A study of the mechanisms of methylglyoxal-induced changes in plasma lipid levels in Sprague-Dawley rats

A thesis submitted to the College of Graduate Studies and Research in partial fulfillment of the requirements for a Master’s degree in the Department of Pharmacology University of Saskatchewan Saskatoon

By

Qian Huang

Supervised by

Dr. Kaushik Desai & Dr. Lingyun Wu

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ABSTRACT

Dyslipidemia, defined as abnormal levels of plasma lipids, is an independent risk factor for cardiovascular diseases, and is increasing worldwide. Methylglyoxal (MG), a reactive glucose metabolite, is a causative factor for type 2 diabetes and is associated with hypertension. We have recently reported abnormal plasma lipid levels in Sprague-Dawley (SD) rats treated chronically with MG. The liver and the adipose tissue are the main organs that contribute to plasma lipid level regulation. The aim of this study was to explore the possible mechanism(s) of MG-induced dyslipidemia. MG (60 mg/kg/day) or 0.9% saline was administered to 12-week-old male SD rats by continuous infusion with a minipump for 28 days and thereafter, the liver, and the adipose tissue were isolated and the plasma was collected. We also used cultured 3T3-L1 adipocytes for molecular studies. We performed Oil-Red O staining and hematoxylin-eosin staining, ELISA, western blotting and quantitative PCR. In the liver of rats treated chronically with MG, there was increased protein expression of sterol regulatory element binding protein-2 (SREBP-2), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and microsomal triacylglycerol transfer protein (MTP), with no change in SREBP-1 and low density lipoprotein (LDL) receptor. In the adipose tissue, MG treated rats showed enlarged abdominal adipocytes, and increased protein expression and/or mRNA of CCAAT-enhancer binding protein (C/EBP)α, C/EBPβ and tumor necrosis factor alpha (TNFα), with no significant change in peroxisome proliferator activated receptor gamma (PPARγ), adiponectin, leptin and lipoprotein lipase levels. The effects of MG were attenuated by aminoguanidine, a
MG and an advanced glycation endproducts scavenger. The findings in adipose tissue were confirmed in cultured 3T3-L1 cells. Our results indicate that the increase in plasma cholesterol and triacylglycerols by MG may be due to increased activity of hepatic SREBP-2 and HMG-CoA reductase, and the subsequent increased cholesterol synthesis; and due to increased activity of MTP and the resultant increased VLDL synthesis. The increase in plasma free fatty acids may be due to increased triacylglycerol accumulation caused by over-expression of C/EBPα and C/EBPβ, increased adipocyte size and up-regulated gene expression of TNFα, and the resultant decreased free fatty acids uptake and increased lipolysis in adipocytes.
ACKNOWLEDGEMENTS

It will be exactly two years for me from entering the gate of the Department of Pharmacology to finishing my master's thesis. Two years is not a long time in life. However these two years is an invaluable journey for me, not only because it’s my first time being abroad, but because of the people who support me, help me, give me guidance or suggestion during this period. I would like to express my sincere appreciation to some of them here.

First, I would like to thank my primary supervisor, Dr. Kaushik Desai. He picked me up from the airport, showed me around the campus, took me to the lab meeting and guided me to the research field. He is a patient, knowledgeable and warm-hearted gentleman. He always offers me help whenever I need, from lending me the Ping-Pong bat to showing me the staining technique; from giving me extra mattress to answering my questions generated from pharmacology course. He always encourages me no matter what results I got and tries to create opportunities for me to gain self-confidence and interests about research. I have felt strong self-growth within these two years which was impossible without him.

Secondly, I would like to thank Dr. Lily Wu, my co-supervisor. Her Chinese background made her close to me and I know this comfortable and sincere relationship is more based on her caring characteristics. She cares about me and other students, cares about our feelings and our future. She leads me to a broad field beyond the research and leads me to think seriously what my goal is and what I should do to pursue it.
Thirdly, I really want to say thanks to my other committee members, Dr. Steve Richardson and Dr. Lixin Liu. Thanks for their invaluable advice all through the period. Dr. Richardson is also a good friend of mine while Dr. Liu is an example of a real hard working person for me. I also want to thank all the colleagues inside or outside of my lab: Arlene Drimme, Tuanjie Chang, Jianghai Liu, Ashley Untereiner, Arti Dhar, Indu Dhar, Ali Banigesh, Cindy Wruck, Donna Dodge, and Bob Wilcox. Thank you for your selfless help and kind support. Because of you, I love this lab, this department and enjoyed the two years here and will definitely miss this place when I leave.

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To my lovely family
# TABLE OF CONTENTS

PERMISSION TO USE ..................................................................................... I
ABSTRACT ....................................................................................................... II
ACKNOWLEDGEMENTS ............................................................................... IV
TABLE OF CONTENTS ............................................................................... VII
LIST OF TABLES ........................................................................................ IX
LIST OF FIGURES ......................................................................................... IX
LIST OF ABBREVIATIONS ........................................................................... XI

## CHAPTER ONE INTRODUCTION

1.1 Dyslipidemia .......................................................................................... 1
  1.1.1 Definition ......................................................................................... 1
  1.1.2 Prevalence ..................................................................................... 1
  1.1.3 Pathogenesis ................................................................................... 3
1.2 Plasma lipid sources and cycling .......................................................... 6
  1.2.1 Types of lipids ................................................................................. 6
  1.2.2 Structure of Lipoproteins ................................................................. 7
  1.2.3 Characteristics of lipoproteins .......................................................... 8
  1.2.4 Lipoprotein metabolic pathways ...................................................... 9
1.3 Adipose tissue and lipid regulation ...................................................... 12
  1.3.1 Adipose tissue types ...................................................................... 12
  1.3.2 Adipocyte differentiation ................................................................. 14
  1.3.3 CCAAT/enhancer binding protein family (C/EBPs) ......................... 15
  1.3.4 Peroxisome proliferator-activated receptor gamma (PPARγ) ........... 16
  1.3.5 Tumor necrosis factor alpha (TNFα) ............................................. 18
  1.3.6 Lipoprotein lipase (LPL) ................................................................. 20
  1.3.7 Leptin ............................................................................................ 21
  1.3.8 Adiponectin .................................................................................. 23
1.4 Liver enzymes / receptors in regulating plasma lipids ....................... 24
  1.4.1 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) ........................................................................ 24
  1.4.2 Low density lipoprotein receptor (LDLR) ...................................... 25
  1.4.3 Sterol regulatory element-binding proteins (SREBP) ...................... 27
  1.4.4 Microsomal triacylglycerol transfer protein (MTP) ....................... 30
1.5 Methylglyoxal ....................................................................................... 33
  1.5.1 Synthesis of MG ............................................................................ 33
  1.5.2 Degradation of MG ....................................................................... 34
  1.5.3 MG and reactive oxygen species (ROS) .......................................... 35
  1.5.4 MG and AGEs .............................................................................. 37
  1.5.5 Role of MG in diabetes ................................................................. 37
  1.5.6 Role of MG in hypertension .......................................................... 38
  1.5.7 Previous findings in MG treated rats ............................................. 40
1.6 Hypothesis ............................................................................................ 45
1.7 Objectives ............................................................................................. 46
1.8 Research Protocol Outline ................................................................. 47
LIST OF TABLES

Table 1-1. Metabolic syndrome criteria defined by the International Diabetes Federation (IDF) and the National Cholesterol Education Program’s Adult Treatment Panel III (NCEP/ATPIII) 3
Table 1-2. Characteristics of Plasma Lipoproteins 8
Table 1-3. Comparison of major features of white and brown adipose tissue 13
Table 1-4. Plasma lipid levels in Sprague-Dawley rats treated with methylglyoxal 42
### LIST OF FIGURES

| Figure 1-1. | Structure of lipoprotein | 7 |
| Figure 1-2. | The exogenous and endogenous lipoprotein metabolic pathways | 11 |
| Figure 1-3. | Tumor necrosis factor-α (TNFα) regulates lipid metabolism in the adipocytes | 19 |
| Figure 1-4. | Sequential Steps in low density lipoprotein (LDL) receptor pathway | 27 |
| Figure 1-5. | Regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol synthesis, and low density lipoprotein (LDL) receptors by sterols | 29 |
| Figure 1-6. | Role of hepatic microsomal triacylglycerol transfer protein (MTP) in the regulation apolipoprotein (apo)B-100 lipoproteins assembly | 32 |
| Figure 1-7. | Structure of methylglyoxal | 33 |
| Figure 1-8. | Synthesis and degradation of methylglyoxal | 35 |
| Figure 1-9. | Distribution of methylglyoxal (MG) in different organs/tissues/urine in Sprague-Dawley rats after intraperitoneal administration. | 40 |
| Figure 1-10. | Methylglyoxal (MG) levels are elevated and reduced glutathione (GSH) levels are decreased in Sprague-Dawley rats chronically treated with MG. | 43 |
| Figure 3-1. | Effects of methylglyoxal (MG) or MG co-treated with aminoguanidine (AG) on adipocytes size in rat abdominal adipose tissue | 56 |
| Figure 3-2. | Effects of methylglyoxal (MG) on mRNA expression of leptin, lipoprotein lipase (LPL) and tumor necrosis factor alpha (TNFα) in adipose tissue | 58 |
| Figure 3-3. | Effects of methylglyoxal (MG) or MG co-treated with aminoguanidine (AG) on the protein levels of CCAAT/enhancer binding protein (C/EBP)α and C/EBPβ in adipose tissue | 60 |
| Figure 3-4. | Effect of methylglyoxal (MG) on the gene expression of peroxisome proliferator activated receptor gamma (PPARγ) in adipose tissue | 61 |
| Figure 3-5. | Effects of methylglyoxal (MG) on the gene expression of adiponectin and lipoprotein lipase (LPL) in adipose tissue | 62 |
| Figure 3-6. | Effect of methylglyoxal (MG) on circulating leptin levels in rat plasma | 63 |
| Figure 3-7. | Effects of methylglyoxal (MG) on lipid accumulation in 3T3-L1 adipocytes | 65 |
| Figure 3-8. | Effects of methylglyoxal (MG) on the expression of leptin, lipoprotein lipase (LPL) and tumor necrosis factor alpha (TNFα) in 3T3-L1 adipocytes | 66 |
| Figure 3-9. | Effects of methylglyoxal (MG) or MG co-treated with | 67 |
aminoguanidine (AG) co-treatment on the protein levels of C/EBPα and C/EBPβ in 3T3-L1 adipocytes

Figure 3-10. Effect of methylglyoxal (MG) on the gene expression of Peroxisome proliferator activated receptor gamma (PPARγ) in 3T3-L1 adipocytes

Figure 3-11. Effects of methylglyoxal (MG) on the expression of adiponectin and lipoprotein lipase (LPL) in 3T3-L1 adipocytes

Figure 3-12. Methylglyoxal (MG) did not alter leptin secretion in 3T3-L1 adipocytes

Figure 3-13. Effects of methylglyoxal (MG) on sterol regulatory element-binding protein (SREBP)-1 and SREBP-2 protein levels in the liver

Figure 3-14. Effects of methylglyoxal (MG) on 3-hydroxy-3-methylglutaryl conenzyme A reductase (HMGCR) and low density lipoprotein receptor (LDLR) gene expression in the liver

Figure 3-15. Effects of methylglyoxal (MG) on microsomal triacylglycerol transfer protein (MTP) expression in the liver.

Figure 4-1. Possible mechanisms for MG-induced increase of FFAs, cholesterol and triacylglycerol levels in the plasma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>Alagebrium</td>
</tr>
<tr>
<td>AG</td>
<td>Aminoguanidine</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>ACS</td>
<td>Acyl-CoA synthetase</td>
</tr>
<tr>
<td>apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSO</td>
<td>L-buthionine sulfoximine</td>
</tr>
<tr>
<td>CEL</td>
<td>Nε-caboxyethyl lysine</td>
</tr>
<tr>
<td>CML</td>
<td>Nε-carboxymethyl lysine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovasuclar disease</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyleneiodonium</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transporter</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>F-1-P</td>
<td>Fructose-1-phophosphate</td>
</tr>
<tr>
<td>F-1,6-P</td>
<td>Fructose-1,6-biphosphate</td>
</tr>
<tr>
<td>FCHL</td>
<td>Familial combined hyperlipidemia</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>G-3-P</td>
<td>Glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>G-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HMGCR/HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A reductase</td>
</tr>
<tr>
<td>HL</td>
<td>Hepatic lipase</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate-density lipoproteins</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MG</td>
<td>Methylglyoxal</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal triacylglycerol transfer protein</td>
</tr>
<tr>
<td>MIX</td>
<td>Methylisobutylxanthine</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide anions</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time-polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley rat</td>
</tr>
<tr>
<td>SSAO</td>
<td>Semicarbazide-sensitive amine oxidase</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rats</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low-density lipoproteins</td>
</tr>
<tr>
<td>VSMCs</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar-Kyoto rats</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

1.1 Dyslipidemia

1.1.1 Definition

Dyslipidemia, characterized as (a) elevated fatty acids in the circulation, (b) raised triacylglycerol (TG) values, (c) low high density lipoprotein (HDL) cholesterol levels, (d) increased small, dense low density lipoprotein (LDL) levels and (e) elevated apolipoprotein (apo) B levels, is widely established as an independent risk factor for cardiovascular diseases (CVD) (Kolovou et al. 2005). However, most individuals who develop CVD have multiple risk factors that commonly cluster together, such as dyslipidemia, hypertension and hyperglycemia and the term metabolic syndrome has been given to this phenomenon (Grundy et al. 2004).

1.1.2 Prevalence

According to the 2009 update of the Canadian Cardiovascular Society (CCS) guidelines for the diagnosis and treatment of dyslipidemia and prevention of cardiovascular disease in the adult (Genest et al. 2009), cardiovascular disease (CVD) is the leading reason for deaths in Canada which accounts for one-third of deaths. The prevalence of CVD in Canada is expected to increase predominantly because of the prevalence of sedentary lifestyles and emergence of obesity and diabetes mellitus in epidemic proportions.

Data from the National Health and Nutrition Examination Survey (NHANES) from 2003-2006 in United States showed that there are approximately 34% of adults who
meet the criteria for the metabolic syndrome. People with older age or higher body mass index (BMI) were much more likely to meet the criteria compared to those aged 20-39 or with normal body weight, respectively (Ervin. 2009).

There are at least 3 organizations that have recommended clinical criteria for metabolic syndrome. They included similar aspects but have notable differences (Grundy et al. 2004). The 2009 CCS guidelines (Genest et al. 2009) have quoted the criteria from the International Diabetes Federation (IDF) while the NHANES used the one from the National Cholesterol Education Program's Adult Treatment Panel III (NCEP/ATP III) guidelines as metabolic syndrome criteria (Ervin. 2009) (Table 1-1).
### IDF

**Central obesity**

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Waist circumference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europeans</td>
<td>Men≥94 cm; Women≥80 cm</td>
</tr>
<tr>
<td>South Asians</td>
<td>Men≥90 cm; Women≥80 cm</td>
</tr>
<tr>
<td>Chinese</td>
<td>Men≥90 cm; Women≥80 cm</td>
</tr>
<tr>
<td>Japanese</td>
<td>Men≥90 cm; Women≥80 cm</td>
</tr>
<tr>
<td>Ethnic South and Central Americans</td>
<td>Use South Asian recommendations until more specific data are available</td>
</tr>
<tr>
<td>First Nations</td>
<td>Use South Asian recommendations until more specific data are available</td>
</tr>
<tr>
<td>Sub-Saharan Africans</td>
<td>Use European data until more specific data are available</td>
</tr>
<tr>
<td>Eastern Mediterranean and Middle East (Arabic) Populations</td>
<td>Use European data until more specific data are available</td>
</tr>
</tbody>
</table>

**Plus two of the following factors:**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma triacylglycerols</td>
<td>&gt;1.7 mmol/L</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>Men&lt;1.03 mmol/L; Women&lt;1.3 mmol/L</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>&gt;130/85 mmHg (or treatment for hypertension)</td>
</tr>
<tr>
<td>Fasting plasma glucose</td>
<td>&gt;5.6 mmol/L</td>
</tr>
</tbody>
</table>

**NCEP/ATPIII**

**At least of three of following factors**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference</td>
<td>Men&gt;102 cm; Women&gt;88 cm</td>
</tr>
<tr>
<td>Fasting triacylglycerols</td>
<td>≥1.7 mmol/L</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>Men&lt;1 mmol/L; Women&lt;1.3 mmol/L</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>≥130/85 mmHg</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>≥6.1 mmol/L</td>
</tr>
</tbody>
</table>

**Table 1-1.** Metabolic syndrome criteria defined by the International Diabetes Federation (IDF) and the National Cholesterol Education Program’s Adult Treatment Panel III (NCEP/ATPIII). The table is modified from 2009 Canadian Cardiovascular Society (CCS) guidelines (Genest et al. 2009) and National Health and Nutrition Examination Survey (Ervin, 2009). HDL, High density lipoproteins.

1.1.3 Pathogenesis

Primary dyslipidemia is mainly due to genetic disorders such as defects in LDL receptor or lipoprotein lipase, whereas secondary dyslipidemia mainly results from
unhealthy lifestyle such as sedentary behavior and diets high in carbohydrates, and saturated fat (Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12e, chapter 31). In addition, environmental factors, medications, drugs, hormones, or even other diseases can cause secondary dyslipidemia.

Familial combined hyperlipidemia (FCHL) is the most common genetic form of dyslipidemia and is associated with 1.7 to 10 times increase in the risk of coronary artery disease (CAD) (Carr and Brunzell. 2004). Compared to the lipid disturbance involved in most metabolic syndrome patients, FCHL patients exhibit exaggerated high plasma triacylglycerol as well as increased plasma apoB and increased number of small dense LDL particles. The reason or the major gene for FCHL has not been clearly defined. However, besides the decrease in lipoprotein lipase (LPL) activity and predisposition of central abdominal fat, researchers also suggest that there exists a gene(s) associated with increased apoB levels (Ayyobi and Brunzell. 2003).

However, the majority of dyslipidemia cases are secondary. Take high-fat and high carbohydrates diets as examples. Saturated fat intake is thought to increase the concentration of LDL cholesterol and therefore increase the risk of CVD. In the United States, the major dietary sources of saturated fatty acids are full-fat dairy products and red meat (Siri-Tarino et al. 2010). The increased consumption of calories, especially in the form of refined carbohydrates is recognized to be highly correlated with the increase of metabolic syndrome (Basciano et al. 2005). Fructose is widely used in sweeteners nowadays and its consumption has increased 5 fold in the last century and doubled in the last 30 years (Lustig. 2010). A numbers of studies have
provided the evidence that over-consumption of fructose is associated with increased TG production and lipoprotein (VLDL and resultant LDL) secretion and the possible mechanisms have been nicely reviewed by Basciano et al (Basciano et al. 2005).

Alarms have been raised on the over-consumption of saturated fats and high carbohydrate diet-induced dyslipidemia as well as obesity and the metabolic syndrome. For reducing the risks and improving health, dietary strategies including reduced postprandial glucose and triacylglycerol, increased intake of dietary antioxidants, potassium (for lowering the blood pressure), and unsaturated fatty acids such as omega-3 fatty acids have been suggested (O'Keefe and Abuannadi. 2010).
1.2 Plasma lipid sources and cycling

1.2.1 Types of lipids

Lipids, insoluble fatty substances, can be classified into simple lipids, such as cholesterol and fatty acids, and complex lipids, including cholesterol esters and glycerol esters such as triacylglycerols. The major function of lipids is to serve as a source of energy for the body. Other important functions include forming cell membranes, synthesis of hormones and vitamins, synthesis of bile acids, protecting vital organs from physical forces and providing insulation.

Cholesterol is a common simple lipid. It is a steroid alcohol comprised of a four-ringed steroid nucleus and a hydroxyl group. Cholesterol exists as a free sterol in cell membranes, but as an ester combined with a long-chain fatty acid in the plasma. Cholesterol is essential for human and other vertebrates because of its important role in creating signaling molecules on the plasma membrane, synthesis of all steroid hormones and bile acids as well as forming the myelin sheath which surrounds axons. (Goldstein and Brown. 2009).

Because of limited production in our body, most fatty acids must be obtained from the diet (Mattson and Grundy. 1985). There are three forms of fatty acids: saturated, monounsaturated and polyunsaturated, which can be distinguished by the number of double bonds between the carbon atoms in the hydrocarbon chain.

There are three basic classes of complex lipids: triacylglycerols, phospholipids, and cholesterol esters. A triacylglycerol is formed by the esterification of glycerol with three fatty acid molecules. Most triacylglycerols are stored in adipose tissue as an
emergency energy source. When carbohydrate energy sources in our bodies are depleted, triacylglycerols will release fatty acids to meet the need of energy.

1.2.2 Structure of Lipoproteins

Since cholesterol and triacylglycerols are insoluble in the water, they must be modified in order to be transported in the blood. Apolipoproteins combine with the lipids to form soluble complexes named lipoproteins (Fredrickson et al. 1972) (Figure 1-1). Each lipoprotein is a sphere with a surface primarily formed by phospholipids and a hydrophobic core containing triacylglycerols and cholesteryl esters. There are two ends in each phospholipid: a hydrophobic end pointing into the lipid core, and a hydrophilic end which is soluble in plasma. An apolipoprotein, which stabilizes the lipoprotein structure, is located in the outer shell. Moreover, apolipoproteins can be recognized by specific receptors that metabolize them.

**Figure 1-1. Structure of lipoprotein.** (Source: Available online at: http://web.campbell.edu/faculty/nemecz/323_lect/fatty_acid/images/struct_2.jpg).
1.2.3 Characteristics of lipoproteins

Six types of lipoproteins are classified by different factors including density, composition and apoproteins. Different types of lipoproteins transport specific lipids from synthesis sites to utilization sites. This is summarized in the table (Table 1-2).

<table>
<thead>
<tr>
<th>Lipoprotein class</th>
<th>Density (g/mL)</th>
<th>Major lipid constituent</th>
<th>TG: CHOL ratio</th>
<th>Significant apoproteins</th>
<th>Site of synthesis</th>
<th>Mechanism(s) of catabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons and remnants</td>
<td>≤1.006</td>
<td>Dietary triacylglycerols and cholesterol</td>
<td>10:1</td>
<td>B-48,E, A-LA-IV, C-I, C-II,C-III</td>
<td>Intestine</td>
<td>Triacylglycerol hydrolysis by LPL, apoE-mediated remnant uptake by the liver</td>
</tr>
<tr>
<td>VLDL</td>
<td>&lt;1.006</td>
<td>“Endogenous” or hepatic triacylglycerols</td>
<td>5:1</td>
<td>B-100,E, C-I, C-II,C-III</td>
<td>Liver</td>
<td>Triacylglycerol hydrolysis by LPL</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>Cholesteryl esters and “endogenous” triacylglycerols</td>
<td>1:1</td>
<td>B-100,E, C-II,C-III</td>
<td>Product of VLDL catabolism</td>
<td>50% converted to LDL mediated by HL; 50% apoE-mediated uptake by the liver</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>Cholesteryl esters</td>
<td>NS</td>
<td>B-100</td>
<td>Product of VLDL catabolism</td>
<td>ApoB-100–mediated uptake by LDL receptor (75% in the liver)</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>Phospholipids cholesteryl esters</td>
<td>NS</td>
<td>A-I,A-II, C-I, C-II,C-III</td>
<td>Intestine, liver, plasma</td>
<td>Complex: transfer of cholesteryl ester to VLDL and LDL; uptake of HDL cholesterol by hepatocytes</td>
</tr>
<tr>
<td>Lipoprotein(a)</td>
<td>1.05-1.09</td>
<td>Cholesteryl esters</td>
<td>NS</td>
<td>B-100, apo(a)</td>
<td>Liver</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 1-2. Characteristics of Plasma Lipoproteins

Abbreviations: apo, apolipoprotein; CHOL, cholesterol; HDL, high-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; NS, not significant
(triacylglycerol is <5% of LDL and HDL); TG, triacylglycerol; VLDL, very-low-density lipoproteins; HL, hepatic lipase; LPL, lipoprotein lipase. (Source: Goodman Pharmacology 12 edition).

1.2.4 Lipoprotein metabolic pathways

Lipoprotein metabolism consists of 3 separate but integrated pathways that involve the liver, the primary organ of lipid metabolism and also other organs such as small intestines, adipose tissue and muscle. The 3 pathways include the exogenous pathway, the endogenous pathway (Fig 1-2) and the reverse cholesterol transport pathway (Schaefer et al. 1978; Kwiterovich. 2000).

Lipids from the food are metabolized via the exogenous pathway (Fig 1-2). Dietary fat breaks down into fatty acids and cholesterol in the gastrointestinal tract and then is converted into triacylglycerols and cholesterol esters, respectively. Within the intestine, triacylglycerols and cholesterol are incorporated into chylomicrons for transport in the blood and delivered to muscles and adipose tissue through capillaries. There, lipoprotein lipase (LPL) breaks down the triacylglycerols into free fatty acids (FFAs). After removing triacylglycerols from the chylomicrons, the resultant chylomicron remnants with relatively high cholesterol concentration are then extracted by the liver from the blood which is mediated by the interactions between apoE on the surface of the chylomicron particles and two receptors on liver cells: the LDL receptor and the LDL receptor-related protein (LRP). Inside the liver cells, the cholesterol from the chylomicron remnants is either stored in the liver or is used to synthesize bile acids, cell membranes, or other lipoproteins.
Lipids synthesized in the liver are metabolized via the endogenous pathway (Fig 1-2). VLDL, the primary lipoprotein produced by the liver, is broken down by LPL into FFAs in a way similar to chylomicron degradation. The resultant IDL then processed in two ways: 1. Binding with the liver LDL receptors which leads to intracellular metabolism. Approximately 50% of the IDL particles are rapidly removed from the circulation in this way; 2. The remaining IDL particles in the blood are gradually taken up by the liver LDL receptors and then converted to LDL by liver hepatic lipase (HL). Most LDL is removed from the blood by the liver LDL receptor and excreted via the enterohepatic circulation (Goldstein and Brown. 1992).

Reverse cholesterol transport is the process by which the cholesterol from the tissues is transported back to the liver, which reuses cholesterol. HDL particles are central to reverse cholesterol transport (Remaley. 2011; Rayner et al. 2011).
Figure 1-2. The exogenous and endogenous lipoprotein metabolic pathways.
LPL, lipoprotein lipase; FFA, free fatty acid; VLDL, very low density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; Apo C’s, Apolipoprotein C-I, C-II, C-III, and C-IV; ApoB, Apolipoprotein B; Apo E, Apolipoprotein E.
1.3 Adipose tissue and lipid regulation

1.3.1 Adipose tissue types

Adipose tissue was first considered simply as a triacylglycerol storage organ. However, studies through recent two decades have demonstrated that the adipocyte is a dynamic cell that plays an important role in energy balance and whole body homeostasis (Deng and Scherer, 2010). The reasons that account for these newly found functions of adipose tissue include the facts that the adipose tissue is now known to secret a series of bioactive adipokines such as leptin, adiponectin and tumor necrosis factor alpha (TNFα) which act both locally and at the systemic level; the adipose tissue expresses a number of receptors such as insulin receptor, leptin receptor and TNFα receptor and therefore it is able to respond to various signals from both the hormonal system and the central nervous system (Kershaw and Flier, 2004).

There are two types of adipose tissues in mammals, white adipose tissue (WAT) and brown adipose tissue (BAT). They differ in several major features (see table). In rodents, most BAT is located in the interscapular region; in humans, large depots of BAT are found in infancy, but only small amounts persist in adults. In addition, BAT expresses almost all the genes that are expressed in WAT, but they also express some distinct genes including uncoupling protein-1 (UCP1), which is located in the mitochondria of BAT and is responsible for its thermogenesis function without generating ATP (Rosen and MacDougald, 2006).

WAT is distributed throughout the body in the form of two major types, subcutaneous adipose tissue and visceral (or abdominal) adipose tissue. Besides the
difference in locations, they possess distinct physiological properties. Increased visceral adipose tissue, that is central obesity, has been found to be associated with an increased risk for developing conditions such as insulin resistance, type 2 diabetes mellitus, dyslipidemia, hypertension, atherosclerosis, hepatic steatosis and cholesterol gallstones. By contrast, increased subcutaneous adipose tissue mass predominantly in buttocks and thighs, that is peripheral obesity, seems to be associated with improved insulin sensitivity and a lower risk of developing type 2 diabetes mellitus compared to those with central obesity (Tran and Kahn. 2010). Various reasons have been proposed to explain the protective and detrimental effect of subcutaneous adipose tissue and visceral adipose tissue, respectively (Tran and Kahn. 2010), and the greater antilipolytic effect to insulin in subcutaneous adipose tissue is one of them.

<table>
<thead>
<tr>
<th>Major feature</th>
<th>WAT</th>
<th>BAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascularization</td>
<td>Some, limited</td>
<td>Extensive</td>
</tr>
<tr>
<td>Distribution</td>
<td>Extensive, many sites</td>
<td>Restricted</td>
</tr>
<tr>
<td>Sympathetic innervation</td>
<td>Some, limited</td>
<td>Extensive</td>
</tr>
<tr>
<td>Fatty acid role(s)</td>
<td>Synthesis, storage, signaling</td>
<td>Storage, oxidation, signaling</td>
</tr>
<tr>
<td>Thermogenesis</td>
<td>Negligible</td>
<td>Highly developed</td>
</tr>
<tr>
<td>Insulin effects</td>
<td>Extensive</td>
<td>Extensive</td>
</tr>
<tr>
<td>Adrenoreceptors</td>
<td>Primarily α₂,β₁,β₂,β₃</td>
<td>Primarily α₁,β₁,β₃</td>
</tr>
<tr>
<td>Droplet size</td>
<td>Large, single</td>
<td>Small, multiple</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>few</td>
<td>Many, densely packed</td>
</tr>
</tbody>
</table>

Table 1-3. Comparison of major features of white and brown adipose tissue. (Source: Dennis E. Vance and Jean E. Vance, Biochemistry of lipids, lipoproteins and membranes 5th edition, chapter 10)
1.3.2 Adipocyte differentiation

Adipocytes derive from mesenchymal stem cells which are pluripotent cells with the capacity to differentiate into limited cell types, including myocytes, chondrocytes, osteocytes and adipocytes. There are two phases of adipogenesis. The first phase is called determination, in which mesenchymal stem cells convert to fibroblast-like pre-adipocytes and lose the potential to differentiate into other cell types. This conversion cannot be distinguished morphologically. The second phase is known as terminal differentiation, in which the pre-adipocytes take on characteristics of the mature adipocytes which are essential for lipid transport and synthesis, insulin sensitivity and adipokine secretion (Rosen and MacDougald. 2006).

Compared to the first phase, the phase of terminal differentiation has been more extensively studied by using both cell line and primary cell models. After growth arrest at confluence, a variety of differentiation protocols which may include endocrine, paracrine regulators and/or transcription factors have been used for inducing differentiation for different cell models (Gregoire et al. 1998; Hwang et al. 1997). For example, the most well developed and frequently used cell lines are mouse pre-adipocyte cell lines 3T3-L1 and 3T3-F422A, which were clonally isolated from Swiss 3T3 cells derived from disaggregated 17- to 19- day mouse embryos (Rosen and MacDougald. 2006; Gregoire et al. 1998). The combination of 10% fetal bovine serum (FBS), insulin, dexamethasone (Dex, a glucorticoid) and methylisobutylxanthine (MIX, an agent that increases the intracellular cAMP level) has been proven to be the most effective for differentiating 3T3-L1 cell lines while
only FBS and insulin are needed for 3T3-F442A (Hwang et al. 1997; Student et al. 1980)

During adipocyte differentiation, chronological changes of expression of numerous genes demonstrate the acquisition of the adipocyte phenotype (Hwang et al. 1997). Moreover, alteration of these genes during differentiation or in the mature adipocyte could lead to physiological dysfunction. These will be explained specifically later.

1.3.3 CCAAT/enhancer binding protein family (C/EBPs)

C/EBPs are expressed in various tissues and are involved in the regulation of several biological processes, including control of energy metabolism. In adipose tissue, C/EBPs were the first transcription factors demonstrated to play a major role in the regulation of adipocyte differentiation. These factors are proteins belonging to the bZIP class, with a transcriptional activation domain and a leucine zipper motif (Hurst. 1995).

C/EBPβ and C/EBPδ are likely functional in the early stage of differentiation. cAMP and glucocorticoid are applied to induce cultured adipocyte differentiation and seem to be directly responsible for the induction of C/EBPβ and C/EBPδ, respectively (Yeh et al. 1995). At the late differentiation phase, the expression of C/EBPβ dropped 50% from its maximal level which it reached within the first 2 days, and C/EBPδ dropped even more sharply (Cao et al. 1991). The major role of these two C/EBPs seems to be the induction of C/EBPα and PPARγ (Wu et al. 1996; Hamm et al. 2001).

C/EBPα is another C/EBP isoform induced relatively late in the adipocyte
differentiation process and just prior to the expression of many adipocyte-specific genes (Cao et al. 1991). C/EBPα is required to induce adipocyte and recognized to play a central role in the regulation of energy homeostasis since the expression of anti-sense C/EBPα in 3T3-L1 pre-adipocytes blocked the differentiation process (Lin and Lane. 1992), and C/EBPα knockout mice also failed to accumulate lipid in adipocyte and died from hypoglycemia within 8 hours of birth (Wang et al. 1995). Moreover, C/EBPα could bind and transactivate the promoter of several adipocyte-specific genes (Gregoire et al. 1998). For example, the mutation of C/EBPα site in leptin blocked its transactivation (Hwang et al. 1996).

Unlike C/EBPβ and C/EBPδ, C/EBPα is expressed at high levels in the mature adipocyte and adipose tissue (Cao et al. 1991). Insulin regulates the transcription of C/EBP genes in mature adipocytes. Adding insulin to 3T3-L1 adipocytes repressed the expression of C/EBPα while it induced the expression of C/EBPβ and C/EBPσ (MacDougald et al. 1995a).

1.3.4 Peroxisome proliferator-activated receptor gamma (PPARγ)

The PPARs belong to a class of nuclear hormone receptors and form heterodimers by binding with the retinoid X receptors (RXRs) (Juge-Aubry et al. 1995). PPARγ, expressed during the early stages of adipocyte differentiation, has been shown to be the most adipose specific and most adipogenic of the three PPAR isoforms: α, σ/β, γ (Braissant et al. 1996). Compared to PPAR γ1, the other PPARγ isoform, PPARγ2 is more predominantly expressed in adipose tissue (Zhu et al. 1995; Tontonoz et al.
PPARγ is thought to be a master regulator of adipogenesis. PPARγ and C/EBPα cross-regulate each other’s expression and govern expression of the entire adipogenic program (Farmer. 2005). A study in type 2 diabetic patients treated with thiazolidinediones (TZDs, PPARγ agonists), showed improved insulin sensitivity, but increased weight gain (Fonseca. 2003). Moreover, an ex vivo study in rats showed that TZDs induce remodeling of visceral adipocytes to a smaller size with higher lipid storage potential (de Souza et al. 2001) and an in vivo study in wild type mice showed that TZD treatment prevented high-fat induced adipocyte hypertrophy but not insulin resistance (Miles et al. 2003).

Failure of insulin to suppress lipolysis leads to elevated blood triacylglycerol levels as well as decreased HDL levels. A review summarized that subjects with mutation in PPARγ have shown elevated triacylglycerols and low HDL levels, without significant change in LDL levels (Gurnell et al. 2003). PPARγ agonists, which improve insulin sensitivity, decrease FFA levels mostly by promoting the storage of FFA in adipocyte (Martin et al. 1998). However, although pioglitazone does reduce triglycerides, rosiglitazone does not lower and may even slightly increase serum triacylglycerol levels (Gurnell et al. 2003).

In addition to the impact on obesity, insulin resistance and dyslipidemia, PPARγ activation in adipose tissue is also associated with the expression of a wide range of adipokines in a beneficial way, including adiponectin, leptin and TNFα. PPARγ activation up-regulates the expression of adiponectin, which appears to be an important mediator of PPARγ agonist-induced improvement of insulin sensitivity.
(Bouskila et al. 2005). Moreover, PPARγ agonists suppress the production of leptin and TNFα (Hammarstedt et al. 2005; Kallen and Lazar. 1996).

1.3.5 Tumor necrosis factor alpha (TNFα)

TNFα, a 17-KDa polypeptide, is an inflammatory cytokine first found in the supernatant of cultured macrophages and later reported to be also highly induced in the adipose tissue in both animals and humans (Kawakami and Cerami. 1981; Kawakami et al. 1982; Hotamisligil et al. 1993; Hotamisligil et al. 1995).

Adipocyte-derived TNFα has been linked to dyslipidemia in both clinical and basic studies. Patients with higher TNFα concentration in plasma showed increased levels of total cholesterol, TG and LDL (Madej et al. 1998). Meanwhile, HMG-CoA reductase inhibitors such as simvastatin and atorvastatin that are used for hyperlipidemia could decrease plasma TNFα levels (Ascer et al. 2004; Marketou et al. 2006).

In the review by Sethi and Hotamisligil (Sethi and Hotamisligil. 1999), three aspects of TNFα involved in the regulation of lipid metabolism have been summarized: 1. Elevated TNFα contributes to elevated basal lipolysis rate which leads to increased FFA levels in the circulation; 2. TNFα could decrease FFA uptake from the circulation and contribute to hyperlipidemia by inhibiting activity and down-regulating the protein expression of LPL and expression of FFA transporters such as fatty acid transport protein (FATP); 3. TNFα decreases the expression of key enzymes involved in lipogenesis including perilipin and hormone sensitive lipase.
(HSL), however this may not happen in mature adipocytes (Figure 1-5). In addition, TNFα could also affect lipid metabolism by altering related adipokines, such as leptin and adiponectin (Finck and Johnson, 2000; Hector et al. 2007; Li et al. 2009). PPARγ, which has been described before, exhibited opposite effects to TNFα on the expression of certain proteins involved in adipocyte lipid regulation. Those proteins are also indicated in Figure 1-5.

**Figure 1-3. Tumor necrosis factor alpha (TNFα) regulates lipid metabolism in the adipocytes.** The net action of TNFα in adipocytes is to decrease FFA uptake and triacylglycerol synthesis (lipogenesis) as well as increase lipolysis. An asterisk indicates proteins whose activity and/or expression is down-regulated by TNFα but up-regulated by PPARγ and its ligands. Lipogenic enzymes such as acetyl-CoA carboxylase and fatty acid synthetase are also included.

Abbreviations: LPL, lipoprotein lipase; FFA, free fatty acids; FATP, fatty acid
transporter protein; aP2, adipocyte fatty acid binding protein; ACS, acyl-CoA synthetase; HSL, hormone sensitive lipase; βAR, β3 adrenergic receptor; AC, adenylate cyclase; PKA, protein kinase A; Glut 4, insulin sensitive glucose transporter.
(Source: Sethi and Hotamisligil. 1999)

1.3.6 Lipoprotein lipase (LPL)

LPL plays a crucial role in lipid metabolism and transport by catalyzing the rate-limiting step in hydrolysis of triacylglycerol, present in the circulating chylomicrons and VLDL, to FFAs which are further synthesized to triacylglycerol in the adipocyte (Weinstock et al. 1997). Besides adipose tissue, other major synthesis sites for LPL are cardiac, skeletal muscle and the lactating mammary gland, with limited expression in many other tissues (Mead et al. 2002). However, LPL is recognized as an extra-hepatic enzyme since the expression of LPL in the liver is terminated soon after birth (Staels and Auwerx. 1992).

The reaction site for LPL-mediated hydrolysis is at the luminal surface of capillary endothelium. However, translocation happens before LPL reaches the site of action (Braun and Severson. 1992). Briefly, LPL mRNA is translated in the rough endoplasmic reticulum (ER), and then transported to the Golgi apparatus. From Golgi apparatus, it is first delivered to the secretory vesicles, and thereafter either to the lysosomes for intracellular degradation or to the parenchymal cell surface. After that, it finally translocated to the capillary endothelial cell surface. In an ex vivo study, Roh et al. (Roh et al. 2001) found that both LPL activity and protein levels are high in the ER with only a small fraction present in low density secretory vesicles in rat
adipocytes.

LPL expression is regulated by a number of factors in physiological and pathophysiological conditions which have been well summarized in the review by Mead et al. (Mead et al. 2002). Take insulin and TNFα as examples, insulin increased LPL activity without affecting its mRNA levels in cultured 3T3-L1 cells while TNFα lowered both LPL activity and mRNA levels in 3T3-L1 cells.

As a central enzyme in lipid metabolism, LPL is implicated in a number of diseases either directly or indirectly, such as chylomicronaemia, obesity, atherosclerosis, Alzheimer’s disease and dyslipidemia associated with diabetes and insulin resistance. In type 2 diabetes and insulin resistance patients, decreased adipose tissue LPL activity was found (Pykalisto et al. 1975; Simsolo et al. 1992). Moreover, elevated production of cytokines such as TNFα results in a reduction in LPL expression and activity, which contributes to the hypertriglyceridaemia (Kern. 1997; Halle et al. 1998).

1.3.7 Leptin

Leptin, the 16-KDa product of obese (ob) gene, was the first adipokine discovered by Friedman and his group in 1994 (Zhang et al. 1994). Leptin is secreted almost exclusively by the adipose tissue. The major role of leptin is to balance body energy by repressing food intake and increasing energy expenditure by signaling to the central nervous system (CNS) (Havel. 2004; Porte et al. 2002; Rosen and Spiegelman. 2006b; Havel. 2001).
Transcription of the *ob* gene appears to be activated by C/EBPα during adipocyte differentiation (Miller et al. 1996). Meanwhile, a number of studies found that the expression of leptin is regulated by a range of factors, such as insulin, cAMP, glucocorticoids, TNFα and PPARγ agonists. To be specific, insulin, glucocorticoids and TNFα increased the leptin expression both in *vivo* and in cultured adipocytes (MacDougald et al. 1995b; Slierker et al. 1996a; Murakami et al. 1995; Medina et al. 1999). As a counter-regulator to the effect of insulin, cAMP lowered leptin mRNA levels (Slierker et al. 1996). TZDs and PPARγ agonists also suppress leptin gene expression (Kallen and Lazar. 1996).

Mutations of *ob* gene or defects in the leptin receptor lead to extreme hyperphagia and obesity. Treatment with recombinant leptin reduces the hyperphagia and induces weight loss in leptin-deficient subjects (Havel. 2004). A lipoatrophic diabetes mouse model exhibits marked insulin resistance and hyperlipidemia, which is also effectively reversed by the administration of leptin (Ebihara et al. 2001). Serum leptin level is positively correlated to total body fat mass in humans which could be explained by increased release of leptin in large fat cells compared to small ones since fat cell size is usually 2-4 times larger in the obese subjects compared to the normal subjects. In addition, leptin has complex interactions with the lipid and lipoprotein metabolism in different glycemic conditions (Singh et al. 2010) and leptin has been found to have an inverse relationship with plasma HDL level in humans (Rainwater et al. 1997).
1.3.8 Adiponectin

Adiponectin, a 30-KDa protein, is found to be produced exclusively by adipose tissue and circulate at extraordinarily high concentrations. Plasma adiponectin levels in humans range from 0.5-30 μg/ml, which is about 1000 fold higher than leptin and most other adipokines and thus is known as the most abundant adipose tissue protein (Rosen and Spiegelman. 2006b; Haluzik et al. 2004). Unlike all the other current known adipokines, clinical studies have shown that adiponectin levels in the plasma are negatively correlated with BMI (Arita et al. 1999; Yatagai et al. 2003).

Several factors were reported to regulate adiponectin gene expression. The expression of adiponectin in WAT is decreased by obesity, TNFα, β-adrenergic stimulation and glucocorticoids, and increased by leanness, cold exposure, and PPARγ agonists (Haluzik et al. 2004; Combs et al. 2002). Insulin is an important regulator of adiponectin gene expression and different effects have been shown under different doses and durations of action by in vitro studies (Haluzik et al. 2004).

Besides decrease in obese patients, plasma adiponectin was also found decreased in type 2 diabetic individuals as compared to non-diabetic individuals and the degree of hypoadiponectinemia is highly correlated to the degree of insulin resistance and hyperinsulinemia (Hotta et al. 2000; Weyer et al. 2001). Another study showed that when given purified recombinant adiponectin treatment to normal and diabetic mice, serum glucose reduced without stimulating insulin was found in the diabetic mice which suggests that adiponectin is a potent insulin sensitivity enhancer (Berg et al. 2001). In addition, growing evidence appears to support that adiponectin is involved
in the regulation of lipid metabolism. Decreased adiponectin levels have been linked to smaller LDL size and high apoB and triacylglycerol levels in young men (Kazumi et al. 2002). Plasma adiponectin levels have also been found to be negatively associated with triacylglycerol levels and positively associated with plasma HDL and the relationships are independent of age and sex (Cnop et al. 2003).

1.4 Liver enzymes / receptors in regulating plasma lipids

1.4.1 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase)

HMG-CoA reductase, found in the endoplasmic reticulum (ER) of the liver and other cells and synthesized on membrane-bound ribosome, is the rate-limiting enzyme in the mevalonate pathway which is vital for hepatic cholesterol biosynthesis (Goldstein and Brown. 2009). The activity of HMG-CoA reductase is regulated by end-product feedback which was demonstrated by its suppression in human fibroblasts in the presence of serum containing high concentration of cholesterol and notably increasing after removing serum from the medium (Brown et al. 1973). Moreover, this study also showed that only LDL, not HDL was effective in suppressing HMG-CoA reductase activity. The most important mechanism of suppressing the activity of HMG-CoA reductase by cholesterol involves an inhibition of the transcription of a reductase gene. Stimulation of the degradation of pre-existing reductase protein is considered to be the second mechanism (Goldstein and Brown. 1984).

There are two forms of HMG-CoA reductase: phosphorylated (inactive) and
dephosphorylated (active). However, a study carried by Brown et al. (Brown et al. 1979) elucidated that long term alteration of cholesterol synthesis in the rat liver is not due to the changes in the state of phosphorylation of reductase, but due to changes in the total amount of the protein. Because of the alteration effect of cholesterol synthesis, HMG-CoA reductase inhibitors such as lovastatin and related compounds have been found to reduce plasma cholesterol levels without serious side effects (Frishman and Rapier. 1989; Todd and Goa. 1990; Jungnickel et al. 1992). Nowadays these agents are widely used clinically as a well-known class of hypolipidemic drugs called “statins” (Goodman & Gilman’s The Pharmacological Basis of Therapeutics, 12e, chapter 31).

1.4.2 Low density lipoprotein receptor (LDLR)

LDLR was first found by Goldstein and Brown in 1973 through the research in a human genetic disease called familial hypercholesterolemia (Goldstein and Brown. 2009; Brown and Goldstein. 1976) and they were awarded the Nobel Prize in physiology or medicine for their discoveries concerning “the regulation of cholesterol metabolism” in 1985 (Hulsmann. 1986). LDLR is first synthesized in the rough ER as a precursor containing at least two high-mannose N-linked oligosaccharide chains, then converted to the complex N-linked chains to become a mature receptor in the Golgi (Goldstein and Brown. 1984).

Receptor-bound LDL transports from the cell surface to the lysosome by endocytosis which facilitates the following rapid internalization and degradation (Fig
1-4) (Anderson et al. 1977). After internalization, the receptors dissociate from their ligands, run back to the cell surface and therefore being recycled. Usually, the time for LDLR going in and out of the cell once is 10 minutes and the lifespan of each receptor is around 20 hours (Brown et al. 1983).

Together with HMG-CoA reductase, LDLR was also subject to the end-product feedback regulation by cholesterol (Fig 1-4) and they showed coordinate regulation in both *in vitro* and *in vivo* studies (Rudling. 1992; Goldstein and Brown. 1990). The balance between *de novo* cholesterol synthesis with HMG-CoA reductase as the rate-limiting enzyme and external cholesterol supply through the LDL receptor mediated endocytosis is important to stay away from intracellular sterol over-accumulation and elevation of plasma LDL levels (Goldstein and Brown. 1984; Goldstein and Brown. 1990). According to the most recent review by Goldstein and Brown (Goldstein and Brown. 2009), LDL derived cholesterol acts at different levels, including suppression of HMG-CoA reductase and LDLR gene expression through the sterol regulatory element-binding protein (SREBP) pathway which will be discussed later; acceleration of the degradation of pre-existing HMG-CoA reductase protein and activation of acyl CoA: cholesterol acyltransferase (ACAT) to esterify excess cholesterol to cholesteryl ester droplets which could be stored in the cytoplasm.
Figure 1-4. Sequential Steps in low density lipoprotein (LDL) receptor pathway. (Goldstein and Brown. 2009). ACAT, Acyl coenzyme A: cholesterol acyltransferase; HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase. Copyright permission has been granted.

1.4.3 Sterol regulatory element-binding proteins (SREBPs)

SREBPs belong to the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors and include three isoforms named SREBP-1a, SREBP-1c and SREBP-2 (Horton et al. 2002). Unlike other family members, SREBPs are synthesized as membrane-bound inactive precursors that require cleavage through a two-step proteolytic reaction which facilitates the entry of SREBP into the nucleus and activation of the genes for both cholesterol and fatty acid synthesis (Brown and Goldstein. 1997; Shimano. 2001). SREBP cleavage-activating protein (SCAP) is required to activate SREBP cleavage and is suggested to act as a sterol sensor in the liver (Korn et al. 1998).

From the studies of transgenic mice overproducing SREBP-2 (TgSREBP-2),
Horton et al. (Horton et al. 1998) found that SREBP-2 is a relatively selective activator of cholesterol synthesis. Likewise, SREBP-1c was reported to be more selective in activating fatty acid synthesis, and over-expression of nuclear SREBP-1a activated both cholesterol and fatty acid synthetic genes (Shimano. 2001). mRNA expression of SREBP-1a and SREBP-1c was different in organs and cultured cells (Shimano et al. 1997). To be specific, SREBP-1a and SREBP-2 are predominantly expressed in most cultured cell lines while SREBP-1c and SREBP-2 are predominantly expressed in the liver and other tissues such as adipose tissue and the brain (Horton et al. 2002).

Expression of SREBPs is also regulated by sterols (Fig 1-5). This has been confirmed by a number of lines of evidence. For example, cholesterol feeding reduced both nuclear SREBP-1c and SREBP-2 as well as LDL receptor protein levels in hamster liver (Shimomura et al. 1997) while deletion of sterols by administration of a bile acid-binding resin (colestipol) and a cholesterol synthesis inhibitor (mevinolin) led to an increase of nuclear SREBP-2 and a decline in the nuclear SREBP-1 in hamster liver (Shimomura et al. 1997; Sheng et al. 1995). Thirty-three genes involved in the synthesis of fatty acids and cholesterol were found to be increased in both transgenic mice overproducing SREBP-1a (TgSREBP-1a) and SREBP-2 (TgSREBP-2) and to be decreased in SCAP-deficient mice (Horton et al. 2003). HMG-CoA reductase and LDL receptor are among these 33 genes and they showed a 38 fold and 3.5 fold increases in mRNA levels in TgSREBP-2 liver, respectively.

In Shimano’s review (Shimano. 2001), he also summarized the effects of insulin
and glucose on SREBP-1 expression. SREBP-1c is up-regulated by insulin mainly in hepatocytes, and there is an increased possibility that the induction of SREBP-1c could mediate the roles of glucose and insulin in the production of fatty acids.

Figure 1-5. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol synthesis, and low density lipoprotein (LDL) receptor by sterols. A reduced availability of sterols causes the translocation of sterol regulatory element binding proteins (SREBPs) to the nucleus where they induce transcription of several genes involved in lipid metabolism, including up regulation of HMG-CoA reductase and LDL receptor. An increase in cell sterols has the opposite effect.
1.4.4 Microsomal triacylglycerol transfer protein (MTP)

MTP, capable of accelerating the transfer of triacylglycerols and cholesteryl esters within the lumen of microsomes, was isolated from the liver and intestinal mucosa, and was first discovered and purified by Wetterau and Zilversmit (Wetterau and Zilversmit. 1984; Wetterau and Zilversmit. 1985; Wetterau et al. 1991b). Two bands of MTP protein were later recognized as two non-covalently bound polypeptides with the molecular weight 58 kDa and 97 kDa. (Wetterau and Zilversmit. 1985; Wetterau et al. 1991b; Wetterau et al. 1990; Wetterau et al. 1991a). The small 58 kDa subunit of MTP, which was identified as protein disulfide isomerase (PDI) later, is a ubiquitous multifunctional protein within the lumen of ER and not essential for the lipid transfer activity (Wetterau et al. 1991a; Wetterau et al. 1997). Unlike PDI, the large 97 kDa subunit is unique and is essential for MTP lipid transfer activity (Lamberg et al. 1996; Hussain et al. 2003a; Hussain et al. 2003b).

The absence of functional MTP, caused by a defect in the gene for the large subunit of MTP was found in individuals with abetalipoproteinemia and it suggests that MTP is required for the assembly and secretion of lipoprotein containing apoB (Wetterau et al. 1992; Sharp et al. 1993). Jamil et al. (Jamil et al. 1995) summarized a well-accepted scheme of lipoprotein assembly and secretion in which initial assembly occurs in the ER. Apolipoproteins, cholesterol, phospholipid and triacylglycerol are synthesized and incorporated into lipoprotein particles there, and then transported to the Golgi and secreted. MTP was proposed to carry the lipids from their synthesis site to nascent lipoproteins within the ER and has a preference for transporting neutral
lipids compared with phospholipids (Jamil et al. 1995).

More studies have been done by different groups to demonstrate the role of MTP in the assembly and secretion of lipoproteins. Through different types of studies, Hussain et al. (Hussain et al. 2003b) suggested that apoB-MTP binding plays a role in lipoprotein biogenesis and may be important for the prevention of degradation and lipidation of nascent apoB. Hui et al. (Hui et al. 2002) demonstrated that the inability to express MTP was associated with a complete block in the secretion of both apoB-100 and apoB-48 without affecting triacylglycerol secretion in L35 rat hepatoma cell. Jamil et al. (Jamil et al. 1996) reported that an inhibitor of the MTP inhibits apoB secretion from HepG2 cells. Leung et al. (Leung et al. 2000) found that heterozygous MTP knockout mice with half-normal MTP levels in the liver reduce apoB secretion. Moreover, Tietge et al. (Tietge et al. 1999) provided the original data that hepatic over-expression of MTP resulted in increased secretion of VLDL-triacylglycerols as well as VLDL-apoB in vivo and also suggest that MTP is a rate-limiting enzyme for VLDL-apoB secretion in wild-type mice under basal chow-fed conditions.

Insulin seems to be an important factor involved in MTP regulation. High insulin levels and high concentrations of glucose were found to decrease MTP large subunit mRNA levels in HepG2 cells (Lin et al. 1995). In the study of fructose-fed Syrian golden hamster, a novel animal model of insulin resistance, hepatic apoB overproduction was found and seems to result from increased intracellular stability of nascent apoB and elevation of MTP expression (Taghibiglou et al. 2000).
Figure 1-6. Role of hepatic microsomal triacylglycerol transfer protein (MTP) in the regulation apoprotein (apo) B-100 lipoproteins assembly. TG, triacylglycerols.
1.5 Methylglyoxal

1.5.1 Synthesis of MG

Methylglyoxal (MG) research started probably in the last decades of the 19th century and the research focus was the synthesis, the chemical characterization and reaction of MG (Kalapos. 1999). MG, also known as pyruvaldehyde, is a highly reactive aldehyde compound with two carbonyl groups (Fig 1-7) and is a yellow liquid with characteristic pungent odor.

![Structure of Methylglyoxal](image)

M.W=72.06

**Figure 1-7. Structure of methylglyoxal**

MG production has been discovered in all mammalian cells and it is mainly generated from the glucose metabolism **via** the glycolytic pathway (Phillips and Thornalley. 1993). In this pathway, the majority of MG is thought to be formed by the non-enzymatic and / or enzymatic elimination of phosphate from dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G-3-P). Triose phosphate isomerase or methylglyoxal synthetase may be involved in the enzymatic formation.

Other important precursors for MG production include aminoacetone and ketone
bodies derived from protein and triacylglycerol, respectively (Desai et al. 2010).
Aminoacetone is a product of glycine and threonine metabolism and the conversion of aminoacetone into MG is catalyzed by semicarbazide-sensitive amine oxidase (SSAO) (Yu et al. 2003). The conversion of acetone into MG goes through two consecutive steps with acetol as an intermediate. Acetol/acetone monooxygenase (AMO) is the enzyme to convert acetol/acetone to MG production and this conversion consumes NADPH+H⁺ (Koop and Casazza. 1985).

Moreover, over-consumption of high carbohydrates and fat food and/or high ethanol level beverages leads to elevated precursors for MG generation and the resultant overproduction of MG in vivo (Wu. 2006).

1.5.2 Degradation of MG

MG is a highly reactive compound and has been proved cytotoxic by both in vivo and in vitro studies (Kalapos. 1999). In healthy bodies, there are several mechanisms for MG detoxification and the glyoxalase system is known as a highly effective one (Kuhla et al. 2005). The glyoxalase system, present in the cytosol of cells and cellular organelles, especially mitochondria, catalyses the conversion of MG to D-lactic acid with the help of two enzymes, glyoxalase I and glyoxalase II (Thornalley. 1990). Glyoxalase I catalyses hemithioacetal formed non-enzymatically from the reaction of MG and reduced glutathione (GSH) to S, D-lactoyl glutathione. Glyoxalase II hydrolyses S, D-lactoyl glutathione to D-lactic acid and regenerates GSH back to the detoxification
system.

Figure 1-8. Synthesis and degradation of methylglyoxal. (Source: Desai et al., 2010). Copyright permission has been granted.

1.5.3 MG and reactive oxygen species (ROS)

ROS is the term indicating free radicals derived from oxygen, such as superoxide (O$_2^-$), hydroxyl radical (OH$^-$), hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$). An excess of ROS production leads to an imbalance with antioxidants and therefore generates oxidative stress. Oxidative stress is the major inducer of the aging process and thus plays a role in many age-related diseases such as CVD and neurodegenerative diseases (Desai et al. 2010).

Elevated ROS production by MG has been reported in numerous studies.
Incubation of rat aorta vascular smooth muscle cells (VMSCs) with MG for 4 hours significantly increased the production of superoxide in a dose dependent manner, which was attenuated by SOD (a superoxide scavenger) or diphenyleneiodonium (DPI, a NAD(P)H oxidase inhibitor) (Chang et al. 2005). Increased production of hydrogen peroxide and peroxynitrite, which is formed by an excess of superoxide reacting with nitric oxide, have also been found in the same study (Chang et al. 2005). Moreover, Over-generation of ROS was also found in other types of cells with incubation of MG, such as neutrophils (Ward and McLeish. 2004), monocytes (Rondeau et al. 2008) and rat hepatocytes (Kalapos et al. 1993).

As mentioned above, MG is mainly degraded by the glyoxalase system whose activity is dependent on the GSH levels. Studies have shown that MG depleted GSH levels, which made the cells more sensitive to oxidative stress (Meister. 1988; Shinpo et al. 2000). Moreover, MG has been shown to inactivate glutathione peroxidase as well as glutathione reductase, which are the key enzymes to remove hydrogen peroxide and recycle GSH (Park et al. 2003; Blakytny and Harding. 1992).

MG derived advanced glycation endproducts (AGEs) are another source for oxidative stress production, which has been found in various cells including endothelial cells (Thornalley. 1998; Wautier et al. 2001). Moreover, cytokines and growth factors such as interleukin 1 beta were proposed to act as mediators for the indirect cause of ROS elevation by MG (Lo et al. 1994; Oya et al. 1999; Westwood and Thornalley. 1996).
1.5.4 MG and AGEs

AGEs, a group of brown colored compounds with intra- or inter-molecular cross linking, are formed by the Maillard reaction of aldehydes and ketones with amino groups of proteins (Singh et al. 2001). The formation of AGEs is age-correlated (Li et al. 1996) and they are found to accumulate slowly in vascular and renal tissues. However, the accumulation of AGEs is accelerated in conditions such as complications of diabetes (Brownlee et al. 1988).

As the most active AGE precursor, MG reacts with residues of different proteins including arginine, lysine and cysteine and therefore forms different AGEs, such as argpyrimidine, $N^\epsilon$-caboxyethyl lysine (CEL) and $N^\epsilon$-carboxymethyl lysine (CML) (Dhar et al. 2008).

Numerous studies, both *in vivo* and *in vitro* have proved that increased MG leads to an increase of AGEs formation. In cultured VSMCs, CEL and CML production detected by using immunohistochemistry increased significantly after treatment with MG for 3 hours (Dhar et al. 2008). In the spontaneously hypertensive rat (SHR) kidney, immunoreactivity to CEL and CML increased at 8, 13 and 20 weeks groups compared to age-matched Wistar-Kyoto (WKY) rats, but not in the 5 week group, following the same pattern as MG levels in the plasma (Wang et al. 2004; Wang et al. 2005).

1.5.5 Role of MG in diabetes

Clinical data has shown that plasma MG concentrations are 5-6 fold higher in
patients with Type 1 diabetes mellitus and 2-3 fold in those with type 2 diabetes mellitus (McLellan et al. 1994).

*In vitro* studies showed that in cultured VSMCs and endothelial cells, MG increased significantly after high concentration of glucose (25 mM) treatment (Dhar et al. 2008; Dhar et al. 2010b). The increased AGEs associated with increased MG under hyperglycemia have been suggested as a causative factor for diabetes mellitus and its complications (Wang et al. 2007).

Argpyrimidine, one of the AGEs formed by MG and arginine residue, has been found to accumulate in atherosclerotic lesions in diabetic patients and has a positive correlation with the severity of atherosclerotic lesions (Oya et al. 1999; Friedman. 1999). Moreover, AGEs can up-regulate vascular cell adhesion molecule-1 (VCAM-1) expression through activation of NF-κB signaling pathway and therefore trigger the first step of atherogenesis (Kunt et al. 1999).

Similarly, elevated AGEs have also been demonstrated to be localized in the kidney and retinal blood vessels in diabetic mice or patients, and these abnormalities have been linked to diabetic nephropathy and diabetic retinopathy, respectively (Soulis-Liparota et al. 1995; Stitt. 2001).

1.5.6 Role of MG in hypertension

Compared to the role of MG and AGEs in diabetes mellitus, their role, if any, in the pathogenesis of hypertension has not been well studied, especially under normoglycemic condition (Wang et al. 2007).
Data in our lab showed that cellular MG levels and MG-induced AGEs levels in cultured rat aorta VMSCs from SHR were significantly higher than those from normotensive WKY rats. The systolic blood pressure was also found to be significantly higher in SHR 8 weeks and older, but not at 5 weeks, and this age-dependent increase coincided with the increase in MG levels in both the aorta and the plasma of SHR. Aminoguanidine, an MG and AGEs scavenger, attenuated the increase of systolic blood pressure accompanied by an attenuation of the increases of MG and AGEs levels (Wang et al. 2007; Wang et al. 2005).

MG also targeted the kidney which is a key organ for regulating the blood pressure. Kidney MG levels were found to be significantly higher in 13 and 20 week old SHR compared to age-matched WKY rats. Kidney AGEs formation also increased significantly in SHR 8 weeks and older. The majority of the AGEs were localized in renal tubules with a small amount in the glomerular vessels. Moreover, GSH levels in the kidney also decreased in SHR at 20 weeks (Wang et al. 2004).

In addition, MG-induced ROS may be another causative factor for hypertension. Increased production of superoxide impairs nitric oxide function and leads to blood vessel tone alteration while increased formation of peroxynitrite leads to endothelial cell dysfunction and therefore results in hypertension (Cuzzocrea et al. 2004). ROS also plays a role in NF-κB activation which is essential for VSMC proliferation during the hypertension development (Wu. 2006).
1.5.7 Previous findings in MG treated rats

Acute and chronic administration of MG has been performed separately in SD rats to investigate the direct effects of MG. In the acute study (Dhar et al. 2010a), a single dose of 17.25 mg/kg MG was given to one group of rats by intraperitoneal administration. Two other groups included a control group and a group pre-treated with alagebrium (ALA), a MG scavenger and an AGEs breaker. MG levels in different tissues and urine were measured by HPLC and a significant increase of the MG levels was found in the different tissues including heart, kidney, liver, lung, aorta and urine and the changes were attenuated by ALA (Fig 1-9). Plasma glucose and insulin levels were significantly increased after acute MG administration and glucose uptake was reduced compared to the control groups.

![Figure 1-9. Distribution of methylglyoxal (MG) in different organs/tissues/urine in Sprague-Dawley rats after intraperitoneal administration.](image)

Saline (control), MG (17.25 mg/kg i.p., MG-17 i.p.) or MG-17 i.p. + ALA (alagebrium 100 mg/kg i.p.) were administered to three groups of rats (n = 6 each). The organs, tissues and urine were collected 3 h after administration of treatment. Data were analyzed with
two-way ANOVA with treatment and tissue as variables. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. corresponding control group, †$P<0.05$, ††$P<0.01$ vs. MG-17 i.p. group. (Source: Dhar et al. 2010a). Copyright permission has been granted.

In the chronic study, an osmotically driven infusion minipump was implanted subcutaneously in the back of the rat to deliver 60 mg/kg/day of MG by continuous infusion for 28 days (Dhar et al. 2011). MG levels in the plasma, pancreas, adipose tissue and skeletal muscle increased significantly after chronic MG treatment and this increase was exacerbated by glutathione synthesis inhibitor, L-buthionine sulfoximine (BSO) and attenuated by the MG scavenger and AGEs breaker, ALA. In contrast, GSH levels in these tissues were decreased after chronic MG treatment (Fig 1-10). Basal plasma glucose levels were significantly elevated, basal plasma insulin levels were decreased with impaired glucose tolerance and glucose uptake in the MG treated group. In this chronic study, plasma lipid profile of rats in different groups was also evaluated (Table 1-4). Significantly elevated plasma free fatty acids, total cholesterol, and triacylglycerols as well as decreased HDL levels were found after MG treatment. Co-treatment with ALA attenuated the alterations of plasma lipid levels induced by chronic MG treatment. The reason for the changes in lipid levels was not pursued in that study. I undertook this exciting project to investigate the mechanisms of these MG-induced changes in plasma lipid levels.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>MG</th>
<th>MG+ALA</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma free fatty acids (μg/L)</td>
<td>393±41</td>
<td>619±21***</td>
<td>405±20†††</td>
<td>389±22†††</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>1.8±0.1</td>
<td>2.4±0.05***</td>
<td>1.9±0.03††</td>
<td>-</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.2±0.05</td>
<td>0.7±0.06**</td>
<td>1.0±0.07†</td>
<td>-</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/L)</td>
<td>0.2±0.03</td>
<td>0.5±0.04***</td>
<td>0.3±0.02††</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1-4. Plasma lipid levels in Sprague-Dawley rats treated with methylglyoxal (MG). 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic pump for 28 days to all groups of rats \(n=6\) each. The MG scavenger, alagebrium (ALA, 30 mg/kg/day in drinking water) (MG+ALA and ALA) was administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days basal fasting plasma levels of substances listed in the table were measured. The values are Mean ± SEM of \(n=6\) experiments. **\(P<0.01, \quad ***P<0.001\) vs respective control group; †\(P<0.05, \quad ††P<0.01, \quad †††P<0.001\) vs respective MG group.

Abbreviations: HDL, high density lipoprotein. (Source: Dhar et al., 2011). Copyright permission has been granted.
Figure 1-10. Methylglyoxal (MG) levels are elevated and reduced glutathione
(GSH) levels are decreased in Sprague-Dawley rats chronically treated with MG. 0.9% saline or MG (60 mg/kg/day) was delivered by continuous infusion with a subcutaneous osmotic minipump for 28 days to all groups of rats (n=6 each). The MG scavenger, alagebrium (ALA, 30 mg/kg/day in drinking water) (MG+ALA) or glutathione synthesis inhibitor, L-buthionine sulfoximine (BSO, 30 mg/kg/day in drinking water) (MG+BSO and BSO) were administered to some groups of rats. The control group received only saline (0.9% by pump). After 28 days MG and GSH levels were determined by HPLC in (A, E) plasma and (B-D, F-H) organs. *P<0.05, **P<0.01, ***P<0.001 vs respective control, †P<0.05, ††P<0.01, †††P<0.001 vs respective MG group, ‡P<0.05, ‡‡P<0.01, ‡‡‡P<0.001 vs. respective MG+BSO group. (Source: Dhar et al., 2011). Copyright permission has been granted.
1.6 Hypothesis

We hypothesized that MG contributes to the pathogenesis of dyslipidemia by affecting one or more of the factors responsible for triacylglycerol and fatty acids metabolism/transport and storage in the adipose tissue and/or key enzymes involved in cholesterol and lipoprotein metabolism/transport in the liver.
1.7 Objectives

The objectives of this study are to investigate the effects of MG on lipid regulation in both the adipose tissue and cultured adipocytes, then further assess the potential role of C/EBPs, PPARγ, TNFα, lipoprotein lipase and adipokines related to MG-induced plasma lipid level alterations. In addition, we also investigated the effects of MG on several key enzymes/receptors related to cholesterol and lipoprotein metabolism/transport in the liver.
1.8 Research Protocol Outline

SD rats (control, MG, MG+AG, AG)

- Adipose tissue
  - Histology study
  - C/EBP-α/C/EBP-6/
  - PPARγ/LPL/leptin/
  - adiponectin/TNFα

- Plasma
  - Leptin

- Liver
  - SREBP-1/
  - SREBP-2/HDLP-R/
  - HMG-CoA reductase/MTP

3T3-L1 cells

- Whole cell
  - Oil-Red O staining
  - (Triacylglycerol)

- Cell lysates
  - C/EBPα/C/EBPβ/
  - PPARγ/LPL/leptin/
  - adiponectin/TNFα

- Cell cultured medium
  - Leptin
CHAPTER TWO

MATERIALS AND METHODS

2.1 Animal treatment

Male SD rats, 12 weeks old, were purchased from Charles River Laboratories (St-Constant, Quebec, Canada). Rats were treated according to guidelines of the Canadian Council on Animal Care and all animal protocols were approved by the Animal Care Committee of the University of Saskatchewan. SD rats were randomly divided into four groups (n=6 each): (i) Control (0.9% saline), (ii) MG treated, (iii) MG+aminoguanidine (AG) treated. MG (60 mg/kg/day) or 0.9% saline was delivered by continuous infusion with a subcutaneously implanted minipump for 4 weeks. AG (100 mg/kg/day) was delivered in drinking water for 4 weeks. After 4 weeks of the treatment, the rats were anesthetized with thiopental sodium (100 mg/kg i.p.) and sacrificed by exsanguination. Blood was collected from the heart and plasma was separated by centrifuging the blood samples at 1000 rpm for 10 minutes at 4°C. The liver and adipose tissue were isolated and rinsed in cold saline, frozen in liquid nitrogen and stored at -70 °C. The liver and adipose tissue were finely ground in liquid nitrogen before performing different assays.

2.2 3T3-L1 cell culture and differentiation

Mouse 3T3-L1 cell line was purchased from American Type Culture Collection (ATTC) and grown to confluence in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma, MO, USA) containing 1% penicillin-streptomycin, 25 mM glucose and 10% heat inactivated bovine serum (Invitrogen, CA, USA). One day after confluence (Day
0), cell differentiation was induced by adding 1.0 µg/mL insulin, 0.25 mM dexamethasone, and 0.5 mM isobutylmethylxanthine to the medium (DMEM containing 25 mM glucose and 10% heat inactivated fetal bovine serum (FBS) (PAA Laboratories, Ontario, Canada) for 3 days and the medium was changed every day. The cells then grew in post-differentiation medium (DMEM containing 25 mM glucose, 10% FBS and 1.0 µg/mL insulin) for 4-6 days and the medium was changed every other day until more than 90% of cells became mature adipocytes. For determination of the effects of MG on mature adipocytes, after 18 hours starvation, 3T3-L1 cells were placed in DMEM (containing 5mM glucose and 10% FBS) and treated with or without MG (30 µM) for 24 h. In oil-red O staining, cells were given treatment from day 0 for 6-8 days until the adipocytes became mature. All the other chemicals were purchased from Sigma.

2.3 Hematoxylin-eosin staining (H-E staining)

Abdominal adipose tissue was collected from rats and kept in buffered 10% formalin solution at 4 °C after rinsing with cold phosphate buffered saline (PBS). A small piece of adipose tissue was embedded in paraffin through a series of processes run by RVG/1 vacuum tissue processor (Belair, NJ, USA). After embedding, each piece was held in a steel embedding ring. After trimming and sectioning (5 µM each section) by using ‘A20’ microtome (Spencer, NH, USA), the tissue sections were finally attached to the slides and ready for staining after overnight drying at 37 °C. For staining, the slides were deparaffinized in xylene, stained with hematoxylin for 3
minutes and eosin for 1 minute, mounted and examined by light microscopy. For each abdominal adipose tissue sample, 3 sections were prepared. Photographs were taken from 4 random areas per section at 20× magnification. The number of adipocytes per area (0.0221 mm²) was counted and the mean value of each sample was calculated upon the number of 4 random areas per section. Mean adipocyte density and adipocyte size was then calculated upon the number per mm².

2.4 Oil-Red O staining

For Oil-Red O staining, cells were gently washed 2 times with PBS, fixed with 10% formalin for at least 1 hour at room temperature and stained with filtered Oil Red O working solution for 10 minutes. After intracellular lipid droplets were fully stained, the Oil Red O solution was removed and cells were rinsed with tap water. Images were taken under a microscope after drying the cells. At last, the dye was eluted by adding 100% isopropanol for 10 minutes and quantified by measuring the optical absorbance at 500 nm (Ramirez-Zacarias et al. 1992) by using a plate reader (Thermo Labsystems, Finland).

2.5 Protein extraction

The cultured cells were washed with cold PBS for 2 times, then scrapped and collected in centrifuge tubes. After 1000 rpm, 5 minutes centrifugation, the supernatant was discarded and the pellet was resuspended in 200 µL lysis buffer (100 volumes of 1×RIPA buffer with 1 volume of 100 mM phenylmethysulfonyl fluoride and 1 volume of protease inhibitor) for 1 hour. After centrifugation at 12000 rpm for
10 minutes, the supernatant was transferred to another eppendorf tube for protein determination. For the tissue protein extraction, a certain amount of fine tissue powder (adipose tissue 100-120 mg, liver 40-50 mg) was weighed and immediately 300 µL of lysis buffer was added into the tube containing the tissue powder. After sonication at 40% power for 15 seconds (5 seconds on, 10 seconds off, 3 times), the tube was left on ice for 30 minutes. Centrifugation and supernatant collection was the same as those for cells.

2.6 Nuclear protein extraction

Triton X-100 lysis buffer was prepared according to online protocol <western blot analysis of sub-cellular fractionated samples using the Odyssey infrared Imaging System> and added to 100-150 mg of liver sample with a volume of 500 µL each. After lysis on ice for 30 minutes and centrifugation (13000 rpm, 4° C, 10 minutes), nuclear and cytosol fractions of the samples were separated. The cytosol part, which formed the supernatant, was discarded, while the nuclear part, which formed the precipitate, was rinsed with the same lysis buffer for 2 times by high speed centrifugation (13000 rpm, 4° C, 5 minutes each). The nuclear fraction was resuspended in 200 µL lysis buffer containing 0.5% SDS and sonicated (5 seconds, 3 times). Nuclear protein was collected from the supernatant after the fourth centrifugation and used for protein determination before western blotting.

2.7 Determination of protein concentration

The protein concentrations of cell or tissue samples were determined by BCA
(bicinchoninate) assay (Ramirez-Zacarias et al. 1992). 10 µL BSA ranging from 0.3125 to 5 mg/mL were used as standards and the same amount of double distilled water or supernatant were added in the plates as blank or samples, respectively. After 30 minutes incubation with 200 µL 50 volumes of bicinchoninic acid solution and 1 volume of copper (II) sulfate solution (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37 °C, the spectrophotometric absorbance of samples was determined using a plate reader (Thermo Scientific, Vantaa, Finland) at 562 nm. The values of samples were calculated according to the standard curve.

2.8 Western blotting

Cell lysate was separated by 8% or 10% SDS-PAGE, electrotransferred onto a polyvinylidene fluoride membrane, blocked with 5% skim milk for 30 minutes and incubated with primary antibodies diluted in skim milk overnight at 4º C. The next day, after 2 hours of thorough washing with PBST buffer (PBS with 0.1% tween-20), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 hours at room temperature. After 1 hour washing, the immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System. Primary antibodies for adiponectin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), C/EBPα was purchased from Abcam (Cambridge, MA, USA), β-actin was purchased from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA), and secondary anti-rabbit and anti-mouse IgG antibodies were from Cell Signaling while other antibodies were from Santa-Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).
2.9  Real-time quantitative polymerase chain reaction (RT-PCR)

Total cellular RNA of 3T3-L1 cells and liver tissue was extracted by using RNeasy mini kit (Qiagen, Valencia, CA, USA) and total cellular RNA of adipose tissue was extracted by using RNeasy lipid tissue mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Total RNA (1 µg) was reverse-transcribed to single-stranded cDNA by using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) after reaction for 5 minutes at 25º C, 30 minutes at 42º C, 5 minutes at 85º C and held at 4º C in iCycler iQ apparatus (Bio-Rad Laboratories, Hercules, CA, USA) associated with the iCycler optical system software (version 3.1). The target cDNAs mixed with primers (Qiagen, Valencia, CA, USA) and SYBR Green (Bio-rad Laboratories, Hercules, CA, USA) were triplicated and run for 45 cycles at 95º C for 30 seconds, 55º C for 1 minutes and 72º C for 30 seconds. 18s rRNA was applied as the internal standard. Crossing point (CP) (cycle threshold (Ct)) of each transcript was determined by iCycler automatically after the cycling and the result was calculated by a mathematical delta-delta method in which the equation is ratio=$2^{-\Delta \Delta CP_{sample}-\Delta CP_{control}}$ (Pfaffl. 2001). For each cycling, the efficiency of amplification was between 90%-110%.

2.10  Leptin assay

Rat’s plasma or 3T3-L1 culture medium were collected to determine leptin concentrations using Quantikine® mouse leptin kit (R&D Systems Inc., Minneapolis, MN, USA) based on an enzyme-linked immunosorbent assay. According to the manufacturer’s instructions, the samples strictly avoided repeated thawing.
2.11 Data analysis and statistics

Data are expressed as or Mean ± SEM from at least three independent experiments. ANOVA or Student’s paired/unpaired t test was used for statistical analysis. \( P < 0.05 \) was considered significant.
CHAPTER THREE

RESULTS

3.1 MG induced hypertrophy of adipocytes in rats

As shown in Figure 3-1A, after 4 weeks of chronic MG treatment, the size of adipocytes from abdominal adipose tissue increased compared to that from control rats while AG co-treatment attenuated this increase (cytosol is stained pink by eosin and nuclei stained blue by hematoxylin, eosin also stained blood vessels). In Figure 3-1B, the adipocyte number was counted in 4 random areas of each section, mean cell number per mm$^2$ was calculated and it could quantitate the difference presented in figure 3-1A, but inversely. Cell size calculation was based upon the cell number per mm$^2$ (Figure 3-1C). A significant decrease ($P<0.001$ vs. control rats) of density and increased cell size ($P<0.05$ vs. control rats) was found in chronic MG treated rats, which was attenuated by co-treatment with AG ($P<0.05$ vs. chronic MG treated rats).
Figure 3-1. Effects of methylglyoxal (MG) or MG co-treated with aminoguanidine (AG) on adipocytes size in rat abdominal adipose tissue. Hematoxylin-eosin staining was applied on paraffin embedded adipose tissue sections. Images are shown (A), and mean adipocytes number per mm$^2$ was calculated (B), and cell size was obtained based on the cell number (C). *$P<0.05$, ***$P<0.001$ vs. control, #$P<0.05$ vs. chronic MG treated rats, $n=3-5$ for each group.
3.2 MG increased TNFα mRNA expression without altering leptin and LPL mRNA expression in adipose tissue

In cultured cells, the majority amount of leptin was found to be secreted into the medium while a small amount of leptin stays in the cells (MacDougald et al. 1995b). Therefore, mRNA expression of leptin was chosen rather than intracellular protein expression. LPL mRNA and TNFα mRNA expression were also measured by real-time PCR to detect the effects of MG on adipocyte lipid metabolism. In the adipose tissue which extracted from chronic MG treated rats, no significant difference was found in leptin and LPL mRNA expression while TNFα mRNA expression significantly increased by 85.1% when comparing to the ones extracted from control rats (*P<0.05 vs. control, Figure 3-2A).
Figure 3-2. Effects of methylglyoxal (MG) on mRNA expression of leptin, lipoprotein lipase (LPL) and tumor necrosis factor alpha (TNFα) in adipose tissue. Leptin, LPL and TNFα mRNA expression in adipose tissue from rats treated with MG was determined by real-time PCR and the results are presented as % of their corresponding controls (A). TNFα mRNA expression in adipose tissue from MG treated rats with or without co-treatment with AG was also measured (B). *P<0.05 vs. control, n=4-6 for each group.
3.3 MG increased C/EBPα, C/EBPβ protein levels in adipose tissue

C/EBPα and C/EBPβ are the transcription factors for adipocyte differentiation and are necessary for adipocyte gene expression and lipid accumulation. To determine whether MG influences expression of these adipocyte differentiation factors, western blotting was applied on adipose tissue lysate from different groups. Protein levels of C/EBPα (Figure 3-3A) and C/EBPβ (Figure 3-3B) in chronic MG treated rats increased by 162.8% and 120.6%, respectively and the effect of MG was counteracted by AG co-treatment (Fig. 3.3B).
Figure 3-3. Effects of methylglyoxal (MG) or MG co-treated with aminoguanidine (AG) on the protein levels of CCAAT/enhancer binding protein (C/EBP)α and C/EBPβ in adipose tissue. Western blotting showing the protein levels of C/EBPα (A) and C/EBPβ (B) in adipose tissue from different groups of treated rats. Data analysis was based on the manual quantification of the western images by Genesnap software from Syngene. **P<0.01 vs. control, #P<0.05 vs. chronic MG treated rats, n=4-5 for each group.
3.4 MG did not change PPARγ protein levels significantly

As an important factor involved in adipogenesis, insulin sensitivity and dyslipidemia in adipose tissue (Gurnell et al. 2003), PPARγ protein levels were examined by western blotting and found to be increased by 34.1%, but the difference was not significant \((P=0.26)\) (Figure 3-4).

![Western blot analysis of PPARγ and β-actin](image)

**Figure 3-4.** Effect of methylglyoxal (MG) on the gene expression of peroxisome proliferator activated receptor gamma (PPARγ) in adipose tissue. Western blotting shows the two isoforms of PPARγ. Results were quantified and normalized after dividing by β-actin. \(n=5\) for each group.
3.5 MG did not alter gene expression of adiponectin and LPL in adipose tissue

Using western blotting, we also compared protein levels of another important adipokine, adiponectin (Figure 3-5B) and LPL (Figure 3-5C) in adipose tissue from rats treated with or without MG. Both adiponectin and LPL did not show any change compared to the control group.

Figure 3-5. Effects of methylglyoxal (MG) on the gene expression of adiponectin and lipoprotein lipase (LPL) in adipose tissue. Western blotting shows the protein levels of adiponectin (A) and LPL (B) in adipose tissue from rats treated with or without MG. n=5-6 for each group.
3.6 MG did not alter leptin levels in rat plasma

Because no alteration was found in leptin mRNA levels in chronic MG treated rats, an enzyme-linked immunosorbent assay (ELISA) was further applied to rat plasma to determine circulating leptin levels. However, same as mRNA levels, no difference was found between the MG treated and untreated group.

![Figure 3-6. Effect of methylglyoxal (MG) on circulating leptin levels in rat plasma.](image)

Leptin levels of plasma collected from control rats and chronic MG treated rats were measured by a commercial ELISA kit. $n=3$ for each group.
3.7 MG increased lipid accumulation in cultured 3T3-L1 adipocytes

To explore the adipogenesis potential of MG, we differentiated 3T3-L1 pre-adipocyte with 10 µM and 30 µM of MG for 6 days (from day 0-6). Lipid accumulation was quantified by Oil-Red O staining. MG increased the optical density (OD) value of Oil-Red O eluted solution at higher concentration (Figure 3-6B) and 30 µM MG exhibited a significantly higher OD value ($P<0.001$ vs. control) which indicated higher lipid accumulation in the cytoplasm.
Figure 3-7. Effects of methylglyoxal (MG) on lipid accumulation in 3T3-L1 adipocytes. Cells were cultured in the medium in the absence or presence of MG from Day 0 to Day 6, and lipid accumulation was measured by Oil-Red O staining. (A) Oil-Red O staining images, $n=6$. (B) Effects of different concentrations of MG on quantitative Oil-Red O. (C) Effects of 30 µm MG on quantitative Oil-Red O. *$P<0.05$ with control, $n=6$ for each group.
3.8 MG increased TNFα mRNA levels without altering leptin and LPL mRNA levels in 3T3-L1 adipocytes

The mRNA levels of leptin, LPL and TNFα in cultured 3T3-L1 adipocytes were affected in a manner similar to the adipose tissue. Thus, leptin and LPL mRNA expression was not altered after 24 hours treatment with 30 µM exogenous MG while TNFα mRNA level was up-regulated by 89.3% (*\(P<0.05\) vs. control).

![Figure 3-8. Effects of methylglyoxal (MG) on the expression of leptin, lipoprotein lipase (LPL) and tumor necrosis factor alpha (TNFα) in 3T3-L1 adipocytes. After 24 hour treatment in the absence or presence of MG (30 µM) on mature adipocytes, the RNA extraction was carried out and further used for leptin, LPL and TNFα mRNA expression determination by real-time PCR. mRNA levels in MG treated 3T3-L1 adipocytes are presented as % of their corresponding controls.* \(P<0.05\) vs. control, \(n=4\) for each group.](image)
3.9 MG up-regulated C/EBPα and C/EBPβ gene expression in 3T3-L1 adipocytes

Expression of adipogenesis transcription factors C/EBPα and C/EBPβ were determined by western blotting in 3T3-L1 adipocyte lysates. After 24 hours, expression of C/EBPα and C/EBPβ in the MG treatment group increased significantly by 99.5% and 101.7% compared to control (***P<0.01 vs. control, Figure 3-9A and *P<0.05 vs. control, Figure 3-9B).

![Western blot images showing increased expression of C/EBPα and C/EBPβ in the MG treatment group](image)

**Figure 3-9. Effects of methylglyoxal (MG) or MG co-treated with aminoguanidine (AG) co-treatment on the protein levels of C/EBPα and C/EBPβ in 3T3-L1 adipocytes.** After 24 hour treatment of mature 3T3-L1 cells in the absence or presence of MG (30 µM), the protein levels of C/EBPα (A) and C/EBPβ (B) were determined by western blotting. *P<0.05 vs. control, **P<0.01 vs. control, n=4-5 for each group.
3.10 MG did not change PPARγ protein levels

Protein levels of PPARγ in 3T3-L1 adipocytes were detected by western blotting and showed results similar to those in the adipose tissue. MG increased PPARγ protein levels by 15.3%, which was not significant.

**Figure 3-10.** Effect of methylglyoxal (MG) on the gene expression of peroxisome proliferator activated receptor gamma (PPARγ) in 3T3-L1 adipocytes. After 24 h treatment with or without exogenous MG, whole cell lysate was used for western blotting and the expression of PPARγ was quantified. n=5-6 for each group.
3.11 MG did not alter adiponectin and LPL expression in 3T3-L1 adipocytes

Same as what we found in adipose tissue, the expression of adiponectin and LPL was also measured in 3T3-L1 adipocytes by the same method. 4-5 samples were collected in each of MG treatment and control groups. After western blotting and quantification, 22.8% increase was found in adiponectin expression in the MG treated group, which was not significant ($P=0.12$, figure 3-9A). No significant difference was also observed in LPL expression.

**Figure 3-11. Effects of methylglyoxal (MG) on the expression of adiponectin and lipoprotein lipase (LPL) in 3T3-L1 adipocytes.** Western blotting showing the expression of adiponectin (A) and LPL (B) in 3T3-L1 adipocytes after 24 hour incubation with or without MG (30 µM). $n=4-5$ for each group.
3.12 MG did not alter leptin secretion in cultured adipocytes

Cell culture medium was collected at the end of 24 hour treatment and assayed for leptin after centrifugation to eliminate any possible cell particles. 269.0 pg/ml was the mean value of leptin levels in the medium from both MG treated group and control group with 4 samples in each group.

Figure 3-12. Methylglyoxal (MG) did not alter leptin secretion in 3T3-L1 adipocytes. Cell culture medium was collected after 24 hour treatment in the absence or presence of MG (30 µM) and used for leptin ELISA assay. \( n=4 \) for each group.
3.13 MG up-regulated gene expression of SREBP-2 without affecting SREBP-1 in the liver

Liver is another important organ involved in lipid metabolism. SREBP-1, the activator which mainly enhances the gene transcription required for fatty acid synthesis but not cholesterol synthesis (Horton et al. 2002), changed negligibly in the liver of MG treated rats compared to the control rats (Figure 3-13A). SREBP-2, the activator for cholesterol synthesis, not fatty acids synthesis, increased significantly by 119.0% (*P<0.05 vs. control, Figure 3-13B).
Figure 3-13. Effects of methylglyoxal (MG) on sterol regulatory element-binding protein (SREBP)-1 and SREBP-2 protein levels in the liver. 40 μg nuclear proteins extracted from liver samples were loaded in western gels and used for western blotting for SREBP-1 and SREBP-2. Lamin B was used as a nuclear protein marker here. Protein levels of SREBP-1 (A) and SREBP-2 (B) are presented after dividing by lamin B levels. *P<0.05 vs. control, n=4-5 for each group.
3.14 MG increased HMG-CoA reductase in the liver without affecting LDL receptor

The level of LDL in plasma could be dictated by the balance between the HMG-CoA reductase and LDL receptor and MG was found to significantly up-regulate HMG-CoA reductase by 47.5% (*P<0.05 vs. control, Figure 3-14B) without affecting both LDL-receptor mRNA (Fig3-14A) and protein levels (Fig 3-14C).
Figure 3-14. Effects of methylglyoxal (MG) on 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and low density lipoprotein receptor (LDLR) protein / gene expression in the liver. LDLR mRNA levels (A) were measured by real-time PCR and protein levels of HMG-CoA reductase (B), and LDLR (C) was measured by applying western blotting on whole protein lysate. *P<0.05 vs. control, n=4 for each group.
3.15 MG increased MTP expression in the liver

MTP is a rate-limiting protein for hepatic apoB secretion which is a major determinant of plasma triacylglycerols and LDL levels (Tietge et al. 1999). In the liver of chronic MG treated rats, the protein level of MTP increased significantly by 79.7% and the effect was attenuated in MG+AG co-treated rats (*$P<0.05$ vs. control).

![Figure 3-15. Effects of methylglyoxal (MG) on microsomal triacylglycerol transfer protein (MTP) expression in the liver. Whole protein lysates from liver samples were used for examining MTP protein levels by western blotting. *$P<0.05$ vs. control, $n=4$ for each group.](image)
### 3.16 Summary of results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chronic MG treated rats</th>
<th>Cultured 3T3 cells MG(30 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adipose tissue</td>
<td>Cultured cells</td>
</tr>
<tr>
<td>Adipocyte</td>
<td>↑ size, ↓ number</td>
<td></td>
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<tr>
<td>Lipid accumulation</td>
<td>↑</td>
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<tr>
<td>TNFα</td>
<td>↑ mRNA</td>
<td>↑ mRNA</td>
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<tr>
<td>Leptin</td>
<td>↔ mRNA</td>
<td>↔ mRNA</td>
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<tr>
<td></td>
<td>↔ plasma level</td>
<td>↔ secretion</td>
</tr>
<tr>
<td>LPL</td>
<td>↔ mRNA and protein</td>
<td>↔ mRNA and protein</td>
</tr>
<tr>
<td>C/EBPα protein</td>
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<tr>
<td>C/EBPβ protein</td>
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<tr>
<td>PPARγ protein</td>
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<td>Adiponectin protein</td>
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<tr>
<td><strong>Plasma leptin</strong></td>
<td>↔</td>
<td><strong>Liver</strong></td>
</tr>
<tr>
<td>SREBP1</td>
<td>↔ protein</td>
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<tr>
<td>SREBP2</td>
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<td>MTP</td>
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<tr>
<td>LDL receptor</td>
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<tr>
<td>HMG-CoA reductase</td>
<td>↑ protein</td>
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Abbreviations and symbols: C/EBP, CCAAT- enhancer binding protein; HMG-CoA reductase, 3-hydroxy3-methyl glutaryl coenzyme A reductase; LPL, lipoprotein lipase; PPARγ, Peroxisome proliferator activated receptor γ; SREBP, Sterol regulatory element binding protein; TNFα, tumour necrosis factor α.

↑ increase; ↓, decrease; ↔, no change.
CHAPTER FOUR

DISCUSSION

A high carbohydrate diet and a high fat diet are being blamed for the recent parallel epidemics of obesity, diabetes and hypertension. Some researchers are even suggesting that a high carbohydrate diet is more harmful than a high fat diet. The mechanisms by which a high carbohydrate diet induces diseases, especially cardiovascular diseases, are not entirely clear. Different research groups all over the world are studying different aspects of high carbohydrate induced metabolic changes. One of the mechanisms implicated for high carbohydrate-induced damage is an increase in oxidative stress (Turan and Vassort. 2011; Robertson and Harmon. 2006), while another mechanism involves O-GlcNAc modification of proteins, which is an reversible post-translational modification depends on glucose concentrations (Issad and Kuo. 2008).

The main suspect and target for my research is MG, mainly generated from the glycolysis pathway, which is a reactive molecule and which is cytotoxic at higher concentrations. The western diet including high consumption of carbohydrates, especially fructose, increases MG production in the human body. Increased MG has been linked to diabetes mellitus and hypertension, and possibly obesity. MG-induced over-production of ROS and AGEs are thought to play important roles in MG-induced pathology.

Studies of acute and chronic MG treatment on SD rats have been performed to investigate the direct effects of MG on diabetes related parameters including plasma
glucose level, plasma insulin level, glucose uptake and some important proteins, such as glucose transporter-4 (GLUT-4) and phosphoinositide-3-kinase (Dhar et al. 2010a; Dhar et al. 2011) and MG was reported to alter these levels significantly and induce type 2 diabetes. Moreover, lipid parameters including free fatty acids, total cholesterol, HDL and triacylglycerols were also measured in the chronic study (Dhar et al. 2011) and significant abnormalities were reported with these parameters. To the best of my knowledge, this was the first study to report changes in plasma lipid levels induced by MG. However, the reason or the mechanism for changes in lipid levels was not investigated by colleagues in our lab who performed that study. This was a novel and exciting observation and I undertook this project to investigate the mechanisms of MG-induced alterations in plasma lipid levels. In the study on acute in vivo effects of MG (Dhar et al. 2010a), MG levels in the liver increased significantly after a single i.p. injection of 17.25 mg/kg MG. In the study on chronic in vivo effects of MG (Dhar et al. 2011), a significant elevation of MG levels in adipose tissue and plasma in MG treated group was reported. Since the liver and the adipose tissue are major organs involved in lipid metabolism and plasma lipid level regulation, we decided to focus on these two organs to study the possible mechanism(s) which might be responsible for the alterations of plasma lipid profiles.

4.1 Effects of MG on adipose tissue and dyslipidemia

Regulation of lipid metabolism is a key function of the adipose tissue according to the energy homoeostasis requirement. There are three levels of regulation: fatty acid
uptake predominantly from circulation, lipogenesis from glucose to intracellular triacylglycerol and lipolysis to glycerol and fatty acid back to the circulation (Sethi and Hotamisligil, 1999).

In my study, abdominal adipocyte size was calculated after H-E staining and enlarged cell size was found in the abdominal adipose tissue from chronic MG treated group compared to control group, and co-treatment with AG prevented the increase in adipocyte cell size (Fig. 3-1). Abdominal adipocyte size itself is associated with substantial metabolic functions. Enlarged abdominal adipocyte size is the evidence of obesity and could also predispose to the onset of type 2 diabetes (Skurk et al. 2007; Weyer et al. 2000). Larger adipocytes are less sensitive to insulin and have lower ability to store triacylglycerols (Chui et al. 2005). Moreover, larger adipocytes were also found to have greater rate of lipogenesis from glucose as well as increased rate of lipolysis which may account for the elevated plasma fatty acid levels (Jacobsson and Smith, 1972).

The adipocyte comes from pre-adipocyte differentiation in which the C/EBPs family of proteins are the essential transcriptional factors to guarantee the successful outcome of this process. High levels of C/EBPα are normally expressed in the liver and the adipose tissue (Birkenmeier et al. 1989). C/EBPα is necessary for inducing differentiation of preadipocytes into mature adipocytes and C/EBPα knockout mice die within 7–12 h after birth, mainly due to defective gluconeogenesis in the liver (Wang et al. 1995). C/EBPα is especially required for the development of WAT (Linhart et al. 2001).
In this study, C/EBPα and C/EBPβ protein levels were measured in both adipose tissue and 3T3-L1 adipocytes. Both C/EBPα and C/EBPβ protein levels increased significantly while the PPARγ expression did not change. Unlike the abundant expression of C/EBPα in the mature adipocyte, C/EBPβ expression dropped 50% by the late differentiation phase compared to its expression in the early stage of differentiation, and therefore our measurement of C/EBPβ in the mature adipocytes or adipose tissue from adult rats may be underestimated. However, the significant over-expression of C/EBPβ as well as C/EBPα could give us a clue that MG increases differentiation of pre-adipocytes. This hypothesis was also confirmed by Oil-Red O staining on 3T3-L1 cells in which we treated the pre-adipocytes with MG for 7 days until most of the cells became fully differentiated. Increased staining on oil droplet which mainly represents triacylglycerols could be the result of increased differentiation induced by MG.

PPARγ is thought to be cross-regulated with C/EBPα and its expression in the adipose tissue begins at the early stage of differentiation and continues through the mature phase with important functions. PPARγ is the central engine of adipose differentiation and its activation initiates the full program of differentiation, including morphological changes, lipid accumulation and the expression of almost all genes related to adipocytes (Rosen and Spiegelman. 2006b). In the mature phase, the activation of PPARγ by its agonists, the TZDs, was found to improve fat storage and insulin sensitivity (Kersten et al. 2000). However, interestingly PPARγ selective antagonist was found to have anti-obesity and anti-diabetic effect by reducing the
adipocyte size and improving insulin sensitivity in the diabetic ob/ob mice (Rieusset et al. 2002). In our study, PPARγ protein levels were measured in both tissue and mature adipocytes by western-blotting and the apparently increased levels found in the MG treated group were not significant. PPARγ agonists are reported to increase the adipocyte cell number and decrease the adipocyte cell size and therefore improve the fat storage (Johnson et al. 2007), which seems paradoxical with our findings on adipocyte cell size. MG-induced effects on other factors, which are involved in regulating fat storage in adipocyte and therefore regulating adipocyte cell size, such as TNFα might be an explanation of this paradox.

TNFα is recognized as an inflammatory cytokine first found in the macrophage. However, TNFα is also highly expressed in the adipose tissue and is involved in lipid metabolism mainly by regulating several key enzymes and receptors, such as decreasing the expression of LPL, FFA receptor, GLUT4, acyl-CoA synthetase (ACS) and increasing the expression of hormone-sensitive lipase (HSL) (Sethi and Hotamisligil. 1999). Over-expression of TNFα leads to a decrease in fatty acid uptake; decrease in lipogenesis and increase in lipolysis, and the resultant elevation of FFAs in the circulation. PPARγ and TNFα have adverse effects on these proteins and thus further investigations on these proteins are important to answer this question clearly. However, in my study, LPL mRNA and protein levels did not show a significant difference between the control and MG treated groups. In a previous study from our lab, GLUT4 was found to be reduced in the chronic MG treated group which would lead to a decrease in lipogenesis and contribute to an increase in FFAs in the plasma. Thus, besides LPL protein expression, LPL activity
and other related protein levels or activity needs to be measured in the future.

Another important group of mediators, which plays a role in lipid metabolism and plasma lipid level regulation, is the adipokines including leptin and adiponectin. Leptin is a hormone to control food intake and energy expenditure by acting on the leptin receptor in the CNS. Increased plasma leptin was found in obese subjects, along with a decrease in plasma HDL level in humans (Rainwater et al. 1997). TNFα is reported to increase leptin secretion while PPARγ to decrease its secretion. Adiponectin is a secreted exclusively by the adipose tissue and unlike other adipokines, the levels of adiponectin decrease in obese subjects. Adiponectin is thought to be an insulin enhancer and increased adiponectin is linked to increased triacylglycerol levels and decreased plasma levels of HDL (Kazumi et al. 2002). Opposite to its effect on the regulation on leptin, TNFα decreases the adiponectin levels while PPARγ increases it. We performed RT-PCR combined with ELISA or western blotting to investigate the levels of leptin in adipose tissue/plasma and adiponectin in adipose tissue. However, no significant difference was found in these two adipokines. The results were confirmed by experiments on cultured 3T3-L1 cells. No significant change in these two important adipokines might be explained by their co-regulation by multiple factors including PPARγ and TNFα.

Insulin resistance is a term that cannot be avoided in evaluating this study. Enlarged adipocyte size and increased expression of TNFα are linked to insulin resistance and nearly all the adipocyte-related proteins are regulated by insulin. Chronic MG treated rats developed insulin resistance with reduced insulin levels as reported previously by our lab (Dhar et al. 2011). In the insulin resistance state, visceral adipocyte is more sensitive to
lipolytic hormones glucocorticoids and catecholamines and therefore increases the release of FFAs into the circulation which then serves as substrates for TG formation in the liver. Moreover, insulin resistance increases the production of apoB which leads to the elevation of apoB-rich lipoproteins (Kolovou et al. 2005). Based on our findings in this study and previous results, we suggest that MG-induced insulin resistance might be a mediator in MG-induced lipid alterations.

AG, an AGEs scavenger, attenuated the effects of MG on adipocyte cell size, C/EBPα, C/EBPβ and TNFα expression in abdominal adipose tissue effectively. Even though AG is not a specific MG scavenger and has other actions (Desai and Wu. 2007), it is one of the most widely used compounds as a MG and AGEs scavenger due to a lack of availability of more specific agents.

4.2 Effects of MG on the liver and dyslipidemia

The liver plays a vital role in lipid metabolism and it is the center for fatty acid and lipoprotein synthesis. There are numerous studies which have investigated the effects of high carbohydrate, especially fructose, diet on lipid metabolism in the liver. When normal rats are fed with the high carbohydrate for a short time, increased triacylglycerol and FFAs levels were found in the rat plasma while insulin resistance also developed in the liver, skeletal muscle and adipose tissue. The possible mechanisms of fructose-induced dyslipidemia have been nicely reviewed by Basciano et al (Basciano et al. 2005) which includes the increased expression of lipoprotein secretion related genes such as MTP, the major fatty acid and cholesterol biosynthesis genes such as SREBP, the
increased lipolysis rates in visceral adipose depots and the decreased protection from lipid peroxidation and the resultant increased VLDL secretion and delayed clearance.

In my study, we first examined the nuclear enzymes SREBP-1 and SREBP-2 which are the activators for mainly fatty acid synthesis and cholesterol synthesis, respectively. The expression of SREBP-2 increased significantly in the liver nuclear extract from chronic MG treated group, which was attenuated by co-treatment with AG. However, the expression of SREBP-1 did not change after MG treatment. SREBPs play a crucial role in maintaining the intracellular free cholesterol content. Thus, when the intracellular cholesterol concentration decreases in the hepatocyte, SREBPs attempt to bring it up to the required concentration in two ways: by increasing HMG-CoA reductase to synthesize more cholesterol, and by increasing LDL receptors on the hepatocyte which will pick up more cholesterol from the circulation into the liver. The SCAP protein described earlier in the introduction acts as a sterol sensor and an escort of SREBPs. When cholesterol level drops in the cell, SCAP enables cleavage of SREBPs to activate them and then guides the active SREBPs to the nucleus, where they switch on genes for cholesterol synthesis (HMG CoA reductase) and LDL receptor synthesis. The increase in HMG-CoA reductase observed in my study can be due to an increase in SREBP-2 or a direct effect of MG on HMG-CoA reductase. HMG-CoA is the rate-limiting enzyme in the cholesterol synthesis pathway. LDLR plays a key role in plasma LDL level regulation. LDLR transports LDL from cell surface to the lysosome through endocytosis. Impaired expression of LDLR and over-expression of HMG-CoA reductase have been linked to hypercholesterolemia (Yu et al. 1996; Pallottini et al. 2006). In this study, MG induced over-expression of HMG-CoA
reductase without affecting both mRNA and protein levels of LDLR. No change in LDLR despite an increase in SREBP-2, which can increase LDLR, needs further studies for an explanation. The increase in plasma cholesterol induced by MG (Dhar et al. 2011) can be explained by increased HMG-CoA reductase and increased cholesterol synthesis in the liver, and also by increased MTP activity and VLDL synthesis.

MTP is the last key enzyme we examined in the rat liver. It is essential for apoB 100-containing lipoproteins assembly and its up-regulation leads to increased secretion of apoB-100 containing lipoproteins such as VLDL. In this study, MTP protein levels increased significantly in the liver of chronic MG-treated rats, which was attenuated by co-treatment with AG. The expression of MTP is negatively regulated by insulin, therefore chronic MG-induced MTP up-regulation might be due to decreased insulin levels in vivo, because chronic MG treated rats had significantly lower basal and glucose-stimulated plasma insulin levels (Dhar et al. 2011).

Lipid metabolism in the liver is a complicated network and involves hundreds of enzymes and receptors. In this study, we chose five important ones to try to get a clue for MG-induced plasma lipid level alterations. Changes in SREBP-2, HMG-CoA and MTP protein levels might be the key mechanisms for MG-induced increased levels of total cholesterol and triacylglycerol in the plasma.

4.3 Overall discussion

In this study, MG was infused continuously into adult SD rats for 28 days by an implanted minipump. MG levels increased in the adipose tissue and also created an
insulin resistance state. In chronic MG treated rats, abdominal adipocyte cell size increased and the expressions of C/EBPα and C/EBPβ as well as TNFα increased significantly. The overall effects of these alterations might result in increased lipolysis and decreased TG storage ability and therefore lead to increased FFAs in the plasma. FFAs in the plasma are substrates for TG synthesis in the liver. With the MG-induced up-regulation of SREBP-2, HMG-CoA reductase and MTP and enough supply of TG, cholesterols including VLDL, LDL production increases and therefore results in triacylglycerols and cholesterol increase in the plasma (Fig 4-1).
Figure 4-1. Possible mechanisms for MG-induced increase of FFAs, cholesterol and triacylglycerol levels in the plasma.

Abbreviations: C/EBPs, CCAAT-enhancer binding proteins; FFAs, free fatty acids; HMGCR, 3-hydroxy-3-methyl glutaryl coenzyme A reductase; LDL, low density lipoprotein; MG, methylglyoxal; MTP, microsomal triacylglycerol transfer protein; SREBP, sterol regulatory element binding protein; TNFα, tumour necrosis factor α; VLDL, very low density lipoprotein.
CHAPTER FIVE

CONCLUSIONS

In chronic MG treated rats, the increase in fatty acids may be due to increased abdominal adipocyte size, elevated gene expression of TNFα and increased triacylglycerol accumulation caused by over-expression of C/EBPα, C/EBPβ and possibly PPARγ. Even though LPL protein and mRNA did not change, the activity of LPL needs to be checked. The increase in plasma cholesterol and triacylglycerols in chronic MG treated rats observed in the previous study from our lab (Dhar et al. 2011) may be due to increased expression of HMG-CoA reductase and the subsequent increased cholesterol synthesis; and due to increased MTP and the resultant increased VLDL synthesis in the liver. Further studies expanding on the current findings reported in my study will clarify the molecular mechanisms of altered plasma lipid levels induced by MG.
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