EFFECTS OF FATTY ACIDS ON ALBUMIN-MEDIATED CHOLESTEROL
EFFLUX FROM ENDOTHELIAL CELLS AND ON THE GROWTH OF
HAMSTER PANCREATIC β-CELLS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAI‘I IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN
MOLECULAR BIOSCIENCES AND BIOENGINEERING

DECEMBER 2010

By
Vivian C. Tuei

Dissertation Committee:
Chung-Eun Ha, Chairperson
Dulal Borthakur
Pratibha Nerurkar
Michael Dunn
Nadhipuram Bhagavan

Keywords: Human serum albumin, fatty acid, β-cell, endothelial cell, cholesterol efflux
DEDICATION

To my late father Walter C. Tuei, my mother Anne Rose Tuei, my late uncle Joseah Tuei, and my siblings Vincent, Kennedy, Beatrice, Gladys, and Winnie for their love, kindness, and support.
ACKNOWLEDGEMENTS

I wish to express my special thanks to my advisor, Dr. Chung-Eun Ha for accepting to be my primary mentor throughout my graduate studies. I learnt a lot from you as well that you gave me a great opportunity to be a scientist, thank you.

I wish to thank Dr. Nadhipuram Bhagavan for his helpful discussions in the course of my research; your expertise on albumin has always been invaluable. I am also thankful for your time and guidance as a member of my committee.

I am indebted to the mentorship and support from Dr. Dulal Borthakur as a member of my committee and also as our graduate program chair. I am also thankful for the opportunity and advice you extended to me in teaching some of your lectures.

I owe special thanks to Dr. Pratibha Nerurkar and Dr. Michael Dunn for accepting to be members of my committee. Your contributions in our discussions in regard to my research, questions, and critiques have helped me in being more articulate, thank you.

I am also indebted to the support from my co-workers in the laboratory, Dr. Ji-Sook Ha and past summer students. I thank Dr. Andre Theriault, Department of Medical Technology, Radioactive Safety Offices, and Dr. Karin Bjorkman for kindly allowing me to use some of their technical facilities at University of Hawai‘i.

I am so grateful to Dr. Geoffrey Maiyoh for his help and advice on pursuing my graduate studies. I sincerely appreciate your mentorship, friendship, and encouragement.

I thank Dr. Duane Stevens and his family for extending a warm friendship to me in Hawaii, and to all my family members and friends for their love and support.

Last but not least, I am very grateful to the American Heart Association-Pacific Mountain Affiliate for awarding me a two-year Predoctoral Fellowship.
ABSTRACT

There is compelling evidence that elevated levels of plasma free fatty acids (FFAs) are associated with insulin resistance and pancreatic β-cell dysfunctions. On the other hand, elevated levels of circulating cholesterol are linked with higher incidence of cardiovascular diseases (CVD). CVD and its complications that several type 2 diabetic patients experience are the leading causes of death. Despite these findings on the elevated levels of FFAs in metabolic diseases, the role that the most abundant serum protein and the sole FFA carrier, human serum albumin (HSA), plays in FFA-induced cellular effects is not well studied. HSA binds to many other ligands of both endogenous and exogenous sources, including cholesterol as evidenced through its potency to mediate cholesterol efflux in cell cultures.

In this study, we examined the effects of HSA mixed with various FFAs on FFA-induced cell viability changes and insulin secretion from the hamster pancreatic insulinoma cell line, HIT-T15. Our study showed that various FFAs mixed with HSA have differential effects on HIT-T15 cell viability, depending on the degree of saturation and chain length of FFAs. Furthermore, we showed that palmitate and stearate had adverse effects on cell viability that cylosporin A (csA) and caspase inhibition could not reverse, regardless of the presence of HSA or bovine serum albumin. However, caspase inhibition blocked apoptotic DNA fragmentation, thus indicating that FFA-induced β-cell death is not due to a single mechanism of cell death. Insulin secretion was also reduced after 24-hour exposure of β-cell to HSA/oleate complexes.

We further examined the effects of FFAs on HSA-mediated efflux in the endothelial cell-line, EA.hy926. We observed significant reductions in cholesterol efflux ($P < 0.05$)
at HSA/oleate or palmitate molar ratios of 1:5.3; this was not elicited by the presence of arachidonate. These results suggest that palmitate and oleate competes with cholesterol for the same binding site on HSA. Lastly, modified HSA/FFA interactions caused by mutations of key amino acids involved in binding of FFA to HSA resulted in β-cell viability changes as well as on changes in HSA-mediated cholesterol efflux. Our study suggests a possible role of HSA polymorphism in FFA-induced cellular changes.
# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS**........................................................................................................ iv  
**ABSTRACT**................................................................................................................................. v  
**LIST OF TABLES**......................................................................................................................... xi  
**LIST OF FIGURES**....................................................................................................................... xii  
**LIST OF ABBREVIATIONS**.......................................................................................................... xiii  
**CHAPTER 1. GENERAL BACKGROUND ..................................................................................... 1**  
1.1 Growing Burden of Chronic Non-Communicable Diseases .............................................. 1  
1.2 Free Fatty Acids (FFAs) ........................................................................................................ 5  
    1.2.1 Distribution and Metabolism of FFAs ....................................................................... 10  
    1.2.2 Trafficking of FFAs ..................................................................................................... 13  
    1.2.3 Modulation of Insulin Secretion by Glucose and FFAs ......................................... 15  
    1.2.4 FFA Metabolism and \( \beta \)-Cell Death ................................................................... 18  
        1.2.4.1 Nitric Oxide and Ceramide Generation ......................................................... 20  
        1.2.4.2 Mitochondrial Perturbations ......................................................................... 21  
        1.2.4.3 Activation of NADPH Oxidase .................................................................... 22  
        1.2.4.4 Endoplasmic Reticulum Stress ..................................................................... 23  
1.3 Human Serum Albumin (HSA) ............................................................................................ 27  
    1.3.1 Functions of HSA ....................................................................................................... 28  
    1.3.2 FFA Binding to HSA ................................................................................................. 30  
    1.3.3 Steroid Binding to HSA .............................................................................................. 31  
    1.3.4 Other Ligand Binding to HSA ................................................................................ 31  
    1.3.5 Mutations and Polymorphisms of HSA .................................................................. 32  
    1.3.6 Site-Directed Mutagenesis and Protein Expression System of HSA .................. 33  
1.4 Cholesterol ............................................................................................................................ 37  
    1.4.1 Pathways of Cholesterol Efflux .............................................................................. 38  
    1.4.2 Cholesterol Efflux and Athero-protection .............................................................. 39
CHAPTER 2. EFFECTS OF HSA COMPLEXED WITH FFA ON β-CELLS........44

2.1 Introduction .................................................................................................................44
2.2 Aims of Study ..............................................................................................................45
2.3 Materials and Methods ...............................................................................................46
  2.3.1 Materials ..............................................................................................................46
  2.3.2 Cell Culture ..........................................................................................................46
  2.3.3 Treatment of Cells with FFAs, csA, ZVAD-FMK, and Staurosporine ...........47
  2.3.4 Measurement of Cell Viability ............................................................................48
  2.3.5 Assay of Apoptotic DNA Fragmentation ............................................................48
  2.3.6 Determination of Insulin Secretion ......................................................................48
  2.3.7 Analysis of Data .................................................................................................49

2.4 Results .........................................................................................................................49
  2.4.1 Differential Effects of FFAs Bound to HSA on β-cell Viability .....................49
  2.4.2 Effects of csA and ZVAD-FMK on FFA-induced β-cell Death ....................50
  2.4.3 Apoptosis is Involved in FFA-induced β-cell Death .......................................51
  2.4.4 Oleate and Linoleate are Cytoprotective Against Palmitate-induced Cell Death .........................................................................................................................52
  2.4.5 24-hour Exposure of Cells to Oleate Decreases Insulin Secretion ............52

2.5 Discussion and Conclusion .........................................................................................59

CHAPTER 3. EFFECTS OF FFA ON HSA-MEDIATED CHOLESTEROL EFFLUX........63

3.1 Introduction ..................................................................................................................63
3.2 Aims of Study ..............................................................................................................64
3.3 Materials and Methods ...............................................................................................65
  3.3.1 Materials ..............................................................................................................65
  3.3.2 Cell Culture ..........................................................................................................65
3.3.3 Preparation of HSA/FFAs Complexes .............................................66
3.3.4 Cholesterol Efflux Assay ...............................................................66
3.3.5 Measurement of Cell Viability .........................................................67
3.3.6 Analysis of Data ...........................................................................67

3.4 Results ............................................................................................67
3.4.1 Effects of FFAs on HSA-mediated Cholesterol Efflux .....................67
3.4.2 Cell Viability after Cholesterol Efflux to HSA/FFA Complexes ..........68

3.5 Discussion and Conclusion ..............................................................71

CHAPTER 4. HSA MUTANTS’ ROLES ON FFA CELLULAR EFFECTS ..........73
4.1 Introduction ......................................................................................73
4.2 Aims of Study ..................................................................................74
4.3 Materials and Methods .................................................................75
4.3.1 Materials ......................................................................................75
4.3.2 Molecular Techniques for Developing Recombinant HSA Mutants ....75
4.3.2.1 Cloning of HSA Coding Region ................................................76
4.3.2.2 Expression of Recombinant HSA .............................................76
4.3.2.3 Verification of DNA Sequence of HSA Clones .........................77
4.3.2.4 Purification of Recombinant HSA ...........................................77
4.3.3 Cell Culture ..................................................................................78
4.3.4 Treatment of β-cells with HSA Mutants/FFA Complexes ..............78
4.3.5 Measurement of β-cell Viability .....................................................78
4.3.6 Cholesterol Efflux Assay ...............................................................78
4.3.7 Determination of Unbound FFA (FFA_u) in Recombinant HSA/FFA
  Complexes ..........................................................................................78
4.3.8 Analysis of Data ...........................................................................80
4.4 Results .............................................................................................80
4.4.1 Various HSA Mutants Show Differential Effects on FFA-induced β-cell Death .................................................................80
4.4.2 Unbound FFA (FFA_u) Levels in Recombinant HSA/FFA Complexes ......81
4.4.3 Effects of HSA Mutants in FFA Effects on HSA-mediated Cholesterol Efflux .................................................................81

4.5 Discussion and Conclusion ..........................................................................................86
CHAPTER 5. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS.........90
REFERENCES ..............................................................................................................92
LIST OF TABLES

Table 1.1. Leading Causes of Death in USA in 2006 ......................................................... 4
Table 1.2. Categories of Lipids ......................................................................................... 7
Table 1.3. Some Naturally Occurring Fatty Acids ............................................................... 8
Table 1.4. Distribution of Total Human Serum Fatty Acids ............................................... 12
Table 1.5. Human Body Fat Content of Major Fatty Acids ............................................... 12
Table 1.6. HSA Polymorphisms Associated with Increased Fatty Acid Binding ............ 36
LIST OF FIGURES

Figure 1.1. Molecular Structures of Free Fatty Acids .................................................9
Figure 1.2. Possible Mechanisms for FFA-induced β-cell Death .................................25
Figure 1.3. X-ray Crystallographic Structure of HSA Complexed to Palmitate ..........35
Figure 1.4. Pathways of Cholesterol Efflux .................................................................42
Figure 2.1. Effects of HSA/FFA Complexes on β-Cell Viability ..................................54
Figure 2.2. Effects of Cyclosporin A (csA) and Caspase Inhibitor (ZVAD-FMK) on Palmitate and Stearate-induced β-cell Death .................................................................55
Figure 2.3. Apoptotic DNA Laddering Analysis in Ethidium-bromide Stained Agarose Gel After FFA Treatments ..................................................................................56
Figure 2.4. Effects of Unsaturated FFAs on Palmitate-induced β-cell Death in the Presence of HSA ..............................................................................................................57
Figure 2.5. Effects of Different HSA/oleate Molar Ratios on Insulin Secretion .........58
Figure 3.1. Effects of FFAs on HSA-mediated Cholesterol Efflux .................................69
Figure 3.2. Cell Viability after Cholesterol Efflux to HSA/FFA Complexes ...............70
Figure 4.1. Effects of Various HSA Mutants Complexed to FFAs on Cell Viability ......83
Figure 4.2. Unbound FFA (FFAu) Concentrations in Complexes of Recombinant HSA (rHSA) and its Variants Bound to FFAs .................................................................84
Figure 4.3. Cholesterol Efflux to Recombinant HSA Mutants /FFA Complexes ........85
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette transporter</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>ADIFAB</td>
<td>acrylodan-labelled intestinal fatty acid binding protein</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>Apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter protein</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid assay</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BiP</td>
<td>immunoglobulin heavy-chain-binding protein</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic-AMP</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>CLA</td>
<td>conjugated linoleic acid</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme-A</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CPT</td>
<td>carnitine palmitoyltransferase</td>
</tr>
</tbody>
</table>
CVD  cardiovascular disease
csA  cyclosporin A
cyt c  cytochrome c
DAG  diacylglycerol
DMEM  dulbecco’s modified eagle’s medium
DMSO  dimethyl sulfoxide
EA.hy926  human umbilical vein cell-line
EDTA  ethylenediaminetetraacetic acid
eIF2  eukaryotic initiation factor 2
ELISA  enzyme-linked immunosorbent assay
ER  endoplasmic reticulum
ERAD  ER-associated protein degradation
FABP  fatty acid binding protein
FABpm  plasma membrane-bound fatty acid binding protein
FAS  fatty acid synthase
FAT/CD36  fatty acid translocase
FATP  fatty acid transporter protein family
F-12 K  kaighn’s modification of ham’s F-12 medium
FBS  fetal bovine serum
FcRn  MHC-related Fc receptor for immunoglobin G
FFA  free fatty acid
FFAu  unbound free fatty acid
FH  familial hypercholesterolemia
FXR  farnesoid X receptor
GLP-1  glucagon-like peptide-1
GLUT  glucose transporter
GPR40  G protein-coupled receptor 40
gp60  albondin
GRP glucose regulated protein
HDL high density lipoprotein
HIT-T15 Syrian golden hamster pancreatic β cell-line
HMG 3-hydroxyl-3-methylglutaryl
HNF-4α hepatic nuclear factor-4α
HUVEC human umbilical vein endothelial cells
HSA human serum albumin
HSL hormone sensitive lipase
I-FABP intestinal-fatty acid binding protein
iNOS inducible nitric oxide synthase
Insig insulin-induced gene
IP₃ 1,4,5 inositol-triphosphate
IRE-1 inositol-requiring ER-to-nucleus signal kinase 1
JNK/SAPK Cjun-NH2 terminal kinase/stress-activated protein kinase
Kd dissociation constant
LC-Acyl-CoA long chain acyl-CoA
LCAT lecithin: cholesterol acyltransferase
LCFA long chain fatty acid
LDL low density lipoprotein
LDL-R low density lipoprotein receptor
LRP LDL-related protein
LXR liver X receptor
MAPK mitogen-activated protein kinase
MCFA medium chain fatty acid
MHC major histocompatibility complex
MPTP mitochondrial permeability transition pore
MUFA mono-unsaturated fatty acid
MTT (3,4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NAD  nicotinamide adenine dinucleotide
NADPH nicotinamide adenine dinucleotide phosphate
NOX  NADPH oxidase
NCD  non-communicable disease
NEFA non-esterified fatty acid
NF-κB nuclear factor-κB
NMR nuclear magnetic resonance
NO  nitric oxide
OxLDL oxidized-LDL
PA  phosphatidate
PBS phosphate buffered saline
PCR polymerase chain reaction
PDI protein disulfide isomerase
PDH pyruvate dehydrogenase
PDX-1 pancreatic and duodenal homeobox-1
PERK PKR-like ER kinase
PKA protein kinase A
PKB/Akt protein kinase B
PKC protein kinase C
PKR double-stranded-RNA-activated protein kinase
PLC phospholipase C
PPAR peroxisome proliferator-activated receptor
PUFA polyunsaturated fatty acid
UPR unfolded protein response
RCT reverse cholesterol transport
rHSA recombinant human serum albumin
RNS reactive nitrogen species
ROS reactive oxygen species
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP-cleavage activating protein</td>
</tr>
<tr>
<td>SCD</td>
<td>stearoyl-CoA desaturase</td>
</tr>
<tr>
<td>SCFA</td>
<td>short chain fatty acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SR-BI</td>
<td>scavenger receptor-class B type I</td>
</tr>
<tr>
<td>SRE</td>
<td>sterol regulatory element</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element binding protein</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>T3</td>
<td>triidothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</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>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZVAD-FMK</td>
<td>carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl) fluoromethylketone</td>
</tr>
</tbody>
</table>
CHAPTER 1. GENERAL BACKGROUND

1.1 Growing Burden of Chronic Non-Communicable Diseases

The World Health Organization (WHO) reported that non-communicable diseases (NCDs) accounted to 60% of the total global deaths in 2008 (2004 update) [1]. Among these NCDs, cardiovascular diseases (CVD) was the main cause of death (13.9 million deaths; mainly ischemic heart disease, cerebrovascular disease, and hypertensive heart disease) followed by cancers (3.0 million), chronic obstructive pulmonary disease (3.0 million), and diabetes mellitus (1.1 million), respectively. In USA, diseases of the heart alone accounted to 26.0% of the total deaths in 2006 while cerebrovascular disease caused 5.7% of the deaths [2] as shown in Table 1.1. While the disease burden is different in many other low income developing countries, where communicable diseases is the major cause of mortality, this is however projected to change due to the rapidly increasing prevalence of NCDs in these regions [3, 4]. For instance, the largest increase in diabetes mellitus prevalence is projected to occur in Asia and Africa, and may represent as much as 70% of all cases by 2030 [5]. It is also important to note that of all diabetes cases reported about 90-95% are of type 2 diabetes mellitus (T2DM) while 10% are of type 1 diabetes mellitus (T1DM) and of other specific types [6]. Since, the leading cause of morbidity and mortality among T2DM patients is mainly due to CVD [7], this rising trend of T2DM might also result in higher cases of CVD complications. Therefore, these global trends of NCDs will pose great health and socioeconomic threats in the 21st century.

The risk factors associated to the development of most of the NCDs, especially CVD and T2DM are interrelated [3]. These include the non-modifiable risk factors of ageing,
On the other hand, the main modifiable risk factors are obesity, physical inactivity, and rapid urbanization. Other classical risk factors mainly for CVD also include smoking, low density lipoprotein cholesterol, and hypertension [8]. Obesity has in fact reached epidemic proportions worldwide [8, 9]. Persistent obesity disrupts metabolic processes controlling blood glucose, blood pressure, and lipids (dyslipidemia) [10]. Dyslipidemia characterized in T2DM, a multiorgan disease that involves both pancreatic β-cell dysfunctions and peripheral insulin resistance [11], is also a major risk factor for CVD [12]. Many studies have shown that increased serum levels of free fatty acids (FFAs), which are a common in obese subjects [13-15] play a crucial role in the development of T2DM.

Since human serum albumin (HSA) is the principal FFA carrier molecule in human serum [16] and since many studies have shown the close relationship between elevated FFA levels and incidence of T2DM [13-15, 17], HSA may play a role in modulating the effects of the unbound FFAs (FFA_u) in the pathogenesis of T2DM. This fraction, FFA_u, exists in monomeric form within the aqueous phase in circulation [18]. FFA_u is important because previous studies have shown its role in pathogenesis of some diseases and adverse cellular effects including in insulin secreting cells [19]. On the other hand, it is also widely known that elevated cholesterol level in the blood is directly related to the increased incidence of CVD [20] and that high-density lipoprotein (HDL) exerts its main cardio-protective effects. This cardio-protection by HDL is believed to be through cholesterol efflux, also known as reverse cholesterol transport (RCT) that involves transport of excess cholesterol from the peripheral tissues to the liver for metabolism and eventually excretion. Although HSA has been shown to mediate cholesterol efflux from
cells [21], little is known about the effects of FFAs on albumin-mediated cholesterol efflux and the competition between cholesterol and FFA to the same binding sites on HSA. Therefore, understanding of the degree of competition between FFA and cholesterol on HSA binding sites is important to estimate the FFA effects on albumin-mediated cholesterol efflux, thereby the effects of elevated serum FFAs in the pathogenesis of CVD among obese subjects. Additionally, studies using HSA variants that have modifications on FFA or cholesterol binding sites on HSA, may provide information on the possible role of HSA variants in modulating cellular effects of FFAs and cholesterol.
### Table 1.1. Leading Causes of Death in USA in 2006

Source: Centers for Disease Control and Prevention/National Centre for Health Statistics [2].

<table>
<thead>
<tr>
<th>Rank</th>
<th>Cause of Death</th>
<th>Deaths</th>
<th>% of Total Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Diseases of heart</td>
<td>631,636</td>
<td>26.0</td>
</tr>
<tr>
<td>2.</td>
<td>Malignant neoplasms (cancers)</td>
<td>559,888</td>
<td>23.1</td>
</tr>
<tr>
<td>3.</td>
<td>Cerebrovascular diseases (stroke)</td>
<td>137,119</td>
<td>5.7</td>
</tr>
<tr>
<td>4.</td>
<td>Chronic lower respiratory diseases</td>
<td>124,583</td>
<td>5.1</td>
</tr>
<tr>
<td>5.</td>
<td>Accidents (unintentional injuries)</td>
<td>121,599</td>
<td>5.0</td>
</tr>
<tr>
<td>6.</td>
<td>Diabetes mellitus</td>
<td>72,449</td>
<td>3.0</td>
</tr>
<tr>
<td>7.</td>
<td>Alzheimer’s disease</td>
<td>72,432</td>
<td>3.0</td>
</tr>
<tr>
<td>8.</td>
<td>Influenza and pneumonia</td>
<td>56,326</td>
<td>2.3</td>
</tr>
<tr>
<td>9.</td>
<td>Nephritis, nephrotic syndrome, and nephrosis</td>
<td>45,344</td>
<td>1.9</td>
</tr>
<tr>
<td>10.</td>
<td>Septicemia</td>
<td>34,234</td>
<td>1.4</td>
</tr>
</tbody>
</table>
1.2 Free Fatty Acids (FFAs)

FFAs or non-esterified fatty acids (NEFAs) are aliphatic monocarboxylic acids that are constituents of the broad biological term ‘lipids’ [22]. Lipids are generally defined as classes of organic compounds that are hydrophobic or amphipathic (both polar and nonpolar) and are soluble in organic solvents [23]. Lipids can further be classified into four categories; simple, complex, derived and, miscellaneous lipids [24] as shown in Table 1.2. FFAs fall under the category of derived lipids, usually obtained from the hydrolysis of triacylglycerols (TAGs) and cholesterol esters. Naturally occurring FFAs are found in animal or vegetable fats and oils [25] contained in esterified forms. The FFAs can be either saturated (no carbon-carbon double bonds) or unsaturated (carbon-carbon double bonds present). The double bonds in most of the naturally occurring unsaturated fatty acids are usually in the cis configuration (hydrogens on the same side on the bond) while the trans isomers commonly arise from partial hydrogenation of vegetable oils [26]. The cis bonds exerts kinks in the fatty acid structure, creating a U-like orientation, thus providing fluidity while the trans bonds assumes a straight shape as that of saturated fatty acids (Refer to Figure 1.1). Unsaturated FFAs that contain one double bond are usually referred to as mono-unsaturated fatty acids (MUFAs) while those with two or more double bonds are called poly-unsaturated fatty acids (PUFAs). Some types of PUFAs also occur as conjugated isomers (double bonds separated by only one single bond versus a methylene group) such as conjugated linoleic acid (CLA), which occurs during food processing and also found in some ruminants [26]. Humans can synthesize all the fatty acids except, linoleic acid (ω-3) and α-linolenic acid (ω-6), which are precursors for the synthesis of important cell signaling molecules such as arachidonic acid [27], thus their
essentiality. In some circumstances, the fatty acids are also classified as short chain fatty acids (SCFAs) if the chain carbons are less than 8, medium chain fatty acids (MCFAs) for 8-14 chain carbons, and long chain fatty acids (LCFAs) for 16 and more chain carbons [24]. The LCFAs are the most common components in dietary fat. Table 1.3 shows some common naturally occurring FFAs, their classification, and main dietary sources.
Table 1.2. Categories of Lipids

Source: [24].

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple lipids</td>
<td>Fatty acids esterified with alcohols</td>
<td>Triacylglycerols (neutral fats and oils), true waxes, cholesterol esters, Vitamin A and D esters</td>
</tr>
<tr>
<td>Complex lipids</td>
<td>Fatty acids esterified with alcohols plus other groups</td>
<td>Phospholipids, glycolipids, cerebrosides, sulfolipids, lipoproteins, lipopolysaccarides</td>
</tr>
<tr>
<td>Derived Lipids</td>
<td>Obtained by hydrolysis of simple or complex lipids</td>
<td>Fatty acids (saturated, monounsaturated, polyunsaturated), monoacylglycerols, diacylglycerols, alcohols (steroids, sterols, Vitamin A and D)</td>
</tr>
<tr>
<td>Miscellaneous lipids</td>
<td></td>
<td>Straight chain hydrocarbons, some wax lipids, carotenoids, squalene, Vitamin E and K</td>
</tr>
</tbody>
</table>
Table 1.3. Some Naturally Occurring Fatty Acids

Numbering of carbon atoms begins at the carboxyl carbon except in ω-series which is based on the carbon atoms present between the terminal methyl group and the nearest double bond; ω-3 and ω-6 are essential fatty acids. n: indicates the “normal” unbranched structure. Source: [23, 25, 28, 29].

<table>
<thead>
<tr>
<th>Saturated Fatty Acids Carbon Skeleton</th>
<th>Common Name</th>
<th>Systematic Name</th>
<th>ω-Series</th>
<th>Food Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>Lauric</td>
<td>n-Dodecanoic</td>
<td></td>
<td>Coconut and palm nuts oils</td>
</tr>
<tr>
<td>C14:0</td>
<td>Myristic</td>
<td>n-Tetradecanoic</td>
<td></td>
<td>Coconut and palm nuts oils</td>
</tr>
<tr>
<td>C16:0</td>
<td>Palmitic</td>
<td>n-Hexadecanoic</td>
<td></td>
<td>Animal and plant fats</td>
</tr>
<tr>
<td>C18:0</td>
<td>Stearic</td>
<td>n-Octadecanoic</td>
<td></td>
<td>Animal fats, some plant fats</td>
</tr>
<tr>
<td>C20:0</td>
<td>Arachidic</td>
<td>n-Eicosanoic</td>
<td></td>
<td>Peanut oil</td>
</tr>
<tr>
<td>C24:0</td>
<td>Lignoceric</td>
<td>n-Tetracosanoic</td>
<td></td>
<td>Most natural fats, peanut oil in small amounts</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Monounsaturated Fatty Acids</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:1</td>
<td>Palmitoleic</td>
<td>9-Hexadecenoic</td>
<td>ω-7</td>
</tr>
<tr>
<td>C18:1</td>
<td>Oleic</td>
<td>9-Octadecenoic</td>
<td>ω-9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polyunsaturated Fatty Acids</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2 Linoleic</td>
<td>9,12-Octadecadienoic</td>
<td>ω-6</td>
<td>Corn, safflower, soybean, cottonseed, sunflower seed, and peanut oil</td>
</tr>
<tr>
<td>18:3 α-Linolenic</td>
<td>9,12,15-Octadecatrienoic</td>
<td>ω-3</td>
<td>Linseed, soybean, and other seed oils</td>
</tr>
<tr>
<td>20:4 Arachidonic</td>
<td>5,8,11,14-Eicosatetraenoic</td>
<td>ω-6</td>
<td>Lean meat and organ lipids</td>
</tr>
</tbody>
</table>
**Saturated Fatty Acids**
- Myristic acid 14:0
- Tetradecanoic acid
- Palmitic acid 16:0
- Hexadecanoic acid
- Stearic acid 18:0
- Octadecanoic acid

**Mono-unsaturated Fatty Acids**
- Palmitoleic acid 16:1 \(\Delta 9\)
- 9-Hexadecenoic acid
- Oleic acid 18:1 \(\Delta 9\)
- 9-Octadecenoic acid
- Elaidic acid 18:1 \(\Delta 9\) trans
- 9-trans-Octadecenoic acid

**Poly-unsaturated Fatty Acids**
- Linoleic acid 18:2 \(\Delta 9,12\)
  (9,12-Octadecadienoic acid)
- Arachidonic acid 20:4 \(\Delta 5,8,11,14\)
  (5,8,11,14-Eicosatetraenoic acid)
- Linoelaidic acid 18:2 \(\Delta 9,12\) trans
  trans, trans-9,12-octadecadienoic acid
- Conjugated linoleic acid (CLA) 18:2 \(\Delta 9,12\) cis, trans
  9-cis-11-trans-octadecadienoic acid

**Figure 1.1. Molecular Structures of Free Fatty Acids**
1.2.1 Distribution and Metabolism of FFAs

Fatty acids in dietary fat (exogenous), mainly as TAGs are usually released by the action of pancreatic lipases in the duodenum and jejunum and absorbed into the enterocytes [30]. In enterocytes, LCFAs are reassembled again into TAG and packaged as chylomicrons which are transported through the lymphatic system into the blood. The apolipoprotein, apo B48 is the primary protein in chylomicrons. In circulation, endothelial lipoprotein lipases mainly in non-hepatic tissues remove FFAs from TAG of chylomicrons into target cells and the remaining glycerol is metabolized in the liver and kidney, thus producing chylomicron remnant. FFAs released into adipocytes in the fed state, are assembled again into TAG. On the other hand, adipocyte TAG depots provide sources of FFAs into circulation for uptake by liver and muscle cells during starvation, stress, and prolonged exercise, through the action of adipocyte hormone-sensitive lipases. FFAs released into the circulation are then usually bound to HSA that facilitates FFA transport in the aqueous environment. Catabolism of the FFAs generate acetyl-CoA and particularly in the liver, ketone bodies are produced which are important for fueling the brain under prolonged fasting, since brain cells cannot utilize FFAs.

The liver plays a central role in metabolism, due to its capacity of processing various metabolites under different body states. In fed state, glucose, amino acids, SCFAs, and MCFAs from the exogenous sources (diet) enter the portal vein and are metabolized into FFAs as a result of an accelerated glycolytic flux in the liver. The FFAs are then incorporated into TAG pools. Also chylomicron remnant is also eventually up taken into the liver releasing FFAs, monoacylglycerols, diacylglycerols (DAGs), glycerol, and cholesterol. This uptake involves the recognition of apo E and lipoprotein lipases in
chylomicron remnants by hepatic LDL-receptors (LDL-R) and LDL receptor-related protein (LRP) [31]. TAGs are again repackaged in the liver and combined with phospholipids, cholesterol, and apo B100 to the very low density lipoprotein (VLDL). VLDL is released into the circulation for the delivery of the various lipid components into non-hepatic tissues by action of endothelium lipoprotein lipases, now representing an endogenous pathway. The remaining VLDL remnants can either be taken up by the liver or are transformed to LDL at which the LDL delivers cholesterol to all body cells via the LDL-R [31]. In fasting, glycogen stores in the liver and FFAs released from adipose tissues are the main precursors for synthesis of VLDL.

Therefore, FFAs also occur as constituents of TAGs in lipoproteins, mainly VLDL as well as in adipocyte TAG depots. TAG is the main storage lipid and thus the principal body fat. Membranes also constitute a number of lipids with esterification to FFAs (glycerophospholipids and glycolipids). Oleate is by far the most abundant FFA in the body lipid content followed by palmitate (Table 1.5). Noteworthy, is that it is in the blood that significant levels of FFAs only occur and are bound to albumin. Under normal physiological conditions, between 1 and 2 moles of FFA are bound to HSA, but the molar ratio of HSA to FFA can rise to 1:6 or greater under pathological conditions such as diabetes mellitus, insulin resistance, and CVD [32, 33]. The distribution of the major total serum fatty acids are shown in Table 1.4.
Table 1.4. Distribution of Total Human Serum Fatty Acids

Source: [34].

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleate</td>
<td>38</td>
</tr>
<tr>
<td>Palmitate</td>
<td>25</td>
</tr>
<tr>
<td>Linoleate</td>
<td>22</td>
</tr>
<tr>
<td>Stearate</td>
<td>10</td>
</tr>
<tr>
<td>Arachidonate</td>
<td>3</td>
</tr>
<tr>
<td>Linolenate</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1.5. Human Body Fat Content of Major Fatty Acids

Data are based on a 70kg adult human. Source: [24].

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Content (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid</td>
<td>6640</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>3320</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>1560</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>550</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>130</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>80</td>
</tr>
<tr>
<td>Total</td>
<td>12280</td>
</tr>
</tbody>
</table>

The oxidation of FFA in cells occurs in the mitochondrial matrix. The process involves; uptake of FFAs into the cytosol, activation to fatty acid acyl-CoA, translocation into mitochondria involving the carnitine transesterification shuttle, and β-oxidation of the acyl-CoA at the β-carbon [23]. The β-oxidation process involves a sequential two carbon removal and reduction of flavoproteins and thus generation of adenosine-5'-triphosphate (ATP) as they are channeled through the tricarboxylic acid cycle (TCA) and oxidative phosphorylation. Other pathways of fatty acids oxidation occur such as peroxidation in peroxisomes (oxidation of very long chain unbranched FFAs) [23].
Fatty acid oxidation is under hormonal regulation. Insulin inhibits adipose tissue lipolysis in fed state, stimulates lipogenesis, and the synthesis of malonyl-CoA. These insulin effects are counteracted by glucagon during fasting. The other regulatory point is on carnitine palmitoyltransferase-1 (CPT1) which is inhibited by malonyl-CoA. The peroxisome proliferator-activated receptors-α (PPARα) belonging to the nuclear hormone receptor family [35], have been also shown to be able to modulate the expression of peroxisomal acyl CoA oxidase and mitochondrial carnitine palmitoyltransferase [36]. These receptors are ligand activated by fatty acids and its derivatives as well as some lipid-lowering drugs such as fenofibrates [37, 38].

Unlike fatty acid oxidation, fatty acid synthesis occurs in the cytosol through the activity of a multienzyme functioning in a sequence. The rate limiting step involves the first enzyme, acetyl-CoA carboxylase (ACC) that converts acetyl-CoA to malonyl-CoA, while fatty acid synthase (FAS) sequentially joins two carbon units of malonyl-CoA, elongating the chain. FAS is highly regulated at transcription level by signals such as insulin and carbohydrates [39], thyroid hormone [40], fatty acids [41], and sterols [42]. FAS have also been shown to be downstream target gene of sterol regulatory element binding protein (SREBP) involved in the nutritional induction of FAS promoter [43]. On the other hand, ACC is mainly regulated by 5′ adenosine monophosphate-activated protein kinase (AMPK) which is activated when AMP:ATP ratio increases in the cells [44].

1.2.2 Trafficking of FFAs

It is a general consensus that FFAs plays a central role in energy homeostasis through processes of energy provision and storage as well as precursors for the derivation of
complex structural membrane lipids. Recent studies have also found that FFAs are involved in the regulation of; a) activation of $K^+$ [45] and $Ca^{2+}$ ion channels in certain cells [46], b) membrane receptors, e.g., G-protein coupled receptors and LDL-R [47], c) kinases [48], d) gene expression [49-51], and e) reversal of arrhythmias in neonatal cardiac myocytes [52]. LCFA metabolites such as those derived from membrane localized arachidonic acid (leukotrienes, prostaglandins, and thromboxanes) are also involved in intracellular signaling during injury [53, 54]. As a consequence of the varied functions of FFAs, they are now majorly implicated in many conditions such as T2DM [55], atherosclerosis [56], inflammation [57], immune response [58], cell differentiation [59], and cancer [60]. Therefore, understanding the circulatory, transmembrane, and intracellular transport of FFAs is important in order to appreciate the various effects of FFAs in the body. These transport processes were extensively reviewed by Hamilton (1998) [61] where he classified them into three main stages; adsorption, transmembrane movement, and desorption. However there is not yet a clear unison among researchers regarding these transport processes especially in the transmembrane transport.

In the first step of adsorption, FFAs adsorb into the outer leaflet of the plasma membrane in circulation and is largely assumed to be a monomeric unbound form, $FFA_u$ (neither associated to albumin nor any other protein). Thus the FFAs must first desorb from albumin. Briefly, the fatty acid exists in two forms; 99.6 % anions and 0.4% protonated [62]. In cell culture, it is difficult to determine this fraction of $FFA_u$ that desorbs off from albumin. The most controversial stage is transmembrane transport of the FFAs, since the involvement of passive diffusion (flip-flop) or plasma membrane protein-mediated translocation are questionable if they could be occurring independently or
The carboxylic head should be re-oriented into the cytosolic face. The form of FFA translocating across the membrane is also not agreed as to whether it is the widely thought unionized form as confirmed by the quick acidification of the cytosol [63] or the ionized form. Despite these contradictions, there has been no data confirming the functionality of translocase in FFA translocation across plasma membranes [62] even though the fatty acid translocase (FAT/CD36), the plasma membrane-bound fatty binding protein (FABPpm) and the fatty acid transporter protein family (FATP) have been identified [64]. In the final step of desorption, the FFAs leaves the cytosolic leaflet into the cytosol to be utilized in intracellular sites. At desorption, the disagreement is on the rate limiting step being flip-flop over dissociation into the aqueous phase [65]. Thus, the abundant intracellular protein family, fatty acid binding proteins (FABPs) have been the speculated in facilitating the transfer of FFAs from the cytosolic leaflet into the cytosol [61, 66, 67] for esterification to fatty acyl-CoA by acyl-CoA synthetase. FABPs are also thought to be involved in the sequestration of LCFAs into cytoplasmic organelles and incorporation into esterified forms [62].

1.2.3 Modulation of Insulin Secretion by Glucose and FFAs

The β-cells are the most abundant endocrine cell-type of pancreatic islets responsible for secreting insulin while α, δ, and φ-cells secrete glucagon, somatostatin, and pancreatic polypeptide, respectively [13]. Normal insulin secretion involves the uptake of glucose into the β-cells via the non-insulin-dependent glucose transporter 2 (GLUT-2) due to increased blood glucose [68]. Glucose is metabolized through the glycolytic pathway initiated by the rate-determining enzyme glucokinase and this enhances the TCA cycle resulting in an overall increase in ATP:ADP ratio. Increase in ATP levels causes
the closure of the ATP-sensitive K+ channels, depolarization of the plasma membrane, opening of the voltage-activated Ca^{2+} channels and influx of Ca^{2+}, and finally fusion of insulin containing granules with the plasma membrane [69, 70]. The secretion occurs in two phases; a first phase that is rapid and transient (2-5min), and a second that progressively increases as glucose amplifies the insulin signal for the duration of the stimulus [13]. The increased insulin secretion enhances glucose transport into muscle and adipose tissue as well as regulating other processes geared at lowering blood glucose to normal levels [13, 71]. On the other hand, β-oxidation of FFA is reduced because of increased citrate and malonyl-CoA from the glycolytic flux leading to increased cytosolic long chain acyl-CoA (LC acyl-CoA), phosphatidate (PA), TAGs, and phospholipids [70, 72] due to the inhibition of CPT-1 by malonyl-CoA [73]. Thus malonyl-CoA plays a role in this shift from FFA to glucose as an oxidative fuel. Elevated levels of LC-CoA activates isoforms of phospholipase C (PLC), promoting the hydrolysis of membrane phospholipids and generation of 1, 4, 5 inositol-triphosphate (IP_{3}) and DAG [69]. DAG activates protein kinase C (PKC) [74-78] and IP_{3} mobilize Ca^{2+} from intracellular stores. LC acyl-CoA may also stimulate insulin secretion by a more direct effect on exocytosis as it facilitates the fusion of insulin-secretory granules with the β-cell plasma membrane [69, 79]. In addition, lipolysis of TAGs stored in β-cell by hormone sensitive lipase (HSL) might also contribute to the LC acyl-CoA pool [80]. Activation of HSL might be through protein kinase A (PKA) which is activated by cAMP e.g., as a result of the incretin gut hormone, glucagon-like peptide-1 (GLP-1) binding on GLP1-receptors [81, 82]. Recently, G protein-coupled surface receptors mainly, GPR40 have been characterized in β-cells and are bound by FFAs activating PLC [83], thus increasing
insulin secretion. Altogether, all the above data supports LC acyl-CoA to be involved in a stimulus-insulin secretion coupling.

Results from both in vitro and in vivo studies support the suggestion that acute exposure of cells to FFA leads to enhanced rates of insulin secretion [69, 84-88] but a more chronic treatment is associated with reduced glucose induced insulin secretion [84, 88-91]. Increasing FFA chain length and degree of saturation has been shown to relate positively to this insulinotropic potential of the FFAs [92, 93]. The chronic FFA exposure is suggested to impair glucose oxidation resulting in a fall in the ATP:AMP ratio and thus activating AMPK [94]. The activation of AMPK enhances inhibition of ACC, thus decreased fatty acid synthesis and increased fatty acid oxidation, impairing glucose-stimulated insulin secretion. In fact, the expression of liver X receptor (LXR) [95], sterol regulatory element binding protein-1c (SREBP1c) [96, 97], and hepatic nuclear factor-4α (HNF-4α) have been shown to be decreased due to AMPK activation, thus decreasing lipogenesis [69]. The reduced glucose oxidation has been linked to a β-cell glucose-fatty acid cycle (Randle cycle) characterized by a higher rate of FFA oxidation reducing glucose oxidation [84, 89, 98-101]. This was supported by the inhibition of β-oxidation of fatty acids by etomoxir that resulted in reversal of secretion in islets cells on long-term exposure of FFAs [84, 91]. Furthermore, the activation of pyruvate dehydrogenase (PDH) kinase, was shown to inhibit PDH (catalyzes conversion of pyruvate to acetyl-CoA) on chronic exposure of FFAs [90, 102]. PDH regulation is by acetyl-CoA:CoA and NADH:NAD ratios which increase during oxidation of FFAs [103]. Other mechanisms have been implicated on the effects of chronic FFA exposures in β-cells including those that lead to changes in membrane fluidity [13, 104], toxic ionic effects [105], GLUT-2
and the β-cell transcription factor PDX-1 expression [106], insulin biosynthesis [107], and generation of free radicals [88, 108]. Of note is the various subtypes of the PPAR family that have been identified in pancreatic β-cells and their involvement in regulation of β-cell fatty acid metabolism [109]. Specifically, PPARα have been shown to activate β-cell fatty acid catabolism while PPARγ directs fatty acids to TAG synthesis and its expression is thus enhanced under hyperglycemic and hyperlipidemic conditions [110]. PPARα agonists have also been shown in β-cells to regulate the expression of PDX-1 and improve glucose induced insulin secretion impaired by palmitate [111]. PDX-1 is involved in the regulation of expression of insulin [112] and other β-cell specific genes such as GLUT-2 [113] as well as in β-cell differentiation [114]. The PPARδ have recently been shown in a rat insulinoma cell line and also pancreatic islets to be the most abundant subtype and through which the unsaturated fatty acids mimic synthetic PPAR agonists in enhancing mitochondrial fatty acid oxidation and GSIS [115]. Thus PPARδ and PPARα therapeutic agonists may be important in the prevention and management of fatty acid-induced β-cell defects.

1.2.4 FFA Metabolism and β-Cell Death

In addition to the acute and chronic effects of FFAs on β-cell insulin secretion discussed above, emerging evidence is on the causal effects of FFAs on the reduction of β-cell mass [116]. This is important in the understanding of the pathogenesis of T2DM which is characterized by both insulin resistance and β-cell dysfunctions [117]. Insulin resistance seems not to be sufficient to cause T2DM since many obese people with insulin resistance do not have T2DM [118]. This can be explained by the islets β-cell adaptation to increase β-cell number and/or volume (β-cell mass) thus sufficiently
compensating for the insulin demand by increasing the secretion levels of insulin [119]. However, T2DM ensues when this compensatory mechanism is compromised and this might be caused by mechanisms affecting β-cell transcription response or secretory capacity as well as reduced β-cell viability [118]. This reduction in β-cell viability has been linked to either the toxic elevation of plasma glucose levels (glucotoxicity) [120], or FFAs/dyslipidemia (lipotoxicity) [104], or both (glucolipotoxicity) [11]. Indeed the long term exposure of FFAs in β-cells have been associated with toxicity [76, 86, 87, 121-125]. This toxicity is more pronounced during treatment with long-chain saturated FFA than with the short-chain saturated FFA or longer chain unsaturated FFA [125-131] with a few papers disputing this [124, 132-134]. Furthermore, there are some proposals that the unsaturated fatty acids can counteract β-cell death induced by FFAs, serum removal, and exposure to proinflammatory cytokines [125-127, 130, 135, 136].

FFA are thought to be transported by passive transport into β-cell and esterified to LC acyl-CoA, the activated intracellular form of FFAs and a precursor for TAGs, DAGs and phospholipids [13, 70]. These acyl-CoA moieties are then destined for β-oxidation in the mitochondria and to a small extent in the peroxisomes [137]. However, studies have shown that an excess availability of plasma FFA results in the accumulation of cytoplasmic TAGs and increases the intracellular LC acyl-CoA pool beyond the oxidative requirements of the cell [13, 138]. In support of this, Triacsin-C have been shown to block the initial esterification step of FFAs preventing β-cell death, thus FFA itself does not cause cell death but it is their activated derivatives [69, 87]. These acyl-CoA moieties and related metabolites probably initiate other additional pathways leading to β-cell death such as by nitric oxide (NO) generation [139], ceramide formation [140]
and its inhibition of protein kinase B (PKB/Akt) activity [141, 142], activation of PKC [143, 144], and activation of calpain-10 [145]. Other possible mechanisms are by activation of peroxisome proliferator-activated receptors (PPARs) that might affect the expression of several proteins such as caspases [146], mitochondrial perturbations [147], and endoplasmic reticulum (ER) stress [148] (Refer to figure 1.2). Below is a discussion of some of these possible molecular pathways involved in FFA-induced β-cell death.

1.2.4.1 Nitric Oxide and Ceramide Generation

NO generation has been mainly implicated in β-cell death in T1DM as a result of secretion of cytokines in response to autoimmune reactions [149]. The cytokines increase the expression of inducible nitric oxide synthase (iNOS) that leads to the production of NO, a potent oxidant and mediator of apoptosis [139]. Excess palmitate results in increased de novo synthesis of ceramide, an initiator of apoptosis since palmitate is involved in the first step to ceramide synthesis [150]. Ceramide increases iNOS expression by activation of nuclear factor-κB (NF-κB) leading to NO formation [139, 151]. Ceramide has also been implicated in the inhibition of PKB/Akt activity leading to some of its downstream targets inactivation including glycogen synthase kinase 3, caspase 9, and Bcl-2 family member Bad involved in pro-survival pathways [94]. However, the involvement of ceramide or NO in FFA induced β-cell death is still contentious since other studies have shown minimal cytoprotective effects when inhibitors of ceramide synthase or iNOS were tested in palmitate treated cells [152] and also that NF-κB genes are not upregulated during fatty acid treatment [134].
1.2.4.2 Mitochondrial Perturbations

Mitochondrial perturbations may arise due to a collapse of the mitochondrial inner transmembrane potential, uncoupling of respiratory chain, reactive oxygen species (ROS) generation, calcium and glutathione sequestration that might lead to plasma membrane disruption and/or apoptosis when caspase and caspase activators leak into the cytosol [147]. The mitochondrial permeability transition pore (MPTP) consisting of a dynamic multiprotein complex are formed in the contact site between inner and outer mitochondrial membranes facilitating the efflux of pro-apoptotic proteins such as cytochrome c (cyt c) and apoptosis inducing factor (AIF) [147, 153]. The inner mitochondrial membrane is normally impermeable to molecules and this is a requirement for maintenance of inner transmembrane potential but opening of MPTP pore which is normally in a closed state dissipates this potential as well as enabling the efflux of these pro-apoptotic proteins [147]. Several studies now indicate that elevated FFAs particularly palmitate induce the MPTP formation in pancreatic β-cells and the release of cyt c [125, 130, 133, 154]. Release of cyt c into the cytosol activates the caspase cascade by forming a multimeric complex with apoptotic protease activating factor-1 (Apaf-1) which then activates procaspase 9. Caspase 9 then activates the effector caspases such as caspase 3 causing apoptosis. A recent study in the insulin secreting cell line, INS-1 has further shown that apoptosis arise due to NO-mediated mitochondrial DNA damage [155]. A marked reduction in Bcl-2 mRNA was also noted in human islets on fatty acid-induced apoptosis [121]. Bcl-2 family of proteins are thought to function through selective protein-protein interactions in the mitochondria in either facilitating cell death or survival. Also, increased mitochondrial ROS and/or reactive nitrogen species (RNS) generation
due to elevated glucose or FFAs oxidation in β-cells has been suggested to directly inflict macromolecular damage or indirectly increase oxidative stress [156] that leads to activation of stress-sensitive pathways such as NF-κB, p38 mitogen-activated protein kinase (p38 MAPK), Cjun-NH2 terminal kinase/stress-activated protein kinase (JNK/SAPK), and hexosamine pathways [157]. These reactive species are cleared in the cells by the action of catalase, glutathione peroxidase, and superoxide dismutase which are expressed at low levels in the β-cells, thus making β-cells highly susceptible to oxidative stress [158].

1.2.4.3 Activation of NADPH Oxidase

Noteworthy is recent advances on the role of NADPH oxidase(s) in diabetic pancreatic beta-cell dysregulation and vascular derangements [159]. NADPH oxidase (NOX) family are plasma or sub-cellular membrane-associated enzymes (e.g., in phagosomes) that transport electrons through biological membranes and catalyzes the reduction of molecular oxygen to superoxide (O$_2^-$) [160]. It has generally been known that ROS in pancreatic β-cells originate from mitochondrial electron transport chain, and acutely, it enhances metabolic-stimulus-coupling factors (ATP, citrate, glutamate) for glucose stimulated insulin secretion [160]. However, under conditions of excess exposures of fatty acids, some proinflammatory cytokines, glucose, or angiotensin II to cells, it leads to activation of NOX [159, 161] mediated by PKC increases inducing β-cell dysfunctions, adipocytokine-release dysregulation as well as vascular complications because of the surplus generation of ROS. These excess ROS species reduces insulin secretion levels and are also potent β-cell apoptotic stimuli. In particular the O$_2^-$ species or it subsequent conversion to hydrogen peroxide can combine with NO to generate
peroxynitrites [162] that can cause mitochondrial stress, ER stress, and apoptosis as well as other effects e.g., lipid peroxidation. Thus inhibition of NOX on fatty acid or ROS-induced β-cell defects might be mechanistic targets for pharmacological inventions in prevention of β-cell dysfunctions besides its effects on the vasculature. *In vitro* studies have shown the potency of two compounds, apocynin [161] and diphenylene iodonium [163] in inhibiting NOX in oxidative stress states.

1.2.4.4 Endoplasmic Reticulum Stress

Emerging evidence now shows the involvement of cellular ER stress in FFA-induced apoptosis [132, 164]. ER stress is induced when unfolded proteins accumulate in the ER due to increased input of proteins or decreased capacity of the ER to fold and/or transport proteins to Golgi or when folded proteins accumulate in the cytosol. This protein accumulation triggers the unfolded protein response (UPR) to activate three major ER stress-sensing proteins that are located in the ER membrane. β-cells synthesize and secrete large amounts of insulin to meet metabolic demands and therefore they are highly sensitive to ER stress [132, 165, 166]. Exactly how FFAs cause ER perturbations that ultimately lead to ER stress is still not clear although few proposals point to the accumulation of insoluble TAG within the ER, depletion of Ca\(^{2+}\) stores, and inhibition of normal glycosylation or alterations of ER redox state [132, 167, 168]. The PKR (double-stranded-RNA-activating protein kinase)-like ER kinase (PERK), Inositol-requiring ER-to-nucleus signal kinase 1 (IRE1), and activating transcription factor 6 (ATF6) signaling pathways have been shown to initiate cell-protective mechanisms that restore ER folding capacity [132]. These responses were outlined by Oyadomari et al. (2004) [169] with the first response involving the PERK signaling pathway which inhibit protein translation by
phosphorylation of eukaryotic initiation factor 2 (eIF2), thus preventing further accumulation of unfolded proteins. The second phase involves upregulation of gene expression encoding ER chaperone proteins such as immunoglobulin heavy-chain-binding protein (BiP)/glucose-regulated protein 78 (GRP78), glucose-regulated protein 94 (GRP94), and the protein disulfide isomerase (PDI) to enhance protein folding capacity. In a latter phase, components of ER-associated protein degradation (ERAD) are transcriptionally induced to eliminate misfolded proteins in the ER by the ubiquitin-proteosome pathway. However, under prolonged and extreme ER stress, the signaling pathways lead to apoptosis. The transcription activation of the gene for C/EBP homologous protein (CHOP) is involved in one of the apoptotic pathways and is thought to affect the Bcl-2 protein family [169]. Activation of the JNK has also been described [170, 171] as well as the activation of caspase-9 by caspase-12 that is not cytochrome c or death receptor-mediated [172, 173]. Detailed downstream pathways are now beginning to be characterized [132].
**Figure 1.2. Possible Mechanisms for FFA-induced β-cell Death**

1.3 Human Serum Albumin (HSA)

HSA is the most abundant protein in plasma with the normal concentration of around 0.6 mM, which account for 60-65% of the total protein concentration [174]. It is synthesized in the liver (10% of total liver protein synthesis) and has a half-life of 19 days in circulation [175]. Changes in interstitial colloid pressure is thought to be the major regulatory factor for HSA synthesis [176]. The plasma compartment has 30-40% of the total body HSA while the remainder is distributed in other tissues, mainly in the skin and muscle [177]. Within cells, HSA is found in very low concentrations if at all present [175]. HSA is a 66.5 kDa non-glycosylated, multifunctional, monomeric protein of 585 amino acids [16]. The protein adopts a heart shaped 3-dimensional (3D) structure (Figure 1.3) as determined by X-ray crystallography [16] consisting of three homologous domains (I, II, and III). Each domain consists of two sub-domains, A and B [174, 178]. The three homologous domains are held by a total of 17 disulfide bonds, which are exclusively intra-subdomain that makes HSA highly thermostable [174]. The protein is 67% α-helical [16] and posses a high net negative charge (-17) arising from asymmetric charge distribution at neutral pH that make it highly soluble and stable in circulation [174]. HSA also undergoes reversible conformational changes at different pH namely, ‘N’ form at neutral pH, ‘F’ form that is fast migrating at pH less than 4.0, and ‘B’ form for basic at pH above 8.0 [175]. Ligands such as fatty acids on binding HSA induce conformational changes [174].

The primary sequence of HSA contains high quantities of cysteine (35) and charged amino acids residues, but less of isoleucine, methionine, and tryptophan (only 1) residues [174]. Out of the 35 cysteinyl residues, 34 form the 17 stabilizing disulfide bonds [174].
Thus, HSA is unusual among extracellular proteins in having a free single sulfhydryl group (Cys-34) referred to as mercaptoalbumin [175]. There is high level of amino acid sequence homology of albumin among the species studied so far and the highest sequence homology exist between HSA and BSA (76%) [175].

The albumin gene is located on chromosome 4, near the centromere, at position 4q11-13 and spans 16,961 nucleotides from the putative “Cap” site to the first poly (A) tail [179]. It is split into 15 exons by 14 intervening sequences which are symmetrically placed within the three domains [180]. The mRNA encodes a preproalbumin (609 amino acids residues) which on cleavage of the signal peptide and propeptide of 18 and 6 amino acid residues, respectively yields the mature monomeric protein of 585 amino acid residues [179]. Albumin together with α-albumins (afamin), α-fetoproteins, and Vitamin D-binding proteins belong to the albumin super family [181]. Albumin does not however share immunological properties with the latter two proteins that have interactions with the major histocompatibility complex (MHC) molecules [175].

1.3.1 Functions of HSA

HSA’s multifunctional property arises from its enormous capability to bind to various ligands of both endogenous and exogenous sources in circulation [174, 175]. This perhaps, has made HSA to be one of the most studied protein. Apart from being the principal carrier of fatty acids in circulation, it is also a carrier for bilirubin, hemin, hematin, hormones (thyroxine, progesterone, testosterone, and aldosterone), cholesterol, eicosanoids, bile acids, NO, and tryptophan [174]. It also form adducts with cysteine, homocysteine, reduced glutathione, pyridoxyl phosphate, and various metals (Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Ag^{2+}, and Au^{+}, etc.,) [175]. HSA facilitates the transport of most of
these ligands in circulation since they are mainly hydrophobic molecules. HSA also contribute to 80% to colloid osmotic pressure [174] and clinically it has been employed as a plasma expander as well as part of hemodialysis regimens [177]. Recently, HSA has also been shown to be involved in buffering blood pH [182]. By virtue of its transport capability, it thus forms a significant reservoir for some molecules such as NO [183], as a detoxifying agent for the case of bilirubin [184], and as an antioxidant [185] (binds free radical scavengers, sequester pro-oxidant metal ions, and PUFAs binding are protected from peroxidation etc.). Furthermore, protein ligands also interact with the HSA such as MHC-related Fc receptor for immunoglobin G (FcRn) [186] and albondin (gp60) [187]. HSA has also been shown to possess esterase and enolase activities for certain ligands that might be useful in the activation of prodrugs [188]. In disease states such as diabetes mellitus, non-enzymatic glycation of HSA convert HSA to deleterious advanced glycation end products (AGE) [189]. In other cases such as liver damage, patients exhibit reduced serum albumin levels while microalbuminuria might be indicative of renal failure [177].

Exogenous ligands that bind to HSA include various drugs mainly in their anionic forms [175]. HSA binding to drugs in circulation aids in distribution of the drugs but at the same time the binding affinity determines the amounts of the free fraction of the drugs which is responsible for therapeutic action of the drug [190]. Thus study of HSA-drug interactions has been essential tasks in the pharmaceutical industry in determining therapeutic doses of various drugs. The most widely used drugs with known interactions with HSA include warfarin, digitoxin, diazepam, ibuprofen, anti-retroviral drugs, and various anti-diabetic drugs among others [174, 175, 190].
1.3.2 FFA Binding to HSA

A major hydrophobic ligand of HSA is FFA, and HSA is well known for its ability to bind many FFAs of different chain length at multiple sites [191, 192]. HSA can bind up to 11 molecules of FFAs with binding affinities (Kd) ranges from $10^{-8}$ to $10^{-6}$ M [16, 174, 175]. Under normal circumstances, HSA carries one or two FFAs [174]. Figure 1.3 depicts a crystal structure of HSA loaded with palmitate with numbers 1 to 7 representing seven binding sites of fatty acids but not based on their strength of binding to HSA. Recent studies using X-ray crystallography [193] and NMR spectroscopy [194] has shown that fatty acid binding sites 4 and 5 within domain III represent 2 of the three high affinity fatty acid binding sites on HSA (the third is in site 2 in domain II). SCFAs and MCFAs have been determined to bind to several other sites than LCFAs [192]. From several of these structural studies, it is also known that FFAs bind HSA in their anionic forms [195] and that the carboxyl groups are largely stabilized in the hydrophobic cavities by interacting with positively charged amino acids, arginine and lysine, usually in combination with polar amino acids such as serine and tyrosine, in order to establish bond with the carboxylate moiety [16]. This stabilization is the case for fatty acid binding sites 1-5 but sites 6 and 7 do not involve these binding interactions, and are therefore thought to be low affinity sites [196, 197]. Although the binding pockets seem to be adapted to bind fatty acids, fatty acid site 7 however overlaps with drug site I, while fatty acid sites 3 and 4 overlap with drug site II [190] (Refer to Figure 1.3). Indeed, studies have shown that fatty acids are effective allosteric modulators of various ligands binding to drug sites I and II [197]. Fatty acid binding alone to HSA is also reported to cause conformational changes on HSA [16, 192].
1.3.3 Steroid Binding to HSA

Previous studies have also shown that steroids such as progesterone, bind to HSA with moderate binding affinity and that fatty acids can displace up to 50% of steroids bound to HSA [178, 198], indicating that steroids such as cholesterol are likely to bind in the IIA and IIIA subdomain, that are thought to harbor high affinity sites for fatty acids [194]. Indeed, previous studies have shown that HSA mediates cholesterol efflux from cultured human fibroblasts and endothelial cells [21, 199-201]. Also, an animal study in rats showed that serum albumin played a role in cholesterol transport in circulation and estimated approximately 24% of the non-esterified cholesterol was transported by albumin [202]. Recently Peng et al. (2008) studied the cholesterol binding on HSA and inferred that the binding site for cholesterol on HSA is located in sub-domain IIA due to the observed tryptophan fluorescent quenching and HSA’s conformational alterations [203]. Other cholesterol derivatives that have been shown to bind to HSA are estradiol, testosterone [204], aldosterone [205], and bile acids [206]. Fusidic acid, a steroidal antibiotic has also been shown to bind to HSA [207].

1.3.4 Other Ligand Binding to HSA

Sudlow et al. (1975) first characterized two primary drug binding sites namely, drug sites I and II [208] which are now known to be in subdomains IIA and IIIA [175], respectively (Refer to Figure 1.3). Site I is bound preferentially by large heterocyclic compounds possessing negative charge and the only tryptophan (W214) in the HSA primary sequence is at this site, while site II is bound by small aromatic carboxylic acids [175]. Warfarin and azaproprazone are examples of drugs that bind to site I, while diazepam and ibuprofen bind to site II [190]. Since these drug sites overlap with some of
the endogenous ligands such as fatty acids, bilirubin, and thyroxine [188, 196], physiological changes that alters plasma HSA levels or structural changes that affect affinity (diseases or HSA polymorphisms), competition by endogenous ligands, and other drugs could influence the pharmacokinetics of the drugs and their dosages.

Apart from drugs, metal ions also bind to HSA at mainly the N terminal portion (N-Asp-Ala-His-Lys-) and Cys-34 of HSA [175]. Cu$^{2+}$, Ni$^{2+}$ and Co$^{2+}$ bind with high affinity to the N terminal portion but Zn$^{2+}$ and Ca$^{2+}$ have weaker affinity at this site [177]. Dogs are particularly sensitive to copper and it is thought that this might be due to the lack of His-3 in their N terminal portion of HSA lowering copper binding affinity to albumin [174, 175]. Au$^+$, Ag$^{2+}$, and Hg$^{2+}$ bind to the Cys-34 in subdomain IA and also NO, homocysteine, cysteine, reduced glutathione, and some drugs occur as metabolic adducts at this site [188].

1.3.5 Mutations and Polymorphisms of HSA

Single nucleotide polymorphism (SNP) of HSA less than 100 has been identified with unknown clinical or biological effects [179]. Additionally, 65 mutations causing bisalbuminemia (presence of two circulating forms of HSA) and 49 cases of analbuminemia (absence of the protein) have been reported and updated in the albumin website repository [209]. Many of the bisalbuminemic variants were detected by anomalous electrophoretic migration pattern due to a charge difference [175]. Thus, those mutations involving uncharged and hydrophobic amino acids positioned in the interior of the protein might remain undetected [179]. The cumulative frequency of HSA variants in most population is only about 1 in 3000 and most of the variant HSA carriers are heterozygotes [210]. It important to note that some countries as Italy and Brazil have
reported many of the variants just because they have carried out intensive elecrophoretic surveys, thus existence of possible other variants in other countries are unknown. However, the albumin variants Naskapi and Yanomama have a high polymorphic frequency of \( \geq 1\% \) among the American Indians [211].

The impact of mutations on ligand binding by HSA variants has not generally been linked to any clinical condition [179]. However three mutations namely, Leu66Pro cause familial dysalbuminemic hypertriiodothyroninemia [212], and Arg218His or Arg218Pro cause dysalbuminemic hyperthyroxinemia [213]. These mutations forms strong binding sites for triidothryonine (T3) or thyroxine (T4), which can be identified by the increases in total T3 or T4 in serum of the patients. High fatty acid binding to genetic HSA variants has been observed and is summarized in Table 1.6. Other mutations had no effect on fatty acid binding especially those mutations on the surface of the protein and few variants had reductions on binding [179, 214].

Analbuminemia, although rare, is a condition that occurs in homozygotes [179]. The clinical symptoms include low blood pressure and low albumin levels [209]. A compensatory mechanism for the lack of albumin is through increases in serum globulin levels. Most of the analbuminemic subjects also have edema, hyperlipididemia, and show fatigue but it is still a well tolerated condition [209]. However, there have been reports of several deaths in analbuminemic neonates [215].

### 1.3.6 Site-Directed Mutagenesis and Protein Expression System of HSA

The determination of the crystal structure of HSA has led to a vast interest in examining the protein’s interactions with ligands by using genetic engineering techniques. Previous studies mainly utilized chemical modifications, spectroscopic, and ligand analog
to study ligand binding with HSA [174]. However, they have been largely unsuccessful in providing specific structural and mechanistic information. Since many of the ligands display non-specific binding on HSA, proteolysis was employed to generate HSA fragments [216]. As a similar approach, recombinant HSA fragments expressed in yeast has now been used to study specific binding sites or structural analysis using NMR [196]. However, the authenticity of conclusions drawn from studies using such fragments have been questionable due to issues on the integrity of the protein.

In the contrary, site-directed mutagenesis and a novel protein expression system has a great advantage in allowing use of recombinant protein mutants to examine specific amino acids involved in ligand binding. This has been a success in probing important ligands sites of thyroxine [217], fatty acids [193, 218], bilirubin [219], warfarin [220], and the esterase activity [221] of HSA. The protein expression system enables production of recombinant HSA with no chemical or enzymatic modification and since the procedure does not involve extraneous conditions, the folded protein is likely to be in a native conformation. The properties of recombinant HSA from Pichia pastoris have been well studied and shown to be identical structurally and functionally with commercial HSA [222]. Since most carriers of HSA variants are heterozygotes, isolation of a homogenous HSA mutant is a challenge, thus the expression of HSA mutants enables the preparation of a homogenous protein simulating SNP. Therefore, recombinant HSA provides promising applications [223] e.g., as stabilizing agent in vaccines, in various drug formulations, and as a blood expander from the conventional preparation from pooled human plasma which usually involve expensive and strenuous screening procedures with a possibility of retention of viral/prion contaminants.
Figure 1.3. X-ray Crystallographic Structure of HSA Complexed to Palmitate

Domains are numbered I, II, and III and the subdomains A and B are shown in dark and light shades, respectively. Bound fatty acids are shown in sites numbered 1 to 7 (carbon, grey, oxygen, red).

Source: [192].
Table 1.6. HSA Polymorphisms Associated with Increased Fatty Acid Binding

Subtract 24 amino acid numbers (18 of signal peptide and 6 of propeptide) to convert to starting at the Asp-1 of mature albumin. Source: [179, 209].

<table>
<thead>
<tr>
<th>Mutation Name</th>
<th>Protein Change</th>
<th>Protein Function Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lille</td>
<td>Arg23His</td>
<td>High fatty acid binding</td>
<td>[224]</td>
</tr>
<tr>
<td>Brest</td>
<td>Asp338Val</td>
<td>High fatty acid binding</td>
<td>[225]</td>
</tr>
<tr>
<td>Redhill</td>
<td>Arg23Cys; Ala344Thr</td>
<td>High fatty acid binding; N-glycosylated at Asn342</td>
<td>[226-229]</td>
</tr>
<tr>
<td>Roma</td>
<td>Glu345Lys</td>
<td>High fatty acid binding</td>
<td>[224]</td>
</tr>
<tr>
<td>Casebrook</td>
<td>Asp518Asn</td>
<td>N-glycosylated at Asn518; high fatty acid binding</td>
<td>[228, 230]</td>
</tr>
<tr>
<td>Makes(Wapishana)</td>
<td>Lys565Glu</td>
<td>High fatty acid binding</td>
<td>[211, 228, 231]</td>
</tr>
<tr>
<td>Fukuoka-1</td>
<td>Asp587Asn</td>
<td>High fatty acid binding</td>
<td>[228, 232]</td>
</tr>
<tr>
<td>Bazzano</td>
<td>Cys591AlafsX17</td>
<td>High fatty acid binding</td>
<td>[210, 228]</td>
</tr>
<tr>
<td>Rugby Park</td>
<td>Gly596. Leu609delins7</td>
<td>High fatty Acid biding</td>
<td>[228, 233]</td>
</tr>
</tbody>
</table>
1.4 Cholesterol

Cholesterol is an important class of lipid that is an essential constituent of mammalian cell membranes and a precursor for the biosynthesis of bile acids, steroid hormones, and fat-soluble vitamins. The major site of endogenous synthesis of cholesterol is in the liver but some can be derived from animal-based dietary sources. Apart from utilization of cholesterol by hepatic cells for membrane incorporation and bile acid synthesis, the liver also secretes cholesterol together with TAGs as VLDL into circulation that is further metabolized to LDL-cholesterol and is taken up by peripheral cells through the LDL-R [234]. Most of the peripheral tissues excluding steroidogenic organs and the liver cannot catabolize excess cholesterol [235] and so in order to maintain cellular cholesterol homeostasis, the excess cholesterol in these peripheral tissues must be delivered back to the liver constituting cholesterol efflux [236].

*In vitro* studies have shown that the transcription factor, SREBP in the ER sense the level of intracellular cholesterol [237]. When cellular cholesterol levels are low, the protein Insig1 dissociates from the SREBP/SREBP-cleavage activating protein (SCAP) complex in the ER membrane, and this leads to the migration of the complex to the Golgi. In the Golgi, the complex is cleaved by site 1 protease (SIP) and S2P and is activated by SCAP [238]. SREBP then migrates into the nucleus and binds to the DNA sequence, the sterol regulatory element (SRE) responsible for the expression of LDL-R as well 3-hydroxy-3 methylglutaryl-CoA (HMG-CoA) reductase and HMG-CoA synthase. HMG-CoA reductase catalyze the rate limiting step in cholesterol biosynthesis which converts HMG-CoA to mevalonate [239]. In this way, the cells maintains cholesterol homeostasis by synthesizing cholesterol and also increasing internalization of LDL.
through the increased number of cell surface LDL-R, thus lowering plasma cholesterol levels.

1.4.1 Pathways of Cholesterol Efflux

Various specific cholesterol efflux pathways have been proposed [235, 236, 240] as summarized in Figure 1.4. It is well known that apo AI-mediated cholesterol efflux is the major cholesterol efflux system in the body [20]. Apo AI is the principal protein of HDL and is responsible for the formation of HDL particles in the blood through cholesterol efflux [241]. HDL is secreted by the liver or intestine in nascent discoidal shape but also formed during lipolysis of chylomicrons and VLDL in circulation. On lipidation of the nascent HDL with free cholesterol effluxed from peripheral cells mediated by ATP-binding cassette transporter AI (ABCA1) [242], cholesterol are esterified to cholesterol ester by lecithin: cholesterol acyltransferase (LCAT) [243], enabling the cholesterol esters to be sequestered into the inner core of HDL. Cholesterol esters and cholesterol in the mature HDL is then delivered to the liver via two paths. First, the mature HDL can be taken up through the hepatic scavenger receptor class-B type I (SR-BI) in the liver or through action of hepatic lipases releasing cholesterol on metabolism [244]. Secondly, cholesterol ester in mature HDL can be transferred from HDL to the apoB containing lipoproteins, VLDL or LDL in exchange of TAGs by the action of cholesterylester transfer protein (CETP). Remarkably, patients with CETP deficiency have increased levels of HDL cholesterol but it has been controversial on the net beneficial effect on cholesterol efflux [245]. The hepatic LDL-R or LRP uptake pathway of VLDL and LDL is believed to be more efficient in delivering the excess cholesterol esters back to the liver from the apoB-lipoproteins than in HDL [246]. SR-BI [247] and ABCG1 [248] have been
also shown to mediate efflux of free cholesterol from peripheral cells directly to mature HDL whose fate follows a similar path as described above. Cholesterol in the liver is then secreted into bile through the ABCG5 and ABCG8 transporters [249] and some are reabsorbed in the intestines back to the liver and the rest excreted. It will be interesting to know the findings of ongoing studies on the contribution of transintestinal cholesterol efflux [250] that involves intestinal excretion of cholesterol bypassing the liver.

Aqueous diffusion mechanisms of efflux of free cholesterol from peripheral cells to various cholesterol acceptors do also exist [240]. Free cholesterol can desorb from cell surfaces and bind to HDL depending on cholesterol concentration gradient without facilitation by any membrane protein [240, 251]. HSA has also been shown to mediate this aqueous diffusion-mediated efflux of free cholesterol for delivery to the liver for metabolism [21, 201, 252].

1.4.2 Cholesterol Efflux and Athero-protection

Atherosclerosis is a degenerative disease that is characterized by vascular endothelium dysfunction and impediment in normal arterial blood flow [253]. Atherogenesis is a complex process but it is believed to begin in the endothelium from a response to an injury caused by factors such as hypertension and oxidized-LDL (Ox-LDL) [19, 253]. For instance, Ox-LDL in the arterial intima attracts cells of the immune system such as macrophages which internalize the oxidized lipids and accumulate cholesterol esters, converting the macrophages into “foam cells”. A series of complex degeneration of the endothelium ensues, due to immune aggregation of platelets, proliferation of the macrophage ‘foam cells’ and arterial smooth muscles. Eventually the
proliferation causes atheromatous plaques in the arterial lumen restricting normal blood flow that can lead to ischemia of the heart and brain [253].

The inverse relationship between plasma HDL levels and the risk for coronary heart disease (CHD) [254-257] has led to the explanation that HDL-mediated cholesterol efflux is protective against the development of CHD [258]. Since the cholesterol loaded macrophage foam cells are the cellular hallmark of the atherosclerotic lesion, cholesterol efflux from the intimal foam cells may protect against the further progression of the vascular endothelium damage [235, 259]. HDL may also provide protection against CHD by inhibiting the formation of platelet aggregates [260] at sites of endothelial injury and inhibiting the production of monocyte adhesion molecules [261]. Additionally, apo AI protein of HDL possess anti-oxidant properties [262, 263] which prevents formation of Ox-LDL or neutralizes its effects [264]. PUFAs are more prone to lipid peroxidation and so LDL over enriched with PUFAs might be more atherogenic than MUFAs [265]. These findings have led to HDL being referred to as the “good” cholesterol and LDL as the “bad” cholesterol.

Other studies that have supported the importance of cholesterol efflux include the discovery that the rare familial disease, Tangier disease [266], is caused by mutations in ABCA1 transporters. The individuals with this disease have very low levels of plasma HDL [267], leading to intracellular accumulation of cholesterol esters that increases their risk of developing atherosclerotic vascular disease [264]. ABCA1 expression has been shown to be induced by the nuclear receptors, liver X receptors (α and β), via binding retinoid X receptor [268]. Potent ligands of LXR is oxysterols and 9-cis retinoic acid for
RXR. Induction of ABCA1 expression has also been shown in macrophages to be cAMP dependent [269].

On the other hand, genetic defects in LDL-R have been linked to reduced LDL clearance and classified as familial hypercholesterolemia (FH) [270]. The HMG-CoA reductase inhibitors, statins are used [271] as LDL lowering drugs, by inhibiting cholesterol synthesis and upregulating LDL-R expression. However, patients who are homozygous FH do not respond to statin therapy [272]. Furthermore, statin therapy reduces CVD events only by about a third [259]. Moreover, other available lipid-lowering drugs (such as nicotinic acid, bile acid sequestrants, fibrates, estrogens, and plant sterols) have notable side effects that are not well tolerated by most patients [271]. Therefore, there is growing interests in targeting cholesterol efflux for drug developments [273].

Many epidemiological studies have also shown that high levels of HSA are associated with a reduced risk for CHD and mortality from CHD [274-281]. Although there is no definitive explanation for this observation, HSA being the most abundant protein in blood might contribute to this athero-protection through its notable role in cholesterol efflux. Other HSA’s anti-atherogenic effects might be due to its potential role as an antioxidant in circulation [185]. Also, since obesity is a major risk factor for the development of CVD, the high plasma FFAs in most of these subjects might alter their HSA’s efficiency in cholesterol efflux, which might be modulated in the presence of HSA variants [21]. Thus studies elaborating on the effects of free fatty acids on HSA-mediated cholesterol efflux are important in understanding the high risk to developing atherosclerosis among obese subjects.
Figure 1.4. Pathways of Cholesterol Efflux

Modified from Rader et al. (2009) [235]. Lipid poor apolipoprotein-AI (apo-AI) secreted by the liver acquires free cholesterol (FC) from macrophages via ABC transporter A1 (ABCA1). Mature high density lipoprotein (HDL) is formed by the action of lecithin: cholesterol acyltransferase (LCAT) and promotes efflux from macrophages via the ABC transporter G1 (ABCG1) as well as other peripheral tissues by aqueous diffusion (AD), and scavenger receptor BI (SR-BI)-mediated effluxes. Human serum albumin (HSA) also can mediate efflux through aqueous diffusion. FC is thus transported to the liver mainly for excretion. BA bile acid, CE cholesterol ester, CETP cholesterol ester transfer protein, FFA free fatty acid, HL hepatic lipase, LDR-R low density lipoprotein-receptor, LRP LDL-R related protein, TG triacylglycerols.
CHAPTER 2. EFFECTS OF HSA COMPLEXED WITH FFA ON β-CELLS

2.1 Introduction

Previous studies have shown that elevated levels of plasma free fatty acids in obese subjects are associated with insulin resistance and pancreatic β-cell dysfunctions [19, 282]. Furthermore, there is compelling evidence that chronic exposure of β-cells to FFAs is associated with cytotoxicity [116], particularly with long-chain saturated FFAs compared to mono-unsaturated FFAs. In fact, some studies have further documented that unsaturated FFAs can be cytoprotective against palmitate-induced β-cell death [118]. However, molecular mechanisms of how chronic FFA exposures induce β-cell death or the cytoprotective mechanisms by unsaturated FFAs against lipotoxicity are not yet clearly established. Recent studies suggest that accumulation of LC acyl-CoA in the cytosol activates cell-death signals by generation of NO [139] and ceramide [150] as well as by mitochondrial perturbations [69]. Lately, involvement of endoplasmic reticulum (ER) stress in induction of apoptosis on exposure of β-cells to palmitate has also attracted attention. [164, 283].

Therefore, transport of FFAs across the plasma membrane plays an important role in modulating FFA-induced cellular metabolism. In β-cells, concentration gradients across the membrane are believed to be a major contributing factor for this FFA transport [63]. The free fraction of FFAs, unbound FFAs (FFAu) in the extracellular space, which is available for this transmembrane transport, is greatly influenced by the extent of binding to their transport protein, HSA. HSA is the principal carrier of FFAs in serum; it can bind with up to 11 FFA molecules, depending on the chain length with binding affinity ranging from $10^{-6}$ to $10^{-8}$ M [16, 174, 175]. Under normal physiological conditions, an
average of two FFA molecules are bound to HSA in circulation, but the molar ratio of HSA to FFA can rise to up to 1:6 in disease states such as diabetes mellitus [33]. Although the role of HSA as the principal FFA carrier in the blood has been clearly shown, its role in FFA effects on β-cells has not been studied in depth. Furthermore, previous studies used BSA to measure FFA effects on β-cells. Although HSA and BSA have a 76% sequence homology, they differ in their numbers of FFA-binding sites and binding affinities [18, 284] as well as in their three-dimensional structures [285]. Therefore, use of HSA instead of BSA is relevant in studying the effects of FFA on β-cells.

2.2 Aims of Study

In this study, we used a well-established insulinoma β-cell line, HIT-T15 (Syrian golden hamster pancreatic β-cell line) to:

(a) Determine the effects of different types of FFAs (myristate, palmitate, palmitoleate, oleate, stearate, and elaidate) in the presence of HSA on β-cell viability.

(b) Determine the mechanisms involved in palmitate and stearate-induced β-cell toxicity by using two typical apoptosis inhibitors, cyclosporin A (csA) and the general caspase inhibitor ZVAD-FMK in the presence of HSA and/or BSA.

(c) Determine the effects of different unsaturated fatty acids (palmitoleate, oleate, elaidate, linoleate, linoelaidate, and CLA) in combination with cell-death inducing concentrations of palmitate on β-cell viability in the presence of HSA.

(d) Determine the effects of 24 hours exposure to different molar ratios of HSA/oleate complexes on insulin secretion from β-cells.
2.3 Materials and Methods

2.3.1 Materials

HIT-T15 cell line, horse serum, fetal bovine serum (FBS), trypsin/EDTA, Kaighn’s modification of Ham’s medium (F12-K medium), and MTT \{(3,4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide\} cell proliferation assay kit were obtained from American Type Culture Collection (Manassas, VA). Tissue culture flasks and 96 well plates were purchased from BD Falcon (Franklin Lakes, NJ). Fatty acid free HSA, fatty acid free BSA, FFAs (palmitate, stearate, oleate, elaidate, myristate, linoleate, and palmitoleate), bicinchoninic acid assay kit (BCA), and csA were purchased from Sigma-Aldrich (St. Louis, MO). Conjugated linoleic acid (CLA) and linoelaidate was purchased from Cayman Chemical (Ann Arbor, MI). Antibiotic-antimycotic, and phosphate buffered saline (PBS) were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY). The non-esterified fatty acid kits (WAKO C and WAKO HR series) were supplied by the WAKO chemicals (Richmond, LA). Rat insulin ELISA kit was supplied by Crystal Chem Inc. (Downers Grove, IL). The general caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl) fluoromethylketone (ZVAD-FMK) was purchased from Promega Corporation (Madison, WI). Staurosporine was purchased from EMD Chemicals (Gibbstown, NJ). Apoptotic DNA Laddering kit was purchased from Trevigen Inc. (Gaithersburg, MD). All other chemicals used were of analytical grade.

2.3.2 Cell Culture

HIT-T15 cells was cultured in 87.5% F12-K medium containing 7 mM glucose, 2 mM L-glutamine, 1500mg/L sodium bicarbonate and supplemented with 10% horse serum (v/v), 2.5% FBS (v/v), 1% antimycotic-antibiotic (v/v), at 37°C with 5% CO₂ and
95% air. Cells were grown and maintained in T75 cm$^2$ flasks and subcultured for individual experiments in collagen-coated 96-well culture plates until about 80% confluency.

2.3.3 Treatment of Cells with FFAs, csA, ZVAD-FMK, and Staurosporine

Stock solutions of FFAs were prepared by serial dilutions in 10% methanol in accordance with a previous study by Ha et al. 2006 [286]. Stock solution of palmitate and stearate were prepared at 70°C, myristate at 62°C, elaidate at 50°C, oleate at 37°C and palmitoleate, CLA, linoleate, and linoelaidate at 25°C. Proper amounts of stock solution of FFAs and HSA were dissolved in serum free-F12 K medium to give FFA working concentrations of 0.1mM-0.8mM and HSA concentration of 0.1mM. In some instances, palmitate at 0.4mM was mixed with either of the individual unsaturated FFAs at also the same concentration in the presence of 0.1mM HSA. The mixtures were rotated overnight to ensure fatty acids were completely dissolved. Final concentration of methanol in working HSA/FFA solutions did not exceed 0.05% methanol. Confluent cells were treated with the working solutions and control cells received 0.1mM HSA and 0.05% methanol. In experiments for testing apoptosis inhibitors, cells were treated with the general caspase inhibitor (ZVAD-FMK) and csA at final concentrations of 50μM and 5μM, respectively in serum free-F12-K medium in presence of 0.4mM palmitate and or stearate complexed to 0.1mM HSA and/or BSA as described previously for 24 hours [87, 287, 288]. csA and ZVAD-FMK were dissolved in 100% DMSO with final DMSO concentration not exceeding 0.05% and 0.25% in the working solutions, respectively. Another set of cells were also treated with the apoptotic inducer, staurosporine dissolved in serum free medium containing HSA and/or BSA in the absence or presence of csA and
ZVAD-FMK at a final concentration of 2µM. Untreated control cells received 0.1mM HSA and/or BSA alone or in the presence of 5µM csA or 50µM ZVAD-FMK.

2.3.4 Measurement of Cell Viability

HIT-T15 cells were treated with the FFAs, test compounds and control media as described above. After the 24 hour incubation, cell viability assay was determined using MTT cell proliferation assay kit according to the manufacturer’s instruction with slight modification. Briefly, 20µl of MTT dye was added to the cells and the plates were incubated for 4 hours at 37°C. The medium was then aspirated and the cells were lysed with 100µl of the solubilization reagent. The absorbance was determined at 570 nm after overnight incubation in the dark at room temperature with gentle shaking. The MTT assay is widely accepted way to examine cell proliferation based on the yellow tetrazolium MTT being reduced by viable cells to an intracellular formazan that can be solubilized and quantified colorimetrically. All spectrophotometric readings were made using a microplate reader (Bio-Rad Laboratories).

2.3.5 Assay of Apoptotic DNA Fragmentation

Detection of DNA fragmentation was assayed according to the manufacturer’s apoptotic DNA laddering kit. Briefly, after treatment incubation, cells were lysed and DNA isolated. Agarose gel electrophoresis was performed in 1.5% agarose gel to separate DNA fragments. The gel was then stained with ethidium bromide (0.5 µg/ml) and the DNA bands visualized under UV illumination and photographed.

2.3.6 Determination of Insulin Secretion

Insulin secretion was determined by using an ELISA kit according to manufacturer’s manual. After the 24 hours incubation in appropriate treatment media, cell culture media
were collected for insulin ELISA assay. Cells were washed twice with 1X PBS and then lysed with 0.1N NaOH and cell protein was quantified by BCA assay kit. Values of secreted insulin measured in ng was converted to ng per mg cell protein and expressed as percent of insulin secretion mediated by HSA alone (set as 100%).

2.3.7 Analysis of Data

Statistical differences between results were analyzed by two-tailed unpaired student’s t-test or one way ANOVA and a P value of less than 0.05 was considered significant.

2.4 Results

2.4.1 Differential Effects of FFAs Bound to HSA on β-cell Viability

HIT-T15 cells were treated with different types and amounts of FFAs mixed with HSA for 24 hours and the cell viability was determined as described in methods. Two saturated FFAs, palmitate (C16:0) and stearate (C 18:0), showed significantly lowered cell viability with increasing FFA concentrations. Palmitate showed 12.2%, 26.4%, 40.9%, and 49.5% decreases in cell viability at HSA/FFA molar ratios of 1:1, 1:2, 1:4, and 1:8, respectively (Fig. 2.1) and stearate caused 17.5%, 30.0%, 43.7%, and 46.4% decreases at the same HSA/FFA molar ratios compared to the control group, which was treated with HSA only (P < 0.05), (Fig. 2.1). However, myristate (C14:0), a saturated FFA, showed no significant changes (less than 5%) in cell viability compared to the control at the same HSA/FFA molar ratios (Fig. 2.1).

Elaidate (C18:1), a trans-monounsaturated FFA, showed minimal effects on cell viability (1.9%, 7.7%, 5.0%, and 1.0% decreases at HSA/FFA molar ratios of 1:1, 1:2, 1:4, and 1:8 respectively), (Fig. 2.1), and palmitoleate (C16:1), a cis-monounsaturated FFA, also showed similar results as shown in Fig. 2.1 of 2.0%, 5.6%, 7.2%, and 5.1%
decreases compared to the control, respectively (statistically not significant). Also, the effects of the monounsaturated, oleate on cell viability were considered as minimal at particularly HSA/FFA molar ratios of 1:4 and 1:8 (8.9% and 5.7% decreases, respectively, \( P< 0.05 \) vs. control) when compared to its saturated counterpart, stearate at similar molar ratios (Fig. 2.1).

### 2.4.2 Effects of csA and ZVAD-FMK on FFA-induced \( \beta \)-cell Death

We further examined whether the observed effects of palmitate and stearate complexed with HSA on the cell viability were due to apoptosis or not, by using two apoptosis inhibitors, csA and ZVAD-FMK. Additionally, we conducted a comparative study of these apoptosis inhibitors with FFAs in the presence of HSA and BSA. We used a FFA concentration of 0.4mM mixed with 0.1mM HSA and/or BSA as the treated control, in the presence of the apoptosis inhibitors as experimental variables. After the experiment was conducted, we assayed the cell viability. Treatments with FFAs and apoptosis inhibitors in the presence of HSA versus BSA did not show any significant differences on cell viability changes (Fig. 2.2). As a positive control, cells were treated with the classical apoptotic inducer, staurosperine, which showed significant decreases in cell viability versus untreated control (HSA only), by 40.1% and 36.2% decreases in the presence of HSA or BSA, respectively. This staurosperine-induced cell death was neither prevented by csA nor by the caspase inhibitor, ZVAD-FMK. Cells treated with the two FFAs and csA did not show significant changes on cell viability when compared to cells treated with only the two FFAs, palmitate and stearate (7.4% and 6.2% decreases in cell viability, respectively in the presence of HSA, and 3.1% and 4.6% decreases for BSA, respectively). In contrast, ZVAD-FMK co-treated with FFAs and HSA significantly
enhanced cell viability when compared to the treated controls of palmitate or stearate with HSA, by 16.7% and 13.2% increases, respectively. For the case of BSA, ZVAD-FMK only led to significant 13.2% increase in cell viability in the presence of palmitate when compared to treated control while the 8.5% increase in the presence of stearate was not statistically significant.

2.4.3 Apoptosis is Involved in FFA-induced β-cell Death

To further confirm the possible role of apoptosis in FFA-induced β-cell death, we carried out apoptotic DNA laddering experiments. DNA laddering is indicative of a hallmark of apoptosis. A comparison was also made between the presence of HSA versus BSA in the absence or presence of the various apoptotic inhibitors. We observed no detectable DNA laddering in control cells (Fig. 2.3; lanes C1-C6) that contained either HSA or BSA only (C1 and C2, respectively), or in the presence of csA (C3 for csA and HSA, and C4 for csA and BSA), and or ZVAD-FMK (C5 for ZVAD-FMK and HSA, and C6 for ZVAD-FMK and BSA). As a positive control, the classical apoptotic inducer, staurosporine showed a smear indicative of extensive DNA degradation in the presence of HSA or BSA (C7 for staurosporine and C8 for staurosporine and BSA, respectively). There was faint DNA band smear in the presence of HSA/palmitate (P1) while BSA/palmitate (P2), HSA/palmitate + csA (P3) and, BSA/palmitate plus csA (P4) had observable DNA laddering. A similar result was seen in stearate treatments (S1-S4), except that HSA/stearate (S1) had elaborate DNA laddering in comparison to HSA/palmitate (P1). In the presence of ZVAD-FMK in both the palmitate (P5 and P6) and stearate (S5 and S6) treated cells, there was no observable DNA laddering which was
a similar result as to that exhibited by the control cells treated with either HSA or BSA in the presence of ZVAD-FMK only (C5 and C6, respectively).

**2.4.4 Oleate and Linoleate are Cytoprotective Against Palmitate-induced Cell Death**

In an attempt to simulate physiological conditions where FFAs usually exist as mixtures, we examined the effects of palmitate alone or in the presence of individual unsaturated FFAs (palmitoleate, oleate, linoleate, linoelaidate, or CLA) on β-cell viability. The fatty acid concentrations used was at 0.4mM since at this concentration, palmitate had a notable cell-death inducing effect and thus all other fatty acids were also tested at the same concentration and all mixed with 0.1mM HSA. As shown in figure 2.4, palmitate and linoelaidate caused significant reductions on cell viability by 27.1% and 7.5%, respectively when compared to HSA only treated control cells ($P < 0.05$). However, palmitoleate, elaidate, and CLA had no significant changes while oleate and linoleate when compared to the control caused increases on cell viability by 21.6% and 24.9 %, respectively. The cell-death induced by palmitate was completely negated by the presence of oleate or linoleate when compared to the HSA/palmitate treated cells. Interestingly, HSA/palmitoleate or elaidate mixtures when either was in combination with palmitate caused further reductions on cell viability from the HSA/palmitate treated control cells (8.4% and 15.2% decreases, respectively).

**2.4.5 24-hour Exposure of Cells to Oleate Decreases Insulin Secretion**

Long term (24 hours or longer) exposure of β-cells to fatty acids has been shown to cause reduced insulin secretion [289]. However, most studies used mainly BSA/FFA complexes, so we sought to determine HSA’s effect on HIT-T15 cells, using 0.1mM HSA co-treated with different concentrations of oleate (1:1, 1:2, 1:4, and 1:8 HSA/FFA
molar ratios). We chose oleate because it is one of the most abundant FFA in human plasma [34] and also shows minimal cytotoxicity as observed in our cell viability assays. A significant reduction in insulin secretion was observed (Fig. 2.5) with increasing molar ratios of HSA/FFA (8.8%, 45.1%, 39.0%, 37.7% decreases compared to the control treated with HSA only, \( P < 0.05 \)).
Figure 2.1. Effects of HSA/FFA Complexes on β-Cell Viability

Cells were treated with 0.1mM HSA alone or with the various HSA/FFA complexes at various molar ratios (1:1, 1:2, 1:4, and 1:8) for 24 hours. Cell viability was determined by MTT assay as described in methods. Results are expressed as percentage of HSA only (control) treatment set at 100%. The values represent the mean ± S.D of 3 independent experiments performed in duplicate. *P<0.05 vs. control.
Figure 2.2. Effects of Cyclosporin A (csA) and Caspase Inhibitor (ZVAD-FMK) on Palmitate and Stearate-induced β-cell Death

Cells were treated with palmitate or stearate at 1:4 molar ratios of HSA/FFA and or BSA/FFA for 24 hours in the absence and presence of 5μM csA or 50μM ZVAD-FMK. Also, cells were treated in the absence or presence of 2μM staurosporine in the presence of 0.1mM of HSA or BSA. Untreated cells received 0.1mM HSA and/or BSA in the absence and presence of csA or ZVAD-FMK. Cell viability was determined by MTT assay as described in methods. Results are expressed as percentage of untreated control (HSA and/or BSA only) set at 100%. The values represent the mean ± S.D of 3 independent experiments performed in duplicate. *\(P<0.05\) vs. untreated control, **\(P<0.05\) vs. treated control.
Figure 2.3. Apoptotic DNA Laddering Analysis in Ethidium-bromide Stained Agarose Gel After FFA Treatments

HIT-T15 cells were treated for 24 hours in the presence of 0.1mM HSA only (C1), 0.1mM BSA only (C2) or in the presence of 5μM cyclosporin A (C3 for HSA, and C4 for BSA) or with the caspase inhibitor, ZVAD-FMK at 50μM (C5 for HSA, and C6 for BSA). Lanes C7 and C8 represent cells treated with 2μM of staurosporine in the presence of 0.1mM of HSA and/or BSA, respectively. The middle and last panels represent palmitate (0.4mM) and stearate (0.4mM) treated cells, respectively in the absence or presence of various test compounds (P1, S1 HSA/FFA only; P2, S2 BSA/FFA only; P3, S3 HSA/FFA + csA; P4, S4 BSA/FFA + csA; P5, S5 HSA/FFA + ZVAD-FMK; P6, S6 BSA/FFA + ZVAD-FMK). DNA was extracted after treatment incubations and 2 μg DNA was resolved in a 1.5 % agarose gel. Lane M represent 1 Kb DNA marker.
Figure 2.4. Effects of Unsaturated FFAs on Palmitate-induced β-cell Death in the Presence of HSA

Cells were treated with 0.1mM HSA alone or with the various individual unsaturated FFAs at concentrations of 0.4mM with or without 0.4mM palmitate for 24 hours. Cell viability was determined by MTT assay as described in methods. Results are expressed as percentage of HSA only (control) treatment set at 100%. The values represent the mean ± S.D of 3 independent experiments performed in duplicate. *P<0.05 vs. control, **P<0.05 vs. HSA/palmitate.
Figure 2.5. Effects of Different HSA/oleate Molar Ratios on Insulin Secretion

HIT-T15 cells were treated with 0.1mM HSA alone or the various molar ratios of HSA/Oleate complexes (1:1, 1:2, 1:4, and 1:8 molar ratios) for 24 hours. Insulin secretion was quantified by ELISA method and values of insulin secretion were normalized in ng per mg cell protein. Results are expressed as percentage of insulin secreted from cells treated with HSA only (control) set as 100%. The values represent the mean ± S.D of 3 independent experiments performed in duplicate. *P<0.05 vs. control.
2.5 Discussion and Conclusion

Our study investigated the effects of FFAs and HSA complexes on HIT-T15 cell viability by incubating various amounts of FFAs at concentrations ranges of 0.1-0.8 mM in the presence of a fixed concentration of HSA (0.1 mM). The two principal saturated FFAs of human serum, palmitate (C16:0) and stearate (C18:0), induced cell death under all concentrations tested in a dose-dependent manner, while their cis mono-unsaturated counterparts, palmitoleate (C16:1) and oleate (C18:1, the principal FFA of adipocytes and a major mono-unsaturated FFA in human serum), showed minimal changes on cell viability. Myristate (C14:0, a saturated FFA, and elaidate (C18:1, a trans unsaturated counterpart of stearate), had no significant effects on cell viability. These results are in good agreement with earlier studies that showed that long-term exposure to β-cells of saturated FFAs of 16 carbon chain length or greater induce β-cell death while unsaturated FFAs have minimal changes on the cell viability in the presence of BSA [87, 121, 124, 126, 131, 135]. Furthermore, cytoprotection by the cis unsaturated FFAs against palmitate-induced toxicity was recently shown to be more potent than that by trans unsaturated FFAs [290]. In this respect, we treated cells with mixtures of palmitate and individual unsaturated fatty acids and found that the cis FFAs, oleate and the polyunsaturated fatty acid, linoleate were cytoprotective against palmitate-induced cell death (potentially increasing cell proliferation) while their trans counterparts, elaidate and linoelaidate, were not. In fact, linoelaidate caused further slight reduction in cell viability of palmitate treated cells; palmitoleate, a cis FFA elicited similar results. Our results might be suggestive of a cell line-specific response, as also observed by Lai et al. 2008 [164], and the possible deleterious effects might be due to high levels of FFAs.
Recent studies have suggested that the toxic effects of palmitate are due to its lower esterification rates and higher conversion rates to ceramide than those of oleate [138, 291]. Some studies have also proposed that there are differences in the formation of intracellular (TAG) as well as in the cellular phospholipid pool on FFA entry into cells, with saturated FFAs such as palmitate being implicated in more physical cell morphological disruption because of the nature of the resulting TAG species [116, 292]. Although many studies also agree that the net intracellular TAG pool increases after β-cells are exposed to FFAs and resulted in cytotoxicity [131], others have suggested that formation of TAG plays a role in promoting cell survival through the sequestration of potentially toxic fatty acid derivatives [138, 293]. In total, studies on toxic effects of FFAs causing β-cell mass reduction in vitro will remain as an informative model, since high levels of FFAs under both fasting and post-prandial conditions is a characteristic in T2DM, which might be aggravated by hyperglycemia [69].

Of note, apoptosis induced by FFAs has been previously described in β-cells [87, 121, 133, 134, 140] but the mechanistic pathways leading to apoptosis or cell death are not well established. Since most of these studies utilized BSA in the presence of FFAs, our studies provide comparative effects of cell viability changes and apoptosis by FFAs in the presence of BSA and HSA. Induction of apoptosis are believed to be results of mitochondrial perturbations leading to the leakage of caspase and caspase activators into the cytosol, which in turn cause plasma membrane disruption and/or apoptosis [147]. In the event of apoptosis, the mitochondrial permeability transition pore (MPTP) is known to form in the contact site between inner and outer mitochondrial membranes, which facilitates the efflux of pro-apoptotic proteins such as cytochrome c [147, 153]. Some
studies have also indicated that elevated FFAs, particularly palmitate, induced MPTP formation in pancreatic β-cells and cytochrome c release [125, 130, 133, 154]. Release of cytochrome c into the cytosol activates caspase-induced apoptosis. The immunosuppressive drug csA inhibits MPTP formation by binding to cyclophilin protein, a component of the multiprotein pore complex, thereby causing pore closure and preventing the release of pro-apoptotic signals [287, 288, 294].

Our results showed that treatment of HIT-T15 cells with two saturated FFAs, palmitate and stearate bound to HSA or BSA in the presence of csA, did not significantly lower the cell death induced by the FFAs. Cell-death induced by the classical apoptotic inducer staurosporine was also not prevented in the presence of csA. However, we found increases in cell viability in the presence of the caspase inhibitor ZVAD-FMK co-incubated with the fatty acids, although it did not completely block cell death. To further elucidate these observed effects in the presence of apoptosis inhibitors with FFAs, we assayed for apoptotic DNA fragmentation using agarose gel electrophoresis. We observed that csA did not block DNA fragmentation but ZVAD-FMK abolished the DNA fragmentation, thus indicating that other non-caspase-dependent pathways might be responsible for FFA-induced cell death, accounting for the overall cell death. Some earlier systems have also indicated a switch from apoptosis to necrosis by ZVAD-FMK [87] in FFA-treated cells, while other studies are now examining the possible roles of autophagy [295, 296] in cell death or survival in β-cells under FFA exposures. Furthermore, the involvement of ER stress on exposure of β-cells to FFA and its induction of apoptosis [132, 164, 283, 297, 298] have now been revealed as possible pathways that might be parallel to those stemming from mitochondrial dysfunctions.
On the other hand, as previously stated in the results section that oleate proved to be less cytotoxic in comparison to palmitate, and since oleate is one of the most abundant FFAs in human serum, it was pivotal to show the effects of oleate on insulin secretion from β-cells under the HSA/FFA molar ratios used in the viability assays. Our results showed that HSA/oleate complexes in the presence of a 7 mM glucose concentration caused a significant reduction in insulin secretion. Previous studies have shown that long-term exposure to FFAs inhibited glucose-stimulated insulin secretion by lowering the rate of insulin gene expression [84, 292, 299, 300].

In conclusion, our studies showed that various FFAs mixed with HSA have differential effects on cell viability in HIT-T15 cells, which depends on the degree of saturation and chain length of FFAs with unsaturated FFAs eliciting cytoprotective potential to palmitate-induced cell death. Furthermore, we showed that palmitate and stearate had adverse effects on the cell viability that csA and caspase inhibition could not reverse, irrespective of the presence of HSA or BSA. However, caspase inhibition blocked apoptotic DNA fragmentation, thus indicating that FFA-induced β-cell death is not due to a single mechanism of cell death. Insulin secretion was also reduced after 24-hour exposure of β-cells to HSA/oleate complexes. Further studies on the mechanistic details involved especially in the reduction of β-cell mass by FFA, will be beneficial towards a better understanding of the pathogenesis of T2DM and drug innovation for its management and treatment.
CHAPTER 3. EFFECTS OF FFA ON HSA-MEDIATED CHOLESTEROL EFFLUX

3.1 Introduction

Many studies have shown that increased serum FFA levels play a crucial role in the development of T2DM, and are a common phenomenon in obese subjects [13-15]. Since obesity is a major risk factor for CVD and T2DM, much work in this area has mainly concentrated on the FFA effects on the pathogenesis of T2DM. Therefore, in our study we sought to examine the link between the elevated FFA levels and the development of CVD by investigating FFA’s role in modulating cholesterol transport functions of HSA. It is widely known that elevated cholesterol level in the blood is directly related to the incidence of CVD and HDL exerts its main cardio-protective effects through cholesterol efflux from the peripheral tissues to the liver [258]. Also, cholesterol efflux from macrophages has been shown to be effective in lowering foam cell formation and thus preventing atherosclerosis [235].

Apart from the well studied and generally accepted apo AI-mediated cholesterol efflux, previous studies have also shown that HSA effectively mediated cholesterol efflux from cultured cells mainly through aqueous diffusion mechanisms [21, 199-201]. Also, an animal study in rats showed that albumin played a role in cholesterol transport in circulation and estimated approximately 24% of the non-esterified cholesterol was bound to albumin [202]. This HSA-mediated cholesterol efflux mechanism may be quantitatively superior over apo AI-mediated cholesterol efflux since it has a higher capacity for transport due to relatively high concentrations of serum albumin in human plasma (HSA 660μM vs. apo AI 55μM) [21]. Of note, is also the antioxidant potential of
HSA in circulation which could contribute to lowered atherogenic effects of oxidant compounds [185]. Many epidemiological studies have also shown that high plasma levels of HSA are associated with reduced risk for coronary heart disease (CHD) and mortality from CHD [274-277, 280] but definitive biochemical mechanisms has not been elucidated.

With respect on HSA-mediated cholesterol efflux, little is known about the role of FFAs on albumin-mediated cholesterol efflux and relationship between cholesterol and FFA binding to plasma albumin. Previous studies have shown that HSA harbors high affinity sites for FFAs and that steroids might bind at the same sites [193]. Under normal physiological conditions, average number of fatty acids loaded on HSA is up to 2. Therefore, we hypothesized that albumin-mediated cholesterol efflux will be under the influence of fatty acid binding to HSA since fatty acids bind with high affinity (Kd ~10^{-8} M).

3.2 Aims of Study

Since one of the principal cells involved in cholesterol efflux are endothelial cells, we utilized the immortalized human endothelial cell line, EA.hy926 as the model system to study cholesterol efflux. Specifically our aims of study were to:

(a) Examine the effects of different types of FFAs (palmitate, oleate, and arachidonate) on HSA-mediated cholesterol efflux.

(b) Examine the cell viability after cholesterol efflux to the complexes of HSA to the different fatty acids.

EA.hy926 cell-line has been shown to have retained many of the differentiated endothelial properties [301] and also overcomes the shortcomings of primary human vein
endothelial cells (HUVEC) and of freshly isolated normal endothelial cells that tend to lose differentiation characteristics over passages or of limited lifespan.

3.3 Materials and Methods

3.3.1 Materials

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Invitrogen Life Technologies Inc. (Grand Island, NY). Human apo A1 was purchased from Calbiochem (La Jolla, CA). 1-2n-(3H) cholesterol (specific activity 40-60Ci/mmol) was purchased from Perkin Elmer Life Science Research Products (Norwalk, CT). Hypoxanthine-thymidine concentrate and L-glutamine was purchased from ATCC (Manassas, VA). Fatty acid free arachidonic acid was purchased from Sigma-Aldrich (St. Louis, MO). Scintisafe econo F cocktail was purchased from Fisher Scientific (Hampton, NH). Refer to section 2.3.1 for other materials, while all other chemicals used were of analytical grade.

3.3.2 Cell Culture

EA. hy926 endothelial cells, was seeded in 96 collagen-coated wells and maintained in DMEM containing 1mM sodium pyruvate and 5.6 mM glucose. The media was supplemented with 10% (v/v) heat inactivated FBS (thawed at 37°C then incubated at 56°C for 30min), 1% (v/v) antibiotic-antimycotic, 0.1mM sodium hypoxanthine-16μM thymidine, 1% v/v L-Glutamine at 37°C, 5% CO₂ and 95% air. This specific cell line was derived from umbilical vein endothelial cell (HUVEC) by fusion with relatively undifferentiated A549/8 cell line which has been characterized to possess normal endothelial cell properties [301].
3.3.3 Preparation of HSA/FFAs Complexes

Stock solutions of fatty acids were prepared by serial dilutions in 10% methanol in accordance with a previous study by Ha et al. 2006 [286]. Stock solution of palmitate was prepared at 70°C, oleate at 37°C and arachidonate at 25°C. Proper amounts of stock solution of FFA and HSA were dissolved in serum free-DMEM medium to give fatty acid working concentrations of 0.1mM-0.8mM and HSA concentration of 0.15mM (10mg/ml), which is the optimal HSA concentration shown previously to mediate cholesterol efflux in endothelial cells [21]. The mixtures were rotated overnight to ensure fatty acids were completely dissolved.

3.3.4 Cholesterol Efflux Assay

The labeling media was first prepared by concentrating 1-2n-(^3H)-cholesterol (40-60 Ci/mmol) under vacuum and resolubilizing in ethanol so that the final concentration of ethanol did not exceed 0.1% of the final media concentration. This (^3H)-cholesterol-ethanol solution (20μCi/mL) was dissolved into the culture medium supplemented with 5% FBS and added to the cells for 48 hours to equilibrate cellular cholesterol pools. Efflux was carried out by washing the labeled cells twice with culture media containing 2 mg/mL BSA and twice with media alone. The washed radiolabel led cells were incubated at 37°C with serum-free DMEM medium in the presence or absence of various cholesterol acceptors for 90 minutes, using an orbital shaker. At the end of the incubation time, 50 μl of incubation medium was taken and mixed with 100 μl of PBS containing 2 mg/mL BSA. The mixture was centrifuged at 10,000 rpm, 5 min and 100 μl of supernatant was used for radioactivity counting. At the end of incubation, cells were washed once with PBS containing 2 mg/mL BSA and twice with PBS alone followed by
lyses with 0.2 ml of 0.1N NaOH. Aliquots were taken for radioactivity counting by liquid scintillation counter (Tri-Crab 2800 TR liquid Scintillation System) and cellular cholesterol efflux was expressed as cpm/mg cell protein, as well as percent of total cholesterol released into medium \( \% \text{ efflux}= \frac{\text{medium cholesterol}}{\text{cell} + \text{medium cholesterol}} \times 100 \). Total protein concentration was determined by BCA kit.

3.3.5 Measurement of Cell Viability

Cells were treated as described above and after the end of efflux, cell viability was determined as indicated in section 2.3.4.

3.3.6 Analysis of Data

Statistical differences between results were analyzed by two-tailed unpaired student’s t-test or one way ANOVA and a \( P \) value of less than 0.05 was considered significant.

3.4 Results

3.4.1 Effects of FFAs on HSA-mediated Cholesterol Efflux

In agreement with previous studies on albumin-mediated cholesterol efflux [21], cells treated with HSA showed significant cholesterol efflux when compared to cells without HSA treatment as shown in figure 3.1 (~ 4 fold versus media, \( P<0.05 \)). In the presence of varying concentrations of FFAs, the increased cholesterol efflux mediated by HSA remained significant (\( P<0.05 \)) when compared with the control cells on media only treatment. However, cells treated with HSA/oleate or HSA/palmitate complexes at HSA/FFA molar ratio of 1:5.3 showed significant declines on cholesterol efflux rate (7.5 ± 1.9 %, and 9.9 ± 1.5% efflux, respectively). In the contrary, arachidonate had minimal effects on HSA-mediated cholesterol efflux at all tested concentration.
3.4.2 Cell Viability after Cholesterol Efflux to HSA/FFA Complexes

It was also important that we examined the extent of cell viability changes after efflux studies with the various HSA/FFA complexes in order to arrive at better conclusions on the observed reduction in HSA-mediated efflux at the highest concentrations of fatty acid used. As shown in figure 3.2, we found no significant changes in cell viability after efflux with HSA/FFA of molar ratios ranges 1:0.7-1:5.3 when compared to media only treated cells, except that there was a significant decrease in cell viability with HSA/palmitate complexes at 1:5.3 molar ratio (29.9% decrease).
Figure 3.1. Effects of FFAs on HSA-mediated Cholesterol Efflux

EA.hy926 cells were cultured and labeled with $^3$H-cholesterol (20µCi/ml) for 48 hours. Cells were washed and incubated with serum-free DMEM medium containing HSA only at 0.15mM (10mg/ml) and the various HSA/FFA complexes at various molar ratios (1:0.7, 1:1.3, 1:2.7, and 1:5.3) for 90 mins. Radioactivity released into the media is expressed as a % of total $^3$H-radioactivity \( \% \text{ of total efflux} = \frac{\text{medium cholesterol}}{\text{medium + cell cholesterol}} \times 100 \). The values represent the mean ± S.D of 3 independent experiments performed in duplicate. *\( P<0.05 \) vs. media control, #\( P<0.05 \) vs. HSA only.
Figure 3.2. Cell Viability after Cholesterol Efflux to HSA/FFA Complexes

EA.hy926 cells were cultured and labeled with $^3$H-cholesterol (20µCi/ml) for 48 hours. Cells were washed and incubated with serum-free DMEM medium containing HSA only at 0.15mM (10mg/ml) and the various HSA/FFA complexes at various molar ratios (1:0.7, 1:1.3, 1:2.7, and 1:5.3) for 90 mins. Cell viability was then determined by the MTT assay as described in methods. Results are expressed as percentage of media only (control) treatment set at 100%. The values represent the mean ± S.D of 3 independent experiments performed in duplicate. *$P<0.05$ vs. control.
3.5 Discussion and Conclusion

Our results suggest that oleate and palmitate binding sites on HSA might overlap and that with increasing concentrations of these FFAs, cholesterol efflux rates decreases as FFAs displaces cholesterol loaded on HSA. Arachidonate did not show significant effects on HSA-mediated cholesterol efflux. Most studies on HSA/FFA binding interactions have reported that polyunsaturated fatty acids bind differently on HSA unlike the saturated and monounsaturated fatty acids [18, 191, 302] and this might explain the observed effects. Also, fatty acids have been shown to bind strongly to high affinity sites on HSA which are also believed to be cholesterol binding sites [193] and thus competition between FFA and cholesterol affects HSA’s efficiency in mediating cholesterol efflux. However, we observed significant reduction in cell viability in HSA/palmitate molar ratio of 1:5.3 that elicited also reduced cholesterol efflux. This might question our result on the observed reduction in overall cholesterol efflux, but with the cell viability of the oleate treated cells having not changed from the control, it thus indicate that the reduction in cholesterol efflux in this case is quite valid. These results thus suggest the deleterious effects of higher concentrations of FFAs on endothelial functions and to some extent on its cell viability which might be aggravated with longer periods of FFA exposures. Also noteworthy, is that fatty acids particular MUFAs and PUFAs and not saturated FFAs have been shown to reduce cholesterol efflux to apo AI in macrophages through down regulation of ABCAI transporters [303-305]. Since HSA-mediated efflux are through passive diffusion-mediated mechanisms unlike apo AI-mediated efflux that are mediated through the ABCAI active transporters, free fatty acids will thus compromise both of these efflux mechanisms. As a result of the reduced
alterations in cholesterol efflux efficiency, people on high risk of developing CVD are made more susceptible to the disease. From these results, we went further ahead to study the effects of amino acid substitutions introduced into key cholesterol and FFA binding sites on HSA by treating endothelial cells with various HSA mutants in the presence of FFA and determined the effects of mutations on HSA-mediated cholesterol efflux as is described in the next chapter.
CHAPTER 4. HSA MUTANTS’ ROLES ON FFA CELLULAR EFFECTS

4.1 Introduction

FFAs are transported exclusively by HSA (99.9%) while 0.1% is only the unbound free fatty acids, FFA_u [61]. The solubility of an ordinary fatty acid such as palmitate at pH 7.4 is less than 0.1nM. Therefore, the concentration of HSA in plasma plays key roles in determining the fraction of FFA_u. These FFA_u levels have been implicated in various cellular effects more than just the total fatty acids. Changes in HSA/fatty acid binding interaction will modify the fraction of FFA_u in plasma, thereby resulting in changed availability of FFA_u to cells. Direct determinations of FFA_u levels by using a florescent probe, acrylodan coupled to a fatty acid binding protein from rat intestine (I-FABP), and referred to as ADIFAB has been developed recently [306]. It is therefore important to understand specific interactions of free fatty acids with certain amino acids on the binding sites of HSA.

With the advancement of technologies, new information on specific ligand binding interactions to HSA is now beginning to emerge. In the past, various studies have used chemical modifications, ligand analog studies, etc., to elucidate ligand binding to HSA but they have been somewhat unsuccessful in providing specific ligand properties of HSA. Recently, site-directed mutagenesis and recombinant protein expression techniques have been developed and are widely being applied in addressing biological problems [21, 219, 220, 307-317]. Our study employed site-directed mutagenesis and a novel yeast protein expression system to produce recombinant HSA proteins with specific mutations of key amino acid residues involved in fatty acid and cholesterol binding. Studies with these recombinant HSA proteins may provide specific structural information on the
mechanisms of HSA/FFA and HSA/cholesterol interactions, i.e., which amino acid residues interact with the ligand’s functional groups to provide the ligand binding free energy. Previous X-ray crystallographic studies have shown the presence of seven major fatty acid binding sites on HSA [16]. From the X-ray crystallographic structure it appears that only about 25 amino acids residues in fatty acid binding sites participate specifically in fatty acid binding. We produced specific HSA mutant proteins each containing a particular amino acid substitution in fatty acid binding sites in order to examine mutational effects on cholesterol and FFA bindings.

Our research focuses on two major areas which involve HSA mediated processes: (a) effects of HSA/FFA complexes on β-cell lipotoxicity, (b) effects of HSA mutants on cholesterol efflux in the presence of FFAs. Since the level of FFAs determines the degree of β-cell lipotoxicity, any mutations in the fatty acid binding sites of HSA could influence the effect by modifying FFA binding affinity. We also hypothesized that any competition between FFA and cholesterol at binding sites on albumin will result in modified albumin-mediated cholesterol efflux. Understanding of FFA binding to HSA is therefore important in the estimation of $\text{FFA}_u$ levels and to the overall elucidation of FFA-induced cellular effects associated with pathogenesis of diseases such as T2DM and CVD.

4.2 Aims of Study

Our study investigated the following aims:

(a) Determine the effects of HSA mutants in FFA-induced effects on β-cell viability by using the HIT-T15 pancreatic insulinoma cell line as a model system.

(b) Determine the fraction of unbound FFAs ($\text{FFA}_u$) when complexed with
recombinant HSA and its mutants in the presence of either oleate or palmitate by using the fluorescent probe, ADIFAB.

(c) Determine the effects of HSA mutants complexed with FFAs on HSA-mediated cholesterol efflux by using the EA.hy926 endothelial cell line.

4.3 Materials and Methods

4.3.1 Materials

Acrylodan-labelled intestinal fatty acid binding protein (ADIFAB) was purchased from Molecular Probes (Eugene, OR). Cibacron coupled to sepharose 6B were purchased from Sigma-Aldrich (St. Louis, MO). The plasmid vector, pHIL-D2, and the yeast strain Pichia pastoris were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY). Vent DNA polymerase was from New England Biolabs (Beverly, MA). Lipidex-1000 was from Packard Instruments (Meriden, CT). Refer to sections 2.3.1 and 3.3.1 for other materials, while all other chemicals used were of analytical grade.

4.3.2 Molecular Techniques for Developing Recombinant HSA Mutants

We synthesized recombinant HSA and the following single and double mutant proteins and the selections for these mutations were based on the specific FFA binding data provided by X-ray crystallography studies which showed that up to seven fatty acids can bind to a single molecule of HSA [16, 175, 314]. Also, our recent studies using X-ray crystallography and NMR spectroscopy [193] showed that FFA binding sites 4 and 5 within domain 3 represent two of the three high affinity FFA binding sites on HSA (the third is on site 2 in domain II). The mutants studied were, R410A/Y411A, W214L/Y411W, R410A, Y411A and W214L. Various studies also showed that steroids bind to HSA with moderate affinity and that fatty acids can displace up to 50% of
steroids bound to HSA, indicating that steroids are likely to bind the 2A and 3A subdomain [198]. Specific mutations were introduced into the HSA-coding region in a plasmid vector containing the entire HSA coding region as described previously [284, 314]. HSA mutant proteins as well as the recombinant HSA was expressed in *Pichia pastoris* and purified as previously described [318, 319]. Specifically, the experimental methods consisted of the following sequential steps.

4.3.2.1 Cloning of HSA Coding Region

With human liver cDNA as template, the entire coding region of the HSA gene, including the native signal sequence, was amplified by polymerase chain reaction using Vent DNA polymerase. The resulting DNA fragment was inserted into the plasmid vector pHiL-D2 using standard cloning techniques. pHiL-D2 is a shuttle vector that can be manipulated by cloning in *Escherichia coli* and that can also be used to introduce genes into yeast species *Pichia pastoris* by homologous recombination. Specific mutations were introduced into the HSA coding region using site-directed mutagenesis as described previously [8].

4.3.2.2 Expression of Recombinant HSA

Each pHiL-D2 expression plasmid contained a methanol-inducible promoter upstream of the HSA coding region. For each expression plasmid, a yeast clone that contained the expression cassette stably integrated into the yeast chromosomal DNA was isolated. The native HSA signal sequence, which was left on the HSA coding region, directed high level secretions of mature HSA into the growth medium.
4.3.2.3 Verification of DNA Sequence of HSA Clones

The total genomic DNA from each *P. pastoris* clone used to produce a particular HSA species was isolated using standard techniques. The genomic DNA isolated from each clone was used as template to amplify the entire HSA coding region by polymerase chain reaction. For each clone, the entire HSA coding region was sequenced using the dideoxy chain termination technique.

4.3.2.4 Purification of Recombinant HSA

The secreted HSA was isolated from growth medium as follows. The medium was brought to 50% saturation with ammonium sulfate at room temperature. The temperature was then lowered to 4°C, and the pH adjusted to 4.4, the isoelectric point of HSA. The precipitated protein was collected by centrifugation and resuspended in 10X phosphate-buffered saline. Dialysis was carried out for 48 hours at 4°C against 100 volumes of phosphate-buffered saline (150mM NaCl, 40mM phosphate, pH 7.4). A sample of the precipitant before and after dialysis was run on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 3 hours using commercial HSA as a standard. The sample for SDS-PAGE was prepared by denaturing the protein at 100°C for 5 minutes in a 1X loading dye containing 50μM dithiothreitol. The bands on the gel were visualized by staining with coomassie brilliant blue for one hour. The solution was loaded onto a column of cibacron blue immobilized on sepharose 6B. After washing the column with 10 bed volumes of phosphate-buffered saline, HSA was eluted with 3M NaCl. The eluent was dialyzed into phosphate-buffered saline and passed over a column of Lipidex-1000 to remove hydrophobic ligands possibly bound to the HSA [320].
final protein concentration was checked using the Bicinchonic Acid Protein Assay kit (BCA).

**4.3.3 Cell Culture**

HIT-T15 β-cells were maintained as described in section 2.3.2 while endothelial cells (EA. Hy926) were maintained as described in section 3.3.2.

**4.3.4 Treatment of β-cells with HSA Mutants/FFA Complexes**

The various mutant proteins at 0.1mM were each complexed to either oleate or palmitate at 0.4mM concentration in final working solutions and prepared as described in section 2.3.3. Control cells were treated with the wild type recombinant HSA (rHSA) complexed to either oleate or palmitate.

**4.3.5 Measurement of β-cell Viability**

This was determined as described previously in section 2.3.4.

**4.3.6 Cholesterol Efflux Assay**

This was determined as described in section 3.3.4, only that the various HSA mutants at 0.15mM (10mg/ml) were used in place of commercial HSA. Oleate and palmitate at concentrations of 0.4mM were mixed with the recombinant HSA and its mutants.

**4.3.7 Determination of Unbound FFA (FFA$_u$) in Recombinant HSA/FFA Complexes**

200μg of ADIFAB powder was brought up in 130μl of storage buffer consisting of 50mM TRIS, 1mM EDTA and 0.05% sodium azide at pH 8.0, resulting to a stock of approximately 100μM. This low salt was used only for storage for not more than three months. The sodium salts of the FFAs, palmitate and oleate were used to prepare the aqueous solutions. Stock solutions were prepared at a FFA concentration of 30mM in deionized water plus 4mM NaOH and 25μM butylated hydroxytoluene (BHT). BHT was
dissolved in ethanol at 50mM and the final ethanol concentration in FFA-sodium salt solution did not exceed 0.05% of ethanol. 500μM dilutions of these FFA stocks were made in water plus 4mM NaOH but no additional BHT. Total FFA was determined by WAKO NEFA C kit and aliquots stored in -20°C for no longer than 3 months. Upon excitation at 386 nm, ADIFAB fluorescence at 432 nm in the absence of fatty acid and at 505nm in the presence of fatty acids [321]. Thus the intensity ratio (R) of 505nm to 432 nm is indicative of the amount of FFAu present. Measurements of R values were done with a photon technology international fluorometer using the photon counting mode. ADIFAB was used to determine FFAu levels in HSA/palmitate or oleate complexes. rHSA and its mutants were all treated identically. For each complex, FFAu levels were determined by measuring the fluorescence as described previously by Richieri et al. 1993 [18] from three separate samples containing (1) HSA in buffer (blank), (2) HSA and ADIFAB in buffer (the R₀ value), and (3) FFA, HSA and ADIFAB in buffer (the R value). The measuring buffer was at pH 7.4 containing 20mM HEPES, 140mM NaCl, 5mM KCl and 1mM Na₂HPO₄ and same HSA (4μM) and ADIFAB (0.2μM) concentrations were used in each sample. Appropriate aliquots of FFA were added to the cuvette to give total FFA concentrations of between 0 and 24 μM. Just prior to FFA addition, the concentrated stocks of FFA-sodium salts were warmed to temperatures above the FFA melting point (62°C for palmitate and 37°C for oleate). FFA was then added in small volumes to the third sample which was maintained at 37°C and immediately mixed by drawing the solution in and out of the pipette. Between each FFA addition the cuvette was allowed to incubate for 10 minutes at 37°C. After the 10 minutes incubation the 432 nm and 505 nm intensities were determined from the three samples all
at 37°C. This procedure was repeated for total FFA to total albumin molar ratio values between 0 and 6 and in steps of 1.0. As a consequence, the FFA_u concentration was determined according to [306] from the 505nm and 432nm fluorescence after each titration.

### 4.3.8 Analysis of Data

Statistical differences between results were analyzed by two-tailed unpaired student’s t-test or one way ANOVA and a $P$ value of less than 0.05 was considered significant.

### 4.4 Results

#### 4.4.1 Various HSA Mutants Show Differential Effects on FFA-induced β-cell Death

Having shown the differential effects of fatty acids in the presence of HSA on β-cell viability, we attempted to further characterize the effects of modified interactions between HSA and FFA on cell viability. We hypothesized that the changes in fatty acid binding sites of HSA might change the concentration of FFA_u, which is available to cells and thus responsible for different effects on cell viability. We used various HSA mutant proteins complexed to the two principal FFAs of human serum, oleate and palmitate, at a concentration of 0.4mM (1:4 ratio of HSA/FFA) and assayed cell viability after 24 hours incubation. As shown in figure 4.1, we found that among the 5 HSA mutant proteins tested, the double mutant R410A/Y411A showed significant increase on cell viability when treated with either oleate (28.4% increase) or palmitate (24.8% increase) compared to the control group treated with rHSA/FFA complexes ($P<0.05$). Treatment of mutant proteins, R410A, W214L, and W214L/Y411W complexed with oleate showed significant reductions on cell viability (28.8%, 37.0%, and 14.2% decreases, respectively) compared to the control treated with rHSA/oleate complexes ($P<0.05$). Also, the mutants, W214L
and W214L/Y411W complexed with palmitate showed significant reductions on cell viability when compared to control group treated with rHSA/palmitate complexes (35.3%, and 32.9% decreases, respectively, $P<0.05$). However, R410A complexed to palmitate did not show similar significant reductions on cell viability as shown with oleate treatment (14.7% decreases). Furthermore, the HSA mutant Y411A showed no significant effect whether complexed with oleate or palmitate (3.1% increase, and 6.2% decrease, respectively).

4.4.2 Unbound FFA ($\text{FFA}_u$) Levels in Recombinant HSA/FFA Complexes

We also determined the FFA$_u$ levels in mixtures of three recombinant HSAs (R410A/Y411A, W214L/Y411W, and wild type rHSA) and either with palmitate or oleate by using the fluorescent probe ADIFAB at HSA to FFA molar ratios of 1:1-1:6. As shown in figure 4.2, there was a gradual rise in FFA$_u$ levels with increasing total FFA concentrations without changing HSA concentrations and with sharp increases beyond HSA to FFA molar ratios of 1:2. In correlation with the HSA to FFA molar ratios of 1:4 that were used in cell viability assays, the FFA$_u$ levels in rHSA/oleate, R410A/Y411A/oleate, and W214L/Y411W/oleate complexes were determined to be 71.6nM, 59.0nM, and 36.9nM, respectively. On the other hand the FFA$_u$ levels in rHSA/palmitate, R410A/Y411A/palmitate, and W214L/Y411W/palmitate complexes were 62.6nM, 38.2nM, and 44.0nM, respectively.

4.4.3 Effects of HSA Mutants in FFA Effects on HSA-mediated Cholesterol Efflux

Since the high concentrations of oleate and palmitate caused significant reductions on cholesterol efflux as presented in the previous chapter, we examined further the role of different HSA mutants on the FFA effects on HSA-mediated efflux. We chose 0.4mM
fatty acid concentrations for this study to avoid high cell-death rates at higher concentrations of FFAs and in the presence of 0.15mM HSA (10mg/ml). As shown in figure 4.3, rHSA and its mutants complexed to FFAs were all efficient in mediating cholesterol efflux (about 20-34% cholesterol efflux). Among the 5 HSA mutants studied and complexed to oleate, three had higher efflux rates when compared to rHSA/oleate treatment namely, Y411A, W214L, and R410A (by 10.3%, 6.9%, and 7.2% more in cholesterol efflux, respectively). The same three mutants when mixed with palmitate had no significant changes when compared to rHSA/palmitate; this result was similar for the other two mutants, R410A/Y411A and W214l/Y411W.
Figure 4.1. Effects of Various HSA Mutants Complexed to FFAs on Cell Viability

HIT-T15 cells were treated with 0.1mM HSA mutants complexed to either oleate or palmitate at 0.4mM for 24 hours. Cell viability was determined by MTT assay as described in methods. Results are expressed as percentage of cell viability of cells treated with rHSA/FFA complexes set at 100%. The values represent the mean ± S.D of 3 independent experiments performed in duplicate. *P<0.05 versus rHSA/oleate treated cells, #P<0.05 versus rHSA/palmitate treated cells.
Figure 4.2. Unbound FFA (FFAu) Concentrations in Complexes of Recombinant HSA (rHSA) and its Variants Bound to FFAs

FFAu concentrations were determined as described in methods using the fluorescent probe ADIFAB (0.2µM) in a fixed albumin concentration of 4µM and FFA concentrations between 4-24µM. For clarity, results are shown as averages from six determinations excluding ± S.D.
Figure 4.3. Cholesterol Efflux to Recombinant HSA Mutants /FFA Complexes

EA.hy926 cells were cultured and labeled with $^3$H-cholesterol (20µCi/ml) for 48 hours. Cells were washed and incubated with serum-free DMEM medium containing recombinant HSA (rHSA) only at 0.15mM (10mg/ml) and the various rHSA or mutants/FFA complexes at molar ratio of 1:2.7 for 90 mins. $P$ represents palmitate while $O$ is oleate. Radioactivity released into the media is expressed as a % of total $^3$H-radioactivity ($\%$ of total efflux = (medium cholesterol)/ (medium + cell cholesterol) x 100). The values represent the mean ± S.D of 3 independent experiments performed in duplicate. All treatments versus media only were significant, $P<0.05$. *$P<0.05$ vs. rHSA/oleate.
4.5 Discussion and Conclusion

We postulated that changes in HSA affinity for FFAs might affect the fraction of FFA\textsubscript{u} in plasma which in turn induces different cellular effects. We examined this aspect in β-cells viability by using rHSA proteins with specific mutations of key amino acid residues in FFA-binding sites of subdomains 2A and 3A of HSA. Our results showed that among the five HSA mutant proteins used in the study, the double mutant R410A/Y411A caused a significant reduction in cell death induced by palmitate and oleate compared to the control group treated with rHSA/FFA complexes. This might indicate that this mutant protein strongly binds to FFAs and thus decreases the fraction of FFA\textsubscript{u} available to the β-cells. This was in agreement with an earlier study in that this mutant complexed with oleate showed a significantly reduced rate of oleate transport into HepG2 cells and thereby lowered apoB secretion by 70.4% [286]. In particular, these results suggest that double substitution mutations of the polar amino acids arginine and tyrosine at positions 410 and 411 to alanines enhanced binding, thus indicating that hydrophobicity might be the major force for binding of FFA to HSA. This was an interesting result since X-ray crystallographic data has shown that R410 and Y411 are the key amino acids in the binding pocket of subdomain IIIA of HSA [193]. In contrast, the single mutant Y411A did not produce significant changes in cell viability, suggesting that the single substitution of small hydrophobic alanine alone did not cause significant changes in the FFA-binding pocket. The single mutant R410A complexed to palmitate also had no significant effect, but when it was complexed to oleate, it had no improvement on the viability as seen before with rHSA/oleate treatment. This suggests that the R410A HSA mutant might bind more strongly to palmitate than to oleate. Interestingly, the W214L
HSA mutant showed a significant reduction in cell viability and the double HSA mutant W214L/Y411W in subdomains 2A and 3A also showed a significant reduction, respectively, indicating that these changes were unfavorable for FFA binding. Therefore, lowered affinity to FFAs consequently increased the FFA_u pool to the β-cells which resulted in deleterious effects on the cell viability. Furthermore, our study of FFA_u level determination in rHSA/palmitate or oleate complexes showed a pattern of higher FFA_u concentration profiles when compared to either of the two representative complexes of the rHSA mutants, R401A/Y411A and W214L/Y411W, to FFAs. These FFA_u concentrations were all less than 100 nM at molar ratios below 1:4 of HSA to FFA and approximately in the range of previous determinations [18, 34]. These results are consistent with other results which further support that the enhanced effects on cell viability of the double mutant R410A/Y411A when complexed to FFAs are due to the reductions in cytotoxic FFA availability to β-cells, thus increasing cell viability. However, it is noteworthy that rHSA or its mutants/palmitate complexes were more cytotoxic than those of oleate, and yet their FFA_u profiles were not drastically different, but generally the unbound levels of cytotoxic palmitate profiles were lower than oleate. Previous studies have shown that FFA_u levels are stronger determinants of insulin secretion [322] and degree of β-cell cytotoxicity [138] than total FFA concentrations. Thus, the differential effects of the rHSA mutants on cell viability in the presence of FFAs suggest that HSA variants might play a role in modulating the FFA_u fractions that is more relevant under higher levels of circulating FFAs such as in obese subjects. There have been very few studies that have attempted to study FFA binding to genetic variants of HSA. Although a few noted increased FFA binding [179], majority of the mutations had
none or less effect, especially those on the surface of the protein [214]. Therefore, while the existence of other genetic HSA variants in the populace remains to be screened or unknown, our study presents a possible mechanism on what alterations on HSA might induce changes on FFA-binding since we carried out amino acid substitutions in key FFA-binding pockets of HSA.

Our investigations with the same HSA mutants in oleate and palmitate effects on HSA-mediated cholesterol efflux were not so dramatic. However, we observed that the mutants, Y411A, W214L, and R410A when treated with oleate had higher cholesterol efflux when compared to rHSA/oleate treated cells. These results indicate that these mutants had either weak interactions to oleate or higher interactions to cholesterol, thus eliciting higher efficiency in cholesterol efflux than recombinant HSA. Our previous studies using the same HSA mutants alone on cholesterol efflux [21] showed that Y411A had higher cholesterol efflux rate than rHSA, while W214L and R410A had no significant effects. Thus, the latter two mutants probably elicit lower binding interactions to fatty acids while Y411A higher cholesterol efflux rate is probably due to its enhanced interactions with cholesterol on binding sites. Also, from our previous studies, the two double mutants, R410A/Y411A and W214L/Y411W, both showed higher reduction in apo B secretion in hepatic cells [286] and also enhanced cholesterol efflux in endothelial cells [21] suggesting that they might bind strongly to oleate and cholesterol, but we did not observe any significant changes when they were mixed with fatty acids on cholesterol efflux.

However, these results should not be undermined since we only examined the effects of the FFAs at 0.4mM to avoid lipotoxicity caused by high concentration of FFAs, while
in some disease states the concentrations of serum FFAs can rise above 0.8mM. Therefore *in vivo*, the cholesterol efflux efficiency of some of the genetic variants might be greatly altered in high levels of serum FFAs or when HSA concentrations are near constant while total fatty acid concentration increase, thus increasing the physiologically available FFAn pool in human serum. It is also unknown, how these HSA mutant effects contribute to the desorption rates of cell-surface cholesterol or to conformational changes arising when FFA and cholesterol are loaded on HSA.

In conclusion, modified HSA/FFA and HSA/cholesterol interactions caused by mutations of key amino acids involved in the binding of FFA to HSA resulted in changes in cell viability and on HSA-mediated cholesterol efflux, suggesting a possible role of HSA polymorphism on FFA-induced changes in cellular functions.
CHAPTER 5. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation has provided results on the effects of various types of FFAs on β-cells as well as on endothelial cells. Most important is that our studies examined the effects of these fatty acids in the presence of HSA, which should be more relevant in delineating albumin’s modulating effects of FFAs unlike the commonly used BSA, thus presenting a stronger mimic of in vivo conditions in humans. However, we did not observe significant differences using HSA compared to BSA in the FFA-induced β-cell effects despite the fact that BSA has 76% sequence homology to HSA, suggesting that FFAs that dissociate from albumin-FFA bound forms and enter cells are very potent in relatively low concentrations irrespective of the albumin type.

Specifically, our studies showed differential effects of FFAs on β-cell viability that was dependent on the degree of saturation, chain length, and concentration levels of the fatty acids. The saturated fatty acids, palmitate and stearate had adverse effects on the β-cell viability while we did observe minimal changes on cell viability with the unsaturated FFAs that further exhibited cytoprotective potential, particularly the cis FFAs, oleate and linoleate against these FFA-induced cytotoxic effects. How FFAs cause these differential effects in β-cells remain unknown. Therefore, future studies that can elaborate on the interactions of specific FFAs on either the surface of β-cells (receptors) in signaling or through distinct intracellular signals will be helpful in understanding these FFA-induced β-cell effects. Moreover, since the involvement of apoptosis in causing β-cell death under our FFA treatments was evidenced, signaling by the unsaturated FFAs that cause activation of anti-apoptotic pathways might provide targets for development of drugs to enhance β-cell mass proliferation or slowing the progressive β-cell destruction.
Elaboration of mechanisms that lead to ER stress, oxidative stress, and also mitochondrial stress will be very insightful in understanding the β-cell mass reduction that is characterized in T2DM, in regard to lipotoxicity. Other areas of future elaboration is on the roles of autophagy in β-cell survival or cell death during chronic FFA exposures to β-cells. Most important will also be the contributions from in vivo studies to better support these in vitro cell culture-based studies of FFA effects on β-cells.

Our studies also showed that oleate and palmitate at higher concentrations reduced HSA-mediated cholesterol efflux. Since HSA is the most abundant serum protein, its contributions to cholesterol efflux might be significant together with the well known HDL-mediated cholesterol efflux since HSA can bind cholesterol and also possesses antioxidant properties. Thus in disease states where there is high circulating FFAs, HSA’s efficiency in mediating cholesterol efflux might be compromised. These various cholesterol efflux mechanisms are currently areas of pharmacological attempts to managing lipid-related complications. Furthermore, our studies using HSA variants with key mutations in key amino acids in FFA and cholesterol binding pockets on HSA, showed differential effects of FFA-induced effects in the β-cell viability as well as on HSA-mediated cholesterol efflux. Therefore, future studies on HSA genetic variants to ligand binding, particularly with FFAs and cholesterol might give an overview of how HSA variants modulates these FFA or cholesterol cellular-induced effects. Determination of accurate binding affinities of such HSA genetic variants as well as those genetically engineered will be cornerstone in drawing stronger conclusions and also rationale for undertaking HSA genetic surveys in people or ethnic groups with apparent metabolic disparities associated with FFA and cholesterol metabolism.
REFERENCES


