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PANCREATIC β-CELL FATTY ACID METABOLISM AND MODULATION OF FUNCTION IN RESPONSE TO GLUCOLIPOTOXICITY

By

Christopher D. Green

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Biochemistry & Molecular Biology

2009

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ABSTRACT

PANCREATIC β-CELL FATTY ACID METABOLISM AND MODULATION OF FUNCTION IN RESPONSE TO GLUCOLIPOTOXICITY

By

Christopher D. Green

Type 2 diabetes is associated with gradual diminishment of pancreatic islet β-cell function in response to chronic hyperglycemia and elevated plasma free fatty acids (FFAs), defined as glucolipotoxicity. The effects of glucolipotoxicity on β-cells include decreased insulin gene expression, diminished glucose-stimulated insulin secretion (GSIS), and ultimately decreased β-cell mass. Loss of β-cell GSIS has been linked to increased lipogenic gene expression and triacylglyceride (TAG) accumulation. Activation of the liver X receptor (LXR) transcription factor further increases lipogenic gene expression and TAG accumulation but increases both basal insulin release and GSIS. Here, INS-1 β-cells treated with the LXR agonist T0901317 during chronic hyperglycemia increased lipogenic gene expression, *de novo* synthesis of TAG, and basal and GSIS. LXR-activated INS-1 cells exhibited increased fatty acid (FA) oxidation and expression of genes involved in mitochondrial β-oxidation. Inhibition of fatty acyl-CoA synthesis and mitochondrial β-oxidation blocked the elevated basal insulin release. Thus, together with the rapid turnover of TAG in LXR-activated cells, these results indicate that enhanced basal insulin release involves oxidation of fatty acyl-CoAs generated during turnover of neutral lipid pools. Increased synthesis and turnover of TAG suggested increased lipolysis of complex lipids and the generation of lipid signaling molecules such as diacylglycerol. In this manner, inhibition of TAG turnover and diacylglycerol binding proteins reduced the LXR-mediated increase in GSIS.

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LXR activation in INS-1 cells also increased monounsaturated FA (MUFA) synthesis and elevated stearoyl-CoA desaturases (SCD) 1 and 2 gene expression, rating-limiting enzymes in MUFA synthesis. SCD1 and 2 gene expression were then identified to be elevated in pre-diabetic Zucker diabetic fatty (ZDF) rat islets, whereas diabetic ZDF rat islets had reduced expression of SCD1, SCD2 and Elovl-6, a FA elongase involved in MUFA synthesis. These findings suggested SCDs and Elovl-6 could be involved in the β-cell response to metabolic load. Elevated exogenous FFA levels, particularly saturated FAs, cause toxic effects to β-cells that include altered endoplasmic reticulum (ER) integrity, which is linked to induction of ER stress responses and apoptosis. Using siRNAs and adenoviral constructs, altered SCD or Elovl-6 gene expression in INS-1 cells was examined for its effects on ER stress and apoptosis mediated by exogenous palmitate (16:0). Knockdown of SCDs decreased MUFA synthesis and increased susceptibility of INS-1 cells to palmitate-induced ER stress and apoptosis, whereas over-expression of SCD2 increased palmitate desaturation to palmitoleate (16:1,n-7) and reduced palmitate toxicity. Elovl-6 knockdown decreased palmitate conversion to stearate (18:0) and oleate (18:1,n-9) and tended to reduce palmitate-induced ER stress and apoptosis, while Elovl-6 over-expression increased synthesis of 18:0 and 18:1,n-9 and increased susceptibility to palmitate-induced toxicity. Further studies showed that coordinated expression of SCDs, Elovl-6 and Elovl-5, which elongates 16:1,n-7 to vaccenate (18:1,n-7), is required for maintaining balanced *de novo* synthesis of n-7 versus n-9 MUFAs.

In conclusion, these studies demonstrate that modulation of TAG synthesis and turnover and MUFA synthesis significantly alters the β-cell response to glucolipotoxicity.

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1.3. Exogen
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1.5. Glycer
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AA

ABC

ACC

Ad

ADP

AGPAT

AMP

AMPK

ATF

ATGL

ATP

B-gal

bHLH

CE

C/EBP

CHOP

ChRE

ChREBP

CPT

DAG

Δ5D

Δ6D

List of Abbreviations

AA	arachidonic acid
ABC	ATP-binding cassette
ACC	acetyl-CoA carboxylase
Ad	adenovirus
ADP	adenosine 5'-diphosphate
AGPAT	acylglycerol-3-phosphate acyltransferase
AMP	adeonsine monophosphate
AMPK	AMP-activated protein kinase
ATF	activating transcription factor
ATGL	adipose triglyceride lipase
ATP	adenosine 5'-triphosphate
B-gal	β-galactosidase
bHLH	basic helix-loop helix
CE	cholesterol ester
C/EBP	CCAAT/enhancer-binding protein
CHOP	C/EBP homologous protein
ChRE	carbohydrate response element
ChREBP	carbohydrate response element binding protein
CPT	carnitine palmitoyl transferase
DAG	diacylglyceride
Δ5D	delta-5 desaturase
Δ6D	delta-6 desaturase

Δ^9D

DGAT

DNA

Elovl

ER

FA

FAS

FFA

GK

GLUT2

GPAT

GSIS

HSL

INS-1

IRE1

JNK

KREB

LCAD

LC-CoA

LDL

LPA

L-PK

Luc

Δ9D	delta-9 desaturase
DGAT	diacylglyerol acyltransferase
DNA	deoxyribonucleic acid
Elovl	fatty acid elongase
ER	endoplasmic reticulum
FA	fatty acid
FAS	fatty acid synthase
FFA	free fatty acid
GK	glucokinase
GLUT2	glucose transporter 2
GPAT	glycerol-3-phosphate acyltransferase
GSIS	glucose-stimulated insulin secretion
HSL	hormone sensitive lipase
INS-1	rat insulinoma cell line
IRE1	inositol requiring ER-to-nucleus signal kinase 1
JNK	c-Jun N-terminal kinase
KREB	Kreb's Ringer bicarbonate buffer
LCAD	long chain acyl CoA dehydrogenase
LC-CoA	long chain-acyl-coenzyme A
LDL	low density lipoprotein
LPA	lysophophatidic acid
L-PK	liver pyruvate kinase
Luc	luciferase

L-VGCC

LXR

ME

MLX

mtGPAT

MUFA

NADH

NADPH

PA

PAP

PC

PDH

PERK

PKC

PLA2

PLC

PPARα

PUFA

qPCR

RNA

ROS

RPL32

RXR

L-VGCC	L-type voltage-gated calcium channel
LXR	liver X receptor
ME	malic enzyme
MLX	Max-like protein X
mtGPAT	mitochondrial GPAT
MUFA	monounsaturated fatty acid
NADH	β-nicotinamide adenine dinucleotide
NADPH	β-nicotinamide adenine dinucleotide phosphate
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase
PERK	double-stranded RNA-activated kinase (PKR)-like ER kinase
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PPARα	peroxisome proliferator activated receptor-α
PUFA	polyunsaturated fatty acid
qPCR	quantitative PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RPL32	ribosomal protein L32
RXR	retinoid X receptor

SCAP

SCD

SREBP

TAG

TCA

T0

T2D

VLAD

VLDL

Xbp1

ZDF

ZF

16:0

16:1,n-7

18:0

18:1,n-7

18:1,n-9

18:2,n-6

18:3,n-6

20:3,n-9

20:4,n-6

20:5,n-3

22:6,n-3

SCAP	SREBP cleavage activating protein
SCD	stearoyl-CoA desaturase
SREBP	sterol regulatory element binding protein
TAG	triacylglyceride
TCA	tricarboxylic acid acyle
T0	T0901317
T2D	type 2 diabetes
VLAD	very long chain acyl CoA dehydrogenase
VLDL	very low density lipoprotein
Xbp1	X-box binding protein 1
ZDF	Zucker diabetic fatty rat
ZF	Zucker fatty rat
16:0	palmitic acid
16:1,n-7	palmitoleic acid
18:0	stearic acid
18:1,n-7	vaccenic acid
18:1,n-9	oleic acid
18:2,n-6	linoleic acid
18:3,n-6	linolenic acid
20:3,n-9	mead acid
20:4,n-6	arachidonic acid
20:5,n-3	eicosapentaenoic acid
22:6,n-3	docosahexeanoic acid

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INTRODUCTION

Type 2 diabetes (T2D) accounts for more than 90% of diabetes cases, which is projected to affect 300 million people worldwide by 2025 (1, 2). Whereas type 1 diabetes requires insulin administration due to the complete absence of pancreatic islet β-cell insulin secretion, T2D results, in part, from the inability of β-cells to secrete enough insulin to overcome insulin resistance and maintain glucose homeostasis. As dysregulated glucose metabolism develops, sustained periods of hyperglycemia and elevated plasma free fatty acids (FFAs) contribute to the progressive loss of β-cell function, collectively termed glucolipotoxicity (3). The adverse effects of glucolipotoxicity on β-cells include decreased insulin gene expression, diminished glucose-stimulated insulin secretion (GSIS), and ultimately the loss of β-cell mass (4). A major goal of our lab is to understand how these effects develop and to identify mechanisms to protect from β-cell dysfunction.

Modulation of fatty acid (FA) metabolism in β-cells in response to glucolipotoxicity is essential for maintaining proper function. Chronic exposure of β-cells to elevated levels of glucose or glucose plus FFAs increases the storage of FA in triacylglyceride (TAG) and diminishes GSIS (3). Thus, accumulation of TAG in β-cells has been associated with the pathogenesis of β-cell dysfunction. Evidence has emerged, however, for protection of β-cells from glucolipotoxicity by modulating the expression and activity of genes involved in *de novo* FA synthesis (lipogenesis) and TAG storage. This includes regulation of lipogenic genes via liver X receptor (LXR) activation and genes involved in monounsaturated FA synthesis (5, 6). This dissertation provides insight into the mechanism of elevated insulin secretion from LXR-activated β-cells

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under chronic hyperglycemia, and examines the roles of altered expression of FA desaturase and elongase genes involved in monounsaturated FA synthesis in response of β-cells to exogenous saturated FAs. The findings of this research identify mechanisms that could be utilized to prevent the onset of β-cell failure and T2D.

Chapter

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Chapter 1. Literature Review

1. Fatty Acid Metabolism and Regulation of Genes Involved in Lipogenesis

1.1. Fatty Acid Structure and Classification

Fatty acids (FAs) serve numerous essential biological functions, such as stabilizing cellular membranes, providing energy storage depots, and participating in signal transduction. The efficacy of these functions is largely dependent on the FA structure. FAs are composed of hydrocarbon chains of various lengths with a methyl group and a carboxyl group residing at opposite ends of each chain. FAs with hydrocarbon chains that are completely saturated with hydrogen atoms are termed saturated FAs. An example of a saturated FA is the sixteen-carbon FA palmitic acid, represented as 16:0. Modified FAs containing one or more double bonds are termed unsaturated FAs. The number of double bonds further categorizes these FAs into monounsaturated FAs (MUFAs), containing one double bond, and polyunsaturated FAs (PUFAs), containing two or more double bonds. Unsaturated FAs are characterized by denoting the position of the carbon of the first double bond, counting from the methyl end. For example, the MUFA oleic acid is represented as 18:1,n-9, as it has eighteen carbons, one double bond, and the double bond is nine carbons from the methyl end. A PUFA such as arachidonic acid (20:4,n-6) has four double bonds and only the position of the first double bond is listed. As in all cells, the cellular FA composition of islet β-Cells consists of a broad range of saturated and unsaturated FAs, the concentrations of which are under tight regulation.

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1.2. Modification of Fatty Acid Structure

1.2.1. Desaturation

Modification of FAs by oxidative desaturation uses NADH-cytochrome b5 reductase, cytochrome b5, and a desaturase to convert a single carbon-carbon bond to a cis-double bond (Figure 1.1). In mammals, the FA desaturases capable of enzymatic addition of a cis-double bond include delta 5 desaturase (Δ5D), Δ6D, and Δ9D, also called stearoyl-CoA desaturase (SCD). The 'Δ' refers to the position of the carbon the double bond is added, counting from the carboxyl end. The Δ5D and Δ6D genes are involved in PUFA synthesis. Substrates preferentially desaturated by the Δ6D are oleic acid (18:1,n-9) and the C18 and C24 PUFAs, whereas Δ5D prefers C20 PUFA substrates (7). Δ5D and Δ6D activities are essential for the synthesis of the PUFAs mead acid (20:3,n-9), arachidonic acid (20:4,n-6), and docosahexaenoic acid (22:6,n-3) (7, 8). Δ5D and Δ6D are ubiquitously expressed with the highest level found in liver, followed by brain and heart (9). The Δ5D and Δ6D genes were identified to be expressed in rat islets and β-cells (10), but a unique role in β-cell function has not been demonstrated.

Stearoyl-CoA desaturase (SCD) is the rate-limiting enzyme for MUFA synthesis, particularly palmitoleate (16:1,n-7) and oleate (18:1,n-9). SCD isoforms cloned thus far include four from mouse (SCD1-4) (11-14), two in rat (SCD1 and 2) (15), three in hamster (SCD1-3) (16), and three in human (SCD1, 2, and 5) (17-19). Analysis of substrate specificity using microsomal fractions from cells over-expressing SCD 1, 2, or 4 demonstrated the ability to desaturate C13-C19 saturated FAs with a particular preference towards stearic acid (18:0) (20). SCD3 desaturated C13-C16 FAs but not stearic acid (18:0), suggesting that SCD3 should be redefined as a palmitoyl-CoA

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desaturase (20). SCD1 is highly expressed in adipose tissue and liver, tissues with enhanced capacity for neutral lipid storage (12). The activity of SCD1 is more than 2-fold higher for stearic acid than palmitic acid (20). This in turn increases synthesis of oleic acid (18:1,n-9) and correlates with the requirement of SCD1 activity for neutral lipid storage, as oleic acid is the preferred substrate for TAG storage (21). SCD2 expression in mice is primarily found in the brain, presumed to be important for myelin formation (11), and is required for lipid synthesis during early development (22). Human SCD2 is expressed at high levels in the brain and whole pancreas (19). SCD3 expression is restricted to skin sebaceous glands and is thought to be involved in making wax esters for the skin (14, 23, 24). The mouse SCD4 isoform is expressed specifically in the heart (13).

Although studies have identified direct roles for SCD1 and SCD3 in lipid storage and wax ester synthesis, respectively, the significance of the SCD2 and SCD4 isoforms in lipid metabolism remains unclear. More specifically to the islet β-cell, the gene expression profile of SCDs and the contributions of specific SCD isoforms to normal β-cell function have not been determined.

1.2.2. Elongation

FA elongation by addition of a malonyl-CoA C2 unit to a fatty acyl-CoA is catalyzed in four steps: condensation of the fatty acyl-CoA with malonyl-CoA, reduction using NADPH, dehydration, and reduction to the fatty acyl-CoA product (Figure 1.1). The rate-limiting condensation step is catalyzed by various FA elongases, the activities of which are substrate dependent. Seven subtypes of FA elongases have been identified in mouse, rat, and human, and are referred to as elongation of very long chain fatty acids

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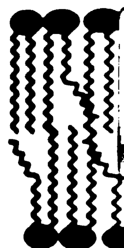
(Elovl-1 to 7) (www.ensembl.org). Elovl-1, 3, and 4 elongate a broad range of very long chain FAs (>C20) and are involved in sphingolipid synthesis, brown adipose and skin barrier function, and retinal function, respectively (25-30). Elovl-2 and Elovl-5 are mainly involved in n-3 and n-6 PUFA synthesis, with Elovl-2 elongating C20 and C22 PUFAs and Elovl-5 elongating C18 PUFAs as well as palmitoleic acid (16:1,n-7) (31-34). The expression of Elovl-2 and Elovl-5 was detected in most tissues with the highest level being in the liver (35). Elovl-6 is capable of elongating C12-16 saturated FAs and palmitoleic acid (16:1,n-7) (31, 32). Although the expression of Elovl-6 is low is most tissues (35), its activity significantly regulates stearic acid (18:0) synthesis, the precursor of oleic acid (18:1,n-9) (36). Elovl-7 substrates and expression have not been characterized.

In regards to MUFA synthesis, FA elongation assays using microsomal preparations demonstrated Elovl-5 and Elovl-6 to elongate C16 FAs for the generation C18 MUFAs. The roles of these genes in the *de novo* synthesis of MUFAs in intact mammalian cells, however, have not been addressed. In addition, a unique role for FA elongases in β-cells is not known.



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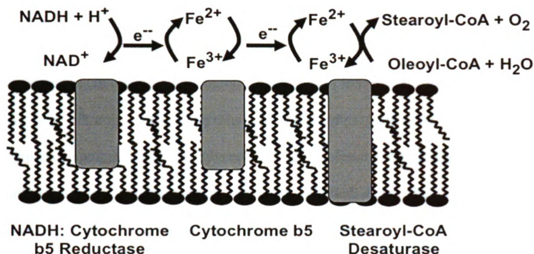

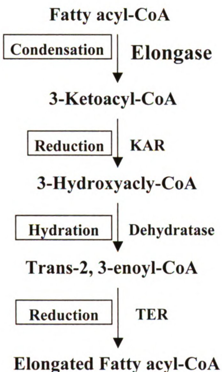

Figure 1.1. Reaction diagrams for FA desaturation through the stearoyl-CoA desaturase complex and general FA elongation. Microsomal FA desaturation occurs through the transfer of two electrons from NADH to oxygen to produce a desaturated FA-CoA and water. Microsomal FA elongation occurs through the following sequential reactions: 1) FA-CoA condensation with malonyl-CoA to form 3-ketoacyl-CoA, 2) 3-ketoacyl-CoA reduction to 3-hydroxyacyl-CoA using NADPH, 3) 3-hydroxyacyl-CoA dehydration to *trans*-2,3-enoyl-CoA, 4) *trans*-2,3-enoyl-CoA reduction to acyl-CoA. KAR, 3-ketoacyl-CoA reductase. TER, *trans*-2,3-enoyl-CoA reductase.

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1.3. Exogenous Fatty Acid Uptake and Essential Fatty Acids

A major source of intracellular FAs comes from transport of exogenous FA derived from circulating chylomicrons, lipoproteins, free FAs (FFAs), and protein bound FAs (e.g. albumin). Chylomicrons and lipoproteins consist of lipid droplet/protein complexes composed of triacylglycerol (TAG), cholesterol esters, glycerophospholipids, cholesterol, and apolipoproteins, and are secreted from the intestine and liver, respectively. FAs incorporated into chylomicrons primarily originate from dietary fat after digesting a meal. TAG, the predominant source of dietary FA (37), is cleaved in the lumen of the intestine by pancreatic lipase at the sn-1 and sn-3 positions to produce FFAs and 2-monoacylglcyerol (2-MAG) (38-40), the latter of which can be further cleaved to glycerol and FFA (41). MAG and FFAs are then transported inside enterocytes where they are used to reassemble TAG for chylomicron formation (42, 43). Lipid droplets within lipoproteins that are secreted from the liver contain a mixture of FAs derived from both exogenous and *de novo* (see next section) sources. Circulating chylomicrons and lipoproteins, particularly very low density and low density lipoproteins (VLDL and LDL), are cleaved by lipoprotein lipase to release FFAs for storage and use in peripheral tissues (44). FFAs are also released into the circulation during fasting by activation of TAG and cholesterol ester hydrolysis in lipid storage depots, such as adipose tissue and the liver (45). Subsequent uptake of exogenous FFAs at peripheral tissues occurs through either facilitated transport by FA transport proteins or by diffusion of the plasma membrane.

Dietary FAs are the source of the essential FAs linoleic acid (18:2,n-6) and linolenic acid (18:3,n-3). Essential FAs are defined as essential because animals lack the

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Δ12D and Δ15D enzymes required for the synthesis of n-6 and n-3 FAs. Conversion of linoleic acid and linolenic acid to other PUFAs, such as arachidonic acid (20:4,n-6) and docosahexeanoic acid (22:6,n-3), is important for normal cellular function. Endogenous synthesis of arachidonic acid from 18:2,n-6 requires sequential desaturation and elongation activities of Δ6D, Elovl-5, and Δ5D, while docosahexeanoic acid synthesis from 18:3,n-3 additionally requires elongation by Elovl-2, desaturation by Δ6D, and peroxisomal β-oxidation (Figure 1.2) (7).

1.4. De Novo Fatty Acid Synthesis

De novo synthesis of FAs characterizes the intracellular, cytosolic production of FAs that are made entirely from intracellular acetyl-CoA and malonyl-CoA, derived mainly from the tricarboxylic acid cycle (TCA). The accumulation of cytosolic acetyl-CoA and malonyl-CoA is highly dependent upon glucose metabolism and the carbon flux in and out of the TCA cycle. Exposure of cells to elevated glucose levels increases transport of the glycolytic product pyruvate into the mitochondria, conversion of mitochondrial pyruvate to citrate via the TCA cycle, and export of citrate from the mitochondria. ATP-citrate lyase (ACL) then catalyzes the cleavage of cytosolic citrate into acetyl-CoA and oxaloacetate, the latter of which can be converted back to pyruvate for entry into the mitochondria. Carboxylation of ACL-derived acetyl-CoA via acetyl-CoA carboxylase (ACC) provides the necessary FA elongation substrate malonyl-CoA. Fatty acid synthase (FAS) performs all the enzymatic reactions necessary for FA elongation and subsequently utilizes one acetyl-CoA and seven malonyl-CoA units to produce the saturated FA palmitic acid (16:0) (46, 47). Together, elevated glucose increases *de novo* FA synthesis through elevated carbon flux through the TCA cycle and

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export of citrate, which can be sequentially converted through ACL, ACC and FAS to palmitate.

De novo or exogenously derived palmitic acid can be used for MUFA synthesis through two pathways: Elovl-6 elongation to stearic acid (18:0) followed by SCD desaturation to oleic acid (18:1,n-9); SCD desaturation to palmioleic acid (16:1,n-7) and either Elovl-6 or Elovl-5 elongation to vaccenic acid (18:1,n-7) (Figure 1.2). Synthesis of oleic acid (18:1,n-9) has been demonstrated to be important for storing excess saturated FA produced after exposure to a high carbohydrate diet, an effect that is blocked by reducing SCD1 gene expression (48). Thus, enhanced *de novo* FA synthesis drives the synthesis and storage of MUFAs as a mechanism to prevent the accumulation of excessive levels of endogenous FFA. During conditions of essential FA deficiency, *de novo* FA synthesis also provides MUFAs as substrates for PUFA synthesis of mead acid (20:3,n-9) through sequential Δ6D desaturation, Elovl-1 elongation, and Δ5D desaturation of oleic acid (Figure 1.2) (8).

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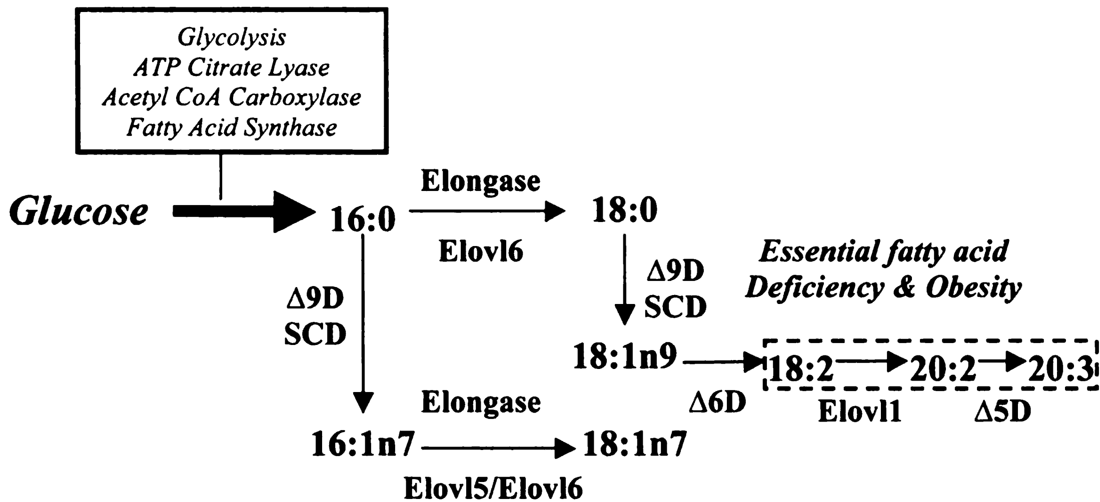

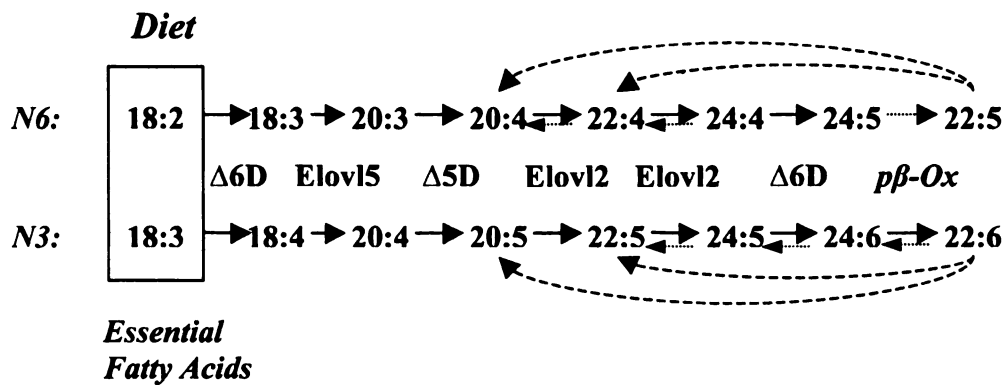

Figure 1.2. Synthesis of FAs *de novo* and from exogenous essential FAs. Glucose-derived palmitic acid (16:0) can be further modified by two pathways: Elovl-6 elongation to stearic acid (18:0) and SCD desaturation to oleic acid (18:1,n-9); or SCD desaturation to palmioleic acid (16:1,n-7) and Elovl-5/Elovl-6 elongation to vaccenic acid (18:1,n-7). Essential FAs from the diet, linoleic acid (18:2,n-6) and linolenic acid (18:3,n-3), are processed to long chain unsaturated FAs through the desaturases Δ6D and Δ5D, the elongases Elovl-5 and Elovl-2, and peroxisomal β-oxidation. During essential FA deficiency, *de novo* synthesized 18:1,n-9 is converted to mead acid (20:3,n-9) by Δ6D, Elovl-1, and Δ5D. Modified from *Fatty Acid Regulation of Gene Transcription* (8).

1.5. Glycerol

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1.5. Glycerol 3-Phosphate Pathway of Triacylglycerol Synthesis

TAG serves as a storage depot for excess intracellular FFAs. In eukaryotes, synthesis of TAG *de novo* proceeds through stepwise FA acylation of glycerol 3-phosphate, a product of the glycolytic intermediate dihydroxyacetone phosphate. In the first step, an activated FFA, fatty acyl-CoA (FA-CoA) formed from an acyl-CoA synthetase, is esterified onto glycerol 3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) at the sn-1 position to form lysophophatidic acid (LPA) (Figure 1.3). Synthesis of LPA occurs at the mitochondrial and endoplasmic reticulum (ER) membranes, as GPAT isoforms are found at both locations (49, 50). Next, another FA-CoA is transferred to LPA by a family of 1-acylglycerol-3-phosphate acyltransferases (AGPATs), located in the ER membrane, to form phosphatidic acid (PA) (51, 52). Dephosphorylation of PA by a PA phosphatase (PAP) 1, also referred to as lipin, produces diacylglyceride (DAG) (53). Both PA and DAG can be utilized for the synthesis of glycerolphospholipids. The final reaction in TAG synthesis involves the addition of a third FA-CoA to DAG, catalyzed by a DAG acyltransferase (DGAT) (53). The rate of *de novo* TAG synthesis is highly affected by substrate availability and, through both direct and indirect mechanisms, substrate-induced transcriptional regulation of genes involved in FA synthesis and storage as described below.

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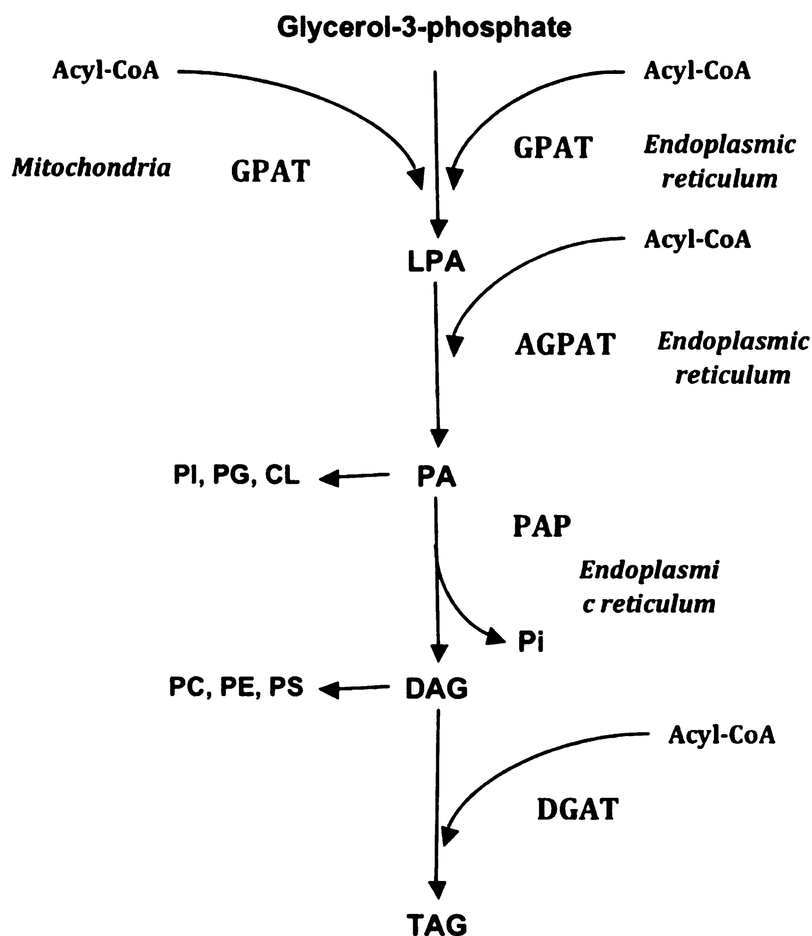

Figure 1.3. Glycerol-3 phosphate pathway for triacylglyceride synthesis. A FA-CoA is esterified to glycolysis-derived glycerol-3 phosphate by glycerol-3 phosphate acyltransferase (GPAT) to form lysophosphatidic acid (LPA) at either the mitochondrial or the ER membrane. A second FA-CoA is esterified to LPA by acylglycerol-3 phosphate acyltransferase (AGPAT) to form phosphatidic acid (PA). PA is then dephosphorylated to diacylglycerol (DAG) by PA phosphatase (PAP). Finally, a third FA-CoA is esterified to DAG by diacylglycerol acyltransferase (DGAT) to form the triacylglyceride (TAG). PA and DAG can also be directly used for phospholipid synthesis (PI, PG, CL, PC, PE, and PS).

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1.6. Transcriptional Regulators of Lipogenesis

1.6.1. Carbohydrate Response Element Binding Protein

Glucose metabolism is initiated through uptake, phosphorylation, and subsequent glycolysis. In addition, glycolytic intermediates can enter the hexose monophosphate shunt. Flux through the hexose monophosphate shunt produces xyulose 5-phosphate, an activator of protein phosphatase 2A that has been proposed to dephosphorylate and activate the carbohydrate response element binding protein (ChREBP) a member of the basic helix loop helix leucine zipper (bHLH/leu zip) transcription factor family (54). Binding of ChREBP and its heteromeric partner Max-like protein X (Mlx) to promoters of genes containing carbohydrate response elements (ChREs) activates transcription of genes involved in glucose metabolism and *de novo* FA synthesis (55, 56). Genes known to contain ChREs include L-pyruvate kinase (LPK), ACC, FAS, and mitochondrial GPAT (mtGPAT) (57-59). Studies in liver using a dominant negative Mlx demonstrated that glucose transporter 2 (GLUT2), malic enzyme (ME), and SCD1 are also regulated by the ChREBP/Mlx complex (55). Enhanced pyruvate production by increased GLUT2, LPK, and ME expression increases pyruvate entry into the mitochondria and generation of TCA cycle intermediates. Accumulation of TCA cycle intermediates favors the efflux of citrate and conversion to acetyl-CoA by ACL that is driven towards synthesis and storage of FAs via increased ChREBP/Mlx-mediated expression of ACC, FAS, SCD1, and mtGPAT.

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1.6.2. Sterol Regulatory Element Binding Protein-1c

Sterol regulatory element binding proteins (SREBPs) are bHLH/leu zip transcription factors and key regulators of lipid synthesis (60). Under non-stimulated conditions, inactive and unprocessed SREBPs reside in the ER membrane and interact with the SREBP cleavage activating protein (SCAP) and Insig-2 (61-63). Activation of SREBP involves translocation of the SREBP/SCAP complex to the Golgi where the bHLH/leu zip domain is cleaved by site 1 and site 2 proteases and subsequently transported into the nucleus to initiate gene transcription (64, 65). Isoforms of SREBP include SREBP-1a, -1c, and -2. SREBP-1c is a major activator of the expression of genes involved in FA synthesis, whereas SREBP-2 induces genes involved in cholesterol synthesis and SREBP-1a activates genes for both FA and cholesterol synthesis (60). Low intracellular sterol levels initiate processing of SREBP-1a and -2 but not -1c (66, 67). SREBP-1c processing is initiated by insulin through decreased gene expression and protein abundance of Insig-2 (68), which releases its interaction with SCAP and allows the SREBP/SCAP complex to translocate to the Golgi (62, 63). Although the mechanism is still unclear, insulin-mediated SREBP-1c processing increases the metabolism of glucose and synthesis of FAs through binding to promoters of glucokinase (GK), ACL, ACC, FAS, Elovl-6, SCD 1 and 2, and mtGPAT (59, 69-76). Elevated GK expression increases the rate-limiting step of glycolysis, while the latter enzymes increase the shuttling of TCA cycle intermediates into FAs that are stored in complex lipids. In this manner, intake of dietary carbohydrates, specifically simple sugars, activates ChREBP to divert glucose to storage as FAs, and this process is enhanced through SREBP-1c by insulin secreted from glucose-stimulated pancreatic β-cells.

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1.6.3. Liver X Receptors

Cholesterol and lipid homeostasis are also regulated by liver X receptors (LXR) α and β, members of the nuclear hormone receptor superfamily of transcription factors. LXRs are activated by intracellular oxysterols (cholesterol intermediates), and recently, LXR was identified to directly bind and be activated by glucose (77). The LXRα isoform is highly expressed in the liver, intestine, adipose tissues, and macrophages, whereas LXRβ is ubiquitously expressed (78). LXR heterodimerizes with its retinoid X receptor (RXR) partner, which then bind LXR response elements and activate transcription of genes involved in cholesterol catabolism and efflux such as CYP7A1, the rate-limiting enzyme for bile acid synthesis, and ATP-binding cassette (ABC) A1 and ABCG1, plasma membrane transporters involved in sterol transport (70, 79). Promoters of genes involved in *de novo* FA synthesis that contain LXR response elements include ChREBP and SREBP-1c as well as FAS, SCD1, SCD2, and others (80-84). The synthetic agonist T0901317 also activates LXRs, and its use in mice reduced atherosclerotic plaques caused by cholesterol accumulation (82). LXR activation in the liver, however, caused a significant increase in TAG accumulation and secretion of very low density lipoproteins (85). In addition, LXR was shown to be required for insulin-induced SREBP-1c transcription (86), and LXR α/β -/- mice fed a high carbohydrate diet have reduced expression of some genes involved in FA synthesis (87). Together this illustrates that LXR is a key mediator of *de novo* synthesis and storage of FAs.

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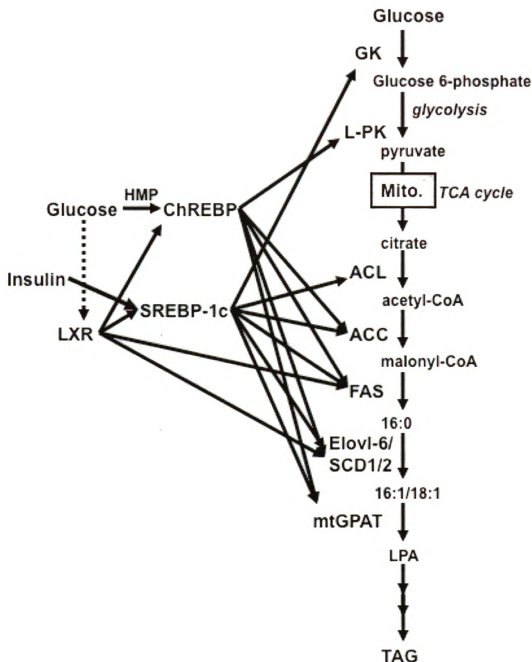

Figure 1.4. Transcriptional regulation of lipogenic gene expression by glucose and insulin. During conditions of elevated glucose, flux through the hexose monophosphate shunt (HMP) activates ChREBP to induce L-type pyruvate kinase (L-PK) and drive the synthesis of pyruvate through glycolysis. Insulin secreted into the circulation from glucose-stimulated β-cells increases SREBP-1c processing, which can induce glucokinase to enhance glucose metabolism. Both ChREBP and SREBP-1c activation lead to the induction of genes involves in *de novo* synthesis and storage of FAs. LXRs can also bind and be activated by glucose and subsequently stimulate lipogenesis idirectly through ChREBP and SREBP-1c and directly through FAS and SCD1/2.

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1.7. Fatty Acid Oxidation

In mammals, mitochondria and peroxisomes carry out chain shortening of FAs through β-oxidation. This process involves the multi-step enzymatic removal of C2 units from a FA-CoA to produce a shortened FA-CoA and acetyl-CoA, the latter of which is utilized by mitochondria to generate ATP. Mitochondrial β-oxidation of FA is dependent on carnitine palmitoyltransferase-1 (CPT-1)-mediated conversion of FA-CoAs to FA-carnitine for transport into the mitochondria. Inside the inner mitochondrial membrane, the FA-carnitine is converted back to FA-CoA by CPT-2, and β-oxidation reduces short to very long-chain FA-CoAs completely to acetyl-CoAs. Peroxisomes, however, reduce medium to very long-chain FA-CoAs to shorter chain FA-CoAs and acetyl-CoA that are transported to the mitochondria for further β-oxidation (88).

During periods of nutrient deprivation or fasting, TCA cycle intermediates decrease, which leads to the induction of FA oxidation. Reduced glycolytic and TCA cycle generation of ATP causes an increase in the AMP/ATP ratio and activation of the cellular energy sensor AMP activated protein kinase (AMPK) (89, 90). AMPK-mediated phosphorylation inhibits ACC and activates malonyl-CoA decarboxylase, effectively reducing cytosolic levels of malonyl-CoA (89, 90). Mitochondrial β-oxidation of FA is dependent on carnitine palmitoyltransferase-1 (CPT-1)-mediated conversion of FA-CoAs to FA-carnitine for transport into the mitochondria. CPT-1 activity is allosterically inhibited by malonyl-CoA (91). Thus, reduced TCA cycle flux and AMPK activation relieve CPT-1 inhibition by malonyl-CoA to allow for FA oxidation and the generation of ATP.

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Genes involved in peroxisomal β-oxidation are induced by the peroxisome proliferator activated receptor-α (PPARα) transcription factor. Natural ligands of PPARα identified thus far include FFAs, FA-CoAs and glucose (92, 93). PPARα also regulates some genes participating in mitochondrial β-oxidation (94). Together, direct glucose regulation of PPARα could possibly serve to prevent accumulation of excess intracellular FFAs during exposure to elevated levels of carbohydrates by elevating mitochondrial and peroxisomal FA oxidation. Similar to LXR, PPARα heterodimerizes with RXR to initiate transcription, suggesting that activation of these opposing pathways may compete for RXR.

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2. Pancreatic β-Cell Insulin Secretion and Role of Fatty Acid Metabolism

2.1. Mechanism of β-Cell Insulin Secretion

Pancreatic β-cells respond to a rise in circulating blood glucose levels by secreting insulin, such as after digesting a carbohydrate-containing meal. The secretion of insulin into the circulation, in turn, signals peripheral tissues to store glucose as glycogen or lipid. Glucose-stimulated insulin secretion (GSIS) requires uptake and metabolism of the sugar and occurs in two phases; an initial first-phase of insulin release lasting up to ten minutes and an amplification second-phase that begins thereafter (95-97). It is well accepted that the first-phase and initiation of the second-phase of GSIS is dependent upon an increased ratio of ATP/ADP, which results from the flux of glucose through glycolysis, the tricarboxylic acid (TCA) cycle, and mitochondrial oxidative phosphorylation (95, 97). The increased ATP/ADP ratio inhibits ATP-sensitive potassium (K+ATP) channel opening and causes depolarization of the plasma membrane, activation of voltage-dependent Ca2+ channels, and influx of Ca2+ that directly and indirectly triggers insulin release (Fig) (95-98). Stimulation of insulin secretion from β-cells in response to glucose can be potentiated by other stimulatory effectors (e.g. fatty acids, amino acids, and incretin hormones) that generate secondary messengers to signal granule exocytosis (99-102). Mechanisms involved in the amplification phase of GSIS are less understood. Evidence has emerged, however, for glucose-stimulated regulation of FA metabolism and the generation of lipid signaling molecules in the mechanism of GSIS. (99, 103, 104).

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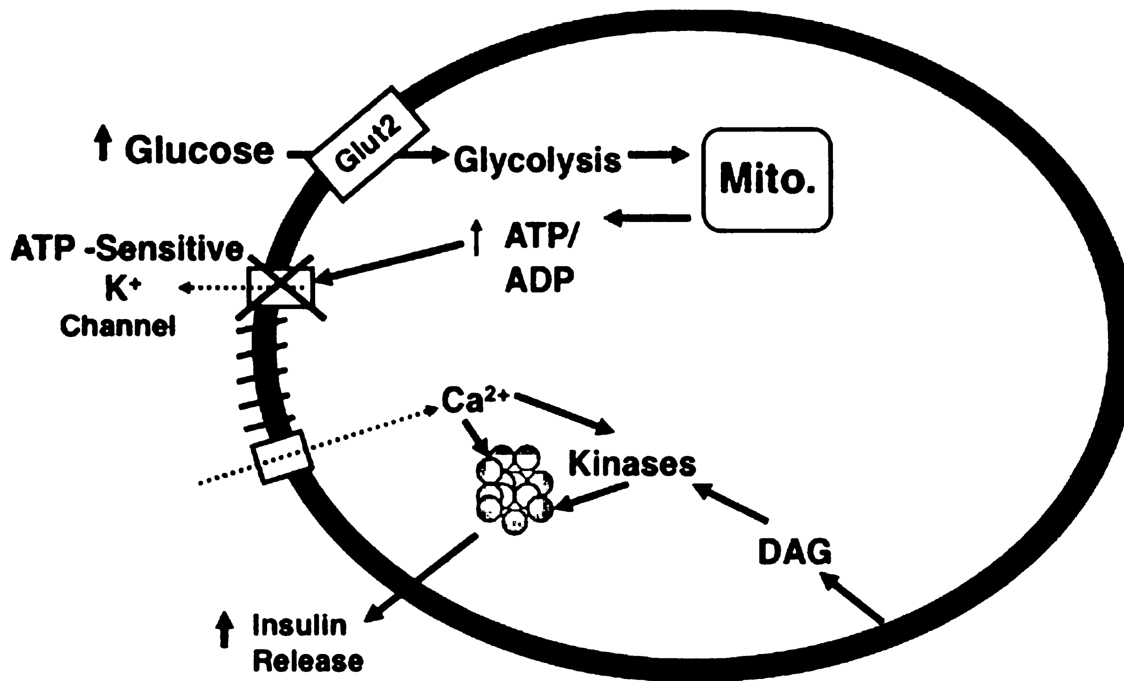

Figure 1.5. Mechanism of the ATP-sensitive K+ channel-dependent pathway of glucose-stimulated insulin secretion (GSIS). Exogenous gluocose is taken up into pancreatic β-cells through a glucose transporter (Glut2). Next, metabolism of glucose through glycolysis and the mitochondrial (Mito.) TCA cycle generates ATP and alters the ratio of ATP to ADP. The ATP-sensitive K+ channel is then inhibited by ATP, which leads to depolarization of the plasma membrane and an influx of Ca2+. Finally, insulin granules are released by both direct and indirect actions of Ca2+ on proteins involved in granule exocytosis.

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2.2. Regulation of Fatty Acid Metabolism in Pancreatic β-cells

The pancreatic β-cell is innately designed to sense and efficiently metabolize glucose over a wide range of physiological concentrations (3-20 mM), due in part to the high Km of both GLUT2 (17 mM) and GK (8 mM) (103). As glucose levels rise, minimal activity through the hexose monophosphate shunt aids in driving the glycolytic production of pyruvate (105). Together with low pyruvate conversion to lactate, due to negligible lactate dehydrogenase (105), pyruvate in the β-cell is directed towards mitochondrial TCA cycling. Studies demonstrate that stimulation of β-cells with elevated glucose subsequently increases mitochondrial efflux of TCA intermediates, ACC-mediated generation of malonyl-CoA, and the synthesis and storage of FAs (106-110). In regards to β-cell FA storage, a considerable portion of glucose is metabolized to glycerol 3-phosphate, from glycolytic dihydroxyacetone phosphate, for use in the backbone of glycerolipids (99). As in the liver, glucose-stimulated *de novo* FA synthesis in β-cells coincides with increased expression of L-PK, ACC, and FAS (110-113). In β-cells elevated glucose has been shown to induce the binding of ChREBP to L-PK as well as ChREBP and SREBP-1c binding to FAS (111, 112).

SREBP-1c activity is essential for glucose induction of lipogenic genes in β-cells (114, 115). Over-expression of SREBP-1c in a β-cell line induced the expression of ACC, FAS, Elovl-6, SCD1, and SCD2 (114), and in rat islets, increased ACC and FAS by SREBP-1c over-expression correlated with elevated TAG synthesis (116). In β-cells, although both LXRα and β are expressed (5), LXRβ is the predominant isoform and is required for maintenance of β-cell function (117). Pharmacological activation of LXRs increased the expression of ABCA1, involved in cholesterol efflux, as well as SREBP-1c,

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GK, ACC, and FAS, resulting in intracellular TAG accumulation (5, 118). In addition, elevated glucose was shown to increase LXRα translocation into the nucleus (119), the activity of which could mediate the glucose-induced increase in ChREBP gene expression observed in β-cells.

Similar to FA synthesis, FA oxidation in β-cells is subject to multiple levels of regulation by glucose. Of particular importance is the interaction of malonyl-CoA with CPT-1 and inhibition of mitochondrial FA uptake. Although high glucose reduces FA oxidation through the generation of malonyl-CoA, FA oxidation can be significantly augmented by over-expression of CPT-1 during exposure to high glucose conditions (120). Another mechanism for inhibiting FA oxidation is through generation of ATP, or reduction of the AMP/ATP ratio through enhanced glycolysis and mitochondrial oxidative phosphorylation. This is associated with decreased β-cell AMPK activity, which leads to reduced ACC phosphorylation and increased ACC-derived malonyl-CoA (121-123). Elevated glucose also causes a significant decrease in PPARα gene and protein expression as well as promoter binding to genes involved in FA oxidation (124), possibly through the decrease in AMPK activity (125). In contrast to glucose, exposure of β-cells to exogenous FAs increases CPT-1 and decreases ACC gene expression, thereby blocking glucose-stimulated repression of mitochondrial FA oxidation.

Overall, these mechanisms allow the pancreatic β-cell to function properly by directing excess exogenous carbon in the form of glucose into FAs for storage or excess FFAs to degradation via FA β-oxidation. The following sections describe the relationship between the regulation of FA metabolism and the mechanism of GSIS from β-cells.

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2.3. Mitochondrial Anaplerosis/Cataplerosis

Enhanced glucose flux through glycolysis generates pyruvate. Mitochondrial pyruvate has two options for entry into the TCA cycle: conversion by pyruvate dehydrogenase (PDH) to acetyl-CoA or conversion by pyruvate carboxylase (PC) to oxaloacetate (105, 126-128). PDH and PC activities provide the necessary substrates for initiating the sequential synthesis of TCA cycle intermediates beginning with citrate and ending with conversion of malate to oxaloacetate to reinitiate the cycle. Anaplerosis describes the replenishment of TCA cycle intermediates. After TCA cycle intermediates are fully replenished, exit of carbons from the cycle is defined as cataplerosis. In β-cells, glucose-stimulated anaplerosis via PC activity is high, metabolizing approximately half of pyruvate to oxaloacetate (105, 126-128). This anaplerosis/cataplerosis of TCA cycle intermediates generates second messengers thought to be involved in signaling for insulin secretion, including the cataplerotic products NADPH, malonyl-CoA and FA (103). The primary production of NADPH via anaplerosis/cataplerosis is pyruvate cycling, the re-synthesis of pyruvate from cycle intermediates. Pyruvate cycling processes include the malate-pyruvate, citrate-pyruvate, isocitrate/alpha-ketoglutarate-pyruvate, and oxaloacetate-pyruvate shuttles (127, 129-135). Studies using [U-13C]-glucose supported this idea showing that two pyruvate "pools" exist in β-cells (127, 132). One pool is derived from glycolytic pyruvate and another is synthesized from a TCA cycle intermediate. It is hypothesized that NADPH serves as a second messenger as the level of NADPH correlates with GSIS, and gluctaredoxin-1, an NADPH target, was recently identified to be involved in GSIS (130, 136, 137). At this time, however, it is still unclear as to which pyruvate cycling process is most important. Nevertheless, it is clear that

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glucose-stimulated cataplerosis activity is high as it drives synthesis of malonyl-CoA (100, 106, 129, 138), the precursor for *de novo* FA synthesis.

2.4. Generation of Lipid Signaling Molecules

2.4.1. Role of Malonyl-CoA

Glucose-stimulated anaplerosis/cataplerosis increases 1) mitochondrial efflux of citrate, 2) conversion of citrate to acetyl-CoA by ACL, and 3) the generation of malonyl-CoA from acetyl-CoA by ACC. The role of malonyl-CoA in GSIS is based on the ability of malonyl-CoA to inhibit CPT-1 activity (91). This blocks mitochondrial β-oxidation of FAs and leads to the accumulation of FFAs, FA-CoAs, DAG and other lipid signaling molecules. ACC gene expression is naturally high in β-cells, allowing for rapid generation of malonyl-CoA prior to GSIS (106-108, 113, 139). Increasing FA oxidation by over-expression of CPT-1 or knockdown of ACC gene expression significantly reduces GSIS (120, 140, 141). Additionally, knockdown and over-expression of PPARα increases and decreases GSIS, respectively (142, 143). Thus, GSIS involves the formation of endogenous FAs and lipid signaling molecules, which are allowed to accumulate when malonyl-CoA levels are sufficient to block FA oxidation (100, 138, 140, 141). In support of this concept, reducing malonyl-CoA levels by over-expressing cytosolic malonyl-CoA decarboxylase (MCDc) increased FA oxidation, decreased FA esterification, and reduced GSIS in the presence of endogenous FFA, a more physiologically relevant state (100). The above studies show that synthesis of malonyl-CoA plays an important role in GSIS by blocking the elimination of FA-CoAs through FA oxidation, resulting in the accumulation of lipid signaling molecules that can participate in insulin secretion.

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2.4.2. Free Fatty Acid, Long Chain Fatty Acyl-CoA, and Diacylglycerol

Inhibition of FA oxidation by malonyl-CoA permits pancreatic β-cell accumulation of lipid signaling molecules, such as FFA, long-chain FA-CoA (LC-CoA), and DAG. Exocytosis of insulin granules is a complex process involving granule synthesis, transport to the plasma membrane, docking, priming, and membrane fusion and release of insulin (144). FFA, LC-CoA, and DAG enhance insulin release through a number of mechanisms.

Intracellular FFA and LC-CoA levels are tightly regulated. Although *de novo* synthesized FAs can be rapidly converted to LC-CoA for incorporation into complex lipids, production of FFAs themselves could affect insulin release (99). Exposure to elevated glucose and exogenous FFA increases synthesis of intracellular lipid signaling molecules as well as activates islet β-cell G protein-coupled receptors, which enhances GSIS (145). GPR40 (or FFA1 receptor) is highly expressed in rodent and human islets (145, 146) and was identified to bind exogenous FFAs. The effect of GPR40 activation by FFA on GSIS involves increased intracellular Ca2+ levels through release of ER Ca2+ stores via the Gαq-phospholipase C pathway (147). Knockdown of GPR40 in mice, however, demonstrated that its activation accounts for only half of the exogenous FFA enhancement of GSIS, supporting the role of FFA-induced increases in additional endogenous lipid signaling molecules (148). In addition to circulating FFA, β-cells have been shown to release FFA when treated with elevated glucose (149). In this way, FFA-mediated GPR40 activation could be a possible mechanism for amplification of GSIS by endogenously synthesized FA.

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Elevated synthesis of LC-CoAs enhance GSIS by increasing the acylation of proteins directly involved in insulin granule exocytosis. Protein acylation is necessary for membrane targeting of specific proteins known to be involved in fusion of granules with the plasma membrane, such as synaptosomal-associated protein-25 and synaptogamin (150, 151). LC-CoAs were further shown to increase islet lipase activity (152). As discussed in the next section, this could provide an additional mechanism for amplifying the production of lipid signaling molecules. Additionally, glucose stimulation of β-cells also alters the specific species of LC-CoAs that are incorporated into phospholipids (110), which could cause membrane remodeling and effect granule fusion.

DAG is also rapidly synthesized *de novo* in β-cells by acute exposure to elevated glucose (108, 110, 153). Generation of DAG by elevated glucose has been implicated in GSIS through activation of various DAG binding proteins. DAG binding proteins include protein kinase C isoforms (PKCs), protein kinase D (PKCμ), chimaerins, Ras guanyl nucleotide-releasing proteins, mammalian homolog of *caenorhabditis elegans* UNC13 protein (Munc-13s), and DAG kinases (154). In β-cells, glucose stimulates the phosphorylation of many proteins, in part, through PKC (155), which occurs through both Ca2+-dependent (classical) and –independent (novel) PKC isoforms. Studies in β-cells have additionally demonstrated that glucose promotes the translocation of PKCs (155-157). Roles both for and against PKC activation in the mechanism of GSIS have been presented. This is in part due to the lack of isoform specific PKC inhibitors and the existence of compensatory mechanisms regulating protein phosphorylation that hinder the determination of which PKCs are directly involved in GSIS (158). Thus, a role for specific PKC isoforms in GSIS cannot be withdrawn. Enhanced activity of Munc-13

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potentiates normal GSIS *in vitro*, and islets from mice lacking Munc-13 had reduced insulin secretion and abnormal glucose tolerance (159, 160). This is in agreement with the role Munc-13 binding to DAG and being involved in synaptic granule priming (161).

2.5. Lipolysis and Glycerolipid/Fatty Acid Cycling

In addition to *de novo* synthesis, lipid-signaling molecules can also be generated from lipolysis of glycerolipids. Lipolysis describes the activity of lipase-catalyzed hydrolytic removal of FAs from a range of complex lipids. The final products of glycerolipid lipolysis are FFAs and glycerol, the latter of which cannot be reactivated to glycerol-3-phosphate for lipid synthesis in β-cells due to the absence of glycerol kinase gene expression (162). Thus, measurement of extracellular glycerol content is used as an index of lipase activity. Interestingly, rat islets and β-cells stimulated with elevated glucose have increased glycerol release (99, 163, 164), which correlates with increased activity of a number of lipases (described below) (164-167). The combination of enhanced lipolysis and increased synthesis of glycerolipids suggested FFAs released by lipolysis could be rapidly reincorporated into glycerolipids, creating a glycerolipid/FA cycle capable of enhancing the generation of lipid signaling molecules (99). In light of the fact that glucose modulates lipase activity, a number of studies have identified various lipases capable of contributing to GSIS.

2.5.1. Neutral Glycerolipid Lipolysis

2.5.1.1. Hormone-Sensitive Lipase

Glycerolipid/FA cycling in β-cells has emerged as a new metabolic pathway possibly involved in GSIS (99). Although it remains to be determined as to which pool of glycerolipids is most important, studies have drawn attention to neutral glycerolipids,

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specifically TAG and cholesterol ester (CE). Lipases known to have TAG lipolysis activity include hormone-sensitive lipase (HSL), adipose tissue TAG lipase (ATGL), adiponutrin, GS2, and carboxylesterase 3, also termed TAG hydrolase (168-173). HSL was the first TAG lipase identified to be expressed in β-cells and regulated by glucose (174, 175). A role for HSL in GSIS has been unclear as independent lines of HSL null mice generated on different genetic backgrounds exhibited inconsistent insulin secretory responses (163, 176). Lipolysis was still present and activated by glucose in HSL null mice, demonstrating the presence of other lipases involved in β-cell function (163). Additionally, HSL has a higher substrate preference for DAG, MAG, and CE than for TAG (168, 177). Studies found the hydrolysis of neutral cholesterol esters rather than DAG was completely blocked in HSL null mice and that its effect on insulin secretion maybe through directly regulating membrane cholesterol levels and granule exocytosis (178, 179). In fact, HSL was found localized on insulin granule membranes (180). Mice with a β-cell specific deletion of HSL were found to have significantly reduced first-phase of insulin release, thus showing that HSL has a direct role in insulin exocytosis (178). Taken together, these studies show that HSL may participate in insulin granule exocytosis, but HSL is not the key TAG lipase linked to GSIS.

2.5.1.2. Adipose Triacylglyceride Lipase

Enzymatic characterization of ATGL, adiponutrin, GS2, and carboxylesterase 3 showed that these lipases have a higher activity for TAG than DAG compared to HSL (168, 170, 172, 173). Recently, ATGL gene expression was identified to be much higher than adiponutrin, GS2, and HSL in both rat islets and the INS832/13 β-cell line (165). Regulation of ATGL gene expression was found to be reduced in islets of fed versus

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fasted rats, and this correlated with reduced ATGL gene expression when β-cells were exposed to long-term elevated concentrations of glucose (165). Knockdown of ATGL gene expression in β-cells reduced TAG lipase activity, increased TAG content, and decreased insulin secretion in response to glucose, exogenous FA, and the incretin hormone glucagon-like peptide-1 (165). Similarly, isolated islets from ATGL null mice displayed increased TAG content and decreased glucose- and exogenous FA-stimulated insulin secretion (165). In both β-cells and mouse islets with reduced ATGL expression, however, there was no change in total glycerol release. The absence of a change in total glycerol release demonstrates a compensatory mechanism that could be activated to maintain lipolytic activity. This is at odds with ATGL having a major role in total glycerol release. The authors propose that β-cells contain fuel–insensitive and –sensitive TAG pools. Fuel-sensitive TAG pools localized close to insulin granules could then be regulated by ATGL and other TAG/FA cycling enzymes and provide lipid signaling molecules that participate in insulin secretion.

2.5.2. Phospholipases C and D

Glycerophospholipids represent a considerable pool for glycerolipid/FA cycling of *de novo* and exogenously derived FAs for participation in GSIS. As described above, Ca2+ influx upon exposure to elevated glucose signals for insulin release through both direct and indirect mechanisms. Of these mechanisms, influx of Ca2+ activates phospholipase C (PLC), which cleaves phosphatidylinositol to generate inositol phosphates and DAG (166, 181). Insulin secretion from glucose-stimulated β-cells parallels an increase in the release of inositol phosphates (166), which can amplify insulin secretion by activating the release of Ca2+ from intracellular stores (182-184).

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Compared to rat islets, the second-phase of insulin release in mouse islets is less pronounced and coincides with lower levels PLC expression and activity (166, 185). This reduced second-phase in mouse islets could be elevated to levels comparable to rat islets by pharmacological activation of PLC or DAG binding proteins (166). From these studies it is apparent that active release of inositol and DAG by PLC lipolysis of phosphatidylinositol has a role in GSIS.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to choline and phosphatidic acid. Subsequent dephosphorylation of phosphatidic acid by phosphatidic acid phosphatase can also generate DAG. Two isoforms of PLD exist in mammals, PLD1 was found in the Golgi apparatus and on intracellular vesicles, while PLD2 was localized primarily to the plasma membrane (186, 187). Intracellular vesicle trafficking, endocytosis, and exocytosis are associated with increased PLD activity (188). The release of phosphatidic acid derived from PLD likely aides in membrane fusion by promoting curvature of the membrane due to the small negatively charged head-group (189). Interestingly, PKC is able to activate PLD by direct interaction rather than phosphorylation (186). Thus, it is feasible that generation of DAG from phosphatic acid phosphatase or PLC could induce translocation of PKC to activate PLD and facilitate membrane fusion. In β-cells, GSIS was elevated and reduced by over-expression and knockdown of PLD1, respectively (167). In addition, PLD1 was partially localized to insulin granules (167). These findings suggest PLD may have two roles, one to provide phosphatidic acid for glycerolipid/FA cycling and another to directly effect insulin granule exocytosis.

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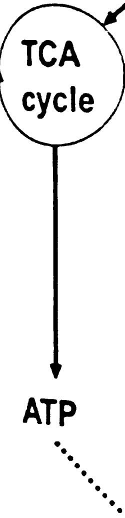
2.5.3. Phospholipase A2

Lipolysis of glycerophospholipids by phospholipase A at either the sn-1 (PLA1) or sn-2 (PLA2) position generates FFA and lysophospholipids, the latter of which can also contribute to glycerolipid/FA cycling and act in signal transduction (190). In rodent and human islets, a Ca2+-independent ATP-sensitive PLA2 (iPLA2β) was identified with specific activity towards arachidonic acid at the sn-2 position (191). Arachidonic acid (AA) comprises approximately thirty percent of the total glycerophospholipid FA mass in rodent islets (192), and free AA accumulates in islets stimulated with glucose (193). Inhibition of potassium channels by ATP is amplified by increased cytosolic AA (194), and *in vitro* studies showed knockdown and over-expression of iPLA2β reduced and amplified GSIS, respectively (195, 196). In mice, however, failure of iPLA2β over-expression to amplify GSIS and inconsistencies in iPLA2β knockdown mice question whether iPLA2β is involved in normal glucose homeostasis (190, 197, 198).



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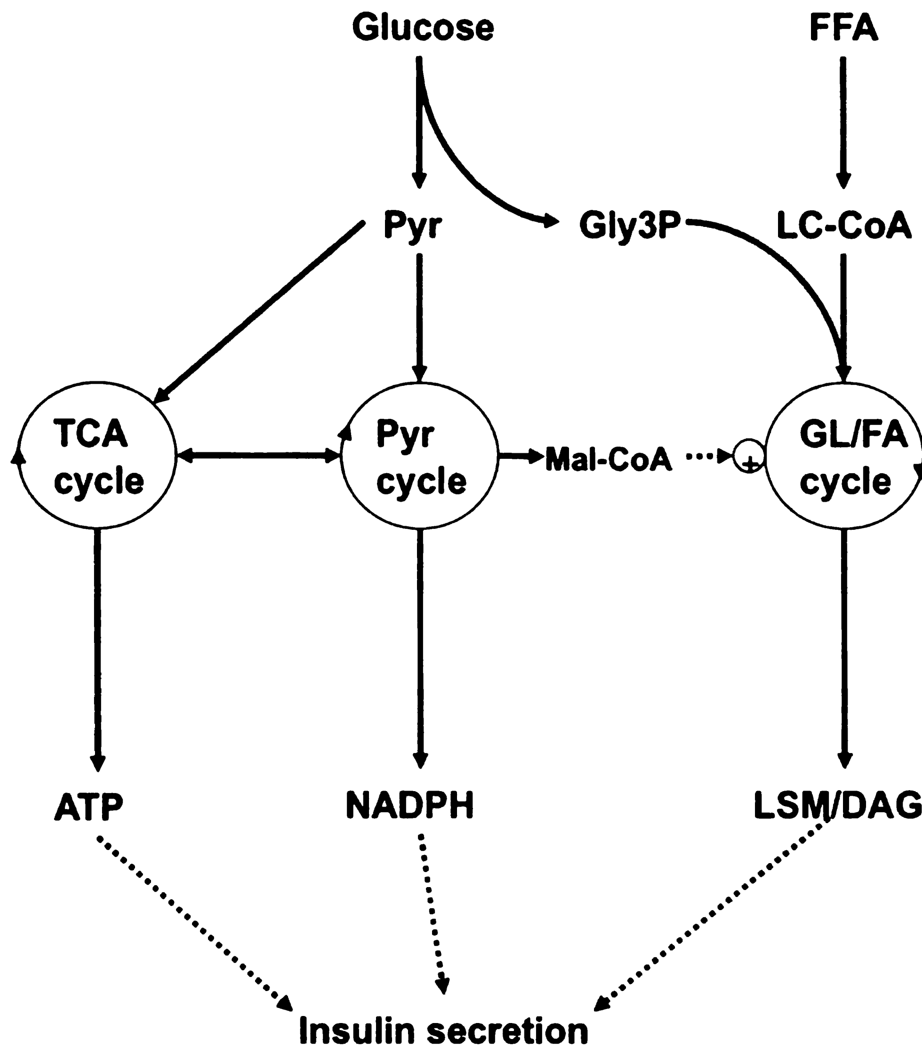

Figure 1.6. Proposed model for the role of FA metabolism in glucose-stimulated insulin secretion. Glucose stimulation drives the generation of pyruvate (Pyr), which enters the TCA cycle to modulate ATP production and undergoes pyruvate cycling to generate NADPH. Mitochondrial cataplerosis and pyruvate cycling increase the level of malonyl-CoA, which blocks FA oxidation. De novo and exogenous FFAs are converted to LC-CoA and esterified to glycerol 3-phosphate (Gly3P) to enter into the glycerolipid/FA cylc (GL/FA cycle). Finally, the combination of GL/FA cycle-mediated generation of lipid signaling molecules (LSM), such as DAG, increased NADPH, and increased ATP/ADP ratio signal the β-cell to secrete insulin.

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3. Glucose and Fatty Acid Mediated Pancreatic β-Cell Dysfunction

3.1. Glucotoxicity vs. Glucolipotoxicity in Type 2 Diabetes

Type 2 diabetes (T2D) is characterized by insulin resistance and pancreatic β-cell dysfunction, due to both genetic and environmental factors, that results in the inability to maintain glucose homeostasis. In the early stages of the pathogenesis of T2D, the onset of insulin resistance causes glucose levels to rise and triggers β-cell compensatory responses that include increased synthesis and secretion of insulin. T2D ensues when β-cells fail to secrete sufficient amounts of insulin to maintain normoglycemia. Glucotoxicity and glucolipotoxicity have been proposed to underlie this progression of β-cells from compensation towards dysfunction and eventually failure (3, 199). Sustained periods of hyperglycemia are associated with adverse effects on the β-cell such as diminished GSIS, decreased insulin gene expression, and apoptosis, collectively referred to as glucotoxicity (4, 199). In obesity-associated T2D, hyperglycemia is often accompanied by hyperlipidemia and elevated levels of plasma FFAs, which also contribute to β-cell dysfunction (200-202). Damage due to chronic elevations in FFAs, referred to as lipotoxicity (203), was found to require FA esterification and to be dependent upon elevated glucose (3). This concept of β-cell dysfunction from the combination of glucose and FAs is defined as glucolipotoxicity (3). The major mechanisms accounting for the effects of both glucotoxicity and glucolipotoxicity recognized thus far are reviewed in the following sections.

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3.2. Mechanisms of β-Cell Failure

3.2.1. Endoplasmic Reticulum Stress

Hyperglycemia signals β-cells to continuously synthesize and secrete large quantities of insulin. Over time, the rate of insulin translation exceeds the protein folding capacity of the β-cell endoplasmic reticulum (ER), causing accumulation of unfolded proteins (204). If sustained, buildup of unfolded proteins causes ER stress and activation of the unfolded protein response (UPR). The UPR acts to reduce ER stress, preserve ER integrity, and prevent cell death. Transduction of this response is mediated by the ER transmembrane proteins inositol requiring ER-to-nucleus signal kinase (IRE) 1, double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK), and activating transcription factor (ATF) 6 (204). ER stress induces IRE1 dimerization and autophosphorylation to gain endoribonuclease activity and splice the mRNA encoding X-box binding protein (Xbp) 1 (204). Spliced Xbp1 (Xbp1s) protein transcriptionally activates genes involved in expansion of the ER, protein maturation, protein folding and export from the ER, degradation of misfolded proteins, and lipid metabolism (205-210). PERK and ATF6 remain inactive in the ER by binding with the ER chaperone Ig heavy chain binding protein (BiP, also known as GRP78 and HSPA5) on the luminal side of membrane and are activated as BiP detaches from the membrane to assist in protein folding (211-214). PERK phosphorylates and inactivates eukaryotic translation initiation factor (eIF) 2α, leading to decreased translation of most proteins except some specific proteins such as ATF4 (215, 216). ATF4 induces genes important for amino acid import and resistance to oxidative stress (217). Activated ATF6 is translocated to the Golgi, cleaved, and transported into the nucleus to initiate transcription of ER chaperone genes

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such as BiP, enhancing ER protein folding capacity (218, 219). Together, IRE1, PERK, and ATF6 pathways act to preserve ER function, which if not maintained can lead to β-cell failure and ultimately cell death (204).

Rat islets cultured in elevated glucose demonstrated that glucotoxic conditions can induce the UPR, exhibiting activation of IRE1 (by increased Xbp1s) and ATF6 (by increased BiP expression) (220). This correlates with increased Xbp1s and BiP expression in T2D human islets cultured in elevated glucose compared to non-diabetic controls (221). Exposure of β-cells to elevated concentrations of exogenous FFAs also activates UPR pathways. Multiple β-cell line models treated with the saturated FA palmitate significantly induces Xbp1s, eIF2α phosphorylation, and protein levels of ATF4 and ATF6 (222-224), whereas only minimal ER stress is elicited by the MUFA oleate. Activation of all three UPR pathways by palmitate was demonstrated in human islets as well (222). These effects in a β-cell line could be partially reversed by over-expressing the ER chaperone BiP (224). In addition to UPR pathways, electron microscopy of β-cells treated with palmitate and pancreatic sections from T2D patients observed alterations in β-cell ER integrity reflected as distention of the ER (221, 223). Thus, the inability to maintain proper ER function in the β-cell could be a significant risk factor for the development of T2D.

3.2.2. Oxidative Stress

Pancreatic β-cells have low levels of antioxidant enzymes compared with other tissues (225, 226), rendering β-cells particularly susceptible to oxidative stress. Elevated production of reactive oxygen species during chronic hyperglycemia is detrimental to β-cells (4). Pathways through which ROS can be produced from elevated glucose include

oxidative phosphorylation, glyceraldehyde autoxidation to methylglyoxal and glycation, α-ketoaldehyde formation and glycosylation, DAG activation of PKCs, hexosamine metabolism, and sorbitol metabolism (227). In β-cell lines and isolated islets, approaches to reduce ROS production by use of antioxidants and over-expression of antioxidant enzymes protected from chronically elevated glucose-induced decreases in insulin gene expression, transcription factor binding to the insulin gene promoter, and GSIS (4, 228, 229). This demonstrates β-cell function is significantly affected by chronic hyperglycemia-induced oxidative stress. The involvement of oxidative stress in FA-mediated β-cell dysfunction, however, remains unclear, as evidence both for and against a role of FAs in oxidative stress have been presented (230, 231).

3.2.3. Malonyl-CoA Inhibition of Fatty Acid Oxidation and Lipid Accumulation

Generation of malonyl-CoA via ACC activation during short-term glucose exposure is essential to normal β-cell GSIS due to the ability of malonyl-CoA to interact with CPT-1 and inhibit FA oxidation. Chronic hyperglycemia, however, continuously drives malonyl-CoA production and subsequent synthesis and storage of FAs into TAG, which has been linked to diminished β-cell GSIS (199, 232). The effects of chronic hyperglycemia on *de novo* lipogenesis, TAG accumulation, and loss of β-cell function was found to correlate with elevated nuclear SREBP-1c in diabetic islets (233, 234). Over-expression of a constitutively active nuclear SREBP-1c in islets and β-cell lines also resulted in increased lipogenesis and reduced GSIS (116, 235). This suggested that sustained glucose-stimulated lipogenesis and TAG accumulation via SREBP-1c activation facilitated diminished GSIS.

SREBP-1c has also been implicated in glucolipotoxicity during β-cell exposure to elevated levels of exogenous FFAs. Treatment of islets with elevated palmitate increased lipogenic gene expression and the nuclear form of SREBP-1c, increased TAG content, and decreased GSIS (236). These effects were prevented by treatment with eicosapentaenoate (20:5,n-3), which is known to reduce SREBP-1c processing (236). More direct evidence of a relationship between malonyl-CoA and FA oxidation comes from examination of palmitate-induced β-cell death. Elevating FA oxidation through AMPK and PPARα activation protected β-cells from palmitate-induced cell death, whereas blocking CPT-1 activity increased susceptibility to cell death from palmitate (237, 238). Together, these studies demonstrate that the intracellular capacity to modulate FA synthesis and oxidation could significantly predispose β-cells to the damaging effects of glucotoxicity and glucolipotoxicity.

3.2.4. Dysregulated Glycerolipid/Fatty Acid Cycling

The role of glycerolipid/FA cycling in normal β-cell function, particularly GSIS, relies on balanced synthesis and turnover of neutral lipid pools. To determine if excessive TAG synthesis was directly implicated in the loss of GSIS during chronic hyperglycemia, the final step of TAG synthesis in islets was increased by over-expression of DGAT1 (239). Elevated DGAT1 increased TAG accumulation and reduced GSIS, demonstrating that a loss of β-cell function may occur without a paralleled increase in TAG turnover (239). In line with this idea, knockdown of the TAG lipase ATGL reduced TAG turnover as well as GSIS (165). Conversely, excessive neutral lipid hydrolysis by over-expression of HSL also impairs GSIS (240), further supporting the importance of balanced lipid synthesis and turnover for normal β-cell function. In

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addition to glucotoxicity, exogenous saturated FAs are incorporated less efficiently into TAGs than MUFAs, which coincides with diminished GSIS and increased cell death caused by saturated FAs (241). These studies show that dysregulation of glycerolipid/FA cycling contributes to β-cell dysfunction.

3.2.5. Apoptosis-Mediated Loss of β-Cell Mass

Reduced β-cell mass, or number of β-cells, that occurs naturally or due to β-cell failure and apoptosis is a critical factor in the development of T2D. In obese individuals, the level of fasting plasma glucose inversely correlates with the average percent of β-cells occupying the whole pancreas (242). Although the mechanisms are not entirely clear, loss of β-cell mass has been attributed to both ER and mitochondrial induction of apoptosis. Unresolved ER stress can induce apoptosis via pathways that include the ATF4-mediated transcription of the proapoptotic genes CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) and ATF3 (243, 244) and IRE1-mediated activation of c-Jun N-terminal kinase (JNK) (245, 246). CHOP induction is increased in islets cultured in hyperglycemic conditions, exposed to elevated palmitate concentrations, and from human T2D patients (220, 222, 224). Protection from β-cell death by CHOP deletion in multiple diabetes models further demonstrated CHOP to be a major component of ER stress-mediated apoptosis (247). Activation of JNK affects multiple pathways, such as CHOP induction, and inhibition of JNK activity in β-cells partially protects from palmitate-induced apoptosis (222). In addition to the IRE1 and PERK pathways, palmitate-induced β-cell ER stress depleted Ca2+ from the ER (222), which can trigger mitochondrial release of cytochrome C and initiation of the caspase-9 cascade (248, 249). This cascade in part causes deoxyribonuclease-mediated DNA degradation

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(250), an effective marker for palmitate-induced β-cell apoptosis (223, 224). Mitochondrial release of cytochrome C has also been associated with generation of ROS (251). Taken as a whole, this illustrates that β-cell dysfunction from exposure to chronic conditions of glucotoxicity and glucolipotoxicity can lead to the induction of downstream apoptotic pathways and contribute to reduced β-cell mass.

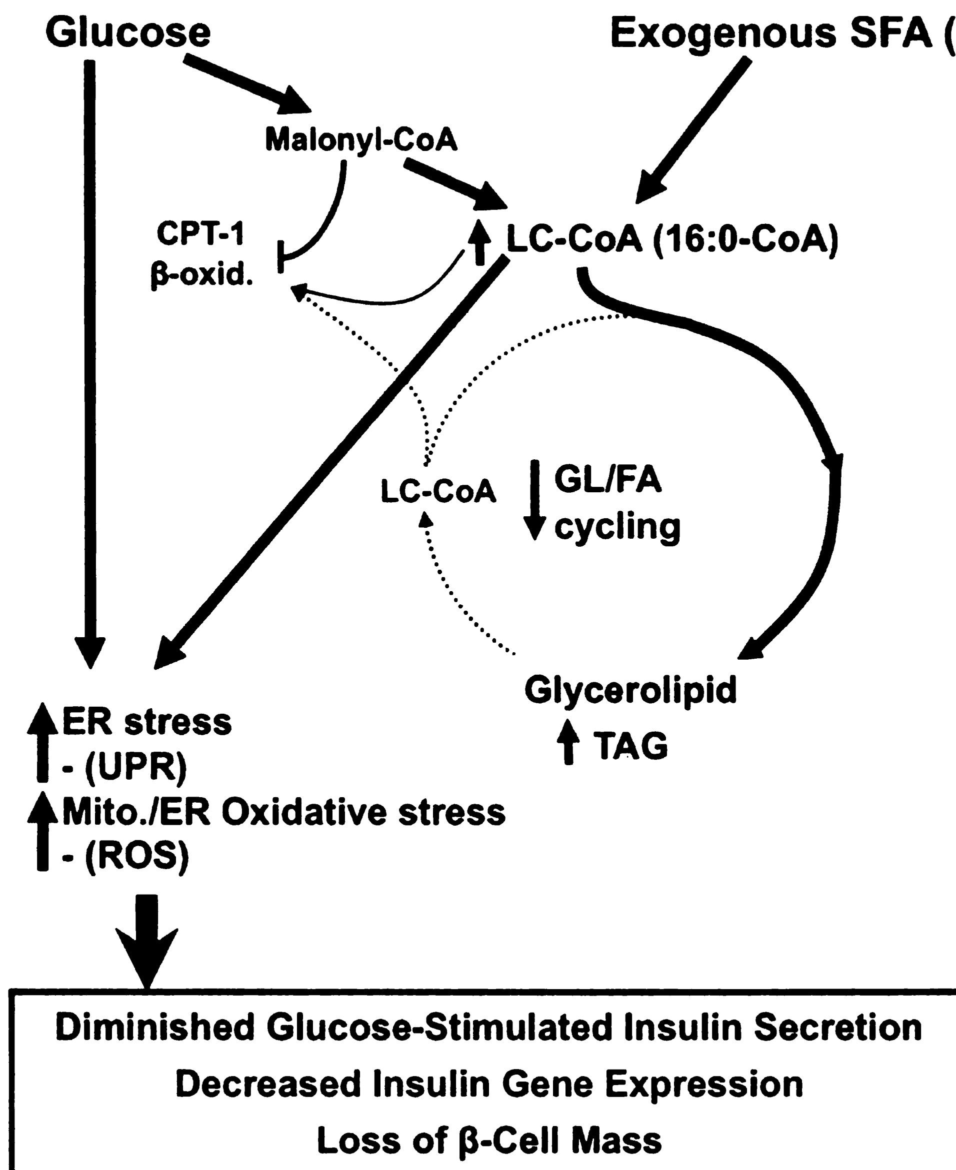

Figure 1.7. Mechanisms involved in β-cell dysfunction from glucolipotoxicity. Chronically elevated glucose drives malonyl-CoA synthesis, which blocks mitochondrial FA oxidation by inhibition of CPT-1. Next, LC-CoAs formed from de novo and exogenous saturated FA (SFA) are mostly driven towards glycerolipid storage as TAG. Accumulation of endogenous glucose metabolites and SFA-CoAs causes ER and oxidative stress to the β-cell, displaying activation of the unfolded protein response (UPR) and generation of reactive oxygen species (ROS). Sustained UPR activation and ROS generation lead to diminished glucose-stimulated insulin secretion, decreased insulin gene expression, and ultimately a loss of β-cell mass.

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3.3. Evidence and Mechanisms of β-cell Compensation

Prior to the onset of T2D, β-cells are capable of activating various compensatory mechanisms to cope with the increased demand for insulin secretion and to prevent β-cell failure from glucolipotoxicity. Of these mechanisms, regulation of FA metabolism through enhanced lipogenesis, glycerolipid/FA cycling, and MUFA synthesis have been identified to participate in β-cell compensation.

3.3.1. SREBP-1c and Liver X Receptors

In animal models of T2D, diminished GSIS has been associated with increased expression of SREBP-1c and TAG accumulation in islets, and this led to the hypothesis that increased neutral lipid storage via SREBP-1c activation was toxic to β-cells (233). The link between SREBP-1c activation and loss of GSIS was shown by over-expression of a constitutively active form of SREBP-1c, which forced lipogenic gene expression, increased TAG synthesis, and reduced GSIS (116). This idea, however, was contradicted by studies showing that the ability of islets to compensate for chronic hyperglycemia by secreting more insulin was dependent on SREBP-1c (234). In the Zucker diabetic fatty (ZDF) rat, increased islet SREBP-1c expression coincides with elevated lipogenesis and decreased GSIS (233). It was recently shown, however, that over-expression of a dominant negative SREBP-1c in ZDF rat islets prevented the increase in lipogenic gene expression and TAG content, but it did not correct for the diminished GSIS (252). In addition to SREBP-1c, activation of LXRs with the synthetic agonist T0901317 in isolated rat islets and β-cell lines increased nuclear SREBP-1c accumulation, lipogenic gene expression, and TAG content as well as elevated both basal and GSIS under normal culture conditions (5, 118). Although elevated insulin secretion from LXR-activated β-

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cells was inhibited by knockdown of SREBP-1c (253), its mechanism is still unknown. Furthermore, it remains to be determined if LXR activation can enhance insulin secretion from β-cells cultured chronically in hyperglycemic conditions. Together, these studies suggest that enhanced *de novo* lipogenesis may have a critical role in β-cell compensation in response to glucotoxic and glucolipotoxic conditions.

3.3.2. Glycerolipid/Fatty Acid Cycling

Dysregulation of glycerolipid/FA cycling in β-cells has been shown in various models to cause reduced GSIS. This implies that balanced incorporation and turnover of FAs into glycerolipids is important for maintenance of β-cell function. Unlike the ZDF rats, obese Zucker fatty (ZF) rats exhibit insulin resistance but maintain normoglycemia. The ability of ZF rats to maintain glucose homeostasis was associated with enhanced β-cell glucose- and FA-stimulated insulin secretion compared to lean control rats (104). ZF rat islets were identified to have increased glucose-responsive capacities for FFA esterification and lipolysis, evidence of enhanced glycerolipid/FA cycling (104). Inhibition of lipolysis by the general lipase inhibitor orlistat blocked the increased insulin secretory response (104). In addition, FA oxidation tended to be elevated as well (104), indicating ZF islets may have enhanced activation of pathways for detoxification of excess FFAs. This *in vivo* evidence demonstrates that enhancing both synthesis and turnover of glycerolipids in β-cells may significantly reduce susceptibility to developing T2D in obese individuals.

3.3.3. Monounsaturated Fatty Acid Synthesis

Elevated TAGs often occur in obesity-associated T2D and has been correlated with high expression and activity of SCD1 in adipose and liver (254). In rodent models

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lacking SCD1 expression, TAG levels remain low and these animals are protected from high fat- and high carbohydrate-diet induced obesity and T2D (48, 255), suggesting that SCDs could be potential pharmacological targets for the treatment of these conditions. One study, however, showed that deletion of SCD1 in mice that are genetically susceptible to obesity and T2D resulted in an earlier onset of T2D (256). This effect was associated with the accumulation of saturated FAs in islets and diminished GSIS (256), suggesting SCD1 expression and activity is important for maintenance of β-cell function. In agreement with this hypothesis, rat islets and β-cell lines with naturally occurring high levels of SCD1 expression are protected from palmitate-induced cell death (6, 257). It is likely, however, that altered expression of other genes could also be involved as well. For example, the subpopulation of palmitate-resistant β-cells from the MIN-6 β-cell line also exhibited increased expression of CPT-1 and enhanced FA oxidation, which would contribute to the detoxification of palmitate (6). Whether over-expression of SCD by itself can protect β-cells from saturated FA-induced dysfunction remains to be determined. Intriguingly, the SCD2 isoform is highly expressed in rat islet β-cells (257), raising the question of whether SCD2 has a significant role in islet β-cell function.

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4. Statement of Problem and Specific Aims

Regulation of FA metabolism significantly affects pancreatic β-cell responses to glucolipotoxicity. The role of SREBP-1c in β-cell compensation versus failure appears to be dependent on the level of its activation. Using a synthetic LXR agonist, LXR-mediated regulation of SREBP-1c provides a way to examine the effects of moderate activation of lipogenesis on β-cell function. Our lab identified LXR activation to elevate both basal insulin secretion and GSIS from INS-1 β-cells chronically cultured in high glucose. In light of this finding, I explored the effects of LXR activation on β-cell *de novo* lipogenesis and the mechanism of elevated insulin secretion. During this research I found that LXR-activated β-cells have an enhanced capacity for *de novo* MUFA synthesis, and this coincided with increased expression of SCD1 and SCD2. Unlike SCDs, the rate-limiting enzymes for MUFA synthesis, role of FA elongases in *de novo* MUFA synthesis is unknown. Thus, based on known substrate specificity elongation assays, I examined the effects of altered Elovl-5 and Elovl-6 expression on *de novo* MUFA synthesis. In contrast to the liver, less is known about FA desaturation and elongation in β-cells. To better understand the role of β-cell FA desaturation and elongation, I characterized FA desaturase and elongase gene expression in INS-1 β-cells and islets from the ZDF rat model of progressive β-cell failure. In addition, I examined the effects of altering SCD2 and Elovl-6 expression on β-cell FA metabolism and function in response to elevated levels of exogenous saturated FAs.

I hypothesized that modulation of de novo lipogenesis and monounsaturated FA synthesis significantly effects pancreatic β-cell compensation in response to glucolipotoxicity.

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The specific aims are:

Aim 1: To characterize the effects of LXR-activation on *de novo* FA and neutral lipid synthesis and to determine which aspects of FA metabolism contribute to elevated basal insulin release and GSIS during chronic hyperglycemia.

Aim 2: To define the roles of the FA elongases Elovl-5 and Elovl-6 in the *de novo* synthesis of MUFAs.

Aim 3: To characterizes the expression of FA desaturase and elongase genes in a setting of progressive β-cell failure and to examine the effects of altering the expression of genes involved in MUFA synthesis on β-cell FA metabolism and viability in response to exogenous saturated FAs.

The findings from this research will contribute to the understanding of how regulation of FA metabolism effects β-cell compensation in response to glucolipotoxicity.

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Chapter 2. Materials and Methods

1. Materials. T0901317 was purchased from Cayman Chemical (Ann Arbor, MI). Palmitic acid was from Nu-Chek Prep, Inc. (Elysian, MN). [1-14C]palmitic acid and D-[5-3H]glucose were from PerkinElmer Life Sciences (Boston, MA). [2-14C]acetic acid and D-[U-14C]glucose were from ICN Pharmaceuticals, Inc (Costa Mesa, CA). Fatty acid-free BSA for insulin secretion studies was from Roche Applied Science (Indianapolis, IN). Fatty acid-free BSA for FA treatments was from Celliance (Kankakee, IL). Primary antibodies used at a 1:1000 dilution, unless noted otherwise, were phospho-JNK (pSAPK/JNK; 9255), total JNK (SAPK/JNK; 9252), and cleaved caspase-9 (9507) from Cell Signaling Technology (Beverly, MA); SREBP-1 (IgG-2A4), GADD153/CHOP (sc-7351) and actin-HRP (horseradish peroxidase-conjugated; sc-1615-HRP) at 1:3300 from Santa Cruz (Santa Cruz, CA). Secondary antibodies used at 1:3300 were goat anti-rabbit-HRP from Vector Laboratories, Inc. (Burlingame, CA) and goat anti-mouse-HRP from Bio-Rad Laboratories (Hercules, CA). Apoptosis ELISA kit was from Roche Diagnostics (Cell Death Detection ELISA; Indianapolis, IN).

2. Fatty acid preparation. Palmitic acid was bound to fatty acid free BSA as described previously (258). Briefly, stock solutions of 100 mM palmitic acid dissolved in 0.1 M sodium hydroxide and 5% fatty acid free BSA dissolved in RPMI-1640 without glucose were heated to 70°C and 55°C, respectively. The palmitic acid solution was then added drop-wise into the BSA solution to make a final stock of 5 mM palmitic acid/5% BSA. The solution was then kept at 55°C for 10 min, vortexed, brought to room temperature, and either used immediately or stored at –20°C for up to 3 three weeks. Aliquots of the 5 mM/5% palmitic acid/BSA stocks were then thawed at 55°C for 15 min, vortexed, and

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brought to room temperature before diluting into culture media to give the final indicated concentrations of fatty acid and BSA (0.5%).

3. Human islets. Human islets were obtained from the Juvenile Diabetes Research Foundation Human Islet Distribution Program at the University of Minnesota, University of Miami, and Northwest Tissue Center, Seattle. Islets were maintained in N2 medium or RPMI-1640 plus 10% FBS containing 100 units/ml penicillin, 100 μg/ml streptomycin and 0.5 μg/ml fungizone. For gene expression experiments, islets were cultured for three days in N2 medium in the absence or presence of 10 μM T0901317. For lipid synthesis experiments, islets were cultured for 36 hrs in RPMI-1640 medium containing 11.1 or 22.1 mM glucose plus 5 μM T0901317 (see below). Then incubated for 12 hrs under the same conditions in the presence of 1 μCi [2-14C]acetic acid.

4. Animals and islet isolation. All animal procedures were approved by the institutional animal care and use committee at Michigan State University. Sprague-Dawley rats were from Charles River Laboratories and fed Harlan-Teklad laboratory chow (No. 8640) prior to islet isolation between 8-10 weeks old. Male *fa/fa* Zucker Diabetic Fatty rats (ZDF-*Leprfa*/Crl and lean controls (*fa*/?) received at 4 and 11 weeks of age were purchased from Charles River Laboratories and fed the Purina 5008 diet. All animals were kept on a 12:12 hr light:dark cycle with food and water ad libitum. At 6 and 13 weeks of age, ZDF rats were weighed and blood glucose measured. Prior to islet isolation, blood was collected from the abdominal aorta and plasma insulin levels were measured using a Rat Insulin Radioimmunoassay (RIA) kit (Millipore, Billerica, MA). Islets were dissociated from pancreatic tissue by collagenase digestion and isolated by hand.

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5. INS-1 cell culture. INS-1 cells were routinely cultured in INS-1 media (RPMI-1640 media containing 11.1 mM glucose, 10% FBS, 1 mM pyruvate, 10 mM Hepes, 50 μM 2-mercaptoethanol, 100 units penicillin/ml and 100 μg streptomycin/ml) as previously described (259). In all experiments, cells were seeded at a density of 0.2x106 cells per cm2 and cultured for 24 or 48 hrs in INS-1 media. Cells were then cultured for 48 hrs in INS-1 media containing 4 or 16.7 mM glucose and vehicle or 10 μM T0901317. For fatty acid treatments, cells were cultured for the indicated times in a modified INS-1 media containing 0.5% FBS and palmitate complexed to BSA as described below.

6. siRNA treatment. Control, Elovl-5, Elovl-6 and SCD2 siRNA were from Dharmacon, Inc. (Lafayette, CO). siRNAs were introduced into cells by electroporation using the Amaxa Nucleofector (program D-026, Gaithersberg, MD) in electroporation buffer (7 mM ATP, 11.6 mM MgCl2-6H2O, 68 mM K2HPO4, 14 mM NaHCO3, and 2.2 mM glucose). Cells were cultured for 24 hrs in INS-1 media and subsequently treated with modified INS-1 media containing increasing concentrations of palmitic acid without or with 1 μM Gö6976 for the indicated times. Cells were then harvested for mRNA and protein analysis, and apoptosis (see below). For FA and complex lipid analyses using palmitate, electroporated cells were cultured for 24 hrs in INS-1 media followed by 12 hr treatment with modified INS-1 media plus 400 μM palmitic acid and 1μCi [1-14C]palmitic acid. For *de novo* FA analysis, cells were cultured for 24 hrs in 5.5 mM glucose and subsequently cultured for 24 hrs in INS-1 media containing 11.1 mM glucose. Cells were then harvested for mRNA analysis or continued culturing overnight with 1 μCi [2-14C]-acetic acid (51 mCi/mmol, ICN Pharmaceuticals, Inc., Coasta Mesa, CA), after which lipids were extracted and analyzed for 14C incorporation.

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7. Adenovirus preparation. Elovl-5 and Elovl-6 cDNA were cloned and used for construction of recombinant adenoviruses as previously described (35). SCD2 cDNA was cloned into TOPO plasmids (Invitrogen) from reverse transcribed INS-1 cell mRNA using the following primers: sense 5'-ATAGTCGACATGCCGGCTCACATACTGCAAGAG-3'; antisense 5'-ATACTCGAGTCAGCCACTCTTGCAGCTCTCCTCCCC-3' (Acc. No. AB032243). A recombinant adenovirus over-expressing SCD2 was constructed using the Adenoviral Vector System (Stratagene). In short, the coding regions of SCD2 were ligated into pShuttle-CMV, recombined with pAdEasy-1 in BJ5183 cells, propagated in XL10 Gold ultracompetent cells, and packaged into adenoviral particles in Ad-293 cells. Adenoviruses were further amplified and then titered in HEK293 cells using the Adeno-X Rapid Titer kit (Clontech, Mountainview, CA). An adenovirus over-expressing β-galactosidase was obtained from Dr Newgard, Duke University, North Carolina. For transduction of genes into INS-1 cells, 90% confluent cell cultures were infected for 2 hrs with 5 or 10 pfu per cell and then cultured for an additional 24 hrs in 5.5 or 11.1 mM glucose INS-1 media to allow for gene expression. Afterwards, cells were treated for the indicated times in modified INS-1 media without or with increasing concentrations of palmitic acid. Cells were then harvested for protein analysis and apoptosis. For FA and complex lipid analyses using palmitate, cells were infected for 2 hrs, cultured for 24 hrs in INS-1 media, and treated with 400 μM palmitic acid with 3 μCi [1-14C]palmitic acid for 12 hrs prior to lipid extraction. For *de novo* FA analysis, cells were cultured in INS-1 media containing 11.1 mM glucose with 1 μCi [2-14C]-acetic acid for 24 hrs prior to lipid extraction.

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8. RNA analysis. Total RNA was extracted using TRIZOL (Invitrogen). Levels of mRNA in human islets were determined by real-time qPCR using Light Upon Extension fluorogenic primers (Invitrogen, Table 2.1) as previously described (260). Relative amounts of mRNA were calculated using the comparative cycle threshold method. Results for human islets were normalized to the abundance of β–actin mRNA. For rodent islets and INS-1 cells, cDNA were synthesized using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time (qRT) PCR was conducted by combining synthesized cDNA and various sets of gene-specific forward and reverse primers (Integrated DNA Technologies, Coralville, IA) with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). qRT-PCR reactions were carried out using the Mx3000P quantitative PCR System (Stratagene, La Jolla, CA). The relative amounts of mRNAs were determined by the comparative cycle threshold method. All samples were analyzed in triplicate. Gene expression is reported relative to cyclophilin or ribosomal protein L32 (RPL32) mRNA levels. Primers for SYBR Green qRT-PCR are listed in (Tables 2.2 and 2.3).

Table 2

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Table 2.1. Oligodeoxynucleotide sequences used for quantitative RT-PCR.

Light Upon Extension Quantitative RT-PCR primers			
Name	Species	Direction	Sequence (5' to 3')
β-actin	Human	Forward	CACGCCACCTTCTACAATGAGCTGCGG#
		Reverse	GGTCATCTTCTCGCGGTTGG
SREBP-1	Human	Forward	GACGGCCTCTGGAACCTCATCCGTC§
		Reverse	TAGCATCCACTCGCAGAGCA
ACC	Human	Forward	CACATGCTCCAAACCAGGCCATGTG§
		Reverse	GCCAGTCCACACGAAGACCA
FAS	Human	Forward	CACCTTAACCTGGTAGTGAGTGGGAAGGTG§
		Reverse	CTTTCCGGGTGGTCGAAGA

#6-carboxy-4',5'-dichloro-2',7'-dimethoxy-fluorescein-labeled ß-actin primer used as internal control for all real-time RT-PCR reactions.
§6-carboxy-fluorescein-labeled forward PCR primers used for real-time RT-PCR reactions.

Table 2.2

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Name
Cyclophi
RPL32
SREBP-1
ACCα
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ABCA1
ABCG1
CPT-1α
CPT-2
VLCAD
LCAD

Table 2.2. Oligodeoxynucleotide sequences used for quantitative RT-PCR.

SYBR Green Quantitative RT-PCR primers			
Name	Species	Direction	Sequence (5' to 3')
Cyclophilin	Rat	Forward	CTTCTTGCTGGTCTTGCCATTCCT
		Reverse	TGGATGGCAAGCATGTGGTCTTTG
RPL32	Rat	Forward	AAACTGGCGGAAACCCAGAG
		Reverse	GCAATCTCAGCACAGTAAGATT
SREBP-1	Rat	Forward	GATTGCACATTTGAAGACATGCTT
		Reverse	GGGTCCCAGGAAGGCTTCCAGAGA
ACCα	Rat	Forward	CGATGTTCTGTTGGACAACGCCTT
		Reverse	TCTCTGATCCACCTCACAGTTGAC
FAS	Rat	Forward	GTGCACCCCATTGAAGGTTCC
		Reverse	GGTTTGGAATGCTGTCCAGGG
ABCA1	Rat	Forward	AGCAGTTTGTGGCCCTCTTGT
		Reverse	TGAAGTTCCAGGTTGGGGTACTTG
ABCG1	Rat	Forward	ATGGAAGGTTGCCACAGCTTCTC
		Reverse	AGTCATGGTCTTGGCCAGGTAGT
CPT-1α	Rat	Forward	AGACCGTGAGGAACTCAAACCCAT
		Reverse	CACAACAATGTGCCTGCTGTCCTT
CPT-2	Rat	Forward	TCCTGCATACCAGCAGATGAACCA
		Reverse	ACAGTGGAGAAACTCTCGGGCATT
VLCAD	Rat	Forward	GTGGGAATGTTCAAAGGCCAGCTT
		Reverse	AAGGAGTCATTCTTGGCAGGGTCA
LCAD	Rat	Forward	AATGGGAGAAAGCCGGAGAAGTGA
		Reverse	GAAACCAGGGCCTGTGCAATTTGA

Table 2

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Elovl-3
Elovl-4
Elovl-5
Elovl-6
Elovl-7
Xbp1s
Xbp1t
ATF3
CHOP

Table 2.3. Oligodeoxynucleotide sequences used for quantitative RT-PCR.

SYBR Green Quantitative RT-PCR primers			
Name	Species	Direction	Sequence (5' to 3')
Insulin	Rat	Forward	GCTTTTGTCAAACAGCACCTT
		Reverse	CTCCAGTGCCAAGGTCTGAAG
SCD1	Rat	Forward	ACATTCAATCTCGGGAGAACA
		Reverse	CCATGCAGTCGATGAAGAAC
SCD2	Rat	Forward	ATGCCGGCTCACATACTG
		Reverse	GACCAGTGTGATCCCGTACA
D5D	Rat	Forward	TGGAGAGCAACTGGTTTGTG
		Reverse	GTTGAAGGCTGACTGGTGAA
D6D	Rat	Forward	TGTCCACAAGTTTGTCATTGG
		Reverse	ACACGTGCAGGCTCTTTATG
Elovl-1	Rat	Forward	CCCTACCTTTGGTGGAAGAA
		Reverse	TCCAGATGAGGTGGATGATG
Elovl-2	Rat	Forward	TTTGGCTGTCTCATCTTCCA
		Reverse	GGGAAACCGTTCTTCACTTC
Elovl-3	Rat	Forward	AATTCTGGTCCTGGGTCTTTC
		Reverse	CCAAAGCTCGTAAACAGTAGCA
Elovl-4	Rat	Forward	GAAGTGGATGAAAGACCGAGA
		Reverse	GCGTTGTATGATCCCATGAA
Elovl-5	Rat	Forward	ACAGCTTCATCCACGTCCTCATGT
		Reverse	AGCTGGTCTGGATGATTGTCAGCA
Elovl-6	Rat	Forward	CAACGGACCTGTCAGCAA
		Reverse	GTGGTACCAGTGCAGGAAGA
Elovl-7	Rat	Forward	TGGCGTTCAGCGATCTTAC
		Reverse	GATGATGGTTTGTGGCAGAG
Xbp1s	Rat	Forward	GAGTCCGCAGCAGGTG
		Reverse	GCGTCAGAATCCATGGGA
Xbp1t	Rat	Forward	GAGCAGCAAGTGGTGGATTT
		Reverse	TCTCAATCACAAGCCCATGA
ATF3	Rat	Forward	GAAGGCACAAAGTCCCGCTTTCAA
		Reverse	TTCAAATACCAGTCTCCACGGGCT
CHOP	Rat	Forward	AACTGTTGGCATCACCTCCTGTCT
		Reverse	TCCTCAGCATGTGCACTGGAGATT

9. Western blot analysis. For analysis of SREBP-1 protein, microsomes and nuclear extracts were isolated as previously described (261). Proteins (30-100 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes as described previously (262). Proteins were detected with specific primary antibodies and the corresponding secondary antibodies and the bands were then visualized on film with SuperSignal West Pico and Dura chemiluminescent kits (Thermo Fisher Scientific, Rockford, IL). Protein bands were quantified by densitometry scanning.

10. Insulin secretion studies. INS-1 cells were preincubated twice for 30 min at 37°C in Krebs Ringer bicarbonate buffer (KRBB) (259) containing 2 mM glucose and subsequently incubated for 60 min in KRBB containing either 2 or 20 mM glucose. For studies using etomoxir (100 μM), orlistat (50 μM) or calphostin C (1 μM), agents were present throughout the incubation period in KRBB. Triacsin C (10 μM) was added 5 hrs prior to secretion studies. Verapamil (100 μM) was only present during the final 1 hr incubation in KRBB. Insulin released into the media and insulin content from acid-ethanol extracted cells were determined by radioimmunoassay (Linco, St. Louis, MO). Total cell protein was determined by Lowry assay.

11. Glucose utilization studies. Glucose usage was measured using a modification of the method of Zawalich and Matschinsky (263) as previously described (259). Briefly, cells were incubated for 30 min at 37°C in KRBB containing either 2 or 16.7 mM glucose and [5-3H]glucose. Duplicate 50 μl samples were added to a tube containing 1 N HCl. The tubes were placed in vials containing 0.5 ml of H2O, sealed, and incubated at 50°C for 18 hrs. Tubes were then removed from vials, scintillation cocktail was added, and

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samples were counted in a Beckman scintillation counter. Glucose utilization was expressed as picomoles of glucose metabolized per min per mg protein.

12. Complex lipid analysis and fatty acid profile. For *de novo* lipid synthesis, INS-1 cells were cultured for 48 hrs in INS-1 media containing 4 or 16.7 mM glucose and vehicle or 10 μM T0901317. During the last 12 hrs, cells were cultured in INS-1 media containing 4 or 16.7 mM glucose, vehicle or 5 μM T0901317, and 1 μCi [2-14C]acetic acid (51 mCi/mmol). Cells from 14C-labeling studies were harvested and lipids extracted as previously described by Pawar et al. (264). For analysis of complex lipids, lipid extracts were separated by thin layer chromatography (TLC) as previously described (264). Quantification of 14C-labeled lipids was determined on a Molecular Dynamics Phosphoimager 820.

Analysis of 14C-labeled FA profile was performed as previously described (35). FA analysis from glucose was determined by culturing INS-1 cells for 48 hrs in 4 or 16.7 mM glucose, and during the last 24 hrs 4 or 16.7 μl of [U-14C]glucose (260 mCi/mmol) was added to the media. Total lipid extracts from labeling studies were saponified (0.5 N KOH in 80% methanol, 1 hr at 50°C), neutralized, extracted in diethyl ether, dried, and resuspended in methanol and 0.1 mM BHT. FAs were then fractionated by reverse phase-HPLC using a J'sphere ODS-H80 (YMC-Waters, Milford, MA) column and quantified by flow-through scintillation counting (IN/US Systems, Inc., Brandon, FL). Total FAs were quantified by evaporative light scatter, and unsaturated FA were quantified by UV absorption at 192 nm.

For total FA synthesis from glucose during the insulin secretion study, cells were incubated for 1 hr with either 2 or 20 mM glucose plus 4 or 40 μCi of [U-14C]glucose

(260 mCi/mmol), respectively. Cells were extracted, extracts saponified, and 14C-labeled FA quantified as described above.

Indexes of elongation and desaturation were determined using the 14C counts incorporated into each specific FA species and calculating the ratios of product(s) to substrate. Elongation of 16:0 and 16:1,n-7 was determined from the ratios of 18:0 plus 18:1,n-9 to 16:0 and 18:1,n-7 to 16:1,n-7, respectively. Desaturation of 16:0 and 18:0 was determined from the ratios of 16:1,n-7 plus 18:1,n-7 to 16:0 and 18:1,n-9 to 18:0, respectively.

13. Palmitate oxidation. INS-1 cells were then incubated for 1 hr in KRBB containing 2 mM glucose, after which cells were incubated for 1 hr in KRBB containing 50 μM palmitate, 2 μCi/ml [1-14C]palmitic acid (56 mCi/mmol), and 2 or 20 mM glucose. Palmitate oxidation was determined by measuring [14C]CO2 released into the medium using the method of Parkera et al. (265).

14. Statistical analysis. Islet studies are representative of 5 to 6 animals per group. All INS-1 cell data represent 3 to 6 independent experiments performed in duplicate. Statistical significance was determined using one-way ANOVA followed by Bonferroni's multiple comparison test for more than two groups or Student's t-test for comparing two groups. *P* values < 0.05 were considered significant.

Chapter 3.

Elevated Insulin Secretion From Liver X Receptor-Activated Pancreatic β-Cells Involves Increased *de Novo* Lipid Synthesis and Triacylglyceride Turnover

Abstract

Increased basal and loss of glucose-stimulated insulin secretion (GSIS) are hallmarks of β-cell dysfunction associated with type 2 diabetes. It has been proposed that elevated glucose promotes insulin secretory defects by activating sterol regulatory element binding protein-1c (SREBP-1c), lipogenic gene expression and neutral lipid storage. Activation of liver X receptors (LXR) also activates SREBP-1c and increases lipogenic gene expression and neutral lipid storage, but increases basal and GSIS. This study was designed to characterize the changes in *de novo* fatty acid (FA) and triacylglyceride (TAG) synthesis in LXR-activated β-cells and determine how these changes contribute to elevated basal and GSIS. Treatment of INS-1 β-cells with a LXR agonist T0901317 and elevated glucose led to markedly increased nuclear localization of SREBP-1, lipogenic gene expression, *de novo* synthesis of monounsaturated fatty acids and TAG, and basal and GSIS. LXR-activated cells had increased FA oxidation and expression of genes involved in mitochondrial ß-oxidation particularly carnitine palmitoyltransferase-1. Increased basal insulin release from LXR-activated cells coincided with rapid turnover of newly synthesized TAG and required acyl-CoA synthesis and mitochondrial ß-oxidation. GSIS from LXR-activated INS-1 cells required influx of extracellular calcium and lipolysis suggesting production of lipid-signaling

molecules from TAG. Inhibition of diacylglyceride (DAG)-binding proteins, but not classic isoforms of protein kinase C, attenuated GSIS from LXR-activated INS-1 cells. In conclusion, LXR activation in β-cells exposed to elevated glucose concentrations increases *de novo* TAG synthesis; subsequent lipolysis produces free fatty acids and DAG, which are oxidized to increase basal insulin release and activate DAG-binding proteins to enhance GSIS, respectively.

Introduction

Type 2 diabetes mellitus occurs when pancreatic β-cells fail to secrete sufficient amounts of insulin necessary to overcome insulin resistance at peripheral tissues and to maintain glucose homeostasis. Loss of β-cell function in type 2 diabetes has been suggested to occur when β-cells are chronically exposed to elevated circulating glucose and free fatty acids (FFA) – a state defined as 'glucolipotoxicity' (3, 4). Sterol regulatory element binding protein 1c (SREBP-1c), a basic helix loop helix transcription factor, plays a major role in inducing lipogenic gene expression in liver and adipose tissue (69, 266, 267), and thereby partitions glucose towards synthesis of lipid. The ability of elevated glucose to increase the nuclear form of SREBP-1c in β-cells (114, 115, 268) has been proposed to serve as a possible mechanism for glucolipotoxicity, and to explain the predominate role of elevated glucose in β-cell dysfunction (268). Consistent with this hypothesis, expression of a constitutively active nuclear form of SREBP-1c in islets or β-cell-lines increased lipogenic gene expression, triacylglyceride (TAG) synthesis and storage, and suppressed glucose-stimulated insulin secretion (GSIS) (115, 116, 235, 268,

269). Islet failure in Zucker Diabetic Fatty (ZDF) rats is also associated with increased expression of SREBP-1c and TAG accumulation (232, 233, 270).

Although activation of SREBP-1c is an attractive mechanism to explain glucolipotoxicity, recent reports suggest that the role of SREPB-1c in β-cell function is more complex. First, SREBP-1c activation and lipid synthesis are required for adaptive changes leading to hypersecretion of insulin from mouse islets exposed to elevated glucose concentrations (234). Second, inactivation of SREBP-1c in ZDF rat islets failed to restore GSIS suggesting that increased SREBP-1c and intracellular TAG are not the principal cause of β-cell secretory dysfunction (252). Third, activation of liver X receptors (LXR) in islets and β-cell-lines increased SREBP-1c, lipogenic gene expression, neutral lipid storage, and basal and GSIS (5, 253).

LXRα (NR1H3) and LXRβ (NR1H2), are nuclear receptors involved in transcriptional control of genes involved in cholesterol, fatty acid, and glucose metabolism (271). LXRα is primarily expressed in liver, kidney, intestine, and macrophages, whereas LXRβ is ubiquitously expressed (78, 272). LXR is activated by oxysterols (273) and glucose (77). In macrophages, LXR regulates reverse cholesterol transport through increased expression of genes encoding ATP-binding cassette (ABC) cholesterol transporters ABCA1 (274) and ABCG1 (275). In liver, LXR controls transcription of genes involved in conversion of cholesterol into bile acids (273, 276) and excretion of biliary cholesterol (277). LXR also directly and indirectly, through increased SREBP-1c expression, activates lipogenic gene transcription including acyl CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl CoA desaturase 1 (SCD1) and 2 (SCD2) (81, 278-280).

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LXRα and LXRβ are expressed in rodent and human pancreatic islets and β-cell lines (5, 281). Islets from LXRβ knockout mice accumulated lipid, have reduced expression of cholesterol transporters, and reduced GSIS (117, 253). Treatment of islets and β-cell lines with the LXR agonist T0901317 increased expression of lipogenic genes and lipid accumulation (5, 118). Importantly, LXR agonists elevated basal and GSIS from islets and β-cell lines through a mechanism dependent upon increased SREBP-1c, and pyruvate carboxylase and ACCα activity (5, 253). These findings suggest that LXR activation promotes insulin release by stimulating anaplerotic and cataplerotic pathways, possibly to supply malonyl-CoA for *de novo* fatty acid and lipid synthesis. The principal aims of the study presented herein were to characterize the changes in *de novo* FA and TAG synthesis in LXR-activated β-cells and determine how these changes in lipid metabolism may contribute to elevated basal and GSIS.

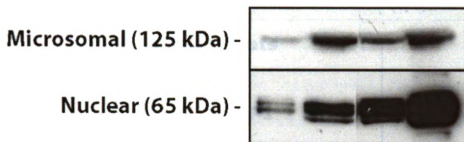
Results

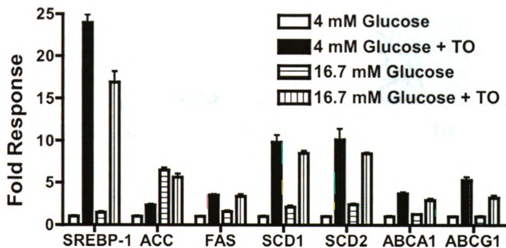
3.1. Effect of glucose and LXR activation on SREBP-1 and lipogenic gene expression.

Culturing INS-1 cells in 16.7 mM glucose for 48 h increased protein levels of both the microsomal precursor (125 kDa) and nuclear active (65 kDa) forms of SREBP-1 (Figure 3.1A) (261). Activation of LXR by the addition of T0901317 further increased microsomal precursor and nuclear forms of SREBP-1 for each glucose concentration tested. These results show that elevated glucose and direct activation of LXR by T0901317 can independently and synergistically activate SREBP-1 synthesis, processing and nuclear localization in β-cells. Since LXR regulates only SREBP-1c and not SREBP-1a (279), these changes in SREBP-1 likely reflect increased SREBP-1c nuclear abundance.

Activation of LXR in a number of tissues results in direct and indirect induction of genes involved in lipogenesis and cholesterol efflux. Culturing INS-1 cells in 16.7 mM glucose for 48 h led to a modest increase (1.4- to 2-fold) in mRNA levels of SREBP-1, FAS, SCD1 and 2, and a large increase in ACCa (6.4-fold) (Figure 3.1B). LXR activation with T0901317 markedly increased the expression of SREBP-1, FAS, SCD1, SCD2, ABCA1 and ABCG1 irrespective of the glucose concentration. T0901317 also led to a 2.3-fold increase in ACCα gene expression in cells culture in low glucose, but did not further increase ACCα expression in cells cultured in elevated glucose. Treatment of human islets with T0901317 (10 μM) for 72 h also led to a 6- to 13-fold increase in expression of SREBP-1, FAS, and ACCα mRNA (Figure 3.1C).

Figure 3.1. Glucose and LXR activation increase SREBP-1 expression and nuclear localization, and lipogenic gene expression. INS-1 cells were cultured for 48 hrs in media containing 4 or 16.7 mM glucose ± 10 μM T0901317. A. Microsomes and nuclear extracts were fractionated by SDS-PAGE and SREBP-1 immunoreactivity was detected by Western analysis. Results shown are representative of four independent experiments. B. Total RNA was isolated and analyzed for SREBP-1, ACC, FAS, SCD1, SCD2, ABCA1 and ABCG1 mRNA expression by real-time RT-PCR. Control genes cyclophilin and ribosomal protein L32 (RPL32) were unaffected by T0901317 or glucose (data not shown). Data are relative to cyclophilin and normalized to cells cultured in 4 mM glucose (mean ± SEM, n = 4). C. Isolated human islets were cultured for three days in medium supplemented without or with 10 μM T0901317. Total RNA was isolated and mRNA expression levels were determined by real-time RT-PCR (mean ± SEM, n = 3).


A
Glucose (mM):
4
16.7
T0901317 (μM):
0
10
0
10
Microsomal (125 kDa) -
Nuclear (65 kDa) -


B
Fold Response
25
20
15
10
5
0
4 mM Glucose
4 mM Glucose + TO
16.7 mM Glucose
16.7 mM Glucose + TO
SREBP-1
ACC
FAS
SCD1
SCD2
ABCA1
ABCG1

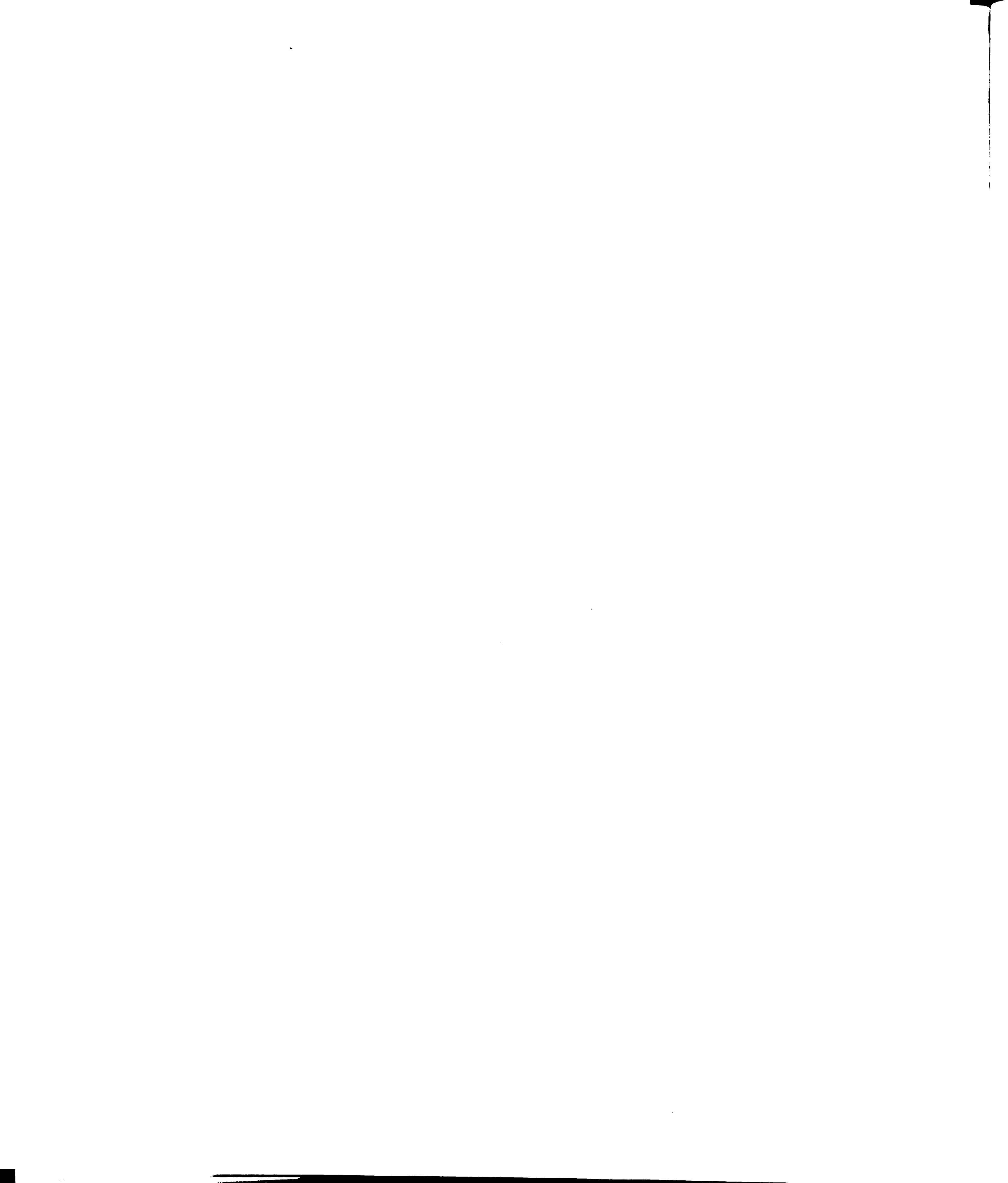
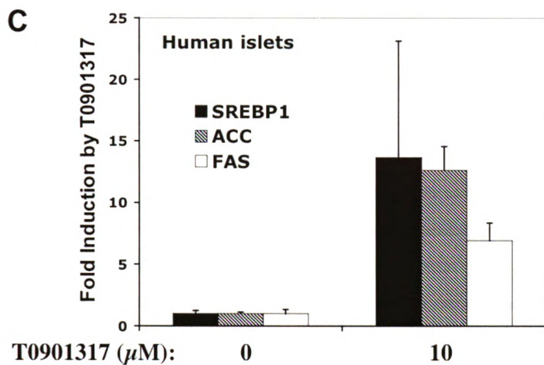


Figure 3.1. Continued



3.2. Effect of glucose and LXR activation on lipogenesis.

To assess *de novo* lipogenesis, INS-1 cells were incubated for 36 hrs in 4 or 16.7 mM glucose ± T0901317 and then incubated for 12 hrs under the same conditions in the presence of [2-14C]acetic acid. Total lipids were analyzed by TLC to measure 14C incorporation into polar lipids (phospho- and sphingolipids), FFA, TAG, cholesterol, and cholesterol esters (264). Figure 3.2A illustrates the fractional distribution of 14C in neutral, polar, and non-esterified lipid fractions. The percent of 14C in the polar lipid fraction ranged from 88 to 71% and was sensitive to both glucose and T0901317. For cells incubated in 4 mM glucose, 83.1 ± 1.5% (N = 4) of 14C-labeled lipid were polar lipids and the fractional distribution was not altered by T0901317 (82.2 ± 1.4%). Treatment of cells with 16.7 mM glucose shifted the fractional 14C distribution from polar lipids (77.6 ± 2.0%) to neutral lipids, and this was further shifted by T0901317 (71.1 ± 1.3%).

In cells cultured in 4 mM glucose, the fractional distribution of 14C assimilation into cholesterol, CE, FFA, and TAG was 7.7 ± 0.6%, 0.6 ± 0.1%, 1.1 ± 0.2%, and 1.7 ±0.3%, respectively, and this was unaltered by treatment with T0901317 (Figure 3.2A). Culturing cells in 16.7 mM glucose led to a 5.4- and 2.5-fold increase in 14C-labeled TAG (9.1 ± 1.6%, $p < 0.01$) and CE (1.4 ± 0.3%, $p < 0.03$), respectively. Addition of T0901317 led to an additional 2-fold increase in 14C-labeled TAG (16.7 ± 1.9%, $p < 0.01$) and a ~50% reduction in 14C-labeled cholesterol (4.0 ± 0.4, $p < 0.03$) and CE (0.7 ± 0.1, $p < 0.04$), respectively. Similar changes in fractional distribution of 14C-labeled polar lipids and TAG were observed in experiments using D-[U-14C]glucose (data not shown). Changes in 14C-labeled TAG were also proportional to changes in TAG mass

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(data not shown). Treatment of human islets with T0901317 also increased 14C-labeled TAG and reduced 14C-labeled cholesterol (Figure 3.3A). Overall, these results demonstrate that activation of lipogenic genes in β-cells by elevated glucose and T0901317 markedly increase *de novo* lipid synthesis and partitioning of carbon from acetic acid or glucose into complex neutral lipids, particularly TAG.

3.3. Effect of LXR activation on fatty acid profile.

Total lipids extracted from [2-14C]acetic acid labeled INS-1 cells were saponified and the FA profile was determined by reverse-phase HPLC. When INS-1 cells were incubated in 4 mM glucose, 4.5%, 53.1%, 23.6%, 5.7%, 5.3%, and 7.8% of 14C-labeled FAs were myristic acid (14:0) palmitic acid (16:0), stearic acid (18:0), palmitoleic acid (16:1,n-7), vaccenic (18:1,n-7) and oleic acid (18:1,n-9), respectively (Figure 3.2B). Incubation of cells in 16.7 mM glucose only modestly affected the fractional distribution of 14C-labeled FAs and this was reflected by a small increase in 14C-labeled palmitoleic acid (16:1,n-7). In contrast, treatment of cells with T0901317 led to a large change in the fractional distribution of 14C-labeled FAs such that 14C-labeled palmitoleic (16:1,n-7), vaccenic (18;1,n-7) and oleic (18:1,n-9) acids increased by ~2-fold. T0901317 also led to a ~25% reduction in 14C-labeled palmitic (16:0) and stearic (18:0) acid. The T0901317-induced change in 14C-labeled FAs occurred with a commensurate increase in MUFA pool size and decrease in saturated FA pool size (data not shown). Treatment of isolated human islets with T0901317 also changed the fractional distribution of 14C-labeled FAs with an increase in palmitoleic acid (16:1,n-7) synthesis and a reduction in stearic acid (18:0) production (Figure 3.3B). These results show that activation of LXR in β-cells increases *de novo* synthesis of MUFA.

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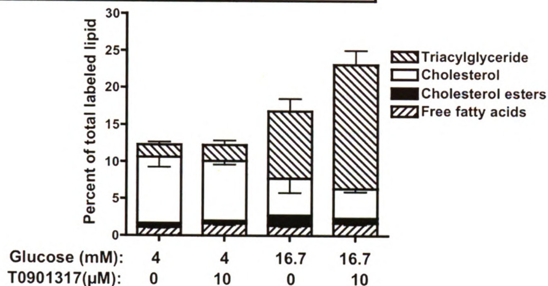
Figure 3.2. Elevated glucose and T0901317 increase *de novo* lipid synthesis in INS-1 cells. INS-1 cells were cultured for 48 h in 4 or 16.7 mM glucose ± 10 μM T0901317. Cells were then incubated overnight under the same conditions in the presence of [2-14C]-acetic acid. A. Total lipids were extracted from the cells and separated on silica TLC plates with hexane:ether:acetic acid (90:30:1). Plates were dried, and radioactivity was detected and quantified on a Phosphoimager. Data are reported as percentage of total labeled lipid. Percent of labeled polar lipid is reported as a numeric value at the top of the graph. B. Total lipids were saponified and incorporation of 14C into fatty acids was determined by reverse–phase HPLC. Data are reported as percentage of total labeled fatty acid. Values are the mean ± SEM for four independent experiments.


A
% as ^{14}C-polar lipid: 83.1 82.2 77.6 71.1
Percent of total labeled lipid
30
25
20
15
10
5
0
Triacylglyceride
Cholesterol
Cholesterol esters
Free fatty acids
Glucose (mM): 4 4 16.7 16.7
T0901317(μM): 0 10 0 10

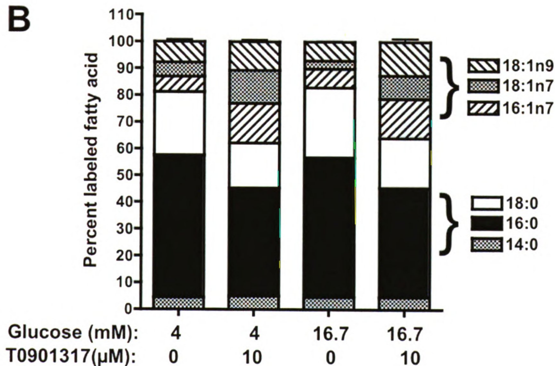

B
Percent labeled fatty acid
110
100
90
80
70
60
50
40
30
20
10
0
18:1n9
18:1n7
16:1n7
18:0
16:0
14:0
Glucose (mM): 4 4 16.7 16.7
T0901317(μM): 0 10 0 10

Figure 3.3.

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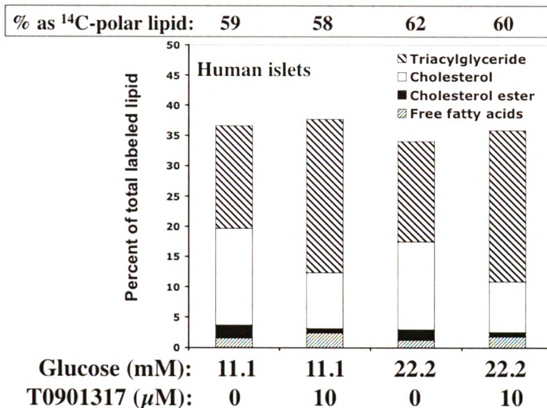
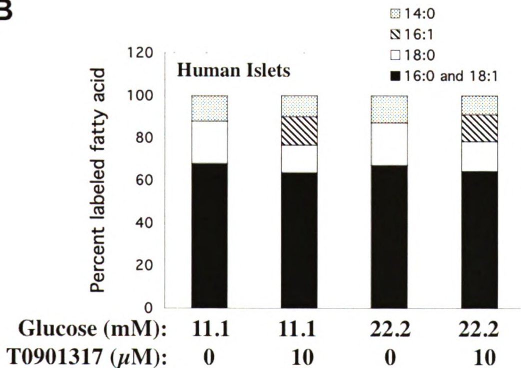
Figure 3.3. T0901317 increase *de novo* lipid synthesis in human islets. Human islets were cultured for 36 hrs in medium containing 11.1 or 22.2 mM without or with 5 μM T0901317. Islets were then incubated for 12 hrs under the same conditions in the presence of 1 μ Ci [2-14C]-acetic acid. Assimilation and distribution of 14C into complex lipids and fatty acids species was determined as described in Figure 3.2. A. Incorporation of 14C into triacylglyceride, cholesterol, cholesterol esters, and free fatty acids. B. Incorporation of 14C into specific fatty acids. Values are means, n = 2.

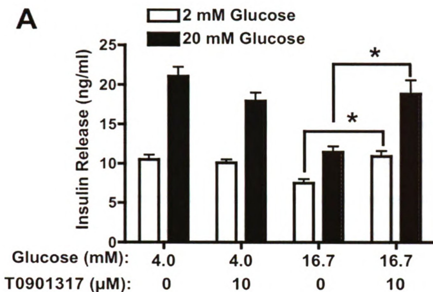

A
% as ^{14}C-polar lipid: 59 58 62 60
Human islets
Triacylglyceride
Cholesterol
Cholesterol ester
Free fatty acids
Percent of total labeled lipid
0
5
10
15
20
25
30
35
40
45
50
Glucose (mM): 11.1 11.1 22.2 22.2
T0901317 (μM): 0 10 0 10

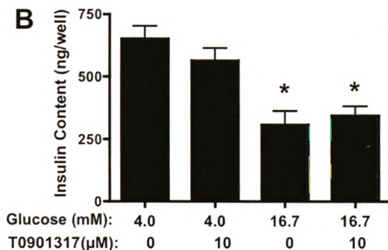

B
14:0
16:1
18:0
16:0 and 18:1
Human Islets
Percent labeled fatty acid
0
20
40
60
80
100
120
Glucose (mM): 11.1 11.1 22.2 22.2
T0901317 (μM): 0 10 0 10

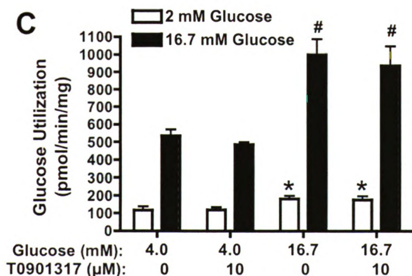
3.4. Impact of LXR activation on basal and glucose-stimulated insulin secretion.

INS-1 cells were cultured for 48 hrs in 4 or 16.7 mM glucose ± 10 μM T0901317, after which insulin release was measured in response to a 1 hr challenge with either 2 or 20 mM glucose. Acute treatment of INS-1 cells, previously cultured in 4 mM glucose ± T0901317, with 20 mM glucose led to an ~2-fold increase in GSIS (Figure 3.4A). In comparison, INS-1 cells cultured in 16.7 mM glucose for 48 hrs had reduced basal insulin release (2 mM glucose) and GSIS. In cells cultured in 16.7 mM glucose plus T0901317, insulin release was increased when cells were stimulated with 2 or 20 mM glucose and there appeared to be a partial recovery of GSIS. Although long-term exposure of INS-1 cells to elevated glucose reduces insulin expression (282), increased insulin release from cells cultured in 16.7 mM glucose plus T0901317 was not due to increased cellular insulin content (Figure 3.4B). Moreover, treatment of cells with T0901317 did not significantly alter glucose utilization at either 2 or 16.7 mM glucose (Figure 3.4C). Similar findings were also observed for glucose oxidation (data not shown). These data indicate that LXR-activated INS-1 cells cultured under an elevated glucose load have increased basal and stimulated insulin release, and this is independent of increased insulin content or changes in acute glucose metabolism.

Figure 3.4. INS-1 cells cultured in elevated glucose and T0901317 have increased insulin release. INS-1 cells were cultured for 48 h in 4 and 16.7 mM glucose ± 10 μM T0901317. Cells were washed and preincubated at 37oC in KRB buffer containing 2 mM glucose for 60 min. A. Acute insulin release was then determined by incubating cells for 60 min at 37oC in KRB buffer containing 2 or 20 mM glucose. Data represent the mean ± SEM for four independent experiments performed in triplicate. *, $p < 0.03$ when compared to cells cultured in 16.7 mM glucose. B. Intracellular insulin content from INS-1 cells that under went an acute glucose (2 mM) challenge as described above. Data represent the mean ± S.E. of four independent experiments. *, $p < 0.05$ when compared to cells cultured in 4 mM glucose. C. Glucose utilization was measured by incubating cells for 30 min at 37o C in KRB buffer containing either 2 or 16.7 mM glucose and [5-3H]-D-glucose. Conversion of [5-3H]-glucose to [3H]-H2O was determined. Data represent the mean ± S.E. of five independent experiments. * and #, $p < 0.04$ or $p < 0.001$, respectively, when compared to cells cultured in 4 mM glucose.


A
2 mM Glucose
20 mM Glucose
Insulin Release (ng/ml)
25
20
15
10
5
0
*
*
Glucose (mM): 4.0 4.0 16.7 16.7
T0901317 (μM): 0 10 0 10


B
Insulin Content (ng/well)
750
500
250
0
*
*
Glucose (mM): 4.0 4.0 16.7 16.7
T0901317(μM): 0 10 0 10


C
2 mM Glucose
16.7 mM Glucose
Glucose Utilization (pmol/min/mg)
1100
1000
900
800
700
600
500
400
300
200
100
0
#
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Glucose (mM): 4.0 4.0 16.7 16.7
T0901317 (μM): 0 10 0 10

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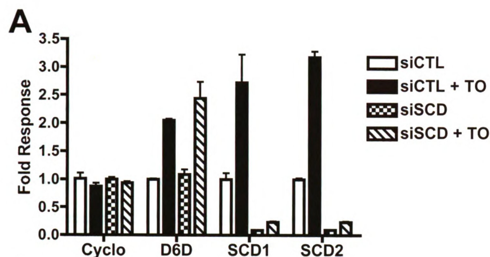
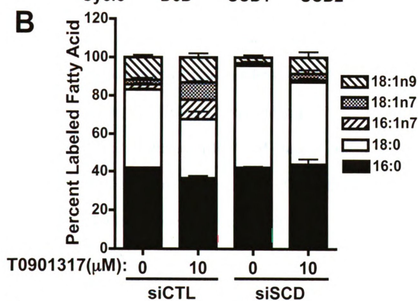
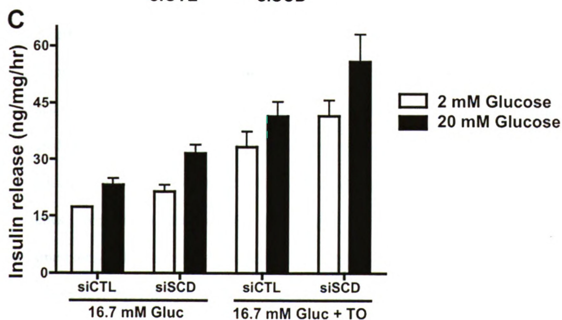
3.5. MUFA synthesis is not obligatory for elevated insulin release from LXR-activated INS-1 cells.

Exposure of human islets to elevated glucose and exogenous palmitate induces apoptosis and diminishes GSIS, whereas these effects are reversed by co-exposure to MUFAs (283). Thus, enhanced insulin secretion from LXR-activated INS-1 cells could be mediated by the increased conversion of *de novo* synthesized palmitate to MUFAs. To test this hypothesis, INS-1 cells were electroporated with SCD targeted siRNA and cultured for 48 hrs in 16.7 mM glucose with or without T0901317. SCD siRNA knocked down both SCD1 and SCD2 gene expression and markedly reduced the induction of SCD1 and SCD2 by T0901317 (Figure 3.5A). The expression of Δ6D was not effected by SCD siRNA. In cells cultured with [1-14C]-palmitate during the last 24 hrs, SCD knockdown significantly reduced the conversion of 14C-labeled palmitate into MUFAs (Figure 3.5B). After culture for 48 hrs in 16.7 mM glucose with or without T0901317, insulin release from INS-1 cells with reduced SCD expression was slightly increased rather than decreased (Figure 3.5C). Nevertheless, this shows that elevated insulin release from LXR-activated INS-1 cells is not dependent on the induction of SCD gene expression.

Figure 3.5. Knockdown of SCD with siRNA does not inhibit insulin release from LXR-activated INS-1 cells. INS-1 cells were electrophorated in the presence of 100 nM control siRNA or siRNA against SCD1 and 2. Cells were cultured for 12 hrs in 11.1 mM glucose and then cultured for 48 hrs in 16.7 mM glucose with or without T0901317. After which mRNA levels, MUFA synthesis from 14C-labeled palmitate, and insulin release were assessed. A. SCD siRNA blocked the ability of T0901317 to increase SCD 1 and 2 mRNA levels. SCD siRNA, however, did not affect T0901317 induction of delta 6 desaturase (D6D) (n=3). B. Knockdown of SCD decreased the conversion of 14C-palmitate (16:0) to palmitoleate (16:1) or oleate (18:1) (n=3). C. Knockdown of SCD failed to decrease insulin release from LXR-activated cells cultured in 16.7 mM glucose (n = 6).


A
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fold Response
Cyclo
D6D
SCD1
SCD2
siCTL
siCTL + TO
siSCD
siSCD + TO


B
120
100
80
60
40
20
0
Percent Labeled Fatty Acid
18:1n9
18:1n7
16:1n7
18:0
16:0
T0901317(μM): 0 10 0 10
siCTL
siSCD


C
60
45
30
15
0
Insulin release (ng/mg/hr)
2 mM Glucose
20 mM Glucose
siCTL
siSCD
siCTL
siSCD
16.7 mM Gluc
16.7 mM Gluc + TO

3.6. Role of fatty acid oxidation in elevated insulin release.

To investigate the role of FA oxidation in insulin release, secretion studies were performed in the presence of an inhibitor of long-chain acyl CoA synthetase (ACS), triacsin C, or an inhibitor of carnitine palmitoyltransferase-1 (CPT-1), etomoxir. Triacsin C prevents conversion of FFA to LC-CoA thereby indirectly inhibiting β-oxidation of FFA, whereas etomoxir directly inhibits the rate-limiting enzyme of β-oxidation. In INS-1 cells cultured for 48 hrs in 16.7 mM glucose, etomoxir (100 μM) had no effect on insulin release in response to a 1 hr challenge with 2 or 20 mM glucose (Figure 3.6A). In contrast, the enhanced basal insulin release (2 mM glucose) from INS-1 cells cultured in 16.7 mM glucose plus T0901317 was reduced 25% ($p < 0.001$, N = 6) by etomoxir. In LXR-activated cells cultured in 16.7 mM glucose, triacsin C reduced basal insulin release by 38% ($p < 0.001$, N = 6) (Figure 3.6A). These data suggest that enhanced basal insulin release from LXR-activated cells cultured in 16.7 mM glucose requires conversion of FFA to LC-CoA and increased β-oxidation. Consistent with this possibility, [14C]palmitate oxidation under basal glucose conditions (2 mM) was elevated ~3-fold in INS-1 cells cultured for 48 h in T0901317 (Figure 3.6B). Although palmitate oxidation was suppressed by elevated glucose (20 mM), [14C]palmitate oxidation remained elevated ~3.5-fold in LXR-activated INS-1 cells. Oxidation of [14C]palmitate in LXR-activated cells was also inhibited ~65% by triacsin C and ~90% by etomoxir. Increased FA oxidation in T0901317-cultured INS-1 cells correlated with increased mRNA levels of genes involved in mitochondrial β-oxidation (Figure 3.6C) including CPT-1α, carnitine palmitoyl transferase-2 and long chain acyl CoA dehydrogenase, but not very long chain acyl CoA dehydrogenase. These data strongly support the hypothesis that

LXR activation increases basal insulin release from INS-1 cells by a mechanism involving increased β-oxidation of fatty acids.

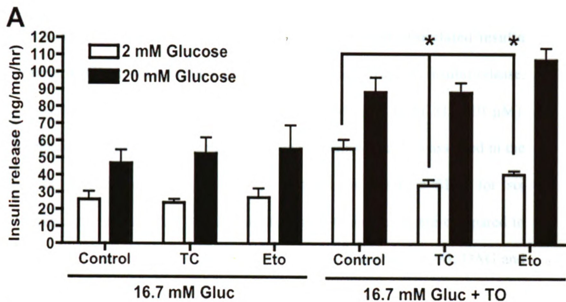
3.7. Role of triacylglyceride turnover in elevated insulin secretion.

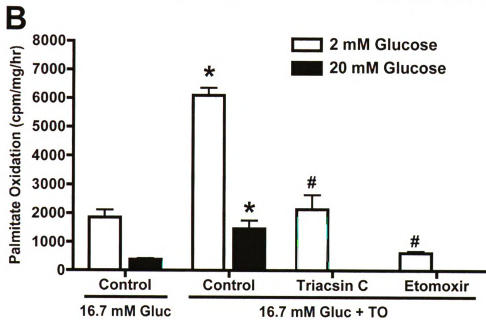
The relationship between enhanced insulin release and the turnover of TAG was examined using the general lipase inhibitor orlistat. Treatment of INS-1 cells cultured in 4 mM glucose with orlistat (5-50 μM) increased basal insulin secretion (data not shown). The mechanism accounting for the elevated basal insulin release is unknown, but may be related to metabolic stress associated with low glucose and the inability to turnover lipid pools. Consistent with this, orlistat (50 μM) had no effect on basal insulin release from INS-1 cells initially cultured in 16.7 mM glucose ± T0901317 (Figure 3.7A). Orlistat, however, completely blocked insulin release in response to 20 mM glucose. These results suggested that enhanced GSIS from LXR-activated INS-1 cells cultured in 16.7 mM glucose was associated with turnover of TAG. To examine TAG turnover, INS-1 cells were cultured in 16.7 mM glucose and T0901317 for 48 hrs, and during the last 6 hrs were labeled with [2-14C]acetic acid. After labeling, cells were subjected to conditions mimicking an acute insulin release assay in the presence of orlistat and turnover of labeled lipid was measured. Incubation of cells for 2 hrs in 2 mM glucose led to an ~65% decrease in TAG and DAG labeling, and an ~50% decrease in FFA labeling (Figure 3.7B). Treatment of cells for 1hr in 2 mM glucose followed by 1 hr in 20 mM glucose tended to slow the turnover of TAG and DAG. Orlistat completely blocked the turnover TAG and led to a precipitous fall in labeled FFA, possibly due to FA oxidation of cellular FFA. Orlistat has also been shown to inhibit FAS (284), suggesting that orlistat may block GSIS from LXR-activated INS-1 cells by inhibiting *de novo* lipid

synthesis. To test this possibility, INS-1 cells cultured for 48 h in 16.7 mM glucose ± T0901317 were subjected to an acute insulin release assay in the presence of 2 or 20 mM glucose containing [U-14C]glucose and incorporation of 14C into FA from methanol-soluble lipids was determined. Incubation of control and LXR-activated cells for 1 h with 20 mM glucose markedly increased 14C-labeled FA and this was further increased by orlistat (Figure 3.7C), suggesting orlistat's action on GSIS is independent of inhibition of *de novo* lipogenesis. These data suggest that a byproduct of TAG turnover such as DAG or FFA may participate in enhanced GSIS from LXR-activated INS-1 cells.

To investigate a potential role for DAG, acute insulin release studies were performed in the presence of calphostin C, an inhibitor of protein kinase C (PKC) that competitively interferes with DAG and phorbol ester binding. GSIS from LXR-activated INS-1 cells was attenuated by calphostin C (1 μM) (Figure 3.8), but not by other PKC inhibitors - GÖ6976 or GÖ6983 (data not shown). Because calphostin C and orlistat only affected GSIS, the role for influx of calcium through the L-type voltage-gated calcium channel (L-VGCC) was tested. The L-VGCC inhibitor verapamil (100 μM) completely inhibited GSIS from LXR-activated INS-1 cells without affecting basal insulin release.

Figure 3.6. Elevated basal insulin release from LXR-activated INS-1 cells involves increased fatty acid oxidation. INS-1 cells were incubated for 48 h in 16.7 mM glucose ± T0901317 (10 μM). A. Insulin release (60 min) in response to 2 or 20 mM glucose was then assessed in the presence or absence of etomoxir (100 μM) or triacsin C (10 μM). Values are mean ± SEM for six independent experiments. *, $p < 0.001$ when etomoxir- or triacsin C-treated cells are compared to control cells. B. FA oxidation was determined by measuring [14C]CO2 production from cells incubated for 1 h in 2 mM glucose followed by a 1 h incubation with palmitic acid (50 μM), [14C]palmitic acid, and 2 or 20 mM glucose. Data are mean ± SEM for 3 independent experiments. *, $p < 0.01$ when cells cultured in 16.7 mM glucose plus T0901317 are compared to cells cultured in 16.7 mM glucose. #, $p < 0.001$ when triacsin C- or etomoxir-treated cells are compared to cells cultured in 16.7 mM glucose plus T0901317. C. Total RNA was extracted and mRNA levels for carnitine palmitoyl transferase-1α (CPT1), carnitine palmitoyl transferase-2 (CPT2), very long chain acyl CoA dehydrogenase (VLAD) and long chain acyl CoA dehydrogenase (LCAD) were determined by real time RT-PCR. Data are mean ± SEM for 3 independent experiments. *, $p < 0.02$ when cells cultured in 16.7 mM glucose are compared to cells cultured in 16.7 mM glucose plus T0901317.


A
Insulin release (ng/mg/hr)
120
110
100
90
80
70
60
50
40
30
20
10
0
2 mM Glucose
20 mM Glucose
*
*
Control
TC
Eto
Control
TC
Eto
16.7 mM Gluc
16.7 mM Gluc + TO


B
Palmitate Oxidation (cpm/mg/hr)
8000
7000
6000
5000
4000
3000
2000
1000
0
2 mM Glucose
20 mM Glucose
*
*
#
#
Control
Control
Triacsin C
Etomoxir
16.7 mM Gluc
16.7 mM Gluc + TO

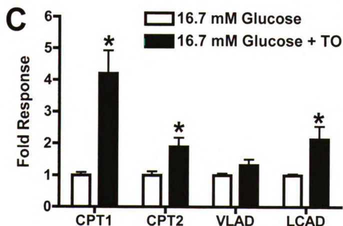
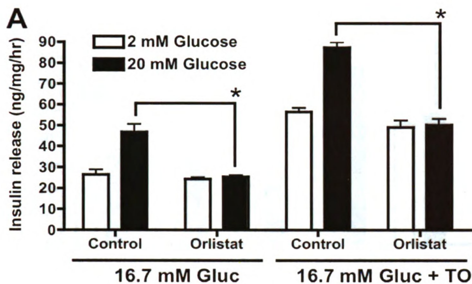
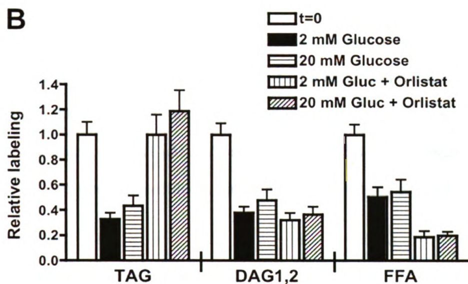
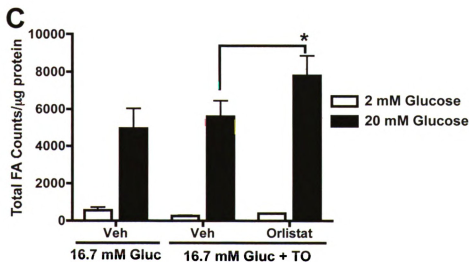

C
Fold Response
6
5
4
3
2
1
0
16.7 mM Glucose
16.7 mM Glucose + TO
*
*
*
CPT1
CPT2
VLAD
LCAD

Figure 3.7. TAG turnover is required for enhanced glucose-stimulated insulin release from LXR-activated INS-1 cells. A. Impact of orlistat on insulin release. INS-1 cells were incubated for 48 h in 16.7 mM glucose ± T0901317 (10 μM). Insulin release (60 min) in response to 2 or 20 mM glucose was then assessed in the presence or absence of orlistat (50 μM). Values are mean ± SEM for six independent experiments. *, $p < 0.001$ when orlistat-treated cells are compared to control cells. B. Impact of orlistat on turnover of *de novo*-derived TAG, DAG and FFA. INS-1 cells were cultured for 48 h in 16.7 mM glucose ± T0901317 (10 μM). During the last 6 h cells were incubated with [2-14C]-acetic acid (t=0), after which cells were subjected to conditions for an acute insulin release study: 1 h incubation in 2 mM glucose followed by an 1 h incubation in 2 or 20 mM glucose. Total lipids were extracted and analyzed as described in Figure 3A. Values are mean ± SEM for 3 independent experiments. Data are presented relative to 14C-labeling at t=0. Phosphoimager intensity values at t=0 for TAG are 482,621 ± 59,573, for DAG are 39,461 ± 4,451 and for FFA are 37,267 ± 3,691. C. Impact of orlistat (50 μM) on *de novo* FA synthesis from glucose. INS-1 cells were incubated for 48 h in 16.7 mM glucose ± T0901317, and subjected to a insulin release assay with 2 or 20 mM glucose containing 4 or 40 μCi of [U-14C]glucose, respectively. 14C-labeled FA were quantified as described in the Experimental Procedures. Values are mean ± SEM of 3 independent experiments. *, $p < 0.001$ orlistat-treated cells are compared to control cells.


A
Insulin release (ng/mg/hr)
2 mM Glucose
20 mM Glucose
*
Control
Orlistat
Control
Orlistat
16.7 mM Gluc
16.7 mM Gluc + TO


B
t=0
2 mM Glucose
20 mM Glucose
2 mM Gluc + Orlistat
20 mM Gluc + Orlistat
Relative labeling
TAG
DAG1,2
FFA


C
Total FA Counts/μg protein
2 mM Glucose
20 mM Glucose
*
Veh
Veh
Orlistat
16.7 mM Gluc
16.7 mM Gluc + TO

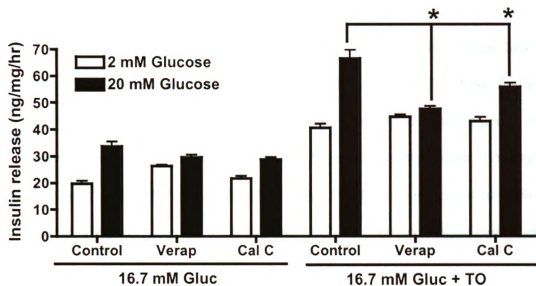


Figure 3.8. Enhanced glucose-stimulated insulin release from LXR-activated INS-1 cells is attenuated by verapamil or calphostin C. Insulin release (60 min) in response to 2 or 20 mM glucose was then assessed in the presence or absence of verapamil (100 μM) or calphostin C (1 μM). Values are mean ± SEM for six independent experiments. *, $p < 0.01$ when verapamil- or calphostin C-treated cells are compared to control cells.

Discussion

Culturing INS-1 cells in elevated glucose led to increased nuclear SREBP-1c, lipogenic gene expression, TAG synthesis, and loss of GSIS. These findings are consistent with reports that SREBP-1c activation, either by elevated glucose or over-expression, in β-cell lines or islets increased lipogenic gene expression, TAG synthesis and decreased GSIS (114-116, 139, 235, 268, 269, 285). Compared to INS-1 cells cultured in elevated glucose, LXR-activated INS-1 cells had significantly elevated microsomal and nuclear forms of SREBP-1c and lipogenic gene expression particularly SREBP-1, FAS, SCD1 and SCD2. These findings are consistent with the role of LXR in regulating SREBP-1 gene transcription, and LXR and SREBP-1c in regulating FAS, SCD1 and SCD2 gene transcription (21, 286). Of the lipogenic genes examined only ACCα mRNA levels were more strongly induced by elevated glucose than by LXR activation. Glucose has been reported to bind and activate LXR (77), but this does not appear to play a prominent role in INS-1 cells because elevated glucose did not induce expression of LXR target genes including ABCA1 and ABCG1. As expected, increased lipogenic gene expression in LXR-activated INS-1 cells cultured in elevated glucose markedly increased *de novo* neutral lipid synthesis. Because LXR activation only affected lipid synthesis and insulin secretion in cells cultured in elevated glucose suggests that the two events are linked. This is likely an adaptive effect because it has recently been shown that metabolic flux through lipogenic pathways is not required for normal GSIS (287, 288). Hypersecretion of insulin, however, has been shown to involve the induction of SREBP-1c and enhanced lipid synthesis in mouse islets cultured in elevated glucose (234).

Fatty acid D9 desaturases (SCD1 and SCD2) function as the rate-limiting step for MUFA synthesis and play an integral role in neutral lipid (TAG and CE) synthesis (21). In agreement with increased SCD1 and SCD2 mRNA levels, LXR-activated INS-1 cells exhibited a 2-fold increase in *de novo* derived MUFA (16:1,n-7, 18:1,n-7, 18:1,n-9) and a commensurate drop in synthesis of saturated FA (16:0, 18:0). The increase MUFA synthesis in LXR-activated INS-1 cells cultured in elevated glucose corresponded with increased MUFA mass (data not shown). Chronic exposure of islets and β-cell lines to oleic (18:1,n-9) or vaccenic (18:1,n-7) acid have been reported to increase basal insulin release (289-291), suggesting that increased MUFA synthesis in LXR-activated cells might be directly involved in basal insulin release. To test this possibility, siRNA targeting SCD1 and SCD2 were introduced into LXR-activated INS-1 cells. SCD1/2 siRNA effectively decreased SCD1 and SCD2 mRNA levels and MUFA synthesis, but did not lower insulin release from LXR-activated INS-1 cells. These data indicate that increased *de novo* MUFA synthesis is not an obligatory step for enhanced insulin release from LXR-activated INS-1 cells, but likely facilitates neutral lipid synthesis.

Enhanced basal insulin release from LXR-activated INS-1 cells was attenuated by triacsin C and etomoxir, indicating a role for increased acyl-CoA formation and FA oxidation. Under our experimental paradigm for insulin release studies, INS-1 cells are first preincubated for 1 hour in 2 mM glucose followed by incubation for 1 hour in either 2 or 20 mM glucose. During this timeframe, newly synthesized TAG is rapidly turned over (Figure 3.7) and likely serves as the source of the FFA for acyl-CoA formation and oxidation. INS-1 cells cultured in elevated glucose also have increased *de novo* synthesized TAG, but do not have elevated basal insulin release. This suggests that INS-

1 cells cultured in elevated glucose either do not synthesize sufficient quantities of TAG to sustain increased basal insulin release or that LXR activation stimulates additional pathways associated with lipid metabolism. Consistent with the later, LXR activation was shown here to increase FA oxidation and this correlated with increased expression of genes involved in mitochondrial ß-oxidation particularly CPT-1α. Recently, Colin et al. showed that activation of LXR with synthetic agonists induced PPARα and subsequently its target CPT-1 in the intestine, but not the liver (292). This suggests that LXR agonists may also induce CPT-1 through PPARα in β-cells. Alternatively, LXR-activation of INS-1 cells may increase *de novo* synthesized FA to levels sufficient to induce CPT-1α. This possibility is supported by the observation that long-term exposure of INS-1 cells to long-chain FA increases CPT-1 gene expression and FA oxidation (290, 293). Our findings also raise the possibility that LXR activation can protect the β-cell from glucose toxicity by shuttling glucose toward FA, which can be oxidized immediately or after release from TAG.

Lipolysis of intracellular TAG and the subsequent generation of lipid signaling molecules including FFA, acyl-CoA and DAG have been proposed to mediate GSIS (reviewed in (99)). TAG turnover produces a FFA and a predominantly less biologically active sn2,3-DAG species, which can be further broken down to monoacylglyceride, glycerol and FFA (294-296). These later products, along with *de novo* derived FA, can be reincorporated into biologically active sn1,2-DAG species through a glycerolipid/FFA cycle (297). Based on this, we hypothesize that enhanced GSIS from LXR-activated INS-1 cells results from elevated lipolysis and formation of lipid products that can directly serve as signaling molecules (e.g. FFA) or used as substrates for the

glycerolipid/FFA cycle to generate sn1,2-DAG. Consistent with this hypothesis, the general lipase inhibitor orlistat blocked turnover of *de novo* derived TAG and GSIS in LXR-activated INS-1 cells, but did not block *de novo* synthesis of FA (Figure 3.7). Mulder et al. have also proposed that orlistat attenuates GSIS by blocking the formation of an acylglyceride-coupling factor (164). If the glycerolipid/FFA cycle is involved in enhanced GSIS from LXR-activated INS-1 cells, one would predict that inhibition of acyl-CoA formation with triacsin C would have also blocked GSIS, which did not occur (Figure 3.6). This might be due to the inability of triacsin C to inhibit all ACS isoforms (298) and that triacsin C is more efficacious at inhibiting FA oxidation than lipid synthesis in β-cells (299). It remains a possibility that enhanced GSIS from LXR-activated INS-1 cells might also involve turnover of phospholipids and direct production of sn1,2-DAG (190). Polar lipid turnover, however, was much slower than TAG turnover in INS-1 cells and not effectively blocked by orlistat (data not shown).

DAG generated from the glycerol/FFA cycle might serve as the coupling factor to enhance GSIS from LXR-activated INS-1 cells. Classic (α, βI, βII, γ) and novel (δ, ε, η, θ) isoforms of PKC are activated by DAG in Ca2+-dependent and –independent manners, respectively. Pharmacologic inhibition of many of these PKC isoforms with GÖ6976 (inhibits PKCα, βI) and GÖ6983 (inhibits PKCα, β, γ, δ, ζ), however, did not attenuate GSIS from LXR-activated INS-1 cells (data not shown). Calphostin C, which competitively blocks DAG-binding sites on classic and novel PKC isoforms, PKD (PKCμ) and DAG-binding proteins, significantly attenuated GSIS from LXR-activated INS-1 cells (Figure 3.8). Blockade of the influx of extracellular Ca2+ with the L-VGCC inhibitor also completely abrogated GSIS from LXR-activated INS-1 cells. Taken as a

whole, these data suggest that enhanced GSIS from LXR-activated INS-1 cells does not involve classic or novel PKC isoforms, but involves activation of a DAG-binding protein that is calcium-dependent or mediates biochemical events upstream from the influx of calcium. There are a number of families of DAG-binding proteins that could be involved including PKD (PKCμ), chimaerins, RasGRPs, MUNC13s or DAG kinases (reviewed in (154)). Further experimentation is necessary to determine the exact DAG-binding protein(s) involved. Straub and Sharp have proposed a similar mechanism to explain how FA depletion of rat islets caused large increases in GSIS (300). In their model, FA depletion is proposed to cause lipid remodeling or increase breakdown of intracellular TAG, which increases DAG production, activates a DAG-binding protein and augments GSIS.

Enhanced insulin release from LXR-activated β-cells has been reported to be associated with increased mRNA levels of Pdx-1, insulin and GLUT2 (253) suggesting a role for LXR or SREBP-1c in augmenting β-cell phenotype and glucose sensing. SREBP-1 is also required for elevated glucose to increase mRNA levels of Pdx-1 and genes involved in glucose sensing including GLUT2 and glucokinase (234). Similar changes in gene expression may play a role in enhanced insulin release from LXR-activated INS-1 cells. Nevertheless, this seems unlikely because glucose utilization and insulin content were not significantly increased in LXR-activated INS-1 cells.

In conclusion, our study shows that LXR activation of INS-1 β-cells exposed to elevated glucose increases TAG synthesis; and subsequent TAG turnover can lead to the production of lipid signaling molecules resulting in elevated insulin release. Similar

mechanisms may account for the ability of SREBP-1c to establish hypersecretion of insulin in some models of hyperglycemia.

Chapter 4.

Stearoyl-CoA Desaturase Modulates Palmitate-Induced Endoplasmic Reticulum Stress and Apoptosis in Pancreatic β-Cells

Abstract

Chronic elevations in exogenous free fatty acids (FFAs) have been implicated in the pathogenesis of β-cell failure and the development of type 2 diabetes. The effects of exogenous FFA, particularly saturated fatty acids (FAs), on β-cells include endoplasmic reticulum (ER) stress and downstream induction of apoptosis. Regulation of monounsaturated FA (MUFA) synthesis through altered FA desaturase and elongase gene expression may serve to protect β-cells from exogenous saturated FAs. In the Zucker diabetic fatty (ZDF) rat model of progressive β-cell failure, islets from 6-week old pre-diabetic ZDF rats showed a 1.5- to 2.3-fold induction in the stearoyl-CoA desaturases (SCD) 1 and 2 mRNA, respectively, compared to control rats. At 13 weeks of age, ZDF rats were hyperglycemic and exhibited decreased plasma insulin levels, an indicator of β-cell dysfunction. This was associated with markedly decreased mRNA levels of insulin, SCD1, SCD2 and Elovl-6, which elongates 16:0 to 18:0 and 16:1,n-7 to 18:1,n-7. These findings suggested enhanced expression of SCD1/2 and other FA modifying genes may protect β-cells from damage caused by exogenous saturated FAs. Next, siRNAs and adenoviral constructs were used to investigate the role of altered SCD and Elovl-6 expression in INS-1 β-cells exposed to exogenous palmitate. Knockdown of SCD gene expression decreased conversion of palmitate to MUFA and increased the susceptibility to palmitate-induced ER stress, as measured by splicing of Xbp1, induction of ATF3 and

CHOP, and JNK phosphorylation. Palmitate-induced apoptosis was also increased by SCD knockdown, as shown by elevated caspase-9 cleavage and DNA fragmentation. Over-expression of SCD2 increased synthesis of n-7 MUFAs and markedly reduced the ER stress and apoptosis induced by palmitate. Elovl-6 knockdown decreased palmitate elongation and tended to reduce palmitate toxicity, whereas Elovl-6 over-expression increased palmitate elongation to stearate (18:0) and increased susceptibility to palmitate-induced JNK phosphorylation and apoptosis. In addition, elevated ER stress in INS-1 cells with decreased SCD expression involved reduced palmitate incorportation into TAG and activation of Ca2+-dependent protein kinase Cs (PKCs). These findings demonstrate that altered expression and activity of SCD2 and Elovl-6 modulate the susceptibility of β-cells to the toxic effect of saturated FAs.

Introduction

Type 2 diabetes arises from an inability of pancreatic β-cells to compensate for insulin resistance in peripheral tissues. The progressive loss of β-cell compensation is likely due to reduced insulin secretory capacity or β-cell mass (301-305). Elevated levels of plasma non-esterified free fatty acid (FFA), a risk factor for insulin resistance and type 2 diabetes (201, 202), have been associated with the pathogenesis of β-cell dysfunction (4, 203). The response of β-cells to long-term elevations in fatty acids (FAs), however, is largely dependent on the FA composition. Saturated FAs, such as palmitate (16:0), cause diminished insulin secretion and insulin gene expression and the induction of apoptosis through multiple processes, including generation of ceramides, reactive oxygen species, and endoplasmic reticulum (ER) stress (223, 237, 306-308). Monounsaturated FAs

(MUFAs), such as palmioleate (16:1,n-7) and oleate (18:1,n-9), and the polyunsaturated FA (PUFA) eicosapentaenoate (20:5,n-3) can protect β-cells from apoptosis and insulin secretory defects induced by saturated FAs (236, 283, 308). In addition to exogenous FA structure, evidence has demonstrated that the intracellular capacity to modulate FA fate has an important role in β-cells.

Alterations in FA metabolism critically affect the response of β-cells to exogenous FAs, particularly the lipotoxicity of saturated FAs. Approaches used to enhance FA oxidation and triacylglyceride (TAG) storage have demonstrated significant alterations in the effects of exogenous saturated FAs on β-cell function (309-311). Studies have also shown that regulation of FA structure may participate in modulating the effects of FAs on β-cells. Subpopulations of MIN-6 and rat β-cells identified to be resistant to palmitate-induced apoptosis were associated with increased expression of the FA delta-9 desaturase, stearoyl-CoA desaturase (SCD) 1, and hence increased conversion of palmitate to MUFAs (6, 257). The level of SCD1 gene expression has also been correlated with the susceptibility of β-cell lines and islets to ER stress *in vitro* and with the severity of diabetes *in vivo* in a mouse model of obesity (256, 312).

FA desaturase and elongase enzymes modify fatty acids by adding a cis-double bond or two-carbons to a fatty acyl-CoA, respectively. These activities are essential for a variety of cellular functions, including maintenance of membrane FA composition and generation of signaling molecules. The desaturase subtypes in mammals include delta 5 desaturase (Δ5D), delta 6 desaturase (Δ6D), and SCD. Isoforms of SCD include four in mouse (SCD1-4) (11-14), two in rat (SCD1 and 2) (15), three in hamster (SCD1-3) (16), and three in human (SCD1, 2, and 5) (17-19). FA elongase (Elovl) subtypes range from

Elovl-1 to 7 in mouse, rat, and human (www.ensembl.org). Synthesis of PUFAs from essential dietary FAs occurs through the desaturases Δ5D and Δ6D and the elongases Elovl-2 and Elovl-5 (7, 31, 32). SCD, the rate-limiting enzyme in C16 and C18 MUFA synthesis, and Elovl-6 synthesize the MUFAs oleate, palmitoleate, and vaccenate (18:1,n-7) (20, 31). Elovl-5 can also elongate palmitoleate to vaccenate (32). Elovl-1, -3, and -4 elongate a broad array of very long chain FAs (>C20) and are involved in sphingolipid synthesis, brown adipose and skin barrier function, and retinal degeneration, respectively (25-30). Unique roles for these enzymes in pancreatic β-cells, however, remain to be defined.

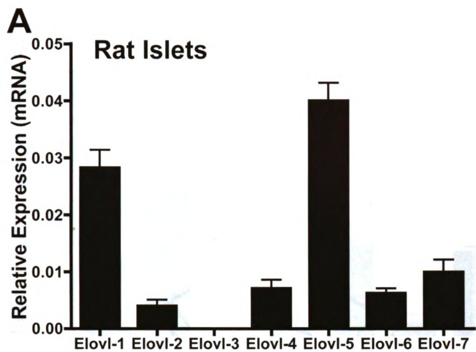
In this study, we first characterized FA desaturase and elongase gene expression in rat islets and INS-1 β-cells. Next, using the Zucker diabetic fatty (ZDF) rat model, FA desaturase and elongase genes were identified to be differentially expressed between pre-diabetic and diabetic ZDF rat islets. Specifically, SCD1 and SCD2 were increased in pre-diabetic islets and, along with Elovl-6, reduced in diabetic islets. Thus, we hypothesize that regulation of genes required for MUFA synthesis may significantly affect β-cell compensation and failure in the pathogenesis of T2D. The results demonstrate that altered expression of SCD2 and Elovl-6 in INS-1 cells modulate the effects of exogenous palmitate on ER stress and apoptosis. In addition, enhanced ER stress in the absence of SCD1 and SCD2 expression may involve altered FA partitioning into neutral lipids and activation of protein kinase C (PKC).

Results

4.1. Rat islet and INS-1 cell FA elongase and desaturase gene expression profiles.

To determine which FA elongase and desaturase genes are expressed in β-cells, elongase and desaturase mRNA levels were characterized in rat islets and INS-1 cells under non-stimulatory glucose conditions. In rat islets and INS-1 β-cells, mRNA expression was detected for the FA elongases Elovl-1, Elovl-2, Elovl-4, Elovl-5, Elovl-6 and Elovl-7, and the FA desaturases SCD1, SCD2, Δ5D and Δ6D (Figure 4.1A-D). There was no difference between Δ5D and Δ6D mRNA levels in either rat islets or INS-1 cells. For SCD mRNA levels, however, the expression of SCD2 was greater than 4-fold higher than SCD1 in both rat islets and INS-1 cells. This suggests that SCD2 may have a larger role in β-cells, whereas SCD1 is highly expressed in tissues with large lipid storage capacities such as adipose and liver.

Figure 4.1. Fatty acid elongase and desaturase gene expression in rat islets and INS-1 cells. Total RNA was isolated from Sprague-Dawley rat islets (A and B) and INS-1 cells (C and D) and analyzed for Elovl-1 to 7, SCD1, SCD2, D5D, and D6D mRNA expression by real-time RT-PCR. Gene expression is reported relative to RPL32 and represents mean ± SEM for three independent experiments.


A
Rat Islets
Relative Expression (mRNA)
0.05
0.04
0.03
0.02
0.01
0.00
Elovl-1
Elovl-2
Elovl-3
Elovl-4
Elovl-5
Elovl-6
Elovl-7

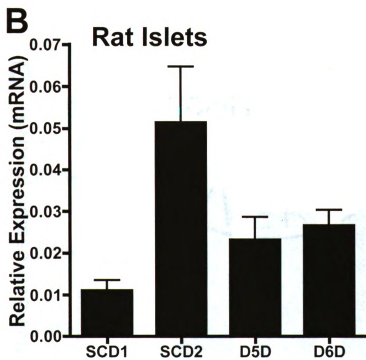
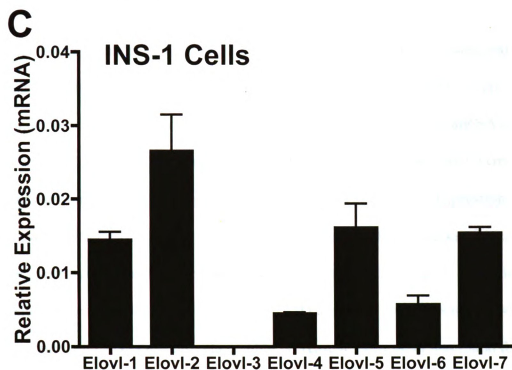
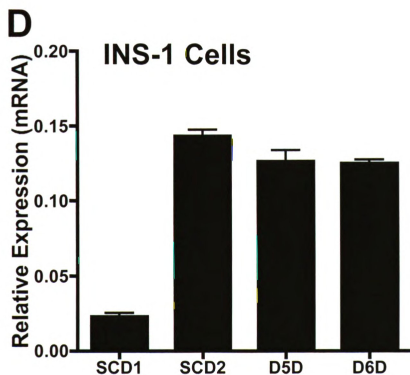

B
Rat Islets
Relative Expression (mRNA)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
SCD1
SCD2
D5D
D6D

Figure 4.1. Continued





4.2. FA elongase and desaturase gene expression in ZDF rat islets.

Before 10 weeks of age ZDF rats are pre-diabetic, displaying obesity and insulin resistance while maintaining normal glucose levels (313). Here, 6-week-old ZDF rat blood glucose levels were normal and plasma insulin levels were elevated (Supplementary Table 2), which coincided with increased islet insulin mRNA expression (Figure 4.2A and B). ZDF islet expression of Elovl-5, SCD1, and Δ6D were modestly increased from 1.4- to 1.6-fold compared to control rats at 6 weeks. Expression of SCD2, however, was increased further to 2.3-fold over control. After 13 weeks of age, ZDF rats were hyperglycemic and both plasma insulin and insulin mRNA levels were diminished, possibly indicating islet failure. Except for Δ5D and Δ6D, which decreased with age in both control and ZDF rat islets, a large 60% decrease in expression was found for Elovl-6 and SCD2 in ZDF islets from 6 to 13 weeks. These results suggest the expression of enzymes involved in monounsaturated fatty acid synthesis may have an important role in islet function.

Table 4.1

Physiological parameters of 6 and 13 week old Control (*fa*/?) and ZDF rats

	Control (*fa*/?)		ZDF (*Leprfa*/Crl)	
	6 week	13 week	6 week	13 week
Body weight (g)	173.8	330.4	179.3	353.5
	+/- 9.0	+/- 5.8 , **	+/- 4.0	+/- 11.1 , **
Insulin (ng/ml)	2.0	3.6	9.2	2.6
	+/- 0.3	+/- 1.1	+/- 1.4 , *	+/- 0.5 , **
Glucose (mg/dl)	83.0	80.6	117.0	316.7
	+/- 3.5	+/- 4.0	+/- 5.0 , *	+/- 25 , * , **

Data are mean +/- SEM. Glucose values are fed blood glucose. *, $p < 0.006$ compared to Zucker control age matched. **, $p < 0.008$ compared to 6 week old with same phenotype.

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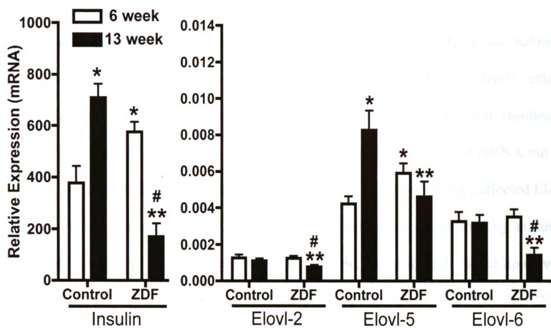
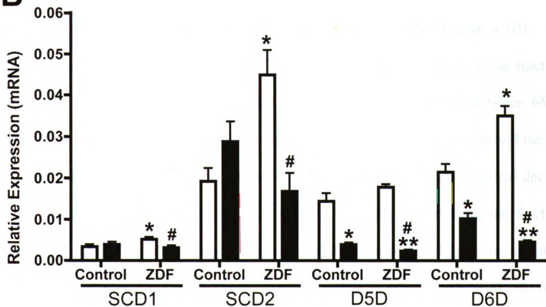
Figure 4.2. Differential expression of fatty acid elongase and desaturase genes in pre-diabetic and diabetic ZDF rat islets. A and B. Total RNA was isolated from control (*fa*/?) and ZDF (*Leprfa*/Crl) rat islets at 6 and 13 weeks of age and analyzed for insulin, Elovl-2, Elovl-5, Elovl-6, SCD1, SCD2, D5D, and D6D mRNA expression by real-time RT-PCR. Data are reported relative to cyclophilin and represent mean ± SEM for five or six animals per group. *, $p < 0.04$ and #, $p < 0.03$ when compared to 6 week control and ZDF islets, respectively. **, $p < 0.04$ when compared to 13 week control islets.

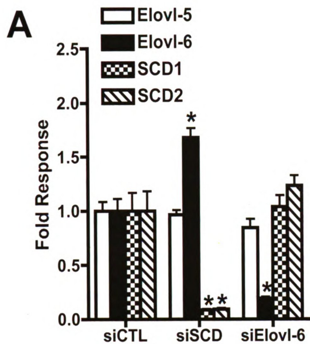

A
6 week
13 week
Relative Expression (mRNA)
1000
800
600
400
200
0
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Control
ZDF
Insulin
Elovl-2
Elovl-5
Elovl-6

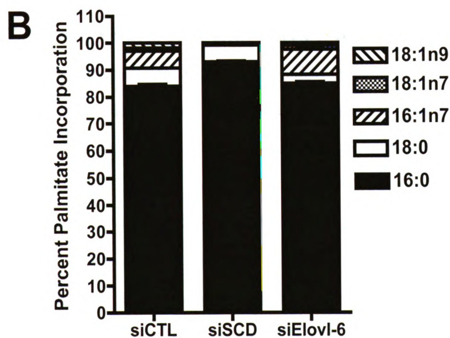

B
Relative Expression (mRNA)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Control
ZDF
SCD1
SCD2
D5D
D6D

4.3. Knockdown of SCD and Elovl-6 gene expression modulate MUFA synthesis.

To determine if SCD and Elovl-6 expression alter FA metabolism in β-cells, siRNAs were introduced into INS-1 cells and examined for effects on palmitate metabolism. In cells cultured for 36 hrs in INS-1 media, SCD siRNA effectively reduced both SCD1 and SCD2 mRNA levels compared to control (siCTL), but resulted in increased Elovl-6 mRNA (Figure 4.3A). Elovl-6 siRNA reduced Elovl-6 mRNA but did not affect SCD1 or SCD2. As a control, neither SCD nor Elovl-6 siRNAs affected Elovl-5 mRNA levels. Effects of siRNAs on FA metabolism were determined by culturing INS-1 cells for 12 hrs in 400 μM palmitate plus [1-14C]palmitic acid. Total lipids were extracted, saponified, and the FA profile analyzed by reverse-phase HPLC. In INS-1 cells with control siRNA, 14C-labeled palmitate was distributed by 83.9, 6.7, 6.4, 1.0, and 1.9% into palmitic acid (16:0), stearatic acid (18:0), palmitoleic acid (16:1,n-7), vaccenic acid (18:1,n-7), and oleic acid (18:1,n-9), respectively (Figure 4.3B). SCD siRNA significantly reduced the ability to convert palmitate into MUFA, as the fractional distribution of 14C-labeled 16:1,n-7, 18:1,n-7, and 18:1,n-9 decreased between 66 and 97%. This resulted in increased accumulation of 14C-labeled palmitic acid and the ratio of saturated FAs to MUFAs (data not shown). Elovl-6 siRNA caused a marked decrease in palmitate elongation, as shown by an approximate 50% reduction in the fractional distribution of 14C-labeled 18:0 and 18:1,n-9. This led to a 1.5-fold increase in labeled 16:1,n-7. These results show that knockdown of SCDs or Elovl-6 effectively decreases the respective desaturation and elongation of palmitate and alters MUFA synthesis.

Figure 4.3. Modulation of SCD and Elovl-6 gene expression alters synthesis of specific MUFAs species derived from exogenous palmitate. A. Effect of SCD2 and Elovl-6 siRNAs on mRNA levels. INS-1 cells were electroporated with siRNAs for control (siCTL), SCD (siSCD) or Elovl-6 (siElovl-6) and cultured for 36 hrs in INS-1 media. Levels for Elovl-5, Elovl-6, SCD1 and SCD2 mRNA were normalized to RPL32 mRNA and reported as fold expression relative to siCTL treated cells. Data are mean ± SEM for three independent experiments. *, $p < 0.02$ when compared to siCTL cells. B. Effect of SCD and Elovl-6 siRNAs on conversion of exogenous palmitate to MUFA. INS-1 cells electroporated with siRNAs for siCTL, siSCD, or siElovl-6 were treated with INS-1 media containing 400 μM palmitate and [1-14C]-palmitic acid for 12 hrs. Total lipids were extracted, saponified, and incorporation of 14C into FAs was determined by reverse-phase HPLC. Data represent the percent of labeled FA. Values are the mean ± SEM for three independent experiments.


A
Elovl-5
Elovl-6
SCD1
SCD2
Fold Response
2.5
2.0
1.5
1.0
0.5
0.0
siCTL
siSCD
siElovl-6


B
Percent Palmitate Incorporation
110
100
90
80
70
60
50
40
30
20
10
0
siCTL
siSCD
siElovl-6
18:1n9
18:1n7
16:1n7
18:0
16:0

4.4. Susceptibility to palmitate-induced ER stress is increased by SCD knockdown.

Loss of β-cell function upon exposure to exogenous FFAs, particularly saturated FAs, involves activation of the ER stress response pathways inositol requiring ER to nucleus signal kinase (IRE)1, PKR-like ER kinase (PERK), and, to a lesser extent, activating transcription factor (ATF)6 (204). In ZDF islets, the response to exogenous FFAs could be affected by altered SCD and Elovl-6 gene expression, described in Figure 4.2. To test whether reduced SCD or Elovl-6 expression affects the β-cell response to exogenous FFAs, INS-1 cells treated siRNAs were cultured for 9 hrs with 0, 200, or 400 μM palmitate and examined for the induction of ER stress. In control cells, only 400 μM palmitate induced a 2.3- to 4-fold increase in splicing of X-box binding protein 1 (Xbp1s) and mRNA levels of ATF3 and CHOP, markers of IRE1 and PERK activation, respectively (Figure 4.4A). Palmitate induction of CHOP mRNA levels corresponded with a 5.5-fold increase in CHOP protein (Figure 4.4B). In INS-1 cells treated with 200 μM palmitate, decreased SCD expression significantly increased the sensitivity to Xbp1 splicing and induction of ATF3 and CHOP mRNA by approximately 2-fold. Compared to control cells, induction of ATF3 mRNA, CHOP mRNA, and CHOP protein by 400 μM palmitate was increased further by SCD siRNA. In contrast, decreased Elovl-6 expression tended to reduce Xbpl splicing and CHOP protein levels induced by palmitate at 400 μM, but it was not significant.

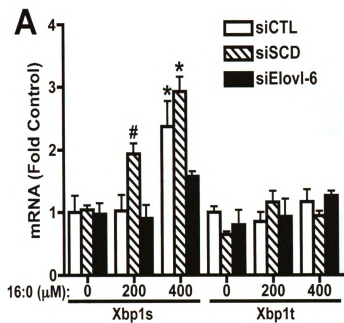
IRE1 also mediates phosphorylation of Jun N-terminal kinase (JNK) (245), which can lead to enhanced CHOP expression and apoptosis (204). In β-cells treated with FFAs, JNK was phosphorylated prior to the induction of CHOP expression (314). INS-1 cells treated for 6 hrs with 400 μM palmitate displayed increased JNK phosphorylation,

and this was markedly increased 2-fold by decreased SCD expression (Figure 4.4C). These findings demonstrate that susceptibility to palmitate-induced ER stress is enhanced in β-cells with a reduced capacity to synthesize MUFAs.

4.5. SCD knockdown impacts susceptibility to palmitate-induced apoptosis.

INS-1 cells with decreased SCD and Elovl-6 expression were then examined to determine whether early changes in sensitivity to ER stress correlated with the induction of apoptosis. In response to increasing concentrations of palmitate, control cells treated for 24 hrs with 300 and 400 μM palmitate had increased caspase-9 cleavage and DNA fragmentation, markers of apoptosis (Figure 4.5A and B). The susceptibility to both caspase-9 cleavage and DNA fragmentation in cells with decreased SCD expression were significantly increased at 200 μM palmitate. Compared to control cells, the induction of apoptosis was further increased at 300 and 400 μM palmitate by SCD knockdown, whereas knockdown of Elovl-6 did not affect either apoptotic marker. Thus, this data confirms a recent report that palmitate induced lipotoxicity is increased by simultaneous knockdown of both SCD1 and SCD2 (257).

Figure 4.4. Sensitivity to palmitate-induced ER stress is increased by SCD knockdown. INS-1 cells electroporated with siRNAs for control (siCTL, C), SCD (siSCD, S), or Elovl-6 (siElovl-6, E) were treated for 9 hrs with modified INS-1 media containing increasing palmitate concentrations. A. Effect of knockdown of SCD and Elovl-6 on levels of spliced Xbp1 (Xbp1s), Xbp1 total (Xbp1t), ATF3 and CHOP mRNA. Data are mean ± SEM for three independent experiments and are expressed relative to siCTL cells. * and #, $p < 0.02$ when compared to 0 μM siCTL cells and siCTL cells at the same palmitate concentration, respectively. B. Effect of SCD or Elovl-6 siRNA on CHOP protein levels. Whole cell protein extracts were fractionated by SDS-PAGE and CHOP protein was analyzed by Western blotting. Data are mean ± SEM for four independent experiments. * and #, $p < 0.03$ when compared to 0 μM siCTL cells and siCTL cells at 400 μM palmitate, respectively. C. Effect of SCD and Elovl-6 siRNA on JNK phosphorylation. INS-1 cells electroporated with siRNAs for control (siCTL, C), SCD (siSCD, S) or Elovl-6 (siElovl-6, E) were treated for 6 hrs without or with 400 μM palmitate. Phosphorylated and total JNK (pJNK and JNKt) were analyzed by Western blotting. Data are mean ± SEM for three independent experiments. * and #, $p < 0.03$ when compared to 0 μM siCTL cells and siCTL cells at 400 μM palmitate, respectively.


A
siCTL
siSCD
siElovl-6
mRNA (Fold Control)
16:0 (μM): 0 200 400 0 200 400
Xbp1s
Xbp1t

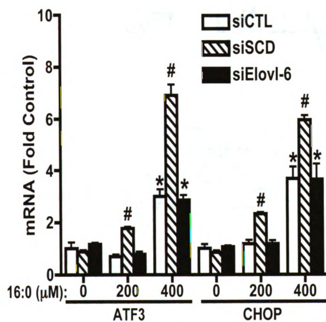
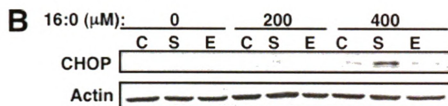
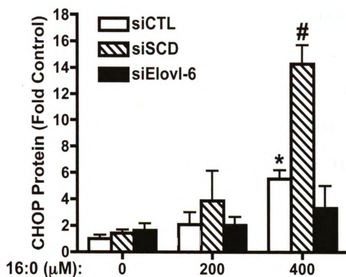
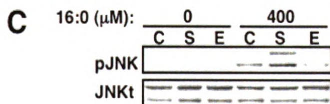

siCTL
siSCD
siElovl-6
mRNA (Fold Control)
16:0 (μM): 0 200 400 0 200 400
ATF3
CHOP

Figure 4.4. Continued







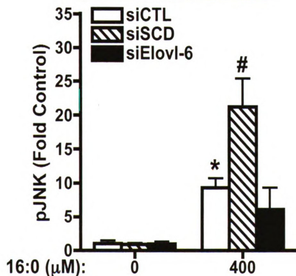
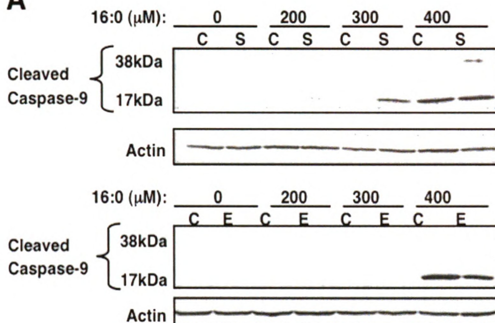
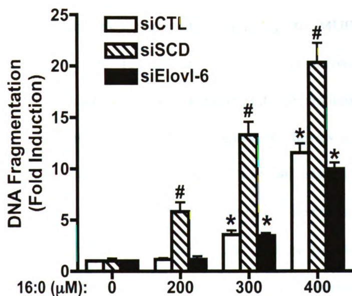


Figure 4.5. Suceptibility to palmitate-induced apoptosis is increased by SCD knockdown. INS-1 cells electroporated with siRNAs for control (siCTL, C), SCD (siSCD, S), or Elovl-6 (siElovl-6, E) were treated for 24 hrs with increasing palmitate concentrations. A. Effect of SCD and Elovl-6 siRNA on caspase-9 cleavage. Cleaved caspase-9 proteins were analyzed by Western blotting. Results shown are representative of six independent experiments. B. Effect of SCD and Elovl-6 siRNA on DNA fragmentation as determined by ELISA. Data represent fold induction and are mean ± SEM for six independent experiments. *, $p < 0.02$ when compared to 0 μM Luc. #, $p < 0.04$ when compared to siCTL at the same palmitate concentration.


A
16:0 (μM):
0
200
300
400
C
S
Cleaved
Caspase-9
38kDa
17kDa
Actin
E


B
siCTL
siSCD
siElovl-6
DNA Fragmentation
(Fold Induction)
16:0 (μM):
0
200
300
400

4.6. Over-expression of SCD2 and Elovl-6 differentially modulate MUFA synthesis and markers of ER stress.

Naturally occurring elevated expression of SCD1 and SCD2 in rat islets and β-cells coincides with reduced palmitate-mediated lipotoxicity, presumably due to SCD1 activity (6, 238). In rat islets and β-cells, however, the SCD2 isoform is expressed much higher than SCD1 (Figure 4.1 and 4.2) (257). To test whether enhanced SCD2 and Elovl-6 gene expression alone affect β-cell FA metabolism and ER stress, adenoviral constructs were used to over-express the respective genes in INS-1 cells treated with palmitate. In cells over-expressing luciferase (Luc) and cultured for 12 hrs in 400 μM palmitate plus [1-14C]palmitic acid, 14C-labeled palmitate was distributed by 71.5, 10.6, 10, 2.5, and 5.3% into palmitic acid (16:0), stearic acid (18:0), palmitoleic acid (16:1,n-7), vaccenic acid (18:1,n-7), and oleic acid (18:1,n-9), respectively (Figure 4.6A). Over-expression of SCD2 significantly increased palmitate conversion to MUFAs, as the fractional distribution of 14C-labeled 16:1,n-7 and 18:1,n-7 was increased 1.3- and 2.1-fold, respectively. This resulted in a marked increase in the accumulation of n-7 rather than n-9 MUFAs (data not shown). Elovl-6 over-expression, however, significantly increased palmitate elongation products, as the distribution of 14C-labeled 18:0 and 18:1,n-9 were increased 2.3- and 1.8-fold, respectively. These results demonstrate that in β-cells over-expression of SCD2 preferentially drives synthesis of n-7 MUFAs, whereas over-expression of Elovl-6 drives synthesis of stearate (18:0) and oleate (18:1,n-9).

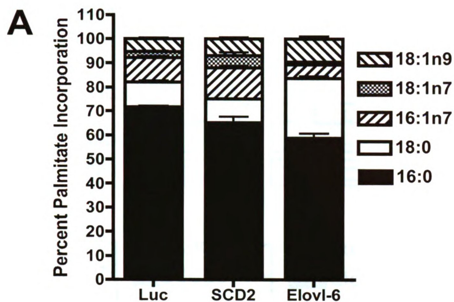
Next, INS-1 cells over-expressing either SCD2 or Elovl-6 were treated with 400 μM palmitate and examined for activation of ER stress. In contrast to SCD knockdown, over-expression of SCD2 resulted in a marked 56% reduction in CHOP protein levels

after 9 hrs of palmitate treatment compared to control cells (Figure 4.6B). Palmitate-induced JNK phosphorylation at 6 hrs was also reduced 40% by SCD2 over-expression (Figure 4.6C). Although Elovl-6 over-expression did not alter CHOP protein, it resulted in a significant 1.6-fold increase in JNK phosphorylation in palmitate treated cells compared to control cells. Together, this shows that enhanced SCD2-mediated synthesis of n-7 MUFAs protects from ER stress induced by exogenous palmitate, whereas ER stress is potentiated by enhanced Elovl-6 expression.

4.7. Effects of SCD2 and Elovl-6 over-expression on palmitate-induced apoptosis.

INS-1 cells with elevated SCD2 and Elovl-6 gene expression were then treated for 24 hrs with increasing concentrations of palmitate and monitored for apoptosis. Control cells over-expressing luciferase exhibited significant capase-9 cleavage at 400 μM palmitate and a dose-dependent increase in DNA fragmentation at 200, 300, and 400 μM palmitate (Figure 4.7A and B). Over-expression of SCD2 showed a marked reduction in cleaved caspase-9 and a 30-60% decrease in DNA fragmentation at each FA concentration tested compared to control cells. Elovl-6 over-expression increased both apoptotic markers at intermediate levels of palmitate but not at the 400 μM level. These results provide the first direct evidence that enhanced SCD2 and Elovl-6 expression modulate lipotoxicity in β-cells.

Figure 4.6. SCD2 and Elovl-6 over-expression on MUFA synthesis and palmitate-induced ER stress. A. Effect of SCD2 and Elovl-6 over-expression on MUFA synthesis. INS-1 cells treated with Ad-CMV-Luciferase (Luc), Ad-CMV-SCD2 (SCD2), or Ad-CMV-Elovl-6 (Elovl-6) were treated with INS-1 media containing 400 μM palmitate and [1-14C]-palmitic acid for 12 hrs. Total lipids were extracted, saponified, and incorporation of 14C into FAs was determined by reverse-phase HPLC. Values are the mean ± SEM for three independent experiments. B. Effect of SCD2 and Elovl-6 over-expression on CHOP protein levels. INS-1 cells treated with Ad-CMV-Luciferase (Luc, L), Ad-CMV-SCD2 (SCD2, S) or Ad-CMV-Elovl-6 (Elovl-6, E) were treated for 9 hrs with INS-1 media containing increasing palmitate concentrations, after which CHOP protein was analyzed by Western blotting. Data are mean ± SEM for six independent experiments. * and #, $p < 0.02$ when compared to control (Luc) cells treated with 0 or 400 μM palmitate, respectively. C. Effect of SCD2 and Elovl-6 over-expression on JNK phosphorylation. INS-1 cells treated with Ad-CMV-Luciferase (Luc, L), Ad-CMV-SCD2 (SCD2, S) or Ad-CMV-Elovl-6 (Elovl-6, E) were treated for 6 hrs without or with 400 μM palmitate. Phosphorylated and total JNK (pJNK and JNKt) were analyzed by Western blotting. Data are mean ± SEM for three independent experiments. * and #, $p < 0.03$ when compared to control (Luc) cells treated with 0 or 400 μM palmitate, respectively.


A
Percent Palmitate Incorporation
110
100
90
80
70
60
50
40
30
20
10
0
Luc
SCD2
Elovl-6
18:1n9
18:1n7
16:1n7
18:0
16:0

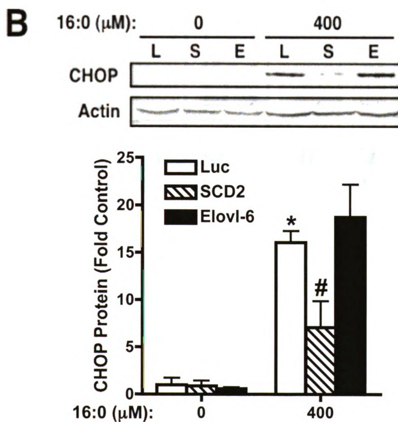

B
16:0 (μM):
0
400
L
S
E
L
S
E
CHOP
Actin
CHOP Protein (Fold Control)
25
20
15
10
5
0
Luc
SCD2
Elovl-6
*
#
16:0 (μM):
0
400

Figure 4.6. Continued

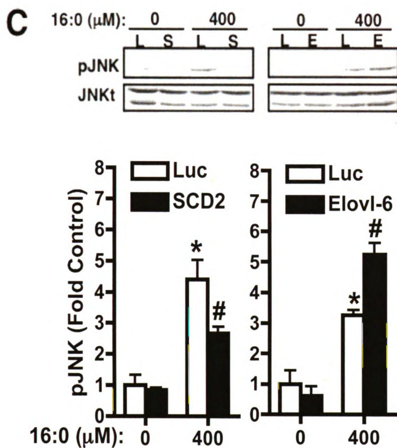
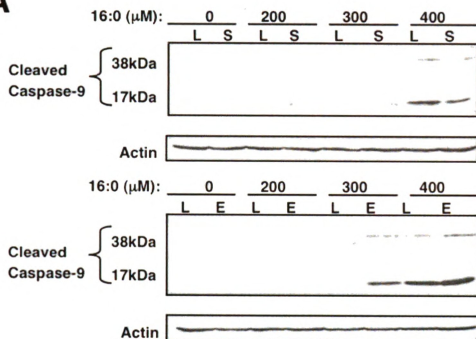
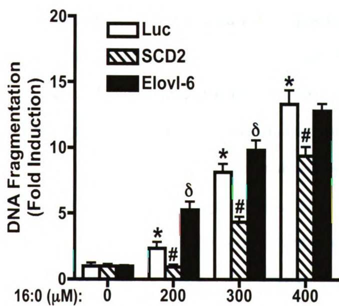


Figure 4.7. SCD2 and Elovl-6 over-expression on palmitate-induced apoptosis. INS-1 cells treated with Ad-CMV-Luciferase (Luc, L), Ad-CMV-SCD2 (SCD2, S) or Ad-CMV-Elovl-6 (Elovl-6, E) were treated for 24 hrs with increasing palmitate concentrations. A. Effect of SCD2 and Elovl-6 over-expression on caspase-9 cleavage. Cleaved caspase-9 proteins were analyzed by Western blotting. Results shown are representative of six independent experiments. B. Effect of SCD2 and Elovl-6 over-expression on DNA fragmentation as determined by ELISA. Data represent fold induction and are mean ± SEM for six independent experiments. *, $p < 0.03$ when compared to 0 μM Luc. #, $p < 0.03$ and δ, $p < 0.05$, when compared to Luc at the same palmitate concentration.


A
16:0 (μM): 0 200 300 400
L S L S L S L S
Cleaved Caspase-9
38kDa
17kDa
Actin
16:0 (μM): 0 200 300 400
L E L E L E L E
Cleaved Caspase-9
38kDa
17kDa
Actin


B
Luc
SCD2
Elovl-6
DNA Fragmentation (Fold Induction)
20
15
10
5
0
16:0 (μM): 0 200 300 400

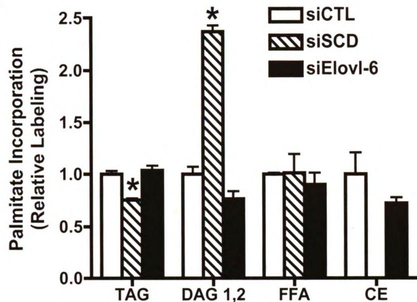
4.8. Elevated CHOP expression by SCD knockdown coincides with increased diacylglycerol formation and involves Ca2+-dependent PKC activation.

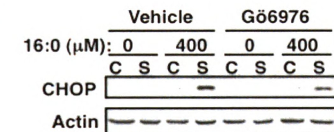
Palmitate-induced lipotoxicity has been proposed to involve reduced incorporation into TAG and cholesterol ester (CE) compared with MUFAs (6, 315). To determine if modulation of palmitate toxicity in INS-1 cells involved changes in neutral lipid synthesis, cells treated for 12 hrs with 400 μM palmitate plus [1-14C]palmitic acid were analyzed for 14C-labeled palmitate incorporation into complex lipids. Compared to control cells, INS-1 cells treated with SCD siRNA had a 25% reduction in palmitate incorporation into TAG, resulting in a significant 2.4-fold increased accumulation of DAG (Figure 4.8A). In cells over-expressing SCD2 complex lipid synthesis was not significantly altered (data not shown), suggesting protection from palmitate toxicity by enhanced SCD2 expression may not involve changes in neutral lipid synthesis. Elovl-6 siRNA did not affect TAG levels but reduced DAG levels by 23%, whereas complex lipid synthesis was not affected by over-expression of Elovl-6 (data not shown).

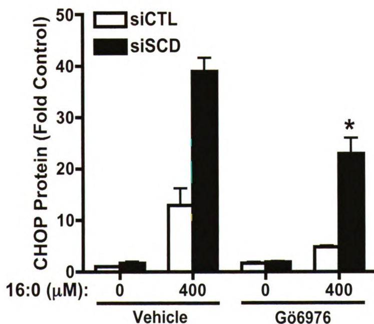
ER stress mediated by exogenous palmitate has been associated with release of ER Ca2+ into the cytoplasm (222). The combination of increased ER Ca2+ release with accumulation of DAG could cause β-cell dysfunction through sustained Ca2+-dependent protein kinase C (PKC) activation. To test this possibility, INS-1 cells treated with control or SCD siRNA were treated for 9 hrs with 400 μM palmitate and without or with the Ca2+-dependent PKC inhibitor Gö6976. In SCD knockdown cells, inhibition of Ca2+-dependent PKCs resulted in a significant 41% reduction of CHOP protein levels (Figure 4.8B). Taken together, increased susceptibility to palmitate-induced β-cell

dysfunction by SCD knockdown involves increased DAG accumulation and PKC activation.

Figure 4.8. Effect of SCD knockdown on palmitate-induced ER stress involves diacylglycerol accumulation and activation of Ca2+-dependent PKCs. A. INS-1 cells electroporated with siRNAs for control (siCTL), SCD (siSCD), or Elovl-6 (siElovl-6) were treated with modified INS-1 media containing 400 μM palmitate and [1-14C]-palmitic acid for 12 hrs. Total lipids were extracted, fractionated by TLC and 14C-labeled palmitate incorporation into complex lipids was determined by densitometry. Data represent fold change and are mean ± SEM for three independent experiments. *, $p < 0.01$ when compared to siCTL. B. INS-1 cells electroporated with siRNAs for control (siCTL, C) or SCD (siSCD, S) were treated for 9 hrs with 400 μM palmitate and without or with 1 μM Gö6976. Whole cell protein extracts were fractionated by SDS-PAGE and CHOP protein was analyzed by Western blotting. Data are mean ± SEM for three independent experiments. *, $p < 0.02$ when compared to siCTL cells.


A
Palmitate Incorporation (Relative Labeling)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
siCTL
siSCD
siElovl-6
TAG
DAG 1,2
FFA
CE


B
Vehicle
Gö6976
16:0 (μM):
0
400
0
400
C S C S C S C S
CHOP
Actin


CHOP Protein (Fold Control)
50
40
30
20
10
0
siCTL
siSCD
*
16:0 (μM):
0
400
0
400
Vehicle
Gö6976

Discussion

Chronic elevations in FFAs are associated with loss of β-cell function and the risk of developing type 2 diabetes (4, 203). Modulation of intracellular FA metabolism in β-cells is essential for preventing the toxic effects of FFAs and maintaining proper function. This study examined whether alterations in FA desaturase and elongase gene expression contribute to β-cell compensation and failure in response to lipotoxicity. Rat islets and INS-1 β-cells were found to express the desaturases SCD1, SCD2, Δ5D, and Δ6D, and the elongases Elovl-1, Elovl-2, and Elovls-4 to -7. In contrast to liver and adipose tissue, SCD2 is the predominant SCD isoform expressed in rat islets and β-cells (Fig 1) (257), and to date, the importance of SCD2 in β-cell MUFA synthesis has not been addressed. In addition, altered expression of SCDs and Elovl-6 in pre-diabetic and diabetic ZDF rat islets emphasizes that the capacity to synthesize MUFAs could significantly affect function, including the susceptibility of β-cells to lipotoxicity.

ZDF rats exhibit gradually increased plasma FFAs levels prior to the onset of overt diabetes (313). Thus, alterations in β-cell FA metabolism likely affect the response of ZDF islets to exogenous FFAs. Pre-diabetic ZDF rats at 6 weeks of age maintained euglycemia but were hyperinsulinemic, which correlated with increased islet insulin gene expression. Islets from pre-diabetic ZDF rats expressed significantly higher levels of SCD1, Δ6D, Elovl-5, and particularly SCD2, than control rats. This is consistent with studies showing elevated expression of SCD1, Δ6D, and Elovl-5 in livers of ZDF rats, Zucker fatty rats, and insulin resistant ob/ob mice (316-318). Increased Elovl-5 expression may serve as a negative feedback mechanism due to the ability of PUFAs to inhibit transcriptional activity of the sterol regulatory element binding protein-1c, a

regulator of lipogenesis (319, 320). More importantly, increased SCD gene expression in pre-diabetic ZDF rat islets may have a protective role by increasing the conversion of lipotoxic saturated FAs into MUFAs as observed in Zucker fatty rat islets (257). SCD and Δ6D gene expression is induced by insulin in liver (318, 321). Thus, elevated plasma insulin levels in ZDF pre-diabetic rats and Zucker fatty rats likely contribute to increased islet desaturase gene expression (Fig. 2) (257). As both control and ZDF rat islets aged, Δ5D and Δ6D gene expression decreased significantly, consistent with decreased PUFA desaturase activity found during aging in other tissues (322, 323). Hyperglycemic, diabetic ZDF rats at 13 weeks had decreased plasma insulin levels that coincided with reduced islet insulin gene expression, indicating islet failure. These islets also had reduced expression of SCD1, SCD2, and Elovl-6. Decreased SCD expression could be a result of islet failure due to reduced plasma insulin levels and a lack of islet insulin signaling. Loss of SCD expression and activity could contribute to islet failure due to increased accumulation of saturated FAs. Reduced Elovl-6 expression may have a protective role by decreasing elongation of palmitate to stearate and allowing it to be immediately desaturated to palmitoleate, a less lipotoxic FA.

Altered SCD and Elovl-6 gene expression in pre-diabetic and diabetic ZDF rat islets raised the possibility that changes in MUFA synthesis may modulate β-cell function. To examine the roles of SCDs and Elovl-6 in β-cells, these genes were knocked down and over-expressed in INS-1 cells and subsequently treated with elevated levels of palmitate. SCDs primarily desaturate the saturated FAs palmitate (16:0) and stearate (18:0). Reduced SCD1 and SCD2 expression in INS-1 cells exposed to exogenous palmitate significantly decreased total MUFA synthesis and increased the

ratio of saturated FA to MUFAs. Over-expression of SCD2, however, selectively increased n-7 MUFAs palmitoleate (16:1,n-7) and vaccenate (18:1,n-7), not oleate (18:1,n-9). This is at odds with increased oleate in cells over-expressing SCD1 (315), and raises the possibility that SCD2 preferentially desaturates palmitate over stearate. Elovl-6 elongates both palmitate and palmitoleate. Reduced Elovl-6 expression in INS-1 cells decreased palmitate elongation and increased palmitoleate accumulation. Over-expression of Elovl-6 strongly drove elongation of palmitate but not palmitoleate, resulting in enhanced synthesis of stearate and oleate. Taken together, conversion of exogenous palmitate into specific MUFAs, n-7 or n-9, in INS-1 cells is dependent on the level of expression and activity of SCD2 and Elovl-6.

ER stress results in activation of the unfolded protein response pathways IRE1, PERK and ATF6 (204). IRE1 induces genes important for ER expansion and reducing protein load by splicing and, in turn, activating the transcriptional activator Xbp1 (206). PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) to inhibit general protein translation while enhancing others such as ATF4 (244). Sustained PERK-ATF4 activation induces the pro-apoptotic genes ATF3 and CHOP (244, 324). Active ATF6 induces ER protein chaperones to aid in protein folding (219). In β-cells, exogenous saturated FAs largely activate the IRE1 and PERK pathways, increasing Xbp1 splicing, eIF2α phosphorylation, ATF4 protein, and mRNA and protein levels of ATF3 and CHOP (222, 223). Here, INS-1 cells treated with palmitate exhibited increased Xbp1 splicing, ATF3 and CHOP mRNAs, and CHOP protein. Decreased SCD gene expression significantly increased the sensitivity to palmitate induction of each of these ER stress markers, whereas decreased Elovl-6 tended to reduce Xbp1 splicing and CHOP protein.

Over-expression of SCD2 reduced palmitate induction of CHOP protein, demonstrating for the first time that SCD2 can modulate ER stress. Liver X receptor (LXR)-activated β-cells display increased SCD1/2 gene expression and protection from lipotoxicity (257, 325). LXR activation, however, did not alter ER stress, suggesting other mechanisms were involved (257). CHOP is also mediated through IRE1 activation of the cJun/cFos pathway by phosphorylation of JNK (204). INS-1 cells exposed to elevated palmitate had increased JNK phosphorylation, and this was significantly enhanced and reduced by knockdown and over-expression of SCDs, respectively. Decreased Elovl-6 expression tended to lower JNK phosphorylation by elevated palmitate, whereas over-expression of Elovl-6 increased palmitate-induced JNK phosphorylation. Thus, altered stearate production could affect ER stress. Overall, these findings directly demonstrate that enhanced MUFA synthesis, particularly through SCD2, reduces the susceptibility to palmitate-induced ER stress.

Accumulation of endogenous palmitate causes the release of ER Ca2+ stores, which could activate the intrinsic apoptosis pathway (204, 222). Increased ER stress in INS-1 cells with decreased SCD expression coincided with increased sensitivity to palmitate-induced caspase-9 cleavage and DNA fragmentation, hallmarks of apoptosis. This confirms that SCD knockdown increases susceptibility to β-cell dysfunction (256, 257). Consistent with a role of SCD to protect β-cells from palmitate-induced ER stress, SCD2 over-expression significantly reduced both markers of apoptosis. Decreased Elovl-6 expression did not significantly affect palmitate-induced apoptosis, whereas it was increased by over-expression of Elovl-6. The minimal effect of reduced Elovl-6 on ER stress and apoptosis could be due to the absence of a simultaneous increase in SCD

gene expression. Thus, Elovl-6 knockdown increases palmitoleate synthesis, but SCD2 over-expression drives it further and additionally increases total MUFA synthesis.

Protection from lipotoxicity in cells with elevated expression of SCD1 has been proposed to involve enhanced palmitate incorporation into neutral lipids (6, 315). Surprisingly, SCD2 over-expression did not enhance storage of exogenous palmitate into TAG or cholesterol ester (data not shown). This absence of an effect of SCD2 on neutral lipid synthesis could be due to enhanced glycerolipid/FA cycling or FA oxidation, thus the role of SCD2 in neutral lipid synthesis is under investigation. INS-1 cells with decreased SCD1 and SCD2 expression had lower palmitate incorporation into TAG and CE but a buildup of DAG, consistent with SCD1 involvement in TAG and CE synthesis (21, 315). Palmitoyl-CoA has been shown to activate PKCs (Corkey 2000). Accumulation of DAG and palmitoyl-CoA combined with release of ER Ca2+ could cause sustained activation of Ca2+-dependent PKCs, which may result in increased β-cell dysfunction such as ER stress. In support of this possibility, treatment with the Ca2+-dependent PKC inhibitor Gö6976 reduced the effect of palmitate on CHOP protein levels in INS-1 cells with decreased SCD expression.

In conclusion, we demonstrate that altered SCD and Elovl-6 expression in INS-1 cells modulates MUFA synthesis and susceptibility to palmitate-induced β-cell lipotoxicity. These findings emphasize that regulation of SCDs and Elovl-6 may significantly contribute to the preservation or loss of β-cell function and the development of type 2 diabetes.

Chapter 5.

Role of Fatty Acid Elongases in Determination of De Novo Synthesized Monounsaturated Fatty Acid Species

Abstract

Enhanced production of monounsaturated fatty acids (FAs) derived from carbohydrate-enriched diets has been implicated in the development of obesity and insulin resistance. The FA elongases Elovl-5 and Elovl-6 are regulated by changes in nutrient and hormone status and have been shown using intact yeast and mammalian microsome fractions to be involved in the synthesis of monounsaturated FAs. Herein, targeted knockdown and over-expression of Elovl-5 or Elovl-6 was used to determine their roles for *de novo* synthesis of specific species of monounsaturated FA in mammalian cells. Treatment of INS-1 cells with elevated glucose increased *de novo* FA synthesis and reduced the ratio of saturated to monounsaturated FAs. Elovl-5 knockdown decreased elongation of 16:1,n-7, whereas Elovl-5 over-expression increased synthesis of 18:1,n-7 but was dependent on stearoyl-CoA desaturase driven substrate availability of 16:1,n-7. Knockdown of Elovl-6 decreased elongation of both 16:0 and 16:1,n-7, resulting in accumulation of 16:1,n-7. In contrast to Elovl-5, Elovl-6 over-expression preferentially drove synthesis of 16:0 elongation products 18:0 and 18:1,n-9 but not 18:1,n-7. These findings demonstrate that coordinated induction of FA elongase and desaturase gene expression is required for balanced synthesis of specific n-7 versus

n-9 monounsaturated FA species. Furthermore, Elovl-6 is identified as a critical regulator in determining *de novo* synthesized FA end products.

Introduction

Diets high in carbohydrates and saturated fat are well established to cause altered fatty acid (FA) metabolism and elevated triglyceride accumulation, contributing to the development of obesity and type 2 diabetes. Elevated levels of carbohydrates specifically enriched in mono- and disaccharides induce the transcription of genes that increase glucose metabolism and lipogenesis in the liver (57, 326, 327), diverting excess catabolic metabolites into FAs for storage as triglycerides and cholesterol esters. FA elongase and desaturase enzymes catalyze the conversion of saturated FAs synthesized *de novo* from glucose into monounsaturated FAs (MUFAs) such as palmitoleate or oleate. The accumulation of MUFAs have been associated with hypertriglyceridemia and adiposity (254, 328, 329), and inhibition of MUFA synthesis decreases triglyceride levels and protects from diet-induced obesity and insulin resistance (36, 48, 255). Interestingly, palmitoleate was recently identified as an adipose tissue-derived lipid hormone capable of enhancing muscle insulin sensitivity (330). These findings emphasize the importance of understanding the mechanisms regulating the production of MUFAs.

Synthesis of *de novo* FAs involves the enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) which carboxylate cytosolic acetyl-CoA to malonyl-CoA and covalently bond malonyl-CoA C2 units to produce the C16 FA palmitate (16:0), respectively (8). After activation to palmitoyl-CoA, a FA elongase adds an additional malonyl-CoA to make stearoyl-CoA (18:0). MUFAs not derived from exogenous sources are synthesized by a delta-9 desaturase addition of a cis-double bond to

palmitoyl-CoA and stearoyl-CoA to form, palmitoleoyl-CoA (16:1,n-7) and oleoyl-CoA (18:1,n-9), respectively. Palmitoleoyl-CoA may also be elongated to vaccenyl-CoA (18:1,n-7).

Stearoyl-CoA desaturases (SCDs) are delta-9 desaturases and rate limiting for MUFA synthesis (20). SCD subtypes identified in mammalian cells thus far include SCDs 1-5 (11-15, 18). Saturated FAs desaturated by SCDs contained chain lengths from C13-19 for SCDs 1, 2, and 4 and C12-16 for SCD3 (20). Synthesis of C18 MUFAs *de novo* from glucose requires chain elongation of C16 FAs by a FA elongase (Elovl), yet it remains uncertain which elongases are involved in synthesis of specific MUFA species (e.g. 18:1,n-7 versus 18:1,n-9).

Substrate specificity analyses using yeast and *in vitro* microsomal preparations identified the elongases Elovl-5 (FAE1, Relo1, Helo1) and Elovl-6 (LCE, FACE, rElo2) to be involved in MUFA synthesis (31-33, 331). Elovl-6 and SCD gene expression are induced by insulin, liver X receptors, sterol regulatory element binding protein-1 (SREBP-1), and glucose induction of the carbohydrate-regulatory element binding protein/MAX-like factor X heterodimer (318, 325). In mice, hepatic Elovl-5, Elovl-6 and SCDs are induced by activation of peroxisome proliferator-activated receptor α and in leptin deficient, obese (ob/ob) mice, whereas they are suppressed by long-term feeding of diets high in saturated fat (318). Coordinated expression of elongases and desaturases by transcription factors that regulate lipogenic pathways, thus control the levels of MUFAs. In vitro assays have shown that Elovl-5 elongates unsaturated FAs, including 16:1,n-7, 18:3,n6 and 18:4,n3 (32, 33, 331). Relative to Elovl-5, Elovl-6 more effectively elongates C12-16 saturated FAs and 16:1,n-7 (31, 32). Because oleate (18:1,n-9) is the

predominate MUFA in cells, Elovl-6 may play a larger role in MUFA synthesis than Elovl-5 by converting palmitate to stearate, a saturated precursor of oleate.

Modulating the expression of FA elongase and desaturase genes has physiological significance, as mice with knockdowns of either Elovl-6 or SCD1 are protected from diet-induced obesity (36, 48, 255). The mechanism regulating the determination of specific *de novo* derived MUFA end products, however, remains to be defined. This study presents a comprehensive analysis of the effects of both decreased and increased expression of Elovl-5, Elovl-6, and SCDs on FAs synthesized *de novo* from glucose in a mammalian cell line. The results demonstrate that altering the expression of each enzyme causes significant changes in select MUFA species synthesized *de novo*.

Results

5.1. Regulation of FA elongase and desaturase genes by glucose

Studies characterizing the substrate specificity of FA elongases in mammalian cells have focused primarily on *in vitro* assays using cellular extracts or fractions. These *in vitro* assays indicated that Elovl-5 preferentially elongates 16:1,n-7 and polyunsaturated FAs, while Elovl-6 elongates 14:0, 16:0, and 16:1,n-7 (31-33, 331). Little is known, however, about their substrate preference in intact cells, particularly in regards to *de novo* derived FAs. To study the roles of Elovl-5 and Elovl-6, INS-1 cells were used to model intracellular *de novo* FA synthesis, as these cells are known to induce lipogenic gene expression in response to glucose (112, 234). INS-1 cells were treated with either 4 mM or 16.7 mM glucose to determine the glucose responsiveness of

lipogenic genes and FA elongase and desaturase genes indicated to be involved in MUFA synthesis (Figure 5.1A).

Genes required for *de novo* synthesized palmitate (16:0), ACC and FAS, were increased 7.9- and 1.8-fold, respectively, by 16.7 mM glucose compared to 4 mM glucose (Figure 5.1B). The expression of Elovl-5 and Elovl-6 was increased 1.3-fold, whereas SCD expression was elevated 2.6-fold for SCD1 and 3.2-fold for SCD2. These results show that between the FA elongases and desaturases involved in MUFA synthesis, the expression of SCDs in INS-1 cells is more responsive to glucose than Elovl-5 and Elovl-6. Further, this suggests that modulating the expression of Elovl-5, Elovl-6, and SCDs could significantly alter the elongation and desaturation status of newly synthesized FAs.

5.2. Elevated glucose increases the abundance of de novo synthesized FAs and alters the ratio of saturated to monounsaturated FAs.

Synthesis of *de novo* FAs in response to glucose was determined by measuring 14C-glucose incorporation into FAs in cells cultured for 48 hrs in either 4 mM or 16.7 mM glucose. INS-1 cells cultured in 16.7 mM glucose showed a 6- to 13-fold increase in 14C-labeled saturated FA and MUFA compared to cells cultured in 4 mM glucose (Figure 5.2A). The percent of labeled FAs was significantly increased for 16:1,n-7, 18:1,n-7 and 18:1,n-9, and decreased for 16:0 and 18:0 (Figure 5.2B). Thus, there was a 38% decrease in the ratio of total saturated FA to MUFAs in 16.7 mM glucose treated cells (Figure 5.2C). These data demonstrate that elevated glucose increases both the abundance of *de novo* synthesized FAs and the conversion of saturated FAs to MUFAs. INS-1 cells cultured in 11.1 mM glucose were subsequently used to examine the role of Elovl-5 and Elovl-6 for synthesis of specific 18:1 FA species.

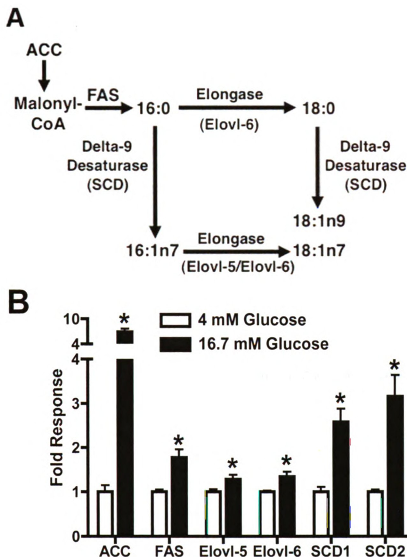
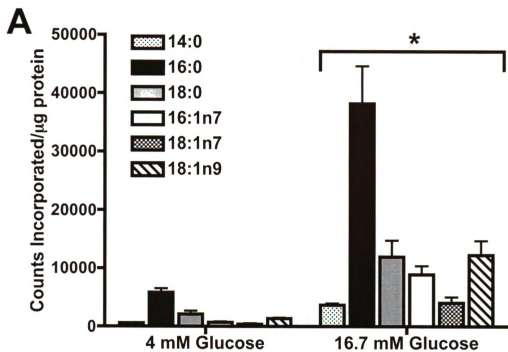


Figure 5.1. Glucose increased mRNA levels of genes involved in *de novo* lipogenesis and FA elongation and desaturation. A. Diagram of genes regulating end products of *de novo* FA synthesis. B. Levels of mRNA of *de novo* FA synthesis genes. Total mRNA was extracted from cells cultured for 48 hrs in 4 mM versus 16.7 mM glucose and analyzed by qRT-PCR. Data are relative to RPL32 expression and normalized to cells cultured in 4 mM glucose. Data represent mean ± S.E. (n=3). *, $p < 0.01$ when compared to 4 mM glucose.

Figure 5.2. Elevated glucose increased *de novo* FA abundance and monounsaturated FA synthesis. Cells were cultured for 48 hrs in 4 mM or 16.7 mM glucose. During the last 24 hrs the culture media was supplemented with [U-14C]-glucose (specific activity of glucose was held constant). Total lipids were extracted, saponified, and 14C incorporation into FAs was quantified by rp-HPLC. A. Newly synthesized FAs are represented as counts incorporated into specific FA species normalized to protein. B. Percentage of total labeled FA. The percentage of labeled 16:1,n-7, 18:0, 18:1,n-7, and 18:1,n-9 in high glucose cultured cells are significantly different when compared to cells cultured in low glucose ($p < 0.05$). C. Ratio of total saturated FAs to total MUFAs. Data are the mean ± S.E. for three independent experiments. *, $p < 0.03$ when compared to low glucose.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Counts Incorporated/µg protein
50000
40000
30000
20000
10000
0
14:0
16:0
18:0
16:1n7
18:1n7
18:1n9
*
4 mM Glucose
16.7 mM Glucose

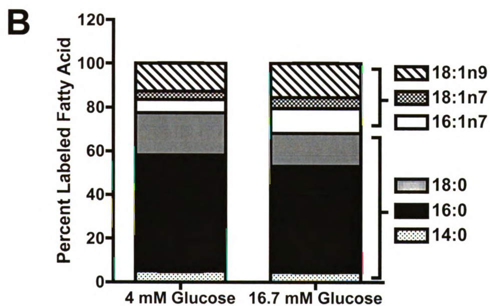
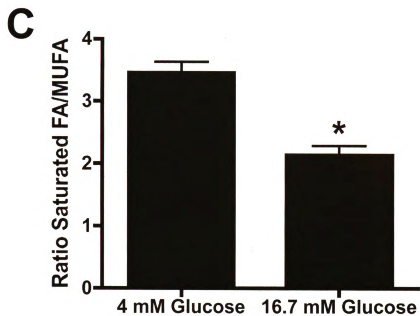

B
Percent Labeled Fatty Acid
120
100
80
60
40
20
0
18:1n9
18:1n7
16:1n7
18:0
16:0
14:0
4 mM Glucose
16.7 mM Glucose

Figure 5.2. Continued



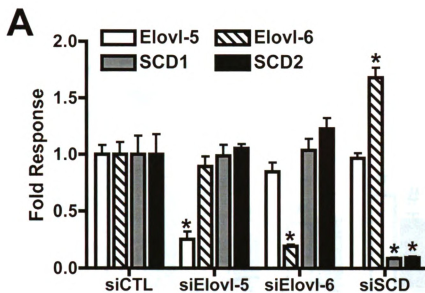
5.3. Selective knockdown of Elovl-5 or Elovl-6 impact synthesis of specific MUFA species.

siRNAs were used to determine the relative contributions of Elovl-5, Elovl-6 or SCD1/2 on the *de novo* synthesis of specific FAs species in INS-1 cells cultured in elevated glucose. siRNAs selective against Elovl-5 or Elovl-6 decreased expression of the target mRNA by 75% and 81% with no significant effect on non-target mRNA levels (Figure 5.3A). SCD siRNA reduced SCD1 and SCD2 by 91% and resulted in a 1.68-fold increase in Elovl-6. Next, *de novo* FA synthesis was assessed in siRNA treated cells using 14C-acetic acid. Decreased expression of Elovl-5 mRNA led to increased 14C-labeling of 16:1,n-7 and decreased labeling of 16:0 (Figure 5.3B). There was no significant change in 14C-labeled 18:0 and 18:1,n-7, but there was a trend for increased 14C-labeled 18:1,n-9. These data are consistent with reduced elongation of 16:1,n-7 and increased flux to 18:1,n-9 production. Decreased expression of Elovl-6 led to decreased 14C-labeling of 18:0 and 18:1,n-9, and increased 14C-labeling of 16:0 and 16:1,n-7. There was no change in labeled 18:1,n-7. These data are consistent with Elovl-6 mediating elongation of 16:0 to 18:0. Reduction of SCD expression decreased 16:1,n-7, 18:1,n-7 and 18:1,n-9 synthesis and led to increased 14C-labeling of 16:0 and 18:0.

Indexes of elongation and desaturation were calculated to determine the effects of decreased Elovl-5, Elovl-6, and SCD expression on the handling of specific FAs. In control cells, the elongation indexes for 16:0 and 16:1,n-7 are the same, whereas the desaturation index for 18:0 is approximately 3-fold greater than 16:0 (Figure 5.3C and D). Elovl-5 siRNA decreased 16:1,n-7 elongation and resulted in increased 16:0 elongation and desaturation, but caused no change in 18:0 desaturation. Elovl-6 siRNA

reduced 16:0 and 16:1,n-7 elongation and 18:0 desaturation. Elovl-6 siRNA had no effect on 16:0 desaturation. Reduced SCD expression decreased 16:0 elongation and caused a greater decrease in 16:1,n-7 elongation. As expected, desaturation of 16:0 and 18:0 with siSCD was also markedly decreased. These results demonstrate that decreased expression of Elovl-5 or Elovl-6 has dramatic effects on the synthesis of specific FA species derived *de novo* from glucose.

Figure 5.3. FA elongase and desaturase siRNA decreased gene expression and modulated MUFA synthesis. Cells electroporated with control (CTL), Elovl-5, Elovl-6 and SCD siRNA were cultured for 24 hrs in media containing 11.1 mM glucose followed by RNA extraction or overnight treatment with the same conditions plus [2-14C]-acetic acid. A. Levels of Elovl-5, Elovl-6, SCD1, and SCD2 mRNA, relative to RPL32 mRNA levels. Data are normalized to siCTL cells and represent the mean ± S.E. (n=3). *, $p < 0.02$ when compared to siCTL. B. Total lipids were extracted, saponified, and 14C incorporation into FAs was quantified by rp-HPLC. Data presented as percentage of total labeled FA species (n=3). C. Elongation index for each specific siRNA. Data are the mean ± S.E. (n=3). *, $p < 0.05$ for 16:0, and #, $p < 0.03$ for 16:1n-7 when compared to siCTL. D. Desaturation index for each specific siRNA. Data are the mean ± S.E. (n=3). *, $p < 0.04$ for 16:0, and #, $p < 0.02$ for 18:0 when compared to siCTL.


A
Elovl-5
Elovl-6
SCD1
SCD2
Fold Response
2.0
1.5
1.0
0.5
0.0
*
siCTL
siElovl-5
siElovl-6
siSCD

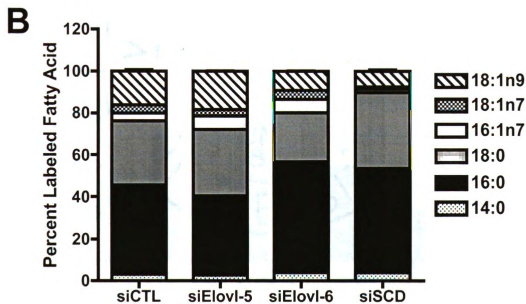
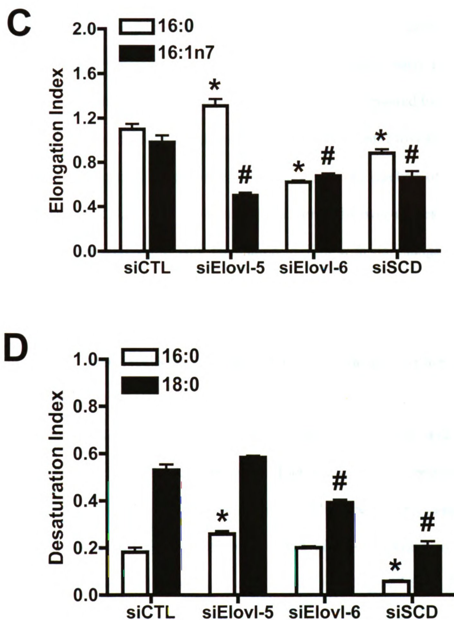

B
Percent Labeled Fatty Acid
120
100
80
60
40
20
0
18:1n9
18:1n7
16:1n7
18:0
16:0
14:0
siCTL
siElovl-5
siElovl-6
siSCD

Figure 5.3. Continued



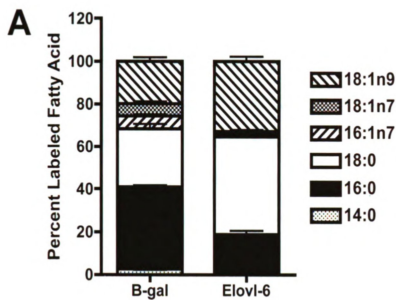
5.4. Over-expression of Elovl-5 or Elovl-6 leads to selective synthesis of specific MUFA species.

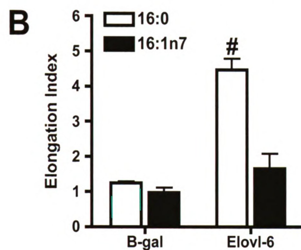
To further examine the selectivity of Elovl-5, Elovl-6, and SCD2 in *de novo* FA synthesis, adenoviruses were constructed to over-express each individual gene and examined for their effect on *de novo* FA end products. Compared to a control adenovirus containing β-galactosidase (Adβ–gal), over-expression of Elovl-6 resulted in a large increase in 14C-labeled 18:0 and 18:1,n-9 and decreased labeling of 16:0, 16:1,n-7, and 18:1,n-7 (Figure 5.4A). The elongation index from cells over-expressing Elovl-6 showed a large 3.6-fold increase in 16:0 elongation to 18:0 with no change in the rate of 18:0 desaturation to 18:1,n-9 (Figure 5.4B and C). The increased 16:0 elongation led to a 54% decrease in 16:0 desaturation and only a small, insignificant increase in 16:1,n-7 elongation. These results are consistent for production of 18:1,n-9 at the expense of 16:1,n-7 and 18:1,n-7.

Elovl-5 over-expression had a limited effect on 14C-acetic acid incorporation into FAs compared to Elovl-6 over-expression. Elevated Elovl-5 expression led to decreased 14C-labeled 16:0 and 16:1,n-7, and increased 14C-labeled 18:0 and 18:1,n-7 (Figure 5.5A). The effect of Elovl-5 on 16:1,n-7 and 18:1,n-7 labeling corresponded with a significant increase in the elongation index of 16:1,n-7 (Figure 5.5B). The minimal effect of Elovl-5 might be due to low 16:1,n-7 substrate availability. To test this possibility, cells were treated with an adenovirus over-expressing SCD2 alone or in combination with Elovl-5 over-expression. SCD2 over-expression caused a decrease in 14C-labeled 16:0 and 18:0 and a marked increase in 14C-labeled 16:1,n-7 and 18:1,n-7. This resulted in a 5-fold increase in the 16:0 desaturation index and a 1.5-fold increase in the 18:0

desaturation index (Figure 5.5C). Compared to SCD2 alone, the combination of Elovl-5 and SCD2 further increased 18:1,n-7 and reduced 16:1,n-7, demonstrating that Elovl-5 elongates *de novo* synthesized 16:1,n-7. These results emphasize a larger role of Elovl-6 and SCD than Elovl-5 in *de novo* FA synthesis in this cell model and point to the elongase Elovl-6 as being critical for regulating newly synthesized FA end products.

Figure 5.4. Effect of increased Elovl-6 expression on de novo FA end product formation. INS-1 cells were infected with adenoviruses expressing β-galactosidase (B-gal) and Elovl-6 and were then cultured for 24 hrs in 11.1 mM glucose and [2-14C]-acetic acid. Total lipids were extracted, saponified, and 14C incorporation into FAs was quantified by rp-HPLC. A. Percentage of total labeled FA species (n=3). B. Effect of β-galactosidase and Elovl-6 on elongation index. Data are the mean ± S.E. (n=3). #, $p < 0.01$ for 16:0 when compared to B-gal. C. Effect of β-galactosidase and Elovl-6 on desaturation index. Data are the mean ± S.E. (n=3). *, $p < 0.006$ for 16:0 when compared to B-gal.


A
Percent Labeled Fatty Acid
120
100
80
60
40
20
0
B-gal
Elovl-6
18:1n9
18:1n7
16:1n7
18:0
16:0
14:0


B
Elongation Index
6
5
4
3
2
1
0
16:0
16:1n7
#
B-gal
Elovl-6

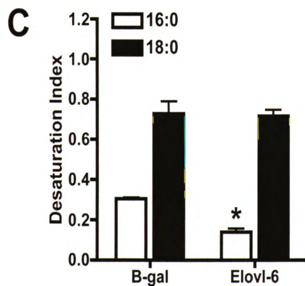
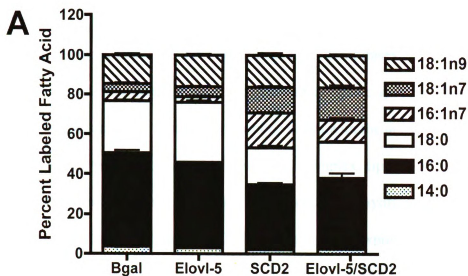
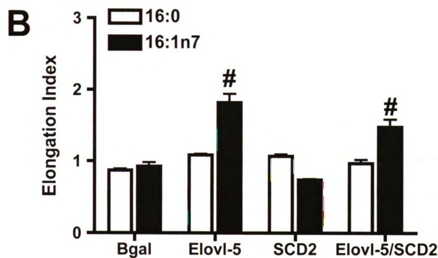
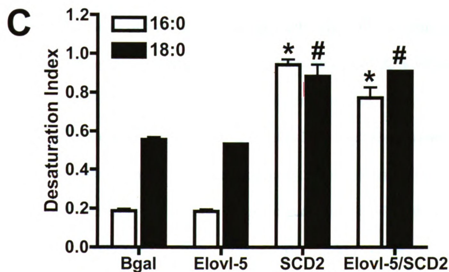

C
Desaturation Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
16:0
18:0
*
B-gal
Elovl-6

Figure 5.5. Effect of increased Elovl-5 or SCD2 expression on de novo end product formation. INS-1 cells were infected with adenoviruses that express β-galactosidase (B-gal), Elovl-5 or SCD2. Cells were cultured for 24 hrs in 11.1 mM glucose and [2-14C]-acetic acid. Total lipids were extracted, saponified, and 14C incorporation into FAs was quantified by rp-HPLC. A. Percentage of total labeled FA species (n=3). B. Effect of B-gal, Elovl-5 and SCD2 on elongation index. Data are the mean ± S.E. (n=3). #, $p < 0.02$ for 16:1,n-7 when compared to B-gal. C. Effect of B-gal, Elovl-5 and SCD2 on desaturation index. Data are the mean ± S.E. (n=3). *, $p < 0.007$ for 16:0, and #, $p < 0.002$ for 18:0 when compared to B-gal.


A
Percent Labeled Fatty Acid
120
100
80
60
40
20
0
Bgal
Elovl-5
SCD2
Elovl-5/SCD2
18:1n9
18:1n7
16:1n7
18:0
16:0
14:0


B
Elongation Index
3
2
1
0
16:0
16:1n7
#
#
Bgal
Elovl-5
SCD2
Elovl-5/SCD2


C
Desaturation Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
16:0
18:0
*
#
*
#
Bgal
Elovl-5
SCD2
Elovl-5/SCD2

Discussion

The expression of FA elongases and desaturases is highly regulated by transcription factors involved in glycolytic and lipogenic gene expression (318). This coordinates changes in nutrient and hormone status with the activation of lipogenic genes and altered synthesis of specific FAs and complex lipids, which can impact the susceptibility to disease. Exposure to elevated carbohydrates activates transcription factors such as SREBP-1c and ChREBP that induce the expression of genes that enhance glucose metabolism as well as the synthesis and storage of FAs (69, 57). As shown here, elevated glucose induced the expression of ACC, FAS, and FA elongases and desaturases, which increased the synthesis of *de novo* derived FAs. The role FA of elongases in determining end products of *de novo* FA synthesis from glucose has been largely speculative. Although substrate specificities of Elovl-5 and Elovl-6 *in vitro* using yeast and microsomal preparations indicated elongation of 16 carbon FAs (31, 32), the effect of altered expression of these enzymes on the specific species of FA synthesized from glucose has not been addressed. This study is the first to characterize the effects of both reduced and enhanced expression of Elovl-5 and Elovl-6 on the intracellular end products of *de novo* derived FAs in mammalian cells. The findings reveal a significant role for FA elongase activity in regulating the synthesis of *de novo* derived MUFAs and establishing the balance between 16:1,n-7, 18:1,n-7, and 18:1,n-9.

Elongation of FA by Elovl-5 is essential for control of hepatic lipid homeostasis as over-expression in liver decreased triglyceride content and knockdown led to activation of SREBP-1c, increased lipogenic gene expression, and hepatic steatosis (320, 332). Elovl-5 substrates include polyunsaturated FAs such as 18:4,n3, a precursor for

20:5,n3 FA synthesis, as well as 16:1,n-7 (32, 33, 331). Elevated levels of carbohydrate leads to increased synthesis of both 18:1,n-7 and 18:1,n-9 FA in INS-1 cells and liver (48). Targeted reduction of Elovl-5 expression decreased the elongation of 16:1,n-7 to 18:1,n-7 and increased elongation of 16:0 to 18:0 and the synthesis of 18:1,n-9, illustrating an ex vivo role for Elovl-5 in the elongation of 16:1,n-7. Findings in INS-1 cells are in stark contrast to Elovl-5 null mice, which have increased rather than decreased hepatic levels of 18:1,n-7 (320). Although 18:1,n-7 levels were unexpectedly elevated in Elovl-5 null mice, PUFA levels were reduced as expected. The increased levels of hepatic 18:1,n-7 maybe associated with reduced synthesis of 22:6,n3, an inhibitor of SREBP-1 processing (8, 320). Indeed Elovl-5 null mice had increased SREBP-1c levels, *de novo* FA synthesis, and Elovl-6 expression, which can elongate 16:1,n-7 to 18:1,n-7 (320). Over-expression of Elovl-5 in INS-1 cells decreased the amount of *de novo* derived 16:1,n-7 but only had a minimal effect on 18:1,n-7. The minimal effect of increased Elovl-5 expression was likely due to limited substrate availability as INS-1 cells have very low concentrations of 16:1,n-7 relative to 16:0. Consistent with this possibility, over-expression of SCD2 significantly increased 16:1,n-7 synthesis, which was available for Elovl-5 to elongate to 18:1,n-7. Under many physiologic states, increased SCD expression occurs with increased expression of Elovl-5 (and Elovl-6) (318). Elovl-5 might function to keep cellular concentrations of 16:1,n-7 low, thereby preventing accumulation of 16:1,n-7 that can serve as a cell-signaling molecule (330). Although the mechanism is unknown, exposure of cells to 16:1,n-7 enhances insulin signaling and its accumulation in the blood has been shown to correlate with increased muscle insulin sensitivity and protection from hepatic steatosis (36, 330).

The preferred substrate for triglyceride storage of excess FAs is the MUFA oleate (18:1,n-9) (21). Synthesis of 18:1,n-9 *de novo* requires the elongation of 16:0 to 18:0 prior to desaturation. Elovl-6 over-expression in INS-1 cells largely drove the elongation of 16:0 to 18:0 and promoted synthesis of 18:1,n-9 rather than elongation of 16:1,n-7 to 18:1,n-7. Conversely, reduced expression of Elovl-6 significantly decreased the products of 16:0 elongation (i.e. 18:0 and 18:1,n-9) while increasing 16:1,n-7. In addition, elongation of 16:1,n-7 to 18:1,n-7 was also decreased with siElovl-6. A role for Elovl-6 in 16:0 elongation is supported by Elovl-6 null mice, which displayed decreased hepatic accumulation of 18:0 and 18:1,n-9 and increased 16:0 and 16:1,n-7 (36). The effect of decreased elongation of both 16:0 and 16:1,n-7 by reduced Elovl-6 expression and activity is a shuttling of *de novo* synthesized FA towards the production of 16:1,n-7. Our data shows expression and activity of Elovl-6 is mostly involved in elongation of *de novo* synthesized 16:0 to produce n-9 MUFA species.

Although Elovl-5 and Elovl-6 activities can influence synthesis of specific MUFA species, SCD activity clearly plays the predominate role in total MUFA synthesis. This was exemplified by the large reduction in 16:1,n-7, 18:1,n-7 and 18:1,n-9 in INS-1 cells with reduced SCD1 and SCD2. Interestingly, Elovl-6 mRNA, but not Elovl-5 mRNA, was induced in SCD deficient INS-1 cells. This finding supports the unique role of Elovl-6 in synthesis of 18:1,n-9, the predominate MUFA in cells, and suggests that MUFA provide negative-feedback control on Elovl-6 expression. Over-expression of SCD2 in INS-1 cells, in the absence of increased Elovl-5/Elovl-6, led to increased 16:1,n-7 and 18:1,n-7, but had little impact on 18:1,n-9 synthesis. These data suggest that

without coordinate regulation of FA elongases, elevated SCD activity will disrupt the balance of 16:1,n-7, 18:1,n-7 and 18:1,n-9.

In conclusion, this study presents a comprehensive analysis of the effects of altered expression of Elovl-5 and Elovl-6 on *de novo* synthesized MUFAs. Our results demonstrate that Elovl-5 preferentially converts 16:1,n-7 to 18:1,n-7, whereas Elovl-6 preferentially elongates 16:0 to 18:0, which can be further desaturated to 18:1,n-9. Loss of coordinate control of Elovl-5, Elovl-6 and SCD can disrupt production of specific MUFA species, which may negatively influence cell function.

Chapter 6. General Conclusions and Future Studies

Chronic hyperglycemia and elevated FFA levels have been associated with the pathogenesis of β-cell dysfunction and T2D (4). Pancreatic β-cell FA metabolism is a growing area of research focused on identifying mechanisms to prevent the adverse effects of glucolipotoxicity. Recent studies demonstrated that β-cells possess innately enhanced regulation of specific FA metabolic pathways that contribute to preserving proper function (6, 104, 234, 257). This dissertation provides novel information into how changes in FA metabolism through activation of LXRs and alterations in MUFA synthesis modulate β-cell function in response to glucolipotoxicity.

Islet β-cells from hyperglycemic animal models of T2D exhibit diminished GSIS in conjunction with elevated lipogenic gene expression, *de novo* FA synthesis, and TAG accumulation (116, 233). This association between diminished GSIS and lipogenesis has been proposed to occur through activation of SREBP-1c, a major transcriptional regulator of lipogenic genes (116, 233). The findings presented here, however, demonstrate that enhanced activation of SREBP-1c, lipogenic gene expression, and TAG synthesis by activation of LXRs results in elevated basal insulin release and GSIS during chronic hyperglycemia. These results conflict with studies showing over-expression of a constitutively active SREBP-1c increases TAGs and causes loss of GSIS (116). The difference could be that β-cells are sensitive to the level of SREBP-1c activation, as its expression is required for LXR-mediated enhancement of insulin secretion (253). In addition, LXR activation may affect other FA metabolism pathways that impact β-cell function. Consistent with this possibility, elevated basal insulin release from LXR-activated INS-1 cells was blocked by inhibition of acyl-CoA formation and FA oxidation,

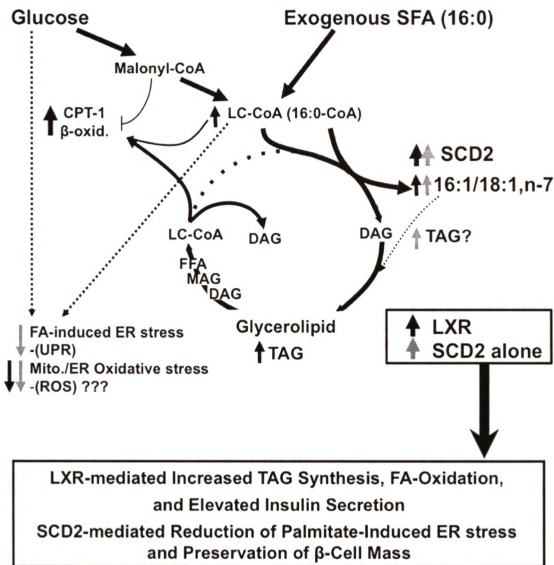


Figure 6.1. Mechanisms of protection from glucolipotoxicity by activation of LXRs and enhanced MUFA synthesis. LXR activation during chronic hyperglycemia drives *de novo* synthesis of 16:0, conversion of 16:0 to n-7 MUFAs, and TAG synthesis. In addition, LXR activation increased CPT-1 gene expression and FA oxidation. Subsequent lipoylsis of glycerolipid pools in LXR-activated β-cells increased basal insulin secretion, via the enhanced FA oxidation, and increased GSIS through generation of DAG. ER stress and loss of β-cell mass from exposure to excess exogenous palmitate was reduced by enhanced SCD2-mediated synthesis of n-7 MUFAs. Whether LXR activation and increased MUFA synthesis affect oxidative stress remains to be determined. Furthermore, how SCD2 protects from palmitate toxicity is still unknown. Overall, utilization of mechanisms designed to enhance LXR and SCD2 activity could provide significant protection of β-cells from glucolipotoxicity.

which coincided with increased FA oxidation and expression of genes involved in mitochondrial β-oxidation. The link between LXR activation and increased CPT-1 gene expression and FA oxidation is unclear, but may involve FA-mediated regulation of AMPK and ACC or an effect of LXR on PPARα as shown in the intestine (292).

Enhanced TAG synthesis and FA oxidation combined with the observation that TAGs are turned over rapidly indicated that the effect of LXR activation on GSIS involved turnover of neutral lipid pools. Inhibition of lipolysis by treatment of INS-1 cells with the general lipase inhibitor orlistat blocked the turnover of TAG and reduced GSIS. Turnover of TAG could, in turn, provide lipid signaling molecules such as DAG to enhance insulin secretion. In support of this hypothesis, elevated GSIS from LXR-activated cells was reduced by inhibition of DAG binding proteins. Thus, elevated GSIS by activation of LXRs involves TAG turnover and signaling through DAG. Our findings indicate that a balance between synthesis and turnover of neutral lipid pools is necessary for enhanced glycerolipid/FA cycling to protect β-cells from chronic hyperglycemia. This is supported by studies showing that altering only synthesis or turnover of neutral lipids causes reduced GSIS (104, 165, 239, 240).

Taken together, LXR activation elevates insulin secretion through a mechanism involving increased *de novo* synthesis and turnover of TAG and enhanced mitochondrial β-oxidation. These findings support the emerging role of increased glycerolipid/FA cycling in β-cell compensation (99). Obese ZF rats maintain normoglycemia, in part, through enhanced islet glucose- and FA-stimulated insulin secretion (104). Interestingly,

ZF rat islets display increased FA esterification, lipolysis, and FA oxidation as well as increased LXRα gene expression (104, 257). Therefore, LXRs could be key regulators of β-cell glycerolipid/FA cycling. Future studies will be necessary to determine if LXR is required for glycerolipid/FA cycling.

In conjunction with elevated TAG synthesis, LXR activation increased MUFA synthesis and expression of the FA desaturases SCD1 and SCD2. Characterization of FA elongase and desaturase genes in rat islets and INS-1 cells identified expression of the elongases Elovls 1, 2, 4, 5, 6 and 7, and the desaturases SCD1, SCD2, Δ5D and Δ6D. In contrast to the liver, SCD2 was the predominant SCD isoform expressed in β-cells, as shown recently (257). In addition, we demonstrated that SCD1 and SCD2 gene expression is elevated in pre-diabetic ZDF rat islets and reduced, along with Elovl-6, in diabetic ZDF rat islets. Prior to the onset of T2D, ZDF rats display gradually increasing concentrations of plasma FFAs (313), which are associated with loss of β-cell function (4). This suggested that regulation of genes involved in MUFA synthesis could be involved in β-cell compensation and failure during the development of T2D. In support of this hypothesis, we show that knockdown of SCD1 and SCD2 increased the susceptibility of INS-1 cells to palmitate-induced ER stress and apoptosis, confirming that SCD expression is required for protection against lipotoxicity (257). Increased palmitate toxicity in SCD knockdown cells was associated with reduced TAG, increased DAG, and Ca2+-dependent PKC activation. This correlates with studies showing that palmitate toxicity is associated with reduced incorporation into neutral lipids compared to MUFAs (241, 315). Although reduced palmitate toxicity in rat islets and MIN-6 β-cells correlates with enhanced SCD1 and SCD2 expression (6, 257), enhanced regulation

of other FA metabolism pathways could account for this protection as well. For example, ZF rat islets and MIN-6 β-cells have increased expression of SCDs and CPT-1, which coincided with increased FA oxidation (6, 104, 257). Here, we show for the first time that elevated expression of only SCD2 protects from palmitate-induced ER stress and apoptosis. Protection by SCD2 over-expression, however, did not coincide with increased TAGs. Further studies are needed to determine how enhanced SCD2 expression modulates lipotoxicity. In addition, palmitate toxicity tended to be reduced by Elovl-6 knockdown, whereas it was enhanced by Elovl-6 over-expression. This correlates with Elovl-6 knockdown having beneficial effects in liver, as Elovl-6 null mice are protected from diet-induced insulin resistance (36).

In the liver, increased MUFA synthesis coincides with increased expression of both SCD and Elovl-6 (318). Here, we show that elevated expression of either SCD2 or Elovl-6 alone alters the conversion of exogenous palmitate into specific MUFA species, n-7 versus n-9. The increased palmitate toxicity in cells over-expressing Elovl-6 is likely due to the significantly increased stearate production in the absence of a simultaneous increase in SCD expression. Interestingly, Elovl-6 drove the synthesis of both exogenous palmitate and *de novo* derived FAs towards stearate and oleate, rather and vaccenate. This demonstrates that although Elovl-6 elongates palmitate and palmitoleate *in vitro* (31, 32), its primary function is to elongate palmitate to provide the precursor for oleate synthesis. The FA elongase Elovl-5 is also involved in MUFA synthesis, as it elongates 16:1,n-7 to 18:1,n-7 (32, 33). Although Elovl-5 knockdown in INS-1 cells decreased elongation of *de novo* synthesized 16:1,n-7, its over-expression had a limited effect on 18:1,n-7 synthesis, which depended on SCD driven substrate availability of 16:1,n-7.

Taken together, our results show that altered expression of either SCD, Elovl-6 or Elovl-5 significantly effects MUFA end products, emphasizing the importance of coordinated regulation of these genes for maintaining balanced synthesis of n-7 versus n-9 MUFAs, as found in liver (318).

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