MOLECULAR MECHANISMS REGULATING THE ASSEMBLY AND SECRETION OF APOLIPOPROTEIN B IN HAMSTER LIVER AND ITS OVERPRODUCTION IN INSULIN RESISTANT STATES

By

Changiz Taghibiglou

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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A Ph.D. thesis by Changiz Taghibiglou submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Laboratory Medicine and Pathobiology, University of Toronto, 2001

ABSTRACT

Insulin-resistant states are commonly associated with lipoprotein abnormalities that are risk factors for coronary heart disease. Hepatic overproduction of apolipoprotein B (apoB)-containing lipoproteins is the hallmark of metabolic dyslipidemia in insulin resistance. We used Syrian golden hamsters to study the mechanisms of hepatic overproduction of apoB-containing lipoproteins, as their lipoprotein metabolism closely resembles that of humans. Our studies revealed that hamster hepatocytes efficiently assemble and secrete apoB-containing very low density lipoprotein (VLDL) particles. We induced an insulin resistant state in the hamster by fructose feeding and studied the effects of insulin resistance on apoB biogenesis.

Cellular stability of apoB was significantly increased in insulin resistant hepatocytes, possibly linked to marked suppression of ER-60, an apoB-associated ER cysteine protease. Furthermore, hepatic mRNA levels, protein mass, and activity of microsomal triglyceride transfer protein (MTP), a key enzyme in assembly of apoB-containing lipoproteins were dramatically increased. Overall, VLDL-apoB overproduction in fructose-fed hamsters appeared to result from enhanced post-translational stability of nascent apoB and an enhanced expression of MTP, leading to facilitated assembly and secretion of VLDL.
We also investigated hepatic insulin signaling in the fructose-fed hamster model to examine the potential contribution of attenuated insulin signaling to the observed VLDL overproduction. Reduced tyrosine phosphorylation of insulin receptor (IR), and its substrates, reduced PI 3-kinase activity, and attenuated serine/threonine phosphorylation of Akt/PKB confirmed induction of hepatic insulin resistance. Elevation of protein mass and activity of protein tyrosine phosphatase (PTP)-1B appeared to be partly responsible for induction of hepatic insulin resistance. Chronic exposure of control hepatocytes to high insulin attenuated IR phosphorylation and increased PTP-1B levels, coinciding with marked suppression of ER-60 and apoB oversecretion. Improvements in phosphorylation status of the IR, in vitro by treatment with sodium orthovanadate, or in vivo by rosiglitazone treatment, attenuated secretion of apoB from fructose-fed hamster hepatocytes.

In summary, induction of whole body and hepatic insulin resistance in the hamster by fructose-feeding is accompanied by a marked hepatic overproduction of VLDL-apoB. The VLDL-apoB overproduction may arise from interplay between hepatic insulin resistance and changes in several key components of the VLDL assembly and secretion process including MTP and ER-60.
ACKNOWLEDGMENTS

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I would also like to extend my appreciation to the Heart and Stroke Foundation of Ontario, The Hospital for Sick Children, and The University of Toronto for their financial and technical supports of my graduate research.
DEDICATION

With love and gratitude to my Mom (Nassibeh), Dad (Yaghoub), Sisters (Soudabeh, Sousan, Homa), Brothers (Yousef, and Gholamreza), and my Nieces (Pegah, and Pardis).
# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii

ACKNOWLEDGMENTS ........................................................................................................ iv

DEDICATION ...................................................................................................................... v

TABLE OF CONTENTS ........................................................................................................ vi

LIST OF FIGURES .............................................................................................................. xiii

LIST OF ABBREVIATIONS .................................................................................................. xvi

CHAPTER ONE .................................................................................................................. 1

INTRODUCTION ................................................................................................................. 1

PART I: General Introduction .............................................................................................. 1

  1.1.1 Lipoprotein Metabolism and Atherosclerosis .......................................................... 1

PART II: Synthesis, Assembly, and Secretion of ApoB-containing Lipoproteins ............. 6

  1.2.1 Introduction ............................................................................................................. 6
  1.2.2 Structure of Apolipoprotein B ................................................................................ 7
  1.2.3 Transcriptional Regulation of Apolipoprotein B Production ..................................... 19
  1.2.4 Translational Regulation of Apolipoprotein B Production ........................................ 20
  1.2.5 Co-translational and Post-translational Regulation of Apolipoprotein B Production ......................................................................................................................... 24
  1.2.6 Assembly of ApoB100-containing lipoproteins ...................................................... 41

PART III: Metabolic Dyslipidemia in Insulin Resistant States ........................................ 44

  1.3.1 Insulin Signaling Pathway: An Overview ............................................................... 44
  1.3.2 Molecular Mechanisms of Insulin Resistance ....................................................... 50
  1.3.3 Pathologic Consequences of Insulin Resistance ................................................... 53
PART IV: Animal Models for Insulin Resistance Related Metabolic Dyslipidemia

1.4.1 Nutrient or Diet-induced Animal Models of Insulin Resistance and Diabetes

1.4.2 Syrian Golden Hamster as an Animal Model of Insulin Resistance

1.4.3 Research Rationale

1.4.4 Overall Hypothesis

1.4.5 General Research Objectives

CHAPTER TWO

Materials and Methods

2.1 Chemicals and Reagents

2.2 Laboratory Supplies

2.3 Apparata

2.4 Procedures

2.4.1 Animal Protocols

2.4.2 Preparation of Collagen-Coated Culture Dishes

2.4.3 Liver Perfusion and Isolation of Primary Hamster Hepatocytes

2.4.4 Metabolic Labeling of Intact Primary Hamster Hepatocytes

2.4.5 Determination of Apolipoprotein B Turnover in Permeabilized Primary Hamster Hepatocytes

2.4.6 Trypsin Digestion of Permeabilized Primary Hamster Hepatocytes

2.4.7 Isolation and Trypsin Treatment of Hepatic Microsomes

2.4.8 Analysis of Luminal and Membrane-associated ApoB Pools

2.4.9 Isolation and Subcellular Fractionation of Hamster Liver Microsomes

2.4.10 Sucrose Gradient Fractionation of the ApoB-containing Lipoproteins
CHAPTER THREE .......................................................................................................................... 88

Intracellular Mechanisms Regulating ApoB-containing Lipoprotein Assembly and Secretion in Primary Hamster Hepatocytes ........................................................................................................ 88

3.1 Rationale, Hypothesis, and Research Objectives ..................................................................... 88

3.1.1 Rationale .............................................................................................................................. 88

3.1.2 Hypothesis .......................................................................................................................... 89

3.1.3 Research Objectives ........................................................................................................... 89

3.2 Results .................................................................................................................................... 90

3.2.1 Primary Hamster Hepatocytes Secrete ApoB-Containing Lipoprotein at the Density of VLDL ................................................................................................................................. 90
3.2.2 Analysis of ApoB Translocational Status in Permeabilized Primary Hamster Hepatocytes .......................................................... 90

3.2.3 Trypsin Sensitivity of Hamster ApoB-100 in Isolated Microsomes .............................. 92

3.2.4 Analysis of ApoB-100 in Subcellular Fractions of Isolated Microsomes ..................... 93

3.2.5 ApoB Stability and Secretion in Primary Hamster Hepatocytes .................................. 95

3.2.6 ApoB Degradation in Permeabilized Primary Hamster Hepatocytes and Detection of Degradation Intermediates ............................................................. 100

3.2.7 The Sensitivity of ApoB Degradation to Various Protease Inhibitors ....................... 103

3.2.8 Evidence for Involvement of the Ubiquitin-Proteasome Pathway in Hamster ApoB Degradation .................................................................................................................. 106

3.2.9 Assembly and Secretion of Hamster ApoB-100 VLDL ............................................ 106

3.2.10 Effect of Oleate on the Stability and Secretion of ApoB in Primary Hamster Hepatocytes .......................................................................................................................... 112

CHAPTER FOUR .................................................................................................................. 117
Mechanisms of Hepatic VLDL Overproduction in Insulin Resistance .................................. 117

4.1 Rationale, Hypothesis and Research Objectives ...................................................................... 117

4.1.1 Rationale ................................................................................................................................................. 117

4.1.2 Hypothesis .......................................................................................................................................... 118

4.1.3 Research Objectives .............................................................................................................................. 118

4.2 Results .................................................................................................................................................... 118

4.2.1 Metabolic Effects of Fructose Feeding in Syrian Golden Hamsters ............................... 118

4.2.2 Effect of Fructose Feeding on Hepatic Synthesis and Secretion of Lipids ................... 119

4.2.3 Overproduction of VLDL-apoB in Hepatocytes from Fructose-Fed Hamsters .... 120
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.4 Turnover of apoB in Control and Fructose-Fed Hepatocytes</td>
<td>120</td>
</tr>
<tr>
<td>4.2.5 Stability of ApoB in Permeabilized Primary Hamster Hepatocytes</td>
<td>125</td>
</tr>
<tr>
<td>4.2.6 Translocational Status of Hamster ApoB in Control and Fructose-Fed Hepatocytes</td>
<td>126</td>
</tr>
<tr>
<td>4.2.7 Effect of Fructose-Feeding on Intracellular Assembly of ApoB-Containing Lipoproteins</td>
<td>135</td>
</tr>
<tr>
<td>4.2.8 Evidence that Direct Incubation with Fructose Does Not Directly Affect Hepatic ApoB Secretion by Primary Hamster Hepatocytes</td>
<td>136</td>
</tr>
<tr>
<td>4.2.9 Evidence for Enhanced Expression of MTP Mass, mRNA Levels, and Increased MTP Activity in Fructose-Fed Hepatocytes</td>
<td>142</td>
</tr>
</tbody>
</table>

**CHAPTER FIVE**

Hepatic VLDL-apoB Overproduction is Associated with Attenuated Hepatic Insulin Signaling in a Fructose-Fed Hamster Model of Insulin Resistance: Evidence for increased expression of PTP-1B and decreased abundance of ER60 protease

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Rationale, Theory, and Research Objectives</td>
<td>145</td>
</tr>
<tr>
<td>5.1.1 Rationale</td>
<td>145</td>
</tr>
<tr>
<td>5.1.2 Hypothesis</td>
<td>146</td>
</tr>
<tr>
<td>5.1.3 Research Objectives</td>
<td>146</td>
</tr>
<tr>
<td>5.2 Results</td>
<td>146</td>
</tr>
<tr>
<td>5.2.1 Effect of Fructose Feeding on the Phosphorylation Status and Protein Mass of the Insulin Receptor, IRS-1, and IRS-2 in Hepatocytes of Syrian Hamsters</td>
<td>146</td>
</tr>
<tr>
<td>5.2.2 PI3-kinase Activity in Hepatocytes Isolated from Control and Fructose-fed Hamsters</td>
<td>148</td>
</tr>
</tbody>
</table>
5.2.3 Evidence that Intracellular Level and Activity of PTP-1B are Enhanced in Hepatocytes Isolated from Fructose-fed Hamsters ................................................................. 149
5.2.4 Impaired Akt/PKB Serine and Threonine Phosphorylation in Hepatocytes Isolated from Fructose-fed Hamsters .................................................................................. 152
5.2.5 Insulin Signaling Status in Hepatocytes Exposed to High Insulin Concentrations Ex Vivo .................................................................................................................. 152
5.2.6 Chronic High Insulin Exposure Induces PTP-1B Expression in Hamster Hepatocytes .................................................................................................................. 156
5.2.7 Chronic Exposure of Hepatocytes to High Insulin Induces ApoB Oversecretion 156
5.2.8 ER-60 Protein Mass in Hepatocytes Isolated from Fructose-Fed Hamsters ....... 157
5.2.9 ER-60 Suppression Can be Induced by High Insulin Exposure and is Accompanied by the Induction of Insulin Resistance ........................................................................ 160
5.2.10 Vanadate Improves Tyrosine Phosphorylation of Insulin Receptor in Hepatocytes Isolated from Fructose-fed Hamsters in a Dose-Dependent Manner .................. 160
5.2.11 Vanadate Reduces Synthesis and Secretion of ApoB from Insulin Resistant Hepatocytes in a Dose-Dependent Manner ................................................................. 161

CHAPTER SIX ................................................................................................................. 167

Effect of treatment with rosiglitazone, an insulin sensitizer agent, on hepatic VLDL-apoB secretion in the fructose-fed hamster model ........................................................................ 167

6.1 Rationale, Hypothesis, and Research Objectives ......................................................... 167

6.1.1 Rationale ............................................................................................................... 167
6.1.2 Hypothesis ........................................................................................................... 168
6.1.3 Research Objectives .............................................................................................. 168
6.2 Results ........................................................................................................................................... 168

6.2.1 Improvement of Hepatic Insulin Signal Transduction in Hepatocytes Isolated from Fructose-Fed/Rosiglitazone-Treated Hamsters ...................................................... 168

6.2.2 Significant Reduction of VLDL-apoB Secretion from Hepatocytes Isolated from Rosiglitazone-Treated Fructose Fed Hamsters ........................................................................ 174

6.2.3 Effects of Rosiglitazone on Turnover of ApoB in Hamster Hepatocytes ...................... 174

6.2.4 Rosiglitazone Significantly Suppresses Hepatic Expression of MTP Protein ..... 180

CHAPTER SEVEN ............................................................................................................................... 185

DISCUSSION AND CONCLUSIONS ................................................................................................. 185

7.1 Intracellular Mechanisms Regulating ApoB-containing Lipoprotein Assembly and Secretion in Primary Hamster Hepatocytes ......................................................... 185

7.2 Molecular Mechanisms of Hepatic VLDL Overproduction in Insulin Resistance ......... 193

7.3 Hepatic VLDL-apoB Overproduction is Associated with Attenuated Hepatic Insulin Signaling in a Fructose-Fed Hamster Model of Insulin Resistance ......................... 198

7.4 Effect of Treatment with Rosiglitazone, an Insulin Sensitizer Agent, on Hepatic VLDL-ApoB Secretion in the Fructose-Fed Hamster Model ........................................ 204

7.5 Final Conclusions ....................................................................................................................... 206

7.6 Future Studies ............................................................................................................................. 208

REFERENCES ................................................................................................................................. 213
LIST OF FIGURES

CHAPTER 1
Figure 1.2.1 Schematic diagram of the distribution of amphipathic α helixes and
amphipathic β strands in apolipoprotein B100 ............................................................... 17
Figure 1.2.2 Some important factors post-transcriptionally modulating hepatic apoB
biogenesis ......................................................................................................................... 22
Figure 1.2.3 Model of proteosome-mediated degradation of apoB ............................... 35
Figure 1.3.1 The insulin receptor tyrosine kinase substrates and downstream signaling
pathways ......................................................................................................................... 48

CHAPTER 3
Figure 3.1 Trypsin sensitivity of hamster apoB-100 in permeabilized hamster hepatocytes
and isolated microsomes ................................................................................................. 96
Figure 3.2 Subcellular distribution of newly synthesized apoB in microsomal membrane and
lumen ................................................................................................................................. 98
Figure 3.3 ApoB stability and secretion in control and MG132-treated intact primary hamster
hepatocytes ...................................................................................................................... 101
Figure 3.4 Intracellular stability of apoB in permeabilized primary hamster hepatocytes ... 104
Figure 3.5 Effect of various protease inhibitors on apoB stability and secretion ............... 107
Figure 3.6 Evidence for ubiquitination of hamster apoB-100 in primary hamster hepatocytes
........................................................................................................................................ 109
Figure 3.7 Analysis of apoB-100-containing lipoproteins in microsomal fractions of primary
hamster hepatocytes ....................................................................................................... 113
Figure 3.8 Effect of oleate on stability and secretion of apoB in primary hamster hepatocytes
........................................................................................................................................ 115
CHAPTER 4

Figure 4.1 Effect of fructose feeding on plasma lipids, insulin, free fatty acids, and glucose ................................................................. 121

Figure 4.2 Synthesis and secretion of newly-synthesized lipids in control and fructose-fed hepatocytes .............................................. 123

Figure 4.3 VLDL-apoB production in control and fructose-fed hepatocytes .............................................................. 127

Figure 4.4 Intracellular stability of hamster apoB in control and fructose-fed hepatocytes .......................................................... 129

Figure 4.5 Posttranslational stability of hamster apoB in permeabilized primary hepatocytes .............................................................. 131

Figure 4.6 Trypsin sensitivity of hamster apoB in permeabilized hamster hepatocytes ................................................................. 133

Figure 4.7 Intracellular distribution of nascent apoB-containing lipoproteins in microsomal lumen of control and fructose-fed hepatocytes ................................................................. 138

Figure 4.8 Effect of in vitro incubation of primary hamster hepatocytes with fructose on synthesis, secretion and stability of hamster apoB ................................................................. 140

Figure 4.9 Evidence for Enhanced Expression of MTP Mass, mRNA Levels, and Activity in Fructose-Fed Hepatocytes ................................................................. 143

CHAPTER 5

Figure 5.1 Insulin receptor, IRS-1, and IRS-2 phosphorylation status and protein mass in hepatocytes isolated from control and fructose-fed hamsters ................................................................. 150

Figure 5.2 Fructose feeding reduces PI 3-kinase activity and Akt/PKB phosphorylation and increases the protein mass and activity of PTP-1B in hamster liver ................................................. 153

Figure 5.3 Chronic High Insulin Exposure of Control Hepatocytes Impaired Insulin Receptor Phosphorylation, Reduced IR Mass and Increased PTP-1B Mass ................................................. 158
Figure 5.4 Chronic High Insulin Exposure of Control Hepatocytes, Caused ApoB Oversecretion and Significant Suppression of ER-60 ....................................................... 162

Figure 5.5 Vanadate treatment stimulates tyrosine-phosphorylation of the insulin receptor and reduces apoB synthesis and secretion in hepatocytes isolated from fructose-fed hamsters ........................................................................................................... 165

CHAPTER 6

Figure 6.1 Improvement of tyrosine phosphorylation of insulin receptor, IRS-1, and IRS-2 in hepatocytes isolated from the fructose-fed/rosiglitazone-treated hamsters ......................... 170

Figure 6.2 Effects of rosiglitazone on hepatic protein mass of IRS-1 and IRS-2 .............. 172

Figure 6.3 Effects of rosiglitazone on protein expression of PTP-1B in hepatocytes isolated from fructose-fed hamsters ........................................................................................................... 175

Figure 6.4 VLDL-apoB production in hepatocytes isolated from fructose-fed and fructose-fed rosiglitazone-treated hepatocytes ........................................................................................................... 177

Figure 6.5 Turnover rate of apoB in hepatocytes isolated fructose-fed and fructose-fed rosiglitazone treated hepatocytes ........................................................................................................... 181

Figure 6.6 Rosiglitazone normalized MTP protein expression levels in hepatocytes isolated from fructose-fed hamsters ........................................................................................................... 183

CHAPTER 7

Figure 7.1 Postulated Molecular Mechanism of VLDL-apoB Overproduction in Hepatocytes Isolated from Fructose-fed Insulin Resistant Hamsters .......................................................... 209
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAT</td>
<td>Acyl coenzyme A: cholesterol acyltransferase</td>
</tr>
<tr>
<td>ALLN</td>
<td>N-acetyl-leucyl-leucyl-norleucinol</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesterol ester transfer protein</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CAAT enhancer-binding protein α</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>ChoRE</td>
<td>Carbohydrate response element</td>
</tr>
<tr>
<td>ChoRF</td>
<td>Carbohydrate response factor</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CSK</td>
<td>Cytoskeletal buffer</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FF</td>
<td>Fructose-fed</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FF + R</td>
<td>Fructose-fed + Rosiglitazone</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoproteins</td>
</tr>
<tr>
<td>HTGL</td>
<td>Hepatic triglyceride lipase</td>
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IDL  Intermediate density lipoprotein
IgG  Immunoglobulin
IR   Insulin receptor
IRS  Insulin receptor substrate
LCAT Lecithin-cholesterol acyltransferase
LDL  Low density lipoprotein
LPL  Lipoprotein lipase
mRNA Messenger ribonucleic acid
α-MEM Minimum essential medium-alpha
MTP  Microsomal triglyceride transfer protein
NF-1 Nuclear factor 1
NF-Y Nuclear factor Y
PBS  Phosphate-buffered saline
PDI  Protein disulfide isomerase
PI 3-kinase Phosphatidylinositol 3-kinase
PIPERES Piperazine-N,N'-bis[2-ethanesulfonic acid]
PKB  Protein kinase B
PKC  Protein kinase C
PMME Phospholipid phosphatidylinmonoethylethanolamine
PMSF Phenylmethylsulfonylfluoride
PPAR Proxisome proliferator-activated receptor
PtdIns Phosphatidylinositol
PTEN Phosphatase with tensin homology
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>PTP-1B</td>
<td>Protein tyrosine phosphatase 1B</td>
</tr>
<tr>
<td>PTPase</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>S.D</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SER</td>
<td>Smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>Serine/Threonine</td>
</tr>
<tr>
<td>Spl</td>
<td>Stimulatory protein 1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-methylene-bis-acrylamide</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)-aminomethane glycine</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>t-octylphenoxy-polyoxyethanol</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
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CHAPTER ONE
INTRODUCTION

PART I: General Introduction
1.1.1 Lipoprotein Metabolism and Atherosclerosis

The relationship between dyslipidemia and atherosclerosis has been well documented (Lusis, 2000). Both genes and the environment modulate plasma lipoproteins. Atherogenesis can be viewed as a "response to injury" with lipoproteins or other risk factors as the injurious agents (Libby, 2000, Ross, 1999). Lipoproteins as energy transport systems have been optimized to transfer energy-rich fatty acids in their core to tissues either utilizing or storing energy as fat. The initial linkage of cholesterol, lipoproteins, and atherosclerosis began in the early 1900s. These studies led to 80 years of investigation to establish the cause-effect relationship of cholesterol and atherosclerosis. In the mid 1980s, it was clinically established that lowering the concentration of plasma LDL reduced risk of CHD (Rifkind, 1984).

Animal models (transgenic and non-transgenic) have significantly advanced our understanding of the mechanisms of atherosclerosis and the evaluation of therapeutic options. Some animal models such as mice, rats, and dogs have been found to be resistant to developing experimental atherosclerosis, while other species, such as rabbits and non-human primates, were found to be atherosclerosis-susceptible animal models, in which apoB-containing lipoproteins are the major cholesterol carrier particles in plasma (Narayanaswamy, et al., 2000). A variety of diets have been used to induce experimental atherosclerosis (Brousseau and Schaefer, 2000, Schaefer and Brousseau, 1998). The development of efficient transgenic technologies in mice and rabbits has allowed the study of the consequences of genetic alterations on atherosclerosis and cardiovascular pathophysiology. Overexpression, as well as deletion, of apoproteins, enzymes, and


lipoprotein receptors dramatically modulate circulating plasma lipoproteins (Brousseau and Hoeg, 1999, Carmeliet, et al., 1998, Kalopissis and Chambaz, 2000). Furthermore, combination of different diets with transgenic animals provides strong models to investigate cardiovascular diseases. Dietary changes interact with the underlying genotypes, greatly modifying the composition of the circulating plasma lipoproteins.

In human, there are three major groups of atherogenic lipoproteins. LDL is the best known and established atherogenic particle. Severe atherosclerosis associated with increased LDL levels (by ten-fold) have been reported in humans and animal models (mice and rabbits) lacking a gene encoding LDL receptor. Boren and collaborators using transgenics in which LDL receptor binding sequences of apoB-100 had been disrupted, demonstrated hypercholesterolemia and atherosclerosis similar to the familial ligand-defective apoB-100 hypercholesterolemic syndrome (Boren, et al., 1996, Boren, et al., 1998a). Both in vitro and in vivo studies in animals have demonstrated that endothelial function may be abnormal within a few hours of exposure to increased levels of LDL cholesterol, indicating a toxic effect of LDL on endothelial cells (Harrison, et al., 1995, Naito, et al., 1994). Subendothelial retention of LDL through the interaction with extracellular matrix, chiefly proteoglycans appears to be a key pathogenic process in atherosclerosis. The link between dyslipidemia, especially hypercholesterolemia, and loss of NO-driven endothelium-dependent dilatation has been demonstrated by numerous investigators (for a recent review refer to Adams, et al., 2000). A reduction of nitric oxide (NO) has been shown in the coronary arteries of hypercholesterolemic pigs (Cohen, et al., 1988). When the plasma LDL levels increase, positively charged apoB-100 bind to the negatively charged glycosaminoglycans of the arterial walls. Recently, Boren and colleagues (Boren, et al., 1998b) identified basic amino
acid residues in delipidated apoB-100 that bind the negatively charged proteoglycans. Upon recruitment of the monocytes to the sites of endothelial disruption, and release of hydrogen peroxide by the scavenger macrophages, apoB-100 present in the tethered LDL particles undergoes oxidative modifications. Oxidized LDL has a profound effect in activating inflammatory process within endothelial cells and other cells within the vessel walls (reviewed in Aviram, 2000). Although the experimental evidence suggests that most of the endothelial functional disturbances are caused by oxidized LDL rather than native LDL, there is some evidence indicating that native LDL has similar deleterious effects (Pritchard, et al., 1995). LDL metabolism by endothelial cells, monocytes, and macrophages within the cell wall, can lead to a variety of modifications including the accumulation of the products of lipid oxidation (such as reactive hydroxy fatty acids) as well as lysophosphatidylcholine, which are highly toxic for the endothelial cell functions (Kugiyama, et al., 1990). Covalently modified apoB-100 loses its affinity for binding to native LDL receptors and then hinders plasma LDL clearance (Steinbrecher, 1987). In human and animal studies, the degree of LDL oxidation has been related to endothelial dysfunction both in large vessels and in the microcirculation. Several lines of evidence suggest that elevated levels of both native and oxidized LDL in the arterial wall increase oxidative stress via the enzymatic actions of NADP/NADPH, NOS, and xanthine oxidase. The excessive generation of the free radicals (\( \cdot O_2 \) and \( \cdot OH \)) deteriorates the impairment of NO, cell permeability, endocytic activity, prostacyclin production, and many other endothelial functions (Adams, et al., 2000). The end result of these modifications is the reversal of normally anticoagulant endothelial surface to a procoagulant condition.
In addition to LDL, other apoB-containing lipoproteins, such as pre-β migrating TG-enriched VLDL, lipoprotein (a), and remnants can potentially promote atherosclerosis. Findings in animal studies and patients with dysbetalipoproteinemia have confirmed the possible role of these TG-rich VLDL particles in the pathogenesis of atherosclerosis. Recent evidence suggests that TG-rich particles may directly damage the endothelium by stimulating the expression of cell adhesion molecules and plasminogen activator inhibitor (Sattar, et al., 1998). Moreover, reduced endothelium-dependent dilatation has been reported in a number of pathologic conditions associated with high TG, such as obesity/insulin resistance syndrome (Steinberg, et al., 1996) and in non-insulin dependent diabetes mellitus (NIDDM) (Clarkson, et al., 1996, McVeigh, et al., 1992). Lipoprotein (a), a particle resembling LDL but containing an additional polypeptide called apolipoprotein (a) that is linked to apoB by a disulfide bridge has been proven to be particularly atherogenic owing to its additional effects on fibrinolysis and smooth muscle cell growth (Grainger, et al., 1994). Lipoprotein (a) is considered an inherited risk factor for premature atherosclerosis.

In contrast to the above-mentioned proatherogenic lipoproteins, HDL is strongly protective against atherosclerosis. An important mechanism underlying this protective effect is the role of HDL in the “reverse cholesterol transport”. HDL is considered an antioxidant particle because it carries an esterase called paraxonase, which can degrade certain biologically active oxidized phospholipids (Hegele, 1999, Shih, et al., 2000). HDL can also inhibit cytokine-induced expression of endothelial cell adhesion molecules (Cockerill, et al., 1995). Despite promising anti-atherogenic effects of HDL shown by numerous reports, results obtained from transgenic animals have been inconclusive suggesting that specific HDL subspecies may confer benefit, whereas others are not protective (Hoeg, 1998).
Tangier disease is a rare genetic disorder, which is characterized by severe plasma HDL deficiency, hypercatabolism of HDL constituents, impaired cellular cholesterol efflux, almost complete absence of plasma HDL cholesterol and phospholipids, and mutations in the gene of ATP-binding cassette 1 (ABC-1). The ABC proteins are plasma membrane localized lipid transporters (Broccardo, et al., 1999). Based on the above observations in Tangier disease, it has been suggested that ABC-1 is involved in both phospholipid and free cholesterol transportation across the plasma membrane. A two-step mechanism for ABC-1-dependent lipid efflux from human vascular cells has been proposed by Fielding et al (Fielding, et al., 2000). Asztalos and colleagues (Asztalos, et al., 2001) have found significant abnormalities in subpopulation of HDL in homozygous and heterozygous Tangier disease patients. These subpopulations were characterized by small size HDL, poor in cholesterol, and different apoA-I contents, which may account for the high risk of CHD in these patients.
PART II: Synthesis, Assembly, and Secretion of ApoB-containing Lipoproteins

1.2.1 Introduction

ApoB100, the major glycoprotein in VLDL, and the only protein in LDL, is an extremely large hydrophobic protein of 4536 amino acids with a molecular mass of approximately 550 kDa. Carbohydrates account for approximately 5% of apo B and include mannose, galactose, fucose, glucose, glucosamine, and sialic acid. Human apoB100 is synthesized in the liver, while in the human intestine, an editing process results in production of apoB48. Liver of some rodents (except rabbit and hamster) produces both apoB100 and apoB48. Except where noted, the term apoB will refer to apoB100 throughout the course of this chapter. In contrast to the classes of soluble apoproteins epitomized by apoA-I and apoE, apoB is insoluble in aqueous buffers. Hence, apoB is incapable of exchange among lipoprotein classes, and its initial biosynthetic association with nascent VLDL particle that occurs in the endoplasmic reticulum (ER) is considered irreversible. ApoB100 is the major apoprotein of VLDL, IDL and LDL, comprising approximately 30, 60, and 95 percent of the proteins in these lipoproteins, respectively. It is essential for the assembly and secretion of VLDL from the liver and is the ligand for the removal of LDL by the LDL receptor. LDL receptor binding domain of apoB is located in the region between amino acids 3000-3700 (Knott, et al., 1986, Yang, et al., 1986). ApoB contains both hydrophobic lipid-binding regions, which probably participate in the assembly of nascent VLDL, as well as hydrophilic sequences, which interact with the polar aqueous environment (Olofsson, et al., 1987).

Elevated plasma concentration of apoB is an important risk factor for CHD (Lamarche, et al., 1996). Overproduction of apoB-containing lipoproteins is a common

1.2.2 Structure of Apolipoprotein B

1.2.2.1 The Human ApoB Gene

The apoB gene is located in the short arm of chromosome 2 in the region from p23 to p24 (2p23-2p24) (Knott, et al., 1986, Mehrabian, et al., 1986). This gene consists of 28 introns and 29 exons, which span a total of 43 kilobases of genomic DNA. The first exon contains the 5'-untranslated region and also codes for the signal peptides. The mature protein sequence starts with exon 2. Most of the exons range in size from 150 to 250 nucleotides, and 24 of these encode the first one-third of apoB (Schumaker, et al., 1994). A single apoB gene produces a single sized mRNA, which is translated into the two different molecular mass forms of apoB. ApoB mRNA has a size of about 14 kb.
Several regulatory sequences have been identified in the apoB gene including a classical TATA box located 29 nucleotides 5' of the transcriptional start site and a CAAT box. 31 nucleotides 5' of the TATA box. Two GC boxes are present on the 3' side of the transcriptional start site within the untranslated portion of the mRNA (Blackhart, et al., 1986). ApoB is constitutively and tissue specifically expressed in liver, heart, and intestine. Expression of apoB gene in the liver is controlled by two positive elements located from –128 to –85 and –84 to –70 (Das, et al., 1988). The regulatory elements of the human apoB gene are located in the proximal promoter region (Kardassis, et al., 1992, Metzger, et al., 1993). Kardassis et al. (Kardassis, et al., 1992) have demonstrated that four elements (named A, B, C, and E) are recognized by nuclear factors. Three of these elements (A, B, and C) are spaced within the apoB promoter –36 to –118, and element E is located in +35 to +53 region within the first exon of the apoB gene. Analysis of the interaction of nuclear proteins with element A (5'-GCGCCCTTTGGACCTTTTGCAATCC-3') localized in the –79 to –63 apoB promoter showed the presence of multiple sequence-specific DNA complexes. Two liver-enriched transcription factors, hepatic nuclear factor-4 (HNF-4) (Sladek, et al., 1990), and CCAAT/enhancer-binding protein α (C/EBPα) (Metzger, et al., 1993), bind to the –81 to –52 region of the apoB promoter; both factors are critical for gene expression in hepatocytes. Furthermore, both transcription factors have an overlapping binding site in the apoB promoter. Zhuang and colleagues (Zhuang, et al., 1992) reported that in the rat liver, nuclear proteins such as BRF-1, BRF-2, C/EBP and hepatic nuclear factor-4 (HNF-4) bind to these regulatory elements. Recently, Novak et al. (Novak, et al., 1998) found that the interaction between HNF-4 and C/EBP alpha in the promoter of apoB occurs through 5'-CCCTTTGGA-3' motif. Brooks and Levy-Wilson (Brooks and Levy-Wilson, 1992) have identified a tissue-
specific transcriptional enhancer containing four distinct protein binding sites in the second intron of the apoB gene from +806 to +952. Several groups have also reported negative regulatory sequences in the 5' region of the apoB gene (Das, et al., 1988, Levy-Wilson, et al., 1991, Paulweber, et al., 1991a, Paulweber, et al., 1991b). Intestinal expression of the apoB gene is governed by a distal intestinal enhancer element that is located between 54 and 62 kb upstream from the apoB gene (McCormick, et al., 1996, Nielsen, et al., 1998a, Nielsen, et al., 1997). Interestingly, expression of apoB gene in the heart of transgenic mice does not require distant gene regulatory sequences and constructs containing as little as 5 kb of 5' flanking sequences and 1.5 kb of 3' flanking sequences were efficiently expressed (Veniant, et al., 1999).

CAD was observed in other studies (Bohn, et al., 1994, Visvikis, et al., 1993). There is also a recent report connecting an apoB gene Xba I polymorphism with high incidence of cholesterol gallstone disease (Han, et al., 2000). ApoB polymorphism also has forensic values and can be used as a useful tool in species identification and molecular evolutionary studies (Latorra and Schanfield, 1996). The role of apoB gene variations in hyperlipidemia has been discussed by Schonfeld (Schonfeld, 1995) and Humphries (Humphries and Talmud, 1995).

1.2.2.2 Apolipoprotein B mRNA

Human apoB mRNA contains 14,121 and 14,112 nucleotide (Cladaras, et al., 1986a), depending on the presence or absence of a 9-nucleotide insertion/deletion polymorphism in the signal sequence (Boerwinkle and Chan, 1989). ApoB mRNA is abundant in the liver, intestine and heart. The two major isoforms of apoB, apoB-100 and apoB-48, both are synthesized from the same gene by a post-transcriptional mechanism known as apoB mRNA editing (Chen, et al., 1987, Powell, et al., 1987). A cytidine to uridine conversion in the apoB transcript at the nucleotide position of 6666 results in editing of a glutamine CAA codon to UAA, at amino acid residue 2153 into an in-frame translational stop codon. This mRNA editing explains the formation of truncated isoform apoB-48. Although intestinal apoB mRNA editing occurs at high levels in nearly all mammals examined, hepatic editing is much less common and occurs only in certain species. Greeve et al. (Greeve, et al., 1993), comparing apoB mRNA editing in 12 different mammalian species reported that intestinal apoB mRNA was edited at high levels in all species, 40% in sheep, 73% in horse, 82% in pig, 84% in dog, 84% in cat, 87% in guinea pig, 88% in rat, 89% in mouse, and >90% in human, monkey, cow, and rabbit. The same group detected the liver apoB mRNA editing as
about 18% in dog, 43% in horse, 62% in rat, 70% in mouse, and <1% in rabbit and guinea pig. In contrast, hepatic mRNA from human, monkey, pig, cow, ship, and cat liver was not edited. Reaves et al. (Reaves, et al., 2000) have also reported editing activity in the small intestine and colon of hamster but not in the liver. This finding may explain in part why hamster lipoprotein metabolism is more similar to humans than that of most other rodents.

The editing reaction occurs in the nucleus and is performed by a multiprotein enzyme complex or "editosome" comprised of a 27-kDa RNA-specific cytidine deaminase, named apoB mRNA editing catalytic subunit 1 (apobec-1) and other auxiliary factors (reviewed in Davidson and Shelness, 2000). Apobec-1 has been cloned from human, rat, rabbit, and mouse (Hadjiagapiou, et al., 1994, Teng, et al., 1993, Yamanaka, et al., 1994). Apobec-1 is a heterodimer enzyme with considerable sequence homology with other cytidine deaminases. Editosome assembly is facilitated by an 11-nucleotide "mooring sequence" that begins five nucleotides downstream of the editing site in the apoB transcript (Smith, et al., 1991); this region has been shown to be critical for editing efficiency (Shah, et al., 1991). The "mooring sequence" also functions to promote editing, albeit at a reduced efficiency, when inserted into heterologous gene (Backus and Smith, 1994, Driscoll, et al., 1993) or alternative sites in apoB mRNA (Backus and Smith, 1992). The mRNA characteristics and structure are important for the efficiency of the editing process. A critical enhancer sequence located immediately 5' of the editing targeted cytidine has been identified. Length and AU content of RNA sequences more distally located from the editing site also contribute a 'bulk RNA context' that enhances editing in vitro (Backus and Smith, 1994). The editing process is also subjected to developmental, environmental, hormonal, and dietary regulation. Intestinal apoB RNA editing is developmentally regulated in humans, with early fetal small intestine
expressing predominantly unedited apoB mRNA and secreting apoB-100 (Patterson, et al., 1992, Teng, et al., 1990). ApoB RNA editing in the intestine starts around the third trimester of pregnancy, and there appears to be little regulation of this process after birth. Research evidence in human, rat, and mouse indicates that intestinal mRNA editing is not responsive to alterations in lipid flux or TG feeding (Higuchi, et al., 1992, Inui, et al., 1994, Lopez-Miranda, et al., 1994). In human, the lack of hepatic editing activity is thought to contribute to our susceptibility to develop atherosclerosis. Consequently, numerous laboratories are developing ways to induce hepatic editing using techniques such as gene therapy.

1.2.2.3 Structural and Functional Domains of ApoB

Apolipoprotein B size and insoluble nature has made it difficult to deduce the structural motifs responsible for its lipid-association properties. On the other hand, the exchangeable apolipoproteins are soluble in aqueous solutions, and the secondary structural motif (amphipathic α helix) responsible for their lipid association has been extensively studied (Segrest, et al., 1994a, Segrest, et al., 1974). The amphipathic α helix is a common secondary structural motif in biologically active peptides and proteins. ApoB has a high affinity for lipids which is reflected by a high average hydrophobicity, hindering studies of its protein structure (0.916 kcal/residue) (Chen, et al., 1986). First success came from data of several different groups using complete apoB cDNA that elucidated primary sequence of the apoprotein (Cladaras, et al., 1986b, Knott, et al., 1986, Law, et al., 1986b, Olofsson, et al., 1987, Yang, et al., 1986). Circular dichroism (CD) analysis indicated that apoB in LDL contained approximately 41 percent α helix, 22 percent β structure, 20 percent β turns, and 17 percent random coil structure (Chen and Kane, 1986, Scanu and Hirz, 1968), in close agreement with secondary structure predicted from cDNA sequence (Yang, et al., 1986).
Segrest and colleagues (Segrest, et al., 1994b) used a computer based program called LOCATE that searched amino acid sequences to identify potential amphipathic structural motifs based on sets of rules for α helix and β strands and identified lipid-associating domains within apoB-100 sequence. According to their model, two dense clusters of putative lipid-associating amphipathic helices were located precisely in the middle and C-terminal end of apoB-100: a cluster of class G* helices was located at the N-terminus. The two regions between the three amphipathic clusters were found to be highly enriched in putative amphipathic β strands, while the three amphipathic helical domains were largely devoid of this putative lipid-associating motif. They finally proposed that human apoB-100 has a pentapartite structure, NH₂-α₁-β₁-α₂-β₂-α₃-COOH, with α₁ representing a globular domain. One of the β sheet domains located between amino acids at about 18 and 43% of full-length apoB-100 appears to be involved in translocation arrest of the protein by a hydrophobic interaction with the ER membrane (Du, et al., 1994, Du, et al., 1996, Thrift, et al., 1992). The β sheet domain has also been suggested to be responsible for lipid binding and VLDL assembly (McLeod, et al., 1996). Liang et al. using human apoB chimeric constructs demonstrated that the translocation efficiency, susceptibility to proteasomal degradation, and lipid responsiveness of apoB were determined by the presence of a lipid binding β sheet domain (Liang, et al., 1998). Chauhan et al. (Chauhan, et al., 1998) based on a series of experiments on delipidated LDL using 28 different anti-apoB monoclonal antibodies proposed that the β strands of apoB-100 may represent a non-flexible lipid-associating backbone, while the amphipathic α-helical domains may represent flexible lipid-binding regions that allow the particle to accommodate varying amounts of lipid. Segrest, et al. (Segrest, et al., 1998) recently reported the ubiquitous presence of the pentapartite structure.
in apoB-100 from eight species of vertebrates (chicken, frog, hamster, monkey, mouse, pig, rat, and rabbit). Figure 1.2.1 shows a schematic diagram of their proposed structural model of apoB-100. Structural resemblance between apoB and microsomal triglyceride transfer protein (MTP), vitellogenin (the ancient egg yolk storage protein and precursor form of lipovitellin), lipovitellin (an oocyte lipoprotein), encouraged researchers to crystallize some of these proteins in order to decipher the mystery surrounding apoB-100 structure. Anderson et al. (Anderson, et al., 1998) by crystallizing lipovitellin which has two large domains conserved in both apoB and MTP, proposed that the lipid binding cavity is formed primarily by a single-thickness β-sheet structure which is stabilized by bound lipid. Mann et al. (Mann, et al., 1999) based on molecular modeling and mutagenesis showed that the globular amino-terminal regions of apoB and MTP are closely related to the vitellogenin. They proposed a model for assembly and secretion of apoB-containing lipoproteins (Mann, et al., 1999). Segrest et al. (Segrest, et al., 1999), reported that the first 200 residues of human apoB-100 (the α1 domain plus the first 200 residues of β1 domain) have sequence and amphipathic motif homologies to the lipid binding pocket of lamprey lipovitellin. They also showed that most of the α1 domain of apoB has sequence and amphipathic motif homologies to MTP. Based on their data, they suggested a "lipid pocket" model for the assembly of apoB-containing lipoproteins.

During its complex biosynthesis, apoB is further modified by disulfide bond formation, glycosylation, phosphorylation, and fatty acylation (Davis, 1996, Havel, 1995). Human apoB-100 contains 25 cysteine residues of which at least 16 are involved in intramolecular disulfide bonds (Cardin, et al., 1982, Yang, et al., 1989). Fourteen of these disulfide linkage are located within the N-terminal portion (Yang, et al., 1990). Secondary
structural analysis has predicted that N-terminal 17% of apoB may form a globular structure owing to the presence of concentrated disulfide bonds. Several groups have shown that the N-terminal disulfide bonds are necessary for proper folding, stability, lipid recruitment and secretion of apoB lipoproteins (Shelness and Thornburg, 1996, Tran, et al., 1998). Callow and Rubin showed that the C-terminal Cysteine at residue 3426 forms the disulfide link of LDL apoB-100 to glycoprotein in the Lp(a) complex (Callow and Rubin, 1995).

Both apoB-100 and apoB-48 are glycoproteins. ApoB-100 contains at least 20 potential N-linked glycosylation sites (Siuta-Mansano, et al., 1982), with concentrations in the N-terminal region and around the putative LDL-receptor ligand domain (Yang, et al., 1989). Although VLDL apoB is secreted containing N-linked high mannose and mature carbohydrate chains, tunicamycin-based studies showed that inhibition of glycosylation decreased net production of apoB by increasing cellular degradation (Liao and Chan, 2001, Macri and Adeli, 1997a).

Early studies have shown that apoB is covalently modified by the fatty acid palmitate via a thioester bond (Hoeg, et al., 1988, Huang, et al., 1988, Kamanna and Lee, 1989, Lee, 1991, Lee and Singh, 1990). Protein palmitoylation is a reversible posttranslational modification that occurs on cysteine residues. Recently, Zhao et al. (Zhao, et al., 2000) demonstrated that palmitoylation of apoB is a structural requirement for proper assembly of the hydrophobic core of the lipoprotein particle and its intracellular sorting.

Data from cultured rat hepatocytes (Davis, et al., 1984) and diabetic rat hepatocytes (Sparks, et al., 1988) indicate that apoB is subjected to phosphorylation on serine and tyrosine residues and phosphorylation occurs early in the secretory pathway. Swift using rat hepatic Golgi apparatus-rich fractions found that the Golgi apparatus is the subcellular site
for apoB phosphorylation (Swift, 1996). Functional significance of apoB phosphorylation is not fully understood.

A tertiary structure of apoB-100 in LDL particle has been proposed based on molecular mapping as well as different approaches including proteolytic digestion (Yang, et al., 1986, Yang, et al., 1989), electron microscopy (Lee, et al., 1987), X-ray studies (Luzzati, et al., 1979) and a battery of monoclonal antibodies (Chatterton, et al., 1991). These data confirmed that there was one molecule of apoB per LDL particle (Milne and Marcel, 1982) which surrounds the LDL neutral lipid core. The results led Chatterton and colleagues (Chatterton, et al., 1995) to propose a three-dimensional model called "ribbon and bow" model. According to this model, the first 89% of apoB100 forms a thick ribbon that completes a circle around the particle at residue 4050 and is in contact with the neutral lipid core. There is also a kink located close to the middle of the proposed ribbon structure. The remaining 11% C-terminal of apoB forms an elongated structure of about 480 residues or "bow" which is stretched back into one hemisphere and then crossing the ribbon into the other hemisphere toward the LDL-receptor binding domain. Further data suggested that the ribbon and bow model might be applicable for the TG-rich particles like VLDL and chylomicrons. The combined data suggest that the circumference of the circle made by apoB is likely to be one criterion that determines the size of the neutral lipid core and thus the capacity to transport TG and cholesterol esters (Davis, 1999). Although the above three-dimensional model is the most comprehensive model proposed for the apoB-100, there are still some questions left unanswered by the model especially regarding the very large TG-rich particles.
FIGURE 1.2.1

Schematic diagram of the distribution of amphipathic α helixes and amphipathic β strands in apolipoprotein B100 (adapted from Segrest, et al., 1998).
FIGURE 1.2.1

APO B-100

Class G*  Class A and Y  Class A and Y

Amphipathic α helices
C-terminal amphipathic helix
Amphipathic β strands
N-terminal amphipathic β strand

LDL Receptor Binding Domain
1.2.3 Transcriptional Regulation of Apolipoprotein B Production

The body of evidence collected from different cell culture systems indicates that acute regulation of apoB secretion is not transcriptional in nature. In Hep G2 cells the level of intracellular apoB mRNA is quite stable and generally does not change when the secretion of apoB-containing particles is changed by metabolic perturbations (Dashti, et al., 1989, Pullinger, et al., 1989). An apoB mRNA has a half-life of 16 h (Pullinger, et al., 1989), making short-term regulation of apoB biogenesis by control of transcription relatively inefficient. In HepG2 and Caco-cells, incubation with fatty acids causes stimulation of apoB secretion without any change in its mRNA levels (Dashti, et al., 1989, Kaptein, et al., 1991, Moberly, et al., 1990, Pullinger, et al., 1989). Similarly, in African green monkey, consumption of high cholesterol diets increased plasma levels of apoB-containing lipoproteins without altering hepatic apoB mRNA levels (Sorci-Thomas, et al., 1989). Srivastava (Srivastava, 1996) using high cholesterol and high fatty acid fed mice and rats showed no alteration of hepatic apoB mRNA despite a significant elevation of plasma levels of apoB-containing particles. Raspe and colleagues (Raspe, et al., 1999) found that 3-thia fatty acids through PPARα activation significantly reduced serum TG, cholesterol, and free fatty acid levels in rats. Although these fatty acids decreased liver mRNA levels of apoA-I, A-II, A-IV, and C-III, they had no significant effect on apoB mRNA levels. It is known that insulin exerts inhibitory effects on apoB secretion through a non-transcriptional control of apoB biogenesis (Dashti, et al., 1989, Pullinger, et al., 1989). Recently, Neele and colleagues (Neele, et al., 1999) using primary cultures of Cynomolgus monkey hepatocytes reported similar results on apoB mRNA levels. Fasting and feeding of animal models also showed no significant effects at the mRNA levels, despite significant alterations of secretion of apoB-
containing lipoproteins (Leighton, et al., 1990). Inui and colleagues (Inui, et al., 1997) in fatty liver of obese rats fed with high sucrose diet reported enhanced apoA-IV mRNA levels without any alteration in apoB gene expression. Gruffat et al. (Gruffat, et al., 1997) using lactating cows reported that apoB synthesis during early lactation was decreased although its mRNA level was unaltered. Nassir et al. (Nassir, et al., 1996) reported that increased plasma levels of apoB-100 in experimentally induced copper deficient rats was not due to apoB gene transcription.

Despite strong evidence indicating stability of transcriptional levels of apoB gene under different metabolic conditions, there are some reports showing that apoB mRNA can be modulated under certain conditions. Dashti reported an increased mRNA level in HepG2 cells incubated with 25-hydroxycholesterol (Dashti, 1992). Theriault and colleagues (Theriault, et al., 1992b) showed a 30% increase in apoB mRNA levels of HepG2 cells incubated with thyroid hormone. Incubation of HepG2 cells with VLDL particles can also increase the level of apoB mRNA (Wu, et al., 1994).

Despite a few reports showing alteration of apoB gene transcription, it appears that in most cases apoB message levels remain relatively constant under different metabolic perturbations. Acute modulation of apoB secretion appears to be mediated by post-transcriptional mechanisms. Fig. 1.2.2 depicts some key factors involved in post-transcriptional regulation of apoB biogenesis in hepatocytes.

1.2.4 Translational Regulation of Apolipoprotein B Production

There is plenty of evidence suggesting that apoB production is mainly regulated through post-translational modifications. However, it appears that in some cases translational level of apoB mRNA may control apoB secretion. Theriault et al. (Theriault, et al., 1992b)
reported an increase in apoB secretion in HepG2 cells incubated with thyroid hormone due to the increased rate of apoB synthesis. Sparks and Sparks (Sparks and Sparks, 1990), in primary rat hepatocytes incubated with insulin showed a reduction in apoB synthesis and secretion. Later, Adeli and Theriault (Adeli and Theriault, 1992), using a HepG2 cell in vitro translation system demonstrated that acute insulin treatment could attenuate apoB mRNA translation. In addition, in hypoinsulinemic streptozotocin-induced diabetic rats, apoB production was reduced due to a decrease in apoB synthesis (Sparks, et al., 1992). In these rats impairment of apoB48 and complete block of apoB100 translation was detected at the level of peptide elongation. They suggested that insulin-dependant factors control apoB mRNA translation through protein-RNA interaction. Wu and colleagues (Wu, et al., 1994) reported that incubation of HepG2 cells with exogenous VLDL, resulted in an increase in apoB synthesis and production. Zhang et al. (Zhang, et al., 1993) demonstrated that apoB mRNA translational modulation was involved in decreased apoB secretion in HepG2 cells incubated with amino acids. Furthermore, Mathur and colleagues (Mathur, et al., 1996) reported a stimulation of apoB synthesis in CaCo-2 cell lines incubated with phosphatidylcholine.

Despite the above-mentioned reports on the role of translational modulation of apoB mRNA, the major mode of regulation of apoB production in most instances appears to be post-translational in nature. Translocation of ApoB across the ER-membrane, apoB degradation, and its assembly in the secretory pathway to form the lipoprotein particle are considered three major processes determining the fate of newly synthesized apoB.
FIGURE 1.2.2

Some important factors involved in post-transcriptional modulation of hepatic apoB biogenesis

Newly synthesized apoB is cotranslationally translocated via translocon channel across the ER membrane. Chaperones such as Bip, calnexin assist the nascent apoB molecule to fold properly. In case of translocation inefficiency such as lipid substrate shortage, apoB polypeptides become exposed to the cytosol and interact with hsp70/90 and are targeted for proteasomal degradation. In the presence of adequate lipid substrates, microsomal triglyceride transfer protein (MTP) transfers core lipids such as TG and cholesterol esters (CE) to the cotranslationally translocated nascent apoB and creates a small dense premordial particle inside the ER-lumen. These precursor particles are converted to mature, secretion-competent VLDL particle by recruiting more lipids. Particles that fail to recruit adequate lipids are secretion-incompetent and are subjected to both intraluminal and proteasomal degradation.
FIGURE 1.2.2

Diagram showing the process of lipoprotein assembly, degradation, and secretion in hepatocytes. The diagram includes the following steps:
- Proteasome
- Membrane Association
- ApoB mRNA (14 kb)
- Translocation
- LDLR
- Lipoprotein Assembly
- Intraluminal Degradation
- Secretion of VLDL particles
- VLDL and CE
- Hepatocyte

The diagram illustrates the flow from the cytosol to the secretion of VLDL particles.
1.2.5 Co-translational and Post-translational Regulation of Apolipoprotein B Production

1.2.5.1 Translocation of Apolipoprotein B across the Endoplasmic Reticulum

It has been shown that translocation efficiency of apoB is a major determinant in apoB secretion (Borchardt and Davis, 1987, Davis, et al., 1990, Du, et al., 1994). The key to this post-translational regulation appears to be the rapid degradation of newly synthesized apoB, which results from inefficient translocation of nascent apoB across the ER membrane. In mammalian cells, all nonorganellar protein biosynthesis is initiated on unbound cytoplasmic ribosomes. Those ribosomes synthesizing proteins destined for secretion or for integration into the ER membrane are identified by a 15-30 residue signal sequence at the N-terminal end of the nascent chain (reviewed by Walter and Johnson, 1994). The sorting of most non-cytoplasmic proteins begins at the membrane of the endoplasmic reticulum (ER). Proteins destined for secretion are translocated across the ER membrane at sites called translocons (reviewed in Johnson and van Waes, 1999). In eukaryotic cells, this translocation occurs co-translationally, at the same time that protein is being synthesized by a ribosome.

Numerous reports indicate that apoB becomes associated with the ER membrane, either co-translationally or very early in the post-translational period. Olofsson and colleagues (Boren, et al., 1990, Bostrom, et al., 1986) demonstrated that in HepG2 cells, the majority of newly synthesized apoB was ER membrane associated. In chick hepatocytes, Bamberger and Lane (Bamberger and Lane, 1988), observed that after alkaline treatment of microsomes, approximately 40% of apoB remained in the membrane fraction. Davis et al. (Davis, et al., 1989) reported that in rat hepatocyte, apoB was exposed to the cytosolic side of ER membrane. This observation was later confirmed in both chicken hepatocytes (Dixon, et
al., 1992) and HepG2 cells (Wilkinson, et al., 1993, Wilkinson, et al., 1992). Several different groups have confirmed the exposure of the apoB to the cytosolic side of the ER membrane, using exogenous protease digestion assays and isolated ER or microsomal vesicles (Du, et al., 1994, Edwards and Grundy, 1989, Furukawa, et al., 1992, McLeod, et al., 1996, Wang, et al., 1996) and later Davis et al. (Davis, et al., 1990) using isolated microsomal fractions obtained from rat liver, determined the susceptibility of apoB-48 and apoB-100 to digestion with trypsin and proteanase K. They showed that approximately 50% of apoB-100 and apoB-48 in rat liver rough microsomes was degraded by exogenous trypsin under conditions in which microsomes remained intact. In addition, Davis et al. (Davis, et al., 1990) used pulse-chase and immunological methods to assay apoB topography. Their combined data indicated that apoB domains are exposed on the cytoplasmic surface of rat liver rough microsomes. In another approach, Lingappa and colleagues found specific sequences called pause transfer (PT) sequences that direct the transient pausing and subsequent restarting of nascent apoB chain across the ER membrane (Nakahara, et al., 1994). Later the same group suggested a pausing-based model of co-translocational exposure of apoB to the cytosol (Hegde and Lingappa, 1996), and also found several PT sequences distributed asymmetrically throughout the apoB molecule (Kivlen, et al., 1997). Research done by Lingappa’s group provides evidence that translocational pausing may play a role in the formation of several different so called “unconventional” secretory and membrane proteins in addition to apoB (Nakahara, et al., 1994).

Although, some investigators have failed to demonstrate the existence of cytosolic exposed apoB (Ingram and Shelness, 1996, Leiper, et al., 1996, Pease, et al., 1995, Shelness, et al., 1999, Shelness, et al., 1994) the majority of reports using different hepatocyte models

More recently, Ginsberg and his colleagues (Pan, et al., 2000) have found a connection between apoB translocation and apoB mRNA translation. They demonstrated that in HepG2 cells, treatment with an MTP inhibitor and ALLN, a proteasomal inhibitor, caused accumulation of newly synthesized apoB in the translocation channel, which subsequently exerted a selective and negative effect on the synthesis of apoB at the stage of elongation.

1.2.5.2 Factors Involved in ApoB Translocation

Several factors are known to contribute to the efficiency of apoB translocation, including protein factors such as microsomal triglyceride transfer protein (MTP) and chaperone proteins, lipid availability, and structural domains of the nascent apoB molecule.

1.2.5.2.1 Microsomal Triglyceride Transfer Protein (MTP)

MTP is a heterodimeric protein consisting of a 97 kDa catalytic subunit and a 58 kDa enzyme protein disulfide isomerase (PDI) (Ferrari and Soling, 1999, Wetterau, et al., 1990). MTP is located in the lumen of ER of apoB secreting cells, hepatocytes, enterocytes, (Gordon, et al., 1994) and cardiac myocytes (Boren, et al., 1998c, Nielsen, et al., 1998b) and acts as neutral lipid transfer protein. PDI is a ubiquitous, multifunctional ER protein that catalyzes the insertion of disulfides into folding proteins and corrects errors in disulfide bonding (Noiva and Lennarz, 1992). The larger subunit of MTP is responsible for its lipid transferring activity. Formation of a heterodimeric complex between MTP and PDI is absolutely required in order to express active lipid transfer activity (Wetterau, et al., 1991).
Wang et al. demonstrated that the role of PDI in MTP complex involved functions other than its known enzymatic activities which was also important for the so-called MTP-independent steps of apoB secretion (Wang, et al., 1997). Although MTP is capable of transferring cholesteryl esters, free cholesterol, and phospholipid, it preferably transfers TG and cholesteryl esters (Jamil, et al., 1995). The $\alpha_1$ domain of apoB and the amino-terminal ~ 65% of the 97-kDa subunit of MTP were found to be homologous with lipovitellin, an ancient lipid transporter in egg-laying animals (Baker, 1988, Shoulders, et al., 1994). MTP, apoB, and lipovitellin share an amino-terminal $\beta$-barrel and an extended $\alpha$-helical domain (Mann, et al., 1999). MTP is thought to transfer lipids to apoB while the apoB polypeptide chain is being translated and translocated into the lumen of ER, allowing apoB to fold properly and assemble into a spherical lipoprotein with a core of neutral lipids (Gordon and Jamil, 2000). Studies in patients with abetalipoproteinemia, an autosomal recessive disease in which MTP activity is virtually absent (Wetterau, et al., 1992 and recently reviewed in Berriot-Varoqueaux, et al., 2000) and dose-dependent inhibition of apoB secretion in animals and cells treated with specific MTP inhibitors (Benoist, et al., 1996, Haghpassand, et al., 1996, Jamil, et al., 1998, Jamil, et al., 1996, Macri, et al., 2000, Wetterau, et al., 1998) have suggested that the presence of active MTP is absolutely essential for the assembly and the secretion of apoB-containing lipoproteins. Furthermore, recent studies in Watanabe rabbits (LDL receptor deficient) have shown that feeding with an MTP inhibitor normalizes plasma cholesterol and TG most likely via extensive presecretory apoB degradation (Wetterau, et al., 1998). In addition, apoB production and secretion in non-hepatic and non-intestinal cells requires co-expression of apoB with MTP (Gordon, et al., 1994, Leiper, et al., 1994, Patel and Grundy, 1996, Wu, et al., 1996). In the absence of MTP, newly synthesized apoB is
rapidly degraded by the proteasomal pathway (Benoist and Grand-Perret, 1997) and essentially none of it gets secreted. Numerous studies (Gordon and Jamil, 2000, Wu, et al., 1996) have demonstrated a physical interaction between MTP and apoB, and in Hep G2 cells the disruption of this interaction inhibited apoB secretion by 70-85% without affecting lipid transfer activity of MTP (Bakillah, et al., 2000, Hussain, et al., 1998). Mutagenesis studies showed that cysteine-enriched amino terminus of apoB was necessary for the MTP responsiveness (Gretch, et al., 1996). N-terminal amino acid residues 430-570 (Hussain, et al., 1998) and 512-721 (Bradbury, et al., 1999) have been proposed to contain the MTP binding sites.

The relationship between translocation of the newly synthesized apoB and MTP activity has been a controversial issue. Thrift et al. (Thrift, et al., 1992) has reported apoB translocation arrest in Chinese hamster ovary (CHO) cells transfected with apoB gene in the absence of MTP. Translocation arrested apoB was later degraded producing an 85-kDa N-terminal fragment (Du, et al., 1994). Wang et al. (Wang, et al., 1996) demonstrated translocation facilitating effects of MTP in COS-7 cells transfected with apoB72 and apoB94. In contrast, other researchers using cell-free systems (Rusinol, et al., 1997), or cell lines such as COS cells (Shelness, et al., 1994), and murine C-127 (Herscovitz, et al., 1995) suggested that MTP may not be required for the translocation of apoB. More recently, Macri et al. (Macri, et al., 2000) using an MTP inhibitor reported that in HepG2 cells, MTP lipid transfer activity did not influence translocational status of apoB, instead it increased susceptibility to proteasome-mediated degradation and reduced assembly and secretion of apoB lipoprotein particles. In agreement with the above issue, Huang and Shelness (Huang and Shelness, 1999) using different truncated apoB appended to a short peptide containing
glycosylation sites expressed in hepatic and non-hepatic cell lines also reported efficient MTP independent translocation of apoB.

It appears that expression of the 97 kDa subunit of MTP is tightly regulated, mostly because of its heterodimeric existence with PDI. Recently, Sato et al. (Sato, et al., 1999) found two sterol response elements, SREBP-1 (amino acids, 1-487) and SREBP-2 (amino acids, 1-481) overlapping with an insulin response element in the promoter of the MTP gene. All these metabolic response elements negatively regulate the expression of the MTP gene.

Lin et al. (Lin, et al., 1994) demonstrated dietary regulation of MTP activity and mRNA levels in Syrian golden hamster intestine and liver. They found that a high fat diet increased MTP mRNA levels in the liver and throughout the small and large intestine whereas a high sucrose diet only increased (~55%) MTP mRNA in the liver. Raabe et al. (Raabe, et al., 1998) first reported the production of MTP knockout mice by gene targeting. Liao et al (Liao, et al., 1999) using adenoviral expression system, overexpressed MTP in HepG2 cells and increased its lipid transfer activity up to five-fold, although most of the overexpressed protein was secreted out of the cells (due to the absence of a KDEL sequence). This MTP overexpression increased apoB secretion and reduced its proteasomal degradation providing more evidence on the crucial role of MTP in apoB production. In addition to overexpression studies, several groups have attempted to develop MTP knock out mice models (reviewed in Chan, et al., 2000). Homozygous MTP knockout mice do not survive embryonically. To overcome this problem, two different groups (Chang, et al., 1999, Raabe, et al., 1999) used conditional MTP gene inactivation methods by inserting loxP sequences and adenovirus-mediated transfer of Cre recombinase. Although the insertion of loxP sequence was not successful, the Cre recombinase system selectively inactivated the hepatic MTP gene.
Interestingly these mice showed marked diminution in plasma apoB-100 levels and variable diminution in plasma apoB48 levels. Homozygous knockout animals did not respond to a high cholesterol diet due to accelerated hepatic apoB degradation and abolition of VLDL secretion. Electron microscopy examination of the livers revealed that, in contrast to normal mice, in MTP knockout hepatocytes, no VLDL size particle was observed while numerous cytosolic fat droplets were detected (Raabe, et al., 1999). Raabe et al. concluded that MTP is essential for transferring the bulk of TG into the lumen of the ER for VLDL assembly and is thus essential for both the first and the second step of apoB100 lipidation. Several recent reviews on MTP function are available (Berriot-Varoqueaux, et al., 2000, Gordon and Jamil, 2000, Murphy and Vance, 1999).

1.2.5.2.2 Chaperone Proteins

Molecular chaperones are defined as a class of proteins involved in correct folding of nascent peptides as well as proper conformation and assembly of the mature forms and even degradation of misfolded proteins (reviewed in Ellis, 2000, Fink, 1999, Freeman, et al., 2000, Ma, et al., 2000, Saibil, 2000). Interactions between apoB and several different chaperones have been documented. In HepG2 cells, calnexin, which is an ER transmembrane protein, has been shown to associate with several glycoproteins, including apoB (Bergeron, et al., 1994, Ou, et al., 1993). Patel and Grundy (Patel and Grundy, 1996) co-immunoprecipitated calnexin with apoB and showed that their interaction was diminished in the presence of MTP. Although they reported that inhibition of N-linked glycosylation prevented the binding of calnexin, this caused no alteration in secretion kinetics and assembly of apoB-containing particles, Chen et al. (Chen, et al., 1998) observed that impairing this association blocked translocation and caused rapid ubiquitin-dependent proteasome degradation. Linnik and
Herscovitz (Linnik and Herscovitz, 1998) have reported the association of apoB with several intraluminal chaperones including Erp72, GRP94, calreticulin, and Bip. The importance of these associations has not been defined.

1.2.5.3 Post-translational Degradation of Apolipoprotein B

The biogenesis of large secretory proteins such as apoB-100 is a complex process with a high chance of production of misfolded or aberrant proteins. Perturbed folding and assembly of secretory proteins within the ER usually results in retarded or blocked anterograde transport and subsequent degradation (Fink, 1999, Hampton, 2000). Co-translational and posttranslational degradation of apoB has been demonstrated and documented using different cell systems. In HepG2 cells approximately one half to two thirds of de novo synthesized apoB is degraded (Adeli, 1994, Borchardt and Davis, 1987, Sato, et al., 1990). In primary cell cultures of rat hepatocytes (Martin-Sanz, et al., 1990, Sparks and Sparks, 1990, Wang, et al., 1995b), 25% to 50% of the newly-synthesized apoB appears to be degraded whereas the extent of degradation is about 44-50% in primary rabbit hepatocytes (Cartwright and Higgins, 1996, Tanaka, et al., 1993). In HepG2 cells (Dixon, et al., 1991) and McA-RH777 cells (White, et al., 1992), stimulation of TG synthesis following addition of oleate protected apoB against degradation and dramatically increased its secretion without affecting synthesis rate. ApoB degradation was initially thought to occur in a pre-Golgi compartment (Adeli, 1994, Borchardt and Davis, 1987, Furukawa, et al., 1992, Sato, et al., 1990), in a process inhibited by ALLN (Adeli, 1994, Sakata, et al., 1993, Thrift, et al., 1992), mostly based on evidence from HepG2 cells. Thus, a large body of evidence supports the notion that apoB is synthesized in surplus and shunted to a pathway involved in lipoprotein
assembly and secretion or degradation. The balance between assembly and degradation determines the secretion rate of apoB.

In HepG2 cells, the site of degradation appears to be localized in a pre-Golgi compartment that does not involve the lysosomal pathway (Adeli, 1994, Dixon, et al., 1992, Du, et al., 1994, Furukawa, et al., 1992, Sato, et al., 1990). However, in most primary hepatocytes examined, degradation of apoB is not confined to the ER (Sparks and Sparks, 1994a) and appears to occur even after an apoB-lipoprotein is formed (Cartwright and Higgins, 1996. Fast and Vance, 1995, Verkade, et al., 1993, Wang, et al., 1995a, Wang, et al., 1993). Overall the available data indicate that apoB degradation can occur in all secretory compartments by proteasomal and non-proteasomal degradative systems (for recent reviews refer to Davidson and Shelness, 2000, Olofsson, et al., 1999, Yao, et al., 1997).

1.2.5.3.1 Proteasomal Degradation of Apolipoprotein B

detected ubiquitinated apoB that was associated with the Sec61 complex and showed that factors including calnexin, which alter translocation, can affect apoB ubiquitination and degradation. The same group reported that in HepG2 cells adenoviral overexpression of MTP caused stimulation of apoB production through downregulation of ubiquitin-proteasome-mediated apoB degradation (Liao, et al., 1999). Sakata and Dixon (Sakata and Dixon, 1999) using rabbit reticulocyte lysate developed an in vitro system to investigate ubiquitin-proteasome-dependent apoB degradation. They also showed that in permeabilized HepG2 cells cytosolic components were required for proteasomal degradation of newly synthesized apoB (Sakata, et al., 1999).

Although a strong body of evidence supports the degradation of newly synthesized apoB by the ubiquitin-proteasome pathway, the mechanism for the targeting of apoB to the cytosolic proteasomal system has not been elucidated. Based on available data on translocational efficiency of apoB, two possible mechanisms have been suggested: First, there is some evidence supporting the notion that the translocation of newly-synthesized apoB across the ER membrane is inefficient and incomplete in the absence of sufficient triglyceride and cholesterol esters which results in a bitropic orientation or bulging of apoB which exposes some domains to the cytosol and subsequent ubiquitination and degradation (Davis, et al., 1989, Davis, et al., 1990, Du, et al., 1994, Du, et al., 1998, Furukawa, et al., 1992, Liang, et al., 1998, Mitchell, et al., 1998). Secondly, a model has been proposed for the early degradation of apoB based on complete and efficient translocation. According to this retrograde translocation model, full-length apoB is retracted through sec 61 p translocational channel, ubiquitinated, and targeted to proteasomal degradation (Huang and Shelness, 1999). Fig. 1.2.3 depicts three possible pathways for retrotranslocation of apoB, based on a model.
proposed by Liao et al. (Chan, et al., 2000, Liao, et al., 1998). More recently, in addition to the two above mentioned models, Ginsberg et al. (Liang, et al., 2000), examined a third possible model involving co-translational retrograde translocation of the N-terminus of apoB to the cytosol via a second nearby translocon as well. They used Chinese hamster ovary (CHO) and HepG2 cells transfected with C and N terminal tagged apoB42 constructs and presented evidence against apoB retrograde translocation (via both original and nearby translocons) and concluded that in the absence of adequate core lipids, partially translocated apoB was exposed to the cytosol and was ubiquitinated and degraded by proteasomes directly from the original translocon channel.

1.2.5.3.2 Non-Proteasomal Degradation of Apolipoprotein B

There is strong evidence supporting post-translational non-proteasomal degradation of apoB by mostly unidentified proteases in the ER (Adeli, et al., 1997a, Wu, et al., 1997) and post ER (Wang, et al., 1995a) compartments. A two step post-translational apoB degradation mechanism has been suggested by Wu et al. (Wu, et al., 1997). They hypothesized that in the first step translocation arrested apoB associated with the ER membrane is degraded by an ALLN-sensitive pathway, while the second step occurs in the ER lumen in a DTT-sensitive process. The exact identity of most of the proteases involved in non-proteasomal apoB degradation is not known and they are classified based on their inhibition by different protease inhibitors. Cartwright and Higgins (Cartwright and Higgins, 1996) found that in freshly isolated rabbit hepatocytes, inhibitors of metalloproteases (o-phenanthroline), serine proteases (aprotinin), serine/cysteine proteases (leupeptin) or cysteine proteases (calpain inhibitor I; ALLN) but not aspartate proteases (pepstatin) resulted in inhibition of cellular degradation of apoB. Although leupeptin did not affect apoB-100
FIGURE 1.2.3

Model of proteasome-mediated degradation of apoB

Three possible pathways have been proposed for the retrograde translocation of apoB to the cytosol for proteasomal degradation. 1, nascent partially synthesized apoB polypeptides are cotranslationally bulged, ubiquitinated and are fed to the proteasomes in a retrograde fashion. 2, full-length glycosylated apoB is retrotranslocated from the ER lumen back into the cytosol for ubiquitination via the same translocon through which the apoB was initially translocated into the ER. 3, the full-length glycosylated apoB is discharged into the ER and is back transported into cytosol for proteasomal degradation via a different translocon channel. Adapted with some modifications from (Chan, et al., 2000, Liao, et al., 1998).
degradation in HepG2 cells (Sato, et al., 1990), in rat primary hepatocytes it inhibited apoB-100 degradation by 20-30% (Wang, et al., 1995a). Wang et al. (Wang, et al., 1995a) also showed that the post-ER degradation of apoB could be inhibited by a cysteine protease inhibitor, EST.

Occurrence of apoB degradation in permeabilized cell systems which has been reported by several groups including our laboratory is a strong evidence supporting the presence of a non-proteasomal apoB degradation pathway (Adeli, 1994, Adeli, et al., 1995, Adeli, et al., 1997b, Du, et al., 1994, Sallach and Adeli, 1995). Degradation of apoB appears to generate proteolytic fragments or intermediates in both intact and permeabilized cells (Adeli, 1994). In permeabilized cells, apoB degradation occurs by a temperature- and pH-dependent and ALLN-sensitive cysteine protease in an ER-related compartment (Adeli, 1994) resulting in generation of an abundant N-terminal 70 kDa fragment (Adeli, 1994, Adeli, et al., 1997b, Sallach and Adeli, 1995). In a recent report, Cavallo et al. (Cavallo, et al., 1999) showed that despite the loss of proteasomal activity in permeabilized HepG2 cells, degradation of apoB occurred generating a 70 kDa fragment, confirming the involvement of non-proteasomal proteolytic systems in apoB degradation.

ER-60 an ER resident cysteine protease has been postulated to be involved in the ER degradation of apoB (Adeli, et al., 1997a). ER-60 is a multifunctional protein with proteolytic activity and disulfide bond-dependent folding activity (Urade and Kito, 1992, Urade, et al., 1992, Zapun, et al., 1998). ER-60 has 98% homology in amino acid sequence to rat phosphoinositide-specific phospholipase C (Urade, et al., 1992). Proteolytic action of ER-60 is inhibitable by p-chloromercuribenzoate (pCMB) and cysteine protease inhibitors such as ALLN, ALLM, ER-64 and leupeptin (Urade and Kito, 1992). Interestingly, C-terminal
truncated ER-60 itself is subjected to the degradation by a non-proteasomal, ALLN-sensitive degradative pathway (Urade, et al., 2000). Although there are striking structural similarities between ER-60 and PDI such as sharing a CGHC motif, which is the active center for proteolytic activity in ER-60, it is not clear whether ER-60 undergoes autodegradation similar to PDI (Urade, et al., 1999). Otsu et al. (Otsu, et al., 1995) reported the association of ER-60 with misfolded human lysozyme (expressed in mouse L cells), while there was no association with the wild-type protein. Furthermore, they also demonstrated in vitro degradation of denatured lysozyme by ER-60, but not the native protein suggesting the possible role of ER-60 as a component of proteolytic machinery of the ER. Adeli et al. (Adeli, et al., 1997a) using cross-linking methods demonstrated association of an ER-60 homologue with apoB in HepG2 cells and hypothesized that ER-60 may be involved in the degradation of apoB within the lumen of microsomes in HepG2 cells. There are reports suggesting chaperonic functions for ER-60 (Lindquist, et al., 2001, Lindquist, et al., 1998, Zapun, et al., 1998). It is not currently known whether apoB is directly degraded by ER-60 or ER-60 acts as a tag chaperone, which facilitates apoB degradation by another unknown proteolytic system.

More recently an interesting and novel form of presecretory non-proteasomal VLDL degradation was reported by Twisk et al. (Twisk, et al., 2000). They observed that apoB secretion from hepatocytes isolated from LDL receptor-knockout mice was 3.5-fold higher than that from wild type hepatocytes. They proposed that the intracellular interaction of LDL receptor with nascent VLDL, via either apoB or apoE target nascent VLDL for presecretory degradation by an unknown proteolytic pathway. The above finding may explain the overproduction of VLDL in genetic LDL receptor deficiency states (familial
hypercholesterolemia) and reduced VLDL production observed when LDL receptors are upregulated by statins (Shelness and Sellers, 2001).

1.2.5.4 Modulation of Apolipoproteins B Translocation and Degradation

There is a strong body of evidence suggesting that the lipid availability and cellular apoB levels are the two major factors influencing apoB-containing lipoprotein production (for the recent reviews refer to Chan, et al., 2000, Davidson and Shelness, 2000, Davis, 1999, Shelness and Sellers, 2001).

1.2.5.4.1 The Effects of Lipid Availability on Apolipoprotein B Production

Several research groups have shown that lipids and especially fatty acids directly stimulate VLDL-TG synthesis and secretion in hepatoma cells (Byrne, et al., 1991, Byrne, et al., 1992, Cianflone, et al., 1990, Dashti and Wolfbauer, 1987, Dixon, et al., 1991, Pullinger, et al., 1989, White, et al., 1992) and primary cultured hepatocytes (Gibbons, et al., 1992, Levinson, et al., 1990). Different lipid substrates exert different effects on hepatic VLDL production. Salter et al. (Salter, et al., 1998a) using Syrian golden hamster fed with either myristic, stearic or palmitic acids found that only dietary palmitic acid significantly increased hepatic apoB mRNA levels. Furthermore, it is also suggested that oleate treatment of the cells facilitates translocation of newly synthesized apoB across the ER-membrane, which in turn reduces early degradation (Macri and Adeli, 1997b). However, whether or not this protection of early degradation stimulates apoB secretion appears to differ among different cell types. In HepG2 cells, exogenous oleate significantly stimulates apoB secretion (Bostrom, et al., 1988, Dixon, et al., 1991, Pullinger, et al., 1989). White et al. (White, et al., 1992) reached similar conclusions using a rat hepatoma cell line. Cartwright and Higgins (Cartwright and Higgins, 1996) reported that in freshly isolated rabbit hepatocytes, oleate, in
addition to prevention of cellular apoB degradation, stimulated its secretion as much as 6-fold. In contrast, no stimulatory effect of oleate on secretion of apoB was observed in rat (Davis and Boogaerts, 1982, Patsch, et al., 1983b), hamster (Arbeeny, et al., 1992) or human hepatocytes (Lin, et al., 1995a). Sparks et al. (Sparks, et al., 1997) reported an inhibitory effect of oleate on the secretion of apoB in McArdle hepatoma cells and no effect on apoB secretion or cellular apoB of primary rat hepatocytes. Gibbons et al. (Gibbons, et al., 1992) using primary rat hepatocytes demonstrated that, the size of the cytosolic TG pool, rather than the availability of extracellular oleate, correlated with VLDL secretion. Salter et al. reached similar conclusions comparing hamster hepatocytes with rat hepatocytes (Salter, et al., 1998b). More recently, Pan et al. found a relationship between lipidation of apoB and translational elongation rates (Pan, et al., 2000). Translational arrested apoB can be targeted for proteasomal degradation, however in the presence of oleic acid, it can be rescued and undergo lipidation and secretion. In HepG2 cells the cytosolic chaperone Hsp70 binds to apoB; oleic acid reduces this association, causing more apoB to be translocated and subsequently secreted as a lipoprotein particle (Zhou, et al., 1995).

1.2.5.4.2 Effect of Conformational Status of Apolipoprotein B on Production of ApoB-containing Lipoproteins

It is widely accepted that any factor that interferes with apoB conformational status can potentially hinder its translocation leading to intracellular degradation. Although the effect of inhibition of N-linked glycosylation on protein conformation and secretion varies with the protein (Bauer, et al., 1985), most of the reports indicate that inhibition of N-linked glycosylation interferes with apoB production in HepG2 cells. Adeli (Adeli, 1994) using permeabilized HepG2 cells reported that inhibition of N-linked glycosylation altered apoB
degradation and its fragmentation pattern. Macri et al. (Macri and Adeli, 1997b) using HepG2 cells reported that disruption of disulfide bond formation using dithiothreitol reduced the percentage of translocated apoB by 63%. Dithiothreitol induced specific changes in the pattern of protected apoB fragments, suggesting a conformational change in apoB that may hinder its translocation. Inhibition of N-linked glycosylation did not significantly alter the rate of apoB translocation but it appeared to stimulate its degradation. Their data suggest that the rate of apoB translocation across the membrane of the ER is determined by both lipid availability as well as the correct conformation of nascent apoB molecules. In a series of experiments in HepG2 cells, Macri and Adeli (Macri and Adeli, 1997a) reached similar conclusions demonstrating that misfolding of apoB prevented the proper association of apoB with lipids, resulting in impairment of the assembly of mature apoB-containing lipoproteins. Furthermore, alteration in the conformation of apoB altered the degradation pathway of apoB. In a recent report Liao and Chan (Liao and Chan, 2001) demonstrated that treating HepG2 cells with tunicamycin and inhibiting N-linked glycosylation decreased apoB biogenesis and increased proteasomal and non-proteasomal apoB degradation without any effect on its assembly and secretion.

1.2.6 Assembly of ApoB100-containing lipoproteins

The basic mechanism for the assembly of apoB-containing lipoproteins appears to involve two relatively well defined steps (Boren, et al., 1994). Based on the effect of Brefeldin A (BFA) and involvement of MTP in the assembly process, Olofsson and colleagues (reviewed in Olofsson, et al., 1999, Rustaeus, et al., 1999, Shelness, et al., 1999) proposed two MTP-dependent (Gordon, et al., 1996, Rustaeus, et al., 1998) and MTP independent steps of VLDL assembly. In the first or early step (MTP-dependent), two VLDL.
precursors including apoB containing pre-VLDL (Alexander, et al., 1976, Boren, et al., 1994) and VLDL-size lipid droplets (Alexander, et al., 1976, Hamilton, et al., 1998) are formed simultaneously and independently in the secretory pathway. The apoB-containing pre-VLDL particles are formed co-translationally and in the posttranslational period (Rustaeus, et al., 1998). The membrane-bound apoB is partially lipidated and resembles HDL-like particles found in the secretory pathway (Boren, et al., 1994, Boren, et al., 1993). In the hepatoma cell lines and transfected non-hepatic cell lines these poorly lipidated particles can be secreted as LDL particles (Bostrom, et al., 1988). In contrast, dense HDL like apoB100-containing particles are secretion incompetent and appear to be degraded post-translationally, unless they are converted to mature secretion competent particles. Evidence using MTP inhibitors and pulse-chase studies indicate that MTP is needed for both co-translational and post-translational lipidation of apoB (Gordon, et al., 1996, Rustaeus, et al., 1998). The MTP dependence disappeared before the newly synthesized apoB acquired the major amount of lipid to form VLDL. In the second or late step, which is MTP-independent VLDL precursors fused with lipid droplets to form bona fide VLDL particles (Boren, et al., 1994, Hamilton, et al., 1998). Recent results of Hamilton et al. have confirmed the existence of apoB-free lipid droplets in the secretory pathway (Hamilton, et al., 1998). It appears that the formation of these lipid droplets is MTP-dependent (Raabe, et al., 1999). To form mature secretion competent VLDL particles, these lipid droplets are fused with pre-VLDL precursors in the secretory pathway in a Brefeldin A-sensitive and MTP independent manner. BFA is a fungal metabolite capable of blocking ER to Golgi transport. BFA inhibits nucleotide exchange on the ADP-ribosylation factor 1 (ARF 1) (Donaldson, et al., 1992, Helms and Rothman, 1992, Morinaga, et al., 1996). A recent in vitro VLDL assembly study confirmed ARF-dependent
inhibition of VLDL assembly by BFA (Asp, et al., 2000). ARF 1 is involved in formation of secretory transport vesicles (Schekman and Orci, 1996). The molecular mechanism behind the formation of these transport vesicles is currently under extensive investigation.
PART III: Metabolic Dyslipidemia in Insulin Resistant States

1.3.1 Insulin Signaling Pathway: An Overview

Upon the binding of insulin to its receptor several important physiologic and metabolic cellular events are triggered. Some of the cellular insulin effects are stimulatory such as glucose uptake, glycogen synthesis, DNA synthesis, amino acid uptake, protein synthesis, ion transport and fatty acid synthesis. Insulin also exerts some inhibitory cellular effects such as inhibition of apoptosis, gluconeogenesis, and lipolysis. Insulin signaling pathway initiates from interaction of the hormone with its receptor on the cell membrane. Insulin receptor is a tetramer $\alpha_2\beta_2$ receptor, its extracellular $\alpha$ subunits containing the insulin binding site, and transmembrane $\beta$ subunits (Van Obberghen, et al., 1981). Insulin binding to the $\alpha$ subunits causes conformational changes and thereby activation of the tyrosine kinase of the insulin receptor $\beta$-subunits (Kasuga, et al., 1983). Insulin receptor kinase activity induces both autophosphorylation and phosphorylation of other cellular substrates. White and colleagues (White, et al., 1985) described the first substrate of the insulin receptor, a 180 kDa protein which was later cloned and named insulin receptor substrate-1 or IRS-1 (Sun, et al., 1991). IRS-2, IRS-3, IRS-4, Gab1 and p62sak are new members of insulin receptor substrate family. All members of this family have common structural features including an NH2-terminal plekstrin homology (PH) and/or phosphotyrosine-binding domain (PTB); multiple tyrosine residues that create SH2-protein binding sites; proline-rich regions to engage SH2 or WW domains; and serine/threonine-rich regions (White, 1998). Tyrosine phosphorylation of IRSs provides docking motifs for the other downstream signaling proteins, among which is phosphoinositide 3-kinase (PI 3-kinase). Receptor tyrosine kinases, including growth factors and insulin receptors, stimulate class 1A PI 3-kinases. In vitro studies have shown that class
PI 3-kinases phosphorylate phosphatidylinositols (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4, 5-biphosphate (PtdIns4,5P2) to produce phosphatidylinositol 3, 4, 5-triphosphate (PtdIns3,4,5P3) as their final products whereas in vivo evidence suggested PtdIns(3,4)P2 as the major substrate for the enzymes (Carter, et al., 1994, Domin and Waterfield, 1997, Hawkins, et al., 1992, Stephens, et al., 1991). Class IA PI 3-kinase is a heterodimer protein consisting of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. PI 3-kinase catalyzes the phosphorylation of phosphoinositides at the 3' position of the inositol ring. Inhibition of PI 3-kinase activity by either dominant-negative mutants or pharmacological agents profoundly abrogates several biological responses to insulin (Cheatham, et al., 1994, Kotani, et al., 1995, Okada, et al., 1994, Quon, et al., 1995). Class IA PI 3-kinases have a very important role in many of insulin regulated physiologic responses including glucose uptake, protein synthesis, membrane ruffling and cell growth and proliferation (for review refer to Alessi and Downes, 1998, Taha and Klip, 1999). Protein kinase B, PKB, is the cellular homologue of the transforming oncogene v-Akt located downstream of PI 3-kinase in the insulin signaling pathway (for a recent review refer to Chan, et al., 1999). In vitro evidence indicate that PKB/Akt can bind to GLUT4-containing vesicles and mediate insulin stimulated glucose transport (Cong, et al., 1997, Kohn, et al., 1996, Kupriyanova and Kandror, 1999, Tanti, et al., 1997). PKB/Akt is a Pleckstrin homology (PH) domain-containing serine-threonine kinase, which can not auto-phosphorylate itself and has three isoforms (Alessi, et al., 1996, Kohn, et al., 1995). The PH domain is a 100- to 120-amino acid motif, which exhibits significant similarity to the structure of the phosphotyrosine-binding (PTB) domain. PH domains are primarily lipid-binding modules, although they are also involved in mediating protein-protein interactions.
(reviewed in Bottomley, et al., 1998). Insulin and growth factors activate Akt-1 and -2, whereas Akt-3 is not activated by insulin in muscle and adipose tissues. Akt-1 and Akt-2 are the main isoforms of Akt activated by insulin in muscle, hepatocytes, and adipocytes (reviewed in Chan, et al., 1999). PI 3-kinase activity is necessary for the activation of PKB/Akt and blocking PI 3-kinase activity abrogates insulin mediated-PKB/Akt activity (Alessi, et al., 1996, Burgering and Coffer, 1995, Datta, et al., 1996). The full activation of PKB/Akt requires phosphorylation of threonine-308 (Thr^{308}) and serine-473 (Ser^{473}). Although it is still controversial, it appears that both PtdIns (3,4)P_2 and PtdIns(3,4,5)P_3 stimulate PKB/Akt activity. Interaction between phosphoinositol lipids and PKB/Akt recruits Akt to the plasma membrane and causes conformational changes so that Thr^{308} and Ser^{473} become accessible to phosphorylation by phosphoinositide-dependent protein kinase 1 and 2 (PDK1, PDK2), respectively (Corvera and Czech, 1998). PDK1 can phosphorylate and activate PKCζ/λ in a similar manner. To date, two potential in vivo substrates of PKB/Akt1 have been identified, namely glycogen synthase kinase-3 (GSK3) and BH3 apoptotic domain (BAD) protein (Alessi and Cohen, 1998). Insulin stimulates glycogen and protein synthesis by Akt-mediated dephosphorylation (activation) of glycogen synthase and initiation factor eIF2B, respectively. Moreover, BAD phosphorylation by Akt1 protects cells from apoptosis (del Peso, et al., 1997). A phosphatase called protein phosphatase 2A (PP2A) acts in opposite direction of PDK and dephosphorylates Akt. Recently Yamada et al. have identified PP2A and not PP1 (protein phosphatase 1) as the phosphatase involved in dephosphorylation of Thr^{308} of Akt1 (Yamada, et al., 2001). Sato, et al. found that binding of heat shock protein 90 (Hsp90) to Akt protected Akt from protein phosphatase 2A (PP2A)-mediated dephosphorylation and concluded that Hsp90 interaction might play an important role in
regulating Akt kinase activity (Sato, et al., 2000). A phosphomonoesterase protein has been recently identified acting in opposite direction of PI 3-kinase signaling cascade. It appears that PTEN (phosphatase and tensin homologue deleted on chromosome ten) a gene located at 10q23, encoding a phosphomonoesterase, negatively controls the PI 3-kinase and Akt signaling pathways for regulation of cell growth and survival by dephosphorylating the 3 position of phosphoinositides (reviewed in Cantley and Neel, 1999). Torres and Pulido (Torres and Pulido, 2001) recently found that protein kinase CK2 to phosphorylate PTEN protein, and modulate both its stability and proteasome-mediated degradation. In general, activation of PKB/Akt by growth factors, such as insulin and growth factors appears to be necessary for various cellular processes including cell growth, differentiation, metabolism, and apoptosis (for a recent review refer to Chan, et al., 1999). Figure 1.3.1 demonstrates the two major insulin receptor signaling pathways, metabolic and mitogenic cascades. For more detail information, refer to the recently published review papers (Baumann and Saltiel, 2001, Le Roith and Zick, 2001, Taha and Klip, 1999).

It is now becoming clear that lipid microenvironment on the cell surface, known as lipid rafts, has an important role in signal transduction including insulin signaling cascade (reviewed in Brown and London, 2000, Dobrowsky, 2000). In insulin signal transduction, the IR-PI 3-kinase-Akt pathway appears to be required for most of the cellular actions of insulin. However, there is a second insulin signaling pathway, which is PI 3-kinase-independent. It involves tyrosine phosphorylation of the Cbl protooncogene, via its recruitment to the receptor by the adaptor protein CAP (c-Cbl-associating protein) (Ribon and Saltiel, 1997). CAP is expressed in insulin sensitive tissues and its level of expression correlates well with insulin responsiveness. Upon phosphorylation, Cbl/CAP complex

47
The binding of insulin to the α subunit of its receptor triggers autophosphorylation of the β subunit on three tyrosine residues. This results in maximal activation of the receptor tyrosine kinase and subsequent tyrosine phosphorylation of a number of intracellular substrates, including the insulin receptor substrates (IRS1-4), Shc and Gab 1. Phosphorylation of c-Cbl requires the adapter protein CAP for the recruitment to the insulin receptor. Phosphorylation of these proteins then facilitates the activation and localization of several downstream effector proteins leading to initiation of multiple signaling pathways that regulates diverse functions including metabolic activity, growth and differentiation. For more detail refer to the text.
translocates to a lipid raft subdomain of the plasma membrane (Mastick and Saltiel, 1997), where it interacts with the SH2 domain of the adaptor protein CrkII and eventually activates the G protein TC10. The activation of this pathway appears to provide a second signal to the Glut4 protein that functions in parallel with the activation of PI 3-kinase-dependent signaling pathway (Baumann, et al., 2000, Chiang, et al., 2001).

1.3.2 Molecular Mechanisms of Insulin Resistance

Insulin resistance is central to the pathophysiology of type 2 diabetes and a number of other related pathologic complications (for review refer to Cefalu, 2001, Saltiel, 2001). Previously, it was thought that insulin resistance simply occurs, because of a reduction in the number or activity of insulin receptors at the cell surface. However, recently published papers indicate that insulin resistance involves dysfunction not only at the cell surface but also intracellularly in the molecular machinery responsible for insulin signal transduction. Goodyear et al. reported that (Goodyear, et al., 1995) in the muscle of severely obese subjects with insulin resistance, PI 3-kinase activation by insulin was reduced. They also reported the reduction in the protein expression level of regulatory subunit of the enzyme in the above subjects. Bjornholm M, et al. (Bjornholm, et al., 1997) reported similar observations in patients with established type 2 diabetes. Both groups also observed reduction in phosphorylation status of insulin receptor and IRS-1 in their patients. More recently, Brown, Goldstein and their colleagues (Zhang, et al., 2001), identified a heptanucleotide sequence (TGTTITG) in the 5' flanking region of the human IRS-2 gene which is a perfect match for the insulin response element reported previously in insulin repressed genes in liver (Hall, et al., 2000). They showed that this sequence was required for insulin mediated repression of IRS-2 gene transcription in hepatocytes. They suggested
insulin-mediated repression of IRS-2 gene transcription as a potential mechanism of hepatic insulin resistance in hyperinsulinemic conditions.

Up-regulation of proteins that negatively modulate signal transduction can also impair signaling pathway. Protein tyrosine phosphatases (PTPs) dephosphorylate tyrosyl phosphoproteins, in a relatively substrate specific manner (specificity of PTPs reviewed in Tonks and Neel, 2001). Several PTPs such as SHP1, SHP2, PTPα, PTP 1B and LAR (Hashimoto, et al., 1992a, Kharitonenkov, et al., 1995, Lammers, et al., 1997, Uchida, et al., 1994) have been investigated for their possible role in attenuating insulin signal transduction and pathogenesis of insulin resistance. Ahmad and colleagues (Ahmad, et al., 1997) reported an elevation of activity and protein levels of protein tyrosine phosphatases in the skeletal muscle of insulin resistant obese and diabetic patients. Protein tyrosine phosphatase 1B (PTP-1B) has been particularly the subject of extensive investigations for its role in pathogenesis of diabetes and different types of cancer (Bjorge, et al., 2000, Warabi, et al., 2000). Recently, Elchebly et al. (Elchebly, et al., 1999) reported that knocking out the PTP-1B gene, increased insulin signal transduction and prevented both insulin resistance and obesity in high fat diet mice. Similar observations were made by Klaman et al. (Klaman, et al., 2000) using homozygotic PTP-1B null mice. In another approach, Egawa et al. (Egawa, et al., 2001) overexpressed PTP-1B in L6 myocytes and Fao cells (liver cell line) and observed that significant blockage of insulin stimulated tyrosine phosphorylation of the IR and IRS-1 resulted in reduced p85 and IRS-1 association, and decreased Akt and MAP kinase phosphorylation. Adenoviral-mediated PTP-1B overexpression in 3T3L1 adipocytes (Venable, et al., 2000) resulted in drastic reduction of IR and IRS-1 phosphorylation, as well as PI 3-kinase and MAP kinase activity, while Akt phosphorylation and activity were
unchanged (Venable, et al., 2000). PTP-1B has been a particularly important candidate for involvement in insulin signaling since it is an abundant intracellular PTPase that is widely expressed in insulin-sensitive tissues (Goldstein, 1993). Early studies of PTP-1B demonstrated its ability to dephosphorylate insulin receptor in vitro (Hashimoto, et al., 1992b, Tonks, et al., 1988). Moreover, microinjection of a truncated form of PTP-1B from human placenta into Xenopus oocytes diminished insulin stimulated oocyte maturation and S6 peptide phosphorylation (Cabibbo, et al., 2000, Cicirelli, et al., 1990). Ahmad et al. (Ahmad, et al., 1995) by using an osmotic loading technique and inhibiting PTP-1B activity with a neutralizing antibody showed that PTP-1B has an essential role in the negative regulation of insulin signaling. Seely et al. (Seely, et al., 1996) demonstrated in vivo association of PTP-1B with the activated insulin receptor and its tyrosine phosphorylation by the insulin receptor kinase. Recently, Cheung et al. (Cheung, et al., 1999) reported that in patients with obesity and type 2 diabetes, insulin resistance was characterized by the increased expression of a catalytically impaired PTP-1B in adipocyte tissue. Goldstein et al. (Goldstein, et al., 2000) studied the in vitro specific activity of four protein tyrosine phosphatases including PTP-1B, SHP-2, LAR, and LRP on IRS-1 dephosphorylation. In their study PTP-1B exhibited the highest specific activity and inclusion of GRB2 in a reaction mixture of IRS-1 and PTP-1B resulted in enhanced dephosphorylation of the substrate. Most recently, Mahadev et al. (Mahadev, et al., 2001) observed that upon the insulin stimulation, a redox signal enhances the early insulin-stimulated tyrosine phosphorylation of signaling proteins by oxidative reversible inactivation of PTP-1B. Furthermore, it has been also suggested that inhibitors of this enzyme may be beneficial in the treatment of type 2 diabetes (Kennedy, 1999, Malamas, et al., 2000, Wagman and Nuss, 2001, Yokomatsu, et al., 1999).
For a recent review on PTP-1B refer to Byon et al. (Byon, et al., 1998). More recently, Zabolotny, et al. reported that overexpression of leukocyte antigen related (LAR) PTP, in the muscle of transgenic mice also induced insulin resistance (Zabolotny, et al., 2001).

There are several recent reports on intracellular localization and compartmentalization pattern of proteins involved in insulin signaling in normal and insulin resistant conditions. Clark, et al. (Clark, et al., 2000) reported that in insulin resistance following okadaic acid or high insulin incubation of adipocytes, dislocation of IRS-1 and IRS-2 from cytoskeleton to the cytosol may be the underlying mechanism of insulin resistance. Calera, et al. (Calera, et al., 2000) studied dynamics of PTPases in rat adipocytes and found that PTP-1B and IRS-1 were present in light microsomes and cytosol, whereas SHPTP2/Syp was exclusively cytosolic. They also found that distribution of PTP-1B in the light microsomes from resting adipocytes was similar to that of IRS-1. Effects of compartmentalization pattern on insulin signal transduction has been comprehensively reviewed by Baumann and Saltiel (Baumann and Saltiel, 2001).

1.3.3 Pathologic Consequences of Insulin Resistance

It is clear that insulin resistance is not simply a problem of deficient glucose uptake in response to insulin, but a multifaceted syndrome. A large number of epidemiological and clinical studies have strongly established consistent correlation between certain anthropometric, metabolic, and hemodynamic variables. These variables include obesity, unfavorable body fat distribution, glucose intolerance or type 2 diabetes, hyperinsulinemia, hypertriglyceridemia, low levels of HDL cholesterol, and hypertension. It is also well established that patients exhibiting these features are at increased risk of atherosclerotic disease (Stern, 1997). Several studies have determined insulin resistance as a common
pathophysiological feature of the above cluster of abnormalities. Reaven (Reaven, 1988) was the first to use the term ‘syndrome X’ to define the cluster of resistance to insulin-stimulated glucose uptake, glucose intolerance, hyperinsulinemia, increased VLDL-TG, decreased HDL cholesterol, hypertension and hypercoagulation.

1.3.3.1 Metabolic Dyslipidemia in Insulin Resistant States

Insulin resistance and diabetic dyslipidemia is characterized by elevation of TG-rich lipoproteins and small dense LDL, and reduced plasma HDL levels. It appears that these anomalies are direct and/or indirect consequence of hyperinsulinemia and hyperglycemia associated with insulin resistance and diabetes. The most common lipid abnormality in patients with insulin resistance and type 2 diabetes is hypertriglyceridemia. Accumulation of TG-rich lipoproteins such as chylomicrons, VLDL, and IDL can cause hypertriglyceridemia. However, hepatic overproduction of VLDL and impaired clearance of VLDL particles due to reduced lipoprotein lipase (LPL) activity are considered the main contributors of hypertriglyceridemia (Garg, 1998). In diabetic patients, the composition of VLDL and IDL are also abnormal by having more TG and unesterified cholesterol. The hypertriglyceridemia associated with insulin resistance further deteriorates dyslipidemia by enhancing cholesteryl ester CETP-mediated exchange (reviewed in Bruce, et al., 1998) of TG from VLDL with HDL cholesteryl esters converting VLDL to atherogenic small cholesterol-rich dense LDL particles. The loss of cholesteryl ester content and TG enrichment are thought to enhance hepatic lipase and LPL-mediated TG hydrolysis. The TG-hydrolyzed HDL is also less protective against oxidative stress and atherosclerosis. Moreover, these small HDL particles shed their surface apoA-1, which is later filtered by the kidney (Horowitz, et al., 1993). Overall, multiple aspects of the commonly observed lipid profile in insulin resistance and
diabetes are atherogenic. Since overproduction of TG-rich-apoB containing lipoproteins is central to diabetic dyslipidemia, we will discuss it here in more details.

1.3.3.1.1 Insulin Resistance and Increased Free Fatty Acids Efflux

Adipocytes are well known for their essential role as energy storage depots for triglycerides, from which energy is called forth at times of need in the form of FFAs and glycerol. Adipose tissue is considered an endocrine organ secreting several peptide hormones and cytokines, including leptin, TNF-α, interleukin-6, plasminogen-activator inhibitor-1, angiotensinogen, etc. Most recently a 12.5 kDa, adipocyte secretory peptide named resistin was identified and shown to be involved in induction of insulin resistance and appears to link obesity to diabetes (Kim, et al., 2001b, Steppan, et al., 2001a, Steppan, et al., 2001b). Adipocyte secreted cytokine, TNF-α, plays a similar role in induction of insulin resistance. TNF-α signaling impairs insulin signal transduction, in part through inducing serine phosphorylation of IRS-1 (Hotamisligil, 1999, Peraldi and Spiegelman, 1998) and can reduce GLUT4 gene expression (for review refer to Kahn and Flier, 2000). Adipocytes can also produce active steroid hormones, including estrogen and cortisol.

It is commonly believed that insulin resistance insult first starts from adipose tissue. In fact, there are strong links between adipose function or mass and insulin resistance. Experimental and clinical evidence suggest that insulin resistance at the level of the fat cells leads to reduced FFA uptake, increased intracellular hydrolysis of TG, release of fatty acids into the circulation and peripheral tissues such as liver and muscle and exposing the target tissues with increased availability of energy. Based on available data (Gavrilova, et al., 2000, Spiegelman and Flier, 2001) both the absence and abundance of fat are associated with increased fatty acid flux to the liver and subsequent overproduction and secretion of VLDL.
Functional impairment of several genes and proteins have been suggested as candidate genes involved in overflow of FFA in insulin resistance, including hormone-sensitive lipase (HSL) (Klannemark, et al., 1998), lipoprotein lipase (LPL) (Babirak, et al., 1992), complement component C3a (Murray, et al., 2000), and various fatty acid binding proteins and transporters (e.g. CD36). Recent studies have shown that significantly elevated FFA levels inhibit glucose uptake by muscle and also impair critical steps in insulin signaling (Dresner, et al., 1999). FFA taken up by muscle and liver are either oxidized or esterified to form TGs.

In hepatocytes, de novo TG pools are stored and some utilized in assembly and secretion of VLDL particles (Dixon and Ginsberg, 1993, Wiggins and Gibbons, 1992). Evidence suggests that high FFA flux to the liver in the insulin resistant state, contributes to overproduction of VLDL by providing core lipid substrates for lipoprotein assembly and/or facilitating translocation of newly synthesized apoB as well as reducing co-translational apoB degradation (Macri and Adeli, 1997b, reviewed in Davis, 1999). Indeed, the response of the liver to the stress of increased FFA flux and lipid synthesis is immediate incorporation of the lipid load into VLDL and its secretion to the circulation (Lewis, 1997). Lewis et al. (Lewis, et al., 1995) have also reported stimulatory effects of elevated plasma FFA on VLDL production in healthy volunteers. In a recent report, Carpentier et al. (Carpentier, et al., 2001) studied the effect of systemic versus portal insulin delivery in pancreas transplant patients on insulin action and VLDL metabolism. They found that chronic hyperinsulinemia and peripheral tissue insulin resistance with the consequent elevation of plasma FFA flux are insufficient per se to cause VLDL overproduction. They concluded that additional factors, such as hepatic insulin resistance, may be an essential prerequisite in the pathogenesis of VLDL overproduction in the insulin resistance syndrome.
1.3.3.1.2 Insulin Resistance and ApoB100 Biogenesis

Insulin has two profound effects on synthesis and secretion of apoB. Acute and short-term incubation of rat hepatocytes (Bjornsson, et al., 1992, Durrington, et al., 1982, Patsch, et al., 1983a, Patsch, et al., 1986, Sparks, et al., 1986, Sparks and Sparks, 1990, Sparks, et al., 1989), isolated human hepatocytes (Salhanick, et al., 1991), and HepG2 cells (Pullinger, et al., 1989) with insulin suppressed apoB synthesis and secretion. Sparks et al. (Sparks, et al., 1994) also found more sensitivity to the inhibitory action of insulin on apoB secretion in hepatocytes derived from partially hepatectomized rats compared to that of normal rats. The inhibitory effect of insulin was attributed to the increased degradation of apoB in cells incubated with the hormone (Jackson, et al., 1990b, Sparks and Sparks, 1990). Patsch et al. (Patsch, et al., 1986) showed that insulin exerted this effect through its receptor. More recently, studies in glucose injected rats confirmed the suppressive effects of insulin on VLDL-TG and apoB secretion (Chirieac, et al., 2000). Studies in our laboratory, using cell-free systems, have shown that insulin attenuates the rate of apoB mRNA translation (Adeli and Theriault, 1992, Theriault, et al., 1992a). It has also been suggested that apoB availability may become a limiting factor in VLDL assembly and secretion in insulin-treated hepatocytes (Wiggins and Gibbons, 1992). Sparks et al (Sparks, et al., 1996), demonstrated that in rat hepatocytes insulin-mediated inhibition of apoB was a PI 3-kinase-dependant process. Phung et al. (Phung, et al., 1997) reported that in rat hepatocytes PI 3-kinase activity was necessary for insulin-dependant inhibition of apoB secretion and insulin induced activation and localization of PI 3-kinase and IRS-1 in an ER fraction containing apoB. Insulin can also affect the phosphorylation of apoB (Jackson, et al., 1990b). Insulin may exert its suppressive effect on VLDL secretion through regulating microsomal triglyceride transfer protein (MTP).
The promoter region of the MTP gene has insulin response elements, which is negatively regulated by the hormone (Lin, et al., 1995a, Sato, et al., 1999).

On the contrary, long-term chronic exposure of primary rat hepatocytes (Bjornsson, et al., 1992), and HepG2 cells (Dashii, et al., 1989) increased secretion of apoB. Inui et al. reported that in obese rats hepatic fatty acid synthesis and apoB transcription increased compared to lean rats (Inui, et al., 1989). The same group later (Inui, et al., 1997) showed that, in obese rats fed with a high sucrose diet, apoB mRNA level remained constant while hepatic fatty acid synthesis and apoA-IV gene expression were elevated. Bourgeois et al. (Bourgeois, et al., 1995) and Sparks and Sparks (Sparks and Sparks, 1994b) reported that hepatocytes prepared from obese Zucker rats were resistant to the inhibitory effects of insulin. Sparks et al. (Sparks, et al., 1989) also demonstrated that in cultured hepatocytes from diabetic rats insulin failed to inhibit apoB secretion. In rat hepatocytes which synthesize both apoB48 and apoB100, insulin exposure increases expression of apobec-1, the catalytic subunit of the apoB mRNA editing complex in favor of more apoB48 synthesis over apoB100 (Thorngate, et al., 1994, von Wronski, et al., 1998). Yamane et al. (Yamane, et al., 1995) reported that in non-insulin-dependent diabetic rats, the apoB48/apoB100 ratio in plasma was increased because of enhanced apoB mRNA editing in the liver. Although some progress have been made in elucidating the underlying mechanisms of apoB overproduction in insulin resistance, the molecular mechanisms connecting insulin signaling pathway to the apoB overproduction is still unclear.
PART IV: Animal Models for Insulin Resistance Related Metabolic Dyslipidemia

Insulin resistance status such as obesity and type II diabetes are serious and growing health concerns (discussed in detail in Chapter 1, Part III). Elucidation of the origin of the defects leading to insulin resistance and consequent metabolic disorders such as dyslipidemia can only be realized by experimentation with appropriate animal models. The major reason for using animal models in biomedical sciences is that experiments can be conducted that are not practically and ethically possible in humans, particularly those involving tissue sampling for evaluating specific morphological, biochemical and metabolic parameters. Programmed breeding of animals with genetic homogeneity, availability of genetically modified animal models especially rodents, possibility of testing different environmental factors such as diet and finally the similarity between animal models and human genes are considered the other advantages of using animal models.

Since dietary and environmental triggers and genetic susceptibility determinants are important etiological factors for human insulin resistance and type II diabetes, the efforts have been focused to manipulate these factors in order to present suitable animal models. Based on these determinants we can categorize animal models of insulin resistance into three categories of genetically modified, diet induced, and surgically induced animal models. Since our current research has focused on diet-induced and specifically carbohydrate-induced insulin resistance, we will discuss these animal models in more detail. There are also other models of insulin resistance based on genetic or surgical manipulations, however they lie outside the scope of our research and will not be discussed here. For more information refer to these reviews (Kadowaki, 2000, McIntosh, 1999, Patti, 1999, Shafrir and Ziv, 1998, Shafrir, et al., 1999).
1.4.1 Nutrient or Diet-induced Animal Models of Insulin Resistance and Diabetes

Growing data support a prominent role for environmental factors such as diet and inactivity, in the pathogenesis and maintenance of insulin resistance. Epidemiological studies have also confirmed that alterations in diet and physical activities of healthy individuals may lead to the development of obesity, insulin resistance, and diabetes (for recent review on epidemiological factors refer to Zimmet, 1999). It appears that nutrient excess and long-term positive energy balance may play a role in the pathogenesis of insulin resistance in genetically predisposed individuals. At the cellular and tissue levels, availability of substrates for cellular energy production may play an important role in metabolic regulation and, in particular, in determining the response to insulin stimulation. Lipids and fatty acids, amino acids, and carbohydrates are the three types of nutrient substrates capable of inducing insulin resistance. Here we will only discuss carbohydrate-induced animal models of insulin resistance. For more information on other nutritionally-induced animal models of insulin resistance refer to these reviews (Patti, 1999, Patti, et al., 1998, Shafrir and Ziv, 1998, Shafrir, et al., 1999).

1.4.1.1 Carbohydrate-induced Animal Models of Insulin Resistance

Carbohydrate and glucose-toxicity have been always synonymous terms with insulin resistance and diabetes suggesting a crucial key role for carbohydrates in pathogenesis of insulin resistance (reviewed in Wolever, 2000). In mammals, excess carbohydrate triggers lipogenesis, which induces the conversion of simple carbohydrates into TG and subsequent lipotoxicity. Lipogenesis occurs predominantly in the liver and adipose tissue and its activation by carbohydrate diet is accompanied by the induction of key metabolic enzymes such as enzymes involved in glycolysis, fatty acid synthesis and fatty acid maturation and
packaging. In fact carbohydrates especially glucose exert their effects on lipogenic genes through a newly identified carbohydrate response element (ChoRE) which is distinct from insulin mediated transcription factor (SREBP) gene regulation (Koo, et al., 2001, Koo and Towle, 2000). Studies conducted in patients with diabetes and rat models have established that chronic hyperglycemia is an independent cause of insulin resistance (Rossetti, et al., 1987). A recent report by Kawanaka et al. (Kawanaka, et al., 2001) indicates that in glucose induced insulin resistance (in muscle cells), neither glycogen accumulation nor impaired insulin signaling is responsible, instead synthesis of a short-lived unknown protein mediates insulin resistance. In a recently published report, McLaughlin et al. (McLaughlin, et al., 2000) showed that feeding healthy volunteers with 60% (total calories) carbohydrates resulted in significantly higher plasma TG and insulin and lower FFA concentrations. At the cellular levels, according to Marshall et al. (Marshall, et al., 1991), metabolism of glucose by the hexosamine pathway generates a signal of “cellular satiety” (reviewed in Rossetti, 2000). In insulin target tissues, this signal leads to desensitization of the glucose transport system to subsequent stimulation by insulin. In fact, prolonged incubation of cultured adipocytes or hepatocytes in the presence of high glucose and insulin resulted in a marked impairment of insulin signal transduction (Marshall, et al., 1991, Sparks and Sparks, 1994a, Traxinger and Marshall, 1992). Indeed, overactivity of the hexosamine pathway has been suggested to be one of the primary mechanisms mediating glucose-induced insulin resistance or “glucose toxicity” (McClain and Crook, 1996).

Fructose alone or as a constituent of the sucrose molecule, has profound metabolic effects. Dietary fructose alters the activity of several important enzymes and regulates hepatic carbohydrate metabolism, leading to hepatic insulin resistance (Blakely, et al., 1981,
Tuovinen and Bender, 1975). Furthermore, fructose promotes metabolic changes that are actually or potentially deleterious, e.g., hyperlipidemia, hyperuricemia, nonenzymatic fructosylation of proteins, lactacidemia, and disturbances in copper metabolism. In fact most of the metabolic effects of fructose are due to its rapid utilization by the liver and its bypassing the phosphofructokinase regulatory step in glycolysis, leading to far reaching consequences to carbohydrate and lipid metabolism. Fructose causes a shift in balance from oxidation to esterification of non-esterified fatty acids resulting in increased secretion of VLDL (Mayes, 1993, Topping and Mayes, 1972, Topping and Mayes, 1976). Acute loading of the liver with fructose causes sequestration of inorganic phosphate in fructose-1-phosphate and diminished ATP synthesis (Reviewed in Mayes, 1993).

Rats fed with a high fructose diet provide an animal model of insulin resistance associated with hyperinsulinemia, hypertriglyceridemia (Sleder, et al., 1980, Tobey, et al., 1982, Zavaroni, et al., 1982, Zavaroni, et al., 1980) and hypertension (Hwang, et al., 1987, Thorburn, et al., 1989). Rats fed with high sucrose diets also exhibited insulin resistance, hypertriglyceridemia, liver TG accumulation, and decreased glucose tolerance, (Kanarek and Orthen-Gambill, 1982, Yamamoto, et al., 1987). Bird and Williams (Bird and Williams, 1982) reported 50% higher TG secretion rates in rats fed with high fructose diet compared with animals fed with high glucose or sucrose. In another study, Blakely et al. (Blakely, et al., 1987) reported that fasting plasma insulin levels were higher in fructose- than in glucose-fed rats. Interestingly, Boivin and Deshaies (Boivin and Deshaies, 1995) reported that in rats fed with a high carbohydrate diet fasting and postprandial hypertriglyceridemia was more prominent than rats fed with a high fat diet, whereas insulin sensitivity was more compromised in the latter group. Recently, Bezerra et al. (Bezerra, et al., 2000) reported a
significantly reduced IR, and IRS-1 phosphorylation as well as decreased PI3-kinase activity in the muscle and livers of rats fed with high fructose-diet.

1.4.2 Syrian Golden Hamster as an Animal Model of Insulin Resistance

The Syrian golden hamster has been used with increasing frequency in recent years to study hepatic lipid metabolism (Hoang, et al., 1993, Hoang, et al., 1995, Hoang, et al., 1992, Jackson, et al., 1990a, Nistor, et al., 1987, Ontko, et al., 1990, Sullivan, et al., 1993). The hamster has attracted increasing attention as a model for lipoprotein research since its lipoprotein metabolism appears to more closely resemble that in humans (Gouliinet and Chapman, 1993, Hoang, et al., 1995, Liu, et al., 1991). In contrast to other rodent models and hepatic cell lines, hamster liver produces apoB-containing lipoproteins with a density close to that of human VLDL (Liu, et al., 1991), which functions as the main plasma cholesterol carrier in this species (Spady and Dietschy, 1983, Spady, et al., 1986). Moreover, hamsters develop hyperlipidemia and atherosclerosis in response to a modest increase in dietary cholesterol and saturated fat (Arbeeny, et al., 1992, Liu, et al., 1991) and can be made obese, hypertriglyceridemic, and insulin-resistant by fructose feeding (Kasim-Karakas, et al., 1996).

*Psammomys obesus*, a desert gerbil living in the sandy arid zone of North Africa (nicknamed sand rat) has been introduced as a diet induced animal model of insulin resistance and diabetes (Hackel, et al., 1965, Shafir and Ziv, 1998, Shafir, et al., 1999). This rodent, in its natural habitat may have only seasonal obesity but no hyperglycemia or hyperinsulinemia. It acquires a diabetic syndrome mimicking human type 2 diabetes only when exposed to a high-energy diet composed of mainly carbohydrates (68% compared to 53.3% carbohydrates in its natural diet). Serum, liver and muscle TG contents of animals under diet progressively increases depending on the duration of exposure to the high
carbohydrate diet. Recently, Bennani-Kabchi et al. reported induction of advanced atherosclerotic lesions and plaques in the same animal model fed with high cholesterol diet and vitamin D2 (Bennani-Kabchi, et al., 2000). Other animals such as dog (Martinez, et al., 1994), and rhesus monkey (de Koning, et al., 1993, Hotta, et al., 1999, Ortmeyer, et al., 1999) have also been used to study effects of carbohydrate induced insulin resistance, diabetes, obesity and hypertension (Syndrome X).

1.4.3 Research Rationale

Insulin resistance is usually defined as reduced insulin mediated whole-body glucose uptake as measured by the glucose clamp technique (DeFronzo, et al., 1979). Insulin resistance is an extremely common pathophysiological trait that is implicated in the development of a number of important human diseases including type 2 diabetes, obesity, atherosclerosis, hypertension, and dyslipidemia (reviewed in Cefalu, 2001, Donnelly and Davis, 2000, Fujimoto, 2000, Ginsberg, 2000, Groop, 1999, Kahn and Flier, 2000, Reaven, 1995, Rett, 1999). Cardiovascular disease (CVD), particularly coronary heart disease (CHD), is a major complication of insulin resistance and type 2 diabetes and in fact over 50% of all these patients die of CHD (Ginsberg, 2000, Matsumoto, et al., 1999, Ounpuu, et al., 2001). Growing evidence supports the notion that insulin resistance-associated dyslipidemia plays a major role in development of CVD in these patients (reviewed in Balkau and Eschwege, 1999, Cullen, et al., 1999, Hayden and Reaven, 2000). The atherogenic dyslipidemia commonly associated with insulin resistant states is characterized by hypertriglyceridemia, increased very low density lipoprotein (VLDL) output by the liver, low HDL cholesterol (Taskinen, 1995), and small, dense LDL (Reaven, et al., 1993). It has been suggested that the most fundamental defect in these patients is resistance to cellular actions of insulin,
particularly resistance to insulin-stimulated glucose uptake leading to hyperinsulinemia, enhanced VLDL secretion and hypertriglyceridemia (Cefalu, 2001, Lewis and Steiner, 1996, Lewis, et al., 1993, Timar, et al., 2000). The increase in VLDL-triglyceride production in insulin resistance appears to result from decreased sensitivity to the inhibitory effects of insulin on VLDL secretion (Betteridge, 2000, Howard, 1987). Resistance to suppressive actions of insulin would thus have important consequences in dysregulation of VLDL production. Surprisingly, A significant gap of knowledge exists currently on the mechanisms that link insulin resistance to hepatic VLDL overproduction. Thus, it appears that significant new efforts are needed to elucidate molecular and cellular mechanisms underlying VLDL-apoB overproduction in insulin resistance states.

1.4.4 Overall Hypothesis

Impairment of hepatic insulin signaling directly contributes to the overproduction of apoB-containing lipoproteins in insulin resistant states.

1.4.5 General Research Objectives

Objective 1: Select a suitable animal model and characterize its apoB metabolism.

Objective 2: Develop insulin resistance in this model by fructose feeding.

Objective 3: Investigate cellular mechanisms underlying apoB overproduction in the fructose-fed hamster.

Objective 4: Investigate hepatic insulin signaling in the fructose-fed hamster model.

Objective 5: Explore potential links between impairment of insulin signaling and hepatic VLDL-apoB overproduction.
CHAPTER TWO

Materials and Methods

2.1 Chemicals and Reagents

Trypsin (tissue culture grade), soybean trypsin inhibitor (tissue culture grade), oleic acid, leupeptin, phenylmethylsulfonylfluoride (PMSF), piperazine-N,N'-bis [2-ethanesulfonic acid] (PIPS), sodium carbonate (monohydrate), glycerol, ammonium sulfate, 4-morpholinepropanesulfonic acid (MOPS), imidazole, cyclohexamide, puromycin, t-octylphenoxyethoxyethanol (Triton X-100), bovine serum albumin (BSA) (Fraction V), fatty acid free BSA (Fraction V), α1-antitrypsin antiserum, albumin antiserum, transferrin antiserum, digitonin (80% pure), brefeldin A, sodium fluoride, ethylenediaminetetraacetic acid (EDTA) (sodium-free and disodium salt), ethylene glycol-bis (β-aminoethyl Ether) N,N,N',N'-tetraacetic acid (EGTA), fructose, glucose, ultra pure sucrose and bromophenol blue (sodium salt), o-phenanthroline, (2S,3S)-trans-epoxysucciny1-L-leucylamido-3-methylbutane ethyl ester (EST), sodium orthovanadate, sodium pyrophosphate, were purchased from Sigma Chemical Company (St. Louis, MO). N-acetyl-L-leucyl-L-leucyl-L-norleucinal (calpain inhibitor I) (ALLN) was obtained from Calbiochem (Lajolla, CA). N-carbobenzoxy-L-leucinyl-L-leucinyl-L-norleucinal (MG132) was from Biomol (Plymouth, PA). Rosiglitazone (5-(4-[2-methyl-N-(2-pyridyl)amino]ethoxy] benzyl)-thiazolidone-2,4-dione) was kindly provided by Dr. Robin Buckingham (GlaxoSmithKline Beecham). Lactacystin was purchased from Dr. E. J. Corey, Harvard University (Boston, MA).

All ultra pure electrophoresis reagents, including 40% acrylamide/bis acrylamide (37.5:1 solution (2.67%C)), tris(hydroxymethyl)-aminomethane (TRIS), glycine, sodium
dodecyl sulfate (SDS), ammonium persulfate (APS), N,N,N,N′-methylenbis-acrylamide (TEMED), were from Bio-Rad Laboratories (Richmond, CA). Tranxylol (aprotinin) was purchased from Bayer (Leverkusen, Germany).

Methanol, ethanol (anhydrous), isopropanol, acetic acid (glacial), hydrochloric acid, perchloric acid, dimethyl sulfoxide (DMSO), hydrogen peroxide, magnesium chloride, potassium chloride, sodium chloride, sodium phosphate (dibasic), potassium phosphate (monobasic), were obtained from British Drug House (BDH, Toronto). pp605-src C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL) and Biomol Green reagent were purchased from Biomol (Plymouth Meeting, PA). The enhanced chemiluminescence detection reagents (ECL™) and fluorographic enhancer Amplify™ were from Amersham Life Sciences (Buckinghamshire, UK). Prestained Rainbow™ protein molecular weight marker was also purchased from Amersham Life Sciences (Buckinghamshire, UK). [35S]protein labelin mixture (Easy Tag EXPRE35S35STM, specific activity of > 1000 Ci/mmol) was from Mandel Scientific (Guelph, ON). Ready Safe™ liquid scintillation cocktail was obtained from Beckman Coulter Inc. (Fullerton, CA). The MTP activity assay kit was obtained from Roar Biomedical Inc. (New York, NY).

L-methionine, L-leucine, insulin, Eagle’s minimum essential medium (alpha) without L-methionine, and L-leucine, fetal bovine serum (FBS) (certified grade), trypsin-EDTA (1 X liquid), penicillin G/streptomycin (100 X) solution, liver perfusion, wash, digest, attachment, and William’s E media were obtained from Life Technologies (Grand Island, NY).

Rabbit anti-human insulin receptor β subunit, rabbit anti-human IRS-1, rabbit anti-p85 subunit of PI 3-kinase, and mouse anti-phosphotyrosine monoclonal antibodies were purchased from Santa Cruz (Santa Cruz, CA). Goat anti-bovine MTP polyclonal antibody
was obtained as a gift from Dr. David Gordon (Bristol-Meyers-Squibb). Anti-IRS-2 rabbit polyclonal IgG was obtained from Upstate Biotechnology (Lake Placid, NY). Mouse anti-PTP-1B monoclonal antibody (Ab-1) was obtained from Oncogene Research Products (Boston, MA). Rabbit anti-total Akt, anti-phospho-Akt (Ser473), and anti-phospho-Akt (Thr308) polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-hamster apoB antiserum was prepared by Lampire Biological Laboratories (Pipersville, USA) using hamster LDL prepared in our laboratory. Specificity of this commercial preparation of anti-apoB polyclonal antibody and lack of any cross-reactivity to other hamster apolipoproteins (apoAI or apoE) was confirmed by immunoblotting analysis of purified plasma lipoprotein fractions. Rabbit anti-rat transferrin antibody was obtained from ICN Pharmaceuticals, Inc. (Aurora, Ohio). Rabbit anti-human ubiquitin antibody was obtained from Dako Diagnostic Inc. (Mississauga, ON). Rabbit anti-rat ER60 was a gift from Dr. Makito Kito (Kyoto University, Japan).

Drugs used for animal surgery were isoflurane from Technilab Inc. (Toronto, ON), ketamine hydrochloride from Vetrepharm Canada (London, ON), Xylazine from Bayer (Etobicoke, ON), Lidocaine from Langford (Guelph, ON), and Acepromazine from Ayerst Laboratories (Montreal, PQ). Normal rodent chow diet and fructose-enriched diet (hamster diet with 60% fructose, pelleted) was obtained from Purina Mills, Inc. (St. Louis, MO) and Dyets Inc. (Bethlehem, PA) respectively.

2.2 Laboratory Supplies

Cell culture dishes (35 mm, 60 mm, and 100 mm) were from either Sarstedt (Newton, NC), and/or Nunc (Kamstrupues, Denmark). Disposable, sterile, polystyrene conical centrifuge tubes (15 ml and 50 ml) were obtained from either Sarstedt or Falcon (Franklin
Lakes, NJ). Disposable sterile serological pipettes (5 ml, 10 ml) were from VWR Scientific (Toronto, ON). Cell scrapers and regular disposable syringes were from Becton Dickinson & Co (Franklin Lakes, NJ). Disposable syringe filters (0.25 μm and 0.45 μm) were obtained from Sarstedt (Newton, NC). Precision 100 μL glass syringe pipettor was from Hamilton Syringe (Reno, NV). Polypropylene microcentrifuge tubes (1.5 ml, 2.0 ml) as well as micropipette tips were from Sarstedt (Newton, NC). Dialysis tubing (molecular weight cut-off 6000-8000) was purchased from Spectrum Medical Instruments Inc (Houston, TX). All surgical disposable materials were obtained from Ethicon (Somerville, NJ) or Johnson & Johnson Medical Inc. (Arlington, TX).

Scientific imaging films were obtained from Eastman Kodak Company (Rochester, NY). All laboratory glassware were from Corning (Corning, NY). Plastic back prepared TLC plates (Silica gel 60 F254) were obtained from EM Science (Gibbstown, NJ). Parafilm laboratory film was from American National Co. (Greenwich, CT). Phosphoimager screen with cassette was purchased from Molecular Dynamics (Sunnyvale, CA). Polyscreen™ PVDF transfer membrane and Hybond™ECL™ nitrocellulose membrane were obtained from NEN™ Life Science Products Inc. (Boston, MA) and Amersham Pharmacia Biotech (Buckinghamshire, UK) respectively.

2.3 Apparata

Micropipettes were from Eppendorf (0.5-10 μL, 2-20 μL, 10-100 μL, 200-1000 μL; Germany) and Gilson (1-20 μL, 10-100 μL; Mandel Scientific, Ville St. Pierre, PQ). Repetitive pipettors were from Eppendorf (Germany), and pipettes aids were either purchased from Drummond Scientific Co. (Broomall, Pa), or Falcon (Franklin Lakes, NJ). All pH measurements were performed using a Corning pH meter (model 240) (Corning,
Before any pH measurement, the electrode was calibrated by pH standard solutions (pH 4, pH 7, pH 10) from BDH (Toronto, ON). A Sartorius CT series electronic balance was used for mass measurements.

All procedures involving cell culture including; preparation of culture media, perfusion solution, cell wash media, and experiments with cultured primary hepatocytes, were conducted under a Nuaire class II, type A/B3 flow-hood cabinet (Plymouth, MN). Long-term incubation of the cells was achieved using a Nuaire Auto flow CO2 incubator (Plymouth, MN). Viability and counting of the cells was checked using a Leica inverted phase-contrast microscope (Leica Microscope Systems, Wetzlar, Germany). Temperature of media and solutions was set by using a Haake type 619 water bath (Haake Circulators, Dieselstrasse, Germany). A small animal anesthetic machine from Med-Vet Anaesthetic System Inc. (Toronto, ON) was used to achieve complete hamster anesthesia. A peristaltic pump (Pharmacia Biotech, Sunyvale, CA) was used to circulate different solutions through the liver during perfusion.

Centrifugation was performed using bench top regular Biofuge-pico™, Heraeus Instruments (USA), or refrigerated Beckman GS-15R centrifuge (Palo Alto, CA). Ultracentrifugation was conducted using either the Sorvall Ultra Pro™ 80 ultracentrifuge in conjunction with the Sorvall TH-641 rotor (Dupont Co, Mississauga, ON) or Beckman Optima LE-80K with SW41Ti, and SW55Ti rotors (Palo Alto, CA). Ultracentrifugation tubes in different sizes were from Beckman (Palo Alto, CA). Mixers, orbital shakers, and rotators were from either VWR Canlab (Mississauga, ON) or Fisher Scientific (Nepean, ON). The glass dounce homogenizer was from Kontes Glass Co. (Vineland, NJ). A Versamax™ tunable microplate reader from Molecular Device Corporation (Sunnyvale,
CA) was used to conduct protein assay. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using either the SE400 Sturdier Vertical Slab Unit from Hoefer Scientific Instruments (San Francisco, CA) or the Protean II xi from Bio-Rad Laboratories (Richmond, CA). The mini SDS-PAGE apparatus was purchased from Bio-Rad Laboratories (Richmond, CA). The gradient gel maker was obtained from Hoefer Scientific Instruments (San Francisco, CA). Transferring step of the western blotting was performed using a Bio-Rad Wet Transfer System (Richmond, CA). The power supply units were either Power Pac 300 from Bio-Rad Laboratories (Richmond, CA), or EC-103, and EC-420 models from Mandel Scientific Co. Ltd. (Ville St. Pierre, PQ). SDS-PAGE gels were dried using either SE540 Slab Gel Dryer, Hoefer Scientific Instruments (San Francisco, CA), or the gel Air Drying System from Bio-Rad Laboratories (Richmond, CA), or Speed Gel™ (Holbrook, NY). Quantification of the radiolabeled band was achieved using either LS 7500 Liquid Scintillation System (Beckman Instruments Inc, Palo Alto, CA) or LKB-Wallace-PerkinElmer™ 1219 Rackbeta liquid scintillation counter (St-Laurent, PQ). In some cases band quantitation was achieved by densitometric scanning of the gels using either the Imaging Densitometer Model GS-670 from Bio-Rad Laboratories (Richmond, CA), or Kodak ds 1D scientific imaging system (New Haven, CT). Phospho-Imaging screens as well as radiolabeled TLC plates were quantified by a Storm 840 phosphorimager™, Molecular Dynamics (Sunnyvale, CA). Quantified bands were statistically analyzed using either Microcal Origins program version 3.5 (Northampton, MA) or Microsoft Excel version 97.
2.4 Procedures

2.4.1 Animal Protocols

Male Syrian golden hamsters (*Mesocricetus auratus*) were obtained from Charles River Canada (Montreal, PQ). All animals were housed in pairs and were given free access to food and water. Normal chow was given for two days to allow acclimatization to the new environment and recovery from the stress of shipping. After blood collection, animals were placed on either the control diet (normal chow) or fructose-enriched diet (hamster diet with 60% fructose, pelleted, Dyets Inc., Bethlehem, PA). The diet was continued for 2-3 weeks and hamster weight was monitored every two days. Plasma glucose, TG, and cholesterol levels were determined on an automated clinical chemistry analyzer (Hitachi 705 or Vitro 950). Plasma insulin levels were determined by radioimmunoassay using a rat insulin kit from Linco Research (St. Louis, MO). This assay has 100% cross reactivity to hamster insulin and the intra- and inter-assay coefficient of variation were 6.8% and 10.6%, respectively.

2.4.2 Preparation of Collagen-Coated Culture Dishes

Type I calf skin collagen was dissolved in 0.05 M hydrochloric acid solvent to make a 100 μg/ml solution. Prepared collagen solution was then sterilized by a syringe-filter system (0.45 μm). For 35 mm, 60 mm, and 100 mm dishes, 1, 3, and 5 ml of filtered collagen solution was added respectively and the dishes left under the tissue culture hood to dry. The dried dishes were then washed twice with sterile PBS, dried and stored at 4°C.

2.4.3 Liver Perfusion and Isolation of Primary Hamster Hepatocytes

At the end of the 3-week feeding period, hamsters were fasted overnight and blood samples were collected for measurement of a number of analytes in plasma. Hamsters were
then fed for another day and were anesthetized by isoflurane or by intramuscular injection of acepromazine (1 mg/kg) and intraperitoneal injection of a mixture of ketamine (200 mg/kg) and xylazine (10 mg/kg). Liver was perfused as described (Miller, 1973) with small modifications which included using commercial liver perfusion and liver digest media to achieve partial liver tissue digestion. Briefly, the animal was placed on a heating pad to reduce loss of body heat and an incision was made along the abdomen exposing the liver and intestines. A 23-gauge butterfly needle was inserted into the abdominal vena cava and sutured in place such that flow to the kidneys was arrested. The liver was isolated from the circulatory system by two further sutures, one blocking the thoracic aorta and caudal vena cava and one below the insertion point of the needle blocking the abdominal aorta and abdominal vena cava. Once the sutures had been tied, the portal vena was cut and perfusion by a peristaltic pump (Pharmacia Biotech) began at a rate of 3.6 ml/min. Solutions used in perfusion were heated to 39 °C and filtered through a 0.22 μm syringe filter. They were also aerated with 100% oxygen and supplemented with penicillin G and streptomycin as well as 10 mM HEPES, pH 7.2, to prevent pH drift. Initial perfusion was with Liver Perfusion Medium (Life Technologies) until no blood was observed exiting the portal vena (~20-30 ml). The liver was then partially digested in situ with Liver Digest Medium (Life Technologies) delivered at 2.6 ml/min. Perfusion was stopped when liver integrity began to deteriorate as evidenced by the appearance of cracks when the tissue was gently probed (~10-15 ml of digest medium). At this point the liver was dissected out of the animal, minced with scissors, and filtered through sterile gauze using Hepatocyte Wash Medium (Life Technologies). Hepatocytes were separated from contaminating cell types by sequential centrifugation in a 50 ml conical centrifuge tube at 720, 300, 115, and 30x g. After each spin,
pelleted cells were resuspended in 25 ml of fresh wash medium. After the final spin hepatocytes were resuspended in 25 ml of Cell Attachment Medium (Life Technologies) supplemented with 5% fetal bovine serum and 10 μg/ml insulin. Cells were placed in an incubator (37 °C, 5% CO2, 95% air, 100% humidity) for 30 min. Cell viability was measured by exclusion of 0.2% trypan blue and 35 mm dishes, previously coated with collagen Type I (100 μg/dish, ICN), were seeded with 1.5 million viable cells. Cell viability varied between 60 and 75%. Dishes were incubated for 4 h to allow attachment of viable cells. Non viable cells were removed by 2 x 1 ml washes with Attachment Medium + 5% fetal bovine serum + 0.0015 μg/ml insulin. Primary hamster hepatocytes were used in experiments either after 4 h or overnight incubation and additional washing.

The viability and tissue specificity of primary hepatocytes were examined by trypan blue exclusion assay, protein synthesis rate, SDS-PAGE analysis of cell extracts and the synthesis and secretion of hepatic specific proteins, albumin and apoB. We consistently obtained a 60-75% cell viability (67.5±6.5%, in four representative animals) based on trypan blue exclusion assay. The average yield of cellular protein recovered was also reproducible in various primary cell cultures (0.44±0.08 mg protein per 10^6 cells, n=8). SDS-PAGE analysis of cell extracts and media from cultured hepatocytes indicated a consistent profile of hepatic proteins over a 3 day period (data not shown). We also examined the ability of cultured hepatocytes to synthesize total as well as liver specific proteins after radiolabeling of primary hepatocytes isolated from various animals with [35S]methionine. Primary hepatocytes were consistently capable of incorporating [35S]methionine into TCA precipitable protein indicating a high degree of viability. Furthermore, all cultures were found to be capable of synthesizing and secreting liver specific proteins, apoB and albumin. The rates of albumin
synthesis and secretion in 3 different cultures of primary hepatocytes were 18137±873 CPM/10^6 cells/h and 34151±1753 CPM/10^6 cells/h, respectively. Primary hamster hepatocytes were found to maintain their synthesis and secretion of albumin for a period of at least three days. Densitometric analysis of the SDS-PAGE profile of media from cultured hamster hepatocytes indicated a consistent rate of albumin secretion (Day 1, 123991±9780, day 2, 114470±4197, and day 3, 102452±4327 scan units/dish).

2.4.4 Metabolic Labeling of Intact Primary Hamster Hepatocytes

Primary hamster hepatocytes were washed twice and then preincubated in methionine-free minimum essential medium (MEM) at 37°C for 1 h and pulsed with 75-100 μCi/ml of [35S]methionine for 45 min. Following the pulse, the cells were washed twice and chased in either Williams’ E media or hepatocyte attachment media supplemented with 10 mM non-radiolabeled (cold) methionine. At various chase times duplicate or triplicate dishes were washed once with PBS (8 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L Na₂HPO₄ and 0.24 g/L KH₂PO₄, pH 7.4) and then harvested, and cells were lysed in solubilization buffer (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM leupeptin, 100 KIU/ml Trasylol, 0.5 μM ALLN) and homogenized using a 3 ml syringe containing a 21G1.5 gauge needle. The lysates were centrifuged at 14000 g for 10 min at 4°C in a microcentrifuge, and supernatants were collected and subjected to immunoprecipitation, SDS-PAGE and fluorography. The media samples were centrifuged at 7,000 g for 1 min and 500 μl of solubilizing buffer containing 0.3% BSA was added to the supernatant. Media samples were also subjected to immunoprecipitation, SDS-PAGE and fluorography.
2.4.5 Determination of Apolipoprotein B Turnover in Permeabilized Primary Hamster Hepatocytes

Primary hamster hepatocytes were pulse-chased and made semi-permeable essentially as described for HepG2 cells (Adeli, 1994). Semi-permeabilization was achieved by incubating radiolabeled cells with cytoskeletal (CSK) buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl$_2$, 1 mM Na-free EDTA, 10 mM PIPES, pH 6.8) containing 50 µg/ml of digitonin for 10 min or 75 µg/ml for 5 min at room temperature and then chased in CSK buffer. At various intervals, duplicate or triplicate dishes were washed with cytoskeletal (CSK) buffer and solubilized in the solubilization buffer. Cell extracts were centrifuged in a microcentrifuge at 14,000 rpm, 4°C, for 10 min, and the supernatant was subjected to immunoprecipitation.

2.4.6 Trypsin Digestion of Permeabilized Primary Hamster Hepatocytes

In order to study apoB translocation, primary hamster hepatocytes were incubated with methionine-free α-MEM for 1 h and then pulsed with 100 µCi/ml [$^{35}$S]methionine, and chased for 10 min in α-MEM or Williams’ E media containing 10 mM cold methionine. The cells were then washed with CSK buffer and permeabilized by incubating in CSK buffer containing 75 µg/ml digitonin for 5 min at room temperature. Permeabilized cells were washed with CSK buffer and then incubated with CSK buffer containing 150 µM puromycin, 50 µg/ml cyclohexamide, and 1.25 µM ALLN in the presence and absence of 200 µg/ml trypsin for 10 min at room temperature (Macri and Adeli, 1997b). In order to stop the reaction, an equal volume of CSK buffer containing 2 mg/ml soybean trypsin inhibitor, 1 mM PMSF, 1.25 µM ALLN and 100 KIU/ml aprotinin was added to all dishes and cells were incubated for 10 min at room temperature. Cells were then transferred on ice and incubated
an additional 10 min. Finally, the permeabilized cells were solubilized and cell lysates subjected to immunoprecipitation and SDS-PAGE as described above. In some experiments, MG132 (25 μM) was added 15 min before the pulse and was present throughout the pulse and chase.

2.4.7 Isolation and Trypsin Treatment of Hepatic Microsomes

Hamster hepatocytes were pulsed for 1 h in the presence and absence of MG132 (25 μM), and were used to prepare a microsomal fraction as described (Borchardt and Davis, 1987, Davis, et al., 1990) with minor modifications. Briefly, cells were washed and homogenized after the pulse to isolate crude microsomes. Microsomes were purified on a step gradient using two sucrose densities (1.3 M and 2.25 M) and centrifuged for 1 h at 4°C and 40,000 rpm. Microsomes collected from the interface between sucrose layers were pooled, aliquoted, and incubated with ±100 μg/ml trypsin for 30 min at room temperature. After the incubation, soybean trypsin inhibitor (1 mg/ml) and PMSF (final concentration 100 μM) were added and the microsomes were solubilized and subjected to immunoprecipitation.

2.4.8 Analysis of Luminal and Membrane-associated ApoB Pools

Isolation of a microsomal fraction and the separation of the luminal and membrane components by carbonate extraction and sucrose gradient ultracentrifugation were performed as described (Boren, et al., 1992, Boren, et al., 1990, Bostrom, et al., 1988, Bostrom, et al., 1986). Briefly, primary hamster hepatocytes were pulsed for 1 h, chased for 0 or 1 h, then homogenized to prepare a crude microsomal fraction. The microsomes were subjected to sodium carbonate treatment to dissociate the luminal and membrane fractions which were separated by ultracentrifugation. Membrane and luminal fractions were then diluted with 800 μl of a solubilization buffer containing 360 μl 5xC buffer (250 mM tris-HCl, pH 7.4, 750
mM NaCl, 25 mM EDTA, 5 mM PMSF, 5% Triton-X100), and 410 μl PBS (containing 450 KIU/ml Trasylol, and 5 mM PMSF) and subjected to immunoprecipitation, SDS-PAGE, and fluorography.

2.4.9 Isolation and Subcellular Fractionation of Hamster Liver Microsomes

Isolation of a microsomal fraction and the separation of the luminal and membrane components by carbonate extraction and sucrose gradient ultracentrifugation were performed as described (Boren, et al., 1992, Boren, et al., 1990, Bostrom, et al., 1988, Bostrom, et al., 1986). Intact cells were pulsed for 1 h in the presence and absence of the protease inhibitor, MG132 (25 μM) and were then scraped in 0.5 ml of 50 mM sucrose solution supplemented with a cocktail of protease inhibitors (1 mM PMSF, 100 KIU/ml Trasylol, 1 μM pepstatin A, and 5 μM ALLN) and homogenized with a glass dounce homogenizer. The homogenate was centrifuged for 10 min at 2200 x g. The supernatant containing the crude microsomes was then treated with sodium carbonate pH 11. The luminal component released by carbonate extraction was isolated from the membrane fraction by ultracentrifugation at 37000 rpm for 90 min at 12 °C in a SW41 rotor. The isolated microsomal membrane was resuspended in 1 ml of PBS, solubilized in 800 μl of a solubilization buffer containing 360 μl 5xC buffer (250 mM tris-HCl, pH 7.4, 750 mM NaCl, 25 mM EDTA, 5 mM PMSF, 5% Triton-X100), and 410 μl PBS (containing 450 KIU/ml Trasylol, and 5 mM PMSF), and then subjected to immunoprecipitation. The luminal fractions were either directly solubilized and immunoprecipitated for apoB, or were subjected to lipoprotein fractionation by sucrose gradient ultracentrifugation.
2.4.10 Sucrose Gradient Fractionation of the ApoB-containing Lipoproteins

ApoB-containing lipoproteins present in luminal contents of microsomes (prepared as above) or secreted into the medium were analyzed by gradient ultracentrifugation. The sucrose gradient was formed by layering from the bottom of the tube: 1.5 ml of 49% sucrose, 3.0 ml of 25% sucrose, 2.0 ml of 20% sucrose, 3.0 ml of sample in 12.5% sucrose, 1.9 ml of 5% sucrose, 0.9 ml of 0% sucrose. All solutions contained 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein inhibitory units/ml aprotinin, 5 μM ALLN, and 3 mM imidazole, pH 7.4. The gradient was ultracentrifuged at 35,000 rpm in a Sorval TH-641 rotor for 65 h at 12 °C, unloaded into 12 fractions, and apoB was recovered from each fraction by immunoprecipitation and SDS-PAGE and fluorography. The band corresponding to the apoB was cut out of the gel and digested and the radioactivity counted.

2.4.11 Determination of the synthesis and secretion of cellular and secreted lipids

Primary hepatocytes were pulsed for 3 h or 18 h with 5 μCi/ml [3H]acetate to assess the rates of synthesis and secretion of cholesterol, cholesteryl ester, and phospholipids. TG synthesis and secretion were monitored by labeling cells for 3-5 h with 5 μCi/ml [3H]oleate bound to bovine serum albumin. Following labeling, cells were extracted with hexane/isopropanol (3:2) and the total lipid extract was dried, suspended in hexane and applied to a thin layer chromatogram. The TLC plates were developed using a two solvent system to separate polar lipids with chloroform/methanol/acetic acid/formic acid/H₂O (70:30:12:4:2) and neutral lipids with petroleum ether/ethyl ether/acetic acid (90:10:1). The lipids were stained with iodine vapor and identified based on the use of a set of known lipid standards (Sigma). The spots identified on the TLC plates were cut and counted using a scintillation counter.
2.4.12 Preparation of Oleate-BSA Complex

Oleate-BSA complex was prepared based on the method described by Van Harken et al. (Van Harken, et al., 1969). A total of 150 mg of fatty acid-free BSA was added to 10 ml of pre-warmed methionine-free MEM, or hepatocyte attachment media, or Williams’ E media and mixed and allowed to incubate for 5 min at 37°C until BSA was completely dissolved. The media-BSA solution was then sterilized by syringe-filtration (0.45 µM). The filtered solution was diluted by adding 40 ml additional media. A solution of oleic acid was prepared by dissolving 34 mg of the oleic acid in 500 µl of anhydrous ethanol. A volume of 75 µl of the prepared oleate stock solution was added in 50 ml of media-BSA solution. The mixture was then either incubated overnight while constantly rotating at room temperature or for 2 h at 37°C. By using this protocol, the final concentration of oleate in solution was 360 µM with an oleate/BSA ratio of 8:1.

2.4.13 Assessment of Insulin-Induced Phosphorylation

In order to detect tyrosine phosphorylation of insulin receptor β subunit, IRS-1, and IRS-2, hepatocytes derived from control, and fructose-fed hamsters were incubated for 5h in a serum and insulin free media. Half of the cells in each group were then stimulated with 100 nM insulin for 10 minutes at room temperature. Cells were then washed once with PBS and lysed with a buffer containing a phosphatase inhibitor cocktail [150 mM NaCl, 10 mM tris (hydroxymethyl)aminomethane (pH 7.4), 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% NP-40, 2mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate] and subjected to immunoprecipitation with a specific polyclonal antibody against either insulin receptor β subunit, IRS-1, or IRS-2 (1 µg antibody/0.5 mg of total cell lysate) using 50 µl of 10%
protein A sepharose (for each sample). Immunoprecipitates were then washed three times at 4°C using wash buffer (PBS containing 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 0.1% NP40, and 0.1% Triton X-100). Immunoprecipitates were used for immunoblotting with mouse monoclonal antibody against tyrosine phosphorylated proteins (1:1000 dilution) using ECL chemiluminescence system as described above.

2.4.14 PI 3-kinase Activity Assays

The PI 3-kinase assays were performed as described (Kido, et al., 2000, Wang, et al., 1998). Briefly, hepatocytes isolated from control and fructose fed hamsters were incubated in serum- and insulin-free media for 5 h and then exposed to 100 nM insulin for 10 min at room temperature. Cells were washed and lysed in solubilizing buffer containing 150 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% NP-40, protease inhibitors (2 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin), and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate). Cell lysates were subjected to overnight immunoprecipitation with anti-p85 or anti-phosphotyrosine antibodies at 4 °C. PI 3-kinase activity was measured on p85 or phosphotyrosine immunoprecipitates. All immunoprecipitates were washed and incubated for 5 min with 20 µg of phosphatidylinositol. The reaction was started by the addition of 5 µl of 1 mM [γ-32P]ATP (10 µCi/5 µl) in 20 mM MgCl₂ and then stopped after 10 min by the addition of 8 M HCl. The lipids were extracted with 160 µl of chloroform/methanol (1:1). Fifty µl of the lower phase was applied to a silica gel 60 F²⁵⁴ thin layer chromatography plastic sheet (E.Merck, Germany) and lipids were separated in a chloroform/methanol/water/ammonium hydroxide
(60:47:12:2) solvent system. Radiolabeled lipids were quantitated using a Storm 840 phosphoimager (Molecular Dynamics, USA).

2.4.15 PTP-1B Activity Assay

The \textit{in vitro} PTP assay was conducted based on a protocol previously published by Cho et al. (Cho, et al., 1993) with some modifications. Hepatocytes isolated from control and fructose-fed hamsters were lysed in solubilization buffer (PBS containing 1% NP40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF, 0.1 mM Leupeptin, 2 μg/ml ALLN). The lysates were centrifuged for 10 min at 4°C in a microcentrifuge, and supernatants were collected for immunoprecipitation. Prior to immunoprecipitation, cell lysates were subjected to preclearing with protein A Sepharose for 15 min at 4°C. Equal amounts of each sample (750 μg of total protein) was then subjected to immunoprecipitation with anti-PTP1B antibody (Ab-1, Oncogene Research Products) at 4°C overnight. PTP-1B immunocomplexes were pulled down by adding protein A Sepharose at 4°C for an additional 2 h. Immunoprecipitates were then washed with the PTP assay buffer (100 mM Hepes, pH 7.6, 2 mM EDTA, 1 mM DTT, 150 mM NaCl, and 0.5 mg/ml BSA). The pp60\(^{c-src}\) C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL, Biomol) was added at 200 μM in a total reaction volume of 60 μl in the PTP assay buffer and the reaction allowed to proceed for 1 h at 30°C. At the end of the reaction, 40 μl aliquots were placed into a 96-well plate, 100 μL of Biomol Green reagent (Biomol) was added and absorbance was measured at 630nm.

2.4.16 Chemiluminescent Immunoblot Analysis

Cell lysates or immunoprecipitates were subjected to chemiluminescent immunoblotting for different proteins including apoB, ubiquitinated apoB, 97 kDa subunit of MTP, insulin receptor β subunit, IRS-1, IRS-2, PTP-1B, Akt/PKB, tyrosine phosphorylated
proteins, and ER-60. Samples were analyzed by SDS-PAGE using mini gels with different percentage (depending on molecular weight of target protein) of polyacrylamide (8 x 5 cm). Following SDS-PAGE the proteins were transferred electrophoretically overnight at 4°C onto nitrocellulose membranes using a Bio-Rad Wet Transfer System. The membranes were blocked with a 5% solution of fat-free dry milk powder, incubated with a relevant antiserum, washed, and then incubated with a secondary antibody conjugated to peroxidase. Membranes were then incubated in an enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech) for 60 seconds and exposed to Kodak Hyperfilm, according to the manufacturer's recommendations, within the limits of linearity of the ECL detection system. Films were developed and quantitative analysis was performed using an imaging densitometer.

2.4.17 RNase Protection Solution Hybridization Assay for Hamster MTP mRNA

Hamster MTP cDNA was kindly provided by Dr. David Gordon (Bristol-Myers Squibb, Princeton, NJ). Two primers GCGCCTCGAGGCCTTCATCCAGCACCTC (XhoI site underlined) and GCGCAAGCTTCAGCCTCAGCATACTTC (HindIII site underlined) were used to amplify a 517 bp fragment of hamster MTP cDNA. After digestion with XhoI and HindIII, this fragment was ligated with XhoI/ HindIII digested pGEM-7Zf vector (Promega). These constructs served as templates to synthesize antisense RNA probes and standard cRNA. Unlabeled cRNA corresponding to the sense DNA strand was prepared for use as hybridization standard. Total RNA from liver tissue were isolated using Trizol reagent (Gibco BRL). RNase protection analyses were performed as described by Azrolan and Breslow (Azrolan and Breslow, 1990). Briefly, riboprobe and either sample or standard cRNA were hybridized overnight in 40 µl hybridization buffer (80% (v/v) formamide; 40

83
mM HEPES, pH6.7; 0.4 M NaCl; 1 mM EDTA) at 63°C. RNase A and RNase T1 in digestion buffer (0.3 mM NaCl; 10 mM Tris-HCl, pH 7.4; 5 mM EDTA) were added to each sample and incubated at 34°C for 1 hr. After incubation, 20% trichloroacetic acid (TCA) and 100 µg salmon sperm DNA were added and incubated for 15 min to precipitate protected RNA, and each sample was filtered using glass fiber filters (Whatman). Filters were washed with 10% TCA and counted with scintillation fluid in a beta counter.

2.4.18 MTP Activity Assay

The assay was carried out using the MTP activity kit (Roar Biomedical, Inc.) according to the manufacturer's recommendations. The MTP activity assay is based on MTP mediated transfer of a proprietary fluorescent neutral lipid entrapped in donor vesicles (self-quenched state) to the acceptor (fluorescent state). The MTP mediated transfer is observed by the increase in fluorescence intensity as the fluorescent neutral lipids is transferred from the self-quenched donor to the acceptor. Briefly, primary hamster hepatocytes isolated from control and fructose-fed hamsters were suspended in a homogenization buffer (150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, 2 mg leupeptin, in 100 ml of 10 mM tris pH 7.4). The suspensions were then sonicated on ice with five-second bursts in a sonicator fitted with a microtip on power setting 4. 100 µg of homogenate protein of each group was used in the assay. The MTP source (0.5 ml total volume) was combined with 10 µl donor and 10 µl acceptor and then incubated for 12 h at 37°C. The assay was read in a fluorescence spectrometer at excitation wavelength of 465 nm and emission wavelength of 535 nm.

2.4.19 Immunoprecipitation

All samples including cell lysates, media, and subcellular or luminal fractions were first subjected to pre-immunoprecipitation by adding 2 µl of an appropriate anti serum plus
30 µl crude immunoprecipitin. All samples were rotated for 1 h at room temperature and then centrifuged for 3 min at 14000 g. Supernatants were collected and subjected to immunoprecipitation by adding 5 µl of antibody and overnight incubation at 4°C. The following day 100 µl of immunoprecipitin was added to each sample and mixed for 1 h on a rotator at room temperature. The immunoprecipitates were separated by centrifugation for 2-3 min at 14000 g and supernatants were discarded. Each immunoprecipitate was washed along with resuspending for 3 times by adding 1 ml of wash buffer (PBS containing, 2 mM EDTA, pH 8, 0.1% SDS, 1% NP-40, 1% DOC). After the third wash, pellets were resuspended in 100 µl of Laemmli electrophoresis sample buffer (dH₂O containing 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.1 mg bromophenol blue). Samples were then boiled on a heat block at 100°C for 5 min, centrifuged at 14000 g for 3 min and supernatants were loaded onto SDS-PAGE gels.

2.4.20 SDS-PAGE

SDS-PAGE was performed essentially as described (Laemmli, 1970). Depending on the molecular weight of the protein(s) under investigation, the percentage of acrylamide in resolving and stacking gels was varied which is indicated in the figure legends. Laemmli sample buffer (0.125 M Tris-HCl, pH 6.8, 5% v/v β-mercaptoethanol, 20% v/v glycerol, 4.1% w/v SDS, and 0.02% w/v bromophenol blue) and the composition of the other major buffers used to perform SDS-PAGE has been indicated below:

Resolving Gel: 4.5%, 6%, 8%, 10%, or 3-15% (gradient gel) (w/v) acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% (W/V) ammonium persulfate, and 0.07% (V/V) TEMED.

Stacking Gel: 3% or 4% (W/V) acrylamide, 0.125 M tris-HCl, pH 6.8, 0.05% (W/V) SDS, 0.1% (W/V) ammonium persulfate and 0.05% (V/V) TEMED.
Running Buffer: 0.02 M Tris, 0.192 M glycine, and 0.01% (W/V) SDS.

Once the gel was cast, it was usually run for 16 h at 60 V or until the blue dye reached the bottom of the gel, depending on the molecular weight of target protein and percentage of acrylamide. Following SDS-PAGE, the gels were removed and incubated in a fixer solution (dH₂O containing 40% V/V methanol and 10% V/V glacial acetic acid) for at least 45 min at room temperature.

2.4.2.1 Fluorography and Scintillation Counting

In order to enhance fluorographical properties of the fixed gels, they were soaked in Amplify™ (Amersham) for 30 min. and then washed with distilled water for 1 min and placed on a sheet of Whatman filter paper and covered with a sheet of plastic wrap. The gel was dried in a gel drier under vacuum at 80°C for 1.5 h. and then exposed to Kodak autoradiographic film at -80 °C for 1-4 days. The film was later developed in an X-ray processor machine. Finally the bands of interest were excised from the gels and placed into scintillation tubes. Perchloric acid (65%, 200 μl) and hydrogen peroxide (400 μl) was added to each vial (on the excised band), and incubated overnight at 60°C. After overnight incubation, 4 ml of scintillation cocktail was added to the digested piece of the gel in each vial and mixed well to ensure that it was completely dissolved. After 1 h incubation at room temperature, the samples were counted for radioactivity in a scintillation counter and band radioactivity was quantified as counts per minute (CPM).

To assess total radiolabeled proteins, 5 μl of radiolabeled cell lysates (before pre-immunoprecipitation) was spotted on a small piece (1.5 Cm x 1.5 Cm) of filter paper and was allowed to air dry at room temperature. The filter papers were then heated twice for 5 min in 10% TCA solution to fix and precipitate labeled protein on the paper. Filter papers were then
soaked in anhydrous ethanol twice for 2 min and then left to dry at room temperature. Finally, 4 ml of scintillation cocktail was added to each vial containing filter paper and subjected to scintillation counting as described above.

2.4.22 Protein Assay

In order to measure total protein content of samples, a small fraction (~20 μl) of cell lysate was aliquoted and subjected to the commercially available Bio-Rad DC Protein Assay kit, according to the manufacturer's recommendations. This method is a colorimetric assay similar to the well-documented Lowry assay (Lowry, 1951) with some improvement and modifications. The assay is based on the reaction of protein with an alkaline copper tartarate solution and Folin reagent. The protein standard (BSA) was made with the same solubilizing buffer used for lysing the cells. Samples were measured using manufacturer's microplate assay protocol and absorbances read at 750 nm. Samples and standards were measured in triplicate and protein mass concentration expressed as mg/ml.

2.4.23 Calculations and Statistical Analysis

All the values are reported as MEAN ± STDV. Statistical significance was calculated by performing 2-tailed paired Student t-test analysis. A p value equal or less than 0.05 was considered to be significant. Each experiment was repeated at least three times in duplicate or triplicate.
CHAPTER THREE

Intracellular Mechanisms Regulating ApoB-containing Lipoprotein Assembly and Secretion in Primary Hamster Hepatocytes

3.1 Rationale, Hypothesis, and Research Objectives

3.1.1 Rationale

Most ex vivo studies on hepatic biogenesis of apoB-containing lipoproteins have been conducted either in cell lines or primary rodent hepatocytes. Although a great deal of data has been collected using the above cellular models, however they have not been without pitfalls. HepG2 cells as the most commonly used cell line has major limitations. The most notable limitation of HepG2 cells in the study of apoB biogenesis is in the type of secreted lipoprotein particles. Little of the neutral core lipids are secreted as VLDL, but are instead secreted as particles in the LDL-density range (Cianflone, et al., 1992, Cianflone, et al., 1990, Gibbons, et al., 1994). The primary rat hepatocyte is the most commonly used primary rodent hepatocyte system. Rat liver secretes both apoB48 and apoB100, making it difficult to extrapolate collected data to humans (Rusinol, et al., 1993, Swift, 1995).

al., 1991), and the total body cholesterol synthetic rates in hamsters are comparable with humans (Spady and Dietschy, 1983, Spady, et al., 1986). Moreover, cholesterol metabolism (Hoang, et al., 1995), LDL binding and degradation (Salter and Sessions, 1993), and bile acid synthesis (Hoang, et al., 1993) in hamster hepatocytes resemble that in humans. Despite the advantages of the hamster as a unique animal model for lipoprotein research, there has not been a comprehensive report published on the intracellular biogenesis and assembly of apoB-100-containing lipoprotein particles in this animal model. In this part of study, we have investigated the intracellular mechanisms regulating apoB biosynthesis, assembly, degradation, and secretion in primary hamster hepatocytes, a primary cell model exclusively expressing apoB-100-containing VLDL particles.

3.1.2 Hypothesis

Primary hamster hepatocytes assemble and secrete apoB-100 containing lipoproteins with VLDL density, and apoB, during its intracellular biogenesis is subjected to co- and post-translational degradation by non-proteasomal and proteasomal proteolytic systems.

3.1.3 Research Objectives

Objective 1- Prepare and culture viable primary hamster hepatocytes

Objective 2- Confirm that primary hamster hepatocytes secrete apoB100-containing particles with the density of VLDL

Objective 3- Investigate apoB synthesis, translocation, degradation, assembly, and secretion in intact primary hamster hepatocytes

Objective 4- Study turnover of apoB100 in permeabilized hamster hepatocytes

Objective 5- Study the effects of exogenous oleate on apoB biogenesis in primary hamster hepatocytes

89
3.2 Results

3.2.1 Primary Hamster Hepatocytes Secrete ApoB-Containing Lipoprotein at the Density of VLDL

The density of apoB-containing lipoproteins secreted by hamster hepatocytes was investigated by density fractionation of isolated lipoproteins in media of cultured hamster hepatocytes labeled with \[^{35}\text{S}\]methionine. In this study, after a 1 h pulse, cells were chased for 1 and 2 h in the presence and absence of ALLN (40 μg/ml). Density of the media samples was adjusted to 1.006 g/ml. After ultracentrifugation, the top fraction (1/10 of total volume of the media) was collected and subjected to immunoprecipitation. Cell lysates were also immunoprecipitated for apoB. After a 1 h chase, 39% of total apoB was recovered in the VLDL fraction of the media; whereas after a 2 h chase, 56% of total apoB was recovered from the VLDL fraction of the media. In ALLN treated cells, at 1 h and 2 h chase, 33% and 42% of total apoB was recovered from media VLDL, respectively. Thus ALLN treatment did not increase the efficiency of VLDL secretion (as estimated by comparing the percentage of total radiolabeled apoB secreted as VLDL-apoB). However, total radiolabeled apoB recovered in ALLN treated cells was about 40% higher at 0 time and by more than 2-fold higher after 1 and 2 h chase times compared to control cells. Increased recovery of labeled apoB in the presence of ALLN suggested protection against co- and/or post-translational degradation of the protein during pulse and chase periods.

3.2.2 Analysis of ApoB Translocational Status in Permeabilized Primary Hamster Hepatocytes

Since our preliminary studies had suggested that hamster apoB may be subjected to intracellular degradation, we investigated the translocational status of hamster apoB in
primary hamster hepatocytes. Translocational status of newly synthesized hamster apoB-100 was assessed based on trypsin digestion of permeabilized hamster hepatocytes. This protocol has previously been used by our laboratory to investigate apoB translocation in HepG2 cells (Macri and Adeli, 1997b) and McRH7777 cells (Cavallo, et al., 1998). The procedure for permeabilizing primary hamster hepatocytes was similar to that previously described for HepG2 cells. Optimization experiments showed that incubation with CSK buffer containing 50 μg/ml digitonin for 10 min or 75 μg/ml digitonin for 5 min at room temperature was sufficient to permeabilize hamster hepatocytes without causing significant damage to intracellular organelles (as assessed by the loss of ER-lumenal proteins). To confirm the integrity of intracellular membranes after the permeabilization process, we monitored the retention of radiolabeled apoB and transferrin in permeabilized cells and any possible leakage into the surrounding medium (CSK buffer). No apoB or transferrin could be recovered by immunoprecipitation from the CSK buffer (pooled solutions of CSK-digitonin and wash buffers). In addition, no significant difference was detectable in the immunoprecipitable apoB and transferrin recovered from permeabilized cells at different concentrations of digitonin. Immunoprecipitable transferrin recovered at 0, 50, and 100 μg/ml digitonin was 11241±62, 9675±554, and 9548±1938 CPM/plate (n=3) respectively; immunoprecipitable apoB recovered was 1373±136, 1257±21, and 1284±43 CPM/plate (n=3), respectively. The absence of labeled transferrin and apoB in the CSK buffer and the lack of a decrease in cellular levels of these proteins strongly argue against any significant leakage of newly-synthesized proteins residing in the secretory pathway of the hepatocytes. This in turn suggests that the integrity of the ER-Golgi component of the secretory pathway remains mostly intact after permeabilization of the cells.
In this and all subsequent protease protection experiments, intact primary hamster hepatocytes were initially pulse-chased to achieve biosynthesis and translocation of apoB across the ER membrane. Permeabilization was only carried out to allow the delivery of trypsin to the cytosolic surface of the ER membrane. Fig. 3.1A shows immunoprecipitable apoB recovered from permeabilized cells (pulsed for 45 min, chased for 10 min, and permeabilized) with and without trypsin digestion (in the presence or absence of Triton X-100). The amount of trypsin resistant apoB was measured as a percentage of the intact apoB immunoprecipitated from control cells not subjected to trypsin treatment. An average of 42.4 ± 10.1% of newly synthesized apoB was trypsin sensitive in permeabilized cells. In the presence of both trypsin and Triton X-100, no immunoprecipitable apoB could be recovered suggesting that the trypsin-resistant apoB detected in permeabilized cells is segregated by intracellular membranes which become accessible to trypsin upon addition of the membrane solubilizing agent, Triton X-100. Finally, percent trypsin sensitivity in permeabilized cells was significantly greater with apoB (42.4 ± 10.1%) than the control protein, transferrin (13.9 ± 6.2%) (Fig. 3.1B), P <0.05, under similar experimental conditions. Addition of Triton X-100 to permeabilized cells caused an almost total degradation of transferrin by exogenous trypsin suggesting that transferrin was not inherently resistant to trypsin digestion (Fig. 3.1B).

3.2.3 Trypsin Sensitivity of Hamster ApoB-100 in Isolated Microsomes

Protease protection assays were also performed with microsomes isolated from hamster hepatocytes to confirm the observations made in permeabilized cells. In these experiments, hamster hepatocytes were pulse-labeled for 1 h and then used to isolate a microsomal fraction by ultracentrifugation. Isolated microsomes were then subjected to in
vitro trypsin digestion. Figs. 3.1C and 3D indicate the amount of apoB recovered from microsomes isolated from control and MG132 (25 μM) treated cells in the presence and absence of exogenous trypsin. In control cells, 27±7.9% of newly synthesized microsomal apoB was trypsin accessible, whereas in MG132 treated cells, 54.7±10.5% was accessible to trypsin digestion. This difference in trypsin sensitivity between the two conditions (nearly 2 times more in MG132 treated cells) can be attributed to the protective effect of MG132 against degradation of the pool of apoB associated with the cytosolic side of the ER membrane. It should be noted that MG132, a proteasome inhibitor, also increased the total amount of immunoprecipitated apoB 2.2-fold compared to control cells. Most of the pool protected by MG132 was thus trypsin accessible. MG132-treated microsomes had 35% more immunoprecipitable apoB than control cells following trypsin digestion. As a control, we also examined the trypsin sensitivity of transferrin in both control and MG132 treated cells. In control cells, 87.6±13.8% (n=3) of labeled transferrin was resistant to trypsin digestion whereas in MG132 treated cells 97.6±5.9% (n=3) of labeled transferrin was trypsin resistant. Trypsin resistance of transferrin in isolated microsomes and the lack of effect of MG132 on trypsin accessibility of this control protein appear to confirm the specificity of the observations with respect to protease sensitivity of apoB in control and MG132 treated cells.

3.2.4 Analysis of ApoB-100 in Subcellular Fractions of Isolated Microsomes

Intracellular distribution of newly synthesized apoB-100 in primary hamster hepatocytes was examined by isolating a total microsomal fraction and preparing luminal and membrane subfractions. Figs. 3.2A and 2B show the results of such an experiment. A small fraction of newly synthesized apoB was consistently found to be associated with microsomal membrane (11.2±4.1%). However, a large pool of radiolabeled apoB could be extracted with
sodium carbonate and was thus detected in the soluble luminal fraction of microsomes. It appeared that the distribution of microsomal apoB in the luminal and membrane pools did not change with MG132 pretreatment of the cells (control cells, 88.8%±0.7 luminal apoB, and 11.2%±4.1 membrane apoB; MG132 treated cells, 86.5%±0.9 luminal apoB, and 12.8%±4.5 membrane apoB). Although the ratio of the pools was similar in control and MG132-treated cells, treatment with the inhibitor increased total and luminal apoB by 18.5%±7.6 and 16.3%±2.6 respectively. Similar observations were made when subcellular fractionation studies were performed in the presence of ALLN as the protease inhibitor. ALLN treatment did not alter the proportion of newly synthesized apoB found associated with the membrane and luminal fractions of microsomes. Following a 1 h pulse in control cells, 80.7% and 19.3% of immunoprecipitated apoB was luminal and membrane-bound respectively, whereas in ALLN-treated cells, 83.2% and 16.8% of apoB was recovered in lumen and membrane fractions, respectively. After a 30 min chase, 65.3%, 11.9%, and 22.8% of immunoprecipitated apoB in control cells, and 67.6%, 14.8%, and 17.6% of apoB in ALLN treated cells were recovered in lumen, membrane and media of hamster hepatocytes, respectively. It should be noted that total immunoprecipitable apoB increased in ALLN treated cells by 11.1% and 19.9% at 0 and 30 min chase times respectively, compared to control cells. Furthermore, after the 30 min chase there was no significant difference in secreted apoB between control and ALLN treated cells, but lumenal and membrane-associated apoB both showed an increase of 24% and 49% respectively in the presence of ALLN (compared to control).
3.2.5 ApoB Stability and Secretion in Primary Hamster Hepatocytes

Pulse-chase labeling experiments were performed to analyze the stability and secretion of apoB in primary hamster hepatocytes. Fig. 3.3A shows the turnover of newly synthesized apoB in the presence and absence of MG132. MG132 induced accumulation of apoB during the pulse (Fig. 3.3B) and appeared to protect apoB during the chase. The kinetics of apoB degradation was further examined in a 6 h chase experiment (Figs. 3.3C and 3.3D). In control cells, the percentage of cellular apoB remaining after 30, 60, 120, 240 and 360 min chase was 72.6%±18 (n=2), 55.4%±9.3 (n=5), 25.8%±8.3 (n=7), 15.6%±1.9 (n=2), and 11.2%±1.5 (n=2) respectively. The percentage of radiolabeled apoB secreted into the media at the same time points was 12%±1.4, 31%±5, 35.3%±2.1, 36%±2.9, and 40.3%±7.9 respectively. Percent apoB degraded after a 30 min chase was 15.8%±14 which increased to 38.8%±6.2 and 48.3%±0.7 after 120 and 240 min chase, respectively. Interestingly, after a 4 h chase, degradation reached a plateau (48.4%±6.6) and no further degradation occurred after a 6 h chase. Treatment with MG132 significantly altered the pattern of apoB degradation kinetics. In these experiments, cells were treated with MG132, 15 min before the pulse, and then chased in the presence of the protease inhibitor. As depicted in Fig. 3.3D, the percentage of cellular apoB remaining was decreased during the chase from 100% at 0 time to 99.4%±26 (n=2), 77.2%±8.5 (n=5, p=0.03), 61.2%±10 (n=7, p= 0.01), 32%±2.2 (n=2), and 25.1%±2.7 (n=2) at 30, 60, 120, 240, and 360 min chase times, respectively. Due to protection of apoB from degradation, the percentage of cellular apoB remaining in MG132 treated cells was significantly higher than the control at various time points. Interestingly, the percentage of radiolabeled apoB secreted into the media of the above cells showed no significant difference.
FIGURE 3.1

Trypsin sensitivity of hamster apoB-100 in permeabilized hamster hepatocytes and isolated microsomes

**Panel A:** Primary hamster hepatocytes (1.5 X 10^6/dish) were pulsed with [35S]methionine for 1 h, chased in hepatocyte attachment medium plus 10 mM L-methionine for 10 min and then incubated in CSK buffer containing 75 μg/ml digitonin for 5 min at room temperature to achieve permeabilization. Permeabilized cells were then washed and incubated in the presence and absence of trypsin (100 μg/ml) or trypsin plus 0.5% Triton X-100, for 10 min at room temperature. Trypsin treatment was stopped by the addition of soybean trypsin inhibitor and other protease inhibitors. Cells were solubilized and cell extracts were subjected to immunoprecipitation by a specific anti-apoB antibody and then analyzed by SDS-PAGE and fluorography. Data shown as mean +/-SE (2 separate experiments in triplicate). **Panel B:** protease protection experiments similar to that in panel A were performed and a control protein, transferrin, was immunoprecipitated to monitor its trypsin sensitivity in the presence and absence of Triton X-100. For all experiments apoB radioactivity recovered were against total TCA-precipitable radioactivity in each dish before converting to percentages. **Panels C & D:** Intact cells (1.5 X 10^6/dish) were pulsed for 1 h in the presence and absence of MG132 (25 μM) and then homogenized to isolate crude microsomes. Purified microsomes were obtained by sucrose gradient centrifugation as described in the methods section. Collected microsomes were treated with 100 μg/ml trypsin for 30 min at room temperature. Trypsin treatment was stopped by addition of soybean trypsin inhibitor. Samples were solubilized and subjected to immunoprecipitation by a specific anti-apoB antibody and then analyzed by SDS-PAGE and fluorography. ApoB-associated radioactivity was quantitated by excision and scintillation counting of the apoB bands. C) a representative fluorograph; D) trypsin resistant apoB (mean ± SD, 3 separate experiments in duplicate, p<0.05 in trypsin-treated cells between +/- MG132) as a percentage of apoB recovered in the absence of trypsin.
Subcellular distribution of newly synthesized apoB in microsomal membrane and lumen

Primary hamster hepatocytes were pulsed (1 h) and chased (5 min) in the presence and absence of 25 μM MG132. Cells were washed, homogenized and subsequently subjected to subcellular fractionation to isolate microsomes. Luminal apoB was extracted from microsomes by carbonate treatment and separated from the membrane fraction by centrifugation (SW41 rotor, 37000 rpm, 90 min). Both the membrane and lumenal fractions were immunoprecipitated with a specific anti-apoB antibody and analyzed by SDS-PAGE and fluorography. ApoB radioactivity was quantitated by cutting and scintillation counting of the apoB band. A) a representative fluorograph; B) distribution of labeled apoB in membrane and lumen of isolated microsomes. In each experiment, two sets of dishes per condition, each set contained five dishes of 1.5 X 10⁶ cells (7.5 X 10⁶ cells) were pulsed and later combined for microsomal membrane and lumen preparation.
FIGURE 3.2

A Lumen Membrane

MG132  -  -  +  +  -  -  +  +

B

Immunoprecipitable ApoB (CPM)

L M L M

Control MG132

P=0.055
between MG132 treated and control cells. In MG132 treated cells, at 30, 60, 120, 240, and 360 min chase time points, 10.4%±1, 22.3%±2.9, 36%±4.6, 35.5%±3.3, and 42.4%±2 of radiolabeled apoB was secreted in the media respectively. ApoB was stabilized considerably with MG132 treatment. In fact, no significant degradation occurred for the initial 2 h of chase (average of 0.5% at 60 and 2.7% at 120 min); but it was dramatically increased to 34.8%±6.3 at 240 min and remained approximately constant up to the end of the chase period (33%±6.6 at 360 min).

3.2.6 ApoB Degradation in Permeabilized Primary Hamster Hepatocytes and Detection of Degradation Intermediates

Experiments were also performed in permeabilized hamster hepatocytes. Cells were pulsed and then permeabilized and chased in CSK buffer for different time points from 0 to 180 min in the presence and absence of ALLN. Fig. 3.4A shows the pattern of apoB degradation in permeabilized hamster hepatocytes. ApoB was gradually degraded in permeabilized cells such that the percentage of apoB remaining was 91.6%±15.2 (n=2), 91.6%±6.3 (n=4), 76.3%±2.4 (n=2), 77.6%±2.4 (n=4), and 51.9%±4.9 (n=4) at 30, 60, 90, 120, and 180 min chase times respectively. Degradation was more prominent after 60 min chase and reached 48.1%±4.9 (n=4) after 180 min chase. The extent of degradation in permeabilized cells was lower than that observed in intact cells during the first 2 h chase (8.2%±6.3 and 22.4%±2.5 in permeabilized cells compared with 17.8%±8 and 38.8%±6.2 in intact cells at 60 and 120 min chase, respectively). Interestingly, several degradation fragments were observed in permeabilized cells with approximate molecular sizes of 167, 70, 57, and 46 kDa. The 70 kDa fragment was surprisingly identical to the fragment previously.
Primary hamster hepatocytes were pulsed with \[^{35}\text{S}]\text{methionine}\) and chased for up to 6 h. MG132 (25 \(\mu\text{M}\)) was added 15 min before the pulse and was present throughout. Media samples and cell lysates were collected at each chase time point, subjected to immunoprecipitation and then analyzed by SDS-PAGE and fluorography. Panel A shows a representative experiment in control and MG132 treated cells (five separate pulse-chase labeling experiments were performed with similar results). Panel B shows the effect of MG132 on apoB accumulation during the pulse (at 0 time) (mean +/-SD of the pulse-chase experiment in Panel A, \(p < 0.05, n=7\)). Panels C and D show the results of several experiments at 0 (n=7), 30 (n=2), 60 (n=5), 120 (n=7), 240 (n=2), and 360 min (n=2) chase times. Panel C shows the distribution of apoB in cells (open squares), media (open circles) as well as the total apoB (open triangles) in control hamster hepatocytes expressed as a percent of labeled apoB at 0 time. Panel D shows the percentage of apoB in cells (closed squares), media (closed circles) as well as the total apoB (closed triangle) in MG132-treated cells. Data shown as mean +/-SD of a representative pulse-chase experiment performed in triplicate. Data were normalized against total TCA-precipitable radioactivity in each dish prior to converting to percentages. * and ** significant differences when comparing control with MG132-treated cells (\(p\) values 0.03 and 0.01, respectively).
FIGURE 3.3

A

Cell

Chase Time (min) 0 0 0 30 30 30 60 60 60 60
MG132 - - + + - - + + +
Media

Chase Time (min) 30 30 30 60 60 60 60
MG132 - - + + - - +

B

P<0.05

Radioiodinated apolipoprotein at 0 Time (CPM/mg)

Control MG132

C

Control

Immunoprecipitable Apol (percent of apol at 0 time)

Chase Time (min) 0 10 20 30 40

MG132

Immunoprecipitable Apol (percent of apol at 0 time)

Chase Time (min) 0 100 200 300 400
observed in HepG2 cells (Adeli, 1994, Sallach and Adeli, 1995). Also shown in Fig. 3.4B is the effect of ALLN on apoB degradation in permeabilized cells. Interestingly, the appearance of the 70 kDa fragment was ALLN sensitive and was almost entirely abolished in ALLN treated permeabilized cells. ALLN caused a significant protection against degradation in permeabilized cells especially at 120 and 180 min chase times (percent apoB-100 remaining was 94.8%±2.2 and 78.8%±0.1, respectively; p values were 0.01 and <0.05, respectively compared to control cells, n= 4). MG132 showed a similar protective effect on apoB-100 degradation although it did not inhibit the generation of the 70 kDa fragment (data not shown). The formation of the 46, 57, and 167 kDa fragments were both ALLN- and MG132-insensitive.

3.2.7 The Sensitivity of ApoB Degradation to Various Protease Inhibitors

To more extensively characterize apoB degradation in hamster hepatocytes, the effect of a number of other protease inhibitors including ALLN (40 μg/ml), MG132 (25 μM), lactacystin (2.5 μM), EST (40 μg/ml), PMSF (1 mM), leupeptin (100 μg/ml), and o-phenanthroline (200 μg/ml), on cellular and secreted apoB as well as the total apoB pool was determined. The results, shown in Fig. 3.5, are expressed as immunoprecipitable apoB recovered/total labeled protein under control and various experimental conditions. Among various protease inhibitors tested, only ALLN, lactacystin, and o-phenanthroline had statistically significant (p<0.05) effects on newly synthesized apoB. Both ALLN and lactacystin increased cellular apoB without affecting its extracellular secretion. Treatment with o-phenanthroline, a metalloprotease inhibitor, significantly increased both cellular and secreted apoB.
Intracellular stability of apoB in permeabilized primary hamster hepatocytes

Primary hamster hepatocytes (1.5 x 10^6 cell/dish) were pulsed with [35S]methionine and then permeabilized with 50 μg/ml digitonin in CSK buffer for 10 min at room temperature. Permeabilized cells were incubated in CSK buffer for 0, 2, and 3 h. ALLN (40 μg/ml) was added 15 min before the pulse and was present throughout the experiment. Cells were solubilized and cell extracts were subjected to immunoprecipitation with a specific anti-apoB antibody and then analyzed by SDS-PAGE and fluorography. The position of the intact apoB-100 and its degradation fragments (167, 70, 57, and 46 kDa) are indicated with arrowheads. The band that appeared between the apoB 167 kDa fragment and intact apoB100 resulted from non-specific protein binding to immunoprecipitin. This was confirmed by immunoprecipitation in the absence of apoB antibody. A) control cells; B) ALLN-treated cells.
FIGURE 3.4

Chase Time (h)  0  0  2  2  3  3  0  0  2  2  3  3
ALLN  -  -  -  -  -  +  +  +  +  +  +  +

ApoB100
167 kDa
70 kDa
57 kDa
46 kDa
3.2.8 Evidence for Involvement of the Ubiquitin-Proteasome Pathway in Hamster ApoB Degradation

Inhibition of hamster apoB turnover by MG132 and lactacystin appeared to implicate the ubiquitin-proteasome system in the degradation process. To further investigate the involvement of the proteasome, hamster hepatocytes pretreated with ALLN (40 μg/ml) or MG132 (25 μM) were solubilized and the cell lysates were immunoprecipitated with anti-apoB antibody and then immunoblotted with anti-apoB and anti-ubiquitin antibodies. As shown in Fig. 3.6, immunoblotting of immunoprecipitated apoB by an anti-ubiquitin antibody revealed a specific and consistent pattern of polyubiquinated apoB. In the absence of MG132, a small amount of ubiquitinated apoB could be detected, most likely as a result of rapid proteasomal degradation. There was considerable increase in ubiquitinated apoB upon pretreatment of the cells with either ALLN or MG132 (Fig. 3.6). Overall these data, as well as the inhibition of apoB degradation by lactacystin (Fig. 3.5), support the notion that hamster apoB undergoes ubiquitination and may act as a substrate for the cytosolic proteasome.

3.2.9 Assembly and Secretion of Hamster ApoB-100 VLDL

In order to investigate the assembly and secretion of apoB-containing lipoproteins in primary hamster hepatocytes, cells were labeled with [35S]methionine, chased for 0 or 1 h, and used to isolate microsomes. Luminal contents of microsomes as well as culture media at each chase time were subjected to sucrose gradient ultracentrifugation as described in the methods section. Fractions 1 through 5 represent high density apoB lipoprotein particles (apoB lipoproteins with a density similar to that of HDL, peak density 1.065 to 1.170 g/ml),
FIGURE 3.5

Effect of various protease inhibitors on apoB stability and secretion

Primary hamster hepatocytes (1.5 x 10^6 cell/dish) were pulsed with 100 μCi/ml [35S]methionine for 2 h, in the presence and absence of different protease inhibitors including ALLN (40 μg/ml), MG132 (25 μM), lactacystin (2.5 μM), EST (40 μg/ml), PMSF (1 mM), leupeptin (0.1 mg/ml), and o-phenanthroline (200 μg/ml). Cell lysates and media were collected and subjected to immunoprecipitation with a specific anti-apoB antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography as described under "Experimental Procedures". The apoB band was excised and associated radioactivity was measured by scintillation counting. Cellular (open bars), and media (solid bars) apoB are expressed as apoB radioactivity recovered, normalized against total TCA-precipitable radioactivity in each dish (mean ± SD, n= 3). * significant difference from control (p <0.05).
FIGURE 3.6

Evidence for ubiquitination of hamster apoB-100 in primary hamster hepatocytes

Primary hamster hepatocytes (4 x 35 mm dishes) were pretreated with proteasome inhibitors, ALLN or MG132 for 1 h, washed, solubilized, and cell lysates were first immunoprecipitated for hamster apoB and the apoB immunoprecipitates were subjected to SDS-PAGE using 4.5% polyacrylamide mini-gels. Following SDS-PAGE, the proteins were transferred electrophoretically onto nitrocellulose membrane, and the membrane was immunoblotted with a primary antibody (anti-apoB or anti-ubiquitin), followed by detection using a secondary antibody conjugated to peroxidase and an ECL detection reagent. The blot with anti-apoB is not shown here, but it has been used to identify the position of apoB. Lanes 1,2, control cells, lanes 3-4, ALLN-treated cells, lanes 5-6, MG132-treated cells. The figure shown is representative of two independent experiments.
FIGURE 3.6

*Immunoprecipitation with anti-ApoB antibody*

*Immunoblotting with anti-Ubiquitin antibody*

ApoB100

<table>
<thead>
<tr>
<th>ALLN</th>
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<td>MG132</td>
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fractions 6-10 represent the lower-density apoB lipoprotein particles (LDL-apoB, peak density 1.011 to 1.045 g/ml) and the top fractions, 11 and 12, contain very low density apoB lipoprotein particles (density <1.011) (see references Boren, et al., 1992, Boren, et al., 1994). Results depicted in Fig. 3.7A indicate that in the microsomal lumen, radiolabeled apoB was distributed in three different density regions of the gradient with the majority of newly synthesized apoB being present in the low density fractions. There was a small pool of labeled apoB associated with the dense fractions at time 0, which increased following a 1 h chase. The lumenal apoB lipoproteins in the low density as well as the very low density fractions of the gradient both decreased following a 1 h chase. Sucrose gradient ultracentrifugation of media samples revealed that the secreted form of apoB lipoprotein particles was present only in the very low density fractions of the gradient (fractions 11 and 12). Very low density lipoprotein particles were present at 0 time in the media but were increased significantly following a 1 h chase (Fig. 3.7B, fractions 10-12). Secretion of apoB-VLDL particles appeared to coincide with their disappearance from the lumenal top light fraction.

The distribution of membrane-associated apoB was also assessed by immunoprecipitating apoB from the membrane pellet recovered during subcellular fractionation of luminal contents and membrane fractions. The results are shown in Fig. 3.7C. Membrane-bound apoB remaining at 1 h chase was 58.5% of that at 0 time. It appears that during the 1 h chase, 42.5% of labeled apoB disappeared from the membrane either as a result of translocation across the ER membrane and/or intracellular degradation.
3.2.10 Effect of Oleate on the Stability and Secretion of ApoB in Primary Hamster Hepatocytes

The effect of exogenous oleate on the stability and secretion of apoB was determined following an overnight pretreatment. Fig. 3.8 shows the immunoprecipitated apoB in cells and media, as well as total apoB remaining in control and oleate treated hamster hepatocytes. Oleate treatment increased cellular apoB at both 1 h and 4 h chase (Fig. 3.8A). Despite the stabilizing effect of oleate on cellular apoB, no stimulation of apoB secretion could be observed at either 1 h or 4 h chase compared to control cells (Fig. 3.8B). In control cells, total apoB remaining decreased from 100% at 0 time to 78.4%±9.2 and 57.2%±12.7 at 1 h and 4 h chase times, respectively (Fig. 3.8C). In oleate treated cells, total apoB remaining decreased from 100% at 0 time to 91.5%±3.9 and 59.7%±6.7 at 1 h and 4 h chase period, respectively (Fig. 3.8C). The protective effect of oleate against apoB degradation was thus evident after the first hour of chase, but not at 4 h.
Analysis of apoB-100-containing lipoproteins in microsomal fractions of primary hamster hepatocytes

Primary hamster hepatocytes were pulsed, chased for 0 and 1 h, and used to prepare a total microsomal fraction. Luminal contents of isolated microsomes as well as culture media were subjected to ultracentrifugation in a sucrose gradient. The gradient was fractionated from the bottom into 12 fractions, and apoB was immunoprecipitated from each fraction. Microsomal membranes were also solubilized and subjected to immunoprecipitation with a specific anti-apoB antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. The apoB band was excised and its radioactivity was determined by scintillation counting. Panels A and B show apoB recovered from luminal and media fractions at 0 time (closed squares) and 1 h chase (closed circles), respectively. Panel C shows membrane apoB recovered at 1 h expressed as a percentage of that present at 0 time. The figure shown is representative of two independent experiments.
FIGURE 3.7

A

B

C

114
FIGURE 3.8

Effect of oleate on stability and secretion of apoB in primary hamster hepatocytes

Cells (1.5 x 10⁶ cell/dish) were treated with hepatocyte attachment medium supplemented with either BSA or an oleate-albumin complex (oleate/BSA ratio of 8:1). After overnight incubation, both oleate-treated and control cells were pulsed with 100 µCi/ml [³⁵S]methionine, and then chased in the presence (oleate-treated cells) and absence (control cells) of oleate for 0, 1, and 4 h. Cell lysates and collected media were subjected to immunoprecipitation and analyzed by SDS-PAGE and fluorography. The apoB band was cut out and counted. Panels A and B show apoB recovered from cells (mean ± SD, n = 6, p values for 1 and 4 h, 0.0023 and 0.19, respectively) and media (mean ± SD, n = 6, p values for 1 and 4 h, 0.08 and 0.0018, respectively) of control (open circles) and oleate-treated (closed circles) cells. In Panel C, total apoB remaining (cell+media) is shown in control (open circles) and oleate treated cells (closed circles) at different periods of chase (mean ± SD, n = 6, p values for 1 and 4 h, 0.04 and 0.38, respectively). Data were normalized against total TCA-precipitable radioactivity in each dish prior to converting to percentages.
Mechanisms of Hepatic VLDL Overproduction in Insulin Resistance

4.1 Rationale, Hypothesis and Research Objectives

4.1.1 Rationale

Insulin resistance is an extremely common pathophysiological trait that is implicated in the development of a number of important human diseases including type 2 diabetes, obesity, atherosclerosis, hypertension, and dyslipidemia (Reaven, 1995). The atherogenic dyslipidemia commonly associated with insulin resistant states is characterized by hypertriglyceridemia, increased very low-density lipoprotein (VLDL) secretion from the liver, increased small, dense low density lipoprotein (LDL) (Reaven, et al., 1993), and decreased high density lipoprotein (HDL) cholesterol (Taskinen, 1995). It appears that hepatic overproduction of apoB-containing VLDL particles is the major pathophysiologic basis of insulin resistance-associated dyslipidemia. Several different animal models have been used to study various pathologic manifestations of insulin resistance including dyslipidemia (for details refer to Chapter 1). Unfortunately, in most of these studies, the metabolic basis of hepatic VLDL-overproduction has not been investigated at the cellular and molecular levels and thus a significant gap of knowledge exists on the molecular mechanisms that mediate hepatic VLDL overproduction. Furthermore, lack of similarity between apoB biogenesis in human liver and liver of more frequently used animal models (rat and mouse) has made it difficult to draw conclusions based on existing data. As it was discussed in Chapter 3, the Syrian golden hamster exhibits close similarity to human in terms of lipoprotein metabolism. Hamsters develop metabolic syndrome including dyslipidemia in
response to fructose feeding and thus fructose-fed hamsters were used to investigate hepatic VLDL secretion.

4.1.2 Hypothesis

Fructose feeding induces insulin resistance and hepatic VLDL overproduction in the hamster. VLDL overproduction is associated with enhanced intracellular apoB stability and facilitated lipoprotein assembly in insulin resistant hepatocytes.

4.1.3 Research Objectives

Objective 1: Induce insulin resistance in male Syrian golden hamsters by feeding them with a high fructose-diet.

Objective 2: Study molecular and cellular mechanisms regulating apoB100 synthesis, degradation, assembly, and secretion in primary hamster hepatocytes isolated from chow-fed and fructose-fed insulin resistant hamsters.

4.2 Results

4.2.1 Metabolic Effects of Fructose Feeding in Syrian Golden Hamsters

Fig. 4.1 shows the physiological changes observed in control and fructose-fed hamsters after a two week feeding period. Fructose-fed hamsters gained body weight at approximately the same rate as that for control hamsters over the two week feeding period. Fructose-fed hamsters showed a significant elevation of plasma TG (p=0.0309) and an elevation of plasma cholesterol level that approached statistical significance (p=0.0550), following a two week period on a fructose-rich diet (Fig. 4.1A and 4.1B). There was also a significant elevation (p=0.0110) of plasma insulin level (Fig. 4.1C), a significant elevation of plasma free fatty acids (p=0.0045) as shown in Fig. 4.1D. However, plasma glucose levels did not differ significantly (p=0.9452) between control and fructose-fed hamsters (Fig. 4.1E).
Overall, fructose feeding induced significant elevation in plasma levels of TG, insulin, and free fatty acids.

4.2.2 Effect of Fructose Feeding on Hepatic Synthesis and Secretion of Lipids

Primary hamster hepatocytes isolated from normal chow-fed and fructose-fed hamsters were used to determine the synthesis and secretion of cholesterol, cholesteryl ester, and TG. Fig. 4.2 shows the effect of fructose feeding on the hepatic synthesis and secretion of total lipids. There was a small decrease in cellular levels of cholesteryl ester, although this change was not statistically significant (Fig. 4.2A). However, the intracellular levels of TG and free cholesterol were both significantly increased in hepatocytes from fructose-fed hamsters (Fig. 4.2A). Analysis of radiolabeled lipids in culture media of primary hamster hepatocytes also revealed no significant change in cholesteryl ester secretion (Fig. 4.2B). Interestingly however the secretion of TG was significantly elevated in fructose fed hamsters (Fig. 4.2B). Conversely, hepatocytes from fructose-fed hamsters secreted significantly lower levels of free cholesterol (Fig. 4.2B). The decline in free cholesterol secretion (p= 0.032, n=4) was accompanied by an increase in its intracellular levels (p<0.05, n=4), suggesting that fructose-feeding of hamsters has an inhibitory effect on the release of free cholesterol from hepatocytes. In the case of TG, both the cellular (p<0.05, n=4) and secreted (p<0.04, n=4) levels were elevated, suggesting that fructose-feeding enhanced the synthesis of TG and its secretion from the cell.

We also analyzed the secreted levels of core lipids associated with VLDL particles secreted by primary hepatocytes. Following radiolabeling of hamster hepatocytes, the cultured media was subjected to ultracentrifugation to isolate the VLDL fraction. The radiolabeled lipids associated with media VLDL were then analyzed by solvent extraction...
and thin layer chromatography. Secretion of VLDL-TG was also significantly induced in fructose-fed hamsters whereas VLDL-cholesteryl ester secretion was unaffected by fructose feeding. The observed increase in VLDL-TG secretion compared well with the increase in the intracellular and secreted levels of total TG reported in Figs. 4.2A and B.

4.2.3 Overproduction of VLDL-apoB in Hepatocytes from Fructose-Fed Hamsters

Primary hepatocytes isolated from hamster liver secrete apoB at a density of VLDL (Refer to the Chapter 3). To determine the effect of fructose feeding on VLDL-apoB secretion, we performed in vitro steady state labeling experiments in which hepatocytes from control and fructose-fed hamsters were radiolabeled for a 2 h period. Culture media containing secreted lipoprotein particles was then collected and subjected to ultracentrifugation to isolate VLDL. Radiolabeled apoB associated with VLDL particles was immunoprecipitated and analyzed by SDS-PAGE and fluorography. Fig. 4.3 shows the immunoprecipitable VLDL-apoB secreted by control and fructose-fed hepatocytes. There was a highly significant (4.6 fold) elevation in the amount of VLDL-apoB secreted into the media in fructose-fed hepatocytes. Increased VLDL-apoB levels suggest the secretion of a considerably higher number of VLDL particles by fructose-fed hepatocytes compared to control hepatocytes.

4.2.4 Turnover of apoB in Control and Fructose-Fed Hepatocytes

We employed pulse-chase labeling experiments to assess the stability and secretion of apoB in hepatocytes isolated from control and fructose-fed hamsters. Isolated hepatocytes were pulsed for 45 min and then chased for 1 and 2 h. Cellular and media apoB was immunoprecipitated and analyzed by SDS-PAGE and fluorography. Fig. 4.4 shows the

120
FIGURE 4.1

Effect of fructose feeding on plasma lipids, insulin, free fatty acids, and glucose

Male Syrian golden hamsters were fed either a control diet (standard chow) or fructose enriched diet for a two-week period. Blood samples were collected in EDTA from the orbital sinus, before and after feeding and plasma levels of lipids, insulin, free fatty acids, and glucose were determined. A) Plasma cholesterol, B) plasma TG, C) plasma insulin, D) plasma free fatty acids, and E) plasma glucose concentrations. All determinations are mean ± SD of 4-7 animals per group.
FIGURE 4.1

A. Plasma Cholesterol (mmol/L) with p = 0.0550

B. Plasma Triglyceride (mmol/L) with p = 0.0309

C. Plasma Insulin (pmol/L) with p = 0.0110

D. Free Fatty Acids (mmol/L) with p = 0.0045

E. Plasma Glucose (mmol/L) with p = 0.9452

[Graphs showing control vs. fructose-fed groups]
FIGURE 4.2

Synthesis and secretion of newly-synthesized lipids in control and fructose-fed hepatocytes

Primary hepatocytes immediately following attachment to culture plates were pulsed for 18 h with 5 μCi/ml [3H]acetate to assess the rates of synthesis and secretion of cholesterol, and cholesteryl ester. TG synthesis and secretion were monitored by labeling cells for 3-5 h with 5 μCi/ml [3H]oleate bound to bovine serum albumin. (A) Cellular levels of cholesterol, cholesteryl ester, and TG; (B) Secreted levels of cholesterol, cholesteryl ester, and TG. (mean ± SD, n=4). * , ** and *** significant differences (p<0.05, p<0.04, and p= 0.032, respectively).
FIGURE 4.2

A Cellular

B Secreted

- Control
- Fructose-Fed

124
intracellular turnover and extracellular secretion of apoB in control and fructose-fed hepatocytes. A large percentage of newly-synthesized, radiolabeled apoB disappeared from control cells over the 2 h chase with a small percentage appearing in the media (Fig. 4.4A and B). The disappearance rate of apoB in fructose-fed hepatocytes was considerably slower, with only about 25% of apoB having been lost during the 2 h chase (Fig. 4.4A). The increased stability of apoB in fructose-fed hepatocytes was accompanied by a dramatic increase in the secreted level of newly-synthesized apoB. As shown in Fig. 4.4B, fructose-fed hepatocytes secreted about 20% of newly-synthesized apoB compared to only about 5% in control cells. Overall, intracellular stability of nascent apoB was found to be significantly enhanced in hepatocytes isolated from fructose-fed hamsters, which appeared to result in a considerable increase in the accumulation of apoB in the extracellular media.

4.2.5 Stability of ApoB in Permeabilized Primary Hamster Hepatocytes

Permeabilized cells have been used previously to investigate posttranslational degradation of apoB, allowing for detection of specific degradation intermediates, including a 70 kDa fragment (Adeli, 1994, Sallach and Adeli, 1995). We also applied the permeabilization protocol to primary hamster hepatocytes and have investigated hamster apoB stability and turnover in this cell model system (see Chapter 3). Utilizing the permeabilized cell model system, we attempted to determine the effect of fructose feeding on the turnover of apoB. Control and fructose-fed hepatocytes were pulse-labeled, permeabilized, and then chased for a 2-3 h period. Fig. 4.5 shows the turnover of full-length hamster apoB100 in permeabilized control and fructose-fed hepatocytes. Hamster apoB100 was significantly more stable in fructose-fed hepatocytes as judged from the considerably higher intracellular level of apoB remaining in permeabilized cells after a 3 h chase. There
was approximately a 2-fold higher level of apoB100 remaining in fructose-fed hepatocytes following completion of the chase period (Fig. 4.5). The higher intracellular stability of apoB in permeabilized cells compared well with the above data in intact cells and further confirms the notion that in fructose-fed hepatocytes post-translational stability of apoB is enhanced.

4.2.6 Translational Status of Hamster ApoB in Control and Fructose-Fed Hepatocytes

The increased stability of hamster apoB100 in fructose-fed hepatocytes as evidenced by the data presented in Figs. 4.4 and 4.5, could result from an enhanced translational efficiency of apoB across the membrane of the ER, as has been argued in a number of previous studies on intracellular apoB biogenesis (Bonnardel and Davis, 1995, Macri and Adeli, 1997b, Thrift, et al., 1992). To investigate this possibility, we assessed the trypsin sensitivity of newly-synthesized hamster apoB100 in control and fructose-fed hepatocytes using a previously published permeabilized cell protocol (Macri and Adeli, 1997b). Hepatocytes were initially pulse-labeled, permeabilized, and then subjected to exogenous trypsin treatment. Fig. 4.6 shows the percent intact apoB100 recovered from cells treated with or without exogenous trypsin. There was no significant difference observed in trypsin sensitivity of hamster apoB100 in control vs. fructose-fed hepatocytes. Since the trypsin resistant apoB pool detected in this experiment represents the proportion of apoB fully translocated across the ER membrane, the data suggest that the translational efficiency of hamster apoB100 is unaltered with fructose feeding. It is thus intriguing that despite a significant increase in stability of hamster apoB in fructose-fed hepatocytes, its translational status does not appear to change under this metabolic condition.
Primary hamster hepatocytes were pulsed for 2 h with 100 μCi/ml [35S]methionine and [35S]cysteine. Culture media was collected, density adjusted to 1.006 g/ml, and adjusted media was subjected to ultracentrifugation for 18 h at 35000 rpm in a SW55 rotor to float the VLDL fraction. The VLDL fraction was then collected and was immunoprecipitated with an specific anti-hamster apoB antibody. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. Quantitation of apoB was performed by scintillation counting of the apoB100 band. (mean ± SD, n=4). Recovered VLDL-apoB was normalized against total TCA-precipitable radioactivity in each condition prior to converting to percentages. *significantly different from control (p<0.05).
FIGURE 4.3

![Graph showing VLDL apoB secretion (% of control) for Control and Fructose-Fed groups.](image-url)
Primary hamster hepatocytes were pulsed for 45 min with 100 μCi/ml [³⁵S]methionine, and the radioactivity was chased for 1 and 2 h in the presence of 5 mM excess cold methionine. Media and cells were collected and apoB was immunoprecipitated with a specific anti-hamster apoB antibody followed by SDS-PAGE and fluorography. Quantitation of apoB was performed by scintillation counting of the apoB100 band. A) apoB stability expressed as percent apoB remaining in cells+media (total apoB) in control and fructose-fed hepatocytes at 0 time (beginning of the chase), 1 h chase, and 2 h chase. * and ** indicates significant difference (p values <0.02 and <0.001, respectively). B) distribution of immunoprecipitable apoB in cells and media expressed as a percentage of radiolabeled apoB in cells at 0 time. (mean ± SD, n=3). Data were normalized against total TCA-precipitable radioactivity in each dish prior to converting to percentages. *Significantly different from control (p<0.05).
FIGURE 4.4

A

Percent ApoB Remaining

Chase Time (h)

Fructose-Fed

Control

B

Percent ApoB in Cells or Media

Chase Time (h)

0 1 2 1 2 1 2 1 2

Cells Media

Control Fructose-Fed

* *
Posttranslational stability of hamster apoB in permeabilized primary hepatocytes

Primary hamster hepatocytes were pulsed for 45 min with 100 μCi/ml [³⁵S]methionine. Cells were then permeabilized with digitonin (50 μg/ml), and the permeabilized cells were incubated in CSK buffer for 2 and 3 h prior to immunoprecipitation with an anti-hamster apoB antibody. Hamster apoB100 radioactivity was quantified by cutting and scintillation counting of the bands and apoB degradation was assessed by calculating the total apoB remaining under various conditions. Data were normalized against total TCA-precipitable radioactivity in each dish. *Significantly different from control (p<0.05).
FIGURE 4.5

[Bar chart showing ApoB100 levels over time for Control and Fructose Fed groups.]

ApoB/Total Protein x 200

Chase Time (h)

0 2 3

Control
Fructose Fed

*
Trypsin sensitivity of hamster apoB in permeabilized hamster hepatocytes

Primary hamster hepatocytes (1.5 x 10^6/dish) were pulsed for 45 min with [35S]methionine, and then permeabilized with digitonin, and the permeabilized cells were incubated with trypsin to digest any untranslocated apoB chains. Hamster apoB was immunoprecipitated and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. A representative fluorograph of the apoB translocation experiment in control and fructose-fed hepatocytes is shown in (A). ApoB radioactivity was quantitated (B) by cutting and scintillation counting of the apoB100 band (mean ± SD, n=4, p= 0.51). Data were normalized against total TCA-precipitable radioactivity prior to converting to percentages.
FIGURE 4.6

<table>
<thead>
<tr>
<th>Trypsin</th>
<th>CONTROL</th>
<th>FRUCTOSE FED</th>
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![Bar chart showing percentage of intact α-galB](chart)

- Untreated Control
- Trypsin-treated (P = 0.5)
4.2.7 Effect of Fructose-Feeding on Intracellular Assembly of ApoB-Containing Lipoproteins

Evidence presented above demonstrating that fructose-fed hepatocytes secreted a significantly higher amount of VLDL-apoB and VLDL-TG, strongly suggest enhanced efficiency of lipoprotein assembly with fructose feeding. To directly investigate the formation of apoB-containing lipoprotein particles in hamster hepatocytes, cells were pulse-labeled, chased for 0 and 1 h and then subjected to subcellular fractionation. Nascent lipoproteins accumulated in the microsomal lumen were fractionated by sucrose gradient centrifugation and immunoprecipitated with anti-hamster apoB antibody. Fig. 4.7 illustrates the pattern of nascent lipoproteins accumulated in the lumen of control hepatocytes compared with that of lipoproteins detected in fructose-fed hepatocytes. Luminal apoB-containing lipoproteins in control hepatocytes were predominantly recovered from the bottom (fractions 1-2) and the top (fraction 12) fractions plus some in the fractions 6-8 which corresponded to densities of HDL, VLDL, and LDL respectively. Whereas in the lumen of the hepatocytes isolated from fructose-fed hamsters only two types of radiolabeled apoB-containing particles were recovered: the minority of counts were found in the LDL fraction (fractions 6-8) and the majority of the counts were recovered from the top fraction or VLDL fraction. The efficiency of this method of fractionation has been well documented (Adeli, et al., 1997b, Boren, et al., 1992, Boren, et al., 1990). There was however a considerable discrepancy as to the ratio of VLDL to HDL-like lipoproteins in control vs. fructose-fed hepatocytes. Control cells had a significantly higher level of HDL-like lipoproteins which are in fact secretion-incompetent (Fig. 4.7A). In contrast, most of the apoB-containing lipoproteins formed in the lumen of microsomes from fructose-fed hepatocytes at 1 h chase had a VLDL-like density
and no HDL-like particle was detected (Fig. 4.7B). This observation suggests that a considerable pool of nascent hamster lipoproteins may form a dense, secretion-incompetent pool in normal hamster hepatocytes as previously reported in HepG2 cells (Boren, et al., 1992, Boren, et al., 1990). The absence of HDL-like apoB-containing lipoproteins in microsomes of fructose-fed hepatocytes may in turn suggest a higher efficiency of lipoprotein assembly under this metabolic condition. It should be noted however that at 1 h chase, there was still a significant amount of VLDL particles in the microsomal lumen of both control and fructose-fed hepatocytes, suggesting delayed secretion of these assembled particles. This observation may however result from the long pulse period (45 min) used in the experiment in order to achieve sufficient synthesis and assembly of apoB-containing lipoproteins in this primary cell system (which requires a much longer pulse period in such experiments compared to established cell lines such as HepG2). A chase period of 3 or more hour is necessary to achieve complete secretion of newly-assembled lipoproteins by hamster hepatocytes after a 45 min pulse. However, even at 1 h chase, there was some secretion of VLDL-apoB in media of fructose-fed hepatocytes arguing against a defect in its secretion.

4.2.8 Evidence that Direct Incubation with Fructose Does Not Directly Affect Hepatic ApoB Secretion by Primary Hamster Hepatocytes

It was important to determine if fructose can directly induce the hepatic synthesis and secretion of apoB100 in hamster hepatocytes since such a direct effect would complicate the interpretation of our data relating apoB overproduction to the development of fructose-induced insulin resistance. Freshly isolated hamster hepatocytes from control, chow-fed hamsters were incubated with different concentrations of fructose for a 24 h period and synthesis and secretion of apoB were monitored by pulse-labeling with [35S]methionine. Fig.
4.8A shows a dose-response study of the effect of fructose on hepatic apoB. Cellular accumulation and extracellular secretion of apoB were unaffected in the presence of increasing concentrations of fructose in the culture media. Even at the highest concentration of 3 mM, there was no significant effect on the synthesis or secretion of apoB in primary hamster hepatocytes. To further confirm a lack of direct effect of fructose on hamster apoB biogenesis, we incubated cultured hepatocytes for a period of up to 3 days with exogenous fructose at the highest concentration (3 mM). Cells incubated for 1, 2, or 3 days were then subjected to pulse-chase labeling (45 min pulse, 1-2 h chase) to determine the extent of hamster apoB secretion and its intracellular stability in hamster hepatocytes. Fig. 4.8B-G show the effects of fructose incubation for periods of 1-3 days. Panels B, D, and F show hamster apoB secretion, while panels C, E, and G show the stability of apoB as assessed by the total apoB remaining in cells and media over a 2 h chase. There was no detectable stimulation of apoB secretion or stability with fructose treatment for up to 3 days. There was actually some inhibition of apoB secretion observed at day 2, but overall the entire experiment revealed no specific effect. Longer incubation of hepatocytes was not attempted due to problems associated with cell lifting and loss of hepatocyte-specific functions when cells are cultured for more than 3 days. We have several lines of evidence however showing that hamster hepatocytes maintain their hepatocyte specific functions during at least the first 3 days in culture.
Intracellular distribution of nascent apoB-containing lipoproteins in microsomal lumen of control and fructose-fed hepatocytes

Cultured primary hamster hepatocytes were pulsed for 45 min with [\textsuperscript{35}S]methionine and the radioactivity was chased for 0 or 1 h. Labeled cells (five dishes of $1.5 \times 10^6$ cells for each chase point) were then combined and subjected to homogenization and fractionation of microsomes. Luminal lipoproteins were extracted from microsomes by carbonate treatment and were separated from the membrane fraction by centrifugation (SW55, 35000 rpm, 93 min). Fractionation of luminal lipoproteins was performed by sucrose-gradient centrifugation (SW41, 35000 rpm, 65 h). After centrifugation, gradient fractions were collected and immunoprecipitated with an anti-hamster apoB antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography and apoB radioactivity was quantitated by cutting and scintillation counting of the apoB100 band. A) Luminal lipoproteins in control hepatocytes at 0 h and 1 h chase; B) Luminal lipoproteins in fructose-fed hepatocytes at 0 h and 1 h chase.
Effect of in vitro incubation of primary hamster hepatocytes with fructose on synthesis, secretion and stability of hamster apoB

A) Control hamster hepatocytes were incubated in the presence of different concentrations of fructose for 24 h. Cells were then pulsed for 2 h with 100 μCi/ml \( ^{35}S \)methionine. Culture media were immunoprecipitated with an anti-hamster apoB antibody and immunoprecipitates were analyzed by SDS-PAGE and fluorography. B-G) control hamster hepatocytes were incubated in the presence (closed circles) and absence (open circles) of fructose (3 mM) for 1, 2, and 3 days. Following 1-3 days of treatment, primary hamster hepatocytes were pulsed for 45 min with 100 μCi/ml \( ^{35}S \)methionine, and the radioactivity was chased for 1 and 2 h in the presence of 10 mM excess cold methionine. Pulse-chase experiments were conducted in the presence and absence of fructose (3 mM). Media and cells were collected and apoB was immunoprecipitated with a specific anti-hamster apoB antibody followed by SDS-PAGE and fluorography. Quantitation of apoB band was performed by cutting and scintillation counting of the apoB\(_{100}\) band. ApoB band counts were normalized against TCA-precipitable radioactivity. Panels B, D, F show secreted radiolabeled apoB at 1 and 2 h chase at 1, 2, and 3 days of incubation. Panels C, E, G show the rates of apoB turnover expressed as total radiolabeled apoB remaining in cell+media at 0, 1, and 2 h chase at 1, 2, and 3 days of treatment (Mean ± SD, n=3). * and ** indicates significant differences (p values, 0.05 and 0.008, respectively).
FIGURE 4.8

- [Graph A] Radiolabeled ApoB (CPM) vs. Fructose Concentration (mM)
- [Graph B] Day 1 - Media ApoB, Labeled ApoB (CPM/Total Protein) vs. Chase Time (h)
- [Graph C] Day 2 - Media ApoB, Labeled ApoB (CPM/Total Protein) vs. Chase Time (h)
- [Graph D] Day 3 - Media ApoB, Labeled ApoB (CPM/Total Protein) vs. Chase Time (h)
- [Graph E] Day 1 - Total ApoB Remaining, Labeled ApoB (CPM/Total Protein) vs. Chase Time (h)
- [Graph F] Day 2 - Total ApoB Remaining, Labeled ApoB (CPM/Total Protein) vs. Chase Time (h)
- [Graph G] Day 3 - Total ApoB Remaining, Labeled ApoB (CPM/Total Protein) vs. Chase Time (h)
4.2.9 Evidence for Enhanced Expression of MTP Mass, mRNA Levels, and Increased MTP Activity in Fructose-Fed Hepatocytes

Facilitated assembly of apoB-containing lipoprotein particles in fructose-fed hepatocytes could be related to an increased mass and/or activity of MTP (microsomal triglyceride transfer protein), the key factor involved in the lipoprotein assembly process. To test this hypothesis, a specific anti-hamster MTP antibody was used to estimate the protein mass of MTP in control and fructose-fed hepatocytes. Equal quantities of total cell lysate (1 μg) were analyzed by SDS-PAGE and then subjected to immunoblotting with the anti-hamster MTP antibody. Fig. 4.9A shows the immunoblotting analysis of lysates from control and fructose-fed hepatocytes. There was approximately two fold higher (p=0.02) cellular protein mass of MTP in fructose-fed hepatocytes compared to control hepatocytes after correction for total protein concentration of the cell lysates analyzed. Fig. 4.9B illustrates the analysis of duplicate aliquots of hepatocyte cell lysates from two different control hamsters and two fructose-fed hamsters. This representative experiment was repeated once with similar results. To further confirm the above observations, MTP mRNA levels were measured using RNase protection assay. MTP mRNA levels in insulin resistant hepatocytes were 47% higher (p<0.02) compared to that in the control cells (Fig. 4.9C). In order to evaluate the impact of elevated MTP protein mass and mRNA on its functionality, MTP activity was measured in hepatocytes isolated from control and fructose-fed hamsters, as described in the Materials and Methods section. As depicted in Fig. 4.9D, MTP activity in hepatocytes isolated from fructose-fed hamsters was significantly higher than control hepatocytes (177.5 ± 14.5% of that of control, n= 3, P= 0.042) suggesting enhanced MTP lipid transfer activity in fructose-fed hamsters.
FIGURE 4.9

Evidence for Enhanced Expression of MTP Mass, mRNA Levels, and Activity in Fructose-Fed Hepatocytes

Panels A & B: Control and fructose-fed hepatocytes were solubilized, and equal amounts of cell protein (1 μg) were subjected to SDS-PAGE (10% (v/v) acrylamide resolving gel) and proteins were then transferred onto nitrocellulose membranes. Immunoblotting was performed to detect the 97 kDa MTP subunit with a rabbit anti-bovine MTP antiserum. (A), the autoradiograph of the MTP immunoblot. In (B), the MTP bands were quantitated by densitometric scanning and the mass of the 97 kDa MTP subunit detected was normalized to the μg of total protein mass and then expressed as a percentage of the MTP mass detected in control cells (P= 0.02). Panel C: MTP mRNA levels were analyzed by RNase protection/solution hybridization assays as described in the Materials and Methods section. mRNA was quantitated using standard curves of cRNA. The results are expressed as pg mRNA per μg of total RNA. Values are given as the mean ±SD from three experiments. P < 0.023 vs. control. Panel D: Equal amounts of microsomal proteins prepared from hepatocytes isolated from control and fructose-fed hamsters were subjected to MTP activity assay as described in the Materials and Methods section. The results are expressed as percentage of MTP activity in control hepatocytes. Values are given as the mean ± SD from four experiments (P= 0.042).
FIGURE 4.9

A

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97 kDa MTP

B

MTP Protein Mass (as a percent of control)

Control | Fructose-Fed

P = 0.02

C

PG.MTP RNA/μg Total RNA

Control | Fructose-Fed

P < 0.02

D

MTP Activity (Percent of Control)

Control | Fructose-fed

P = 0.042
CHAPTER FIVE

Hepatic VLDL-apoB Overproduction is Associated with Attenuated Hepatic Insulin Signaling in a Fructose-Fed Hamster Model of Insulin Resistance: Evidence for increased expression of PTP-1B and decreased abundance of ER60 protease

5.1 Rationale, Theory, and Research Objectives

5.1.1 Rationale

Acute insulin exposure is known to suppress hepatic apoB production in ex-vivo cellular experiments as well as human and animal model studies (Sparks and Sparks, 1994a). It is believed that insulin exerts its effects through the insulin receptor signaling pathway. In fact, this suppressive action of insulin on apoB production is PI 3-kinase-dependent (Phung, et al., 1997). Acute insulin exposure of hepatocytes reduces apoB secretion by attenuating mRNA translation, increasing its cellular apoB degradation and possibly by interfering its assembly into lipoproteins (by negative regulatory effects on MTP) (refer to the Chapter 1). On the contrary, in case of insulin resistance, where hepatocytes are chronically exposed to a high level of insulin, liver overproduces apoB-containing lipoproteins. Indeed, overproduction of hepatic apoB-containing lipoproteins is considered a hallmark of insulin resistance associated dyslipidemia (Betteridge, 2000). Despite numerous in vivo animal and human studies of insulin resistance-associated dyslipidemia, literature search shows that molecular and cellular aspects of apoB overproduction in insulin resistant hepatocytes have been surprisingly understudied.

Data presented in Chapter 4 suggested that in hepatocytes isolated from insulin resistant hamsters, higher cellular stability and facilitated assembly (MTP elevation) contributed to the overproduction of apoB-containing lipoproteins. Here we have attempted
to define hepatic insulin resistance at the level of insulin signaling proteins in order to link chronic high insulin exposure, insulin resistance to the observations reported in Chapter 4.

5.1.2 Hypothesis

Impairment of the hepatic insulin signal transduction pathway may contribute to the overproduction of apoB-containing lipoproteins in hepatocytes isolated from fructose-fed hamsters.

5.1.3 Research Objectives

Objective 1: In order to show impaired insulin signal transduction in fructose-fed hamsters, conduct comparative assessment of insulin signaling pathway (phosphorylation, mass, and/or activity) in hepatocytes isolated from control and fructose-fed hamsters, by examining insulin receptor (IR), IRS-1, IRS-2, PTP-1B, PI 3-kinase, and Akt/PKB.

Objective 2: To demonstrate if chronic high insulin exposure of hepatocytes is the determinant factor in induction of insulin resistance, conduct ex vivo studies by chronic incubation of control hepatocytes with high insulin levels and monitor insulin signaling pathway (IR-tyrosine phosphorylation and mass, PTP-1B mass)

Objective 3: To determine if (ex vivo) induction of insulin resistance as a consequence of high chronic insulin exposure of control hepatocytes results in apoB overproduction

Objective 4: Demonstrate if improvement of insulin resistance in hepatocytes isolated from fructose-fed hamsters coincides with reduction of hepatic apoB secretion.

5.2 Results

5.2.1 Effect of Fructose Feeding on the Phosphorylation Status and Protein Mass of the Insulin Receptor, IRS-1, and IRS-2 in Hepatocytes of Syrian Hamsters

Phosphorylation status of the insulin receptor and its substrate proteins were assessed
in hepatocytes derived from control and fructose-fed hamsters. As depicted in Figure 5.1A, basal insulin receptor phosphorylation (in the absence of insulin) in hepatocytes isolated from control and fructose-fed hamsters was too weak to be detected by immunoprecipitation and immunoblotting methods. As expected, insulin treatment in vitro caused a significant elevation of IR phosphorylation in both control and FF hepatocytes. However, in control hepatocytes, insulin-induced phosphorylation of its receptor was about 2-fold (n=3, p=0.001) higher than that induced in hepatocytes isolated from fructose-fed hamsters. To examine endogenous substrate phosphorylation, immunoprecipitated IRS-1 was subjected to Western blotting with an anti-phosphotyrosine antibody as described in Materials and Methods. Densitometric analysis (Fig. 5.1B) revealed that in hepatocytes isolated from fructose-fed hamsters, both basal and insulin-induced phosphorylation of IRS-1 were lower by more than 5-fold (n=3, p=0.01) and 11-fold (n=3, p=0.009) respectively, compared to that in control hepatocytes. Moreover, in control hepatocytes, insulin increased IRS-1 phosphorylation approximately two fold over basal (199.7 ± 34.5% of basal, n=3, p= 0.04), whereas in hepatocytes isolated from fructose-fed hamsters insulin failed to induce phosphorylation of IRS-1 (91.35 ± 12.2% of basal, n=3, p= 0.73). We also examined the effects of fructose feeding on the protein mass and phosphorylation level of IRS-2. As depicted in Fig. 5.1C, in control hepatocytes, stimulation with 100 nM insulin caused a 54.5 ± 3.4% (n=3, p=0.023) increase in tyrosine phosphorylation of IRS-2 compared to its basal level, whereas in hepatocytes isolated from fructose-fed hamsters insulin increased phosphorylation only by 20 ± 7.4 % (n=3, p= 0.29) of basal, suggesting a significant impairment in IRS-2 mediated insulin signal transduction following fructose feeding. Reduced IRS-1 and IRS-2 phosphorylation in hepatocytes from fructose-fed hamsters is consistent with the data on
insulin receptor tyrosine phosphorylation and further supports the induction of hepatic insulin resistance in this model.

To investigate whether down-regulation of insulin receptor, IRS-1, and IRS-2 phosphorylation in hepatocytes isolated from fructose-fed hamsters was related to changes in the intracellular mass of these signaling molecules, we examined cellular levels of insulin receptor, IRS-1, and IRS-2 by immunoblotting. As shown in Figure 5.1D, in hepatocytes isolated from fructose-fed hamsters, the mass of insulin receptor was 92.4 ± 12% of that in control hepatocytes, suggesting no significant changes in receptor protein mass with fructose feeding. Figure 5.1E shows protein expression levels of IRS-1. Compared to control hepatocytes, IRS-1 protein levels were significantly lower (31.8± 1%, n=3, p=0.01), indicating a significant downregulation of IRS-1 in fructose-fed hamster hepatocytes. We also determined protein expression levels of IRS-2 in hepatocytes isolated from control and fructose-fed hamsters. As shown in Fig. 5.1F, IRS-2 protein mass in insulin resistant hepatocytes was dramatically reduced to 17.5 ± 0.4% (n=3, p=0.01) of that in control hepatocytes.

5.2.2 PI 3-kinase Activity in Hepatocytes Isolated from Control and Fructose-fed Hamsters

PI 3-kinase activity was assessed in two ways. Total activity was measured in immunoprecipitates generated using an antibody specific to the p85 subunit of PI 3-kinase. Figure 5.2A demonstrates total PI 3-kinase activity (normalized to total protein) as a percentage of the activity in the control hepatocytes. There was no significant difference in total PI 3-kinase activity between cells derived from control and fructose-fed livers. The second assay was designed to assess the PI 3-kinase activity associated with insulin receptor
substrates (i.e. the pool activity recruited to the insulin signaling pathway). These experiments were performed on cell lysates immunoprecipitated with an anti-phosphotyrosine antibody. As shown in Fig. 5.2B, PI 3-kinase activity associated with tyrosine phosphorylated proteins was about 25% lower in hepatocytes isolated from fructose-fed hamsters (74.7 ± 3.3% of control, n=3, p=0.03).

5.2.3 Evidence that Intracellular Level and Activity of PTP-1B are Enhanced in Hepatocytes Isolated from Fructose-fed Hamsters

Protein-tyrosine phosphatases, particularly PTP-1B, play an important role in regulating the phosphorylation status of proteins involved in insulin signaling (for a recent review see Byon, et al., 1998). To investigate the possible role of PTP-1B in the impairment of signal transduction in liver of fructose-fed hamsters, immunoblot experiments were conducted using a specific anti-PTP-1B polyclonal antibody. Figure 5.2C shows a representative Western blot for PTP-1B and the quantification of data obtained from 3 independent experiments. These experiments revealed that PTP-1B protein levels in hepatocytes isolated from fructose-fed hamsters were significantly higher (147.03 ± 22.6%, mean ± SD, p= 0.004, n= 3) compared to that of control hepatocytes.

We also conducted experiments to measure PTP-1B activity in hepatocytes isolated from control and fructose-fed hamsters. PTP-1B activity in hepatocytes isolated from fructose-fed hamsters was significantly increased by almost two fold (193 ± 51.9%) compared to that in control hepatocytes (n=8, p= 0.0021) (Fig. 5.2D). These results parallel the above observation of an elevated protein mass of PTP-1B and together suggest enhanced expression and activity of this phosphatase in liver of fructose-fed hamster.
FIGURE 5.1

Insulin receptor, IRS-1, and IRS-2 phosphorylation status and protein mass in hepatocytes isolated from control and fructose-fed hamsters

Hepatocytes freshly isolated from control and fructose-fed hamsters were incubated in serum- and insulin-free media for 5 h. Cells were then divided into two groups, one group (baseline) was lysed immediately after 5 h incubation while the second group was subjected to 10 min stimulation with 100 nM insulin before being solubilized. Cell lysates were first immunoprecipitated for either insulin receptor β subunit, IRS-1, or IRS-2 and then immunoblotted using a monoclonal antibody against phosphotyrosine groups as described in Materials and Methods. Each panel depicts a representative immunoblot along with combined densitometric quantitation of multiple experiments. Net intensity of the bands was normalized for the total protein content of the samples. A) Phosphorylated insulin receptor. Data were collected from 5 experiments performed in duplicate or triplicate. *significantly different (p = 0.001). B) Phosphorylated IRS-1. Data were collected from 3 experiments performed in duplicate or triplicate. C) Phosphorylated IRS-2. Data were collected from 3 experiments performed in duplicate. D) Insulin receptor β subunit protein mass (basal level). Data were collected from 5 experiments performed in duplicate or triplicate. E) IRS-1 protein mass (basal level). Data were collected from 3 experiments performed in duplicate or triplicate. *significantly different (p = 0.01). F) IRS-2 protein mass. Data were collected from 3 experiments performed in triplicate and normalized for the total protein content of the samples prior to converting to percentages. *significantly different (p = 0.001). All data are shown as mean ± SD.
FIGURE 5.1

A

Control Fructose-fed

Insulin

- - + - - +

Phosphorylated Insulin Receptor Mass (pmol/mg protein x 10^-6)

- - + - - +

B

Control Fructose-fed

Insulin

- - + - - +

p=0.04 p=0.009

Phosphorylated IRS-1 Mass (pmol/mg protein x 10^-6)

- - + - - +

C

Control Fructose-fed

Insulin

- - + - - +

p=0.02 p=0.03

p=0.029

Phosphorylated IRS-2 (relative to basal level of control)

- - + - - +

D

Control Fructose-fed

Insulin

- - + - - +

E

Control Fructose-fed

Insulin

- - + - - +

F

Control Fructose-fed

Insulin

- - + - - +
5.2.4 Impaired Akt/PKB Serine and Threonine Phosphorylation in Hepatocytes Isolated from Fructose-fed Hamsters

In order to investigate insulin signaling status downstream of PI 3-kinase, we examined the phosphorylation status of serine473 and threonine308 of Akt/PKB, a key serine/threonine kinase, which mediates many metabolic effects of insulin. Figures 5.2E and F show serine and threonine phosphorylation of Akt/PKB in hepatocytes isolated from control and fructose-fed hamsters. Fructose feeding reduced insulin stimulated phosphorylation levels of serine473 and threonine308 to $29 \pm 15\%$ (n=4, p= 0.002) and $42.6 \pm 20.8$ (n=5, p= 0.001) of that of control hepatocytes respectively, indicating that phosphorylation, and therefore activity, of Akt/PKB with fructose feeding were significantly compromised. Immunoblotting for Akt mass (Fig. 5.2G) showed a small but significant increase ($39.3 \pm 1\%$, n=3, p= 0.03) in Akt/PKB protein expression levels in hepatocytes isolated from fructose-fed hamsters, suggesting a possible compensatory response to the suppressed phosphorylation status of the protein.

5.2.5 Insulin Signaling Status in Hepatocytes Exposed to High Insulin Concentrations Ex Vivo

Fructose-fed hamsters were previously shown to have an elevated plasma insulin level (see above), apparently due to peripheral insulin resistance as documented by the euglycemic-hyperinsulinemic clamp technique (Taghibiglou, et al., 2000). Such hyperinsulinemia may in turn be responsible for the downregulation of insulin signaling in the liver and the induction of hepatic insulin resistance observed in experiments above. To further examine this hypothesis, we directly incubated control hepatocytes with high insulin (1.0 $\mu$g/ml), for up to 3 days and measured the basal phosphorylation level of the insulin
FRUCTOSE FEEDING REDUCES PI 3-KINASE ACTIVITY AND AKT/ PKB PHOSPHORYLATION AND INCREASES THE PROTEIN MASS AND ACTIVITY OF PTP-1B IN HAMSTER LIVER

Panels A-B: Cell lysates were subjected to immunoprecipitation with anti-p85 or anti-phosphotyrosine antibodies. PI 3-kinase activity was measured on p85 (panel A) or phosphotyrosine immunoprecipitates (panel B), as described in Methods. The PI 3-kinase activity was normalized for total protein content of the samples and expressed as a percentage of activity detected in control hepatocytes. Data represent mean ± SD, n=3. *significant difference (p= 0.03). Panel C: Hepatocytes isolated from control and fructose-fed hamsters were solubilized and 20 μg of total cell lysate of each sample was subjected to SDS-PAGE and immunoblotting for PTP-1B as described in Materials and Methods. Corresponding bands were quantified by densitometry and expressed as a percentage of PTP-1B in control hamster hepatocytes. Shown is one representative immunoblot and combined quantitation of immunoblots from 3 experiments performed in triplicate. Mean ± SD, **significant difference (p= 0.004). Panel D: Hepatocytes isolated from control and fructose-fed hamsters were lysed in solubilization buffer. The lysates were centrifuged and supernatants were collected and 1 mg of total cell lysate of each sample was subjected to immunoprecipitation with anti-PTP-1B antibody. PTP-1B immunocomplexes were used to measure phosphatase activity using the pp60	cyt C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL, Biomol) as substrate. PTP-1B activity is expressed relative to the activity detected in control hepatocytes. Activity was measured in 5 control and 8 fructose-fed hamster hepatocyte preparations and is expressed as mean ± SD. *** significant difference (p= 0.0021). Panels E-F: Lysates from basal and insulin-stimulated cells were immunoblotted using polyclonal antibodies against phospho-Ser³⁸³-Akt (panel E) or phospho-Thr³⁰⁸-Akt (panel F). A representative immunoblot is shown along with densitometric quantification. Net intensity of the bands was normalized for the total protein content of the samples prior to converting to percentages. Data were collected from 4 experiments performed in duplicate. All data are shown as mean ± SD.*** significant difference (p= 0.0021) and **** significant difference (p= 0.001). Panel G: Whole cell lysates (30 μg) from control and fructose-fed animal hepatocytes were subjected to immunoblot analysis for total Akt mass and data were expressed as a percentage of Akt mass in control hamster hepatocytes. A representative immunoblot is shown with combined data from 3 experiments performed in duplicate or triplicate. Mean ± SD. *significant difference (p= 0.03).
receptor following each day of incubation. Figure 5.3A shows a representative immunoblot of the tyrosine phosphorylated insulin receptor following various periods of insulin exposure. For the purpose of these experiments the basal level of insulin receptor phosphorylation was taken to be that measured in freshly isolated, and therefore untreated, hepatocytes (Day 0). In cells incubated with high insulin, insulin receptor phosphorylation initially decreased from day 0 to day 1 and then increased from day 1 to day 2, but was substantially reduced by day 3 (856 ± 24, 520 ± 9, 852 ± 48, and 285 ± 69 arbitrary densitometric units/mg of total cell protein x 10^{-3} on days 0, 1, 2, and 3 of incubation, respectively). As depicted in Figure 5.3A, following 3 days of high insulin exposure, phosphorylation of the insulin receptor in high insulin-incubated cells, decreased to 33.3 ± 8.1% of control, suggesting possible desensitization of the insulin receptor under this metabolic condition.

Since the observed differences in insulin receptor phosphorylation status may be attributed to possible changes in the expression level of the insulin receptor, we also measured insulin receptor protein levels under the above experimental condition. Phosphotyrosine immunoblots were stripped and reprobed with a polyclonal antibody against the insulin receptor β subunit as described in Materials and Methods (Fig. 5.3B). Insulin receptor band intensity under the control condition (day 0) was measured as 1136 ± 16 arbitrary units/mg protein. High insulin incubation resulted in a slight but consistent reduction in the receptor protein, suggesting possible down-regulation of the receptor. The insulin receptor protein mass significantly decreased on the third day of incubation and reached 56.8 ± 14.1% (p= 0.025) of the basal level (day 0).

An arbitrary index (Specific Insulin Receptor Phosphorylation Index, SIRPI) was also calculated to better compare the phosphorylation status of the insulin receptor under control
and high insulin exposed conditions. The index is calculated as the ratio of phosphorylated insulin receptor to the total insulin receptor protein mass, as measured by densitometric quantification of Western blots, normalized for total cellular protein content. Figure 5.3C re-evaluates the data presented above in terms of the SIRPI. The SIRPI value for untreated, day 0 cells was 0.75 ± 0.01. After exposure to high insulin concentrations, the index remained constant and approximately equal to control (0.62 ± 0.02, and 1.01 ± 0.24) on days 1 and 2 of incubation, respectively, however, it dropped significantly to 0.4 ± 0.003 (p= 0.01) at day 3 of incubation, suggesting the induction of insulin resistance.

5.2.6 Chronic High Insulin Exposure Induces PTP-1B Expression in Hamster Hepatocytes

In order to investigate whether changes in the insulin signaling pathway may be related to alterations in expression of PTP-1B in hamster hepatocytes, we investigated protein levels of PTP-1B in control hamster hepatocytes incubated with 10 μg/ml insulin for 3 days. As shown in Figure 5.3D, immunoblotting of equal amounts of cell lysate (10 μg total cell protein) revealed that PTP-1B levels in hepatocytes incubated with high insulin gradually increased on day 1, reached a plateau on day 2 and day 3 of incubation. Cellular levels of PTP-1B on days 1, 2, and 3 were 108.3 ± 0.15%, 138.3 ± 2.76% (p= 0.003), and 134.8 ± 4.33% (p= 0.008) of the basal level (day 0), respectively. Thus elevation in cellular levels of PTP-1B occurred earlier on day 2 and appeared to precede the down-regulation of insulin receptor phosphorylation in hamster hepatocytes on day 3 (as shown above).

5.2.7 Chronic Exposure of Hepatocytes to High Insulin Induces ApoB Oversecretion

In order to assess the effect of long term high insulin incubation of control hamster hepatocytes on apoB biogenesis, hepatocytes were incubated with culture media
supplemented with 5% FBS and 1 μg/ml insulin for up to 3 days. After each day of treatment, cells were pulsed with \(^{35}\text{S}\)methionine and apoB secreted into the media was analyzed by immunoprecipitation, SDS-PAGE, and fluorography. As depicted in Fig. 5.4A, secreted apoB was slightly increased day 0 to day 1 and then remained unchanged on day 2 of exposure (0.124 ± 0.006 labeled apoB/ total labeled protein x 300). The apparent elevation in secreted apoB from day 0 to days 1 and 2 was not statistically significant (p=0.22). On the third day of incubation secreted apoB was significantly increased to 0.235 ± 0.006 labeled apoB/total labeled protein x 300), an increase of approximately 2.5 fold (p=0.04) over the basal level (day 0). Importantly, total protein synthesis did not change significantly during the incubation period (5649.6 ± 581, 4287.8 ± 811, 6456.8 ± 865, 5852.8 ± 622.4 CPM/μg cell protein on days 0, 1, 2, and 3 respectively). Thus, long term exposure to high insulin levels appeared to cause a significant hypersecretion of apoB, which coincided with desensitization of the cells to insulin and downregulation of the insulin signaling pathway.

5.2.8 ER-60 Protein Mass in Hepatocytes Isolated from Fructose-Fed Hamsters

We have previously shown that ER-60, a cysteine protease localized in ER-lumen localized, is associated with apoB intracellularly and may be involved in apoB degradation (Adeli, et al., 1997a). In order to investigate possible down-regulation of this important protease in hepatocytes isolated from fructose-fed hamsters, we conducted immunoblotting experiments using a rabbit polyclonal antibody against rat ER-60 that readily cross-reacts with hamster ER-60. As depicted in Fig. 5.4B, ER-60 protein levels in hepatocytes isolated from fructose-fed hamsters (n=8) were reduced to 14.7 ± 6.8% of that in control hepatocytes suggesting drastic down-regulation of ER-60 in insulin resistant hamster livers. In order to determine whether the suppression of ER-60 expression was a result of an impairment of
Chronic High Insulin Exposure of Control Hepatocytes Impaired Insulin Receptor Phosphorylation, Reduced IR Mass and Increased PTP-1B Mass

Primary hepatocytes isolated from control hamsters were incubated in culture medium containing high insulin (1.0 μg/ml), and 5% FBS for up to 3 days and phosphorylation status and mass of insulin receptor were assessed on a daily basis. Hepatocytes were solubilized and immunoprecipitated for insulin receptor β subunit and immunoprecipitates were then subjected to immunoblotting with a monoclonal antibody against phosphotyrosine. The same membrane was then stripped of antibody and reprobed for insulin receptor mass using a polyclonal antibody against the insulin receptor β subunit. Panel A: Representative immunoblot for tyrosine phosphorylated insulin receptor and its quantification (expressed as scanning units/ mg total protein). Data are shown as mean ± SD. *** significant difference (p= 0.001). Panel B: Representative immunoblot for insulin receptor β subunit and its quantification (per mg protein). Data are shown as mean ± SD. * and ** significant differences (p= 0.045 and p= 0.025, respectively). Panel C: SIRPI, an arbitrary parameter used to assess the phosphorylation status of the insulin receptor, taking into account variability in insulin receptor protein mass levels. The value consists of the ratio of phosphorylated insulin receptor (measured as described above) to total insulin receptor mass, as measured by densitometric quantitation of Western blots, normalized for total cellular protein mass. SIRPI is a dimensionless arbitrary parameter. Data are shown as mean ± SD. ** significant difference (p= 0.01). Panel D: Equal amounts of cell lysate (10 μg) were subjected to SDS-PAGE (8%) and immunoblotting for PTP-1B as described in Methods. Corresponding bands were scanned, quantified and normalized to the amount of protein loaded in each lane. This figure shows a representative immunoblot for PTP-1B and densitometric quantification of immunoblots from 3 experiments. Data are shown as mean ± SD. *** significant difference (p= 0.003).
insulin signal transduction in fructose-fed hamsters, we repeated the above experiments using hepatocytes isolated from fructose-fed hamsters treated with rosiglitazone, an insulin sensitizing drug (20 μmols/kg body weight, once daily for 3 weeks). As depicted in Fig. 5.4B, rosiglitazone significantly increased ER-60 protein levels relative to untreated fructose-fed animals, although it could not restore the ER-60 levels to the level observed in control hepatocytes. Control experiments showed that rosiglitazone treatment was also capable of enhancing insulin receptor phosphorylation in hamster livers.

5.2.9 ER-60 Suppression Can be Induced by High Insulin Exposure and is Accompanied by the Induction of Insulin Resistance

To further investigate whether ER60 protein levels change in response to insulin exposure, we incubated control hamster hepatocytes with high concentrations of insulin for up to 3 days and monitored expression levels of ER-60. As shown in Fig. 5.4C, ER-60 protein levels increased from 100 ± 14.4% on day 0 to 149.17 ± 13.27% (p= 0.01) on day 1 and remained constant on day 2 (153.72 ± 8.18%). Interestingly, on day 3 of incubation, where we have observed high PTP-1B levels and induction of insulin resistance (see above), ER-60 protein mass was dramatically decreased to 18.39 ± 1.9% of basal (day 0) levels (p= 0.007) demonstrating an association between the induction of insulin resistance and ER-60 suppression.

5.2.10 Vanadate Improves Tyrosine Phosphorylation of Insulin Receptor in Hepatocytes Isolated from Fructose-fed Hamsters in a Dose-Dependent Manner

Sodium vanadate is a known phosphatase inhibitor and insulin-mimetic agent, which improves insulin signal transduction. We investigated whether ex vivo treatment of hepatocytes from fructose-fed hamsters with vanadate can improve insulin signaling and
reduce apoB secretion. Hepatocytes isolated from fructose-fed hamsters were incubated in serum- and insulin-free Williams' medium E containing 0, 10, 40, and 80 µM sodium orthovanadate for 6 h and then subjected to 100 nM insulin stimulation for 10 minutes. Equal amounts of cell lysates (0.5-1 mg) were immunoprecipitated for insulin receptor β subunit and then immunoblotted with anti-phosphotyrosine antibody as described in the Materials and Methods section. Fig. 5.5A depicts the dose-response curve of the effect of vanadate on insulin receptor phosphorylation and a representative immunoblot. Exposure to vanadate increased tyrosine phosphorylation of the insulin receptor from 100 ± 6.4% in untreated cells to 365.2 ± 20.5% (p=0.0009), 474.3 ± 9.9%, and 488.4 ± 19%, at 10, 40, and 80 µM vanadate respectively. These results indicate that vanadate improved phosphorylation of the insulin receptor in a dose-dependent manner, reaching a plateau at 40 µM. Membranes were later stripped and reprobed for insulin receptor mass. As depicted in Fig. 5.5B Insulin receptor protein mass was detected 106 ± 3.6, 107 ± 7.5%, and 95 ± 6.2% of that of control in hepatocytes incubated with 10, 40, and 80 µM vanadate, respectively suggesting no significant change in insulin receptor mass.

5.2.11 Vanadate Reduces Synthesis and Secretion of ApoB from Insulin Resistant Hepatocytes in a Dose-Dependent Manner

To investigate whether the vanadate-induced enhancement in insulin receptor phosphorylation influences the synthesis and secretion of apoB, we incubated hepatocytes isolated from fructose-fed hamsters with culture media containing 0, 10, 20, 40, and 80 µM vanadate for 6 h. Cells were then pulsed with [35S]methionine for 90 minutes. Vanadate (0-80 µM) and insulin (1 µg/ml) were present throughout the experiment. Cellular, secreted, and total (secreted + cellular) apoB, are shown in Fig. 5.5B-D. Exposure of the cells to vanadate
FIGURE 5.4

Chronic High Insulin Exposure of Control Hepatocytes, Caused ApoB Oversecretion and Significant Suppression of ER-60

Panel A: Control hamster hepatocytes were incubated with a high concentration of insulin (1 μg/ml) for up to 3 days. Cells from each day of incubation were pulsed 90 minutes with [35S]methionine (100 μCi/ml) as described in the Methods section. Culture media were collected and subjected to immunoprecipitation using an anti-hamster apoB antibody. Immunoprecipitates were subjected to SDS-PAGE and fluorography. ApoB bands were excised and their radioactivity was quantified by scintillation counting and normalized against TCA-precipitable radioactivity in each dish and expressed as CPM/Total labeled protein counts. Shown is a representative gel and quantification of apoB bands. Data are presented as mean ± SD. *significant different from day 0 (p= 0.04). Panel B: Equal amounts of hepatocyte cell lysates (30 μg) from control, fructose-fed, and fructose-fed/rosiglitazone-treated animals were subjected to SDS-PAGE (8%) and immunoblotting for ER-60 as described in Methods. Corresponding bands were scanned and quantified. Shown is a representative immunoblot for ER-60 and densitometric quantification of immunoblots from 4 experiments performed in duplicate or triplicate, expressed as mean ± SD.* and ** indicate significant differences (p= 0.004 and p= 0.04, respectively). Panel C Control hamster hepatocytes treated with 0-3 days exposure to insulin 1 μg/ml insulin as in panel A were analyzed for ER-60 protein mass at each time point. 20 μg of cell lysate of each sample was subjected to SDS-PAGE and immunoblotting. A representative immunoblot is show along with combined data from 3 experiments. Mean ± SD. * indicates significant difference (p< 0.008).
FIGURE 5.4

A

ApoB

Day 0 1 2 3

Incubation Time (Days)

B

Control Fructose-fed FF + Rosiglitazone

ER-60

ER-60 Protein Mass (% of control)

Control FF FF + Rosiglitazone

C

ER-60

Day 0 1 2 3

Incubation Time (Days)
reduced cellular apoB from 100 ± 7% at 0 μM, to 97.6 ± 14.1% (p=0.788), 92.9 ± 9.3% (p=0.26), 75.9 ± 13.2% (p=0.050), and 62.8 ± 7.1% (p=0.003) of control, at 10, 20, 40, and 80 μM respectively, indicating that vanadate did not affect cellular apoB significantly at doses up to 40 μM but caused about a 25% suppression of cellular apoB at 40 μM which increased to 37.2% at 80 μM. However, vanadate had a more significant suppressive effect on apoB secretion at lower doses such that at 10 μM vanadate reduced apoB secretion to 52 ± 19% (p=0.015) of control. ApoB secretion was further reduced at 20 μM vanadate (48.7 ± 13.5% of control, p= 0.007), 40 μM (36.9 ± 11.7% of control, p=0.003), and 80 μM (35.0 ± 9.1% of control, p=0.001). Vanadate also caused a dose-dependent reduction in total labeled apoB (total labeled apoB was 100 ± 4.8%, 87.8 ± 11.2% (p=0.122), 81.2 ± 4.3% (p=0.009), 65.1 ± 7.6% (p=0.006), and 55.4 ± 3.8% (p=0.0006), with 0, 10, 20, 40 and 80 μM vanadate respectively), suggesting reduced intracellular apoB stability at doses above 20 μM.
FIGURE 5.5

Vanadate treatment stimulates tyrosine-phosphorylation of the insulin receptor and reduces apoB synthesis and secretion in hepatocytes isolated from fructose-fed hamsters

Panels A and B: Hepatocytes isolated from insulin resistant fructose-fed hamsters were incubated with serum- and insulin-free William's Medium E media containing 0, 10, 40, and 80 μM activated vanadate for 6 h and cells were then subjected to 100 nM insulin stimulation for 10 minutes. Equal amounts of cell lysates (0.5-1 mg) were immunoprecipitated for the insulin receptor β subunit and then immunoblotted with an anti-phosphotyrosine antibody, as described in the Materials and Methods section. In order to measure insulin receptor protein mass, the above membranes were stripped and reprobed for the insulin receptor β subunit, as described in the Materials and Methods section. Corresponding bands were quantified by densitometry and normalized for total protein and expressed as percentage of control. One representative immunoblot is shown along with densitometric quantification. Data were collected from 3 experiments performed in duplicate. All data are shown as mean ± SD.*significantly different from control (p<0.01). Panels C-E: Tissue culture dishes (60 mm) containing 3 x 10^6 hepatocytes isolated from fructose-fed hamsters were incubated with vanadate for 6 h as above and pulsed with [35S]methionine for 90 minutes. Vanadate (0-80 μM) and insulin (1 μg/ml) were present throughout the experiment. Cell lysates and media were collected and subjected to immunoprecipitation for apoB and fluorography. Quantitated labeled apoB was normalized against total radiolabeled protein (CPM) prior to converting to percentage. Panel C: Representative autoradiogram of cellular apoB as well as combined quantitated data for 3 experiments performed in duplicate. *significantly different from control (p<0.05). Panel D: ApoB detected in the media. Panel E: Sum of cellular and secreted apoB (total apoB). All data are shown as mean ± SD.*significantly different from control (p<0.01).
FIGURE 5.5

A. IR-pY

B. Vanadate (µM)

C. ApoB

D. Vanadate (µM)

E. Total ApoB

Vanadate (µM) 0 10 20 40 80

Insulin Receptor Phosphorylation (percent of control)

Insulin Receptor Protein Mass (percent of control)

Cell

Media

Total

0 20 40 60 80

Vanadate (µM)

Recoverd Cellular ApoB (percent of control)

Recovered Labeled Media ApoB (percent of control)

166
CHAPTER SIX

Effect of treatment with rosiglitazone, an insulin sensitizer agent, on hepatic VLDL- apoB secretion in the fructose-fed hamster model

6.1 Rationale, Hypothesis, and Research Objectives

6.1.1 Rationale

Dyslipidemia is an important component of metabolic syndrome observed in patients and animal models of insulin resistance and type 2 diabetes (Betteridge, 2000). Peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors that regulate the expression of genes involved in lipid and glucose homeostasis (Torra, et al., 2001). Dysregulation of PPAR activity modulates the onset and evolution of metabolic disorders such as obesity, insulin resistance, and dyslipidemia (Torra, et al., 2001). Recent evidence indicates that the thiazolidinediones (e.g. rosiglitazone and pioglitazone), which are PPARγ agonists, exert direct effects on lipid and glucose homeostasis and improve insulin sensitivity and glycemic control with reduced insulin requirement (reviewed in Mudaliar and Henry, 2001, Sunayama, et al., 2000). Although the exact molecular mechanism of insulin sensitizing action of thiazolidinediones is not yet known, some reports suggest that they may exert their effects partly by increasing expression of insulin signaling proteins such as p85 (Rieusset, et al., 2001), and IRS-2 (Smith, et al., 2001).

In Chapter 5, we demonstrated that induction of hepatic insulin resistance in fructose fed hamster coincided with hepatic overproduction of apoB-containing lipoproteins. We also showed that sodium vanadate as a phosphatase inhibitor and insulin-mimetic agent increased insulin receptor phosphorylation and reduced synthesis and secretion of apoB in hepatocytes isolated from fructose-fed hamsters. In this part of our research we have attempted to
investigate whether improvement of hepatic insulin signal transduction in fructose-fed hamsters treated with rosiglitazone, can result in improvement in apoB oversecretion.

6.1.2 Hypothesis

Improvement of hepatic insulin signal transduction in hepatocytes isolated from fructose-fed rosiglitazone-treated hamsters, results in attenuation of hepatic apoB oversecretion.

6.1.3 Research Objectives

Objective 1. Assess tyrosine phosphorylation and mass of insulin receptor, IRS-1, and IRS-2 in hepatocytes isolated from fructose-fed and fructose-fed/rosiglitazone treated hamsters.

Objective 2. Investigate VLDL-apoB production in hepatocytes isolated from fructose-fed and fructose-fed/rosiglitazone-treated hamsters.

6.2 Results

6.2.1 Improvement of Hepatic Insulin Signal Transduction in Hepatocytes Isolated from Fructose-Fed/Rosiglitazone-Treated Hamsters

In order to study the effect of rosiglitazone treatment on hepatic insulin signal transduction, hepatocytes prepared from control, fructose-fed, and fructose-fed rosiglitazone-treated hamsters were subjected to immunoprecipitation for insulin receptor β subunit, IRS-1, and IRS-2 and subsequent immunoblotting for tyrosine-phosphorylated proteins, as described in Materials and Methods. As depicted in Figure 6.1A, in hepatocytes isolated from fructose-fed hamster, insulin-stimulated insulin receptor β subunit tyrosine phosphorylation was reduced to 34.1 ± 2.6% (n=3, p=0.033) of that in control hepatocytes and this was restored to the control levels (98.3 ± 0.5%, n=3) following rosiglitazone treatment, indicating complete restoration of insulin receptor phosphorylation by the drug. Insulin-stimulated IRS-1 phosphorylation vs. basal was 184.3 ± 22.6% in controls (n=4, p=0.002), 130.3 ± 5.3% in FF
(n=4, p=0.007), and 188.9 ± 8.5% in FR (n=4, p=0.001) (Figure 6.1B), indicating improvement of IRS-1 phosphorylation to the control levels in hepatocytes isolated from drug-treated hamsters. The effect of insulin on phosphorylation of IRS-2 was similar to that of IRS-1. As shown in Figure 6.1C, insulin-stimulated IRS-2 phosphorylation over basal was 325.7 ± 70.5% in controls, 122.7 ± 17.7% in FF, and 319.5 ± 43.9% in FR, indicating significant reduction (n=3, p=0.01) in insulin-stimulated IRS-2 phosphorylation with fructose feeding and a marked improvement (n=3, p=0.004) after treatment with rosiglitazone. As shown in Fig. 6.2A, and also previously mentioned, fructose feeding had no significant effect on IR protein mass (100 ± 14.1% in control Vs. 88.3 ± 29.6% in FF, n=4, p=0.3). However, in rosiglitazone-treated FF hepatocytes, IR protein mass was significantly increased to more than two-fold of that in control and FF cells (212.6 ± 47% of control, n=4, p=0.0001). As depicted in Figure 6.2B, fructose feeding drastically reduced protein mass of IRS-1 from 359.7 ± 23.9 scanning units/mg of total protein in control hepatocytes to 80 ± 11.5 in hepatocytes from FF animals (n=3, p=0.0002), indicating a significant 77.7% reduction in IRS-1 mass following fructose feeding. Rosiglitazone-treatment partially restored IRS-1 mass to 190 ± 40 scanning units/mg of total protein or 52.8 ± 11.1% of that in control (n=3, p=0.003). IRS-2 protein mass in hepatocytes isolated from FF hamsters was reduced to 57.8 ± 7.1% (p<0.001) of the levels in control cells, whereas rosiglitazone treatment increased it to 74.1 ±8% of that of control hepatocytes (n= 4, p=0.013) (Figure 6.2C). These data suggest that the observed changes in IR, IRS-1, and IRS-2 phosphorylation in hepatocytes isolated from rosiglitazone-treated hamsters may be partially due to changes in protein expression.
FIGURE 6.1

Improvement in tyrosine phosphorylation of insulin receptor, IRS-1, and IRS-2 in hepatocytes isolated from the fructose-fed/rosiglitazone-treated hamsters

Hepatocytes freshly isolated from control, fructose-fed, and fructose-fed/rosiglitazone-treated hamsters were incubated in serum- and insulin-free media for 5 h. Cells were then divided into two groups, one group (baseline) was lysed immediately after 5 h incubation while the second group was subjected to 10 min stimulation with 100 nM insulin before being solubilized. Equal amounts of cell lysates (1 mg) were first immunoprecipitated for either insulin receptor β subunit, IRS-1, or IRS-2 and then immunoblotted using a monoclonal antibody against phosphotyrosine groups as described in Materials and Methods. Each panel depicts a representative immunoblot along with combined densitometric quantitation of multiple experiments. Net intensity of the bands was normalized for the total protein content of the samples. A) Phosphorylated insulin receptor. Data were collected from 3 experiments performed in duplicate or triplicate and expressed as percent of control. P values between control and FF, and FF and FF+R were <0.05 and 0.001, respectively. B) Phosphorylated IRS-1. Data were collected from 4 experiments performed in duplicate or triplicate and expressed as scanning units/mg of total protein. * and ** significant differences (p= 0.002 and p= 0.001, respectively). C) Phosphorylated IRS-2. Data were collected from 3 experiments performed in duplicate and expressed as percent of control. P values between control and FF, and FF and FF+R were 0.01 and <0.005, respectively. All data are shown as mean ± SD.
FIGURE 6.1

A) Control FF FF+R

Insulin — + — + — +

B) Control FF FF+R

Insulin — — + +

Phosphorylated IRS-1 (Scanning Units/Total Protein)

C) Control FF FF+R

Insulin — — + +

Stimulatory IRS-2 Tyrosine Phosphorylation (% of control)
FIGURE 6.2

Effects of rosiglitazone on hepatic protein mass of IR, IRS-1 and IRS-2

Equal amounts of cell lysates (20, 20, and 30 μg for IR, IRS-1, and IRS-2, respectively) prepared from hepatocytes isolated from control, fructose-fed, fructose-fed rosiglitazone-treated hamsters were subjected to SDS-PAGE (8% v/v) and immunoblotting for IR, IRS-1, and IRS-2 as described in Materials and Methods. Panels A, B and C depict a representative immunoblot along with combined densitometric quantitation of multiple experiments for IR, IRS-1 and IRS-2, respectively. Net intensity of the bands was normalized for the total protein content of the samples and is either expressed as scanning unit/mg total protein (panel B) or percent of control cells (panels A and C). Solid, open, and gray bars represent IR, IRS-1, and IRS-2 protein mass in control, FF, and FF+rosiglitazone treated hepatocytes, respectively. Data were collected from 3-4 experiments performed in duplicate or triplicate. A) P values between control and FF, and FF and FF+R were 0.3 and 0.001, respectively. B) P values between control and FF, and FF and FF+R were <0.001 and 0.02, respectively. C) P values between control and FF, and FF and FF+R were 0.001 and 0.002, respectively. All data are shown as mean ± SD.
FIGURE 6.2

A

Control FF FF+Rosi

P = 0.001

P = 0.3

Insulin Receptor Protein Mass

(Percent of control)

Control Fructose-Fed Fructose-Fed + Rosiglitazone

B

Control FF FF + R

P < 0.001 P < 0.02

IRS-1 Protein Mass (Scanning Units/mg Total Protein)

Control Fructose-Fed Fructose-Fed + Rosiglitazone

C

Control FF FF + R

P = 0.001

P = 0.002

IRS-2 Protein Mass (Percent of control)

Control Fructose-Fed Fructose-Fed + Rosiglitazone
levels of these proteins.

Since PTP-1B plays a crucial role in modulating insulin signal transduction and our previous data showed its enhanced protein mass and activity in insulin resistant hepatocytes, we investigated the effects of rosiglitazone treatment on protein expression levels of PTP-1B. Figure 6.3 shows that PTP-1B protein mass increased to 169.9 ± 13.2% (n=3, p = 0.0002) of that of controls in hepatocytes of the fructose-fed animals. Rosiglitazone treatment of fructose-fed hamsters markedly reduced the level of PTP-1B to 24.4 ± 12.9% (n=3, p = 0.0004) of that in control cells.

6.2.2 Significant Reduction of VLDL-apoB Secretion from Hepatocytes Isolated from Rosiglitazone-Treated Fructose Fed Hamsters

To determine the effect of rosiglitazone treatment on VLDL-apoB secretion, we performed in vitro steady state labeling experiments in which hepatocytes from fructose-fed and fructose-fed/rosiglitazone-treated hamsters were radiolabeled for a 2 h period. Culture media containing secreted lipoprotein particles were then collected and subjected to ultracentrifugation to isolate VLDL. Radiolabeled apoB associated with VLDL particles was immunoprecipitated and analyzed by SDS-PAGE and fluorography. As depicted in Fig. 6.4, rosiglitazone treatment significantly reduced VLDL-apoB secretion to 38± 32% (mean ± SD, n=4, p < 0.001) of that of fructose-fed hepatocytes. Decreased VLDL-apoB levels suggest a considerable reduction in the number of secreted VLDL particles from hepatocytes isolated from drug-treated/fructose-fed hamsters.

6.2.3 Effects of Rosiglitazone on Turnover of ApoB in Hamster Hepatocytes

Pulse-chase labeling experiments were conducted to assess the stability and secretion of apoB in hepatocytes isolated from fructose-fed, and fructose fed/ rosiglitazone-treated
Effects of rosiglitazone on protein expression of PTP-1B in hepatocytes isolated from fructose-fed hamsters

Hepatocytes isolated from control, fructose-fed and fructose-fed/rosiglitazone-treated hamsters were solubilized and equal amount of cell lysates (25 µg) was subjected to SDS-PAGE and immunoblotting for PTP-1B as described in Materials and Methods. Corresponding bands were quantified by densitometry and expressed as a percentage of PTP-1B in control hamster hepatocytes. Shown is one representative immunoblot and combined quantitation of immunoblots from 3 experiments performed in triplicate. Mean ± SD, ***significant difference (p<0.001).
FIGURE 6.3

Control  FF  FF+R

P < 0.001  P < 0.001

PTP-1B Protein Mass (% of control)

Control  FF  FF+R

0  50  100  150  200
FIGURE 6.4

VLDL-apoB production in hepatocytes isolated from fructose-fed and fructose-fed rosiglitazone-treated hepatocytes

Primary hamster hepatocytes were pulsed for 2 h with 100 µCi/ml [35S]methionine and [35S]cysteine. Culture media was collected, density adjusted to 1.006 g/ml, and adjusted media was subjected to ultracentrifugation for 18 h at 35000 rpm in a SW55 rotor to float the VLDL fraction. The VLDL fraction was then collected and was immunoprecipitated with a specific anti-hamster apoB antibody. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. Quantitation of apoB was performed by scintillation counting of the apoB100 band. Data were normalized against TCA-precipitable radioactivity prior to converting to percentages. (mean ± SD, n=4). ***significantly different from control (p<0.001).
Radiolabeled VLDL-apoB secreted
(% of control)

FF

FF + Rosiglitazone

P < 0.001
hamsters, as described in Materials and Methods. Isolated hepatocytes were pulsed for 45 min and then chased for 1 and 2 h. Cellular and media apoB was immunoprecipitated and analyzed by SDS-PAGE and fluorography. Fig. 6.5 shows the intracellular turnover, extracellular secretion, and total apoB remaining in fructose-fed and fructose-fed/rosiglitazone-treated hepatocytes. As depicted in Fig. 6.5A, in hepatocyte isolated from fructose-fed hamsters, intracellular apoB was reduced from 100 ± 20% at the beginning of the chase to 60 ± 30%, and 45 ± 16% at 1 and 2 h chase time points, respectively; whereas in rosiglitazone-treated hepatocytes cellular apoB dropped from 100 ± 21% at T0 to 44 ± 16%, and 38 ± 16% at 1 and 2 h chase time points, respectively. Although rosiglitazone treatment appeared to cause a reduction in cellular apoB, the observed differences were not statistically significant (n=4 for FF, and n=6 for FF+R). In FF hepatocytes after 1 and 2 h chase, 46 ± 16% and 48 ± 13% of cellular labeled apoB (at T0) was secreted into the media, respectively. Rosiglitazone-treatment reduced secretion of apoB to 33 ± 9%, and 40 ± 15% of cellular labeled apoB after 1, and 2 h chase, respectively (Fig. 6.5B), however only the change at 1 h was statistically significant (p= 0.026). Total apoB remaining in FF hepatocytes changed from 100 ± 20% at the beginning of the chase to 106 ± 28%, and 95 ± 20% at 1, and 2 h chase, respectively, suggesting no significant apoB degradation in FF hepatocytes; whereas in rosiglitazone-treated hepatocytes, total apoB remaining decreased from 100 ± 21% at T0 to 77 ± 24%, and 78 ± 23% at 1, and 2 h chase, respectively. Comparison between the two conditions showed a significant difference in total apoB remaining at 1 h chase time (p= 0.02) suggesting that rosiglitazone treatment led to destabilization and increased degradation of nascent apoB-containing particles (Fig. 6.5C). There was also a trend toward a reduction
in the fraction of labeled apoB recovered in the FF+R vs. FF animals after the 2 h chase (78 ± 9% vs. 95 ± 10% in the FR vs. FF animals respectively, p = 0.12).

6.2.4 Rosiglitazone Significantly Suppresses Hepatic Expression of MTP Protein

In Chapter 4, we documented the elevation of MTP mRNA, protein mass and activity in hepatocytes isolated from fructose-fed hamsters which may partly be responsible for the facilitated assembly and overproduction of apoB-containing lipoproteins. Furthermore, the MTP gene promoter has an insulin response element, which negatively regulates its expression. Taken together, we hypothesized that improvement of hepatic insulin signal transduction in rosiglitazone-treated hamsters may decrease expression levels of MTP and subsequently contribute to a reduction in apoB production. To test this hypothesis, equal quantities of total protein (cell lysate) were analyzed by SDS-PAGE and then subjected to immunoblotting with the anti-bovine MTP antibody. Fig. 6.6 shows the immunoblotting analysis of lysates of hepatocytes isolated from control, fructose-fed and fructose-fed/rosiglitazone-treated hepatocytes. As depicted in Fig. 6.6, cellular protein mass of MTP in hepatocytes from fructose-fed hamsters was 153.3 ± 6.6% (n=4, p=0.0002) of that of controls and rosiglitazone treatment led to normalization of cellular protein mass of MTP in fructose-fed hamsters to 107.0 ± 9.4% of that in control cells (n = 4, p < 0.005).
Turnover of apoB in hepatocytes isolated fructose-fed and fructose-fed rosiglitazone treated hepatocytes

Primary hamster hepatocytes isolated from fructose-fed and fructose-fed/rosiglitazone treated hamsters were pulsed for 45 min with 100 μCi/ml [35S]methionine, and the radioactivity was chased for 1 and 2 h in the presence of 5 mM excess cold methionine. Media and cells were collected and apoB was immunoprecipitated with a specific anti-hamster apoB antibody followed by SDS-PAGE and fluorography. Quantitation of apoB was performed by scintillation counting of the apoB100 band. Data were normalized against TCA-precipitable radioactivity prior to converting to percentages. A) distribution of immunoprecipitable apoB in cells expressed as a percentage of radiolabeled apoB in cells at 0 time. B) distribution of immunoprecipitable apoB in media expressed as a percentage of radiolabeled apoB in cells at 0 time. C) apoB stability expressed as percent apoB remaining in cells+media (total apoB) in fructose-fed and fructose-fed rosiglitazone treated hepatocytes at 0 time (beginning of the chase), 1 h chase, and 2 h chase. (mean ± SD, n=4-6). *significantly different from fructose-fed hepatocytes (p=0.026). **significantly different from fructose-fed hepatocytes (p=0.019).
A. Cellular ApoB

B. Secreted ApoB

C. Total ApoB

![Graphs showing Cellular, Secreted, and Total ApoB levels over time for Fructose Fed and Fructose+Rosi groups.](image)
FIGURE 6.6

Rosiglitazone normalized MTP protein expression levels in hepatocytes isolated from fructose-fed hamsters

Hepatocytes isolated from control, fructose-fed and fructose-fed/rosiglitazone-treated hamsters were solubilized, and equal amounts of cell protein (20 µg) were subjected to SDS-PAGE (8% (v/v) acrylamide resolving gel) and proteins were then transferred onto nitrocellulose membranes. Immunoblotting was performed to detect the 97 kDa MTP subunit with a rabbit anti-bovine MTP antiserum. (A), the autoradiograph of the MTP immunoblot. In (B), the MTP bands were quantitated by densitometric scanning and the mass of the 97 kDa MTP subunit detected was expressed as a percentage of the MTP mass detected in control cells (n=4, mean ± SD, P<0.005).
FIGURE 6.6

Control   FF   FF + R

P < 0.005

MTP Protein Mass (Percent of Control)

Control    FF    FF + Rosiglitazone
CHAPTER SEVEN

DISCUSSION AND CONCLUSIONS

Insulin resistance, a central pathophysiologic feature of type 2 diabetes and abdominal obesity, is commonly associated with atherosclerosis, hypertension and dyslipidemia (Reaven, 1995). Indeed, insulin resistance-associated dyslipidemia plays a major role in the pathogenesis of CAD. Since the major lipid abnormality of insulin resistance is hypertriglyceremia due to VLDL overproduction, we have focused our studies on the metabolism of apoB-containing lipoproteins.

First, we confirmed the similarities of apoB biogenesis in hamster and human liver (as discussed in 7.1). We then focused on the impact of insulin resistance on VLDL-apoB overproduction. We induced whole body insulin resistance in hamsters by feeding them a high fructose diet and studied overproduction of VLDL-apoB in primary hamster hepatocytes isolated from the insulin resistant hamsters (as discussed in 7.2). Since hepatic insulin signaling is known to play an important role in the regulation of apoB production, we investigated the effect of fructose feeding on hepatic insulin signaling and explored the links between induction of hepatic insulin resistance and VLDL-apoB overproduction (discussed in 7.3). To further study this connection, we examined the effects of insulin sensitization both ex vivo (vanadate experiments, discussed in 7.3) and in vivo (rosiglitazone-treatment of insulin resistant hamsters, discussed in 7.4) on VLDL biogenesis.

7.1 Intracellular Mechanisms Regulating ApoB-containing Lipoprotein Assembly and Secretion in Primary Hamster Hepatocytes

In the first phase of our research, we determined the suitability of using primary hamster hepatocytes as a model of human VLDL production. This model would allow us to
characterize three important processes involved in apoB production: translocation, degradation and lipoprotein assembly. Apolipoprotein (apo) B100 is an atypical secretory protein in that its translocation across the endoplasmic reticulum membrane is inefficient, resulting in the partial translocation and exposure of apoB100 on the cytoplasmic surface of the endoplasmic reticulum. Cytosolic exposure leads to its ubiquitination and proteasomal degradation.

To examine ER membrane translocation of the newly synthesized apoB, we used different approaches including trypsin sensitivity (protease protection) assays of apoB in permeabilized hepatocytes and purified microsomes as well as microsomal lumen and membrane fractionation. Protease protection assays showed different translocational efficiencies for the newly synthesized apoB depending on the methodology employed, although the observed differences were not statistically significant. One possible factor contributing to the variability observed in trypsin accessibility of hamster apoB in permeabilized cells vs. isolated microsomes may be the different pulse and chase times used in these experiments and may reflect differential sensitivity of apoB to trypsin at different times during its transit in the secretory pathway. This was in agreement with previous report by Cavallo et al. in McRH7777 cells and HepG2, respectively (Cavallo, et al., 1998). Our observations suggested that in primary hamster hepatocytes apoB translocation through the ER membrane was inefficient. In an alternative approach we examined newly synthesized apoB associated with ER membrane and lumen (carbonate extraction method). In these experiments, the majority of labeled apoB was recovered in the luminal fraction, which did not correlate well with the data obtained from the protease protection experiments. This discrepancy may have been due to the methodological variation. In fact, there are large
differences in carbonate extractable apoB reported in various published studies (Boren, et al., 1992, Boren, et al., 1994, Bostrom, et al., 1988). Indeed, using a revised extraction protocol, Olofsson and colleagues (Rustaeus, et al., 1998) showed that most of the labeled apoB could be extracted from HepG2 microsomes in the form of lipoproteins with sodium carbonate.

Interestingly, we observed an increased trypsin sensitivity of newly synthesized apoB in MG132-treated microsomes, suggesting that inhibition of proteasomal degradation may cause accumulation of cytosolically exposed apoB molecules. Under these conditions, analysis of a control protein, transferrin did not reveal any significant change in sensitivity to exogenous trypsin treatment, suggesting that the effect of MG132 was specific to apoB. Our observation was in agreement with Bonnardel and Davis (Bonnardel and Davis, 1995) who previously reported membrane accumulation of apoB in ALLN-treated microsomes.

Overall our protease protection studies in both permeabilized hamster hepatocytes and isolated microsomes appear to indicate that a fraction of newly synthesized hamster apoB may be trypsin accessible suggesting cytosolic exposure. However, since carbonate extraction studies of isolated microsomes do not support a large degree of membrane association and there was some variability in trypsin accessible apoB in various experiments, we can not make definitive conclusions as to the nature of membrane association of hamster apoB, or whether it adopts a transmembrane topology in these primary hepatocytes.

1995, Ingram and Sheness, 1996, Leiper, et al., 1996). Our data on membrane association of endogenous apoB in hamster hepatocytes appears to compare well with primary rabbit hepatocytes (Cartwright and Higgins, 1995), which also exclusively synthesize and secrete apoB100-containing lipoproteins.

Intracellular degradation of the newly synthesized apoB has been reported to various extents depending on the different cell types employed (for detailed information, refer to page 31). The extent of apoB degradation in our primary hepatocyte model was similar to that reported in freshly isolated rabbit hepatocytes (Cartwright and Higgins, 1996), and was significantly lower than the commonly used cell line, HepG2 cells (Adeli, 1994, Borchardt and Davis, 1987, Sato, et al., 1990). It should be noted that high levels of apoB degradation in HepG2 cells has been attributed to the abnormally low TG pool in this cell line. It thus appears that the extent of apoB degradation in primary hamster hepatocytes resembles more closely the physiologic conditions than those in HepG2 cells. The presence of MG132 (proteasome inhibitor) during the pulse induced significant accumulation of apoB suggesting protection against proteasomal degradation. To further investigate the mechanisms of apoB degradation, we employed permeabilized hamster hepatocytes. Degradation of apoB in permeabilized hamster hepatocytes coincided with the appearance of a number of apoB fragments ranging in size from 46 to 167 kDa. Consistent detection of these fragments suggests that the intact apoB may be rapidly clipped by a protease(s) to yield several degradation intermediates. Interestingly, generation of the 70 kDa fragment was ALLN sensitive, while the appearance of the 46, 57, and 167 kDa fragments was insensitive to both ALLN and MG132 indicating the involvement of different proteases in the degradation of hamster apoB-100. Although some of the proteolytic fragments detected in our study may be
similar to those detected previously, this is the first observation of both ALLN and MG132-insensitive fragments in permeabilized hamster hepatocytes.

In most primary hepatocytes examined, degradation of apoB is not confined to the ER (Sparks and Sparks, 1994a) and appears to occur even after an apoB-lipoprotein is formed (Cartwright and Higgins, 1996, Fast and Vance, 1995, Verkade, et al., 1993, Wang, et al., 1995a, Wang, et al., 1993). Our present results also indicate that degradation of hamster apoB can occur post-translationally during its transit through the secretory pathway. To identify proteases involved in intracellular degradation of apoB, several research groups have employed different protease inhibitors and cell types (for more information, refer to page 34). In our study, EST, PMSF, and leupeptin showed no effect on apoB stability. Our observation using leupeptin was in agreement with Sato, et al. in HepG2 cells (Sato, et al., 1990), whereas it was in contrast with the reports of Wang et al. (primary rat hepatocytes) (Wang, et al., 1995a) and Cartwright and Higgins (rabbit hepatocytes) (Cartwright and Higgins, 1996). Our EST observation was also in disagreement with a previous report in rat hepatocytes (Wang, et al., 1995a). These controversies may be due to different cell models or methodological differences. In contrast, proteasomal inhibitors, ALLN, and lactacystin exerted significant protective effects on cellular apoB. Interestingly, while ALLN and lactacystin stabilized cellular apoB levels, no stimulation of apoB secretion was observed. Bonnardel and Davis (Bonnardel and Davis, 1995) reported similar observations on the effect of ALLN in HepG2 cells and concluded that translocation, but not degradation determines the intracellular fate of de novo synthesized apoB. In the present study, o-phenanthroline also inhibited apoB turnover and appeared to increase its extracellular secretion suggesting the involvement of metalloproteases in post-translational degradation of apoB. The inhibition of
apoB degradation by $\alpha$-phenanthroline was also reported by Cartwright and Higgins (Cartwright and Higgins, 1996) in freshly isolated rabbit hepatocytes. Overall our observations in primary hamster hepatocytes indicate that apoB degradation can occur in all secretory compartments by proteasomal and non-proteasomal degradative systems. Although this was the first study on the role of degradative systems in hamster apoB biogenesis, further studies are needed to identify nature and structure of these protease systems.

Based on evidence obtained from density gradient fractionation of luminal lipoproteins, hamster apoB-100 appears to form LDL-like particles in the microsomal lumen. Such LDL-like particles appeared to gradually recruit more lipids forming lipid rich VLDL particles, which were subsequently secreted into the media in the form of mature VLDL. Similar observations have been reported in rat hepatocytes (Swift, 1995). In our study we found a small pool of radiolabeled apoB in the high density fraction of the lumen. Interestingly, the size of this pool increased after a 1 h chase. It appears that after formation of the first apoB pool (LDL-like particles) and its gradual conversion to the TG-rich VLDL, a proportion of newly translated and translocated apoB forms high density particles possibly due to unavailability of lipids and/or misfolding of the apoB molecule. Based on previous observations in HepG2 cells (Boren, et al., 1992), HDL-like particles may either be converted to VLDL particles by recruiting lipids and secreted, or may be sorted to a degradative pathway. Cartwright et al. (Cartwright, et al., 1993) also reported that in freshly isolated rat hepatocytes, the majority of apoB in the RER and SER fractions was associated with HDL-like particles. The apoB of VLDL density was predominantly found in trans- and cis-Golgi fractions (Cartwright, et al., 1993). Our findings on VLDL assembly in primary hamster hepatocytes are partly in agreement with the observations made by Cartwright and
Higgins in isolated rabbit hepatocytes (Cartwright and Higgins, 1995). They suggested that assembly of apoB into complete VLDL is not a co-translational process and most lipids become associated with apoB late in the ER compartment and are further modified in the Golgi lumen. Rustaeus et al. (Rustaeus, et al., 1998) also reported that in McA-RH7777 cells, membrane associated apoB-100 is partially lipidated and can be converted to VLDL.

In conclusion, it appears that in HepG2 cells, McA-RH7777 cells, and rabbit and rat hepatocytes, a significant amount of newly synthesized apoB-100 is incorporated into a dense fraction. Primary hamster hepatocytes appear to form a smaller pool of HDL-like particles, although our studies suggest that the lipoprotein assembly process in these cells may be similar to that in other model systems examined.

In contrast to HepG2 cells, treatment with exogenous oleate did not stimulate apoB secretion by primary hamster hepatocytes. Instead, oleate increased stability of cellular apoB (over the first hour of chase) without affecting its extracellular secretion. These observations compare well with a previous report on the effects of oleate on apoB secretion by hamster hepatocytes (Arbeeny, et al., 1992). The effect of oleate on the stability and secretion of apoB appears to be controversial. The effect may depend on the cell type, turnover of TG/fatty acid in the cells, size of cellular TG pool, and duration of incubation with oleate. In our study, after a 12 h incubation with oleate, no stimulatory effect on apoB secretion was observed. Similar results were obtained for longer incubations (data not shown). In a recent report, Salter et al. (Salter, et al., 1998b) compared the TG turnover of hamster and rat hepatocytes, and found that hamster hepatocytes have a larger intracellular TG pool and a lower rate of VLDL-TG secretion compared to rat hepatocytes. In the hamster liver, a larger proportion of newly synthesized TG is retained within the cell, rather than secreted as VLDL.
Furthermore, Bennet et al. (Bennett, et al., 1995) using hamsters fed with triolein, tristearin, and tripalmetin showed that triolein had no effects on VLDL secretion whereas tripalmetin increased both hepatic apoB mRNA and plasma VLDL levels. It is also believed that oleate treatment of the cells facilitates translocation of newly synthesized apoB across the ER-membrane, which in turn reduces early degradation (Macri and Adeli, 1997b). However, whether or not this protection of early degradation stimulates apoB secretion appears to differ among different cell types (for more information, refer to pages 39-40). Our observations were in agreement with previous reports in rat (Davis and Boogaerts, 1982, Patsch, et al., 1983b), McArdle hepatoma cells (Sparks, et al., 1997), hamster (Arbeeny, et al., 1992) and human hepatocytes (Lin, et al., 1995b). Our results were also in contrast with previous reports on the stimulatory effects of oleate on apoB secretion in HepG2 cells (Bostrom, et al., 1988, Dixon, et al., 1991, Pullinger, et al., 1989), rat hepatoma cell line (White, et al., 1992) and freshly isolated rabbit hepatocytes (Cartwright and Higgins, 1996). Overall, our finding that treatment with exogenous oleate failed to stimulate hamster apoB-100 secretion appears to correlate with data obtained in rat and human primary hepatocytes.

In conclusion, the hamster presents a unique model for further investigation of the hepatic assembly and secretion of apoB-containing lipoproteins and their hormonal and pharmaceutical modulation, without many of the drawbacks associated with the use of other primary or established cell culture model systems. The data presented and discussed above helped to characterize some of the mechanisms governing the intracellular biogenesis and subsequent secretion of hamster apoB-100, and provided the basis for further studies in this animal model. Our observations and data strongly suggest that the hamster is a suitable animal model in which to study pathophysiologic conditions associated with VLDL-apoB
dysregulation. Indeed, the susceptibility of hamster to diet-induced insulin resistance, obesity, atherosclerosis and diabetes provides a unique opportunity to study molecular and cellular mechanisms of insulin resistance-associated VLDL-apoB overproduction.

7.2 Molecular Mechanisms of Hepatic VLDL Overproduction in Insulin Resistance

Although overproduction of VLDL-TG and VLDL-apoB has been well demonstrated in the insulin resistant state in both humans and animal models, few data are available on the underlying cellular mechanisms involved, particularly those directly affecting the apoB protein itself. The majority of studies have focused on the acute effects of insulin, while the role of chronic hyperinsulinemia and insulin resistance in VLDL overproduction has been understudied. In this phase of our study, we have simultaneously examined the specific impact of inducing an insulin resistant condition on apoB biogenesis at the potential regulatory steps of synthesis, ER translocation, intracellular degradation, and lipoprotein assembly. We employed a fructose-fed hamster model to investigate the above mechanisms in the state of insulin resistance. This model offers advantages over the more commonly used fructose-fed rat model, in that the metabolism of its apoB-containing lipoproteins is more similar to that of humans.

It has been well documented that fructose feeding in rodents including hamsters (Kasim-Karakas, et al., 1996) results in chronic hyperinsulinemia, an insulin resistant state, and hyperlipidemia. A previous study (Kasim-Karakas, et al., 1996) clearly demonstrated the feasibility of inducing insulin resistance and chronic hyperinsulinemia in fructose-fed hamsters. The fructose protocol employed in the current study was also similar to those previously shown to induce insulin resistance in the rat (Kazumi, et al., 1985, Kazumi, et al., 1986). The data from our collaborating laboratory (Dr. Gary F. Lewis, UHN) using in vivo
hyperinsulinemic-euglycemic clamp studies supported the induction of an insulin resistant condition in the fructose-fed hamster model (Taghibiglou, et al., 2000). Overall, these data combined with the resulting hyperinsulinemia in fructose-fed hamsters suggest that the fructose feeding protocol was successful in inducing an insulin resistant condition.

Our in vitro (in collaboration with the laboratory of Dr. G.F. Lewis, University of Toronto) and ex vivo experiments with primary hamster hepatocytes confirmed both VLDL-TG and VLDL-apoB overproduction in hepatocytes from fructose-fed hamsters. These abnormalities closely resemble those observed in insulin resistance and Type 2 diabetes in humans, in which increased VLDL production is the main abnormality of lipoprotein metabolism (Cummings, et al., 1995, Lewis, et al., 1995, Malmstrom, et al., 1997, Riches, et al., 1998) and is felt to be an important contributor of the increase in cardiovascular disease seen in these patients (Ginsberg, 2000). This observation is important because some animal models of insulin resistance and hyperlipidemia, such as the ob and the db mouse have been shown not to overproduce VLDL in vivo, an observation which limits the usefulness of these animals as a model of human pathophysiology (Li, et al., 1997). Although fructose feeding has been shown to induce an increase in VLDL-TG production in vivo in rats (Kazumi, et al., 1985, Kazumi, et al., 1986) these previous studies did not investigate the VLDL-apoB production.

The insulin resistant, fructose-fed hamster model thus provided an excellent system to investigate the intracellular mechanisms that may mediate the considerable VLDL-apoB overproduction observed. A number of important observations were made which appear to explain the VLDL-apoB overproduction in this model. First, there was a significant enhancement of intracellular stability of newly synthesized apoB with only a minor fraction
being sorted to intracellular degradation. This observation was initially thought to be related to an enhanced rate of apoB translocation across the ER membrane. However, analysis of apoB translocational status in normal and fructose-fed hepatocytes failed to reveal a change in translocational efficiency of apoB in response to fructose feeding. This observation appears to suggest that the increased stability of apoB may occur post-translocationally and may predominantly affect the pool of apoB fully translocated into the ER lumen. Thus, fructose feeding does not appear to affect the extent of association of nascent apoB with the microsomal membrane and thus its trypsin sensitivity. However, increased intracellular stability of apoB in fructose-fed hepatocytes was evident both in intact cells as well as in permeabilized cells. Turnover of nascent apoB was slowed in intact fructose-fed hepatocytes compared with control cells. This increased post-translocational stability could also be demonstrated in permeabilized cells. Further studies are needed to evaluate cellular degradative systems including proteasomal, endoplasmic reticulum associated degradation (ERAD), and non-proteasomal systems in insulin resistant hepatocytes.

Further analysis of lipoprotein formation in hepatocytes derived from control and fructose-fed animals revealed considerable differences in the distribution of apoB-containing lipoproteins. In the ER-lumen of control hepatocytes, three types of apoB-containing lipoproteins were detectable (HDL, LDL, and VLDL-like particles) with a great amount of radiolabeled apoB recovered in the secretion-incompetent HDL-like particles. These particles (HDL-like) are thought to be destined for intracellular degradation. In contrast, in fructose-fed hepatocytes, radiolabeled apoB was only recovered in LDL and VLDL fractions with a large amount of secretion-competent VLDL particles. These observations argue for enhanced efficiency of VLDL assembly in the microsomal lumen of fructose-fed hepatocytes.
Facilitated assembly of hamster VLDL may be related to an increased availability of core lipids, an increased availability of freshly translated apoB, and/or increased activity of MTP. Analysis of intracellular lipid biosynthesis revealed a significant increase in intracellular TG levels, which may in turn contribute to increased assembly of VLDL. In addition, post-translational stability of nascent apoB was also increased, making a higher pool of nascent apoB molecules available for VLDL assembly. More studies are needed to examine the possible changes in sizes of different intracellular lipid pools especially those accessible for VLDL assembly. Most interesting however was an increased mRNA level, protein mass and activity of MTP detected in fructose-fed hepatocytes. MTP catalyzes the transfer of lipids to the apoB molecule and is an important factor involved in the assembly of apoB-containing lipoproteins (Gordon, et al., 1994, Wetterau, et al., 1992, Wetterau, et al., 1997). Thus it is reasonable to conclude that an increased intracellular mass, mRNA, and activity of MTP can enhance the VLDL assembly process, leading to formation and secretion of an increased number of mature particles. Furthermore, the combination of an increased abundance of MTP in the presence of both higher availability of TG as well as apoB, strongly favors the formation of VLDL particles and their secretion from the cell. Our current observations in fructose-fed hepatocytes suggest that chronic hyperinsulinemia and resistance to insulin action in the hamster model may be responsible for an increase in the expression of MTP and thus an enhanced activity of this critical factor in VLDL assembly. Insulin is known to acutely diminish both the MTP mRNA level as well as the mass of MTP protein (Wetterau, et al., 1997). It appears that elevation of MTP levels in fructose-fed insulin resistant hamsters arose from the resistance against negative regulatory action of insulin on MTP gene promoter (Hagan, et al., 1994, Lin, et al., 1995a). A similar observation was made in Otsuka Long-
Evans Tokushima Fatty (OLETF) rat, an animal model of Type 2 diabetes, characterized by visceral obesity and hyperlipidemia (Kuriyama, et al., 1998). Whether increased MTP causes the increased stability and assembly of VLDL in insulin resistance or is merely secondary to the increase in intracellular lipid synthesis is currently unknown.

Hepatic overproduction of VLDL in the state of insulin resistance may result from direct hepatic effects of insulin as well as indirect metabolic effects, such as increased availability of free fatty acids (FFA) for TG secretion (Lewis, et al., 1993). In the present study, we found significantly elevated plasma levels of FFA in fructose-fed hamsters, suggesting that increased flux of FFA into the liver may contribute to VLDL overproduction. However, we did not measure in vivo FFA flux in fructose-fed hamsters and can not confirm the impact of plasma FFA elevation on in vivo VLDL production rates. It is also important to note that the rate of VLDL-apoB secretion from primary hamster hepatocytes was measured under identical concentrations of FFA in the culture media, for both control and fructose-fed hepatocytes.

In conclusion, the fructose-fed hamster model has allowed us to address a number of important questions regarding the intracellular mechanisms that modulate hepatic VLDL assembly and secretion. The evidence obtained in this model suggest that the hyperbetalipoproteinemia observed in the metabolic condition of insulin resistance may be caused by the combined effect of an increased expression and activity of MTP, increased hepatocyte neutral lipid availability, and reduced degradation of apoB, which can in turn facilitate the assembly and secretion of apoB-containing lipoprotein particles. Precisely which of these factors occurs directly as a result of hepatic hyperinsulinemia or insulin resistance and which are secondary to the extrahepatic effects of insulin is not currently
known. The hepatic effects may be due to a direct action of insulin or may be secondary to an increased lipid availability. Enhanced activity of MTP may contribute to post-translocational stability of apoB, but whether it is sufficient by itself to explain the VLDL overproduction is unknown. Further studies are required to fully investigate the mechanisms by which insulin resistance can influence either the expression or intracellular stability of MTP and thus exert a stimulatory effect on VLDL assembly and secretion. Of particular interest is the interaction of MTP abundance/activity, post-translocational apoB stability, and core lipid availability in determining the efficiency of the VLDL assembly process.

In this part of our research, we clearly demonstrated the coincidence of whole body insulin resistance with overproduction of VLDL-apoB. However, in order to connect hyperinsulinemia and insulin resistance with VLDL-apoB overproduction at the cellular and molecular levels, we needed to examine insulin signal transduction in the liver, the tissue where VLDL-apoB is produced. In fact, it was important to determine whether resistance to insulin action developed in livers of fructose-fed hamsters and whether hepatic insulin resistance plays a direct role in dysregulation of VLDL-apoB production.

7.3 Hepatic VLDL-apoB Overproduction is Associated with Attenuated Hepatic Insulin Signaling in a Fructose-Fed Hamster Model of Insulin Resistance

We have previously documented the induction of whole body insulin resistance in the fructose-fed hamster model following fructose-feeding (Taghibiglou, et al., 2000). Induction of insulin resistance at the level of adipocytes and muscle tissues has also been well documented by others (for reviews refer to Bergman and Mittelman, 1998, Fujimoto, 2000, Withers and White, 2000), whereas the induction of hepatic insulin resistance is less well established. It was important to determine whether resistance to insulin action develops in
livers of fructose-fed hamsters and whether hepatic insulin resistance plays a direct role in deregulation of VLDL-apoB secretion.

In order to document the induction of hepatic insulin resistance, we quantified the tyrosine phosphorylation status of the insulin receptor, IRS-1, and IRS-2 ex vivo using hepatocytes from control and fructose-fed hamsters. Data obtained from these experiments showed a significant reduction in the phosphorylation of these key proteins of the insulin signaling pathway, suggesting downregulation of insulin signaling in the liver. Our observations on insulin receptor and IRS-1 phosphorylation are in agreement with Bezerra et al. (Bezerra, et al., 2000) who reported a reduction in IRS-1 and insulin receptor phosphorylation in muscle and livers of high fructose-fed rats. Our data are also in agreement with the findings of Jiang et al. who reported significant reductions in phosphorylation and protein mass of both IRS-1 and IRS-2 in the aorta and microvessels of obese Zucker (fa/fa) rats (Jiang, et al., 1999). Kerouz et al. also observed significant reductions in IRS-1 and IRS-2 mass and phosphorylation in muscle and liver of the ob/ob mouse (Kerouz, et al., 1997). Anai et al. reported similar observations in the liver and muscle of Zucker fatty rats (Anai, et al., 1998).

To determine whether or not these changes result in downstream effects, we examined both phosphotyrosine- and p85-associated PI 3-kinase activity. We found that PI 3-kinase activity associated with tyrosine phosphorylated proteins decreased in fructose-fed insulin resistant hepatocytes, while p85-associated activity remained unchanged. These observations indicate that, although the intrinsic activity of the enzyme was intact, recruitment to the insulin signaling pathway was decreased, most likely as a result of the reduced phosphorylation of upstream signaling components. We also examined the impact of
fructose feeding on phosphorylation of Ser\textsuperscript{473}-Akt and Thr\textsuperscript{308}-Akt a key enzyme downstream of PI 3-kinase. In hepatocytes isolated from fructose-fed hamsters, insulin-stimulated phosphorylation of both Ser473 and Thr308 was significantly reduced suggesting suppressed activity of Akt in fructose-fed hamster hepatocytes. This was most likely due to the observed decrease in tyrosine phosphorylation of upstream insulin signaling proteins and reduced PI 3-kinase activity. Our observations are in agreement with the findings of Krook et al. and Rondinone et al. who reported impaired Akt phosphorylation in muscle and adipocytes of insulin resistant diabetic subjects respectively (Krook, et al., 1998, Rondinone, et al., 1999). Several other groups have reported similar observations in skeletal muscle of insulin resistant rats (Kim, et al., 1999, Storz, et al., 1999) and muscle and adipose tissues of db/db mice (Shao, et al., 2000). However the effect on hepatic insulin signaling was not investigated in these previous studies.

We also investigated whether the suppression of insulin signaling in hepatocytes could be related to the hyperinsulinemia observed in fructose-fed hamsters. We found that exposure to high insulin initially reduced receptor expression and eventually caused a significant downregulation of insulin receptor phosphorylation. These data appear to suggest that hepatic insulin resistance in fructose-fed hamsters may be secondary to elevated plasma insulin levels. In support of this hypothesis, Yoshino et al. (Yoshino, et al., 1992) showed that in streptozotocin-treated, diabetic, hypoinsulinemic rats, fructose feeding increased plasma insulin levels. More recently, Suga et al. (Suga, et al., 2000) reported that dietary fructose caused substantial insulin resistance and hyperinsulinemia in both ventromedial hypothalamic-lesioned obese and sham-operated lean rats. Dirlewanger et al. (Dirlewanger,
et al., 2000) also showed that in healthy humans, fructose infusion induced hepatic and extrahepatic insulin resistance.

Since protein tyrosine phosphatases play a crucial role in the insulin signaling pathway as negative regulators of signal transduction, we examined both protein levels and activity of PTP-1B in the current model and found significant elevations in the cellular mass and activity of this protein in hepatocytes isolated from fructose-fed hamsters. Our findings are in agreement with previous reports in livers of obese, diabetic ob/ob mice (Sredy, et al., 1995), STZ-induced diabetic rats, and genetically diabetic BB rats (Meyerovitch, et al., 1989) as well as muscles of non-diabetic, glucose-intolerant subjects (McGuire, et al., 1991). Several reports indicate that PTP-1B is capable of dephosphorylating the insulin receptor and IRS-1 (Hashimoto, et al., 1992b. Seely, et al., 1996. Tonks, et al., 1988) with higher efficiency than other tyrosine phosphatases (Goldstein, et al., 2000). In the FF hamster model, PTP-1B overexpression in the liver may contribute to dephosphorylation of the insulin receptor and IRS-1, leading to hepatic insulin resistance in the fructose-fed hamster.

In the current study, incubation of insulin resistant hepatocytes with increasing doses of vanadate improved the phosphorylation status of the insulin receptor in a dose-dependent manner. The improvement of insulin receptor phosphorylation coincided with a marked reduction in cellular, secreted, and total apoB, suggesting possible involvement of protein tyrosine phosphatases such as PTP-1B in modulating apoB production. Our observations confirmed previous observations by Jackson and colleagues about inhibitory effects of vanadate on apoB biogenesis in rat hepatocytes, although they used the phosphatase inhibitor in normal hepatocytes rather than insulin resistant hepatocytes from fructose-fed animals (Jackson, et al., 1988). Further studies are needed to examine the possible effects of vanadate
exposure on other important factors in apoB biogenesis such as MTP, Hsp70, Hsp90 and other chaperones.

We also monitored the effects of chronic high insulin incubation of control hamster hepatocytes on protein expression levels of PTP-1B and found that chronic exposure of control hepatocytes to high levels of insulin caused a gradual elevation of PTP-1B levels reaching a plateau on day 2 of exposure. This finding was consistent with our observation of elevated PTP-1B levels in hyperinsulinemic fructose-fed hamsters. More interestingly, upregulation of PTP-1B levels appeared to precede the decline in insulin receptor phosphorylation in hamster hepatocytes. In light of these findings, we hypothesize that chronic exposure to insulin results in elevated PTP-1B and subsequent dephosphorylation of the insulin receptor, IRS-1, and IRS-2 inducing a state of insulin resistance. Since we have not studied other protein phosphatases we can not rule out their possible involvement in impairment of insulin signal transduction.

In experiments involving chronic exposure of normal hepatocytes to high insulin concentration, desensitization of the insulin signaling pathway and overexpression of PTP-1B appeared to coincide with an increase in the synthesis and secretion of apoB. This observation compares well with our previous in vivo and ex vivo observations in the fructose-fed hamster model (Taghibiglou, et al., 2000) and supports the hypothesis that induction of hepatic insulin resistance may play an important role in VLDL-apoB overproduction. It is unclear however, how changes in the insulin signaling pathway lead to alterations in hepatic apoB metabolism. Insulin may directly alter the phosphorylation status of apoB as shown previously in rat hepatocytes (Jackson, et al., 1990b). It is also possible
that reduced phosphorylation of apoB due to impaired insulin signaling may protect apoB from degradation in insulin resistant hepatocytes.

It was previously reported that in HepG2 cells, an ER-localized cysteine protease named ER-60 may be involved in the degradation of apoB in the lumen of the ER (Adeli, et al., 1997a). Interestingly, we observed a drastic suppression of ER-60 protein expression in hepatocytes isolated from fructose-fed hamsters compared to that of control counterparts, suggesting that increased stability of apoB in insulin resistant hepatocytes may be partly attributed to the suppression of ER-60 levels. This suppression was partially restored in hepatocytes isolated from fructose-fed hamsters treated with rosiglitazone, an insulin sensitizing drug, suggesting that ER-60 may normally be positively regulated by insulin, an effect which may be lost in insulin resistance leading to suppression of the cellular levels of this protein. In support of this view, we observed that the expression of ER-60 protein was drastically suppressed with chronic high insulin exposure of control hamster hepatocytes. Overall, our observation that both elevation of PTP-1B and induction of insulin resistance was concomitant with a marked suppression of ER-60 and overproduction of apoB, provides a direct link between impairment of hepatic insulin signaling and apoB oversecretion.

In summary, hepatic VLDL-apoB overproduction in the fructose-fed hamster model appears to be closely associated with the development of insulin resistance in hepatocytes. Downregulation of hepatic insulin signaling was linked to overexpression of PTP-1B and may be secondary to changes in the cellular level of this phosphatase. Hepatic insulin resistance was in turn associated with suppression of ER-60 and elevated secretion of apoB. Further studies are needed to more directly link changes in insulin signaling status to key components of the VLDL assembly and secretion process.
The availability of a highly specific and effective new generation of insulin sensitizing drugs called thiazolidinediones (TZDs) provided a good opportunity to examine and support our observations made with vanadate (a general insulin mimetic agent) that improvement of hepatic insulin signaling attenuates apoB overproduction. Moreover, in vivo and ex vivo experiments with TZDs may help to obtain further additional evidence linking hepatic insulin resistance to VLDL-apoB overproduction.

7.4 Effect of Treatment with Rosiglitazone, an Insulin Sensitizer Agent, on Hepatic VLDL-ApoB Secretion in the Fructose-Fed Hamster Model

In this part of our study, we treated fructose-fed hamsters with rosiglitazone, a member of PPARγ agonist family of insulin sensitizing antidiabetic drugs, to investigate whether improvement in insulin resistance can attenuate hepatic apoB overproduction.

In order to examine whether rosiglitazone treatment of fructose-fed insulin resistant hamsters improved hepatic insulin resistance, we quantified tyrosine phosphorylation of the insulin receptor, IRS-1, and IRS-2 in hepatocytes isolated from control, fructose-fed and fructose-fed rosiglitazone-treated hamsters. Data obtained from ex vivo experiments showed a significant enhancement in the phosphorylation of these key proteins in hepatocytes isolated from rosiglitazone-treated hamsters compared to those in fructose-fed hamsters. These data suggest an improvement of insulin signaling in the liver of the drug-treated hamsters. We also studied protein mass of these key signaling proteins and found that protein levels of IR, IRS-1, and IRS-2 increased in hepatocytes isolated from rosiglitazone treated hamster compared to those in fructose-fed hepatocytes suggesting that rosiglitazone may improve hepatic insulin signal transduction partly by increasing mass of key insulin signaling proteins. Similar effects of thiazolidinediones (TZDs) on IRS-2 in 3T3-L1 and human
adipocytes (Smith, et al., 2001) and p85 subunit of PI3-kinase in human adipocytes (Rieusset, et al., 2001) have been recently reported, however, this is the first report on the elevation of hepatic IR, IRS-1, and IRS-2 mass in an animal model. We also examined the effect of rosiglitazone on the protein expression level of PTP-1B. It appears that significant increases in protein mass and activity of PTP-1B partly contributes to the induction of hepatic insulin resistance in the fructose-fed model. Interestingly, rosiglitazone suppressed cellular protein levels of this negative regulator of the insulin signaling pathway to levels even lower than that of control hepatocytes, suggesting that this drug may exert its insulin sensitizing effects partly through direct or indirect modulation of PTP-1B protein levels. Further studies are needed to elucidate whether there is an active PPAR response element in the promoter of the PTP-1B or this effect is secondary to a decrease in FFA efflux to the liver. Shao, et al. (Shao, et al., 1998) reported that incubation of skeletal muscle and hepatic cells with FFAs such as palmitate or oleate significantly increased protein expression of PTP-1B. Moreover, evidence has suggested that accumulation of lipid in liver or muscle leads to development of insulin resistance and it appears that TZDs exert their insulin sensitizing effects by their lipid lowering action and the sequestering of lipids in adipocytes which ultimately reduces lipid accumulation in liver and muscle (Kersten, et al., 2000, Spiegelman, 1998, Yakubu-Madus, et al., 2000, Ye, et al., 2001). Kim et al. (Kim, et al., 2001a) using tissue specific overexpression of lipoprotein lipase recently demonstrated that intracellular accumulation of TG and fatty acid-derived metabolites in muscle and liver can alter the insulin signaling pathway and induce tissue specific insulin resistance.

The effects of insulin signal transduction on hepatic overproduction of VLDL-apoB was also investigated. Interestingly, rosiglitazone treatment significantly reduced the
secretion of VLDL-apoB containing lipoproteins. Pulse-chase studies showed reduced intracellular stability of apoB in hepatocytes isolated from drug treated FF hamsters compared to that of fructose-fed hamsters. Furthermore, immunoblotting studies revealed that rosiglitazone reduced cellular protein mass of MTP to the level of control hepatocytes. Since insulin has a negative regulatory effect on expression of MTP gene (Lin, et al., 1994, Sato, et al., 1999), the observed effects of rosiglitazone on MTP protein mass may be exerted via an insulin response element to produce an improvement in hepatic insulin signal transduction. In Chapter 4, we showed a significant increase in MTP mRNA level, protein mass and activity in insulin resistant hepatocytes which may be a compensatory response to meet the increasing efflux of FFA into the liver, and/or a consequence of induction of hepatic insulin resistance. It has yet to be determined whether reduced hepatic lipid oversupply/accumulation (reduced demand) and/or insulin signaling improvement induced normalization of hepatic MTP levels. Consequently, reduced MTP protein mass may also contribute to destabilization of cellular apoB and reduce its secretion from rosiglitazone-treated hepatocytes.

In summary, improvement in hepatic insulin signal transduction in rosiglitazone-treated fructose-fed hamsters coincided with increased in apoB degradation and a reduction in VLDL-apoB secretion. Thus, our observations support our hypothesis that the induction of hepatic insulin resistance causes dysregulation and overproduction of VLDL-apoB.

7.5 Final Conclusions

In conclusion, our observations indicated that Syrian golden hamster is a suitable model to study metabolism of apoB100-containing lipoproteins as well as dysregulation of hepatic apoB biogenesis in insulin resistant states leading to VLDL-apoB overproduction.
Based on our observations, we postulate (Fig. 7.1) that in the fructose-fed hamster, reduced expression of insulin receptor substrates, partly due to hyperinsulinemia, together with significant elevation of PTP-1B mass and activity, downregulates phosphorylation of IR, IRS-1, and IRS-2 and consequently reduces PI 3-kinase activity. Reduced PI 3-kinase activity causes a reduction in serine- and threonine-phosphorylation of Akt/PKB and thus a drastic attenuation of the hepatic insulin signal transduction. Impairment of insulin signal transduction decreased and increased expression levels of ER-60 and MTP, respectively. Decreased ER-60 protease level and increased MTP level may partly contribute to the enhanced apoB stability and facilitated apoB particle assembly, respectively. Finally, higher stability of apoB, together with increased FFA flux, and consequently enhanced lipid substrate availability, resulting in facilitated lipoprotein assembly may contribute to the hepatic VLDL-apoB oversecretion. Further studies are needed to link insulin signaling pathway to the apoB biogenesis at the subcellular levels.

Although our animal model has significant advantages over other rodent models, it appears that before extrapolating our finding in hamster to human, more comparative studies on different aspects of lipid metabolism in hamster are needed. One pitfall of our model is our use of extremely high fructose as the only source of carbohydrate to induce insulin resistance, which does not resemble the dietary regimen of humans. There are also some differences in lipoprotein profiles between hamster and human. Moreover, long-term fructose feeding of hamsters in our laboratory indicated that the insulin resistance and dyslipidemia that was induced after 2-3 weeks was transient in nature and reverted back to normal with longer term feeding (based on observed plasma lipid levels). Our recent preliminary data has shown that fructose rapidly enters the TG synthesis pathway in hamster liver making it
difficult to attribute our observations in this model to the induction of insulin resistance alone, although our ex vivo chronic fructose incubation studies showed no significant effects on apoB biogenesis. Overall, although the FF model has allowed us to obtain significant new insights into the link between insulin resistance and metabolic dyslipidemia, further studies in other dietary and genetic models are needed to fully elucidate the physiological mechanisms involved in the development of dyslipidemia in insulin resistant states.

7.6 Future Studies

In order to pursue this research, several different issues are left to be addressed in future research efforts: As mentioned in Chapter 1 (Part IV) on diet induced insulin resistance, lipid and amino acid feeding can also induce insulin resistance. By feeding hamster with a high lipid and amino acid diet, and comparing the results with the present research project one can study the net effects of insulin resistance on apoB biogenesis without any possible nutrient substrate interference. On the other hand, it will provide opportunity to study any extra or specific contribution of diet substrate (carbohydrate, lipid, and amino acid) in pathogenesis of insulin resistance-associated dyslipidemia. Furthermore, Our animal model can potentially be very useful to study other aspects of insulin resistance-related dyslipidemia such as low HDL-cholesterol, small dense LDL particles, high oxidative stress, and etc. In insulin resistance, FFA efflux from adipose tissue to the liver, may directly contribute to the VLDL-apoB overproduction. Furthermore, adipocytes as hormone producing (e. g. leptin, resistin, TNFα) cells are involved in energy balance regulation and insulin signal transduction. A comprehensive functional study of adipose tissues (brown and white) in our model may help to reach a better understanding of the molecular and cellular mechanism involved in the metabolic disturbances causing apoB overproduction.
Based on data presented in this thesis, as well as published literature, we postulate that in the fructose-fed hamster, reduced expression of insulin receptor substrates partly due to hyperinsulinemia, together with significant elevation of PTP-1B mass and activity, downregulates phosphorylation of IR, IRS-1, and IRS-2 and consequently reduces PI 3-kinase activity. Reduced PI 3-kinase activity causes a reduction in serine- and threonine-phosphorylation of Akt/PKB and thus a drastic attenuation of the hepatic insulin signal transduction. It appears that expression and activity of MTP and ER-60, two key enzymes involved in apoB biogenesis are under the control of insulin signal transduction.

Impairment of insulin signal transduction decreased and increased expression levels of ER-60 and MTP, respectively. Decreased ER-60 protease level and increased MTP level may partly contribute to the enhanced apoB stability and facilitated apoB particle assembly, respectively. Finally, higher stability of apoB, together with increased FFA flux, and consequently enhanced lipid substrate availability, resulting in facilitated lipoprotein assembly may contribute to the hepatic VLDL-apoB oversecretion.
Recently, a second insulin signaling pathway (PI 3-kinase-independent pathway) has been identified which functions through flotillin-CAP-Cbl complex and promotes GLUT4-mediated glucose uptake (Baumann, et al., 2000). A reliable means to introduce D-3 phosphorylated phosphoinositides (PI 3-kinase products) into the intact insulin resistant hepatocytes would be of great value to check whether the newly discovered insulin signaling pathway is also involved in insulin resistance-associated apoB-overproduction. The incubation of insulin resistant hepatocytes with the membrane permeant esters of phosphatidylinositol 3,4,5-triphosphate (Jiang, et al., 1998) may answer the above question.

We have shown that hamster apoB is also subjected to ubiquitination and proteasomal degradation. Furthermore, apoB is significantly more stable in hepatocytes isolated from fructose-fed hamsters. Recently, Bennett et al. (Bennett, et al., 2000) reported that insulin inhibits the ubiquitin-dependent degrading activity of the 26S proteasome. It would be interesting to evaluate proteasomal activity in hepatocytes isolated from both control and fructose-fed hamsters and to find out whether insulin resistance compromises proteasomal activity.

It has been reported that in vitro attachment of fructose to the lysine amino acid of the proteins (Miyazawa, et al., 1998) increased their stability against ATP-dependent proteolytic systems (Suarez, et al., 1995, Suarez, et al., 1989). It would be interesting to investigate whether high fructose feeding in our animal model can cause fructation of key proteins and enzymes such as apoB, MTP, and PTP-1B, and thereby stabilize and prolong their intracellular presence leading to the types of observations reported in the present study. As reported in our study, apoB showed significantly higher stability in hepatocytes isolated from insulin resistant hamsters and we postulated that suppression of ER-60 protease might
contribute to this stability. ApoB ubiquitination and proteasomal degradation play major roles in regulating its stability. Evaluation and assessment of proteasomal activity in insulin resistance in general, and specifically in relation to apoB, and PTP-1B turnover may address some of these unanswered questions.

We have recently found some metabolic response elements such as insulin response elements (E-box), sterol response elements (SRE), and nuclear factor Y (NF-Y) elements in the promoter of ER-60 gene as well as SRE in the promoter of PTP-1B using sequence alignment method. Further study is needed to characterize the roles and functions of these metabolic response elements.

Since there is an interplay between bile acid secretion and VLDL-cholesterol assembly and secretion, the study of function and expression of cholesterol 7α-hydroxylase, the rate limiting enzyme in the bile acid biosynthesis in hepatic insulin resistance will provide more information on the apoB overproduction in this pathologic condition. A recently published report suggested a link between bile acid formation, cholesterol 7α-hydroxylase activity and the c-Jun N-terminal kinase pathway (Gupta, et al., 2001). It would be interesting to investigate the role of pathway in hepatic apoB biogenesis.

As a final note, although in this thesis, we have attempted to shed some light on the molecular and cellular mechanisms of apoB overproduction in the insulin resistant state, we believe that many questions are still left unanswered and further efforts are needed to address those important issues.
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236


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250


254


