussions in the overall project and intellectual input in the direction of the manuscript. Dr. Katherine Cianflone provided overall supervision of the project and assisted in the preparation of the manuscript.

Chapter 5: Chylomicron-specific enhancement of factor B and adipsin production in differentiated human adipocytes.

I undertook all the set-up, preparation and execution of the experiments. In addition, I developed the factor B sensitive sandwich ELISA and performed the factor B measurements. Jumana Saleh performed the remainder of the factor B measurements for the experiments with retinoic acid and transthyretin combinations. Hai Vu, developed the adipsin ELISA and performed all the adipsin measurements. Dr. Katherine Cianflone provided helpful discussions in the overall project and assisted in the preparation of this chapter.

Here is a summary of all the work performed during the course of my Ph.D. training and appeared in the following co-authored abstracts and publications:

Abstracts:

1. **Scantlebury T**, Sniderman AD, and Cianflone K, (2000): Retinoic Acid Mediated Regulation of Acylation Stimulating Protein Generation in Human Adipocytes via Chylomicrons. *Obesity Res.* 8(1):52s.
2. Bower JF, **Scantlebury T**, Phelis S, Barakat HA, and Cianflone K (2000): Plasma Acylation Stimulating Protein and Lipid Levels in Lean, Obese, and Diabetic Women: A Comparison of African and Caucasian Americans. *Obesity Res.* 8(1):61s.
3. **Scantlebury T**, Maslowska M, Sniderman AD, and Cianflone K (1998): Chylomicron specific enhancement of Acylation Stimulating Protein and precursor C3 production in differentiated human adipocytes. *Int J. Obesity* 22(3):s70.
4. **Scantlebury T**, Maslowska M, Sniderman, AD, and Cianflone K (1997): Postprandial adipocyte regulation of the C3 and ASP production. Canadian Lipoprotein Conference.

Publications:

1. **Scantlebury T**, Sniderman AD, and Cianflone K, (2001): Retinoic Acid Regulation of Acylation Stimulating Protein (ASP) and Complement C3 in Human Adipocytes. *Biochem. J.* 356:445-452.
2. Sniderman AD, **Scantlebury T**, and Cianflone K (2001): Pathogenesis of Dyslipidemia in Diabetes. *Ann. Int. Med.* (in press)
3. **Scantlebury T**, Maslowska M, and Cianflone K (1998): Chylomicron specific enhancement of Acylation Stimulating Protein (ASP) and precursor C3 production in differentiated human adipocytes. *J. Biol. Chem.* 273 (33):20903-20909.
4. Maslowska M, **Scantlebury T**, Germinario R and Cianflone K (1997): Acute *in vitro* production of ASP in differentiated adipocytes. *J. Lipid Res.* 38:1-11.

Section 1.1: Obesity and Energy Balance

Obesity has been medically defined as a state of increased body weight due to a specific increase in adipose tissue of a significant magnitude that would produce adverse health consequences (1). The severity of obesity and its complications has only come to light over the past 15 years. With economic modernization of developed and developing countries, there has been an increase in the rate of obesity as a consequence of changing nutritional and physical activity patterns (2). Obesity is now a common problem in western society and as a result more and more individuals will suffer from complications such as heart disease, diabetes, hypertension, and stroke (3), thus, increasing significantly morbidity and mortality. In addition, the prevalence of childhood obesity has dramatically escalated and there is concern over the predicted medical problems for these children in the decades to come (4). It has been estimated that 30-60% of the variation in obesity-related phenotypes can be explained by environmental influences (5), while 40-70% is heritable (6), as suggested by studies on closed populations such as the Pima Indians (6). Thus, the major factors influencing obesity are the genetic background and dietary and physical activity habits that influence energy balance.

The basic components of the energy balance equation are energy intake and energy expenditure. Energy intake is the dietary component of the

equation. Energy expenditure encompasses basal metabolism, adaptive thermogenesis and physical activity. Basal metabolism refers to all the biochemical processes necessary to sustain life while adaptive thermogenesis is the energy dissipated in the form of heat in response to temperature changes in the environment. For energy storage, energy intake must exceed energy expenditure. Thus excess fat deposition is a result of chronic positive shift of the energy equation due to increases in energy input, decreases in energy output or both (7). The ability to store energy in the form of fat in times of nutritional abundance may have been a survival trait selected over the thousands of years of human evolution. In the last decade, it is becoming increasingly evident that different individuals have different genetic predispositions to store excess caloric intake as fat. In a study performed on Pima Indians (8), those with a lower metabolic rate had a greater incidence and extent of obesity development. In an overfeeding study on pairs of monozygotic twins (9), the different sets of twins showed a similar tendency toward an increase in adiposity, even though there were differences in the degree to which calories were stored as fat. Since overfeeding above basal caloric needs was controlled, the likely difference in fat storage between the sets of twins was probably due to differences in some component of energy expenditure such as adaptive thermogenesis or basal metabolism. This further suggests that differences in the metabolic rates are relevant in the development of obesity.

Section 1.2: Lipid Storage and Metabolism in Adipose Tissue

Of the basic fuel sources (carbohydrates, proteins and fats), the majority of energy storage is in the form of fat. During the anabolic phase, the body stores energy; whereas during the catabolic phase, the body mobilizes and utilizes the stored fat. Triacylglycerol (TG) is an energy dense substrate stored primarily in adipose tissue and to a lesser extent in the liver. There are two sources of TG that can be stored, a) from the diet and b) *de novo* lipogenesis primarily from glucose. Due to the abundant fat content in our western diet (approx 35%), the major source of TG storage is dietary. After ingestion of a mixed meal, TG absorbed through the intestine is packaged into chylomicrons (CHYLO), secreted into the lymphatic system and subsequently released into the circulation. Chylomicrons (CHYLO) are the largest members of the lipoprotein family. The other members are very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). These lipoproteins function to transport TG and cholesterol throughout the body.

Lipoprotein particles have a hydrophobic lipid core that consists of TG and cholesteryl ester and a hydrophilic outer surface that is made up of a phospholipid monolayer and free cholesterol. In addition, each particle also contains one or more apolipoproteins that interacts with both the hydrophobic and hydrophilic domains of the particle and acts as structural components, cofactors for enzymes and receptor ligands. The different lipoproteins differ in

their tissue origin and in the composition of lipids and various apolipoproteins. The lipid composition of CHYLO is composed of 90% TG, 5% cholesterol, 4% phospholipids and apolipoproteins B48, AI, AII, CI, CII, CIII, E (10). The apolipoprotein B48 component distinguishes the chylomicron from that of VLDL; while both are large TG rich lipoproteins, VLDL is made by the liver and contains apolipoprotein B100 and does not contain B48.

Due to the fact that TG is hydrophobic, it cannot easily pass from the lipoprotein particle to the intracellular TG stores and must be enzymatically broken down to fatty acids, enter the cell, and then be reformed to TG intracellularly. As CHYLO pass through capillaries of adipose tissue, the TG is hydrolyzed by the enzyme lipoprotein lipase (LPL) to release free fatty acids (FFA). Consequently, the CHYLO particle shrinks and the particle remnants are taken up by the liver via a receptor-mediated mechanism. LPL may also help facilitate cellular uptake of lipophilic vitamins (11). LPL is synthesized by adipose tissue and translocated to the surface of the endothelial cells of the capillaries where it is non-covalently bound to chains of the complex glycosaminoglycan, heparin sulphate (12,13). Interaction of LPL with its cofactor apoCII on the lipoprotein surface activates LPL (14,15). The non-esterified fatty acid products (NEFA) diffuse into the interstitial space and then are taken up by the adipocytes through both passive diffusion (16) and membrane fatty acid transporters (17,18). In turn, glucose is taken up by the adipocytes by certain glucose transporters (GLUT) that are located on the cell surface (19,20).

Section 1.2.1: Lipogenesis

The intracellular process of TG storage (Figure 1.1) involves the re-esterification of the FFA (FFA-acyl-CoA) onto a glycerol-3-phosphate backbone by the various enzymes in the pathway of esterification to form storage TG. These enzymes are glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol phosphate acyltransferase (AGPAT) or monoacylglycerol acyltransferase (MGAT), phosphatidate phosphohydrolase (PPH), and diacylglycerol acyltransferase (DGAT). DGAT mediates the final step in the glycerol phosphate pathway of TG synthesis. With the recent cloning of human (21,22) and mouse (23) DGAT, it has been shown that DGAT mRNA increases eightfold during differentiation of 3T3-L1 adipocytes and the activity also increases 60-fold in these cells (22). To date, hormonal regulation of these enzymes is still undetermined, and thus further studies are needed to elucidate their detailed regulation in TG synthesis in adipose tissue.

Section 1.2.2: Lipolysis

Utilization of the stored TG as an energy source requires mobilization of these depots and release into the circulation (Figure 1.1). Hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) both function in the release of fatty acids and glycerol into the circulation (24). HSL is synthesized by adipocytes and acts at the surface of the intracellular lipid droplet to hydrolyze

two fatty acids from the TG molecule. MAGL then removes the last fatty acid from the glycerol backbone (10,24). These fatty acids can then be utilized by other tissues as a source of energy. Recently, perilipin, an intracellular adipocyte protein was shown to regulate HSL (1). In perilipin knockout mice, HSL is constitutively increased and the mice are lean despite an increase in food intake and manifest an increase in metabolic rate (25). In addition, it has been shown that the absence of perilipin significantly reverses obesity of *db/db* mice (25). In summary, it is clear that there is an array of hormones that work to regulate TG homeostasis in response to the various energy requirements of the body.

Interestingly, HSL deficient mice are not obese and show residual adipose tissue lipolysis (26,27), which suggests the existence of another triglyceride lipase. It is of added interest that in DGAT knockout mice (CD36) (28,29), mice lacking LPL (30) or HSL knockout mice (26,27), all animals can still accumulate adipose tissue. Therefore, although these enzymes are necessary for TG synthesis, there appears to be an as yet undefined compensatory system that still allows TG synthesis to occur, such as DAG transacylase that makes TG from two DAGs in a CoA-independent manner (31).

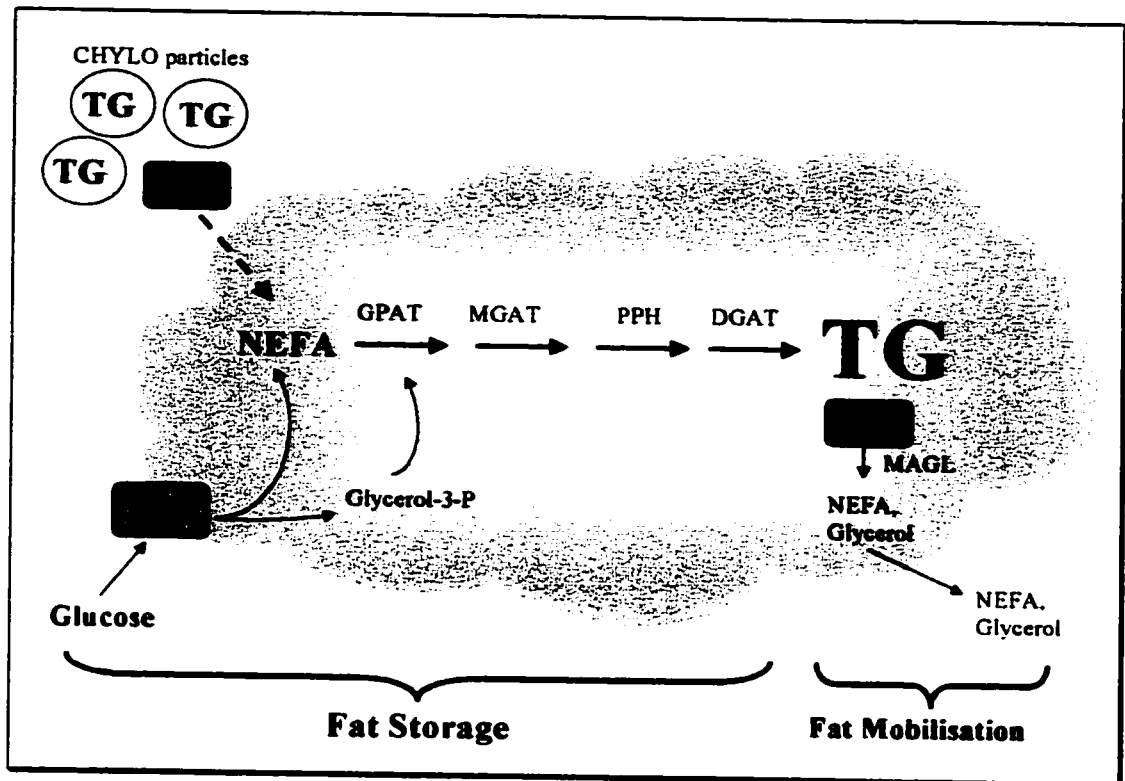


Figure 1.1: Outline of Lipid Metabolism in Adipose Tissue

Several enzymes are involved in the triacylglycerol (TG) synthesis from glucose and non-esterified fatty acids (NEFA) that are taken up by the adipocyte. Abbreviations: glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol phosphate acyltransferase (AGPAT) or monoacylglycerol acyltransferase (MGAT), phosphatidate phosphohydrolase (PPH), and diacylglycerol acyltransferase (DGAT), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), monoacylglycerol lipase (MAGL) and glucose transporter 4 (GLUT4).

Section 1.3: Adipose Tissue as a Dynamic Organ

There are two major types of adipose tissue: white and brown. The role of brown adipose tissue is to generate heat and is especially important in hibernating mammals. In adult humans, most of the adipose tissue is white. Adipose tissue is now widely recognized as a dynamic organ that also synthesizes and secretes a variety of enzymes and hormones. These factors can act in an endocrine and autocrine/paracrine manner to regulate the metabolic activity of adipose tissue as well as other tissues (brain, liver and muscle), thus establishing adipose tissue as an endocrine organ. Table 1.1 summarizes many of the factors recognized to date that are synthesized or secreted by adipose tissue. Directly relevant to obesity, adipose tissue synthesizes and secretes leptin, which acts in the hypothalamus as a potent satiety factor to regulate body weight (32,33). Tumor necrosis factor alpha ($\text{TNF}\alpha$) is an example of both an autocrine and endocrine factor (immune response). In an autocrine manner, $\text{TNF}\alpha$ has multiple actions in adipose tissue such as: a) inducing phosphorylation of insulin-receptor-substrate-1 and thus affecting insulin resistance (34,35), b) positively modulating leptin secretion from a preformed pool (36), c) decreasing the expression of glucose transporter 4 (37), and d) decreasing hormone sensitive lipase (38). Very recently, it has been demonstrated that protein tyrosine phosphatase-1b (39) and pantophysin (40,41) may play a role in insulin resistance. It is important to note that complement proteins C3, factor B and adipsin have also been shown to be synthesized and secreted by adipocytes and the product of the interaction of

these three proteins, acylation stimulating protein (ASP), acts back on the adipocytes to stimulate TG synthesis (42-45). While the recognition of adiponectin dates back many years, work in our lab was instrumental in ascribing a function in lipid metabolism to these immunological proteins in adipose tissue.

Secreted Proteins	Reference
Angiotensinogen(AGT)	(46)
Dehydroepiandrosteronesulfate (DHEA-S)	(47)
Estrogen	(47-49)
Plasminogen activator inhibitor-1 (PAI-1)	(50)
IGF-binding protein 3 (IGFBP3)	(51)
Insulin-like growth factor-1 (IGF-1)	(52)
Interleukin-6 (IL-6)	(53)
Monobutyryl	(54)
Prostaglandin E2	(55)
Prostaglandin I2	(56)
Retinol-binding protein	(57)
Transforming growth factor beta (TGF β)	(58)
Tumor necrosis factor alpha (TNF α)	(59)
Vascular endothelial growth factor (VEGF)	(60)
Acrp30	(61)
Proteins involved in lipid metabolism	
Adipsin	(20,62-64)
Adiponectin	(65,66)
Agouti	(67-69)
Apoprotein E	(70)
Cholesterol ester transfer protein (CETP)	(71,72)
Complement C3	(43,64,73-77)
Factor B	(43,64,73)
Leptin	(32)
Lipoprotein lipase (LPL)	(78,79)
Phospholipid transfer protein (PLTP)	(72)
Intracellular Proteins	Reference
Protein tyrosine phosphatase-1B	(39)
Pantophysin	(40,41)
Uncoupling protein 1 (UCP1)	(80-82)
Uncoupling protein 2 (UCP2)	(83)
Peroxisomal proliferator activated receptor γ	(84)
C/EBP α , C/EBP β , C/EBP γ (in differentiation)	(85,86)
ADD1/SREBP1	(87,88)

Table 1.1: Summary of factors that are synthesized or secreted by adipose tissue.

Section 1.4: Complement Proteins

The adipsin gene was originally isolated as a differentiation-dependently expressed mRNA in the murine cell line 3T3-F442 (89,90). It was subsequently shown to encode a serine protease based on sequence analysis (62). In both humans and mice, adipsin is expressed abundantly in adipose tissue. In humans, macrophage/monocyte cells also express adipsin to a small extent. The human homologue of mouse adipsin has been shown to be complement factor D, a key regulatory enzyme of the alternate complement pathway (63). Adipsin is required for the initial catalytic step of the activation of the alternate complement pathway (63,77,91,92). Studies looking at the synthesis of adipsin in 3T3 adipocytes show that there are two forms of adipsin that are synthesized and secreted (62). These proteins are of 44 and 37 kd and are both converted to a protein of 25.5 kd by enzymatic deglycosylation. Unlike other eukaryotic serine proteases, adipsin (factor D) is secreted and circulates in the blood in its enzymatically active form devoid of an activation peptide (63,93), and devoid of proteolytic activity against uncomplexed factor B (93). It has been proposed that a conformational change in the catalytic center of adipsin is induced by the natural substrate C3b-B, resulting in the expression of the full catalytic potential of the enzyme (93,94).

The third component of the human complement cascade is a protein that has a molecular mass of 185 kDa (95). The C3 polypeptide has an α chain and

a β chain that are covalently linked (96) and are synthesized and secreted by adipocytes and other cells (62,74,97-107). Using 3T3-L1 adipocytes, it has also been shown that upon differentiation there is a fivefold increase of C3 mRNA levels (43,73-75,108).

Factor B is a single chain glycoprotein of 90 kDa found in plasma (77). Factor B is synthesized and secreted by adipocytes as well as other cells (43,62,73,74,77,108) and is also increased with adipocyte differentiation (43).

Section 1.5: ASP Generation

ASP is produced (Figure 1.2) via the initial step of the activation of the alternate complement pathway (43,73-75,91,92). The production of ASP proceeds with the binding of factor B to C3b in the presence of Mg^{2+} to form a Mg^{2+} -dependent reversible complex of C3bB (77). Upon binding, factor B is rendered susceptible to proteolytic cleavage of a single lysine-arginine bond by adipsin, forming active C3bBb and a Ba fragment (77,91). This complex is localized to the cell surface and attachment is mediated through the C3b portion (109,110). This C3bBb complex, termed C3 convertase, expresses serine esterase activity and cleaves a single arginine-serine bond near the amino-terminal of the α chain of additional complement C3 molecules (94,111) forming products, C3a (8.9 kD) and C3b. It is believed that the esterolytic activity of the

C3bBb complex resides in the Bb portion (109) and the carboxy-terminal chain shares homology to serine proteases (77,109,112,113). The terminal arginine of the C3a fragment is rapidly and efficiently cleaved by carboxypeptidase E (114,115) to produce a seventy-six amino acid basic peptide, ASP (or C3adesArg).

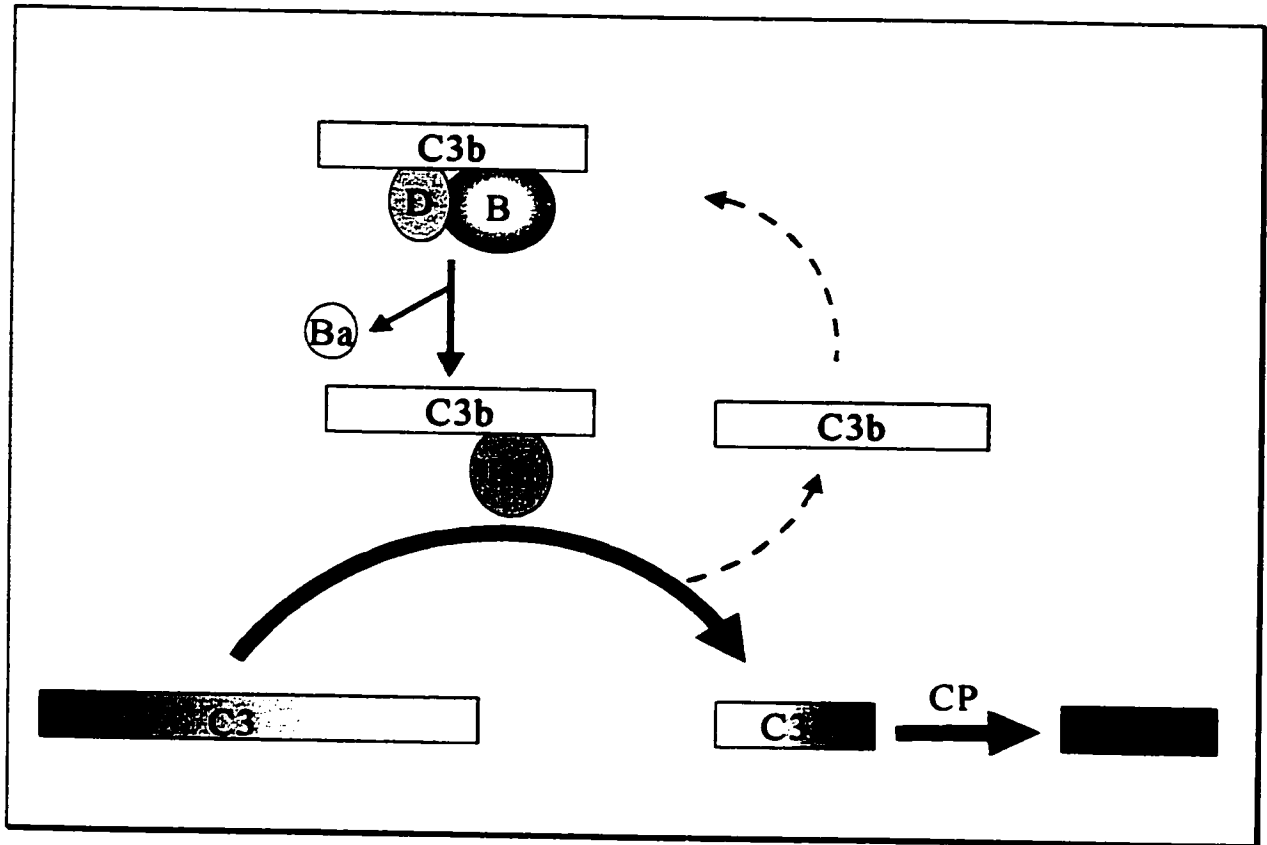


Figure 1.2: Overview of ASP Generation

The interaction of complement C3, factor B and adipsin to generate ASP. Abbreviations: complement C3 (C3), factor B (B), adipsin (D), factor Ba fragment (Ba), C3 convertase (C3bBb), complement C3b fragment (C3b), complement C3a fragment (C3a), and acylation stimulating protein (ASP).

Section 1.6: Regulation of TG Storage

As discussed above, the major function of adipose tissue is to store TAG in periods of energy excess and to mobilize energy during periods of deprivation. Both the short-term control of these lipogenic and lipolytic processes and long-term differentiation (size and number of adipocytes) are modulated by hormonal signals from the bloodstream and the adipose tissue. Both of these processes can be affected by autocrine and endocrine hormones at specific steps. Figure 1.3 gives an overview of the hormonal regulation of TG storage. Insulin, glucocorticoids (116), and 1,25 dihydroxy-vitamin D (117,118) stimulate LPL in adipose tissue. By contrast, glucagon (10), estrogen (119), growth hormone (120), thyroid hormone (121), catecholamines (11,122), TNF alpha (38) and hormones of the adrenergic system down regulate LPL (118).

The enzyme diacylglycerol acyltransferase (DGAT) mediates the final step in the glycerol phosphate pathway of TG synthesis. It has been previously demonstrated that ASP can significantly increase overall TG synthesis in adipocytes (43,123). More specifically, ASP was shown to stimulate DGAT activity in adipocytes and ASP may directly interact with DGAT to accomplish this (42). Further studies by Baldo *et al.* suggest that ASP may be acting through the PKC pathway (124). In addition to FFA uptake, the adipocyte must also take up glucose to be used as the backbone of the TG molecule. Insulin

and ASP both stimulate glucose uptake in adipocytes via increased translocation of the glucose transporter (GLUT1 and GLUT4) to the cell surface (46).

While insulin stimulates LPL after a meal, it simultaneously inhibits lipolysis of TG by decreasing the activity of HSL. Recently, both ASP and insulin were shown to play a role in regulating basal and catecholamine-stimulated FFA release from adipocytes, through the stimulatory effects on FFA re-esterification and inhibitory effects on HSL (125). While insulin affected both processes, ASP had a more pronounced effect on the FFA re-esterification. ASP appears to function by reducing cAMP levels through the activation of phosphodiesterase IV (PDE IV) (125), thus inhibiting the activation of HSL, while insulin acts via the PDE III signal pathway. It is important to note that while both insulin and ASP stimulate TG storage and inhibit FFA release, their effects are additive suggesting that they act via different pathways.

On the other hand, glucagon, catecholamines, and leptin (126) oppose those actions of insulin and ASP by stimulating HSL lipolysis in adipose tissue. While a number of hormones have been shown to affect LPL or HSL, little is known of the regulation of the TG synthesis enzymes (i.e. FATS, GPAT, MGAT, PPH and DGAT) and the direct effect of ASP on these enzymes has as yet to be examined.

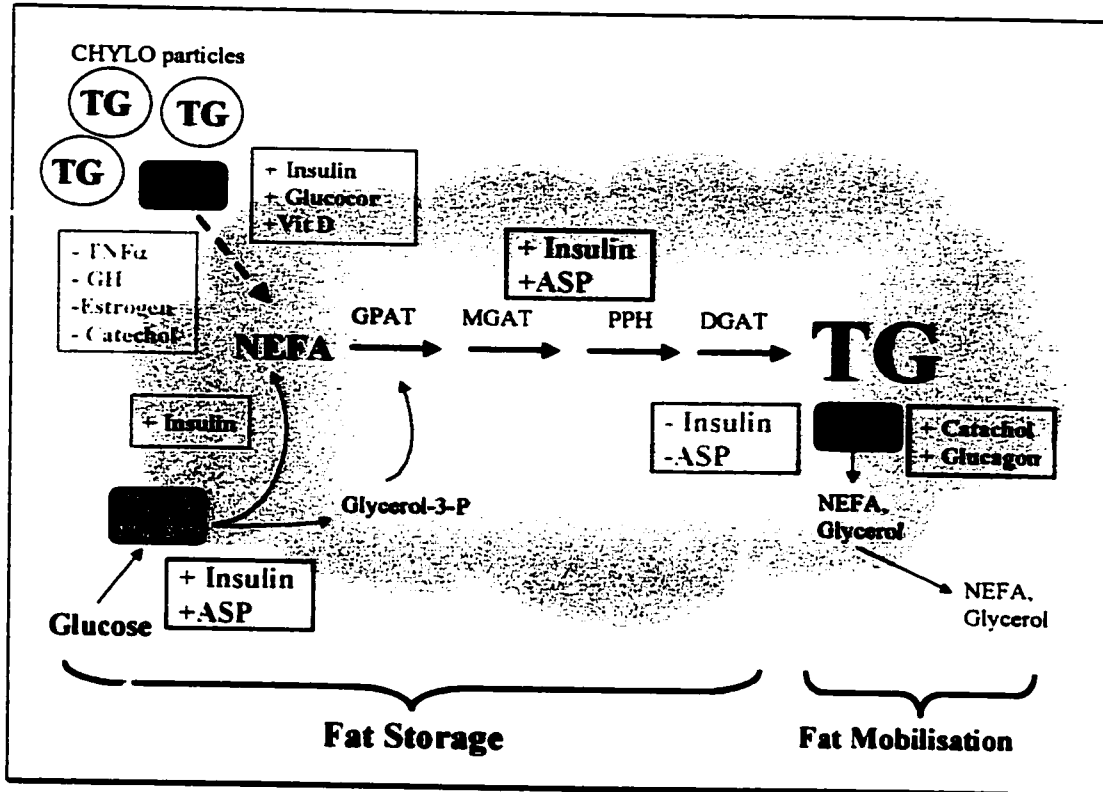


Figure 1.3: Hormonal Regulation of TG storage

Several hormones/factors regulate at the different steps involved in TG synthesis. Abbreviations: non-esterified fatty acid (NEFA), glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol phosphate acyltransferase (AGPAT) or monoacylglycerol acyltransferase (MGAT), phosphatidate phosphohydrolase (PPH), and diacylglycerol acyltransferase (DGAT), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), glucose transporter 4 (GLUT4), catecholamines (Catechol), growth hormone (GH), glucocorticoids (glucocor), vitamin D (vit D), and tumor necrosis factor α (TNF α).

Section 1.7: Rational

In vitro, ASP can directly regulate TG synthesis and may play a significant role in lipid metabolism. An accumulation of population studies (127-131) performed clearly shows that ASP levels are increased with obesity and are directly proportional to body mass index (BMI). An *in vivo* study by Saleh *et al.* (132) sampled venous and arterial blood from an adipose tissue depot after giving obese and controls subjects an oral fat load. The analysis of the blood taken over a period of eight hours demonstrated that ASP is generated in the micro-environment of the adipose tissue and that the rise in ASP was concurrent with an increase in TG clearance from the blood. Thus, it was hypothesized that ASP should have increased TG removal from the blood by adipose tissue due to the role of ASP in augmenting the TG synthetic capacity of the adipose tissue.

Earlier *in vitro* studies performed by Magdalena Maslowska demonstrated that CHYLO has the most pronounced stimulatory effect on ASP generation. It then was of interest to further investigate the postprandial signal for this increase in ASP. Thus the major objectives of my thesis project were a) to identify the CHYLO postprandial signal responsible for the increase of ASP, b) to measure the precursor protein, complement C3, c) to determine if the increase in ASP and C3 are a result of transcriptional regulation, *de novo* protein synthesis and/or secretion, d) to investigate the changes in the other proteins (factor B and

adipsin) involved in ASP production using the same previously identified stimulant.

ASP has been discovered only fairly recently and there still remains a considerable amount of research to be done to understand the physiological role and mechanism of action of ASP in the body. In this research lab, there are three main avenues of ASP research: 1) regulation of ASP generation, 2) mechanism of action of ASP, and 3) *in vivo* studies in humans and ASP deficient mice. I am responsible for the studies on the regulation of ASP generation, which are presented in this thesis. We believe this work is a key step in achieving a better understanding of ASP and its function. In addition, I am also responsible for developing a new transgenic mouse model that will allow this lab to examine, in the near future, the effects of ASP on obesity.

CHAPTER TWO
ACUTE INVITRO PRODUCTION OF AGYLATION STIMULATING PROTEIN
IN DIFFERENTIATED HUMAN ADIPOCYTES

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Maslowska, Magdalena; Scantlebury, Thea; Germinario, Ralph*; Cianflone, Katherine.

McGill Unit for the Prevention of Cardiovascular Disease, Royal Victoria Hospital; Lady Davis Institute*, Jewish General Hospital, McGill University, Montreal, Quebec, Canada.

Address for correspondence:

Dr. K. Cianflone
Division of Cardiology, Rm. M4.76
Royal Victoria Hospital
687 Pine Avenue West
Montreal, Quebec H3A 1A1

Tel: (514) 842-1231 ext. 5426
Fax: (514) 982-0686

Short title: ASP Production in Human Adipocytes

Section 2.1: ABSTRACT

We have previously shown that in normolipidemic healthy adults, plasma ASP increases postprandially and is produced *in vitro* by cultured differentiated human adipocytes. The present studies were undertaken to examine the influence of specific plasma components on endogenous ASP production in cultured human adipocytes. The results demonstrate that neither glucose nor fatty acids (over a wide range of concentrations) had any substantial effect on ASP production. Insulin increased ASP production up to two-fold ($208\% \pm 18\%$, $p < 0.01$). However the most profound increase in ASP was generated by the addition of chylomicrons to the cell culture medium. Chylomicrons (CHYLO) obtained from postprandial plasma increased ASP production in a time- and concentration dependent manner, producing up to a 150 fold increase in ASP at the highest concentration of CHYLO tested ($500 \mu\text{g triacylglycerol/mL medium}$ ($p < 0.001$)). By contrast, VLDL, HDL and LDL had only marginal effects. The effects on ASP paralleled the changes in adipocyte C3 secretion (the precursor protein of ASP). As with ASP, glucose, oleate, insulin and hepatic lipoproteins (VLDL, LDL and HDL) had little or no effect on C3 secretion. In contrast, CHYLO had an even greater effect on C3 secretion than on ASP generation. Finally, the effects of CHYLO on generation of ASP and C3 were not dependent on lipolysis of CHYLO by LPL. These results are consistent with the changes in plasma ASP seen postprandially, and suggests a role of ASP as a positive feedback regulator of triacylglycerol synthesis in adipose tissue.

Supplementary key words: triacylglycerol, postprandial metabolism, adipose tissue, complement C3

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Abbreviations:

ASP	:	acylation stimulating protein
BSA	:	bovine serum albumin
C3	:	complement C3
CHYLO	:	chylomicrons
DMEM/F12	:	Dulbecco's minimum essential medium / Ham's F12
FBS	:	fetal bovine serum
HDL	:	high density lipoprotein
LDL	:	low density lipoprotein
LPL	:	lipoprotein lipase
MEM	:	minimum essential medium
PBS	:	phosphate buffered saline
RIA	:	radioimmunoassay
VLDL	:	very low density lipoprotein

Section 2.2: Introduction

The role of adipose tissue in the storage and release of energy is well-known. Fatty acids from adipose tissue are mobilized in response to specific stimuli through the action of hormone-sensitive lipase. The activity and regulation of this enzyme have been well characterized (133). Classically, interaction of a lipolytic hormone with the cell surface of the adipocyte results in activation of adenylate cyclase, increasing the production of cAMP, which activates protein kinase A. This enzyme, in turn, phosphorylates hormone sensitive lipase, thus activating it. To oppose this action, insulin stimulates phosphodiesterase which catabolizes cAMP, preventing activation of hormone sensitive lipase.

In contrast to lipolysis, although the enzymatic sequence for triacylglycerol synthesis is well known, none of the enzymes have been purified, nor is their regulation well characterized (134). Triacylglycerol molecules are formed through sequential enzymatic reactions in which fatty acid molecules are esterified to a glycerol-3-phosphate backbone. Both phosphatidate phosphohydrolase (which catalyses the dephosphorylation of phosphatidate to form diacylglycerol) and diacylglycerol acyltransferase (which catalyses the final esterification reaction to form triacylglycerol from diacylglycerol) have been implicated as the rate-limiting step (135,136). To date, insulin has been considered to be the main factor responsible for stimulation of triacylglycerol synthesis in adipose tissue. In fact, insulin does have a profound effect on glucose transport and on inhibition of lipolysis

mediated by hormone-sensitive lipase, but has little effect on the fatty acid esterification process itself, which ultimately produces triacylglycerol (137).

Although triacylglycerol storage has usually been considered to be the main function of adipose tissue, more recently, it has been recognized as a secretory organ (138). In addition to lipoprotein lipase (LPL), adipocytes are also a source of cholesterol ester transfer protein (139), apoE (70), estrogen (140), angiotensinogen (141) and tumor necrosis factor (TNF) (142) as well as the widely publicized discovery of leptin, the obese gene product responsible for the mutation in ob/ob mice (143,144).

The discovery that adipose tissue also produces and secretes discrete proteins of the alternate complement pathway evolved from the work of Spiegelman and colleagues on murine 3T3-L1 clonal adipocyte differentiation (63,64). They demonstrated that murine cultured adipocytes synthesize and secrete a novel protein, in a differentiation dependent manner, which was named Adipsin (63). Cloning from human adipocytes indicated that adipsin is homologous to human plasma factor D and is one of the proteins involved in the alternate complement pathway (64). Similarly, complement C3 and factor B are also expressed and secreted in a differentiation dependent manner in cultured adipocytes and mouse adipose tissue (64). These observations did not, however, define any specific function for the system, but interestingly, coincided with the molecular identification of Acylation Stimulating Protein (ASP) by our group (145).

Upon purification, ASP, a human plasma protein, was found to be identical to C3adesArg (145), a cleavage product generated through the specific action of factor B and adipsin (factor D) on C3 protein (64). ASP is produced in differentiated human adipocytes to a much greater extent than in human preadipocytes (43,74). The increase in ASP production is differentiation dependent, is associated with increased mRNA of the three factors needed: C3, adipsin and factor B, and precedes the profound increase in triacylglycerol synthetic capacity seen in these cells (74). The production of ASP is correlated with the size of the adipocytes (43) and is dependent both on the secretion of all three proteins (C3, factor B and adipsin) from adipocytes and on the appropriate activation of the enzymatic cleavage.

ASP was initially discovered based on its functional activity (145,146). ASP actively stimulates triacylglycerol synthesis in human skin fibroblasts and to a much greater extent in human adipocytes (145). ASP also stimulates glucose transport in fibroblasts (19), adipocytes (46), and muscle cells (147) and does so through translocation of the glucose transporters (GLUT 1, GLUT 4, and GLUT 3) from intracellular pools to the plasma membrane. ASP action is achieved through interaction with the cell membrane, which results in stimulation of the second messenger diacylglycerol (124). Diacylglycerol then mediates stimulation and translocation of protein kinase C producing a downstream stimulation of triacylglycerol synthesis (124). Differentiated adipocytes are more responsive to ASP stimulation than are preadipocytes (43).

These findings suggest that the storage of triacylglycerols in adipocytes may, in fact, be regulated at the cellular level by the tissue itself and therefore provide a positive feedback stimulus. We have previously shown that plasma ASP is modulated through dietary intake. Prolonged fasting in obese subjects results in profound decreases in plasma ASP (148). Postprandially, ASP increases following a fatload (132), and the present studies were undertaken to examine the influence of specific plasma components on endogenous ASP production in cultured human adipocytes.

Section 2.3: Materials and Methods

Oleic acid (sodium salt), bovine serum albumin-essentially fatty acid free (BSA), collagenase Type II and all other tissue culture grade compounds were from Sigma (St Louis, MO). General chemicals were from Fisher Scientific (Nepean, Canada). All tissue culture medium, Dulbecco's-phosphate buffered saline (D-PBS), fetal bovine serum (FBS) and all other tissue culture supplies were from Gibco (Gaithersburg, MD) or Flow Laboratories (Mississauga, Ontario).

Section 2.3.1: Culture of human differentiating adipocytes

Human adipose tissue was obtained with informed consent from patients undergoing reduction mammoplasty and then processed as previously reported (43). Briefly, adipose tissue was cleaned of connective tissue and small blood vessels, then minced and treated with collagenase. The cell suspension was centrifuged to pellet the stromal-vascular cells (containing the preadipocytes) and was subsequently treated with the lysing buffer for 10 min to lyse the red blood cells. After filtration through a 50 μ m filter and gentle centrifugation, the cell pellet was resuspended in minimum essential medium (MEM) containing 10% FBS. Preadipocytes were plated out on 24 well culture plates (10 g of cleaned tissue per 24 well plate). After 24 hours, cells were changed to serum-free Dulbecco's Minimum Essential Medium/Ham's F12 medium (DMEM/F12) supplemented with 7.5 mg/L insulin, 1 μ M dexamethasone, 33 μ M biotin, 17 μ M pantothenate and 0.2 nM triiodothyronine (74). Differentiating adipocytes were maintained in a 37°C incubator with 5% CO₂ and the medium was changed on the cells twice a week for a

total of three weeks at which time the cells exhibited clear adipocyte morphology with multiple fat droplets.

Section 2.3.2: Experimental Incubation

On the 21st day of culture, differentiated adipocytes were changed to serum-free, supplement-free DMEM/F12 overnight prior to initiation of experiments. The next day, cells were exposed to the medium supplemented with different plasma components as indicated. Basal ASP production was linear for up to 8 hours (data not shown); hence, the incubation time chosen for the experiments was 6 hours. For the oleate experiments, oleic acid complexed to BSA in a 5:1 molar ratio as described by Van Harken (149) was added to the cells up to a final concentration of 0.8 mM. For the chylomicron (CHYLO) time course experiments, medium was changed anew to serum- and supplement-free medium. To this medium, CHYLO were then added at a concentration of 50 µg triacylglycerol/mL medium for the last 0, 2, 4, 6, 8 and 24 hours of the 24 hour incubation period.

In all experiments, following incubation the medium was removed and frozen immediately at -70°C for later analysis of medium ASP and C3 levels. The cells were washed twice with ice cold PBS, 0.5 mL of 0.1 N NaOH was added to the cells and cell proteins were measured by the method of Bradford (150) using a commercial kit (Bio-Rad, Hercules, CA).

Section 2.3.3: Medium ASP Determination

ASP was measured in the medium of cultured human differentiating adipocytes following incubation of the cells in serum- and supplement-free medium with various additions using a radioimmunoassay (RIA) kit specific for C3adesArg (Amersham, Oakville, Canada) with values expressed as nmol ASP/mg cell protein. The use of the commercial RIA for C3adesArg for ASP determination has been validated in detail previously (43,74).

Section 2.3.4: Medium C3 Determination

Medium C3 was determined by sandwich ELISA immunoassay. Murine monoclonal antibody to the C3d fragment of C3 (Quidel, San Rafael, CA) was coated at 1 µg/mL in PBS (100 µL per well) overnight at 4°C and blocked with 1.5% BSA for 2 hours. The plate was washed three times with wash solution (0.05% Tween 20 in 0.9% NaCl) between every step. A standard solution (0 to 10 ng/mL) of C3 (Calbiochem, San Diego, CA), as well as test samples (conditioned culture media diluted appropriately) and in house control samples were added at 100 µL per well. The plate was incubated for 1 hour at 37°C, followed by incubation for 1 hour at 37°C with 100 µL goat polyclonal anti-C3 (Quidel), diluted appropriately (1:5000) in PBS-0.05% Tween 20. The plate was then incubated for 30 minutes at 37°C with 100 µL rabbit anti-goat IgG conjugated to horseradish peroxidase (1:1250, Sigma, St Louis, MO) diluted in PBS-0.05% Tween 20. Following the final wash, the color reaction was initiated with 100 µL o-phenylamine dihydrochloride (1 mg/mL) in 100

mM Na citrate, 0.05% Tween 20. After visual development the reaction was stopped with 50 μ L of 4 N H_2SO_4 and absorbance was read at 490 nm.

Section 2.3.5: Lipoprotein Isolation

Blood was obtained from healthy subjects with normal lipoprotein profiles and collected on ice into Vacutainer Tubes containing EDTA as anticoagulant. Plasma was immediately isolated by low-speed centrifugation at 4°C and the lipoproteins were subsequently separated by discontinuous preparative ultracentrifugation according to the procedure of Havel *et al.* (151). In summary, fresh plasma was initially layered under a salt solution of density 1.006 g/mL. CHYLO were isolated after centrifugation for 30 minutes at 30 000 rpm at 11°C. The infranate from the initial step was again overlaid with 1.006 g/mL solution and centrifuged for 18 hours at 40 000 rpm (100 000 x g) at 11°C. VLDL was recovered in the top 2 mL fraction. LDL was isolated from the infranate obtained in the second step by increasing solution density from 1.006 g/mL to 1.063 g/mL. The supernatant fraction was removed after centrifugation at 40 000 rpm (100 000 x g) for 20 hours. Finally, the remaining infranate was used for HDL isolation after increasing the solvent density to 1.21 g/mL and centrifuging for 48 hours at 40 000 rpm (100 000 x g) at 11°C. LDL and HDL fractions were dialysed overnight in PBS at 4°C. Triacylglycerol and cholesterol concentration of the lipoprotein fractions were measured using commercially available assays from Boehringer Mannheim (Laval, Quebec).

Section 2.3.6: Statistics

Values are reported as means of experiments (with all determinations for each point in each experiment performed in triplicate) \pm standard error of the mean (sem). Statistical significance was set at $p=0.05$ and was determined using either one-way ANOVA or paired Student's t-test as indicated in the results or figure legends where $p=NS$ indicates not significant.

Section 2.4: Results

We have previously demonstrated that ASP is produced by cultured human differentiated adipocytes and that its production increases proportional to differentiation of adipocytes (43,74). The aim of the present study was to determine whether ASP production could be influenced and identify potential physiological stimulatory factors for this.

Human preadipocytes were obtained from adipose tissue and differentiated into adipocytes in culture over the period of three weeks as described in Materials and Methods. At this point, the adipocytes were changed to fresh serum- and supplement-free medium for the indicated times and the amount of ASP produced by control cells was then measured. ASP production was found to be linear up to 8 hours and for all incubations, unless otherwise indicated, medium ASP was measured after a 6 hour incubation period.

Since fatty acids and glucose provide essential building blocks for triacylglycerols and ASP is very effective in stimulating both triacylglycerol synthesis and glucose transport in human adipocytes we first examined the effects of these two substrates on ASP production by cultured human differentiated adipocytes. As shown in Figure 2.1, ASP generation in culture medium was measured by RIA in the presence of increasing concentrations of oleic acid and glucose. These concentrations represent physiological values in humans ranging from fasting to postprandial levels. Figure 2.1 (left panel) represents data obtained from cells that

were exposed for 6 hours to increasing concentrations of glucose (5.0 mM to 55.0 mM) where basal ASP = 35.2 ± 2.1 pmol/mg cell protein. The amount of ASP generated by the differentiated adipose cells in the medium under different glucose concentrations did not differ overall from the control values at any glucose concentration as determined by ANOVA. There was a slight decrease ($-49\% \pm 12$) at a glucose concentration of 16 mM ($p < 0.05$). The results from experiments in which increasing concentrations of oleic acid complexed to BSA (up to 0.8 mM) were added to the culture medium are also shown in Figure 2.1 (right panel) where basal ASP = 31.1 ± 10.4 pmol/mg cell protein. Again, the amount of ASP generated by the cells differed little from the baseline other than the slight, but significant increase at a very low concentration. At 0.025 mM the increase was 42% ($p < 0.05$) and at 0.8 mM it was 25% ($p < 0.05$) as determined by ANOVA.

The capacity of cultured human adipocytes to generate ASP was also examined under different concentrations of insulin ranging from fasting to postprandial to pharmacological (up to 100 mU/mL) concentrations. Insulin has a well documented effect on triacylglycerol synthesis through its action on glucose transport and inhibition of hormone-sensitive lipolysis (137). It is also an essential component necessary for differentiation of both mouse and human adipocytes (152). With increasing medium insulin concentrations there was a slow but steady increase in ASP in the medium where basal ASP = 11.3 ± 3.9 pmol/mg cell protein. The ASP increased a maximum of 2.1 ± 0.2 fold ($208 \pm 18\%$) at a concentration of 50 mU/mL of insulin, $p < 0.01$ as determined by ANOVA (Figure 2.2).

We have previously shown that plasma ASP increases following an oral fatload in normolipidemic subjects concomitant with an increase in plasma triacylglycerol (153). We therefore tested the ability of various lipoprotein fractions to stimulate ASP production. Lipoprotein particles were isolated by sequential discontinuous ultracentrifugation from normolipidemic plasma as described in the Materials and Methods section. Each lipoprotein fraction was assayed for the concentrations of triacylglycerol and cholesterol. CHYLO and VLDL were added to the cultured cells at a concentration of 50 μg of lipoprotein triacylglycerol/mL and LDL and HDL were added at a concentration of 25 μg of lipoprotein cholesterol/mL. These concentrations represent low plasma levels. Table 2.1 shows the changes in the medium ASP levels following a 6 hour incubation with the indicated concentrations of CHYLO, VLDL, LDL and HDL. As compared to control values there was on average a 12 fold increase ($p < 0.025$) in the amount of ASP generated by the cells after their exposure to CHYLO whereas all other lipoprotein fractions had no significant effect on the cells as compared to CHYLO. Nonetheless, the effects of VLDL, LDL and HDL on ASP generation were greater than the effects of fatty acids and comparable to those of insulin. It should be pointed out that there is plasma ASP associated with the CHYLO lipoprotein fraction, but background ASP was subtracted from the total medium ASP. In all cases, the amount of plasma ASP associated with the CHYLO fraction was not greater than 10-20% of the total medium ASP generated by cultured adipocytes. There was no plasma ASP associated with the other lipoprotein fractions.

This profound stimulatory effect of CHYLO on ASP production was further examined. The effects of varying concentrations of CHYLO on cultured human adipocytes are shown in Figure 2.3. The data demonstrate that increasing concentrations of CHYLO increase the amount of ASP that is generated in the medium of human adipocytes. Concentrations up to 500 $\mu\text{g}/\text{mL}$ are shown in the graph, lower concentrations (up to 50 $\mu\text{g}/\text{mL}$) are shown in the inset. At both low and high concentrations, the increase in ASP is proportional to the amount of CHYLO reaching 150 fold at the highest concentration of 500 μg lipoprotein triacylglycerol/ mL ($p < 0.001$). We did not observe a plateau at the CHYLO concentrations tested. However, one must keep in mind that the concentrations chosen were still well within the range of physiological postprandial levels; 500 $\mu\text{g}/\text{mL}$ is equivalent to 50 mg/dL plasma triacylglycerol and postprandial increases can be substantially larger than that. It should also be noted that in each experiment, cells were derived from a different subject. Therefore there is a certain amount of variability in the extent to which the cells differentiate, and thus in the basal amount of ASP produced (43,74). Similarly, the responses to CHYLO stimulation vary from 4 fold to 20 fold, although they are significantly increased in all cases.

Next, to investigate the response rate to the CHYLO stimuli, the adipocytes were exposed to CHYLO at a selected concentration of 50 μg triacylglycerol/ mL for varying incubation periods. In this experiment the cells were changed to fresh serum-free and supplement-free medium 24 hours before the medium was collected

for ASP determination. The CHYLO were then added during the final 0, 2, 4, 6, 8 and 24 hours of the incubation period. Therefore, the 0 time point represents the amount of ASP generated basally over the 24 hour time period. As shown in Figure 2.4 the amount of ASP generated when CHYLO were present for the last 2 hours of the 24 hour time period was almost the same as the baseline or 0 time point. The amount of ASP present increased rapidly when CHYLO were present during the last 4 to 6 hours incubation reaching a plateau thereafter which represents a 4 fold increase over baseline in these experiments ($p < 0.01$ as measured by ANOVA).

Effects on ASP accumulation in the medium could be the result of two actions: an increase in the cellular secretion of C3, the precursor molecule from which ASP is generated through enzymatic cleavage, or an increase in the proportion of C3 which is enzymatically converted to ASP. We therefore examined C3 production in the adipocytes and the effects of various postprandial factors. As shown in Figure 2.5, as with ASP, glucose, oleic acid and insulin had only minimal but significant effects on C3 production in the adipocytes. The amount of C3 produced was, on average, much greater than the amount of ASP produced on a molar basis. Therefore, under these incubation conditions, only a portion of the C3 secreted from adipocytes is converted to ASP.

The effect of various lipoproteins on C3 production in adipocytes was tested as for ASP. As shown in Table 2.2, neither VLDL, LDL nor HDL had any significant effect on C3 production. As with ASP, CHYLO had a profound stimulatory effect on

C3 production ($p < 0.0025$). There was a small amount of endogenous C3 associated only with the CHYLO, and this was subtracted from the total medium C3. Background CHYLO C3 levels did not exceed 10-20% of total medium C3.

We then tested the effects of a range of CHYLO concentrations on C3 production and these results are shown in Figure 2.6. Again, as with ASP, CHYLO had a profound and significant stimulation on C3 production at all concentrations $p < 0.0025$. The amount of C3 produced appeared to increase linearly up to 100 μg CHYLO TG/mL, and then began to level off at higher concentrations of CHYLO. Overall, the proportion of C3 that was converted to ASP was on average 12%.

LPL is made and secreted by adipose tissue and is necessary for hydrolysis of plasma lipoprotein triacylglycerol. In some cases, LPL was also added to the cultured adipocytes at a concentration of 0.25 U/mL. Addition of LPL alone had no effect on ASP generation by the cells ($p = \text{NS}$ as determined by two mean t-test, results not shown). Addition of LPL to the incubations with the lipoproteins (CHYLO, VLDL, LDL or HDL) also had no additional effect on generation of ASP. This is not surprising, since the differentiated adipocytes are capable of secreting active lipoprotein lipase. We then tested to see if lipolysis was necessary for the CHYLO effect on ASP and C3 production. As shown in Figure 2.7, the addition of BSA alone (2 mg/mL) to the culture medium had no effect on basal C3 or ASP production. As well, addition of BSA with CHYLO had no effect on the CHYLO induced increase in C3 and ASP. We also tested the effect of adding increasing concentrations of

tetrahydrolipstatin (THL). THL is an effective lipoprotein lipase inhibitor which acts via binding to the active site (154). THL was added to the adipocytes at 2 different concentrations concurrently with the CHYLO, and both C3 and ASP production were assessed. As shown in Figure 2.7, addition of THL did not prevent the CHYLO induced increase in C3 and ASP, nor did THL have any effect on basal C3 and ASP production. This suggests that the release of C3 and production of ASP is not secondary to a detergent effect caused by the hydrolytic production of large amounts of fatty acid. Finally, we also examined the interaction of insulin and CHYLO on C3 and ASP production. Although the addition of insulin with CHYLO had no additive effect on ASP production, there was an additive effect with respect to C3 production as compared to CHYLO alone ($p < 0.01$).

Section 2.5: Discussion

The striking effects of ASP on triacylglycerol synthesis as well as on glucose transport in human fibroblasts and particularly in human adipocytes (19,43,46,74,145-147) have made it clear that the triacylglycerol synthetic pathway is regulated and that ASP is a key regulator of this pathway. This is particularly relevant since ASP not only has marked effects on adipocyte lipid metabolism, but is also produced by mature adipocytes (43,74). Based on these observations, the goal of this study was to define the particular stimuli that modulate ASP production. The present results provide the first evidence that ASP generation from human adipocytes can be driven by specific postprandial plasma components.

Neither glucose nor oleate had significant effects on ASP generation in the culture medium. The fact that glucose and free fatty acids did not cause increases in medium ASP levels was initially surprising since both factors increase postprandially. However, it should be noted that fatty acids are also generated through the action of hormone sensitive lipase in adipocytes for the purpose of releasing fatty acids for transport to other tissues (133). An increase in ASP and C3 generation at this point would indeed be paradoxical and would result in a futile cycle of lipolysis/reesterification. The results indicate that insulin does significantly increase ASP and C3 production. Increases in insulin may be one mechanism by which plasma ASP increases postprandially, particularly at the early stages (1-2 hours) when insulin levels are elevated.

We have shown in fatload studies that plasma ASP levels rise concurrently with the rise of plasma triacylglycerols (147). We speculated, therefore, that the triggering signal might lie within the lipoprotein particles which carry dietary fat in the form of triacylglycerols. To test this hypothesis, experiments were performed in which different plasma lipoprotein fractions (CHYLO, VLDL, LDL and HDL) were tested for their effect on ASP and C3 production. Addition of CHYLO to the cultured adipocytes caused dramatic elevations in both medium ASP and the precursor molecule to ASP, C3, and these effects were both time and concentration dependent. This suggests that the signaling mechanism is on the CHYLO particle itself. Certainly, the apoprotein composition of CHYLO is different from that of the other lipoproteins. One obvious difference is the presence of apoB48 on CHYLO, whereas the other apoB containing lipoproteins have only apoB100 (155). But there are many other differences as well, including differences in lipid composition and lipid soluble factors such as retinol ester (156) and future experiments will focus on defining the key components in CHYLO responsible for the effects on ASP and C3 production. Based on the magnitude of the increase in ASP achieved with the addition of *in vivo* concentrations of CHYLO, this mechanism is most likely the major physiological source of the postprandial increase in ASP.

What, then, is the mechanism by which insulin and CHYLO stimulate ASP production? Production of ASP is dependent on three protein factors (C3, factor B and adipsin), two of which are consumed during the reaction (C3 and factor B) whereas adipsin, as a catalytic enzyme, is not. Therefore the levels of bioactive ASP

could be increased by increasing cellular synthesis and secretion of any one of these three proteins (substrates or enzyme), or by enhancing the catalytic cleavage reaction itself. In murine adipocytes, preformed adipsin is stored in intracellular secretory vesicles and translocation and secretion of adipsin is triggered through the action of insulin (157). In human adipocytes, insulin may affect not only the secretion of C3 (as shown here) but also the acute secretion of adipsin and factor B, although an effect on catalytic activity cannot be ruled out. Insulin appeared to have only minimal effects when added alone to cells (maximum two-fold increase in ASP), although it enhanced markedly the CHYLO effect on C3 production.

CHYLO, also, may be acting via an effect on either catalytic conversion of C3 to ASP, or on the secretion of C3/B/Adipsin through effects at the mRNA or protein level. The present study clearly documents a profound stimulatory effect of CHYLO on C3 production by adipocytes. However, we cannot rule out an additional effect on catalytic conversion of C3 to ASP. Certainly, catalytic conversion cannot occur in the absence of an "activated" C3-B complex and it has been suggested that this complex can only be formed through specific cell or membrane interaction (158); C3, B and Adipsin mixed together in solution will not produce ASP without artificial activation (43,64,159). Thus CHYLO may also provide the specific surface interaction which is required for activation. In addition, however, a key component of CHYLO may interact directly with adipocytes and stimulate increased secretion not only of C3, but also of B or Adipsin. Clearly, all these potential mechanisms must be investigated in future experiments.

These *in vitro* experiments are not without physiological relevance. Postprandial generation of ASP will result in activation of adipose tissue and increase triacylglycerol synthesis as well as glucose transport (19,43,46,145,147). In this way, through autoregulation, the adipose tissue is primed to sequester the excess plasma glucose and dietary fatty acids much more efficiently via the ASP action on triacylglycerol synthesis. If this adipocyte-ASP positive feedback loop was ineffective, the resulting fatty acids would not be removed as quickly from plasma and the build-up of fatty acids in the microenvironment would result in inhibition of LPL action (160) and detachment of the LPL from the cell surface (161). Consequently, an increased flux of fatty acids or partially hydrolyzed lipoprotein particles containing detached LPL could flood the liver causing increased hepatic lipoprotein production (162,163).

Modulations of plasma ASP in response to dietary fat intake as well as the metabolic state of the individual both *in vitro* and *in vivo*, suggest that ASP may play a role in positive feedback regulation of adipose tissue fat mass. The present data, therefore, provide new and important insights into how the ASP pathway is integrated into the complex process of energy storage in adipocytes.

Section 2.6: Tables and Figures

Table 2.1: Effect of lipoproteins on ASP generation by human differentiated adipocytes

Addition	ASP Pmol/mg cell protein	p
PBS	38.1±2.1	
LPL	38.6±1.8	ns
CHYLO	474.0±94.0	p<0.025
VLDL	51.6±6.9	Ns
LDL	47.8±10.0	Ns
HDL	38.0±6.7	Ns

Human preadipocytes were differentiated in serum-free hormone supplemented medium over a period of three weeks. Differentiated adipocytes were then exposed to CHYLO or VLDL (at 50 µg lipoprotein triacylglycerol/mL medium), LDL or HDL (at 25 µg lipoprotein cholesterol/mL medium) for 6 hours. LPL was added to all lipoprotein fractions at 0.25 U/mL. Medium ASP was measured by RIA. The results are shown as pmol/mg cell protein ± sem for an average of 3 different subjects from 3 experiments each assayed in triplicate (n=9). Statistical significance was calculated using two mean t-test where p ns = not significant.

Table 2.2. Effect of lipoproteins on C3 generation by human differentiated adipocytes:

Addition	C3 Pmol/mg cell protein	P
PBS	10±3	
LPL	14±4.3	Ns
CHYLO	3009±670	p<0.0025
VLDL	26±13	Ns
LDL	18±5.6	Ns
HDL	0	Ns

Human preadipocytes were differentiated in serum-free supplemented medium over a period of three weeks. Differentiated adipocytes were then exposed to CHYLO or VLDL (at 50 µg lipoprotein triacylglycerol/mL medium), LDL or HDL (at 25 µg lipoprotein cholesterol/mL medium) for 6 hours. LPL was added to all lipoprotein fractions at 0.25 U/mL. Medium C3 was measured by ELISA. The results are shown as pmol/mg cell protein ± sem, for an average of 3 different subjects from 3 experiments each assayed in triplicate (n=9). Statistical significance was calculated using two mean t-test where p ns = not significant.

Figure & Legends:

Figure 2.1: Concentration dependent effect of glucose and oleate on ASP generation by human differentiated adipocytes: Human preadipocytes were differentiated in serum-free supplemented medium for 3 weeks. Cells were then exposed to increasing concentrations of glucose (left panel) or oleic acid complexed to BSA (right panel) over a period of 6 hours. Medium ASP was measured by RIA (pmol/mg cell protein) at each of the concentrations. The results are expressed as fold change in ASP \pm sem as compared to the control, where control values are defined as 1.0, * $p < 0.05$ by one way ANOVA for $n=3$ experiments from 3 different subjects each assayed in triplicate at each concentration ($n=9$). Basal ASP concentration was 35.2 ± 2.1 and 31.1 ± 10.4 pmol/mg cell protein for glucose and oleate experiments, respectively.

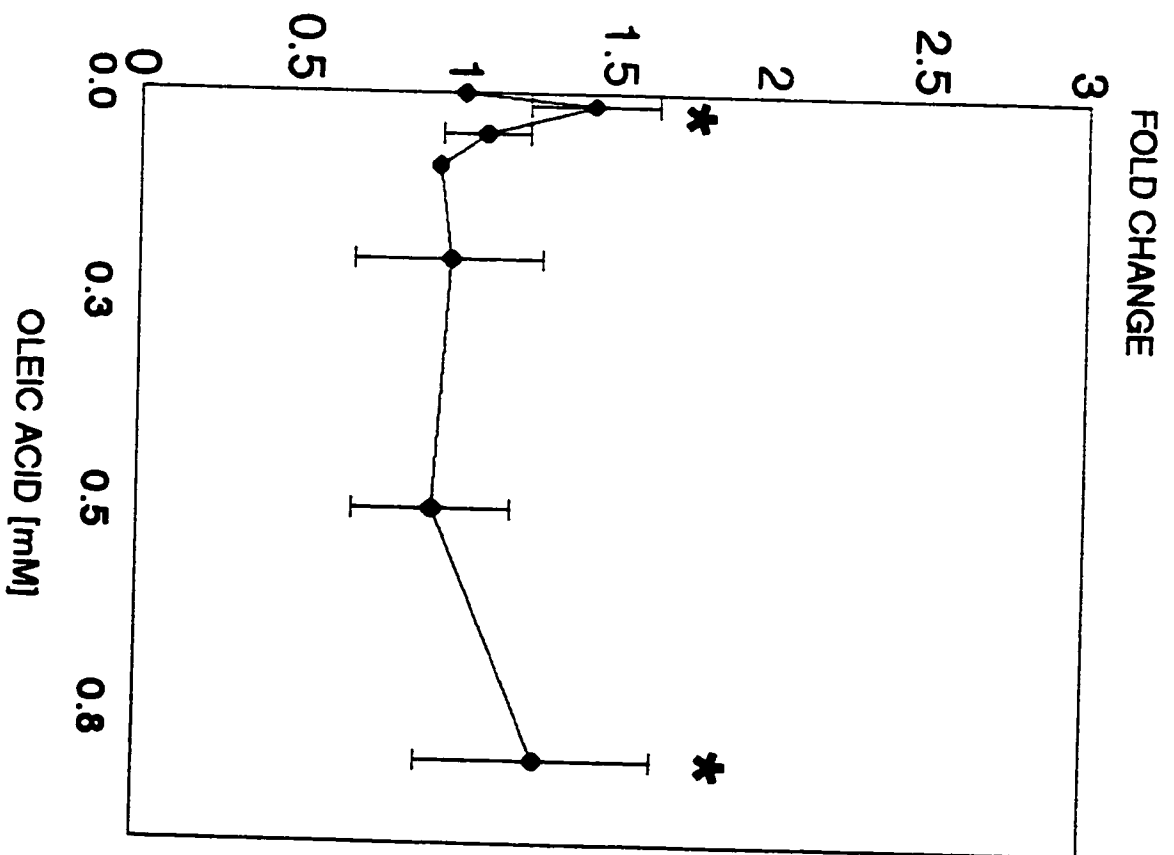
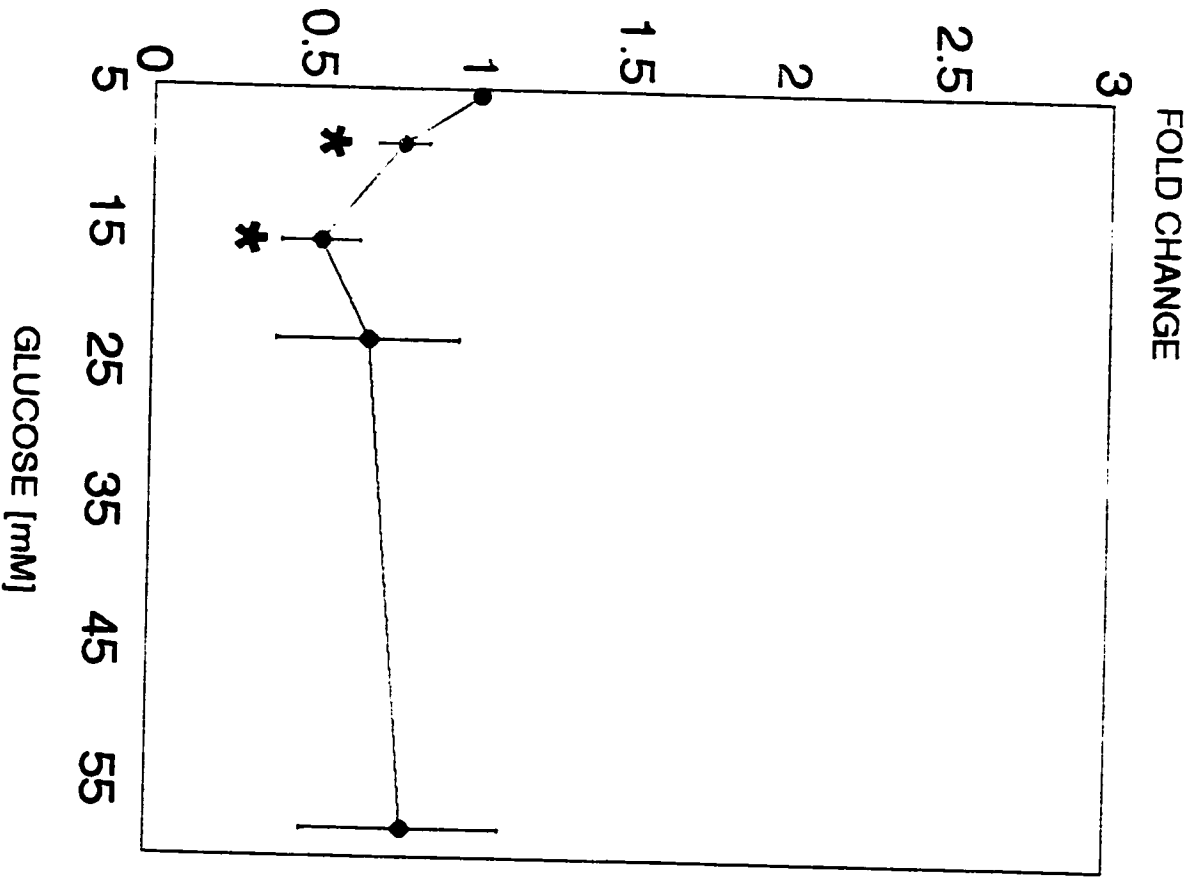


Figure 2.2: Dose dependent effect of insulin on ASP generation by human differentiated adipocytes: Human preadipocytes were differentiated in serum-free supplemented medium over a period of 3 weeks. Differentiated adipocytes were then exposed to increasing insulin concentrations for 6 hours. Medium ASP was measured by RIA as described in Methods and the results are expressed as fold change in medium ASP \pm sem as compared to the control, where control values are given as 1.0. n=3 experiments from three different subjects each assayed in triplicate (n=9), * p<0.05 and ** p<0.01. Basal ASP concentration was 11.3 \pm 3.9 pmol/mg cell protein.

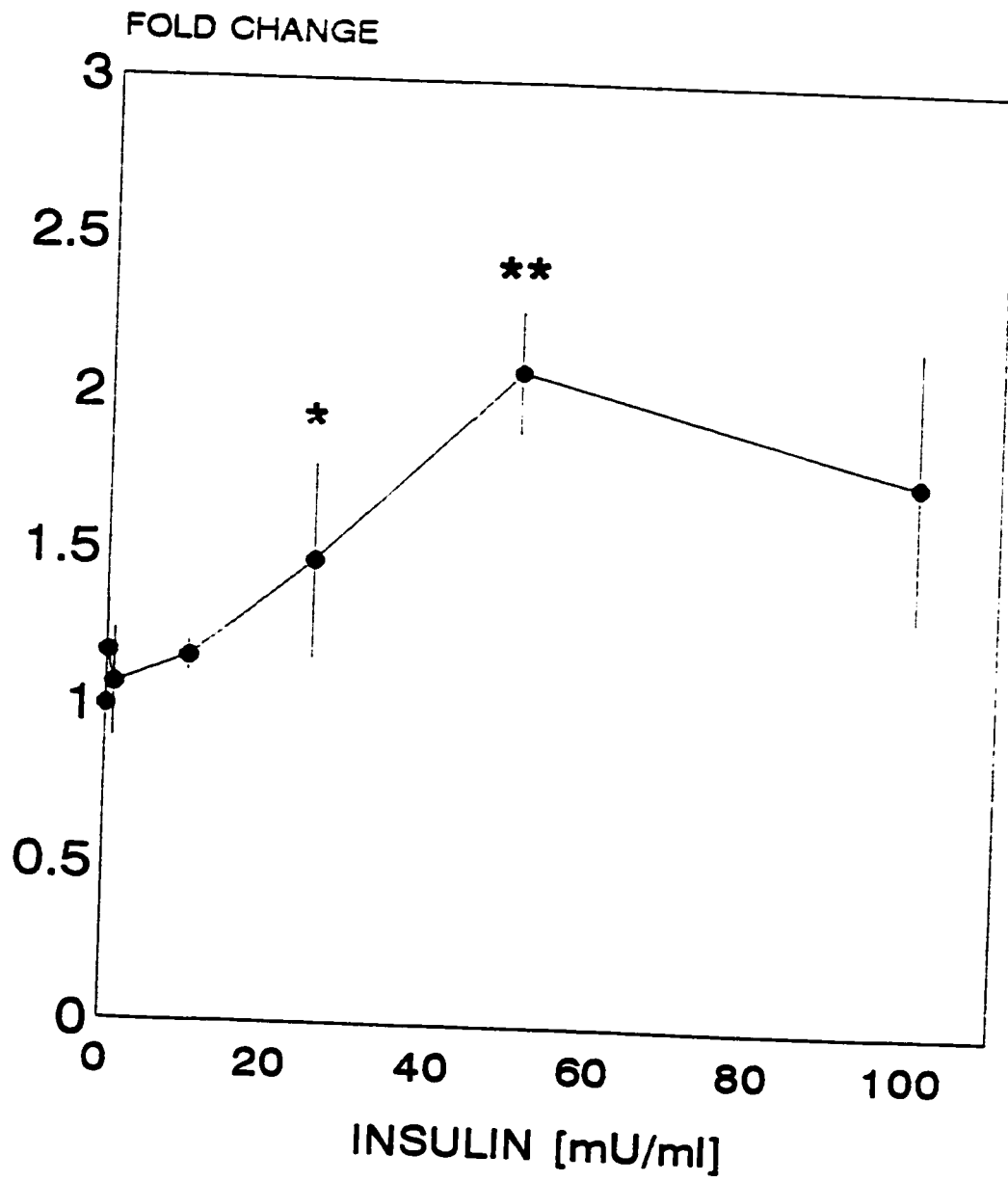


Figure 2.3: Dose dependent effect of chylomicrons on ASP generation by human differentiated adipocytes: Human preadipocytes were differentiated in serum-free supplemented medium over a period of three weeks. Differentiated adipocytes were then exposed to increasing concentrations of CHYLO (in μg lipoprotein triacylglycerol/mL medium) for 6 hours and ASP generated was measured in the cell medium by RIA. LPL was added to all CHYLO concentrations at 0.25 U/mL. The results are shown as pmol ASP/mg cell protein \pm sem for an average of 3 different subjects from 3 experiments each assayed in triplicate (n=9). Statistical significance was calculated by one-way ANOVA. Significance for the individual CHYLO concentrations was calculated by paired t-test where * $p < 0.05$.

ASP (pmol/mg cell protein)

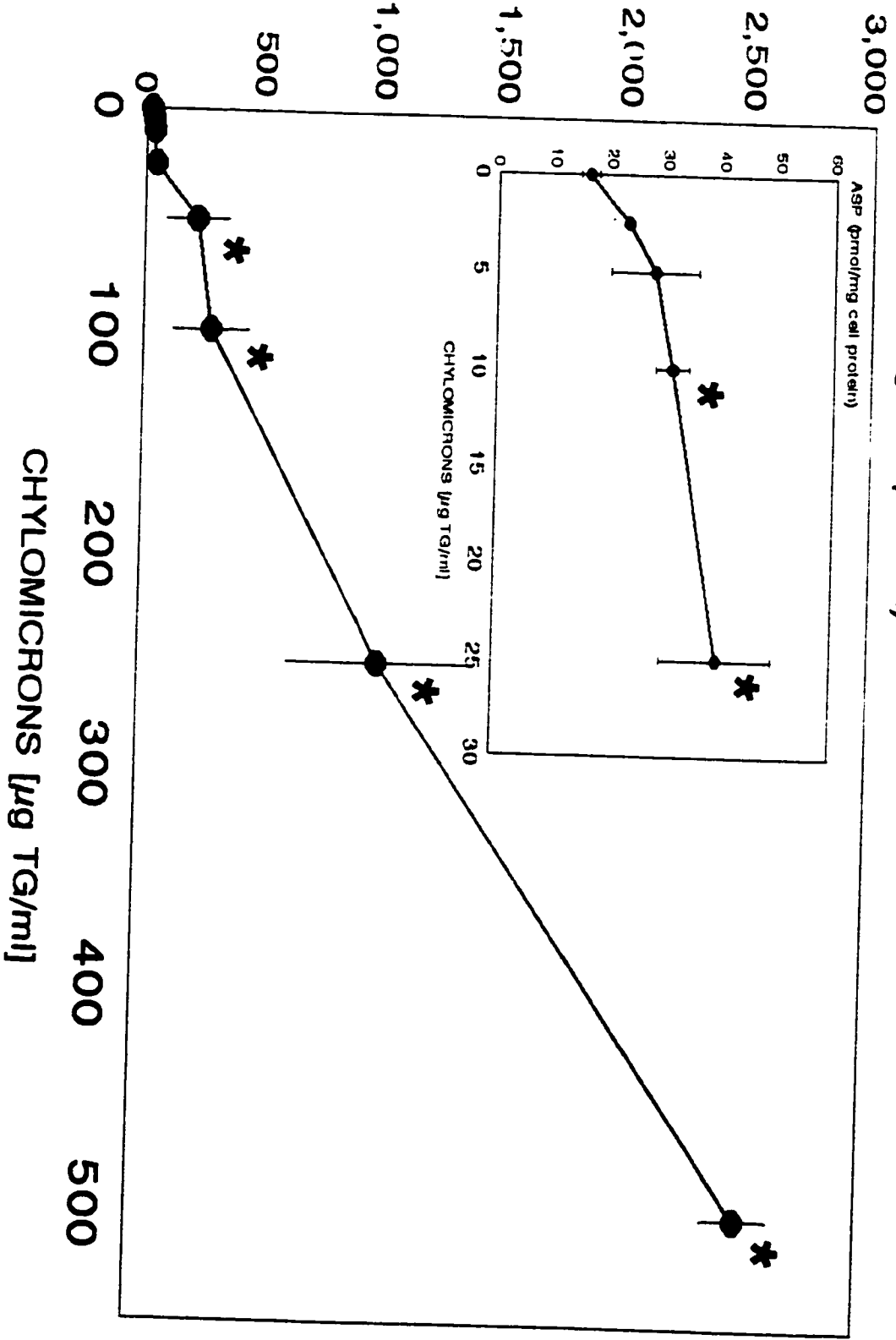


Figure 2.4: Time course of the chylomicron effect on ASP generation by human differentiated adipocytes: Human preadipocytes were differentiated in serum-free supplemented medium over a period of 3 weeks. Differentiated adipocytes were then changed to serum-free medium and exposed to CHYLO at 50 μ g lipoprotein triacylglycerol/mL medium for the indicated times. ASP levels were measured in the cell medium by RIA. The results are expressed as pmol/mg cell protein \pm sem for 2 experiments from 2 different subjects assayed in triplicate (n=6), * p<0.01 determined by ANOVA for all points except at 2 hours.

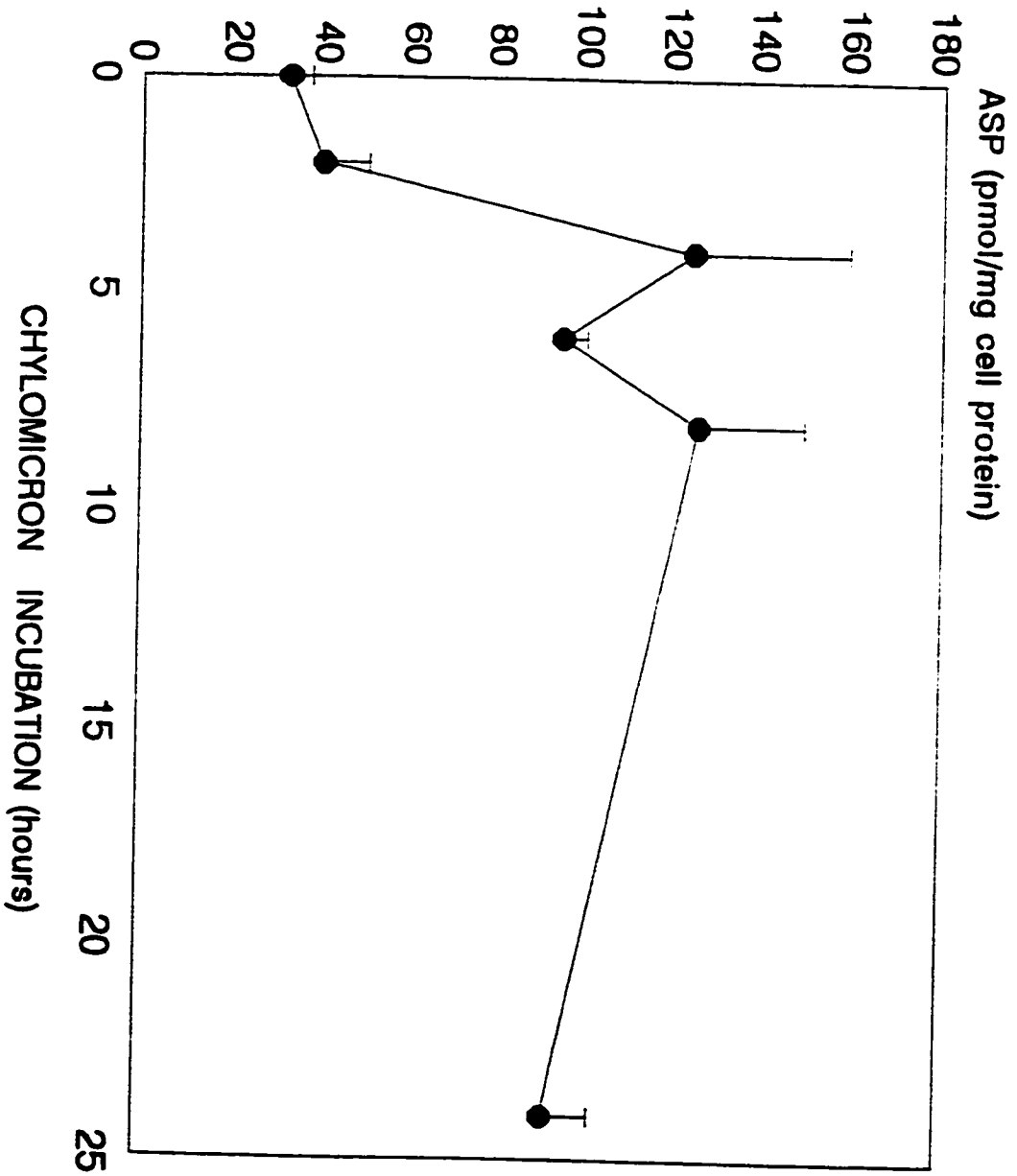


Figure 2.5: Concentration dependent effect of glucose, oleate and insulin on C3 generation by human differentiated adipocytes: Human preadipocytes were differentiated in serum-free supplemented medium for 3 weeks. Cells were then exposed to increasing concentrations of glucose (top panel, n=6), oleic acid complexed to BSA (middle panel, n=6) or insulin (bottom panel, n=9) over a period of 6 hours from 2-3 different subjects. Medium C3 was measured by ELISA (pmol/mg cell protein) at each of the concentrations. The results are expressed average C3 \pm sem (pmol/mg cell protein), * p<0.01 for all concentrations of glucose, oleic acid and insulin (except 10 mM glucose, p ns).

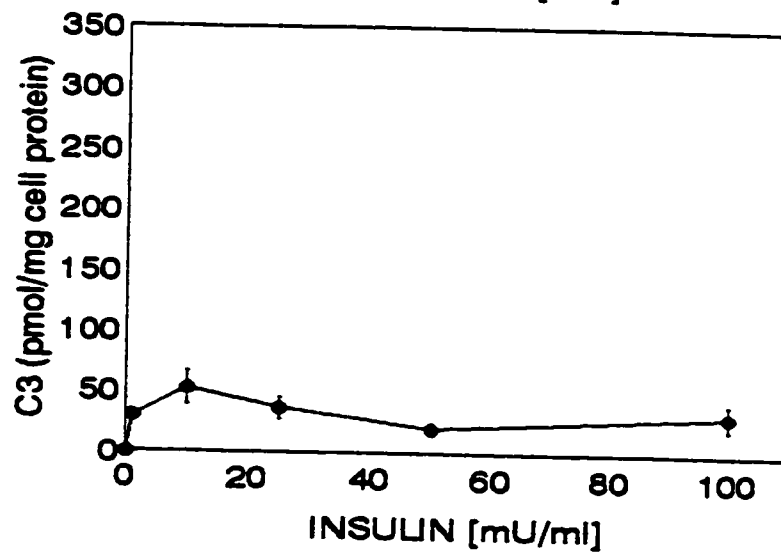
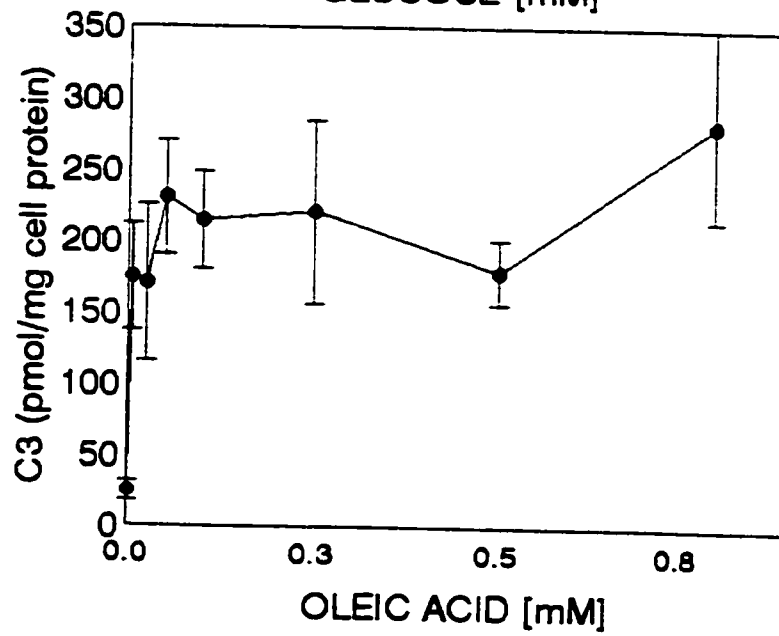
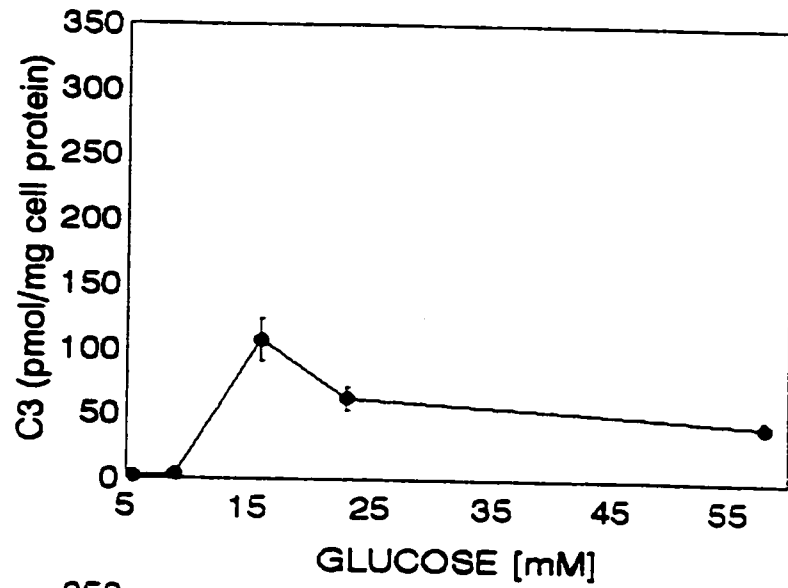


Figure 2.6: Dose dependent effect of chylomicrons on C3 generation by human differentiated adipocytes: Human preadipocytes were differentiated in serum-free supplemented medium over a period of three weeks. Differentiated adipocytes were then exposed to increasing concentrations of CHYLO (in μg lipoprotein triacylglycerol/mL medium) for 6 hours and C3 generated was measured in the cell medium by ELISA. LPL was added to all CHYLO concentrations at 0.25 U/mL. The results are shown as pmol/mg cell protein \pm sem for an average of $n=6$ from 2 different subjects. Significance for the individual CHYLO concentrations was calculated by t-test of CHYLO where * $p<0.0025$ for all concentrations.

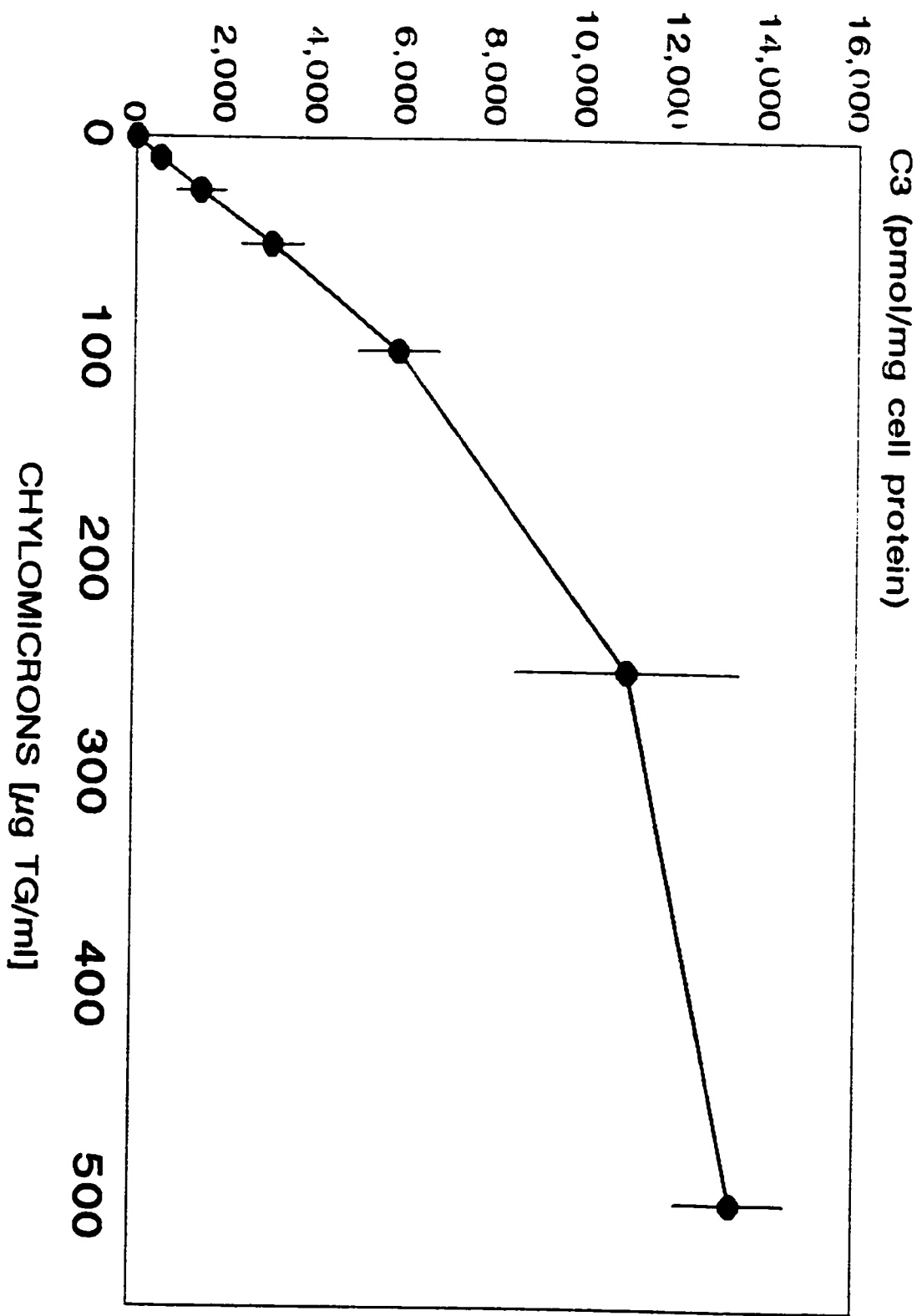
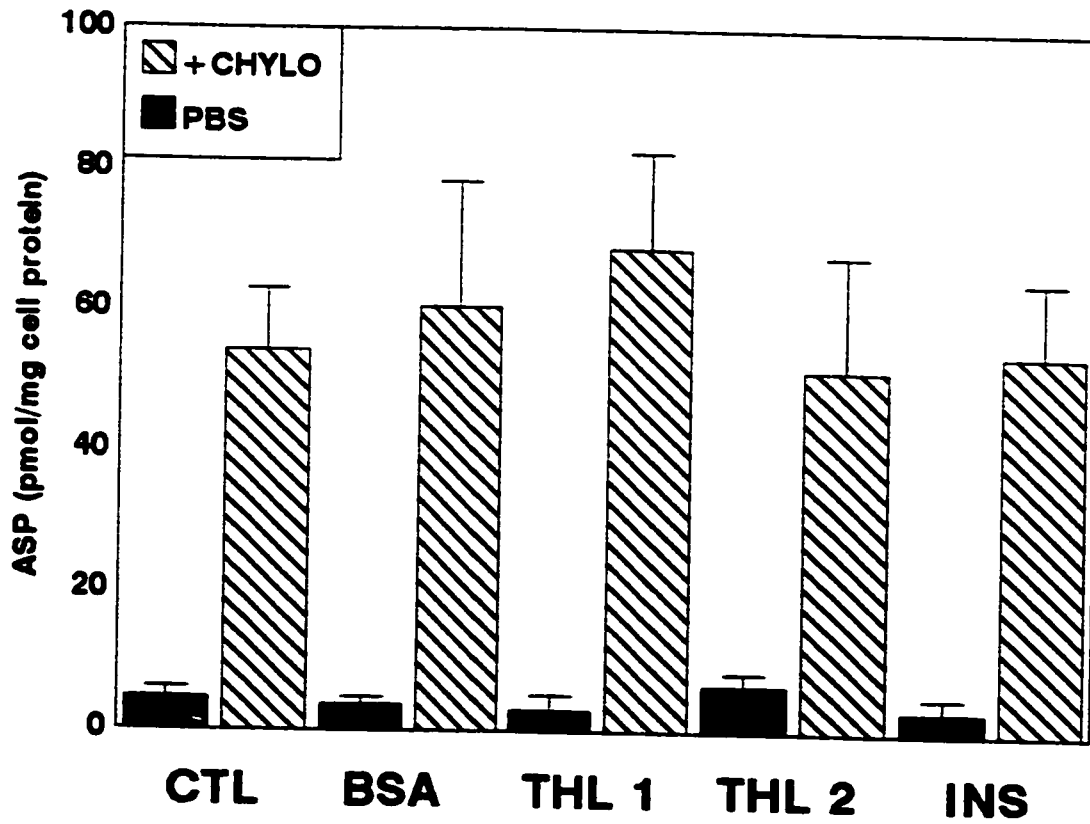
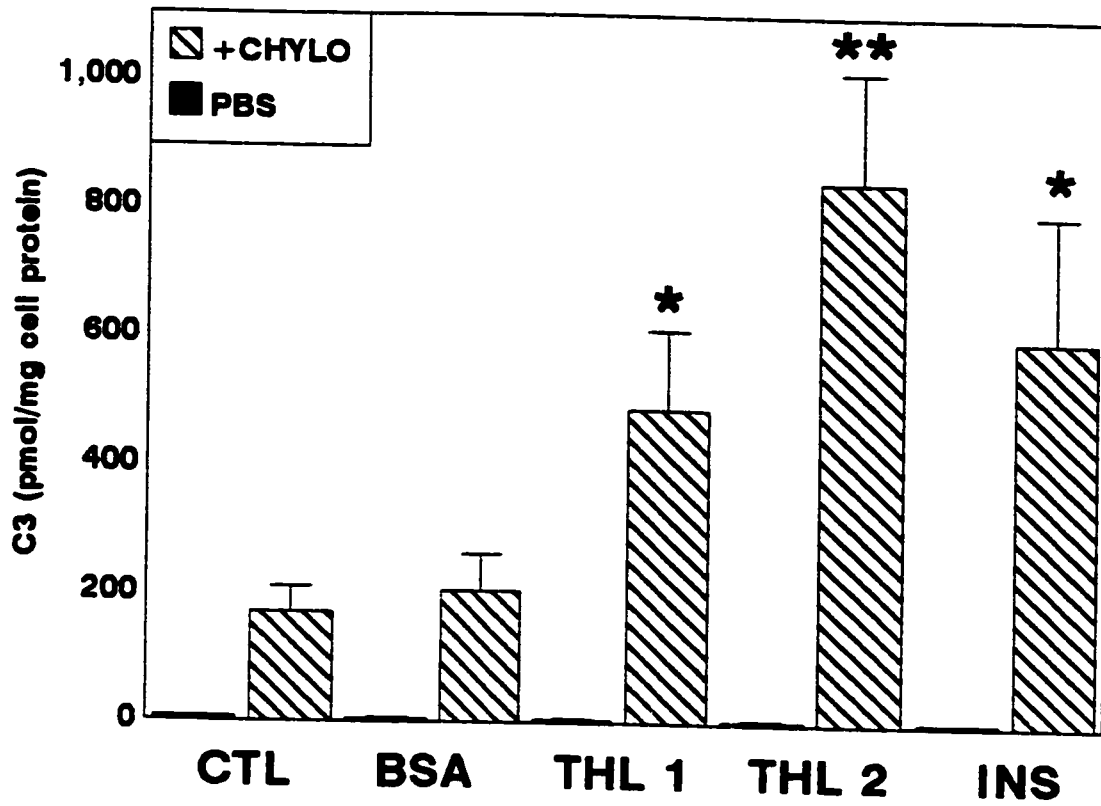


Figure 2.7: Modulation of the CHYLO mediated increase in medium C3 and ASP in human differentiated adipocytes: Human preadipocytes were differentiated in serum-free supplemented medium over a period of three weeks. Differentiated adipocytes were then exposed to the indicated conditions: insulin (50 mU/mL), BSA (2 mg/mL), or tetrahydrolipstatin (THL, 1 or 2 μ M) with (hatched bars) or without (solid bars) CHYLO (50 μ g lipoprotein triacylglycerol/mL medium) for 6 hours. C3 (top panel) and ASP (bottom panel) were measured in the medium as indicated in Methods. The results are shown as pmol/mg cell protein \pm sem for an average of $n=7$ from 2 different subjects from 2 experiments. All additions with CHYLO were significantly increased vs. no CHYLO for each set of additions ($p<0.005$) for both C3 and ASP. Significance for the individual CHYLO + additions vs. CHYLO alone was calculated by paired t-test where * $p<0.01$ and ** $p<0.005$.



PRELUDE ONE

Clearly CHYLO had the greatest effect on ASP and C3 production, more pronounced than any other effect. This raises two issues: (i) is this effect physiologically relevant? and (ii) how is this effect mediated? With respect to the first question, it is certainly relevant since it has been demonstrated in humans, *in vivo*, that there is a direct adipose tissue production of ASP, and this lab has also demonstrated that this production increases following a mixed meal reaching a maximal production 3-5 hours postprandially (132). Finally, the ASP production is increased of in obese subjects (127-131) and a net flux is apparent in the fasting state. To address the second issue, what is the physiological trigger that mediates the effect, we dissected the components of the CHYLO in order to identify the factor(s) responsible for the postprandial increase in C3 and ASP.

CHAPTER THREE
**CHYLOMICRON SPECIFIC ENHANCEMENT OF ACYLATION STIMULATING PROTEIN (ASP)
AND PRECURSOR PROTEIN C3 PRODUCTION IN DIFFERENTIATED HUMAN ASIPOCYTES**

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Thea Scantlebury[†], Magdalena Maslowska, Katherine Cianflone

Mike Rosenbloom Laboratory for Cardiovascular Research, McGill University Health Center, McGill University, Montreal, Quebec, Canada

[†] and Chemistry and Biochemistry Department, Concordia University, Montreal, Quebec, Canada

Short Title: CHYLO Specific Production of ASP in Adipocytes

Address Correspondence to:

Dr. Katherine Cianflone
Mike Rosenbloom Laboratory
for Cardiovascular Research
Room H7.35
McGill University Health Center
Royal Victoria Hospital
687 Pine Avenue West
Montreal, Quebec
H3A 1A1
Tel: 514-842-1231 ext. 5426
Fax: 514-982-0686
Email: mdkc@musica.mcgill.ca

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Abbreviations:

ACN	acetonitrile
ASP	Acylation Stimulating Protein
BSA	bovine serum albumin
CHYLO	chylomicron
FBS	fetal bovine serum
MEM	minimum essential medium
PBS	phosphate buffered saline
sem	standard error of the mean
T4	thyroxine
TTR	transthyretin

Section 3.1: ABSTRACT

Acylation Stimulating Protein (ASP) is a potent stimulator of adipocyte triacylglycerol storage. *In vivo* studies have shown that ASP production by adipocytes increases locally after a fat meal. Initial *in vitro* studies demonstrated increased production of ASP in the presence of chylomicrons. The present aim was to define the CHYLO component responsible. None of the apoproteins tested (AI, AII, AIV, CI, CII, CIII, E) were capable of stimulating C3 (the precursor protein) or ASP production. Rather, the active component is a non-lipid, loosely associated, trypsin sensitive molecule. HPLC fractionation of the CHYLO infranate proteins identified the critical protein as transthyretin (TTR) which binds retinol-binding-protein and complexes thyroxine and retinol. Addition of TTR alone, with lipid emulsion, or with respun CHYLO to human differentiated adipocytes had little effect on C3 and ASP production. By contrast, when transthyretin was added to CHYLO, C3 and ASP production were substantially enhanced up to 75 fold and 7.5 fold respectively, compared to the effect of native CHYLO alone. Finally, a polyclonal antibody against TTR could inhibit stimulation of C3 and ASP production by CHYLO (by 98% and 100% respectively) and by CHYLO infranate proteins (by 99% and 94% respectively). We hypothesize that TTR mediates the transfer of the active components from CHYLO to adipocytes which then stimulates increased C3 and ASP production. Thus the CHYLO provides the physiologic trigger of the ASP pathway.

Section 3.2: Introduction

Adipose tissue is recognised as an active organ releasing hormones, enzymes and proteins such as lipoprotein lipase (138), cholesteryl ester transfer protein (139), apo E (70), angiotensinogen (141), estrogen (140), tumor necrosis factor (143), and leptin (164). Both human and murine adipose tissue have been shown to synthesize and secrete complement C3, factor B and adipsin, proteins involved in the alternate complement pathway (43,64,73-75). Furthermore, complement C3, factor B, and adipsin have been shown to be synthesized and secreted in a differentiation dependent manner by adipocytes (64,73,74). The interaction of these three proteins results in the cleavage of complement C3 generating the bioactive protein Acylation Stimulating Protein (ASP/C3adesArg) (43,64,74) which was initially recognized as a small basic protein present in human plasma (145). ASP can actively stimulate triacylglycerol synthesis in human adipocytes through a co-ordinated effect on translocation of the glucose transporters (GLUT1, GLUT3, and GLUT4) (19,46,147) and increases in the activity of the enzyme diacylglycerol acyltransferase. These effects of ASP are mediated through the diacylglycerol protein kinase C pathway (124) via specific interaction with the cell surface. The most responsive target to the action of ASP is adipose tissue, which is also known as the primary tissue for storage and release of energy. Within the cell, free fatty acids are enzymatically esterified to a glycerol-3-phosphate backbone to form triacylglycerol, the main storage fuel for the body (165). The hormone sensitive lipase pathway then allows the release of energy in the form of free

fatty acids in response to appropriate stimuli. Thus, the physiological role of ASP is to regulate the synthesis and storage of triacylglycerol in adipose tissue (166).

Triacylglycerol-rich lipoproteins play a major role in the transportation of fatty acids to the tissues. Chylomicrons (CHYLO) are intestinally derived postprandial lipoprotein particles, which carry dietary fat in the form of triacylglycerols (132). In humans, venous and arterial measurements of ASP, triacylglycerol, and CHYLO triacylglycerol across an adipose tissue bed at fasting and after a meal suggest that the postprandial production of ASP is coordinated with the increase of triacylglycerol clearance (132). By what mechanism is this ASP pathway activated? *In vitro* studies have identified CHYLO as a plasma component that greatly augments the production of complement C3 and ASP in adipocytes (75). The other lipoproteins such as VLDL, LDL, and HDL had no significant effect on C3 and ASP production while insulin had a 2 fold effect on ASP although this is small compared to the increases of both C3 and ASP by CHYLO (75). This *in vivo* and *in vitro* data associating ASP production to CHYLO clearance lend importance to identifying the active component of the CHYLO which is responsible for the increase in both C3 (the precursor protein) and ASP.

Section 3.3: Materials and Methods

Bovine serum albumin-essentially fatty acid free (BSA), collagenase Type II, lipid substrate, cholesterol and human transthyretin (TTR) were from Sigma (St. Louis, MO). All tissue culture medium, Dulbecco's-phosphate buffered saline (PBS), fetal bovine serum (FBS) and tissue culture supplies were from Gibco (Gaithersburg, MD) or Flow Laboratories (Mississauga, Ontario). Antiserum to human TTR was from Cedarlane Laboratories (Hornby, Ontario). Apoprotein AII, apoprotein CII, and apoprotein CIII were purchased from Calbiochem (La Jolla, CA). Apoprotein CI was obtained from PerImmune (Rockville, Maryland). Apoprotein AI and apoprotein E were a gift from Dr. J. Westerlund. Apoprotein AIV was a gift from Dr. J.C. Fruchart (Pasteur Institute, Lille, France).

Section 3.3.1: Culture of human differentiating adipocytes

Human adipose tissue was obtained with informed consent from patients undergoing reduction mammoplasty and then processed as previously reported (75). Briefly, adipose tissue was cleaned of connective tissue and small blood vessels, then minced and treated with 0.1% collagenase. The cell suspension was centrifuged to pellet the stromal-vascular cells (containing the preadipocytes) and the resuspended pellet was subsequently treated with buffer for 10 minutes to lyse the red blood cells. After filtration through a 50 μ m filter and gentle centrifugation, the cell pellet was resuspended in minimum essential medium (MEM) containing 10% FBS. Preadipocytes were plated out on 24 well

culture plates (cells from 10 g of cleaned tissue per 24 well plate) at a concentration of 3×10^4 cells per cm^2 . After 24 hours, cells were changed to serum-free Dulbecco's Minimum Essential Medium/Ham's F12 medium supplemented with 7.5 mg/L insulin, 1 μM dexamethasone, 33 μM biotin, 17 μM pantothenate and 0.2 nM triiodothyronine (152). Differentiating adipocytes were maintained in a 37°C incubator with 5% CO_2 and the medium was changed twice a week for a total of two to three weeks at which time the cells exhibited adipocyte-like morphology with multiple fat droplets.

Section 3.3.2: Experimental Incubation

On the 21st day of culture, differentiated adipocytes were changed to serum-free, supplement-free Dulbecco's Minimum Essential Medium/Ham's F12 medium overnight prior to initiation of experiments. The next day, medium was changed to fresh medium supplemented with components as indicated. All additions were diluted with PBS to a final volume of 125 μL and added to the cells with 375 μL medium Dulbecco's Minimum Essential Medium/Ham's F12 medium. Lipoprotein lipase (Sigma) was added to all CHYLO containing samples at a concentration of 0.25 IU/mL. Apoproteins, TTR and polyclonal antibody to TTR were added to CHYLO and incubated (1 hour, 37°C) prior to addition to cells. Following incubation (6 hours, unless otherwise indicated) the medium was removed and frozen immediately at -70°C for later analysis of medium ASP and C3 levels. The cells were washed twice with ice cold PBS, 0.5

mL of 0.1 N NaOH was added to dissolve cells and cell proteins were measured by the method of Bradford (150) using a commercial kit (Bio-Rad, Hercules, CA).

Section 3.3.3: Medium ASP Determination

ASP was measured via a sandwich ELISA immunoassay. A murine (in house) monoclonal antibody raised to the last eight amino acids of the carboxy terminal of ASP was used as capture antibody (as described previously) (132). The monoclonal antibody was coated at 7 µg/mL in PBS (100 µL per well in a 96 well plate) overnight at 4°C and blocked with 1.5% bovine serum albumin (BSA) for 2 hours. The plate was washed three times with wash solution between every step (0.05% Tween 20 in 0.9% NaCl). Standard solutions (0 to 2.13 ng/mL) of ASP, purified as described previously (132), as well as samples (conditioned culture media diluted appropriately) and control plasma samples (precipitated and diluted as described for plasma ASP assays (132) were added at 100 µL per well. The plate was incubated for 1 hour at 37°C, washed, followed by an incubation for 1 hour at 37°C with 100 µL rabbit antiserum to human ASP (raised against the holoprotein), diluted appropriately (1:2000) in PBS-0.05% Tween 20. The plate was then incubated for 30 minutes at 37°C with 100 µL goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000, Sigma) diluted in PBS-0.05% Tween 20. Following the final wash, the color reaction was initiated with 100 µL O-phenylenediamine dihydrochloride (1 mg/mL) in 100 mM Na citrate, 0.05% Tween 20. After visual development the reaction was stopped with 50 µL of 4 N

H₂SO₄ and absorbance was read at 490 nm. ASP concentration vs absorbance was graphed and calculated by linear regression.

Section 3.3.4: Medium C3 Determination

Medium C3 was also determined by sandwich ELISA immunoassay as described previously (75). Murine monoclonal antibody to the C3d fragment of C3 (Quidel, San Rafael, CA) was coated at 1 µg/mL in PBS (100 µL per well) overnight at 4°C and blocked with 1.5% BSA for 2 hours. The plate was washed three times with wash solution (0.05% Tween 20 in 0.9% NaCl) between every step. A standard solution (0 to 10 ng/mL) of C3 (Calbiochem), as well as test samples (conditioned culture media diluted appropriately) and in house control plasma samples (diluted 1:10⁵) were added at 100 µL per well. Subsequent steps were identical to those found in the ASP sandwich ELISA. Note that goat polyclonal anti-C3 (1:5000, Quindel) and antiserum IgG conjugated to horseradish peroxidase (1:1250, Sigma) were used for the C3 sandwich ELISA. Linear C3 concentration was plotted against absorbance and sample C3 concentration calculated by linear regression.

Section 3.3.5: Chylomicron Preparation and Fractionation

Blood was obtained from healthy subjects with normal lipoprotein profiles 3 hours following a fat meal and collected on ice into Vacutainer Tubes containing EDTA as anticoagulant. Plasma was immediately isolated by low-speed centrifugation at 4°C and the CHYLO were isolated by discontinuous

preparative ultracentrifugation according to the procedure of Havel *et al.* (151). Plasma was layered under a salt solution of density 1.006 g/mL and CHYLO were isolated after centrifugation for 30 minutes at 30,000 rpm (40000 x g) at 11°C. Where indicated, CHYLO were again overlaid with 1.006 g/mL solution and re-centrifuged for 18 hours at 40,000 rpm (100 000 x g) at 11°C. The respun CHYLO were recovered in the top 2 mL fraction. The loosely associated CHYLO proteins in the infranate (lower layer) were collected and fractionated by hydrophobic interaction HPLC on a C4 column (Vydac C4, S.P.E. Ltd, Ontario) from 0% to 80% acetonitrile in 0.1% fluoroacetic acid. Polyacrylamide SDS gel electrophoresis (4.8%/10%/16.5% discontinuous gradient gel) was performed on the isolated fractions according to Schagger and von Jagow (167). Triacylglycerol and cholesterol concentrations of the CHYLO were measured using commercially available colorimetric enzyme assays (Boehringer Mannheim, Laval, Quebec). The protein concentrations were measured by Lowry method (168).

Section 3.3.6: Chylomicron Modifications

CHYLO were gently delipidated with twenty-five volumes of ethanol/ether (3:1 v/v) added to a known concentration of CHYLO. After incubation on ice for two hours, the CHYLO were centrifuged at 1200 rpm (500 x g) for 15 minutes at 4°C. The CHYLO pellet was reconstituted to its original volume using the concentration of lipid substrate (Sigma) or lipid substrate/cholesterol (50 µg cholesterol/mL lipid substrate) equal to that of the original CHYLO triacylglycerol

concentration or with a comparable volume of PBS and incubated for 1 hour (37°C) prior to addition to cells. For heat inactivation of CHYLO, a known concentration of CHYLO was incubated at 70°C for 30 minutes then quenched on ice. CHYLO were trypsinized with an equal concentration of trypsin incubated at 37°C for one hour after which soybean trypsin inhibitor was added in 4x excess concentration.

Section 3.3.7: Statistics

Values are reported as means of experiments (with all determinations for each point in each experiment performed in triplicate) \pm standard error of the mean (sem). Statistical significance was set at $p=0.05$ and was determined using either Student's t-test or one-way ANOVA (with multiple comparisons by Dunnett's method) as indicated; where $p=NS$ indicates not significant.

Section 3.4: Results

The secretion of C3 in human differentiated adipocytes was first examined. Medium C3 was measured by sandwich ELISA. We have shown previously that ASP production was linear up to eight hours (75) and was stimulated by CHYLO. In Figure 3.1 (top panel), C3 production in cultured medium was measured in the presence and absence of CHYLO up to a 24 hour incubation period. Cells were changed to fresh serum-free and supplement-free medium 24 hours before the medium was collected for C3 measurements. The CHYLO were then added during the final 0,2,4,6,8, and 24 hours of the incubation period. Hence, the 0 time represents the amount of C3 generated basally over the 24 hour time period. In the absence of CHYLO, relatively little C3 was produced over the time period assessed (up to 24 hrs). In the presence of CHYLO, C3 production increased markedly and reached a maximum between the last 4 to 6 hours of incubation, which parallels that of ASP production (Figure 3.1, bottom) as shown previously (75). Thus, the CHYLO effect on C3 production, as with ASP (75), is time-dependant. As well, as shown previously, the CHYLO effect on C3 and ASP production was concentration dependent (75). Based on these data, the remaining experiments utilized a six-hour incubation period containing 50 µg/mL CHYLO triacylglycerol following which medium C3 and ASP were measured. Overall, under these conditions, the average stimulation of C3 was 43 fold and the effect on ASP was 6 fold where the basal level of C3 was 3.40 ± 1.09 pmol/mg cell protein and of ASP was 2.40 ± 0.90 pmol/mg cell protein.

In order to characterize the component of the CHYLO that caused this effect on ASP and C3, the CHYLO were manipulated in several ways. As shown in Figure 3.2 (top panel), delipidation of CHYLO diminished the stimulation of C3 by CHYLO. The results for ASP (bottom panel) were similar to those for C3. The stimulation of both C3 and ASP production were reinstated when the triacylglycerol-phospholipid (lipid substrate) cholesterol emulsion plus cholesterol was added back to the delipidated CHYLO although the lipid emulsion alone (ls/ch) had no effect on C3 or ASP production.

As shown in Table 3.1, mild heat treatment of the CHYLO resulted in a slight decrease in C3 production and a more pronounced decrease in ASP production as compared to the positive (CHYLO) controls. Protein digestion with trypsinization of CHYLO eliminated the CHYLO-induced stimulation of both C3 and ASP production by 92% ($p < 0.003$) and 100% ($p < 0.002$), respectively. This would suggest that the protein component of the CHYLO is essential for C3 and ASP production; the lipid component may play a role in maintaining the proteins in the appropriate conformation for the effect.

Many apoproteins are associated with CHYLO particles (165). As shown in Table 3.2, addition of individual apoproteins alone to the tissue culture medium had no effect on C3 production. When the apoproteins were preincubated with CHYLO, then added to the cells, again there was no significant increase in C3 production over and above that of the CHYLO positive

control. Only apoprotein AIV had a small but significant effect on C3 production. Similarly, with the same treatments, there was no significant increase in ASP production following incubation alone, or in combination with CHYLO (Table 3.2). Thus, the apoproteins tested do not appear to be the active protein component of the CHYLO responsible for the increase in C3 and ASP production.

To further investigate the protein component of the CHYLO, an additional ultracentrifugation step was performed to reisolate CHYLO ($d < 1.006$) and remove loosely associated proteins ($100,000 \times g$ for 18 hours). Following re-isolation of CHYLO, there was a loss of C3 and ASP stimulation as compared to the CHYLO alone as shown in Table 3.3 (84% loss for C3, $p < 0.0001$ and 83% loss for ASP, $p < 0.0001$). By contrast, the loosely associated proteins now present in the infranate (lower layer) following ultracentrifugation maintained partial capacity to stimulate the production of both C3 and ASP as compared to CHYLO (45% C3, $p < 0.0001$; 59% ASP, $p < 0.0001$). Therefore, it appears that the loosely associated protein(s) initially associated with the CHYLO are required for the stimulation of C3 and ASP production.

The CHYLO infranate proteins were fractionated using hydrophobic interaction C-4 HPLC (5 fractions on a gradient from 0% to 80% acetonitrile (ACN) in 0.1% trifluoroacetic acid). Fractions were collected, lyophilized and reconstituted in PBS. SDS-polyacrylamide gel electrophoresis demonstrated

distinct protein bands in each fraction isolated. The activity of the fractions was tested in the same manner as with the infranate proteins (above). An equivalent concentration of each fraction was added to the adipocytes for a period of 6 hours, after which the medium was removed and medium levels of ASP and C3 were measured by ELISA. One fraction (55% to 59% ACN on HPLC) demonstrated activity (283%, $p < 0.05$) with a later fraction (69% to 73% ACN on HPLC) having lesser activity (184%, pNS) on C3 production. Of note, the activity of the CHYLO infranate (Table 3.3) could not be fully reconstituted by the addition of the HPLC fraction alone. Therefore, it appears that this active fraction containing TTR requires the presence of other protein components to have the affect. However no other fractions tested possessed significant activity and the fraction with the most pronounced effect was further analysed. This fraction contained two distinct bands on SDS polyacrylamide gel electrophoresis under reducing conditions. An 83 kD molecular weight band was present in several fractions and co-migrated with the albumin standard. We have shown previously that albumin had no stimulatory effect on C3 and ASP production alone or in the presence of CHYLO (75). The second band had an apparent molecular weight of 20 kD. The 20 kD band was transferred to PVDF, sequenced and identified as TTR based on the amino terminal 19 amino acids.

TTR is a plasma protein that complexes to retinol-binding-protein and transports both thyroxine (T4) and retinol (169) in plasma. The TTR used in the following experiments is the purified un-complexed protein (95% purified human

protein, Sigma, St. Louis, MO). The addition of purified TTR alone or with triacylglycerol-phospholipid (lipid substrate) cholesterol emulsion to the tissue culture medium did not result in any significant effect on C3 and ASP stimulation as compared to the basal level of C3 and ASP production (Table 3.4), although at higher concentrations there was some effect (Figure 3.3). As well, addition of TTR back to respun CHYLO had little effect on C3 and ASP production. However, when an increasing concentration of TTR was added to a constant amount of CHYLO there was an enhancement (up to 75 fold) of the CHYLO effect on C3 stimulation where the CHYLO effect alone was already 16 fold (Figure 3.3). The results of ASP (not shown) parallel those of C3. This data suggests that although TTR plays a role in stimulating C3 and ASP production it does not function alone.

To further examine the role of TTR in stimulating C3 and ASP production, a polyclonal antibody to TTR was tested. As shown in Figure 3.4, the addition of a TTR antiserum to the culture medium (+pAb) resulted in blockage of the CHYLO induced stimulation of both C3 and ASP production by 98% for C3 (left panel, $p < 0.001$) and 100% for ASP (right panel, $p < 0.001$). Similarly, addition of the TTR antiserum also blocked the increases in both C3 and ASP production induced by the CHYLO infranate proteins to the same extent, 99% for C3 ($p < 0.001$) and 94% for ASP ($p < 0.001$). Finally, addition of TTR to CHYLO, which enhanced the CHYLO effect, could also be blocked by addition of the TTR antiserum (86% decrease for C3, $p < 0.05$ and 80% decrease for ASP, $p < 0.05$). A

non-immune preparation had no inhibitory effect on the TTR-CHYLO or CHYLO infranate protein stimulation (data not shown). Thus the data indicate that a loosely associated protein, TTR, found associated with CHYLO is critical to the overall mediation of the CHYLO-induced stimulation of both C3 and ASP production.

Section 3.5: Discussion

In our species, as in most others, energy intake is intermittent and therefore the rate at which energy must be stored in adipose tissue varies markedly. Storage of dietary fatty acids in adipocytes is a two step process: first, there must be release of fatty acids from CHYLO triacylglycerol by lipoprotein lipase; and second, there must be uptake of fatty acids and incorporation into triacylglycerol by adipocytes (165). The first takes place in the capillary space, the second in the subendothelial space. However, unless the fatty acids liberated from CHYLO triacylglycerol are rapidly taken up by the adipocytes, lipoprotein lipase activity will be inhibited and triacylglycerol clearance from plasma will be reduced (170). By increasing the rate at which fatty acids are incorporated into adipose tissue triacylglycerol, the ASP pathway allows the sudden influx of the dietary fatty acids from CHYLO to be transferred, rapidly and efficiently, from the capillary space into adipocytes which lie in the subendothelial space (166). Thus, the rate of adipocyte triacylglycerol synthesis *in vivo* appears to govern the rate of CHYLO lipolysis by lipoprotein lipase.

The present data add powerfully to the model of the ASP pathway. *In vivo* studies in humans have shown that ASP was released into the systemic circulation by subcutaneous adipocytes of the anterior abdominal wall (132). This release was steady during fasting and during the first three hours after an oral fat load. However, the production of ASP increased markedly thereafter. CHYLO triacylglycerol clearance by subcutaneous adipocytes also markedly

increased in the second half of the postprandial period as did fatty acid storage in adipocytes. These data suggested that CHYLO stimulated adipocytes to increase their production of ASP. This hypothesis is in accord with previous *in vitro* studies which demonstrated that CHYLO had a profound stimulatory effect on the production of ASP, as well as its precursor protein C3, from human adipocytes (75). This effect was both concentration and time dependent and was distinctive in that other lipoproteins (VLDL, LDL and HDL) had minimal effect. Other postprandial components such as fatty acids or glucose had little effect and insulin had only a moderate effect (75). Based on these results, our aim was to identify the CHYLO component that was responsible for the acute stimulatory effect on C3 and ASP production.

The results of the present study indicate, first of all, that the effect of CHYLO is not only on ASP, the bioactive protein, but is manifested through a marked increase in the precursor protein, C3. Under all experimental conditions, parallel trends in both C3 and ASP were evident. The few instances where ASP does not fully parallel C3 production may result from changes in the activation of the enzymes (adipsin and C3 convertase) involved in the conversion of C3 to ASP. The CHYLO component responsible for the increase in ASP and C3 is a protein, loosely associated with the lipoprotein particle. Although the lipid free mixture of proteins possesses most of the stimulatory capacity, reassociation with a lipid substrate increases its potential suggesting that a change in conformation with lipid association may enhance its capacity to stimulate. The

active protein component was demonstrated to be TTR: (i) by the identification of TTR as the active protein in the CHYLO infranate protein mixture, (ii) by the demonstration that addition of TTR to CHYLO increases the stimulatory capacity and, finally, (iii) by the ability of a polyclonal antiserum to TTR to obliterate the stimulatory capacity of both native CHYLO and the active loosely-associated protein complex. Thus, TTR appears to be necessary but not sufficient, since addition of TTR alone (purified uncomplexed protein), in the presence of lipid substrate or respun CHYLO, does not produce any increase in either medium ASP or C3. Thus an additional substance, that remains associated with TTR during the additional ultracentrifugation, or remains with the native CHYLO particle, appears to be required.

How then, can TTR play a role in mediating increases in C3 and ASP production? TTR is a plasma protein that is found associated in a complex with retinol-binding protein (169). This TTR-RBP complex binds both T4 and retinol and transports them through plasma to the sites of action of the active forms: T3 and retinoic acid. Both T3 and retinoic acid are regulators of gene transcription (171,172). Both have been implicated in adipocyte differentiation (173) and acute gene regulation of adipsin and phosphoenolpyruvate carboxykinase (174-176). Crosstalk has been demonstrated between thyroid hormone, peroxisome proliferator-activated receptors and retinoid X receptors (177,178). Retinol is an exogenously derived dietary compound. It is a fat-soluble vitamin (vitamin A) and is absorbed and delivered to tissues through incorporation into the lipid core of

the dietary triacylglycerol-rich CHYLO in intestinal villus cells (179). We propose that the TTR associated with the CHYLO may serve as the vehicle to shuttle the hormones to the adipocyte. Preliminary studies suggest that retinoic acid associated with TTR play a role in the stimulatory effect of TTR on C3 and ASP production (manuscript in preparation). As the CHYLO docks with lipoprotein lipase on the endothelial surface, TTR may disassociate from the CHYLO and mediate transport of the hormones to the adipocytes. This result in initiation of increased secretion of C3, which is converted to ASP. ASP will then activate triacylglycerol synthesis within the adipocyte by coordinately stimulating both glucose transport and esterification to generate storage triacylglycerol.

By this means CHYLO, once they bind to lipoprotein lipase on the adipose tissue capillary endothelium, would activate the ASP pathway. ASP, by increasing the rate of fatty acid storage in adipocytes, would allow rapid hydrolysis of CHYLO triacylglycerol to continue. Chylomicron triacylglycerol hydrolysis is then coupled to adipocyte triacylglycerol synthesis and the ASP pathway would link events in the capillary space to events in the subendothelial space. It appears, therefore, that the ASP pathway constitutes a novel model of microenvironmental metabolic regulation, which allows effective and rapid storage of energy in adipose tissue.

Section 3.6: Tables and Figures

Table 3.1: The Effects of Modified Chylomicrons on C3 and ASP Production in Human Differentiated Adipocytes

{PRIVATE } Addition	C3 (pmol/mg cell protein)	ASP (pmol/mg cell protein)
PBS	3.4±0.9	0.14±0.12
CHYLO	371.7±92.9	3.39±1.15
CHYLO + heat treatment	229.3±156.0	0.41±0.28
CHYLO + trypsinization	7.4±4.2	0.0±0.0
p value (ANOVA)	p<0.003	p<0.002

Differentiated adipocytes were exposed to a concentration (50 µg triacylglycerol/mL medium) of chylomicrons (CHYLO), heat treated CHYLO, or trypsinized CHYLO for a period of 6 hours. The modifications are described in methods. C3 and ASP levels were measured in cell media and the results are expressed as pmol/mg cell protein ± sem (n=7 to 9). One way ANOVA was performed vs PBS control and p values are indicated accordingly.

Table 3.2. The Effect of Apoproteins on C3 and ASP Production by Human Differentiated Adipocytes

Addition	C3 (pmol/mg cell protein)		ASP (pmol/mg cell protein)	
	- CHYLO	+ CHYLO	- CHYLO	+ CHYLO
PBS	6.8±2.5	407±143	2.26±0.81	11.2±3.6
AI	2.3±1.7	763±357	0.24±0.21	8.9±4.7
AII	0.5±0.3	918±289	0.0±0.0	8.9±4.5
AIV	16.4±4.2*	892±205	—	—
CI	3.0±1.8	520±224	1.14±0.35	7.2±2.6
CII	5.8±1.3	855±420	1.56±0.79	17.0±7.7
CIII	6.5±2.1	523±174	1.30±0.47	8.4±2.9
E	5.8±1.6	910±374	0.0±0.0	6.7±5.0
All apoproteins	8.0±3.2	423±15	1.10±0.35	8.5±4.2
p value (ANOVA)	p<0.01	pNS	pNS	pNS

Differentiated adipocytes were exposed to a constant concentration of the selected apoprotein (10 µg/mL medium) or a mixture of all apoproteins (10 µg/mL medium), with or without chylomicrons (CHYLO : 50 µg lipoprotein triacylglycerol/mL medium) for a period of 6 hours. C3 and ASP levels were measured in cell media and the results are expressed as pmol/mg cell protein ± sem (n=4 to 8). Significance was determined by one way ANOVA (as indicated) vs the PBS control, where * p<0.05 by multiple comparison analysis vs PBS control.

Table 3.3: Effect of Chylomicron Components on C3 and ASP Production in Differentiated Human Adipocytes

Treatment	C3 (pmol/mg cell protein)	ASP (pmol/mg cell protein)
PBS	2.24±0.34	1.45±0.47
CHYLO	90.79±13.18*	11.98±1.88*
CHYLO respun	14.34±4.17	2.87±1.13
CHYLO infranate	41.10±10.11*	7.05±2.54*
p value (ANOVA)	p<0.0001	p <0.0001

Differentiated adipocytes were exposed to a constant concentration of chylomicrons (CHYLO : 50 µg lipoprotein triacylglycerol/mL medium), recentrifuged CHYLO (CHYLO respun : 50 µg lipoprotein triacylglycerol/mL medium), or CHYLO protein infranate (CHYLO infranate: 2.5 µg protein/mL medium) for a period of 6 hours. C3 and ASP levels were measured in cell media and the results are expressed as pmol/mg cell protein ± sem from eleven and nine different subjects for C3 and ASP respectively (n=31 to 66). Significance was calculated by one way ANOVA (as indicated) vs PBS control, where * p<0.05 vs PBS control by multiple comparison analysis.

Table 3.4: The Effect of Transthyretin on C3 and ASP Production by Differentiated Human Adipocytes

Addition	C3 (pmol/mg cell protein)		p value	ASP (pmol/mg cell protein)		p value
	-TTR	+TTR		-TTR	+TTR	
PBS	3.48±1.35	1.02±0.34	ns	1.96±0.55	2.53±1.70	ns
Lipid Substrate + Chol	1.17±0.70	0.98±0.38	ns	1.65±0.73	1.30±0.64	ns
CHYLO respun	2.25±0.44	3.23±0.72	ns	1.31±0.58	3.68±1.65	ns
CHYLO	55.33±10.53	352.15±123.19	p<0.0001	13.07±1.69	18.01±2.90	p<0.0002

Differentiated human adipocytes were exposed to a constant concentration of chylomicron (CHYLO), CHYLO respun (CHYLO respun: 50 µg lipoprotein triacylglycerol/mL medium), PBS or lipid substrate, with or without transthyretin (TTR: 1.25 µg/mL medium) for a period of 6 hours. The results are expressed as pmol/mg cell protein ± sem. C3 and ASP levels were measured in cell media for three experiments assayed in quadruplicate (n=12). Multiple comparison analysis (ANOVA) was analyzed comparing in the presence of TTR vs absence of TTR where ns = not significant.

Figure 3.1: Chylomicron Stimulation of C3 and ASP Production by Human Differentiated Adipocytes. Differentiated human adipocytes were exposed to a constant concentration of chylomicrons (50 μ g lipoprotein triacylglycerol/mL medium) for the indicated times (PBS was added for control incubations). Cells were changed to fresh serum-free and supplement-free medium 24 hours before the medium was collected for C3 (top panel) and ASP (bottom panel) measurements. The CHYLO were then added during the final 0,2,4,6,8 and 24 hours of the incubation period. C3 and ASP levels were measured in media by a sandwich ELISA immunoassay. The results are expressed as pmol/mg cell protein \pm sem for four experiments assayed in quadruplicate CHYLO: $p < 0.003$, PBS: p :NS by ANOVA, * $p < 0.05$ vs zero time point for multiple comparison analysis.

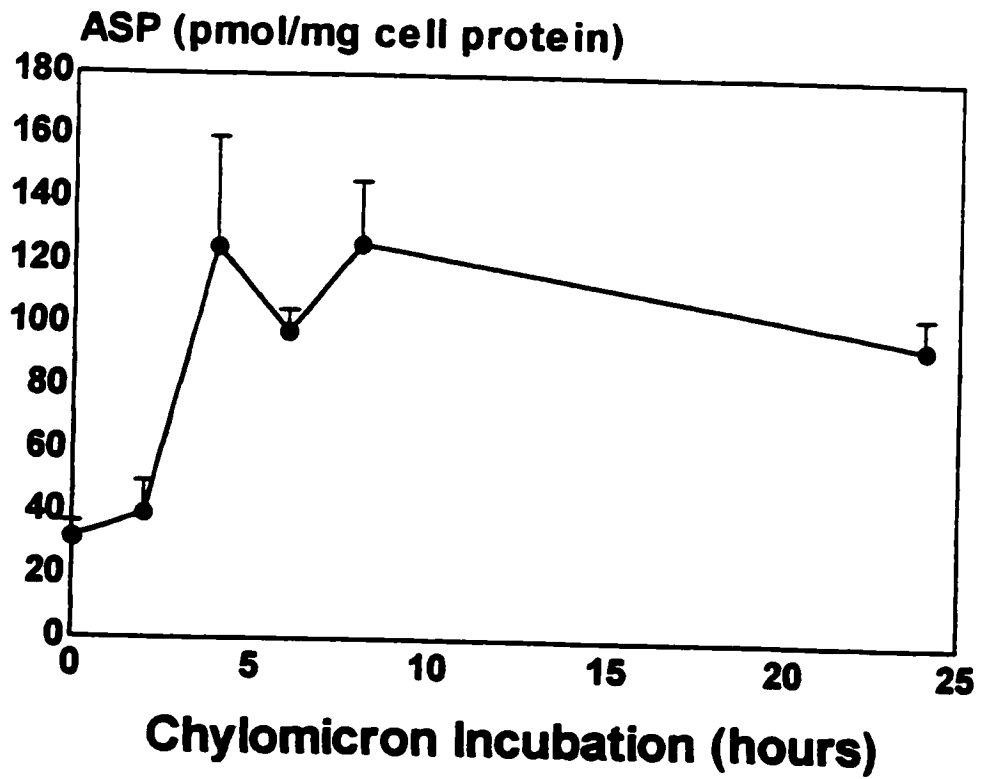
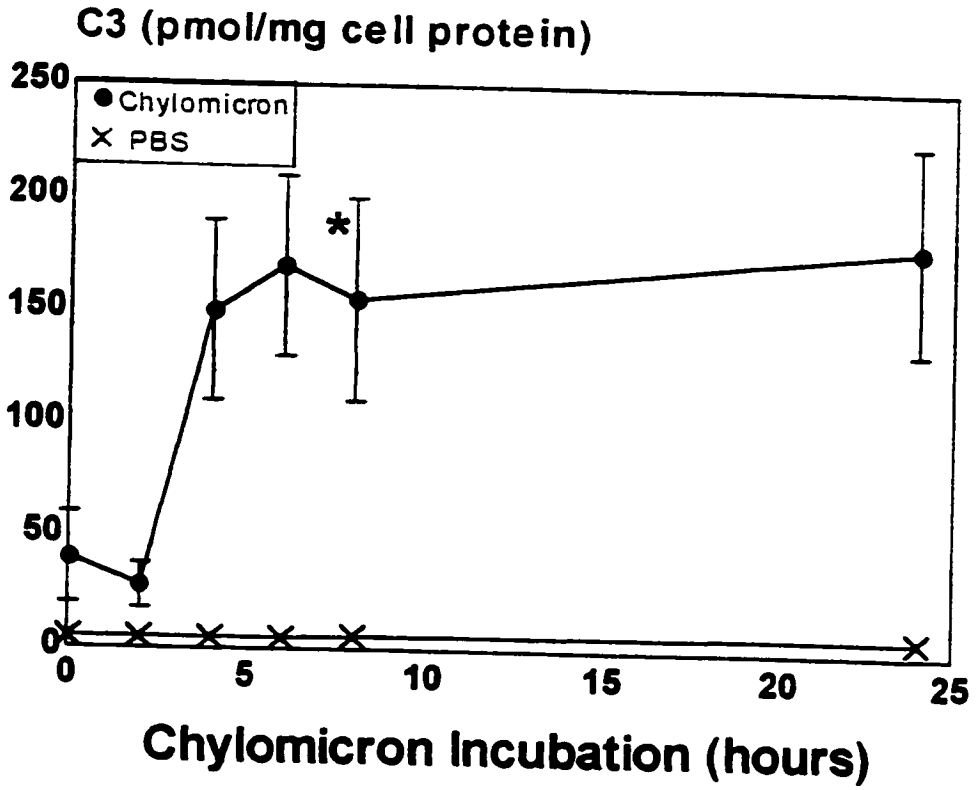


Figure 3.2: The Effect of Lipid Modified Chylomicrons on C3 and ASP Production by Human Differentiated Adipocytes. Differentiated human adipocytes were exposed to a concentration (50 µg triacylglycerol/mL medium) of chylomicrons (Chy), delipidated chylomicrons (dChy), delipidated chylomicrons with lipid substrate (dChy/l_s) or delipidated chylomicrons with lipid substrate and cholesterol (dChy/l_s/ch) for a period of 6 hours. PBS or lipid substrate plus cholesterol (l_s/ch) were used for the control incubations. C3 (top panel) and ASP (bottom panel) levels were measured in cell media and the results are expressed as pmol/mg cell protein ± sem (n=3) where p<0.0001 for C3 and ASP by ANOVA, and *p<0.05 vs PBS for multiple comparison analysis.

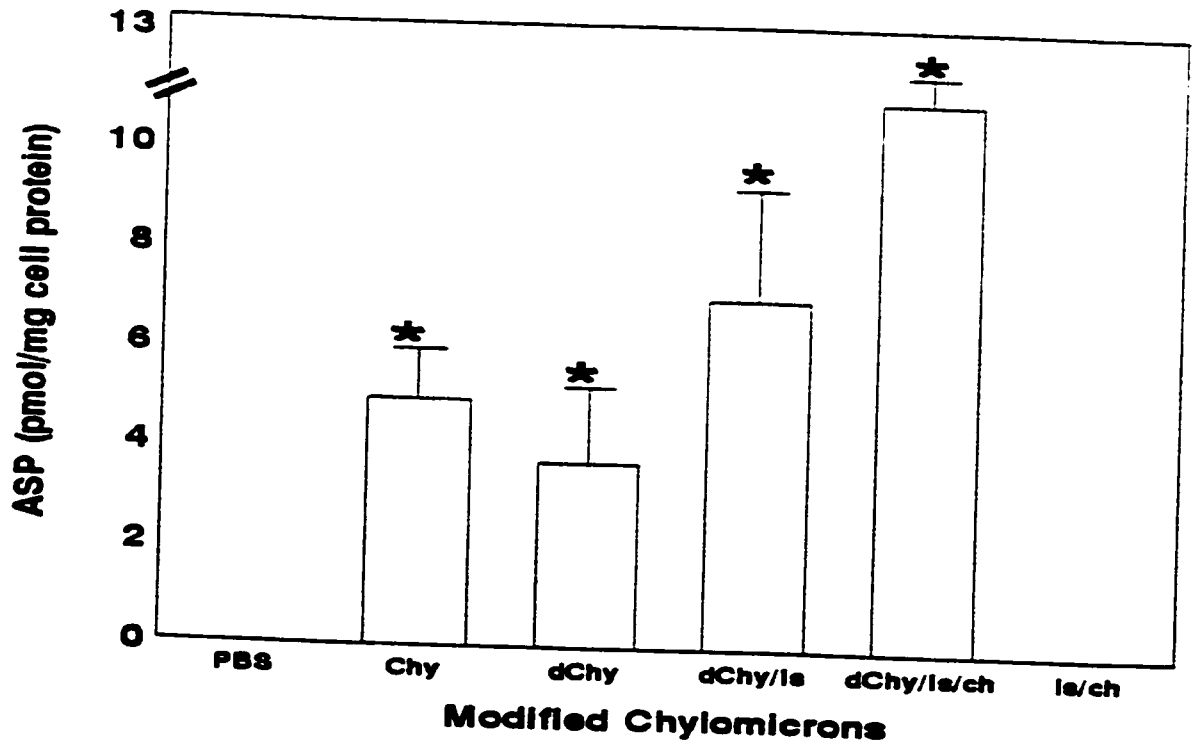
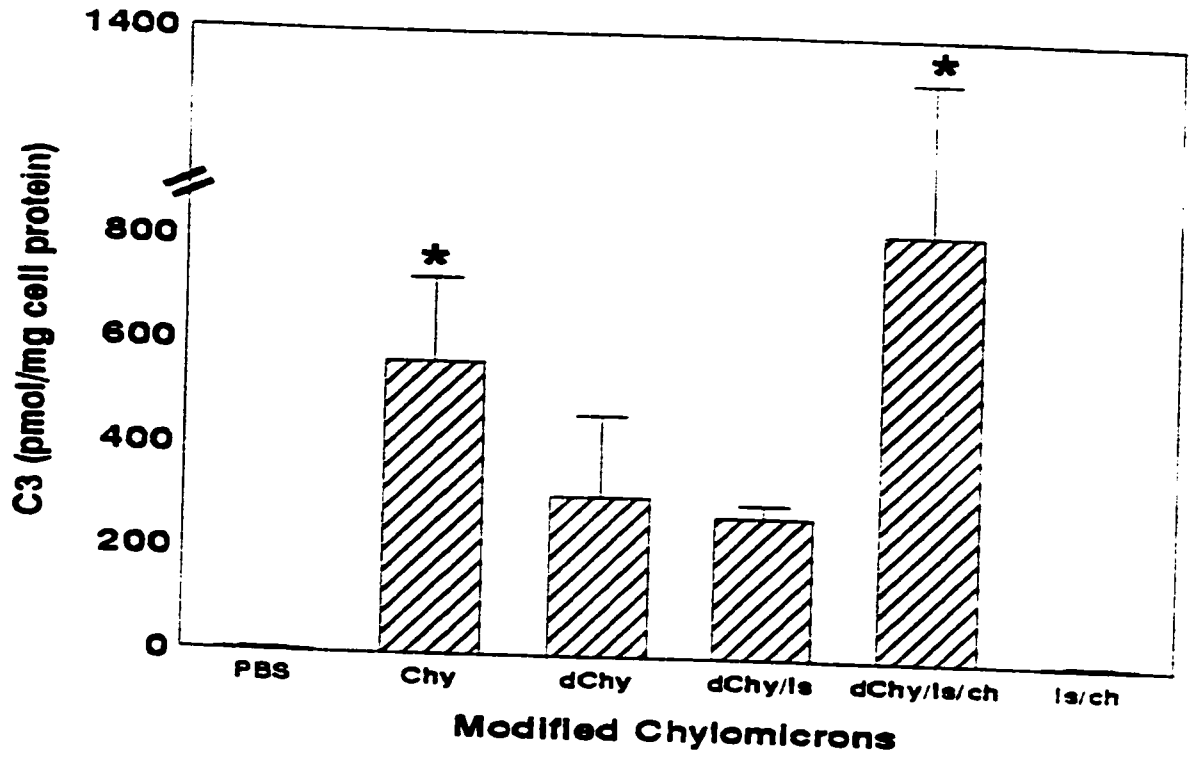


Figure 3.3: Concentration-Dependant Enhancement of C3 Production by Transthyretin in the Presence of Chylomicrons in Differentiated Human Adipocytes. Differentiated human adipocytes were exposed to increasing amounts of transthyretin (TTR) in the presence of a constant concentration of chylomicrons (50 μ g lipoprotein triacylglycerol/mL medium) and in the absence of chylomicrons over a period of 6 hours. C3 levels were measured in cell media and the results are expressed as pmol/mg cell protein \pm sem. The TTR concentration curve without chylomicrons (linear Y1 axis) was performed in duplicate (n=2). The TTR concentration curve with chylomicrons (logarithmic Y2 axis) was performed in triplicate in two experiments (n=6). One way ANOVA was performed, *p<0.0001 vs no TTR additions where CHYLO stimulation (no TTR) is 15 \pm 0.2 fold increase vs PBS alone.

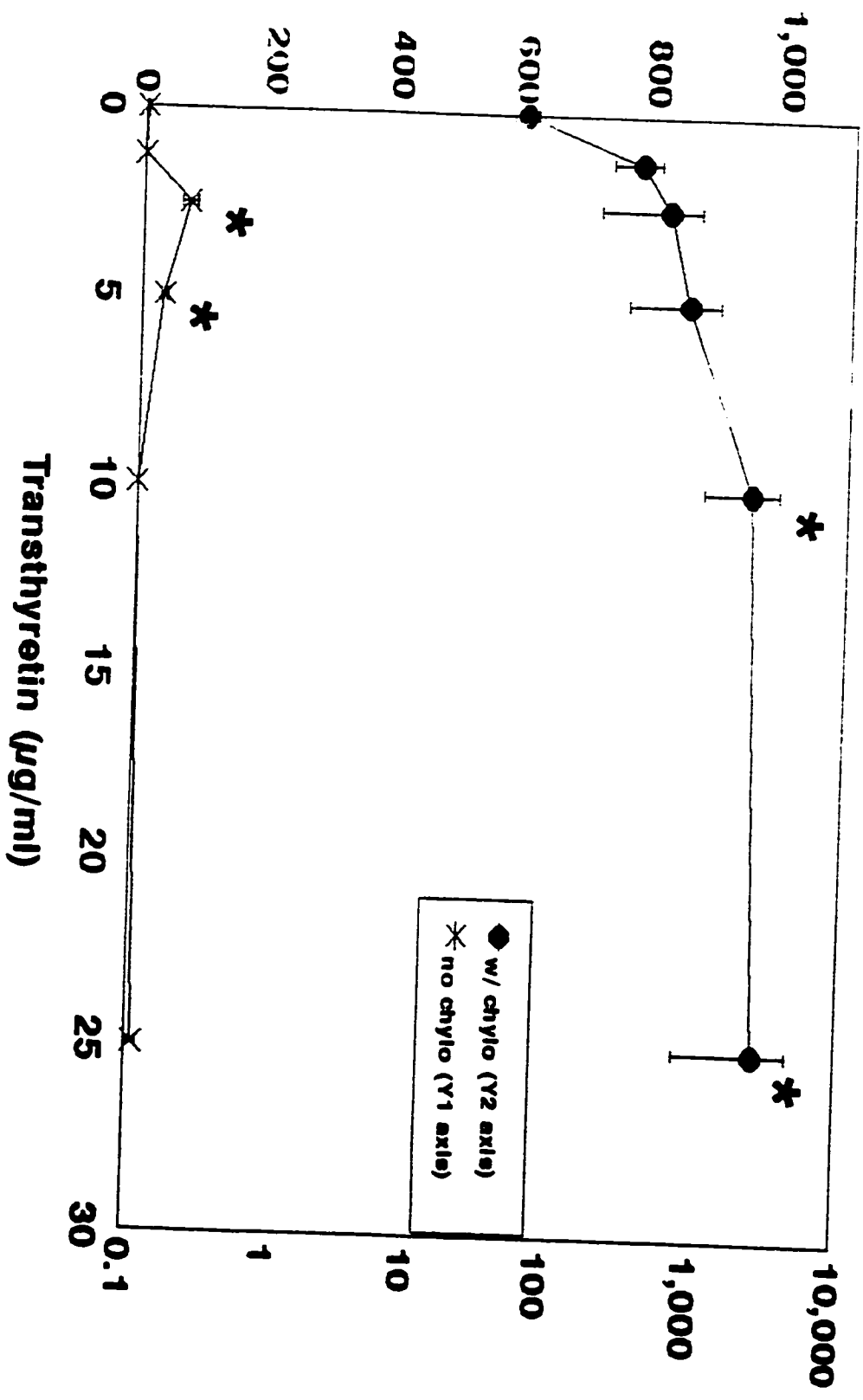
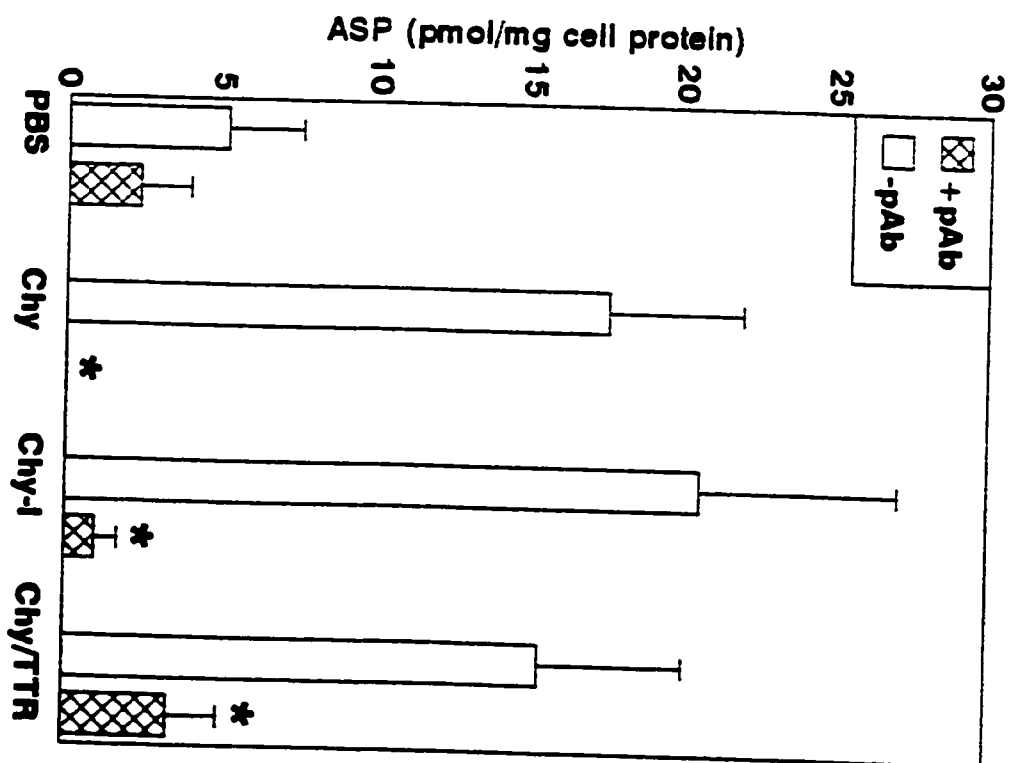
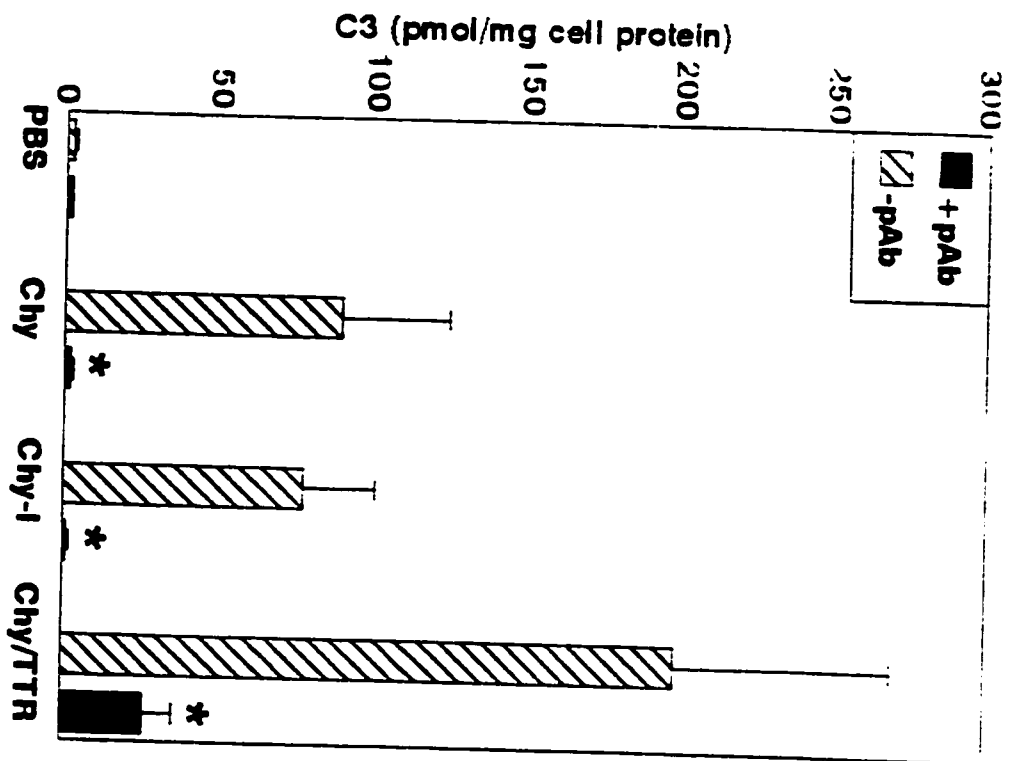


Figure 3.4: Immunospecific Blocking of Transthyretin Enhancement of Chylomicron Stimulation of C3 and ASP Production. Differentiated human adipocytes were exposed to a constant concentration of PBS, chylomicrons (Chy: 50 μg triacylglycerol/mL medium), chylomicron infranate proteins (Chy-I): 2.5 $\mu\text{g}/\text{mL}$ medium), or chylomicrons plus transthyretin (Chy/TTR: 1.25 μg transthyretin/mL medium). Antiserum to TTR was added as indicated (+pAb: 2.5 $\mu\text{g}/\text{mL}$ medium). C3 (left panel) and ASP (right panel) levels were measured in cell media and the results are expressed as pmol/mg cell protein \pm sem (n=8 in 3 separate experiments). Multiple comparison analysis was performed comparing results in the presence of antiserum vs the absence of antiserum where * $p < 0.001$.



PRELUDE TWO

From the above data, TTR is involved in mediating the stimulatory response of CHYLO on C3 and ASP production. TTR is a carrier protein that is complexed to retinol binding protein in circulation. Because this carrier-protein complex transports thyroid hormone and retinol, we proposed that the observed stimulation may be caused by the action of one or both of these hormones. Preliminary experiments with thyroid hormone and retinol did not reconstitute the stimulatory effect seen with CHYLO (data not shown). It was not clear in the *in vitro* cell model if these prohormones could be appropriately taken up and converted to the active forms by the cells. So we opted to use the active form of retinol, which is retinoic acid to study the effects on C3 and ASP production.

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Chapter Four is the last version of the manuscript that was submitted to the *Biochemical Journal* for publication. The journal holds the right to make editorial changes resulting in a few small differences when comparing the version presented in chapter four with respected to the final published version in the *Biochemical Journal*.

CHAPTER FOUR
RETINOIC ACID REGULATION OF ACYLATION STIMULATING PROTEIN (ASP) AND
COMPLEMENT C3 IN HUMAN ADIPOCYTES

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Thea Scantlebury[†], Allan D. Sniderman, and Katherine Cianflone

Mike Rosenbloom Laboratory for Cardiovascular Research, McGill University
Health Centre, McGill University, Montreal, Quebec, Canada

[†] and Chemistry and Biochemistry Department
Concordia University, Montreal, Quebec, Canada

Short Title: Retinoic Acid Effect on C3 and Acylation Stimulating Protein

Address Correspondence to: Dr. Katherine Cianflone
Mike Rosenbloom Laboratory
for Cardiovascular Research
Room H7.30
McGill University Health Centre
Royal Victoria Hospital
687 Pine Avenue West
Montreal, Quebec
H3A 1A1
Tel: 514-842-1231 ext. 5426
Fax: 514-843-2843
Email: mdkc@musica.mcgill.ca

Keywords: chylomicrons, C3adesArg, adipose tissue, retinoic acid

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Abbreviations:

ASP	Acylation Stimulating Protein
BSA	bovine serum albumin
DMEM/F12	Dulbecco's minimum essential medium/Ham's F12
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
MEM	minimum essential medium
PBS	phosphate buffered saline
PPAR	peroxisome proliferator-activated receptor
RA	retinoic acid
sem	standard error of the mean
TG	triacylglycerol
TMAC	tetramethylammonium chloride
TTR	transthyretin

Section 4.1: SUMMARY

Acylation stimulating protein (ASP) a product of complement C3, stimulates triglyceride synthesis in adipocytes. Previous studies identified transthyretin (TTR) associated with chylomicrons, as a stimulator of C3 and ASP production. Since both transport retinyl ester / retinol, our goal was to investigate whether retinoic acid (RA) could be a potential hormonal mediator of the effect. Protein synthesis and secretion inhibitors eliminated the chylomicron stimulatory effect on both C3 and ASP production in human differentiated adipocytes, suggesting that *de novo* protein synthesis and secretion are required. Chylomicron incubation increased C3 mRNA ($37\% \pm 1.5\%$). RA alone or with chylomicrons had a stimulatory effect on C3 production (29 fold at 16.6 nM RA) and ASP production. RA receptor antagonist blocked both RA and chylomicron stimulation of C3 mRNA and C3 secretion. Finally, RA and chylomicrons activated a 1.8 kb C3 promoter-luciferase construct transfected into 3T3-F442 and 3T3-L1 ($41\% \pm 0.2\%$ and $69\% \pm 0.3\%$, respectively), possibly via the RA receptor half-sites identified by sequence analysis. This is the first evidence documenting RA stimulation of the C3 gene. Thus we propose RA as a novel cellular trigger from chylomicrons that subsequently results in increased ASP production by adipocytes after a meal.

Word count 198

Section 4.2: Introduction

Complement C3 is a plasma protein that plays a major role in the complement system (180). C3 is not only synthesized in the liver but is also synthesized in extra-hepatic tissues: fibroblasts mononuclear phagocytes endothelium myoblasts and adipocytes (43,64,73,74). Furthermore, complement factor B and adipsin (factor D) are also produced by adipocytes (43,64,73,74). These three proteins are synthesized and secreted in a differentiation-dependent manner in both murine and human adipocytes.

Complement C3 interacts with factor B and adipsin (Factor D) (43,64,74,145) to generate the cleavage product C3a. The carboxyl terminal arginine is removed by carboxypeptidase to produce C3adesArg, which is identical to Acylation Stimulating Protein (ASP) (145). *In vitro*, ASP is a potent stimulant of triacylglycerol (TG) synthesis in human adipocytes (146), and *in vivo* accelerates clearance of postprandial TG in both lean and obese (*db/db*) mice when administered intraperitoneally (181,182). C3 knockout male mice (which are thus ASP deficient) have delayed TG clearance, which can be partially corrected by administration of ASP (182). In contrast, Wetsel *et al.* (183) did not observe any difference in TG clearance with C3 knockout mice. These differences may be due to the influence of the background strains of these mice (manuscript in preparation). Further we have also demonstrated a decrease in adipose tissue mass in female mice, even when TG clearance is normal (184). These opposing viewpoints have been discussed in two recent review articles

(185,186). In spite of the fact that adipsin and factor B are also involved in ASP production via the alternate complement pathway, to date there is no information published on lipid profiles and fat metabolism in factor B or adipsin knockout mice (187,188).

Although in humans, ASP levels do not increase in the general circulation postprandially (132,189,190,191), ASP production from subcutaneous adipose tissue increases in the local environment, concurrently with increased TG lipolysis after a meal (189,192), and elevated circulating ASP levels are found in obese subjects (129,130). These findings all reinforce a connection between the alternate complement pathway and adipose tissue metabolism.

Regulation of C3 production has been examined in various tissues and cells. Cytokines such as IL-1 α and β (193), IFN γ (193) and IL-6, have been shown to increase C3 production. Hormones such as insulin (75), glucocorticoids (194) and estradiol (195) are also involved in augmenting C3 production. By contrast, TNF- α (196) has been shown to decrease C3 production. We have shown previously that chylomicrons had a profound stimulatory effect on the production of C3 (the precursor to ASP) and ASP in cultured human differentiated adipocytes (75,76). The effects of chylomicrons on ASP and C3 production are time- and concentration-dependent (75,76). Recently, transthyretin (TTR) was identified as a protein loosely associated with

chylomicrons that was involved in mediating the stimulatory chylomicron effect on C3 and ASP production (76).

TTR is a plasma protein that is found complexed with retinol-binding protein (169). This complex binds both thyroxine and retinol, and transports the prohormones throughout the plasma compartment to specific tissue sites. It has been proposed that there is receptor-mediated uptake and internalization of TTR; however no specific receptor has been identified. Intracellularly, thyroxine T4 and retinol are activated to T3 and retinoic acid (RA), bind to specific DNA binding sites and regulate gene transcription. Since TTR alone was not sufficient to stimulate C3 and ASP production (76), we have proposed that it acts as a vehicle to shuttle specific hormones to the adipocyte. In the present study, we examined the potential role of RA as the hormonal mediator of the chylomicron effect on regulation of C3 secretion and ASP production.

Section 4.3: Materials and Methods

Bovine serum albumin-essentially fatty acid free (BSA), collagenase Type II, human TTR, *all-trans* RA, and 17 β estradiol were from Sigma (Oakville, Ontario). All tissue culture medium, Dulbecco's-phosphate buffered saline (PBS), fetal bovine serum (FBS) and tissue culture supplies were from Gibco (Burlington, Ontario). Antiserum to human TTR was from Cedarlane Laboratories (Hornby, Ontario). Trizol reagent was purchased from Gibco BRL. RA antagonist (Ro 41-5253) was a gift from Dr. L. Forni (Hoffman-LaRoche Ltd., Mississauga, Ontario). C3-Luciferase plasmid was a gift from Dr. D.P. McDonnell (Duke University, North Carolina, USA). All chemicals used for the transfection assay were purchased from Sigma (Oakville, Ontario). 3T3-F442 cell-line was a gift from Dr. R. Germinario (Lady Davis Institute, McGill University, Montreal, Quebec) and 3T3-L1 cell-line was purchased from ATCC (Rockville, MD). The 100 bp DNA ladder (N3231S) was purchased from New England BioLabs Ltd. (Mississauga, Ontario).

Section 4.3.1: Culture of human differentiating adipocytes:

Human adipose tissue was obtained with informed consent from patients undergoing reduction mammoplasty and then processed as previously reported (43). Briefly, adipose tissue was cleaned of connective tissue and small blood vessels, then minced and treated with 0.1% collagenase. The cell suspension was centrifuged to pellet the stromal-vascular cells (containing the preadipocytes) and the resuspended pellet was subsequently treated with buffer

for 10 minutes to lyse the red blood cells. After filtration through a 50 μm filter and gentle centrifugation, the cell pellet was resuspended in minimum essential medium (MEM) containing 10% FBS. Preadipocytes were plated out on 24 well culture plates (cells from 10 g of cleaned tissue *per* 24 well plate) at a concentration of 3×10^4 cells per cm^2 . After 24 hours, cells were changed to serum-free Dulbecco's Minimum Essential Medium/Ham's F12 medium (DMEM/F12) supplemented with 7.5 mg/L insulin, 1 μM dexamethasone, 33 μM biotin, 17 μM pantothenate and 0.2 nM triiodothyronine. Differentiating adipocytes were maintained in a 37°C incubator with 5% CO_2 and the medium was changed twice a week for two to three weeks at which time the cells exhibited adipocyte-like morphology with multiple fat droplets.

Section 4.3.2: Experimental Incubation for C3 and ASP Secretion

On the 21st day of culture, differentiated adipocytes were changed to serum-free, supplement-free DMEM/F12 medium overnight prior to initiation of experiments. The next day, medium was changed to fresh medium supplemented with components as indicated. All additions were diluted with PBS to a final volume of 125 μL and added to the cells with 375 μL medium (DMEM/F12). Lipoprotein lipase (Sigma) was added to all chylomicrons containing samples at a concentration of 0.25 IU/mL. Monensin, colchicine, cycloheximide, TTR, RA, and RA receptor antagonist were added to chylomicron and incubated (1 hour, 37°C) prior to addition to cells. RA was solubilized in ethanol (8.32 mM stock) and RA receptor antagonist was solubilized in

dimethylsulfoxide (1 mM stock). Both were then diluted in the media to a maximum 0.1% of ethanol or dimethylsulfoxide, respectively. This was performed according to the product information supplied by commercial supplier of RA (Sigma Product No. R2625), and according to product information supplied by Dr. L. Furni, Hoffman-LaRoche Ltd. (197) respectively. Following incubation (6 hours, unless otherwise indicated) the medium was removed and frozen immediately at -70°C for later analysis of medium ASP and C3 levels. The cells were washed twice with ice cold PBS, 0.5 mL of 0.1 N NaOH was added to dissolve cell proteins which were measured by the method of Bradford (150) using a commercial kit (Bio-Rad, Mississauga, Ontario).

Section 4.3.3: Medium ASP Determination

ASP was measured via a sandwich ELISA immunoassay (as described previously) (75,76,132). A murine (in-house) monoclonal antibody to the eight amino acid carboxy terminal of ASP was used as capture antibody (76,132). The monoclonal antibody was coated at 7 µg/mL in PBS (100 µL per well in a 96 well plate) overnight at 4°C and blocked with 1.5% BSA for 2 hours. The plate was washed three times with wash solution between every step (0.05% Tween 20 in 0.9% NaCl). Standard ASP solutions (0 to 2.13 ng/mL), samples (conditioned culture media diluted appropriately) and control plasma samples were added at 100 µL per well. The plate was incubated for 1 hour at 37°C, washed, followed by an incubation for 1 hour at 37°C with 100 µL rabbit antiserum to human ASP (raised against the holoprotein), diluted appropriately

(1:2000) in PBS-0.05% Tween 20. The plate was then incubated for 30 minutes at 37°C with 100 µL goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000, Sigma) diluted in PBS-0.05% Tween 20. Following the final wash, the color reaction was initiated with 100 µL O-phenylenediamine dihydrochloride (1 mg/mL) in 100 mM Na citrate, 0.05% Tween 20. After visual development the reaction was stopped with 50 µL of 4 N H₂SO₄ and absorbance was read at 490 nm. ASP concentration vs absorbance was graphed and calculated by linear regression.

Section 4.3.4: Medium C3 Determination

Medium C3 was also determined by sandwich ELISA immunoassay as described previously (75,76). Murine monoclonal antibody to the C3d fragment of C3 (Quidel, San Rafael, CA) was coated at 1 µg/mL in PBS (100 µL per well) overnight at 4°C and blocked with 1.5% BSA for 2 hours. The plate was washed three times with wash solution (0.05% Tween 20 in 0.9% NaCl) between every step. A standard solution (0 to 10 ng/mL) of C3 (Calbiochem), as well as test samples (conditioned culture media diluted appropriately) and in-house control plasma samples (diluted 1:10⁵) were added at 100 µL per well. Subsequent steps were identical to those in the ASP sandwich ELISA. Goat polyclonal anti-C3 (1:5000, Quidel) and anti-goat IgG conjugated to horseradish peroxidase (1:1250, Sigma) were used for the C3 sandwich ELISA. C3 concentration was plotted against absorbance and sample C3 concentration calculated by linear regression.

Section 4.3.5: Chylomicron Preparation and Fractionation

Blood was obtained from healthy subjects with normal lipoprotein profiles 3 hours following a fat meal and collected on ice into Vacutainer Tubes containing EDTA as anticoagulant. Plasma was immediately isolated by low-speed centrifugation at 4°C and the chylomicrons were isolated by discontinuous preparative ultracentrifugation according to the procedure of Havel *et al.* (151). Plasma was layered under a salt solution of density 1.006 g/mL and chylomicrons were isolated after centrifugation for 30 minutes at 30,000 rpm (40000 x g) at 11°C. TG and cholesterol concentration of the chylomicrons were measured using commercially available colorimetric enzyme assays (Boehringer Mannheim, Laval, Quebec).

Section 4.3.6: mRNA Analysis via Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells were treated as described above for experimental conditions. Following incubations, the medium was removed and total RNA was isolated using Trizol reagent (Gibco BRL) as described by manufacturer. Reverse transcription was performed as described previously using a random hexa-oligomer primer (Gibco) to generate cDNA (43,64,73,74). Total RNA (3 µg) was denatured in the presence of RNase Inhibitor (Gibco) for 5 minutes at 65°C. The RT reaction mix [final concentration/reaction: 1 x buffer, 10U RNase inhibitor in 200 U MMLV, 0.01mM DTT, 0.5 mM each of dATP, dCTP, dGTP, and dTTP]

was added to the denatured RNA and incubated for 2 hours at 37°C. The reaction was quenched at 95°C (5 minutes) and diluted to 100 µL.

cDNA was amplified using in-house primers for C3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (housekeeper gene) as described previously (43,64,73,74). We have previously reported that product signal for GAPDH was linear up to 29 cycles, and for C3 was linear up to 37 cycles in differentiated adipocytes. The cycle number utilized was 35 cycles for C3 and 25 cycles for GAPDH. The cDNA (4 µL of RT reaction) was added to the PCR reaction mix when the final concentrations/tube: 0.5 U Taq polymerase, 1x buffer for Taq, 2 mM MgCl₂, 10 µM tetra methyl ammonium chloride (TMAC), 1 µM each of 5' and 3' primers and amplified [1 minute 95°C, 1 minute 60°C, 1 minute 72°C]. The PCR product generated was linear from 1 µL to 5 µL of cDNA from RT reaction. The PCR products were separated on a 7% PDA polyacrylamide gel with 100 bp DNA mass ladder (New England Biolabs, N3231S). The gels were stained using silver staining (Bio-Rad Kit, Hercules, CA) and scanned using a densitometer to quantitate. The PCR product signal was linear over a 4 fold range (5 µL to 20 µL) and 10 µL was routinely applied to the gel. The DNA mass ladder was used to construct a standard curve which was linear from 18 ng to 97 ng ($r^2 = 0.90$ to 0.98). All samples quantitated were within the range of the mass standards utilized. Values were expressed as a ratio of C3 to GAPDH.

Section 4.3.7: 3T3 Cell Culture and C3 Promoter Activity

3T3-F442 and 3T3-L1 cells were maintained in DMEM/F12 medium plus 10% FCS. Cells were plated in 24-well plates 24 hours prior to transfection. DNA was introduced into the cells using lipofectAMINE reagent (Gibco) as described by manufacturer. The C3-Luciferase plasmid contained a 1.8 kb fragment of the C3 promoter (-1807 to +58 bp). Transfection of the DNA (189 ng/well) into 3T3-F442 and 3T3-L1 cells was allowed to proceed for 5 hours, then the cells were washed, fresh phenol red-free medium with 10% FBS was added, and the cells were cultured for 36 hours. Subsequently, the cells were lysed and assayed for luciferase. On each plate, triplicate wells were transfected with pCMV β galactosidase (62 ng/well) to assess transfection efficiency, which ranged from 28% to 37% (average 32%).

Section 4.3.8: Statistics

Values are reported as means of experiments (with all determinations for each point in each experiment performed at least in triplicate) \pm standard error of the mean (sem). Statistical significance was set at $p=0.05$ and was determined using either Student's t-test or one-way ANOVA (with multiple comparisons by Bonferoni's method) as indicated; where $p=NS$ indicates not significant.

Section 4.4: Results

Section 4.4.1: Effect of Protein Synthesis and Secretion Inhibitors

In previous work we demonstrated that chylomicrons stimulate the production of ASP as well as the precursor C3 in cultured human adipocytes (75,76). The specific mechanism might result from increased synthesis of C3 or secretion of a preformed pool of C3. To examine these issues we first looked at the effect of protein synthesis or secretion inhibitors on C3 and ASP production. Based on the fact that the chylomicron effect is both concentration- and time-dependant (75,76), an incubation time of 6 hours using 50 µg/mL chylomicron TG, which gave linear maximal stimulation previously, was utilized (75,76). In Table 4.1 cultured differentiated adipocytes were incubated with protein secretion inhibitors (monensin and colchicine) in the presence and absence of chylomicrons. Monensin and colchicine alone had little effect on basal C3 and ASP production, which were already very low. In the presence of chylomicrons, C3 and ASP production are markedly increased; however the ASP constituted only a small portion of the total C3. The addition of monensin (with chylomicrons) decreased C3 secretion by $90\% \pm 1\%$ ($p < 0.001$) as compared to the chylomicron positive control. Similarly, the addition of colchicine (with chylomicrons) decreased C3 by $72\% \pm 1\%$ ($p < 0.001$). For ASP, there was a complete loss of production as a result of the addition of monensin or colchicine as compared to the chylomicron control ($-99\% \pm 1\%$, $p < 0.001$ and $-96\% \pm 0.5\%$, $p < 0.001$ respectively).

Protein synthesis inhibitor (cycloheximide) was also tested in the presence and absence of chylomicron as shown in Table 4.2. The addition of cyclohexamide (with chylomicrons) decreased C3 production by $75\% \pm 5\%$, $p < 0.001$ of the chylomicron positive control. Cycloheximide almost completely inhibited ASP production ($-92\% \pm 2\%$, $p < 0.001$). Overall, both the inhibition of *de novo* protein synthesis and secretion of C3 (precursor protein) resulted in diminished ASP production. Therefore, the stimulatory effect of chylomicrons on C3 and ASP production requires both protein synthesis and secretion.

Section 4.4.2: Effect of RA on C3 and ASP Secretion

In plasma, TTR and retinol-binding protein form a transport complex for thyroxine and retinol. The thyroxine is bound to TTR and the retinol to retinol-binding protein. Although we have previously shown that TTR significantly increased C3 and ASP production, addition of TTR alone was not sufficient to produce stimulation equivalent to chylomicrons (76). Accordingly, we proposed that TTR act as a vehicle to shuttle hormone(s) from chylomicrons to the adipocyte. It has been previously demonstrated that retinol is transported in the lipid core of chylomicrons as a fatty acid ester (198), and can be translocated into adipocytes (199) and converted to RA, the bioactive form of retinol. RA was therefore investigated as the possible active component of chylomicrons responsible for the stimulation of C3 and ASP production, and was used within a range of concentrations previously used in adipocytes (85,175,176).

At low concentrations of RA (Figure 4.1, left panel), there was a concentration-dependant effect on C3. A RA concentration of 15 μM had the most pronounced effect on C3 production as compared to basal secretion (70.8 ± 36.7 RA vs 2.4 ± 0.8 CTL pmol C3/mg cell protein). However, the effects of RA on ASP production were much smaller and not significant. When RA was added to chylomicrons (Figure 4.1, right panel), both the patterns for C3 and ASP were similar. There was no further stimulation over the effect of chylomicrons alone, and an inhibitory effect was seen at the higher RA doses. There was no change in cell protein measured for the different conditions when compared to the controls, suggesting that the RA is not toxic.

Section 4.4.3: Effect of RA Antagonist on C3 and ASP Secretion

The effect of RA on the stimulation of C3 and ASP production was further investigated using an antagonist to the RA receptor. The antagonist (Ro 41-5253) is a synthetic retinoid that binds RA receptors $\text{RAR}\alpha$ (high affinity), $\text{RAR}\beta$ and $\text{RAR}\gamma$ (low affinity); and subsequently blocks binding of RA (197). Adipose tissue expresses mRNA for $\text{RAR}\alpha$, β and γ , and $\text{RXR}\alpha$, β and γ ; with $\text{RAR}\alpha$, $\text{RAR}\gamma$, $\text{RXR}\alpha$, $\text{RXR}\beta$ being the most abundant in mature adipocytes (200). Addition of antagonist blocked RA stimulation of C3 production at RA concentrations of 7.5 μM ($-86\% \pm 28\%$, $p=\text{ns}$) and 15 μM ($-99\% \pm 1\%$, $p=0.002$) (Figure 2, left panel). As well, the antagonist effectively blocked the chylomicron stimulation of C3 production even in the presence of RA or TTR: chylomicron ($-88\% \pm 15\%$, $p=0.039$), chylomicron + RA 3.75 μM ($-97\% \pm 1\%$, $p<0.001$), and

chylomicron + TTR (-69% \pm 8%, $p < 0.001$). There was also a noticeable decrease in ASP production with addition of antagonist to RA (Figure 4.2, right panel: -91% \pm 26%, $p < 0.05$ at 7.5 μ M). However, there was only partial inhibition by the antagonist of the chylomicron stimulation of ASP production. Thus, the stimulatory effect of chylomicrons on C3 appears to be mediated through RA while the subsequent changes seen in ASP concentration are likely an indirect effect of change in substrate (C3) concentration.

Section 4.4.4: Effect on C3 mRNA Expression in Human Adipocytes

From the above data, chylomicrons stimulate both *de novo* C3 synthesis and C3 secretion and RA appears to be involved in this effect. Since RA executes its actions via a family of nuclear receptors (RAR's and RXR's) (199), thus regulating gene transcription, the chylomicron stimulatory effect was investigated at the level of gene transcription for C3. Cells were incubated with and without chylomicrons for 2, 6, and 7 hours and C3 mRNA analyzed by semi-quantitative RT-PCR (Figure 4.3). In the control cells there was no significant change in C3 mRNA over the 7 hour incubation period. However, there was a significant increase in C3 mRNA at 6 hours of chylomicron stimulation vs the control at 6 hours (87 \pm 14 (n=12) vs 45 \pm 4 (n=5) C3/GAP ratio, $p < 0.05$). In addition, C3 mRNA at 6 hours of chylomicron stimulation was also increased as compared to the chylomicron incubation at 2 and 7 hours. This increase of mRNA levels at 6 hours further suggests that *de novo* synthesis of C3 was involved. This time profile is consistent with the increase in C3 secretion in the

media, which was minimal at 2 hours, maximal at 6 hours and had plateaued at 7 hours (no change from 6 hours) (76). Inhibitors of the chylomicron effect were also tested for their effect on C3 mRNA at 6 hours. We have previously shown that a polyclonal antibody to TTR inhibits the chylomicron stimulation of C3 (76). With addition of a polyclonal antibody to TTR, the chylomicron induced increase in C3 mRNA was blocked (Figure 4.3). As well, addition of RA antagonist also blocked the effect. These results are consistent with chylomicron stimulation of C3 production acting via gene up-regulation and stimulation of *de novo* protein synthesis and both TTR and RA are involved in the chylomicron stimulation of C3.

Section 4.4.5: Effect on C3 Promoter-Luciferase Reporter Gene Expression in 3T3 Cells

Analysis of the 5' flanking region (1018 bp of exon 1) of the C3 gene has previously identified putative response elements for IFN γ , IL-6, estrogen, glucocorticoid, NF- κ B, and thyroid hormone (201). Our analysis of this C3 promoter region (1018 bp of exon 1) using the Mac Vector version 6 program (Oxford Molecular Group), indicated that there are also two putative RXR binding half-sites at -268 to -275 and -205 to -212 bp. These sites have not been tested to our knowledge. A C3 promoter (-1807 to +58 bp) fused to firefly luciferase reporter gene was used to test the activation of the C3 promoter by RA in transiently transfected into 3T3-F442 and 3T3-L1 cells. As seen in Table 3, following a 36 hour incubation, chylomicrons demonstrated a slight activation

of the C3 promoter in both 3T3-F442 and 3T3-L1 cells. RA (3.75 μ M) incubation for 36 hours had a significant effect in both 3T3-F442 (left panel) and 3T3-L1 (right panel) cells as compared to the control (3T3-F442: $69.2 \pm 5.8 \times 10^3$ RA vs $40.8 \pm 4.8 \times 10^3$ CTL in arbitrary units, $p < 0.05$, and 3T3-L1: $9.0 \pm 1.9 \times 10^3$ RA vs $2.8 \pm 1.4 \times 10^3$ CTL in arbitrary units, $p < 0.05$). Estradiol, which has been shown to stimulate the C3 promoter-luciferase activity in hepatocytes (202), also increased C3 promoter activity in preadipocytes by $22\% \pm 7\%$ in 3T3-F442 and $92\% \pm 11\%$ in 3T3-L1 vs the control. Stimulation of the C3 promoter for 6 hours demonstrated a similar trend and the addition of RA receptor antagonist to chylomicrons resulted in a complete loss in the activation of the C3 promoter by chylomicrons (results not shown). Thus, this evidence confirms the involvement of RA in the chylomicron stimulatory effect on C3 production in adipocytes.

Section 4.5: Discussion

These data demonstrate that the stimulatory effect of chylomicrons occurs through moderate up-regulation of mRNA and larger increases in *de novo* protein synthesis and secretion of C3, thus resulting in an increase in ASP production. Although antibody blockage of TTR can inhibit the C3 mRNA increase as well as the secretion of both C3 and ASP (76), when TTR was added alone to adipocytes there was little stimulatory effect (76). Therefore we proposed that TTR acts as a transporter of a hormonal signal, which we believe is RA. Of note, RA does not enhance the effect of chylomicrons (suggesting a similar and non-additive mechanism); and RA antagonist can effectively block the effects of both RA and chylomicron (individually and together) on gene expression, protein synthesis and secretion of C3. It has been previously demonstrated that increases in protein secretion occur without large changes in mRNA and RA is capable of prolonging mRNA stability (203). Thus minimal changes in C3 mRNA could result in increases in secreted C3, and retinol/RA is likely one of the bioactive components of chylomicrons which stimulates C3.

Although the chylomicron effect was comparable for both C3 and ASP, the RA stimulation and RA antagonist inhibition were less pronounced for ASP as compared to C3. As well the absolute level of ASP was much lower than C3. Thus the level of ASP will depend not only on the level of its precursor, C3, but also on the enzymatic activity of the conversion process which includes C3, adipsin, factor B and cell attachment activators. How this overall process, which

generates ASP, may be influenced by RA is unknown. Although RA has been previously shown to modulate adipsin expression in adipocytes (174), a key enzyme in the ASP pathway, this is the first evidence to date that demonstrates that RA has a direct regulatory effect on complement C3 in adipocytes although the role of RA in adipocytes has been studied extensively.

Retinol is an exogenously derived fat soluble vitamin (Vitamin A) that is incorporated into the lipid core of the dietary TG rich lipoproteins, chylomicrons, in intestinal villus cells. Most of the dietary retinol, as retinyl ester in chylomicrons, is taken up by the liver, where the majority of the retinoid reserves are stored (179). Retinol is then transported in the circulation bound to RBP-TTR and transferred to cells. However, it has been demonstrated that retinyl ester is also transferred from chylomicrons to adipocytes (199) and, in fact, adipose tissue is the second largest storage site of retinoids in the body (204). Retinol binding protein (RBP), a plasma transport protein for retinol, has been shown to be synthesized and secreted by adipocytes (57). In addition, adipocytes express cellular retinol binding protein, the intracellular protein thought to have a primary role in retinol uptake, esterification as well as oxidation to RA (199). Within the cells, the retinol is esterified by lecithin:retinol acyltransferase for storage as retinyl ester, or enzymatically oxidized to the bioactive forms of RA: *all-trans* RA and *9-cis* RA (199).

RA regulates the expression of several genes by binding to specific nuclear receptors, RA receptors (RAR α , RAR β and RAR γ) and retinoic X receptors (RXR α , RXR β , and RXR γ) (for review see (199)). These nuclear receptors are all found in adipocytes, are ligand-dependant and function as transcription factors that recognize specific response elements on DNA. RAR's can form homodimers or heterodimers with RXR's and RXR's can also form homodimers or heterodimers with thyroid hormone receptor peroxisome proliferator-activated receptors (PPAR) and vitamin D receptor (199). Crosstalk has also been demonstrated between thyroid hormone, PPARs and RA receptors. In addition, it has been reported that RXR can be activated in the RAR/RXR heterodimer in the presence of RAR ligands (205) and as a heterodimer it can bind to imperfect repeats of a hexamer consensus (85).

While RA acutely increases expression in adipocytes of (176), RAR β and γ (200), acyl CoA oxidase (206), S14 (207), uncoupling proteins UCP1 and UCP2 (208), phosphoenol pyruvate carboxy kinase (175), fatty acid transport protein (FATP-1) (209) and acylCoA synthetase (209), it decreases expression of adipsin (174), lipoprotein lipase (206) and glycerol-3-phosphate dehydrogenase (210). RA also affects gene expression and/or protein secretion in other cell types. Low dose RA has been implicated in stimulation of adipogenesis (199,205), whereas high dose RA blocks the C/EBP β -dependant activation (early differentiation signal) of PPAR α and C/EBP α expression, which leads to inhibition of adipose differentiation (199,205). From our data, RA also

appears to have a stimulatory effect at low dose and an inhibitory effect at high dose on C3 and, to a lesser extent, on ASP production. It has been suggested that the diversity of effects depends not only on RA concentration but also on isomer availability and retinoid receptor subtype expression (199).

Taken together with the above evidence, we propose that in the postprandial state, chylomicrons transport dietary TG and retinol/retinyl ester to the adipocyte. The retinol associated with TTR-RBP and/or chylomicrons is then presented to the adipocyte (198,199). This retinol is transferred to the cell and further metabolized to the bioactive form of RA and/or stored as retinyl ester (199). Thus we propose that RA can function as a postprandial signal that increases the synthesis and secretion of C3 and, consequently, affects ASP levels. The augmentation in ASP stimulates the adipocyte to synthesize and store TG. This is consistent with the *in vivo* evidence that demonstrates a similar temporal profile of ASP production and TG uptake in the micro-environment of the adipose tissue (132,189).

In summary, retinol/retinyl ester is a novel postprandial signal that is transferred from chylomicrons and partly explains the stimulatory effect of chylomicrons on C3 production and subsequently ASP. Taken together with the previous observation that insulin is also capable of stimulating C3 and ASP production (75); we believe the effects of insulin, chylomicrons and other factors will all be relevant in regulating ASP production and, consequently, increase TG

storage after a meal. We hypothesize that the increased activity of the ASP pathway may play a pathophysiological role in the development of obesity and the potential regulation of RA may provide possible strategies for future pharmacotherapy.

Section 4.6: Tables and Figures

Table 4.1. The Effect of Protein Secretion Inhibitors on C3 and ASP Production in Human Differentiated Adipocytes:

(-/+CHYLOS)	C3 pmol/mg cell protein		ASP pmol/mg cell protein	
	-	+	-	+
Basal	2.1±0.9	111.2±83	0.2±0.5	24.1±3.6
+ Monensin	2.3±0.9	11.5±0.9*	1.0±0.5	1.0±0.5*
+ Colchicine	4.2±1.1	32.0±0.9*	0.2±0.5	1.7±0.5*

Differentiated human adipocytes were exposed to monensin (10 μ M), or colchicine (10 μ g/mL) in the absence and presence of chylomicron (50 μ g TG/mL medium) for a period of 6 hours. PBS was used for the basal incubations. Medium C3 and ASP levels were measured by a sandwich ELISA immunoassay. The results are expressed as pmol/mg cell protein (c.p.) \pm sem for n=12 where $p < 0.001$ for C3 and ASP by ANOVA, * $p < 0.001$ vs chylomicron for multiple comparison analysis.

Table 4.2. The Effect of Protein Synthesis inhibitors on C3 and ASP Production by Human Differentiated Adipocytes:

	C3 pmol/mg cell protein	ASP pmol/mg cell protein
Basal	2.07±0.40	0.23±0.09
Cycloheximide	2.03±0.75	0.94±0.79
Chylomicron	111.23±8.29	24.13±3.62
Chylomicron + cycloheximide	27.53±12.30*	1.86±0.67*

Differentiated human adipocytes were exposed to cycloheximide (10 µg/mL) in the absence and presence of chylomicrons (50 µg TG/mL medium) for a period of 6 hours. C3 and ASP levels were measured in cell media and the results are expressed as pmol/mg cell protein ± sem for n=12 where *p<0.001 for C3 and ASP by ANOVA vs chylomicrons alone for multiple comparison analysis.

Table 4.3. RA Activation of the C3 promoter:

	3T3 – L1	3T3 – F442
CTL	2.78±1.38	40.81±4.81
+ Chlyo	5.38±1.00	55.20±7.07
+RA	9.01±1.86*	69.16±5.81*

Transcriptional activity of the C3 promoter was assessed in 3T3-L1 and 3T3-F442 cells following transient transfection of C3 promoter- Luciferase construct. Cells were stimulated with chylomicrons (50 µg TG/mL) or RA (3.75 µM) for 36 hours following transfection. Subsequently, the cells were lysed and assayed for luciferase activity. The results are expressed in arbitrary units ± sem for n=9 where *p=0.009 vs control).

Figure 4.1: RA Effect on C3 Production in Differentiated Human Adipocytes. Differentiated human adipocytes were exposed to increasing amounts of RA in the absence of chylomicrons (left panel, n=15) and in the presence of a constant concentration of chylomicrons (right panel, 50 μ g lipoprotein TG/mL medium, n=9) over a period of 6 hours. C3 and ASP levels were measured in cell media and the results are expressed as pmol/mg cell protein (c.p.) \pm sem. Left Panel: C3 p=0.006 (ANOVA) where *p<0.001 vs no addition; ASP p=NS. Right panel: C3 p=0.008 by ANOVA and *p=0.012 vs chylomicron alone; ASP p<0.001 by ANOVA and *p<0.001 vs chylomicron alone.

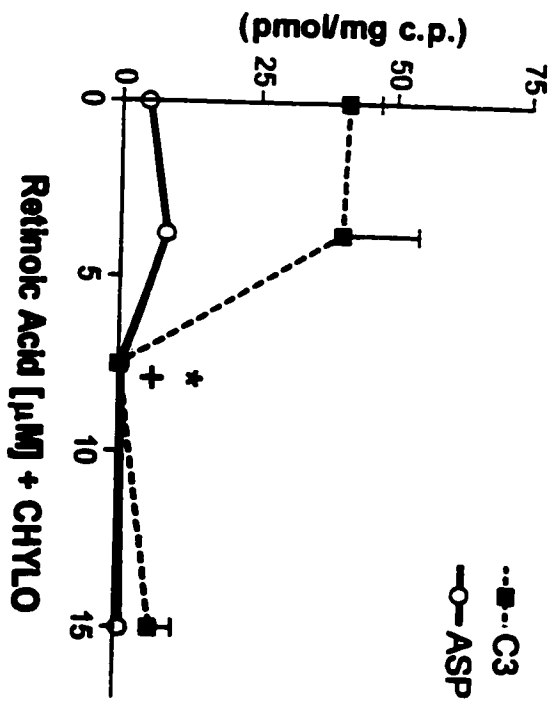
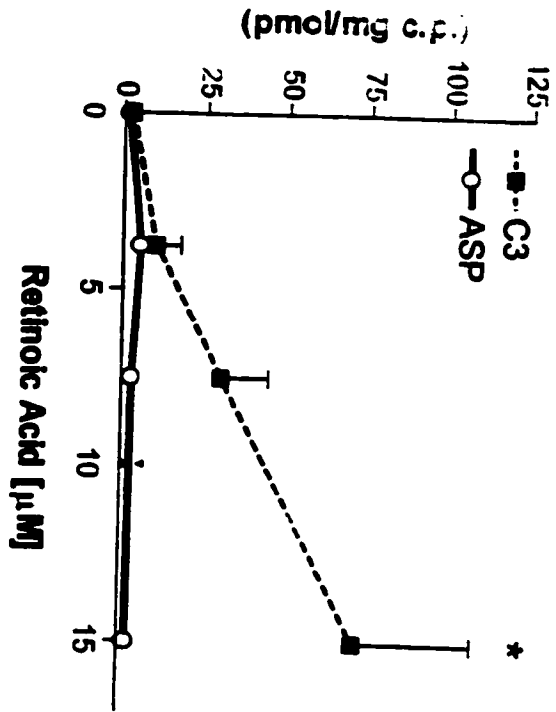


Figure 4.2. RA Receptor Antagonist Blocks the Stimulation by Chylomicrons and RA on C3 and ASP Production. Differentiated human adipocytes were exposed to RA (at the indicated concentration in μM), chylomicrons (50 μg TG/mL), or TTR (1.25 μg TTR/mL) for 6 hours. RA receptor antagonist was added as indicated (+RA Antag: 3.3 μM). C3 (left panel) and ASP (right panel) levels were measured in cell media and the results are expressed as pmol/mg cell protein (c.p.) \pm sem for n=12. Multiple comparison analysis was performed (ANOVA) comparing results in the presence of RA receptor antagonist vs the absence of RA receptor antagonist where * $p < 0.005$.

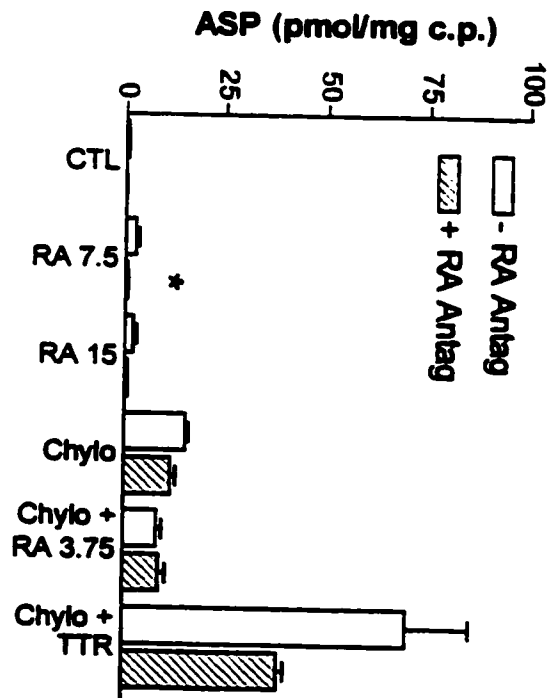
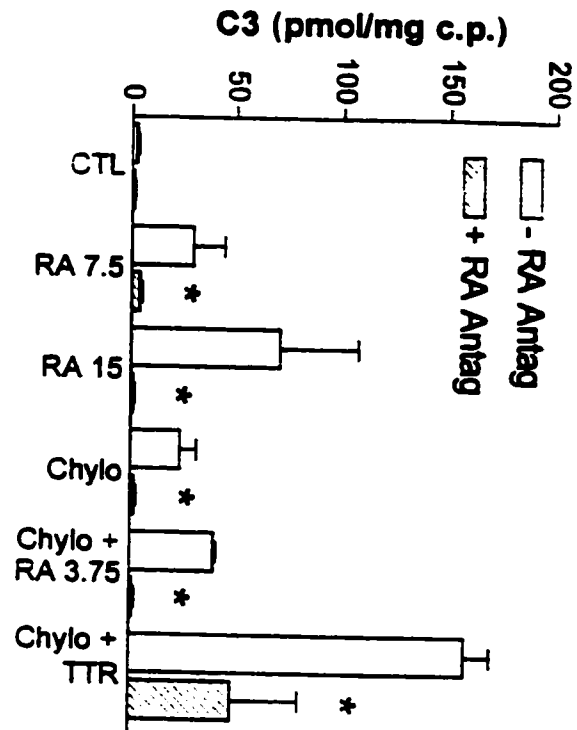
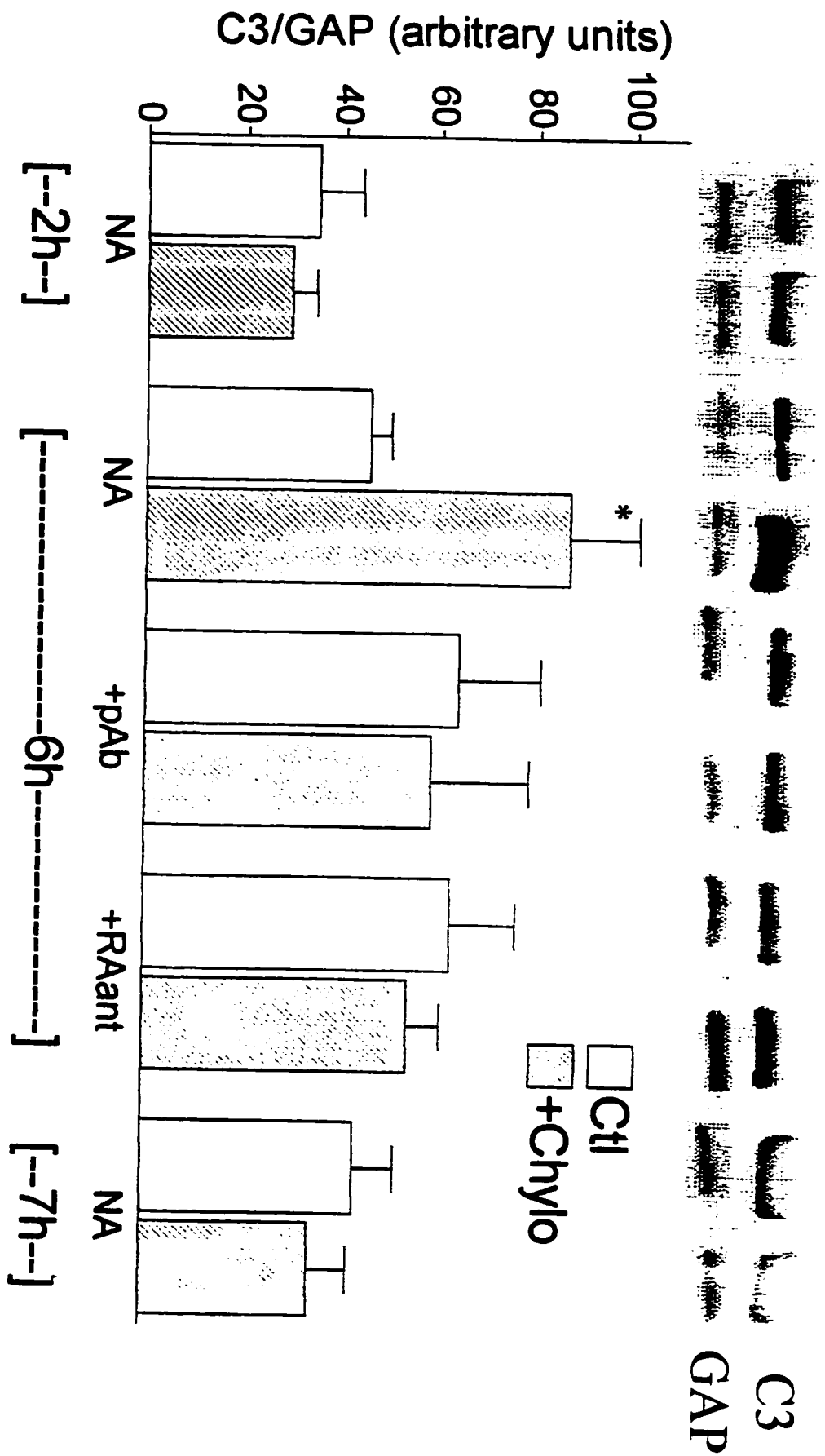


Figure 4.3: RA Receptor Antagonist and TTR Antibody Blocks the Chylomicron Stimulation of C3 mRNA. Differentiated human adipocytes were incubated with or without chylomicrons (50 μg TG/mL) for 2, 6 and 7 hours with no other additions (NA) and, at 6 hours with the addition of polyclonal antiserum to TTR (pAb; 2.5 $\mu\text{g}/\text{mL}$) or RA receptor antagonist (RA ant: 3.3 μM). The media was removed from the cells and total RNA was isolated. Using in-house primers for C3 and GAPDH, RT-PCR was performed. The results are expressed as C3/GAPDH ratio (arbitrary units) for an average of at least $n=5$ where $*p=0.04$ for chylomicrons at 6 hours vs CTL at 6 hours and $^{\dagger}p<0.001$ for chylomicrons at 6 hours vs chylomicrons at 2 and 7 hours by multiple comparison analysis (ANOVA).



PRELUDE THREE

In addition, the data suggested the involvement of TTR in mediating the transfer of the retinoic acid trigger. We have since expanded these studies to ascertain if CHYLO stimulated the other required proteins involved in ASP production, such as factor B and adipsin.

CHAPTER FIVE
CHYLOMICRON-SPECIFIC ENHANCEMENT OF FACTOR B AND ADIPSIN
IN DIFFERENTIATED HUMAN ADIPOCYTES

Section 5.1: Introduction

There is a large body of evidence that demonstrates that in various tissues complement C3 and factor B can be regulated. Unlike complement C3 and factor B, there are few studies that investigate the regulation of adipsin.

The C3 gene is 42 Kb in length and is comprised of 41 exons ranging in size from 52 bp to 213 bp (201,211-213). Analysis for homologies between sequences in the 5' flanking region (1018 bp of exon 1) of the C3 gene (77,201,211,212) identified a number of putative response elements (chapter 4.4.5).

Factor B is encoded by a single gene in the major histocompatibility complex and is closely linked to the gene of C2 (112). Genebank analysis of the promoter region (1605 bp) of the factor B gene using the Mac Vector version 6 program identified putative IFN γ response elements, glucocorticoid response element, IL-6 response element, thyroid hormone response element (TRE), and estrogen response element (ERE). In mouse and human HepG2 cells transfected with the factor B promoter fused to CAT reporter gene, IL-1 and IFN γ are capable of inducing the gene, (112,214).

To date, there is no data on the promoter sequence of adipsin or the identification of putative response elements. Earlier studies using DNA mapping and gel retardation demonstrated that specific regions of the promoter were necessary for adipsin expression (215,216). Taken together with my analysis of the promoter regions of C3 and factor B, and the published experimental data, it appears that different hormones could regulate these three proteins transcriptionally.

Not only are complement C3, factor B and adipsin synthesized and secreted by a variety of cells, but they are also regulated by a number of factors, and in some cases in a cell specific manner (Table 5.1).

Aside from the putative response elements identified in the promoter regions of the C3 or factor B genes, it has been shown directly that IL-1 β in epithelial cells upregulates C3 and factor B at the transcriptional level (217). Other studies looking at complement C3 synthesis have demonstrated that increases in C3 levels by factors such as TNF α (217,218), interferon γ (217,219,220), 1 α -25-dihydroxyvitamin D3 (221) and transferrin (222) are due to transcriptional regulation.

The data on factor B suggest that there is both transcriptional and translational regulation of factor B. Interferon α increases transcriptional rates

of factor B in a dose dependant manner (223). On the other hand, several studies demonstrate that interferon γ increases post-transcription regulation of factor B by increasing mRNA stability (219,224). It has been shown that there are two transcripts of factor B (long and short) and that these transcripts are translated at a two-fold difference in rate in murine kidney and intestinal cells (225). Changes in the ratio of the long to the short transcript would affect the net production of factor B. Thus these results suggest that the synthesis of factor B can be regulated by both transcriptional and translational control mechanisms.

While many tissues produce C3 and factor B, adipose tissue synthesizes the majority of whole body adipsin (62). However, it recently has been demonstrated that adipsin is also synthesized and secreted by hepatocytes (226), U937 cells (human macrophages) (227), blood monocytes (228) and sciatic nerves (62). There is a controversy in the studies that investigate the regulation of adipsin by insulin. In some instances (229,230), insulin has a negative effect and in other instances insulin has a positive effect (20,157,231). More specifically, insulin decreases adipsin gene expression by affecting the rate of transcription (232), but has a stimulatory effect on the release of adipsin (157). In the literature there have been hormonal regulators identified for adipsin, more will likely be added as research in potential drug targets expands. Only a few other factors to date have been shown to regulate adipsin production (Table 5.1). With the above evidence and the fact that there may be internal processing of two transcripts of different sizes yielding the detection of one

molecular weight species of adipsin (62,228), this would suggest that adipsin also could be regulated at both the transcriptional and post-transcriptional levels.

Since complement C3, factor B and adipsin can also be regulated at multiple levels in other cells prior to secretion (RNA synthesis, RNA stability, RNA transcript length, post translational modification), it is feasible that these proteins would also be regulated in adipocytes. As adipose tissue do not synthesize complement C2 or C5 proteins (personal correspondence by Dr. RA. Wetsel), which are part of the classical or alternative complement, the generation of ASP by adipose tissue cannot be seen as simply an immune response. This is unique and differs from the response by other tissues in the body that respond to a toxin by stimulating the classical or alternative complement pathway. From the previous studies (chapters 2, 3, 4), it is clear that CHYLO stimulated both C3 and ASP production and this effect is mediated through a direct increase in C3 mRNA. We have extended these studies to look at the other two factors involved as well (factor B and adipsin), since the production of ASP is dependent on extracellular interaction of the three components (C3, factor B and adipsin). As well it is believed that cell anchorage of the activated convertase is important for the enzymatic action (77,233,234), and we have also examined this facet of ASP production.

Section 5.2: Methodology

Methodology for adipocyte cell culture, ASP and C3 determination, mRNA isolation, and statistical analysis are described in the previous chapters. Additional methodology is described below.

Section 5.2.1: Sandwich Elisa for Factor B

Murine monoclonal antibody to Bb fragment of factor B (Quidel, San Rafael, CA) was coated at 1 µg/mL in PBS (100 µL per well) overnight at 4°C and blocked with 1.5% BSA for 2 hours. The plate was washed three times with wash solution (0.05% Tween 20 in 0.9% NaCl) between every step. A standard solution (0 to 100 ng/mL) of factor B (Calbiochem), as well as test samples (conditioned culture media diluted appropriately) and in-house control plasma samples (diluted 1:10³) were added at 100 µL per well. Subsequent steps were identical to those in the ASP sandwich ELISA (Chapter 2). Goat polyclonal anti-B (1:5000, Quidel) and anti-goat IgG conjugated to horseradish peroxidase (1:5000, Sigma) were used for the factor B sandwich ELISA. The log of factor B concentration was plotted against the log of absorbance and sample factor B concentration calculated by linear regression.

Section 5.2.2: Sandwich ELISA assay for Adipsin

Adipsin was measured using a sandwich ELISA, as previously described (129). Briefly, monoclonal 101.1 (Dr. T. White, Scios Nova, LaJolla, Ca, USA) was used as capture antibody for adipsin and monoclonal 2.1A (Dr. T.

White, Scios Nova, Lajolla, Ca, USA) was used as the detecting biotinylated antibody for adipsin. Monoclonal 101.1 was coated at 3 $\mu\text{g/mL}$ in PBS (100 μl per well in a 96-well plate) overnight at 4°C and blocked with 1.5% BSA for 2 hours at room temperature. The plate was washed three times with wash solution (0.05% Tween 20 in 0.9% NaCl) between every step. A standard solution (0.0156 nM to 1 nM) of adipsin (Scios Nova), as well as test samples (conditioned culture media diluted appropriately) and in-house control plasma samples (diluted 1:300) were added at 100 μL per well. Subsequent steps were identical to those in the ASP sandwich ELISA found in Chapters 2, 3, and 4. Biotinylated monoclonal 2.1A (1.5 $\mu\text{g/mL}$, Scios Nova) and NeutrAVIDIN conjugated to horseradish peroxidase (0.1 $\mu\text{g/mL}$, Pierce, Rockford, IL, USA) were used for the adipsin sandwich ELISA. Adipsin concentration was plotted against absorbance and sample adipsin concentration calculated by linear regression.

Section 5.2.3: Factor B and Adipsin RT PCR

As previously described in the methods section of chapter 4, mRNA was isolated from differentiated human adipocytes that were exposed to the experimental incubations. Reverse transcription was performed to generate cDNA. cDNA was amplified using in-house primers for factor B, adipsin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (housekeeper gene) as described previously (43,74). The cycle number utilized was 35 cycles for both factor B and adipsin, and 25 cycles for GAPDH. Tetramethyl ammonium

chloride (TMAC) was excluded from the PCR reaction mix when amplifying adipsin. PCR products were separated on a 7% piperazine diacrylamide polyacrylamide gel with a 100 bp DNA ladder as size marker. All samples were within the range of the mass standards used and values are expressed as a ratio of factor B to GAPDH and adipsin to GAPDH.

Section 5.2.4: Statistics

Values are reported as means of experiments (with all determinations for each point in each experiment performed at least in triplicate) \pm standard error of the mean (sem). Statistical significance was set at $p=0.05$ and was determined using either Student's t-test or one-way ANOVA (with multiple comparisons by Bonferoni's method) as indicated; where $p=NS$ indicates not significant.

Section 5.3: Results

We first investigated the effect of CHYLO on factor B secretion. Differentiated adipocytes were incubated with increasing concentrations of CHYLO for an incubation period of 6 hours. From Figure 5.1, not only do CHYLO stimulate factor B production, this occurs in a concentration dependant manner. The fold increase in the levels of factor B was comparable to the CHYLO effect on C3 as shown previously (Chapter 2, 3, 4) although the absolute concentration of protein was lower. CHYLO also stimulated the secretion of adipsin (9.24 ± 1.60 fold with respect to basal). Since we had previously demonstrated that the CHYLO effect on C3 and ASP production was mediated through TTR transfer of retinoic acid to the adipocytes, this was examined for factor B and adipsin as well (Table 5.2). C3 and ASP levels are presented for comparison.

From the data in Table 5.2, it is clear that CHYLO significantly stimulate factor B and adipsin secretion. We chose to present this preliminary data in percentage with respect to basal so as to better compare the increases in stimulation for each protein. Stimulation of factor B by the various combinations is quite similar to the pattern of stimulation of C3 in that the stimulation by CHYLO/TTR is significantly over and above the stimulatory effect of CHYLO alone. The addition of RA with CHYLO produced a dramatic effect on factor B levels over and above the effect of CHYLO alone, while this trend was not observed for C3 protein levels. By contrast, the addition of TTR to CHYLO did

not enhance adipsin stimulation when compared to the CHYLO. Overall, the data demonstrate that the stimulation pattern of the product ASP generally follows that of the substrates C3 and factor B but differs from the pattern of stimulation of the enzyme adipsin.

mRNA levels of factor B and adipsin were also determined. The same conditions were used as with the mRNA studies on C3 (chapter 4). Differentiated adipocytes were incubated for 2, 6 or 7 hours with and without CHYLO and the mRNA was assessed by semi-quantitative RT-PCR (Figure 5.2). There is a significant increase in factor B mRNA at 6 hours when compared to the control at 6 hours ($67\% \pm 16$ increase ($p < 0.001$)). There was no stimulation by CHYLO at early (2 hours) or later (7 hours) time points. The stimulatory effect of CHYLO on factor B mRNA is also blocked in the presence of polyclonal antibody to transthyretin (pAbTTR) and retinoic acid receptor antagonist (RA ant) (Table 5.3). Again, this pattern parallels that of C3 as previously shown. By contrast, if anything, the CHYLO effect on adipsin mRNA appeared to be inhibitory with a significant decrease by 7 hours ($p = 0.051$). The addition of pAb TTR or RA antagonist did not have any effect on adipsin mRNA (Table 5.3).

While secretion of C3, factor B and adipsin can influence the production of ASP, extracellular anchorage of the C3 convertase has also been suggested to play a role (235). We have examined this in preliminary experiments by attempting to disrupt the cell surface interaction. Human adipocytes were

exposed to CHYLO in the presence or absence of heparin. Heparin, a proteoglycan, interferes with cell surface interaction (236). From the data in Figure 5.3 heparin alone does not affect basal secretion levels of C3 or ASP production. Although the addition of heparin to CHYLO did not change the chylo-stimulated C3 levels, the addition of heparin to chylo did abolish the CHYLO-stimulatory effect on ASP levels ($91\% \pm 2\%$ inhibition, $p < 0.001$). This preliminary evidence suggests that the association to the cell surface of one or more factors appears to be important in the generation of ASP.

Section 5.4: Discussion

From the literature, it is apparent that complement C3, factor B and adipsin are regulated by different factors/hormones and this regulation can differ with respect to the cell type. Previously, we have shown that ASP and C3 (precursor) are produced by adipocytes and CHYLO is the postprandial plasma component that dramatically stimulated C3 and ASP production. We postulated that the other proteins (factor B and adipsin) involved in ASP production would also be stimulated. From the data, it is evident that CHYLO can also significantly stimulate factor B and adipsin. In addition, the CHYLO effect on factor B is concentration dependent. The overall pattern and extent of factor B stimulation was similar to that of C3 and ASP, while the extent of adipsin stimulation was less. It must be emphasized that the conversion of C3 to ASP is not one to one. ASP levels were on average 15 percent of that of C3 levels. In most enzymatic reactions, the substrates (C3 and factor B) are in excess of the enzyme (adipsin) because the enzyme is usually reused in several cycles for the generation of products, thus the decreased effects on adipsin levels might well not affect the overall level of level of ASP production. This is the first evidence that demonstrates the co-ordinated regulation of C3, factor B, adipsin and ASP via the same postprandial signal.

The addition of TTR to CHYLO resulted in a level of stimulation that was above the stimulation of CHYLO for the substrates (C3 and factor B) and product (ASP), but not adipsin. The addition of RA to CHYLO significantly reduced

adipsin production, this was consistent with the effect of RA down-regulation of the adipsin gene (237). By contrast, there was a noticeable increase in factor B, C3, and ASP production over that of CHYLO stimulation alone. While fold stimulation of factor B was seemingly high, the amount of factor B protein secreted basally is very low and close to background, therefore a small increase in protein secretion would generate a noticeable fold change, making accurate determinations difficult.

Because we were able to demonstrate that the CHYLO-stimulatory effect on C3 involves an increase in mRNA levels and *de novo* protein synthesis (chapter 4), we then investigated whether CHYLO also increased mRNA of factor B and adipsin. From the data, it is clear that CHYLO increase factor B mRNA levels at the same time point and there is an inhibition of the CHYLO stimulatory effect when either the polyclonal antibody to TTR or RA receptor antagonist is present as seen with C3. By contrast, there was a significant reduction in adipsin mRNA levels at 7 hours in the presence of CHYLO. The above data hints at a regulation of adipsin by CHYLO but further investigation with additional time points is needed. As previously discussed in chapter 4, due to the limitation of the availability of cultured human adipocytes, these time points were selected based on the CHYLO time courses (chapters 2, 3).

The analysis of the C3 and factor B promoters identified a number of putative response elements that could possibly regulate *de novo* protein

synthesis of these proteins, while detailed analysis of the adipsin promoter still remains to be performed. It is important to note that the majority of the putative response elements have not as yet been investigated to determine their regulation of C3 and factor B. We have shown that retinoic acid can regulate factor B, yet there are no known retinoic acid receptor response element sequences identified on the promoter region, suggesting that there are new retinoic acid response element sequences present that remain to be identified.

As previously discussed, there are two enzymes involved in ASP generation, adipsin and C3bBb convertase. Adipsin cleaves factor B to release of Ba portion of C3bB generating the active convertase, C3bBb. C3bBb can now cleave additional C3 molecules to produce C3a and C3b. To date, functional studies on adipsin have demonstrated that the sole substrate for adipsin is complexed factor B (93,94) and it was proposed that the active conformation of adipsin is induced by its natural ligand. This is not without precedent as matrilysin is an example of a serine protease that requires conformational changes to induce proteolytic activity (238). Muscle glycogen phosphorylase b (239) is another enzyme where conformational changes are induced by specific ligands (239-241). Thus, ligand-induced conformational changes may play an integral part of the regulation of adipsin activity.

Early studies of the convertase indicated that enzymatic activity of the assembled complex (C3bBb) decays with a half-life of 10-20 mins at 37°C (242-

244). The addition of antibody to the Bb portion (245) or complexing of nickel cation (Ni^{2+}) (246) to the convertase substantially increases the stability of the complex. *In vivo*, it is believed that properdin maintains activation of this complex by binding to and stabilizing the convertase (247). Assuming that there is a sufficient amount of all three factors present, the conformational changes as a result of ligand binding to adipsin or stabilization (properdin-convertase) are additional factors that could affect product (ASP) levels. Although C3 was still secreted, the inhibition of cell surface contact caused by heparin may have resulted in two situations: i) hindering the proper formation of the initial C3bB complex or interaction with adipsin and hence preventing the ligand-induced conformational changes hypothesized for adipsin, or ii) preventing the anchoring of the active convertase complex (C3bBb) to the cell surface that may stabilize the convertase and increase its half life, thus consequently affecting the conversion of C3 to generate ASP. The preliminary studies with heparin are the first data in a new experimental direction that will examine the regulation of the conversion of C3 to ASP.

Section 5.5: Tables and Figures

Table 5.1: Regulation of Complement Proteins by Various Proteins

Regulatory Factor	Complement C3	Factor B	Adipsin
IL-1α	↑ Keratinocytes (248) ↑ HepG2reporter (249) ↑ Epithelial cells (250) ↑ Endothelial cells (217)	↑ Keratinocytes (248) ↑ HepG2reporter (249) ↑ Epithelial cells (250) ↑ Endothelial cells (217)	
IL-1β	↑ myocytes (251) ↑ Epithelial cells (218,251,252)	↑ Myocytes (251) ↑ Epithelial cells (101)	
IL-4	↑ Fibroblasts (253)	↑ Fibroblasts (253)	
IL-6	↑ Fibroblasts (254,255) ↑ HepG2reporter (256)	↑ Keratinocytes (248) ↑ Fibroblasts (254,255) ↑ Epithelial cells (101)	
IL-17	↑ Fibroblasts (257)	No change in Fibroblasts (257)	
IFNγ	↑ Keratinocytes (248) ↑ Myocytes (251) ↑ Epithelial cells (220) or No change (218) ↓ Fibroblasts (219) ↓ Endothelial cells (219) ↑ Hepatocytes (249)	↑ Keratinocytes (248) ↑ HepG2reporter (219,249) ↑ Fibroblasts (219,224,258) ↑ Endothelial cells (219) ↑ Myocytes (251)	↓ Adipocytes (259) ↓ Astroglioma cells (260)
TNFα	↑ Keratinocytes (248) ↑ Fibroblasts (255) ↑ Epithelial cells (101,218)	↑ Adipocytes (73) ↑ Mesangial cells (261) ↑ Fibroblasts (255)	
C/EBPβ & δ over expression	-	-	↑ Adipocytes (262)
Retinoic acid	-	-	↓ Adipocytes (237)
Insulin	↑ Adipocytes (75)	-	↓ Adipocytes (229,230) ↑ Adipocytes (20,157,231)
Growth Hormone	-	-	No change Adipocytes (263)
Estrogen	↑ Epithelial cells (264) ↑ HepG2reporter (256)	-	-
Glucocorticoids	↓ Endothelial cells (250) ↑ Epithelial cells (265)	↓ Endothelial cells (250)	↑ Adipocytes (230)
Aldosterone	↑ Epithelial cells (265)	-	-
Vitamin D	↑ Stromal cells (221)	-	-
Transferrin	↑ Epithelial cells (222)	-	-

Table 5.2: Effects on C3, Factor B, Adipsin and ASP secretion

	ASP	C3	Factor B	Adipsin
Basal	100 % (1.3 ± 0.32 mg/c.p.)	100 % (2.3 ± 0.79 mg/c.p.)	100 % (0.3 ± 0.02 mg/c.p.)	100 % (0.7 ± 0.2 mg/c.p.)
+ TTR	128 ± 46	29 ± 33	1187 ± 83*	26 ± 20
+ RA	378 ± 142	418 ± 102*	No data	7 ± 5
CHYLO	2255 ± 225*	1752 ± 120*	3180 ± 550*	924 ± 160*
+ TTR	5260 ± 115**	6724 ± 170**	6011 ± 69**	846 ± 70*
+ RA	4067 ± 2571*	1709 ± 187*	19023 ± 24**	398 ± 130**

Differentiated human adipocytes were exposed to combination of chylomicrons (CHYLO, 50 µg TG/mL medium), transthyretin (TTR, 1.25 µg/mL medium) or retinoic acid (RA, 1.25 µg/mL medium) for 6 hours. C3, ASP, factor B and adipsin were measured in cell media and the results are expressed as percentage ± percent SEM with respect to the basal level. Basal was set at 100 percent. Three to four experiments were performed with duplicate or triplicate conditions (n ≥ 9) for C3 and ASP. Preliminary results are presented for factor B and adipsin where one to two experiments were performed with duplicate or triplicate conditions (n ≥ 3). Multiple comparison analysis (ANOVA) was performed where * p<0.001 for comparison with basal control, and ** p<0.001 for comparisons with chylomicron alone.

Table 5.3: Effect of Transthyretin antibody and Retinoic Acid Receptor Antagonist on C3, Factor B and Adipsin mRNA.

	C3	Factor B	Adipsin
Basal	100%	100%	100%
CHYLO	191 ± 31*	167 ± 16*	117 ± 21
+ pAb TTR	106 ± 9	126 ± 25	83 ± 10
+ RA Ant	110 ± 8	120 ± 7	112 ± 17

Differentiated human adipocytes were incubated with or without chylomicrons (50 µg TG/mL), polyclonal antiserum to transthyretin (pAb TTR: 2.5 µg/mL) or retinoic acid receptor antagonist (RA Ant: 3.3 µM) for 6 hours. The media was removed from the cells, and total RNA was isolated. Using in-house primers for C3, factor B, adipsin and GAPDH, RT-PCR was performed. Results are expressed as a percentage ± percent SEM with respect to basal for an average of at least 5 experiments where *p=0.04 vs CTL by ANOVA. Basal was set at 100 % . C3 results presented can be found expressed as a ratio over GAPDH (arbitrary units) in chapter 4 (Figure 4.3) and in chapter 5 (Figure 5.2).

Figure 5.1: Chylomicron concentration curve for Factor B

Differentiated human adipocytes were exposed to increasing concentrations of chylomicrons (0 - 500 μg TG/mL medium) for a period of 6 hours. Factor B was measured in cell media and the results are expressed as pmol/mg cell protein \pm sem for n=9 where significance for the individual CHYLO concentrations was calculated by t-test of CHYLO where * $p < 0.002$ for all concentrations.

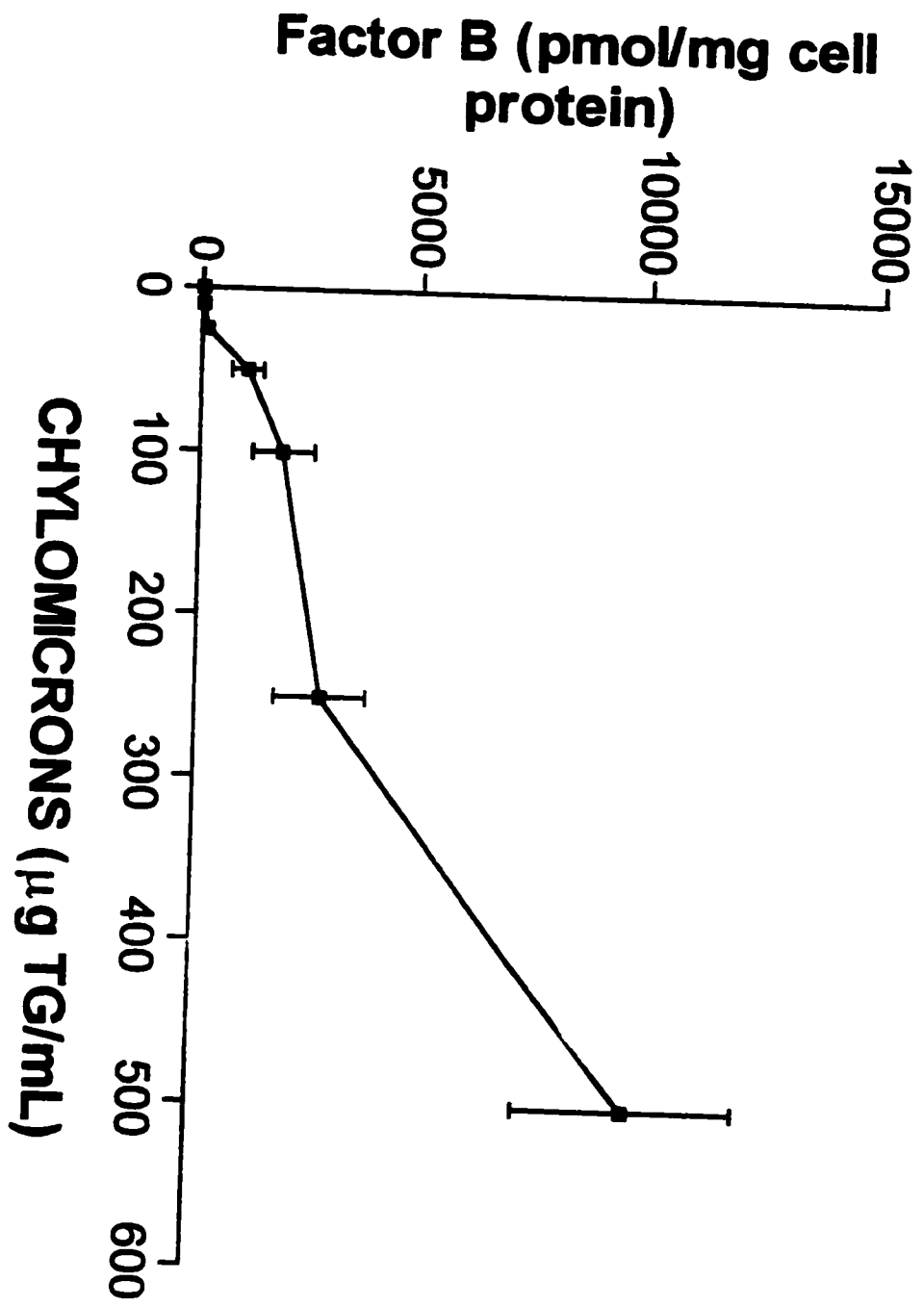


Figure 5.2: The Effect of CHYLO on C3, Factor B and Adipsin mRNA levels.

Differentiated human adipocytes were incubated with or without chylomicrons (50 µg TG/mL) for 2, 6 and 7 hours. The media was removed from the cells and total RNA was isolated. Using in-house primers for C3, factor B, adipsin and GAPDH, RT-PCR was performed. The results are expressed as ratio over GAPDH (arbitrary units) for n=5 or more where *p=0.04 vs CTL at 6 hours, *p<0.001 vs chylomicrons at 2 and 7 hours, and "a" represents p=0.051 by multiple comparison analysis (ANOVA).

Note: C3 results are reproduced from Chapter 4 (Figure 4.3).

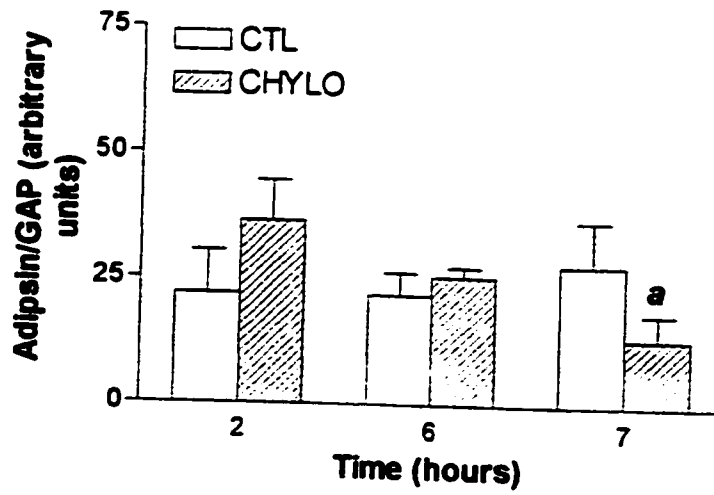
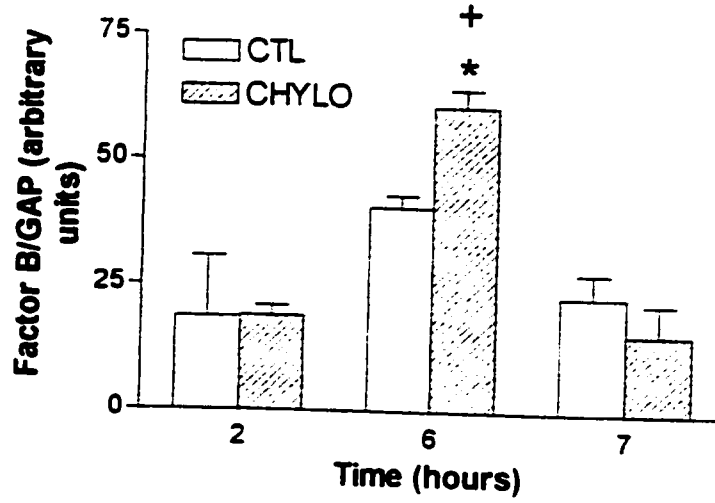
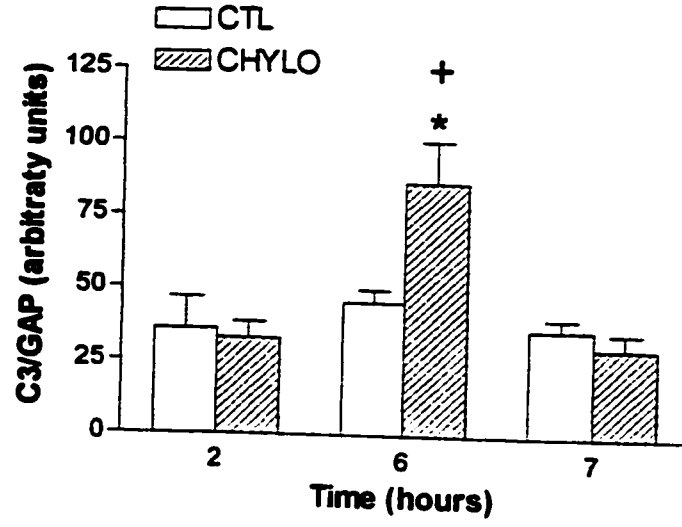
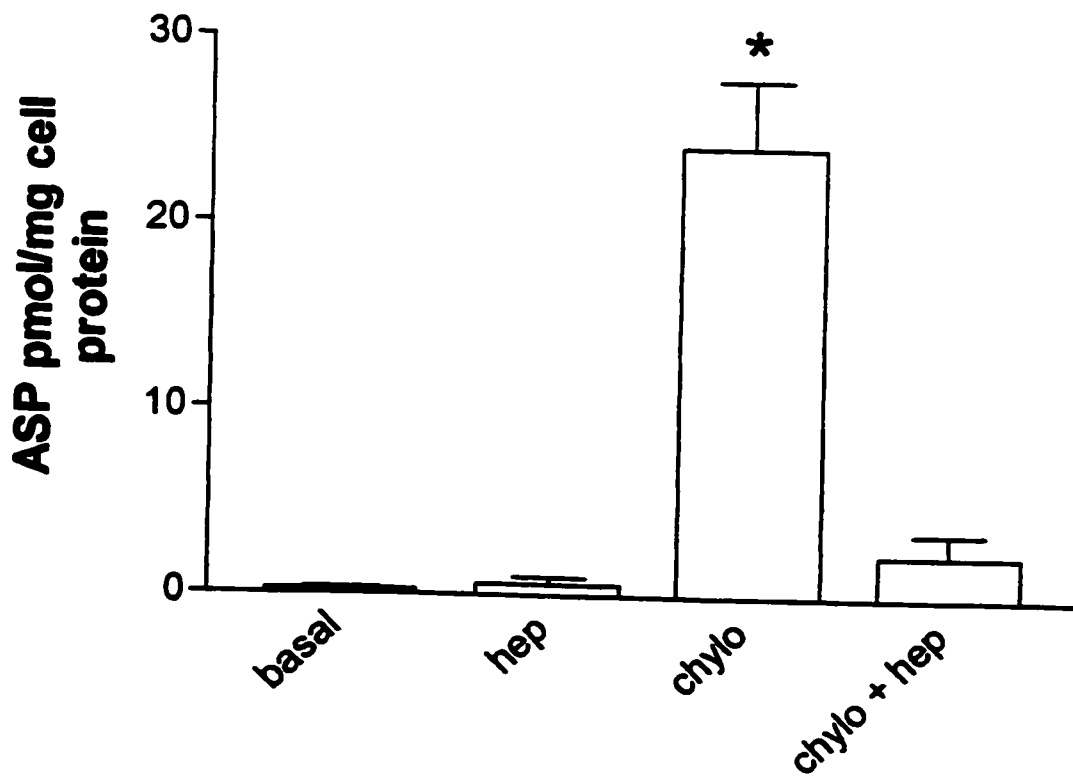
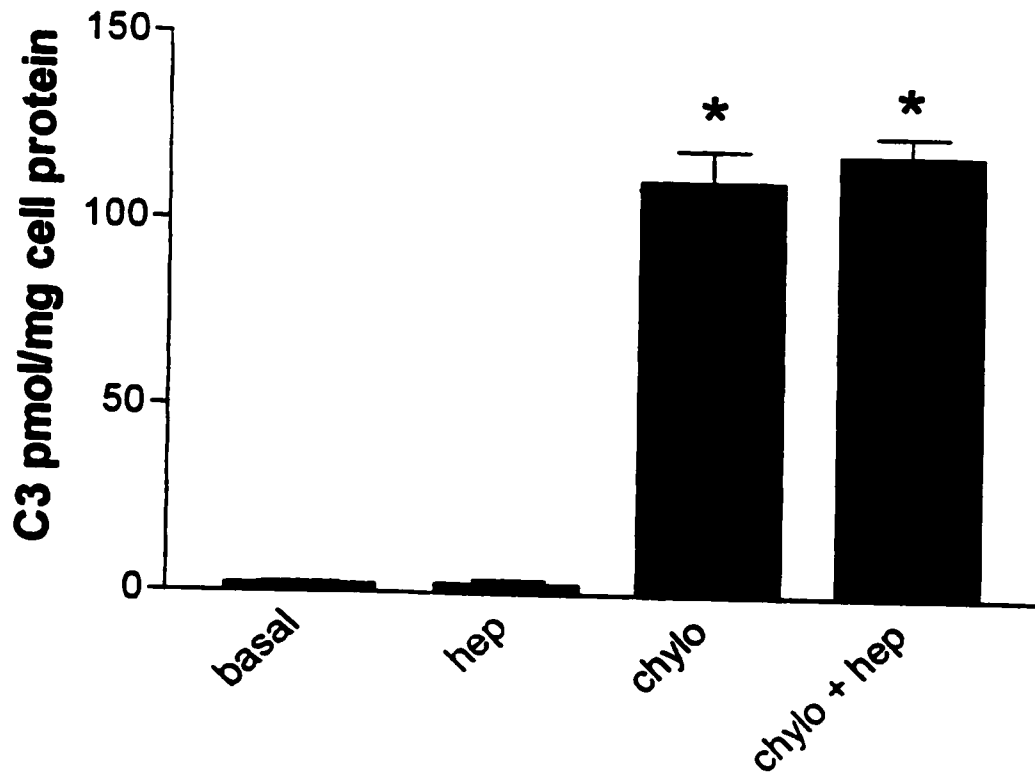


Figure 5.3: Heparin Inhibits ASP Production but not C3 production

Differentiated human adipocytes were exposed to heparin (10 U/mL) in the absence or presence of chylomicrons (50 µg TG/mL) for a period of 6 hours. C3 was measured in cell media and the results are expressed as means ± SEM (n=8), where * p<0.001 vs basal was calculated by t-test.



Abbreviations:

ASP	Acylation Stimulating Protein
BSA	bovine serum albumin
DMEM/F12	Dulbecco's minimum essential medium/Ham's F12
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
MEM	minimum essential medium
PBS	phosphate buffered saline
PPAR	peroxisome proliferator-activated receptor
RA	retinoic acid
sem	standard error of the mean
TG	triacylglycerol
TMAC	tetramethylammonium chloride
TTR	transthyretin

Section 6.1: Summary of Research Presented

The above body of evidence from this thesis demonstrates that CHYLO stimulated ASP, C3, factor B and adipsin production in adipocytes. The levels of ASP and C3 were at a maximum at four to six hours of CHYLO incubation, which is concurrent with the latter part of the postprandial period *in vivo*. While the TTR mediated retinoic acid transfer is involved in the stimulatory effect on C3 and factor B secretion (and subsequently ASP production), the CHYLO mediated effect on adipsin appeared to be mediated differently. Finally, while the focus of this thesis was on the transcriptional and secretory regulation of the precursor proteins C3, factor B and adipsin, extracellular cell interaction factors also appear to be important in the actual generation of ASP.

As mentioned previously, various cells produce C3, factor B and adipsin. The fact that these are regulated by different factors and the regulation is specific to cell type, supports the hypothesis that the synthesis and secretion of these proteins are likely regulated in adipocytes in a specific manner. The fact that insulin increases *in vivo* early in the postprandial period (2 hours) and has been shown *in vitro* to stimulate the release of adipsin (20,157,231) is concordant with the mRNA data that suggest early protein synthesis of the necessary enzyme. This in turn is consistent with the previous evidence that insulin can stimulate ASP production. In this way, insulin may initiate the cycle of

ASP generation in the first part of the postprandial period. This production cycle is then further enhanced by the increasing presence of retinol that is transferred from the CHYLO and activated to RA (Figure 6.1). It is conceivable that RA, in combination with other factors, directly activates *de novo* protein synthesis and secretion of C3, factor B and adipsin; and thus regulate ASP production. The interaction of these proteins on the cell surface leads to the generation of ASP in the microenvironment of the adipose tissue. ASP will then act in an autocrine manner to increase TG synthesis by enhancing glucose transport, FA-esterification and reducing FA release, resulting in a net increase in TG storage. Taken to the extreme, an excess of ASP might even shift the energy balance equation in the direction of energy storage and thus aid in the development of obesity.

Section 6.2: Novelty of the Results

It is important to note that this body of evidence identified one dietary component (retinol/retinyl ester) responsible for the CHYLO stimulatory effect and the mechanism of action. These data also demonstrate that a dietary factor can directly affect adipose tissue lipid metabolism, which is in contrast to other dietary factors that first provoke the release of intestinal hormones which then act on adipose tissue. It must also be emphasized that this is the first documentation of the co-ordinated regulation of ASP and the necessary proteins (C3, factor B, adipsin) involved in ASP synthesis by a novel postprandial signal (retinoil/retinyl ester) that is transferred from CHYLO. Because CHYLO TG

hydrolysis is now coupled to adipocyte TG synthesis, one can then link the events of the capillary space to the events of the subendothelial space via the ASP pathway. These data also demonstrate novel transcriptional regulation of C3 and factor B in adipocytes by retinoic acid/CHYLO. It must be further pointed out that this is also the first evidence that presents a more defined hormonal regulatory model of ASP in the adipocyte micro-environment that subsequently allows effective and efficient energy storage via an autocrine protein, ASP, stimulated by a postprandial signal.

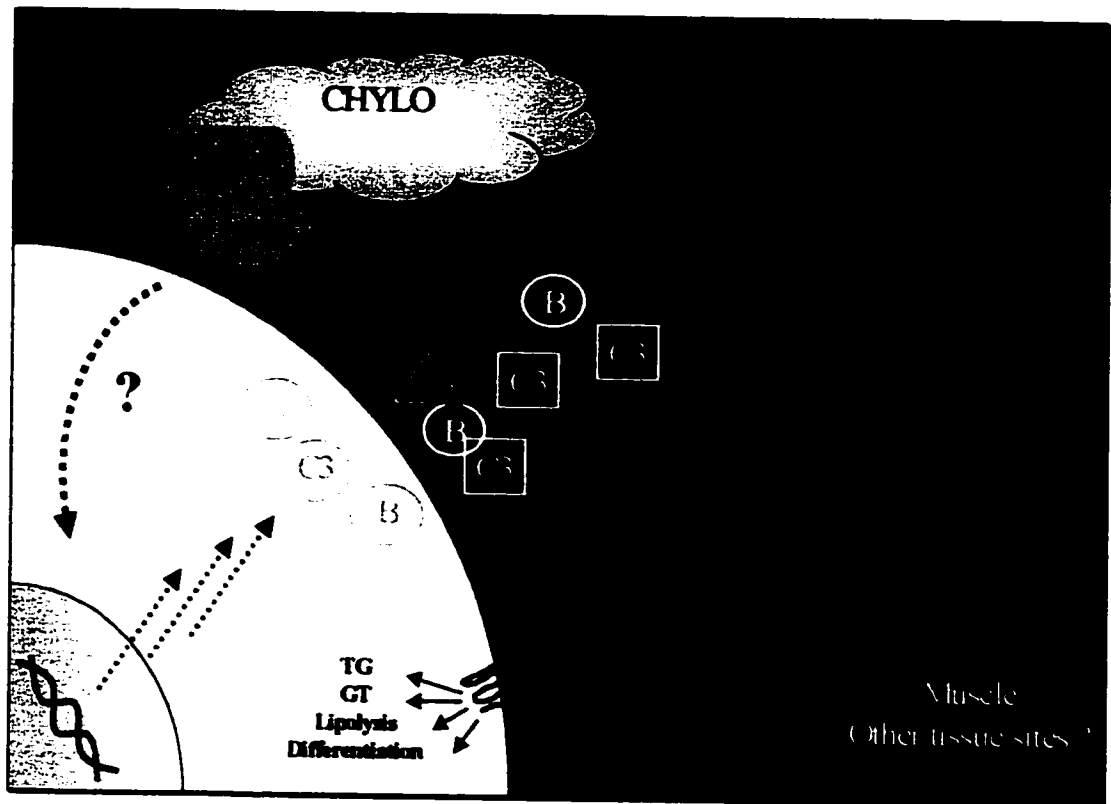


Figure 6.1: Postprandial Mechanism of ASP Production

CHYLO transports the postprandial signal that results in the stimulation of the synthesis and secretion of complement C3, factor B and adipsin subsequently generating ASP. Abbreviations: complement C3 (C3), factor B (B), adipsin (D), transthyretin (TTR), chylomicrons (CHYLO), acylation stimulating protein (ASP), triacylglycerol (TG), and glucose transport (GT).

Section 6.3.1: Limitations of the *In vitro* Experimental Cell Model

It is important to note that the cell model used does have limitations. Because we used human differentiated adipocytes, this limits the amount of adipose tissue obtained, thus limiting the number of conditions that we could test on cells from one patient source. Because of patient to patient variation this results in a larger standard error. It is important to note that CHYLO were also isolated from different human sources, since we needed to use freshly isolated CHYLO within a time frame of two weeks to minimize degradation of the lipoprotein particles. Because postprandial blood is needed in order to obtain CHYLO, we could not use the same individual every two weeks, again generating unavoidable variation in the experiments. However, it is also important to note that while there was variation in the basal and stimulated concentrations of proteins measured, CHYLO clearly stimulated consistently with a similar magnitude. The different adipose tissue and CHYLO sources likely explain the variability noticed both in the basal and stimulated protein levels throughout the thesis. Notwithstanding the limitations, we believe that this experimental system most closely represents the *in vivo* situations, since continuous human adipocytes cell lines do not exist. While embryonic mouse clonal cell lines exist that can be differentiated, no mouse ASP assay was available. Finally, a CHYLO substitute was also not available, and the lipid substrate used for LPL activity did not mimic the CHYLO stimulation.

Finally, it is important to note that we used isolated human adipocytes as a simplified model to directly determine the events in the postprandial increase of ASP. As a result, this isolated adipocyte cell model lacks the *in vivo* environmental influences from other tissues such as hormones, and other factors that are physiologically present. By eliminating some of the factors that may normally contribute to cell response, we may have overlooked some influences. However, in spite of these limitations we believe the results to be valid and important, and there is a notable amount of *in vivo* evidence that supports the human differentiated adipocyte cell data.

Section 6.3.2: Limitations of the Data

We tried to dissect both the specific factor in CHYLO responsible as well as the mechanism of action. Although we did find that the RA/TTR mediated an increase on C3, factor B and ASP via a transcriptional mechanism, it should be pointed out that the limitations were: i) lack of full reconstitution of the stimulatory activity, and ii) the transient transcriptional effect was of smaller magnitude than the cumulative effect on protein secretion. This suggests that there may be additional circulating factors (thyroid hormone, properdin) and additional mechanisms (formation of C3bB complex, cell surface anchorage, C3 convertase stability) that may explain the CHYLO mediated stimulation.

Within the C3 data presented in this thesis, we observed where a 2 fold increase in mRNA levels with CHYLO stimulation resulted in an increase in

protein levels up to 17 fold. It is not clear whether this large increase in protein is a result of increased transcriptional rate, increased RNA stability, or increased translational rate of C3. Since it has been previously discussed that C3, factor B and adipsin maybe regulated at several steps starting from gene regulation to protein synthesis (chapter 5: introduction), it is plausible that upregulation at a few or all of these steps can result in a large increase in absolute protein levels. Further studies to investigate the regulations of these steps would address this issue in more details. In addition, it is important to note that mRNA processing involves a turnover where there is regulated synthesis and degradation of the mRNA. Since there may not be a regulatory degradation process of these proteins in the *in vitro* cell model, the proteins (C3, factor B, and adipsin) measured in the media represent the accumulation of proteins synthesized from the basal and stimulated mRNA amounts present, which may further help to explain the fold differences in mRNA with respect to protein levels.

Even though RA is one of the key stimulating components of the CHYLO, it is not functioning alone but in conjunction with TTR. CHYLO are also a source of both TTR and RA (retinyl ester and/or retinol). If CHYLO are isolated from one human source that already contains a sufficient amount of TTR and/or RA to have an effect, one may not observe an effect with further addition of these proteins to CHYLO. In the same way, the addition of TTR and/or RA to a CHYLO preparation that contains lesser amounts of these proteins can result in an additional stimulatory response over the CHYLO alone. I believe that this

may explain the variability in the magnitude of the stimulatory response when TTR or RA is added to CHYLO. However, it is important to emphasize that the CHYLO effect, while ranging in magnitude, always had an effect.

Published results on RA effects are somewhat controversial with respect to TG. Systemic administration of retinol and RA to humans (high doses) have resulted in hypertriglyceridemia. Although this seems counterintuitive to the above presented results, it is difficult to compare the two observations since concentrations used in the thesis are extremely low. RA has also been shown to have opposing effects when comparing high versus low concentration treatments to cells. . High pharmacological concentrations of RA decrease differentiation but low doses (physiological) increase differentiation (199,205). It is, therefore, plausible that at physiological concentrations of RA have the above demonstrated effects Further experiments using high doses would address this issue.

Section 6.4: *In vivo* Postprandial ASP Metabolism

Firstly the time course for the postprandial ASP increase is similar *in vitro* and *in vivo*. While the general circulating levels of ASP do not increase postprandially after a fat meal (132,189,190), there is evidence of a direct release of ASP from the adipose tissue bed in humans (132,189). The time period and magnitude of stimulation of ASP released was comparable *in vivo* and *in vitro* (maximally between four to six hours), and synchronous with maximal

TG clearance (132). These data are consistent with ASP function within the micro-environment of the adipose tissue.

Section 6.5: Mice Models of ASP Metabolism

While concurrent increases in ASP production and TG clearance do not prove cause and effect, we do have direct *in vivo* and *in vitro* evidence that ASP enhances TG clearance. Murray *et al.* (182) and Saleh *et al.* (266) demonstrated that the intraperitoneal injection of ASP significantly enhanced TG clearance by two- to five-fold in wild type (182) and two obese mouse models (*ob/ob* and *db/db*) (266). In studies with mice that lack functional ASP (C3 knockout mice), the males had slower postprandial TG clearance than their wild-type counterparts. TG clearance could be normalized in the male mice (C3 knockout) with intraperitoneally injected ASP at the beginning of the fat meal challenge (182). A striking characteristic of the ASP deficient mice for both males and females was a noticeable decrease in adipose tissue mass, and higher postprandial FFA when compared to their wild-type counterparts (182,267,268). The fact that postprandial FFA is increased in these ASP deficient mice suggests that LPL hydrolyzes the dietary TG as expected but that there is a problem with FFA trapping by the adipose tissue. Although mice may present differences from humans, this evidence suggests that lack of ASP results in an inefficient FFA trapping by the mice.

Section 6.6: Importance of ASP in Fat Metabolism

There is an expanding body of data that has investigated the relationship of complement C3, factor B, adipsin and ASP levels with obesity and related pathophysiological conditions. It is apparent from Table 6.1 that most of the studies in humans and all of the studies in rodents only analyzed one or two of the proteins. Additionally, most of the studies are comprised of small sample groups.

	ASP	C3	Factor B	Adipsin
Human: BMI	↑ (129,130,191,269)	↑ (130,270-272)	↑ (272)	↑ (129,272-275), no change (276)
Obesity	↑ (129,191,269)	↑ (272,277)	↑ (272)	↑ (129,273-275), no change (272)
Diabetes	↑ (278-280)	↑ (130,281)	-	-
FCHL or HyperTG	↑ (128,270)	↑ (270,271,282,283)	-	-
Weight Loss	↓ (269,284,285)	↓ (272,286,287)	↓ (272)	↓ (272), no change (274)
Anorexia nervosa	-	↓ (272)	↓ (272)	↓ (272,275)
Rodents: Obesity	-	↑ (192)	-	↓ (288-291) ↑ (292), no change (293)
Diabetes	-	-	-	↑ (293)

Table 6.1: Changes in ASP, C3, Factor B and Adipsin levels with obesity and related pathophysiological conditions.

To date there is only one human study that has simultaneously examined C3, factor B and adipsin with respect to BMI, anorexia nervosa and obesity (272). However, ASP was not measured. Pomeroy *et al.* (272) showed that the plasma levels of C3, factor B and adipsin are higher in obese subjects when compared to their matched controls. With weight loss, there was a significant decrease in C3, factor B and adipsin levels towards normal levels. In a similar fashion, this study also demonstrated that C3, factor B and adipsin are lower in starving anorectics; and their levels are normalized with weight gain. This

further strengthens the relationship between complement C3, factor B, and adipsin with obesity. Unfortunately, this study did not examine plasma ASP levels, and there was no direct correlational analysis between the three parameters. In the same instance, although C3 deficient patients clinically present with lipodystrophy (294), to date there is no available data on the lipid profile of these patients. Nonetheless, this observation further adds to the importance of ASP in lipid metabolism.

There are other studies in the general population (129) and amongst specific ethnic populations (130,191) that, taken together, demonstrate that increases in ASP, C3, factor B and adipsin are associated with obesity (Table 6.1). Nonetheless, further studies are really required to determine whether or not the increase in ASP levels is a cause or consequence of the development of obesity and associated diseases. However difficult to study, we believe that micro-environmental generation of ASP plays an essential role in the regulation of the adipose tissue and this is proposed as the site of action for ASP. In order to examine this directly, it would be of great interest to develop a transgenic ASP overexpressor mouse model to address this aspect.

CHAPTER SEVEN
CONSTRUCTION OF ASP PSEUDOGENE USED TO GENERATE THE ASP
OVEREXPRESSOR TRANSGENIC MOUSE

Section 7.1: Introduction

Disorders in gene expression in adipocytes may lead to pathological conditions such as obesity, lipodystrophy and subsequent associated complications such as diabetes and cardiovascular disease. For that reason adipocyte gene expression is of great biological and medical interest to finding a treatment of obesity. Because ASP levels are increased in obesity and ASP actively plays a role in enhancing TG storage, an ASP overexpressor transgenic mouse could provide evidence of a direct effect of ASP on obesity, and subsequently provide a drug target to control obesity.

In order to overexpress ASP directly in adipose tissue in mice, we needed to construct a gene by which ASP would be directly synthesized and secreted by adipocytes eliminating the normal route via the precursor C3, which is first secreted and then cleaved to generate ASP. To accomplish this we constructed an ASP pseudogene (Figure 7.1). For ASP to be synthesized directly, we used only that portion of the C3 gene that encodes the ASP sequence. We ligated this to the signal peptide sequence of the C3 gene so as to ensure that directly synthesized ASP would be secreted. To ensure the overexpression of this ASP

protein specifically by adipose tissue, we used the adipose tissue specific promoter adipocyte P2 (aP2) (295).

The aP2 gene encodes a fatty-acid binding protein whose expression increases with differentiation and is specific to adipose tissue (90,295-297). Studies of aP2 identified a functional adipose-specific enhancer element within the 5' flanking region at -5.4 Kb which is the major determinant of tissue specificity of the aP2 (295). Adipose tissue specificity of aP2 includes both white and brown adipose tissue. This aP2 promoter (5.4 kB) has been previously linked to both the GLUT4 gene (298) and the uncoupling protein gene (299,300) to generate transgenic adipose overexpressor mice for these proteins.

Section 7.2: Strategy

In brief, the two portions of the complement C3 gene representing the signal peptide and the ASP sequence were amplified. These two sequences were joined and a unique engineered restriction enzyme cut site was incorporated at each end (two different restriction cut sites in total). The engineered restriction enzyme cut sites facilitated the insertion of the ASP mini gene into the pBk-CMV phagemid. Because the CMV promoter lacks tissue specificity and would promote non tissue selective expression of ASP in the mice, it was removed and the aP2 promoter was inserted into this phagemid (pBk-aP2-SP-ASP). Finally the prokaryotic portion of this phagemid was removed and the pseudogene linearized for microinjection.

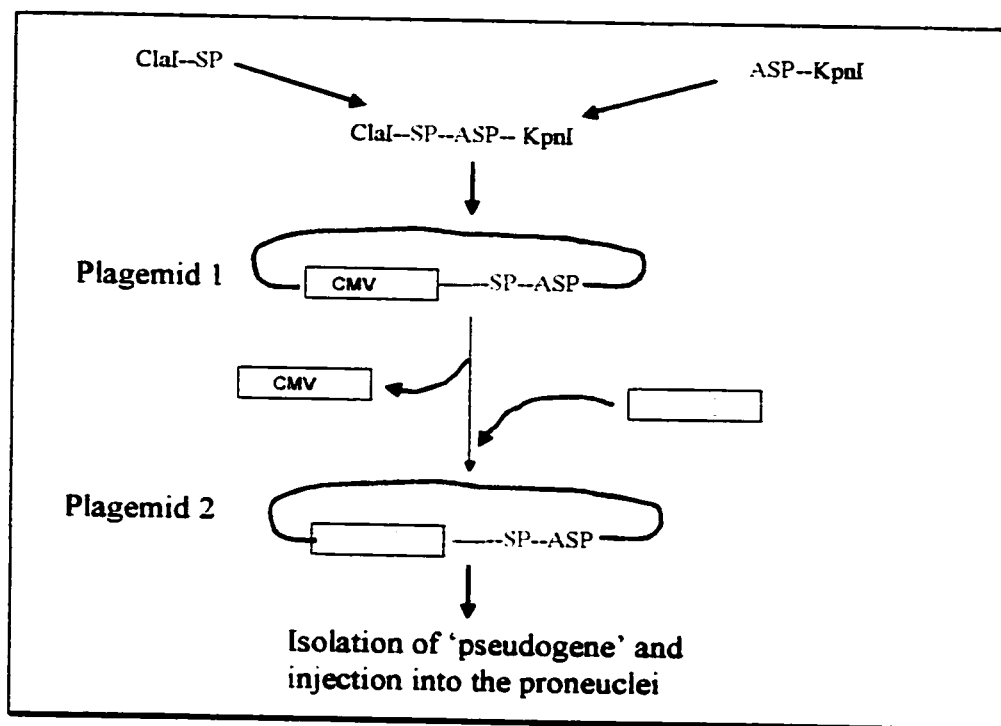


Figure 7.1: Construction of ASP 'pseudogene'

Two engineered cut sites were engineered onto the SP-ASP to facilitate insertion into the phagemid. The original promoter of the phagemid (CMV) was removed and the aP2 promoter was inserted. Abbreviations: complement C3 signal peptide sequence (SP), portion of complement C3 gene that represents ASP (ASP), constitutive eukaryote expression promoter cytomegalovirus (CMV), and adipocyte P2 (aP2).

Section 7.3: Methods

Section 7.3.1: ASP minigene

Reaction 1: Production of Complement C3 Signal Peptide Portion:

Human adipose mRNA was isolated using Trizol reagent (Gibco BRL). RT-PCR with random primers to generated stock cDNA. The entire complement C3 mRNA sequence (accession: K02765) was obtained from the NCBI nucleotide Genbank. The signal peptide template was amplified from the human stock cDNA using the primers (5') AGA AGT CGA TCG ATC TCC TCC CCA TCC TCT CCC TC and (3') GCT TCT CCG TGA GCT GCA CGG ACC CCA GAG CCA GGG GGA GGTG. At the 5' end of primer 1A was an engineered *Cla* / restriction enzyme cut site and at the 5' end of primer 1B was the first 22 bases of the C3 gene that corresponded to initial sequence that encoded the ASP protein. High fidelity PCR was carried out with Elongase enzyme mix (Gibco) that contained a mixture of Taq and pyrococcus species GB-D thermostable DNA polymerases. Because this system contains a proofreading enzyme, it limits nucleotide substitution errors in the new strands and allows amplification of gene targets larger than 5kb (product information, Gibco). The cDNA (4 μ L of RT reaction) was added to the PCR reaction solutions (final concentrations per tube: 200 μ M of each dNTP, 400 nM of each 5' and 3' primers, 1 μ L elongase enzyme mix, 10 μ M tetramethylammonium chloride, and 1.6 mM Mg^{2+}) and amplified 35 cycles (30 seconds at 94°C, 30 seconds at 60°C and 1 min at 68°C).

The Mg^{2+} concentration of 1.8 mM was chosen based on a range of concentrations tested (1.0 mM to 2.0 mM). The expected PCR product (162 bp) was separated on a 2% low melting point agarose gel and purified using the Qiagen agarose gel extraction kit.

Reaction 2: Production of ASP Portion of Complement C3:

The ASP template was amplified from the human stock cDNA using the primers (5') CCT CCC CCT GGC TCT GGG GTC CGT GCA GCT CAC GGA GAAG and (3') TCA TGG ACG GTA CCT CAT TAA GCC AGA CCC AGG TG. At the 5' end of primer 2A were the terminal 19 bases of the C3 signal peptide sequence and at the 5' end of primer 2B was an engineer *Kpn* I restriction enzyme cut site. The cDNA (8 μ L of RT reaction) was added to the PCR reaction solutions (final concentrations per tube: 200 μ M of each dNTP, 400 nM of each 5' and 3' primers, 1 μ L elongase enzyme mix [Gibco], 10 μ M tetramethylammonium chloride, and 1.8 mM Mg^{2+}) and amplified for 35 cycles (1 min at 94°C, 1 min at 60°C and 1 min at 68°C). The Mg^{2+} concentration of 1.8 mM was chosen based on range of concentrations tested (1.0mM to 2.0mM). The expected PCR product (267 bp) was separated on a 2% low melting point agarose gel and purified using the Qiagen agarose gel extraction kit.

Reaction 3: Ligation of the Signal Peptide with ASP Portion

The signal peptide template (50 pg, reaction 1) and ASP template (50 pg, reaction 2) were added to the PCR reaction solutions (final concentrations per tube: 200 μ M of each dNTP, 1 μ L elongase enzyme mix, 10 μ M tetramethylammonium chloride, and 1.8 mM Mg^{2+}) and amplified for 8 cycles (1 min at 94°C, 1 min at 60°C and 1 min at 68°C). These preamplification cycles allow the hybridization of the two templates and then their subsequent elongation to generate the ASP mini gene template. Primers (400 nM of primer 1A and primer 2B) were then added and the signal peptide-ASP mini gene template was amplified for 30 cycles (1 min at 94°C, 1 min at 60°C and 1 min at 68°C). The expected PCR product (382 bp) was separated on a 2% low melting point agarose gel and purified using the Qiagen agarose gel extraction kit. The ASP minigene contained the following: *Cla* I cut site, C3 signal peptide, ASP and *Kpn* I cut site.

Section 7.3.2: Insertion of SP-ASP into the pBk-CMV Phagemid (PBk-CMV-SP-ASP)

Phagemid pBk-CMV (3 μ g) and the ASP minigene (0.5 μ g) were individually digested with *Cla* I (2.5 U, MBI Fermentas) and *Kpn* I (2.5 U, MBI Fermentas) in 1x γ /Tango buffer (MBI Fermentas) for three hours at 37°C. The enzymes were heat-inactivated at 65°C for 20 minutes. The cut products of interest were separated on a 0.7% low melting point agarose gel and purified using the Qiagen agarose gel extraction kit. The restriction digested ASP

minigene (0.03 μg) and pBk-CMV phagemid (0.01 μg) were added to the ligation reaction mixture (1x ligase buffer, and 0.1 units of T4 DNA ligase [Gibco]) for 16 hours at 16°C. The enzyme was inactivated at 75°C for 15 minutes. The ligation reaction was diluted five times and 2.5 μL was used to transform TOP10F' Ecoli cells by standard electroporation techniques (117). Insertion of the ASP minigene would yield white colonies. Transformation of a phagemid without an insert would yield blue colonies, while transformation with unligated DNA would give no colonies. pBk-CMV-SP-ASP (white colony) was then amplified in liquid culture and purified. The unligated cut phagemid was used as the negative transformation control.

Section 7.3.3: Removal of the Cytomegelo virus Promoter (PBk-SP-ASP)

Phagemid pBk-CMV-ASP (1 μg) was digested by *Vsp* I (5 U, Promega, Madison, WI) and *Nhe* I (5U, Promega, Madison, WI) in 1x Buffer C (5 U, Promega, Madison, WI) for three hours at 37°C. The enzymes were heat-inactivated at 65°C for 20 minutes. The cut products of interest were separated on a 0.7% low melting point agarose gel and purified using the Qiagen agarose gel extraction kit. For a blunt end ligation, the restriction digested pBk-SP-ASP phagemid (0.07 μg) was added to the ligation reaction mixture (1x ligase buffer, and 0.1 units of T4 DNA ligase [Gibco]) for 16 hours at 16°C. The enzyme was inactivated at 75°C for 15 minutes. The ligation reaction was diluted five times and 2.5 μL was used to transform TOP10F' Ecoli cells by standard electroporation techniques (96). The unligated cut phagemid was used as the

negative transformation control. pBk-SP-ASP was then amplified in liquid culture and purified and high fidelity PCR with 5' primer 1A from reaction 1 and 3' primer 2B from reaction 2 were used to verify the presence of the ASP minigene.

Section 7.3.4: Insertion of the Adipocyte P2 Promoter (PBk-aP2-SP-ASP)

Phagemid pBk-SP-ASP (3 µg) and pBluescript II SK + with aP2 5.4 Kb promoter insert (1 µg) (gift from Dr. G.A. Mitchell, University de Montreal, with the permission of Dr. Spiegelman, Harvard University) were individually digested with *BssH* II (5 U, New England BioLabs) and *Xho* I (5 U, 3 (New England BioLabs) in 1x NEbuffer 3 (New England BioLabs) for two hours at 37°C, and then at 50°C for two hours. The enzymes were heat-inactivated at 80°C for 20 minutes. The cut products of interest were separated on a 0.7% low melting point agarose gel and purified using the Qiagen agarose gel extraction kit. The restriction digested aP2 promoter (0.03 µg) and pBk-SP-ASP phagemid (0.01 µg) were added to the ligation reaction mixture (1x ligase buffer, and 0.1 units of T4 DNA ligase [Gibco]) for 16 hours at 16°C. The enzyme was inactivated at 75°C for 15 minutes. The ligation reaction was diluted five times and 2.5 µL was used to transform TOP10F' Ecoli cells by standard electroporation techniques (117). Insertion of the ASP minigene would yield white colonies. pBk-aP2-SP-ASP was then amplified in liquid culture and purified. The unligated cut phagemid was used as the negative transformation control.

It is important to note that because the aP2 promoter has not yet been sequenced to date, the restriction enzymes chosen are a result of testing and assessing several restriction enzymes to obtain the most optimal ones.

Section 7.3.5: Linearization of the ASP Pseudogene (aP2-SP-ASP)

For the preparation of the DNA for microinjection, in addition to linearizing the pseudogene, the prokaryotic expression and selection sections should be removed so as to increase the probable insertion of the pseudogene into the genomic DNA of the mice (119). To do this, pBk-aP2-SP-ASP was first digested with *Mlu* I (Promega, Madison, WI) in 1x Buffer D (Promega, Madison, WI) for two hours at 37°C. The enzymes were heat-inactivated at 65°C for 20 minutes. The digestion mixture was separated on a 0.7% low melting point agarose gel and purified using the Qiagen agarose gel extraction kit. *Mlu* I only generates one band since there is only one cut site in the pBk-aP2-SP-ASP phagemid. This phagemid was further digested with *BssH* II (New England BioLabs) in 1x NEBuffer 3 for two hours at 50°C. The enzymes were heat-inactivated at 80°C for 20 minutes. The pseudogene (aP2-SP-ASP, 289 µg/mL) for microinjection were isolated by extraction of low-melting-point agarose 0.7% gels and redissolved in TLE (10mM Tris pH 7.4, 0.1 mM EDTA).

Section 7.3.6: Generation of Transgenic Mice

Pronuclear microinjections were performed at the McIntyre Transgenic Core Facility by Janice Penney (principal investigator: Dr. Michel Tremblay, McGill University) by standard techniques (119). The F2 embryos of the CBAxC57bl/6 cross mice were injected with the pseudogene. Positive transgenic animals with the ASP gene are identified by polymerase chain reaction using primers (5') CTC CTC CCC ATC CTC TCC CTC and (3') TTA GGC CAG GCC CAG GTG GCTG that amplifies the ASP minigene (signal peptide included) from the genomic DNA. Genomic DNA was isolated from the tail cuts of the mice by standard methods (267). Genomic DNA (100 ng) was added to the PCR reaction solutions (final concentrations per tube: 200 μ M of each dNTP, 400 nM of each 5' and 3' primers, 1 μ L elongase enzyme mix, 10 μ M tetramethylammonium chloride, and 1.8 mM Mg^{2+}) and amplified for 35 cycles (1 min at 94°C, 1 min at 60°C and 1 min at 68°C). Phagemid pBk-aP2-SP-ASP and the ASP pseudogene are used as positive controls and the genomic DNA from wild-type mice are used as a negative control. For a positive result, a band of 385 bp is expected. As a second test to confirm the presence of the transgene, Southern blot will be performed with a labeled probe of the SP-ASP fragment. Copy number can be determined from the Southern analysis.

Section 7.4: Future Experiments

Section 7.4.1: *In vitro*

3T3-F442 adipocytes (murine) will be stably transfected with pBk-CMV-SP-ASP to assess successful synthesis and secretion of ASP directly by: i) determining the presence of the mRNA for the ASP minigene (SP-ASP), ii) determining the level of gene expression by quantitative mRNA analysis, and iii) measuring ASP protein levels in the medium and in cell extracts (to determine the proportion secreted).

pBk-aP2-SP-ASP will also be stably transfected into 3T3-F442 cells so as to determine i) the presence of the mRNA for the ASP minigene (SP-ASP), ii) the level of gene expression by quantitative mRNA analysis, iii) the levels of ASP in the medium, iv) the effects of increased levels of ASP on adipocyte differentiation, and v) the effects of increased ASP levels on TG synthesis and TG intracellular mass.

Section 7.4.2: *In vivo*

Firstly, we will need to verify that ASP is expressed, secreted, and detectable in plasma. It is important to note that because the pseudogene contains the sequence for human ASP, we can use the human ASP ELISA to measure the plasma levels of ASP since the antibodies in this ELISA do not recognize murine ASP. This new mouse model of ASP adipose tissue overexpression will be characterized by i) determining basal plasma parameters (such as insulin, FFA, TG, glucose, ASP, cholesterol, and apo B), ii) following the changes in the basal parameters (glucose, insulin, FFA, TG and ASP) after a fatmeal challenge, iii) monitoring food intake, iv) determining basal metabolic rate and energy expenditure, v) identifying if there are any differences in the fatpad size when compared to controls and if there are any changes in fat distribution, and vi) studying the effects of aging and progression of obesity.

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