

Long-Term Treatment with Insulin and Retinoic Acid Increased Glucose Usage in L6 Muscle Cells via Glycogenesis

A Dissertation Presented for the

Doctor of Philosophy

Degree

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DEDICATION

Dedicated to those who have shown me unconditional love and support: my wife Jordan and our beautiful little girl Audrey Grace, my parents (Dorbie and Donna Goff), and to the rest of my family and friends.

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My time at the University of Tennessee, Knoxville (UTK) has been an overall rewarding experience that has provided me with not only a scholastic education, but a worldly education as well. Through the stress and times of challenge associated with obtaining a doctoral degree I have not only persevered, but I have come out of this process as a stronger and more dedicated individual. I completed not only my graduate degree at UTK, but also my undergraduate degree, therefore I must thank all of those whose courses I have taken as in some way you have guided me to this point.

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ABSTRACT

Skeletal muscle glucose metabolism can affect whole body glucose homeostasis significantly. Vitamin A (VA) plays a role in a number of physiological functions including glucose metabolism. However, its role in skeletal muscle glucose metabolism has not been well established. Insulin controls glucose metabolism in the skeletal muscle via the regulations of glucose uptake, glycogenesis, and glycolysis. We hypothesize that insulin and VA signaling pathways may converge to regulate glucose metabolism in skeletal muscle. Here, the effects of retinoic acid (RA) alone and in combination with insulin on glucose utilization in rat L6 muscle cells were studied. L6 cells were treated with 1 μ M [micromole] RA and 10 nM [nanomole] insulin for a period of 6 days. Compared to control, cells treated with RA utilized significantly more glucose at days 4 and 6. RA synergized with insulin to increase glucose usage at 4 and 6 days after treatment. RA and insulin synergistically increased the protein expression levels of glycogen synthase and glycogen synthase kinase-3 phosphorylation, while decreasing glycogen synthase phosphorylation. Similar results were seen in VA deficient (VAD) and sufficient (VAS) rats with the VAS rats who received insulin injections having decreased levels of glycogen synthase phosphorylation and increased levels of glycogen synthase. To determine possible nuclear receptors responsible for the RA effect, L6 cells were treated with specific agonists for retinoic acid receptor, retinoid-X-receptor, or liver-X-receptor. Only the retinoic acid receptor specific agonist mimics the effects of RA to increase glucose usage and protein expression level of glycogen synthase in the presence of insulin. In addition, glycogen content was significantly increased in L6 cells treated with RA + insulin and TTNPB + insulin. Interestingly, this increased glucose usage in L6 cells was associated with a reduction of glucose transporter

(GLUT) 1 and GLUT4 expression, and induction of GLUT3 and GLUT6 expression, a novel observation that has not been reported. We conclude that RA and insulin signaling pathways work cooperatively to enhance muscle cell glucose utilization through the induction of expression level of glycogen synthase, increase of glycogenesis, and alteration of expression profile of GLUTs in L6 cells.

TABLE OF CONTENTS

CHAPTER I. REVIEW OF LITERATURE ON VITAMIN A, INSULIN SIGNALING, METABOLIC DISORDERS, MUSCLE METABOLISM, AND GLYCOGEN REGULATION.....	1
1. Vitamin A.....	2
2. Insulin signaling, glucose transporters, and metabolic disorders.....	20
3. Skeletal muscle.....	35
4. Glycogen metabolism and regulation.....	51
5. Conclusion.....	81
CHAPTER II. RA AND INSULIN PROMOTE GLUCOSE USAGE IN L6 SKELETAL MUSCLE CELLS	82
1. Introduction.....	83
2. Materials and methods.....	84
3. Results.....	88
4. Discussion.....	108
5. Conclusion.....	113
CHAPTER III. REGULATION OF GLYCOGEN SYNTHASE AND GLUCOSE TRANSPORTERS BY VITAMIN A AND INSULIN IN L6 CELLS AND RAT SKELETAL MUSCLE TISSUE.....	114
1. Introduction.....	115

2. Materials and methods.....	118
3. Results.....	124
4. Discussion.....	152
5. Conclusion.....	160
CHAPTER IV. CONCLUSIONS AND FUTURE DIRECTIONS.....	162
1. Conclusion.....	163
2. Future directions.....	166
3. Summary.....	171
LIST OF REFERENCES.....	172
APPENDIX.....	203
VITA.....	205

LIST OF TABLES

Table 1.1	Skeletal muscle fiber classification and characterization.....	43
Table 1.2	Factors positively impacting GS regulation in studies using human materials.....	65
Table 1.3	Factors positively impacting GS regulation in animal studies.....	68
Table 1.4	Factors negatively impacting GS regulation in studies using human materials.....	75
Table 1.5	Factors negatively impacting GS regulation in animal studies.....	77
Table 3.1	List of real-time PCR primers used in this dissertation.....	204

LIST OF FIGURES

Figure 1.1	Chemical structure of molecules that exert VA activity.....	5
Figure 1.2	Schematic graph of the digestion, transport, absorption, and storage of molecules with VA activity.....	8
Figure 1.3	Schematic depiction of the retinoid signaling pathway within target cells.....	12
Figure 1.4	Schematic depiction of a typical nuclear receptor.....	15
Figure 1.5	Schematic depiction of glucose stimulated insulin secretion within pancreatic β -cells.....	24
Figure 1.6	Schematic depiction of skeletal muscle regeneration and differentiation process....	39
Figure 1.7	Differential regulation of glycogen metabolism within the (A) liver and (B) skeletal muscle.....	54
Figure 1.8	Hormonal regulation of glycogen metabolism within skeletal muscle.....	57
Figure 2.1	Changes of pH and glucose levels in media of L6 cells treated with ROL, RAL, or RA.....	90
Figure 2.2	Changes of pH and glucose levels in media of L6 cells treated with increasing doses of insulin and 1 μ M RA.....	92
Figure 2.3	Changes of pH and glucose levels in medium of L6 cells treated with increasing doses of RA and 10 nM insulin.....	95

Figure 2.4 Changes of pH and glucose levels in media of L6 cells treated with 1 μ M each RA, LG268, TTNPB, or T1317 with or without 10 nM insulin	99
Figure 2.5 Medium pH and glucose levels in L6 cells following 2 days of treatment with 1 μ M each RA or TTNPB with or without 10 nM insulin at increasing glucose concentrations.....	102
Figure 2.6 Medium pH and glucose levels in L6 cells following 4 days of treatment with 1 μ M each RA or TTNPB with or without 10 nM insulin at increasing glucose concentrations.....	104
Figure 2.7 Medium pH and glucose levels in L6 cells following 6 days of treatment with 1 μ M each RA or TTNPB with or without 10 nM insulin at increasing glucose concentrations.....	106
Figure 2.8 Decreases of GLUT1 and GLUT4 protein levels in L6 cells treated with insulin and RA.....	110
Figure 3.1 Abundance levels of key proteins in insulin signaling pathway in L6 cells treated with RA, LG268, or TTNPB in the absence or presence of insulin for 6 days.	126
Figure 3.2 Abundance levels of key proteins in glycogen signaling pathway in L6 cells treated with RA, LG268, or TTNPB in the absence or presence of insulin for 6 days.	129
Figure 3.3 Abundance levels of key proteins in glucose usage pathway in L6 cells treated with RA, LG268, or TTNPB in the absence or presence of insulin for 6 days.	133
Figure 3.4 Treatment with insulin enhances <i>Gys1</i> gene expression in L6 cells.....	135
Figure 3.5 Increased glycogen content expressed as fold induction (A) and glucose release (B) in L6 cells treated with insulin, RA + insulin, and TTNPB + insulin.....	138

Figure 3.6 Abundance levels of key proteins in insulin signaling pathway in gastrocnemius muscle of VAD and VAS STZ-induced diabetic rats treated with saline or insulin.....	139
Figure 3.7 Abundance levels of key proteins in glycogen signaling pathway in gastrocnemius muscle in VAD and VAS STZ-induced diabetic rats treated with saline or insulin.....	143
Figure 3.8 Abundance levels of key proteins in glucose uptake and usage pathway in gastrocnemius muscle in VAD and VAS STZ-induced diabetic rats treated with saline or insulin.....	147
Figure 3.9 GLUT1 and GLUT4 protein levels in L6 cells following one-hour treatment of RA, LG268, or TTNPB in the presence or absence of insulin.....	150
Figure 3.10 Changes in glucose transporter protein levels in L6 cells treated with RA, LG268, or TTNPB in the presence or absence of insulin.....	153
Figure 3.11 Schematic representation of proposed mechanisms involved in the long-term presence of RA and insulin effecting glucose utilization in L6 muscle cells.....	161
Figure 4.1 Proposed future experiments to be performed.....	167

LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Name</u>
ACC	acetyl-CoA carboxylase
ACh	acetylcholine
ACSM	American College of Sports Medicine
ADA	American Diabetes Association
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide-1-beta-D-ribose
AMPK	adenosine monophosphate activated protein kinase
ARAT	acyl-CoA:retinol acyltransferase
ATP	adenosine triphosphate
BMI	body mass index
BMP-2	bone morphogenetic protein-2
cAMP	cyclic adenosine monophosphate
CDK	cyclin-dependent kinase
CK	creatine kinase
CNS	central nervous system
COUP-TFII	chicken ovalbumin up-stream transcription factor II
CP	creatine phosphate
CRABP	cellular retinoic acid binding protein
CRALBP	cellular retinaldehyde binding protein
CRBP	cellular retinol binding protein

CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1
DAG	diacylglycerol
DBD	DNA-binding domain
DHP	dihydropyridine
DMEM	Dulbecco's modification of eagle medium
DNA	deoxyribonucleic acid
EAR	estimated average requirement
EDL	extensor digitorum longus
ER	endoplasmic reticulum
ER α	estrogen receptor alpha
FA	fatty acid
FAS	fatty acid synthase
FBS	fetal bovine serum
FFA	free fatty acid
G6P	glucose-6-phosphate
GAA	lysosomal acid alpha-glucosidase
GCK	glucokinase
GDP	guanine diphosphate
GDS	GLUT1 deficiency syndrome
GLUT	glucose transporter
GP	glycogen phosphorylase
GR	glucocorticoid receptor
GS	glycogen synthase
GSD	glycogen storage disease
GSIS	glucose-stimulated insulin secretion

GSK	glycogen synthase kinase
GSP	glycogen phosphatase
GTP	guanine triphosphate
HbA1C	glycated hemoglobin
HDL	high density lipoprotein
HGF	hepatocyte growth factor
HGFR	hepatocyte growth factor receptor
HPLC	high performance liquid chromatography
HRE	hormone response element
HS	horse serum
IFN- γ	interferon gamma
IGF-1	insulin-like growth factor-1
IKK	inhibitor of kappa kinase
IL	interleukin
IOM	institute of medicine
IP3	inositol 1,4,5-triphosphate
IRS	insulin receptor substrate
IU	international unit
JAK	Janus kinase
JNK	c-jun N-terminal kinase
LBD	ligand-binding domain
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LPL	lipoprotein lipase
LRAT	lecithin:retinol acyltransferase

LXR	liver-X-receptor
MEF	myocyte enhancer factor
MHC	myosin heavy chain
MRF	myogenic regulatory factor
MS	mass spectrometry
mTORC	mammalian target of rapamycin complex
NADPH	nicotinamide adenine dinucleotide phosphate
NF- κ B	nuclear factor-kappa B
NK	natural killer
OA	oleanolic acid
PA	physical activity
PBS	phosphate buffered saline
PCOS	polycystic ovary syndrome
PDK1	phosphoinositide-dependent protein kinase-1
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PKB/Akt	protein kinase B
PLC	phospholipase C
PP1	protein phosphatase 1
PTG	protein targeting to glycogen
RA	retinoic acid
RAE	retinol activity equivalent
RAL	retinal

RALDH	retinal dehydrogenase
RAR	retinoid acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
RBP4	retinol binding protein 4
RBPR2	retinol binding protein 4 receptor 2
RDA	recommended dietary allowance
11-RDH	11-cis- retinol dehydrogenase
RDH	retinol dehydrogenase
RDR	relative dose response
RE	retinyl ester
REH	retinyl ester hydrolase
RER	rough endoplasmic reticulum
R _{GL}	muscle-specific regulatory subunit
rhGAA	recombinant human lysosomal acid alpha-glucosidase
RPE	retinal pigment epithelium
ROL	retinol
ROS	reactive oxygen species
RXR	retinoid-X-receptor
SKIP	skeletal muscle and kidney-enriched inositol phosphatase
SLC2	soluble carrier family 2
STRA6	stimulated by retinoic acid 6
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
T4	thyroxine

TAG	triacylglycerol
TBC1D4	Tre-2/USP6, BUB2, Cdc16 domain family, member 4
TFE3	transcription factor E3
TGF- β	transforming growth factor–beta
T _H	T-helper
TNF- α	tumor necrosis factor alpha
TR	thyroid hormone receptor
TTR	transthyretin
UDP	uridine diphosphate
UTP	uridine diphosphate
VA	vitamin A
VADD	vitamin A deficiency disorders
VAD	vitamin A deficient
VDR	vitamin D3 receptor
WAA	whey amino acid
WPH	whey protein hydrolysate

CHAPTER I. REVIEW OF LITERATURE ON VITAMIN A, INSULIN SIGNALING, METABOLIC DISORDERS, MUSCLE METABOLISM, AND GLYCOGEN REGULATION

1. Vitamin A

1.1 Discovery of VA

A nutrient is known to be essential if it is required for normal physiological function. With the progression of modern nutritional science, the roles of nutrients and other dietary components in the prevention, and treatment of various diseases have been gradually revealed. However, the role of nutrients in the development of chronic metabolic disorders such as obesity, diabetes, and metabolic syndrome are still not fully understood. It was originally shown in studies investigating the feeding patterns of experimental animals that researchers discovered that macronutrients were not the only dietary components essential to life¹. The first lipid-soluble micronutrient to be described was termed fat-soluble factor A which eventually became known as vitamin A (VA, retinol) in 1920²⁻⁴. It was shown that when rats were fed a VA-deficient (VAD) diet, their growth ceased, but growth resumed once VA was added back to the diet³. For over 100 years now, research on VA has shown its importance on prevention and treatment of diseases as well as its role in immunity⁵⁻⁷. Clinically, a VA deficiency will manifest in symptoms related to neurological disorders as well as vision impairment^{8,9}. Likewise, VA toxicity resulting from either dietary intake, supplementation, or topical administration has been associated with bone abnormalities as well as alterations in lipid profiles¹⁰⁻¹³.

1.2 VA Sources

VA is an essential micronutrient since mammals cannot synthesize this fat-soluble vitamin, therefore, it is required that we obtain molecules with VA activity through dietary sources. They exist as either preformed VA which is classified as retinol (ROL) or retinyl esters (RE) and provitamin A which is also termed carotenoids (Figure 1.1)^{14,15}. Preformed VA is

derived from animal sources primarily in the form of RE whereas provitamin A is derived from plant sources. Provitamin A is found in a variety of colored fruits and vegetables that are enriched with the various carotenoids. The characterization of carotene revealed three isomers termed α -carotene, β -carotene, and γ -carotene, all of which are found in a variety of plants. Of these three isomers, β -carotene is the most abundant and exhibits the most biological activity¹⁶. Another provitamin A molecule called cryptoxanthins can be found along with carotenes in orange and yellow plants. Similar to α - and γ -carotene, cryptoxanthins possess about only half of the biological activity that β -carotene exhibits¹⁶.

1.3 Nomenclature and Properties of VA and VA Derivatives

VA activity refers to any retinoid exhibiting the biological activity of all-*trans*-retinol (commonly referred to as retinol, ROL)¹⁷. ROL is typically considered the parent retinoid compound that encompasses both preformed and provitamin A compounds¹⁸. Retinoids are compounds that share structural components such as a β -ionone ring, an isoprenoid side chain, and a functional group at the acyclic terminus (Figure 1.1). The isoprenoid side chain contains five carbon-carbon double bonds that allows for the structures to exist in *cis*- or *trans*- isomers. Isomerization of these compounds is common in nature as 11-*cis*-RAL is readily converted into all-*trans*-RAL in the rod and cone cells of the eye. Likewise, all-*trans*-ROL will go through a process requiring esterification, oxidation, and isomerization to form 11-*cis*-RAL during the vision cycle, which due to the polar end of the molecule it will bind to opsin via a Schiff base¹⁹. It is important to note that due to the spatial arrangement of chemical groups in the *cis*- and *trans*- isomers, these compounds will exhibit different biological activity with the *trans*-configuration often exhibiting more activity compared to its *cis*- counterpart^{20,21}.

Due to the presence of the β -ionone ring and isoprenoid side chain, retinoids display hydrophobicity which contributes to the “fat-soluble vitamin” title. VA molecules share amphipathic characteristics such as micelle formation when in aqueous environments, but in vivo experiments have shown that ROL and its derivatives will bind to proteins to prevent micelle formation²². The presence of double bonds in retinoids allows for oxidation reactions to produce compounds such as retrovitamin A, isoanhydrovitamin A, and anhydrovitamin A²³.

1.4 VA Absorption, Transport, and Storage

1.4a Preformed VA: REs released from the digestion of animal products will be enzymatically hydrolyzed to yield ROL and free fatty acids (FFA) within the small intestine lumen (Figure 1.2). This action is thought to be mediated by a combination of enzymes including intestinal phospholipases, pancreatic lipases, and esterases with the products, ROL and FFA, being transferred to the enterocytes for further processing²⁴. Within the enterocytes, two enzymes termed lecithin-retinol acyltransferase (LRAT) and acyl-CoA: retinol-acyltransferase (ARAT) will reesterify a portion of the newly arrived ROL back to RE. The reesterified REs are then packed into chylomicrons for delivery throughout the body via lymph circulation while a portion of ROL is transported throughout the body via portal circulation²⁵. Once REs arrive to the liver and are taken up by hepatocytes they are hydrolyzed once again to yield ROL and FFAs. This ROL can either be reesterified again at its hydroxyl group to produce RE to be stored in stellate cells in the liver or ROL can be released from the hepatocytes and enter into the circulation or it can be further processed to yield RAL (also referred to as retinaldehyde), RA, and other metabolites²⁶. This storage of REs in stellate cells serves as a reserve for periods of starvation or insufficient VA intake, since the REs can be released into the hepatocytes again for further

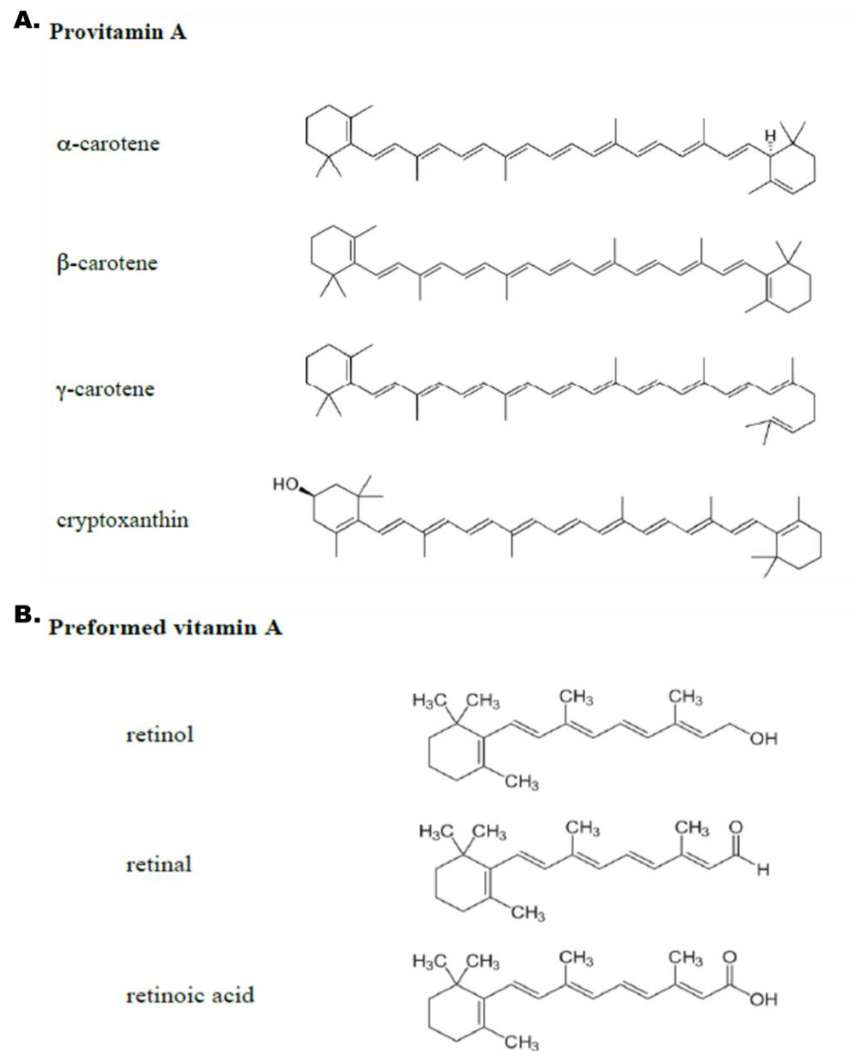


Figure 1.1 Chemical structure of molecules that exert VA activity. See sections 1.2 and 1.3 for details. (A) Chemical structures of Provitamin A and (B) Preformed vitamin A molecules.

processing. ROL will proceed through two oxidation steps to be converted to RAL and then RA²⁷. The conversion of ROL to RAL is a reversible process whereas the conversion of RAL to RA is irreversible²⁸. The process of converting ROL to RAL requires two families of enzymes termed alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs). RAL will then be irreversibly oxidized into RA by retinaldehyde dehydrogenases (RALDHs)²⁹. A specific subset of RALDHs encompassing RALDHs 1-4, are thought to be the primary dehydrogenases responsible for all-*trans*- and 9-*cis*-RA production in a number of tissues²⁹⁻³². RA synthesis seems to be primarily regulated by the actions of RALDH1 as this enzyme is downregulated when elevated levels of RA are detected^{33,34}. RA formation has also been postulated to occur in reactions not involving RALDHs including the oxidation of RAL by cytochrome p450 as well as by the cleavage of β -carotene to produce RA directly, bypassing the typical retinoid intermediates³⁵. It has been shown that cytochrome p450 26A1 (CYP26A1) does produce hydrophilic products from RA in hepatocytes and the expression of CYP26A1 is induced by RA treatment. For these reasons, CYP26A1 is commonly used as an indicator of RA production³⁶⁻³⁹.

1.4b Provitamin A: Ingested provitamin A carotenoids can be converted to RAL then to ROL in enterocytes and hepatocytes (Figure 1.2). This process is mediated by two pathways using the cleavage enzymes β,β -carotene-15,15'-dioxygenase (central cleavage) or β,β -carotene-9',10'-dioxygenase (eccentric cleavage)^{25,26}. Once RAL is formed it can be reduced to form ROL. ROL can bind to retinol-binding proteins (RBPs) to form a complex referred to as holo-RBPs. This complex is released into the blood circulation and once in the plasma it can interact with transthyretin (TTR) which will bind to thyroxine (T4). This complex will now transport both ROL and T4 to tissues expressing RBP receptors. Hepatocytes express the receptor RBP4

receptor-2 (RBPR2) and peripheral tissues express STRA6 to mediate the uptake of ROL from the plasma^{40,41}.

1.5 VA Assessment, Recommendations, Deficiency, and Toxicity

1.5a VA Assessment: To determine VA status in humans, both circulating levels of plasma ROL as well as RE reserves in the liver are analyzed, with the latter considered the best indicator of VA status. Of course, there are challenges in determining the “universal” levels of VA in humans such as variations in diets due to culture and geographic region⁴². With the advancements in biomedical technology, tools such as high performance liquid chromatography (HPLC) and mass spectrometry (MS) has become more readily available in clinical settings for the analysis of plasma ROL in individuals. VA sufficiency has been defined as a plasma ROL level $> 1.05 \mu\text{mol/L}$ while deficiency is set at $< 0.7 \mu\text{mol/L}$ ⁴³. Serum RBP4 has also been used to determine VA status, but to a lesser degree compared to serum ROL due to evidence showing that RBP4 levels decrease during periods of infection⁴⁴. A newer method termed the relative dose response (RDR) test has been developed to determine VA levels within the liver. In this test, the serum ROL concentration of a subject is measured and then the subject is given a dose of ROL, retinyl palmitate, or a ROL derivative. After 4-6 hours, serum ROL concentrations are measured once again to compare to baseline with a value greater than 20% considered a suboptimal VA level⁴⁵. This method is capitalizing on the concept that the liver will store exogenous VA under a fed state or VA sufficient state, but these compounds will be released into the circulation during a fasting or VA deficient state⁴⁴.

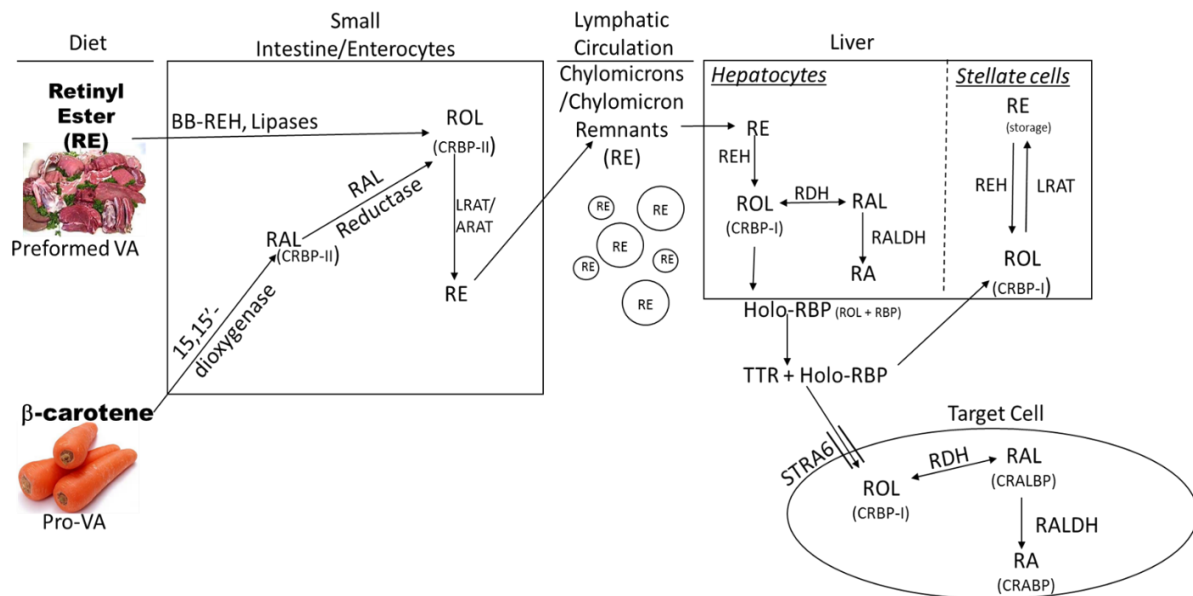


Figure 1.2 Schematic graph of the digestion, transport, absorption, and storage of molecules with VA activity. See section 1.4 for description. Abbreviations: BB-REH, brush-border retinyl ester hydrolase; LRAT, lecithin:retinol acyltransferase; ARAT, acyl-CoA:retinol acyltransferase; CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; CRALBP, cellular retinaldehyde binding protein; RA, retinoic acid; RAL, retinal; RALDH, retinaldehyde dehydrogenase; RBP, retinol binding protein; RDH, retinol dehydrogenase; REH, retinyl ester hydrolase; ROL, retinol; STRA6, stimulated by retinoic acid 6; TTR, transthyretin.

1.5b VA Recommendations: For decades, VA recommendations were expressed in international units (IU), but it was the Institute of Medicine in 2001 that introduced retinol activity equivalent (RAE) to replace IU. One RAE is approximately equal to 1 µg ROL or 2 µg of supplemental β-carotene and 12 µg of dietary β-carotene. One RAE is also equal to 24 µg of both α- and γ-carotene. The Institute of Medicine (IOM) has set the estimated average requirement (EAR) for VA at 627 µg RAE/day for adult men and 503 µg RAE/day for adult women. The recommended dietary allowance (RDA) for VA is set at 900 µg RAE/day for adult men and 700 µg RAE/day for adult women.

1.5c VA Deficiency: VA is known an essential micronutrient which is evidenced by the clinical symptoms of VA deficiency such as a decrease in growth, impaired immunity, retinopathies including night blindness, and anemia⁴⁶. Collectively, these conditions and others are referred to as vitamin A deficiency disorders (VADD), which is proposed to affect 140-200 million individuals worldwide⁴⁶. It is in developing countries that VADD continues to be of utmost concern, particularly in infants and children, resulting in health issues that last a lifetime⁴⁷. VA deficiency is a chronic process, as the deficiency continues the clinical symptoms will become more apparent and severe. Clinically, VA deficiency is diagnosed with a plasma ROL level below 0.7 µmol/L and/or a RDR value above 20%^{43,45}. Large doses of VA is given to those who are confirmed to be deficient, usually in a combination of foods rich in VA as well as in the form of VA supplements. This form of medical nutrition therapy will continue until the liver VA reserve is established and plasma levels remain within normal range⁴⁸.

1.5d VA Toxicity: Like a diet deficient in VA, having excessive VA intake, either from acute or chronic VA overdose, can lead to adverse health effects. Acute toxicity results from

intakes $\geq 40,000$ IU daily or roughly 150,000 μg within 1-2 weeks⁴⁹. Typical symptoms include headache, nausea, vertigo, blurred vision, muscle aches, alopecia, increased cerebral spinal fluid pressure, and lack of coordination. Chronic hypervitaminosis A usually becomes apparent 3 months to years after starting moderate high levels ($\sim 30,000$ $\mu\text{g/day}$) of VA consumption/supplementation⁴⁹.

1.6 Retinoid Signaling

1.6a Retinoid Signaling Overview: It is known that VA exerts its physiological functions by modulating gene expression in cells mainly in the form of RA⁵⁰. As previously mentioned, ROL becomes reversibly oxidized to RAL by RDHs, and then, RAL is irreversible oxidized to RA by RALDHs (Figure 1.3). The reaction of converting ROL to RAL has a low V_{max} value, making this reaction the rate limiting step in the production of RA⁵¹. It has been shown that RDH is anchored within the endoplasmic reticulum (ER) of the cell and cellular retinol binding protein I (CRBP I) facilitates the transfer of ROL to RDHs by functioning as a chaperone protein⁵²⁻⁵⁴. The RALDHs that catalyze the formation of RA from RAL are expressed and regulated in a tissue specific manner. RALDH1 is an enzyme that is expressed in all tissues and has been suggested to be a key regulator of adipogenesis in rodents⁵⁵. RALDH1 has been shown not to be required for rodent embryonic development, whereas it is lethal for mice to be RALDH2 deficient^{56,57}. The cytochrome p450 family of enzymes can contribute to the alteration of RA by using NADPH and oxygen to form various end products including, but not limited to 4-hydroxy-RA and 18-hydroxy-RA⁵⁸. CYP26A1 is the most investigated cytochrome p450 enzyme in regards to VA homeostasis due to its immense induction by RA⁵⁹.

RA molecules will interact with transcription factors once they move into the nucleus and play a role in regulating gene transcription (Figure 1.3). Metazoans contain a class of ligand-

activated transcription factors termed nuclear receptors that play a role in key physiological processes such as development, reproduction, differentiation, and metabolism⁶⁰. Retinoid acid receptors (RARs) and retinoid-X-receptors (RXRs) are two members of the nuclear receptor family that are believed to play a key role in the RA-induced effects on gene expression⁵⁰. RAR and RXR share a common ligand in 9-*cis*-RA whereas all-*trans*-RA only binds to RAR⁶¹. While 9-*cis*-RA binds to both RAR and RXR, this ligand seems to be only produced endogenously and there is no proof that all-*trans*-RA goes through isomerization to form 9-*cis*-RA⁶². 9-*cis*-RA has been shown to be the preferred substrate for RALDH4, but all-*trans*-RA is typically referred to as the naturally occurring form of RA that is vital for physiological actions⁶³. Once a ligand binds to its respective nuclear receptor a conformational change occurs that allows the receptor to interact with a number of transcriptional cofactors that can alter the regulation of their target genes⁶⁴. Within the promoter regions of RA responsive genes, there are short consensus DNA sequences, which are termed retinoic acid response elements (RAREs) that are typically recognized by RAR/RXR heterodimer or RXR/RXR homodimer⁶⁵. But, it has been shown that depending on the specific gene RXRs can bind to RARE in a heterodimer with other nuclear receptors such as thyroid hormone receptors (TRs), liver-X-receptors (LXRs), vitamin D3 receptors (VDRs), and chicken ovalbumin up-stream transcription factor II (COUP-TFII)^{66,67}.

1.6b Nuclear Receptors: With the advances in cloning and bioinformatics, it has been shown that humans encode 48 nuclear receptor family members while mice encode 49⁶⁸⁻⁷⁰. With the discovery of the full length sequences of the two founding members of the nuclear receptor family, the human glucocorticoid receptor (GR) and estrogen receptor α (ER α), it was shown that most nuclear receptors share a common structural configuration: a variable N-terminal region, a central conserved DNA binding domain (DBD), a non-conserved hinge domain, and a

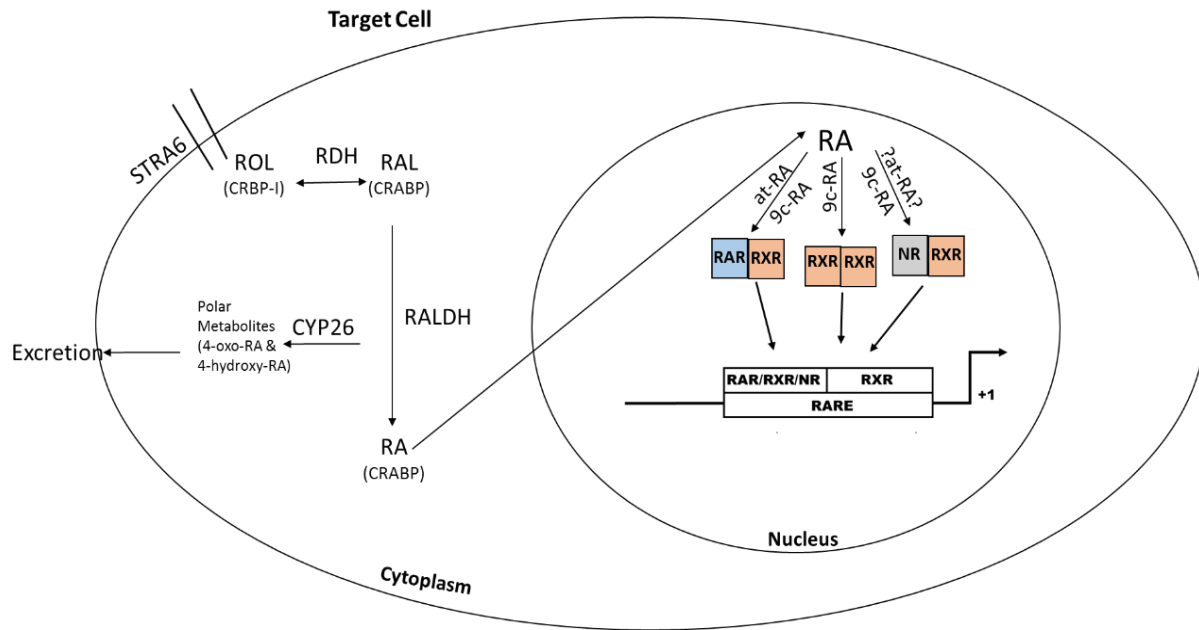


Figure 1.3 Schematic depiction of the retinoid signaling pathway within target cells. See section 1.6 for description. Abbreviations: CRBP, cellular retinol binding protein; CRABP, cellular retinoic acid binding protein; CYP26, cytochrome p450, family 26; NR, nuclear receptor; RARE, retinoic acid response element; RAR, retinoic acid receptor; RXR, retinoid-X-receptor; RA, retinoic acid; RAL, retinal; RALDH, retinaldehyde dehydrogenase; RDH, retinol dehydrogenase; ROL, retinol; STRA6, stimulated by retinoic acid 6.

C-terminal region that serves as a ligand binding domain (LBD) (Figure 1.4)⁶⁰. The DBD contains two highly conserved zinc finger motifs that allows the receptors to bind to specific DNA sequences termed hormone response elements (HRE). The non-conserved hinge domain allows for structural flexibility in the receptor which results in the receptor being able to interact with multiple HRE sequences. The LBD functions not only for binding of ligands, but also leads to receptor dimerization. The receptors will bind to a variety of ligands including FAs, RA, steroid hormones, and thyroid hormones⁷¹. A number of nuclear receptors that have been recognized as to having no identified natural ligand, thus these receptors are termed nuclear orphan receptors. When not bound to a ligand, the receptors will be found either in the nucleus bound to a HRE resulting in a repressive complex with a corepressor or in the cytoplasm bound to heat shock and chaperone proteins^{72,73}.

A characteristic shared by both RAR and RXR is that they both contain three members of their respective family: α , β , and γ . Each of these subtypes of nuclear receptors have been found to be encoded by separate genes in the human and rodent genomes with each subtype susceptible to alternate isoforms due to alternative splicing⁷⁴. Both of these classes of nuclear receptors will bind to a RARE that is located in the promoter of various genes (Figure 1.3). The RARE sequence consists of two consensus half sites (AGGTGA) that are separated by nucleotides that arrange the site as either palindromic, inverted palindromic, or direct repeats⁷⁵. The regulation of gene expression depends on a variety of factors including the receptor specificity for the promoter region, the cofactors that bind with the receptors, and the arrangements of the RARE sequences⁷⁵⁻⁷⁷. Cofactors that can promote or suppress transcription typically interact with RARs and RXRs through their LBD domains. Once a cofactor binds to the LBD it can induce a

conformational change either allowing or preventing the nuclear receptor and coactivator to bind other cofactors to induce or suppress transcription^{75,78-80}.

1.7 Physiological Roles of VA

1.7a Cell Proliferation and Differentiation: Retinoids have been known for decades now for their ability to slow cell growth to promote the differentiation of many cell types⁸¹⁻⁸³. Retinoids primarily regulate gene transcription to alter cell proliferation and differentiation through the activation of their nuclear receptors (RAR and RXR)⁷⁴. This can be done either by the receptors binding to specific response elements in the promoter or enhancer region of target genes or by interfering with other transcription factors^{84,85}. In particular, all-*trans*- and 9-*cis*-RA seem to be the primary regulators of gene expression and possess the dynamic role in the modulation of cell proliferation and differentiation⁸⁶. The retinoids elicit their effects on cells by either directly altering the expression of proteins involved in proliferation and differentiation or indirectly by altering the expression of their receptors and/or binding proteins⁸⁷. A deficiency in VA can result in cell abnormalities and defects in cell maturity as evidenced by proper stem cell differentiation not occurring in the absence of VA⁸². Furthermore, VA is required for the proper progression of spermatogenesis in the testis for male fertility by all-*trans*-RA suppressing Sertoli cell proliferation and assisting in differentiation, particularly during puberty⁸⁸.

RA has been shown to be a regulator in the proliferation and differentiation of cancer cells by altering the progression of the cell cycle. The treatment of RA in various cancer cell lines have shown that RA can have anti-proliferative effects by down-regulating the expression of cyclin-dependent kinase (CDK) and c-myc while up-regulating their inhibitors to halt the cell cycle at the G0/G1 phase⁸⁹. HL-60 cells are a cell model system commonly used to study acute

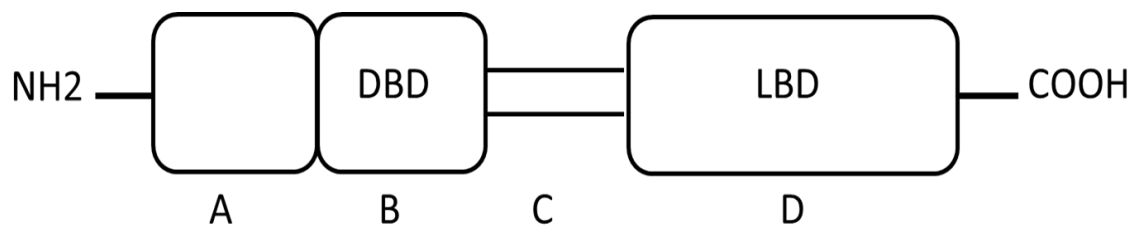


Figure 1.4 Schematic depiction of a typical nuclear receptor. See section 1.6 for description.

A – variable N-terminal region; B – central conserved DNA binding domain; C- non-conserved hinge domain; D- carboxy-terminal ligand binding domain.

myeloid leukemia and in this cell line it has been shown that RA can induce their differentiation from a promyelocytic leukemic state into granulocytes which could have therapeutic benefits^{90,91}. *In-vitro* studies have shown that all-*trans*-RA has the capability to induce not only the differentiation, but also the apoptosis of a variety of tumor cells including glioma cells^{92,93}. Similar results have been found in HepG2 cells, hepatoma cell line commonly used to investigate liver cancer, also by regulating cyclin-cdk activities^{94,95}. RA and cAMP when used in combination have been shown to be more effective at slowing the growth and inducing the differentiation of hepatocellular carcinoma cells than either of the molecules when used independently⁹⁶. Interestingly, all-*trans*-RA has shown to induce the proliferation and differentiation of brain tumor stem cells, but the differentiation is incomplete allowing the cells to form brain tumor spheres⁹⁷.

RA has also been shown to be an inducer of skeletal muscle as well as smooth muscle cell differentiation. In a study optimizing the differentiation of human epithelial stem cells into skeletal muscle, it was found that RA enhanced skeletal myogenesis by increasing the expression of the myogenic regulatory factors (MRFs) MyoD and myogenin which are two key transcriptional mediators of muscle cell differentiation⁹⁸. Transforming growth factor β (TGF- β) is a known inhibitor of myogenic differentiation by inhibiting the expression of MyoD and myogenin⁹⁹. However, in the mouse C2C12 skeletal muscle satellite cell line, RA alleviated the anti-myogenic effect of TGF- β 1, allowing for myoblast differentiation¹⁰⁰. RA has been shown to not only enhance skeletal myogenesis in myoblasts, but also in various stem cell lines as well as in chick limbs¹⁰¹⁻¹⁰³. The treatment of mouse embryonic stem cells with RA led to increases in skeletal myogenesis by upregulating the expression of skeletal muscle progenitor factors as well as MyoD and myogenin¹⁰⁴. *In-vitro* smooth muscle cell data has shown that all-*trans*-RA

modulates the proliferation, migration, and actin expression in the cells through an RAR α -dependent signaling pathway¹⁰⁵. Human arterial smooth muscle cells treated with all-*trans*-RA displayed decreases in their proliferation and migration attributed to the RA influencing differentiation as well as extracellular matrix synthesis and degradation¹⁰⁶.

1.7b Growth and Development: Early studies investigating VA supplementation and deficiency in animals showed that VA status affects growth and development¹⁰⁷⁻¹⁰⁹. Studies in the 1930s had shown that maternal deprivation of VA would result in severe congenital malformations or in fetal death^{109,110}. RA is known to be involved in the signal transduction pathways regulating embryonic development as well as the development of the central nervous system (CNS)^{110,111}. Sufficient levels of VA are required for the proper segmentation and patterning of the hindbrain as well as the forebrain¹¹². The absence of VA during development can have detrimental effects such as allowing epithelial stem cells to proliferate, but not differentiate into the normal phenotype for proper epithelial lining¹¹³. VA deficiency has been shown to lead to abnormalities in the heart, liver, ocular tissues, and reproductive system¹¹⁴⁻¹¹⁷. It is important to note that the opposite is also detrimental during developmental periods. When teratogenic levels of RA are administered to pregnant mammals it has been shown to affect the CNS leading to disorders such as microcephaly, microphthalmia, and spina bifida^{118,119}. Overall, teratogenic doses of VA has been shown to have detrimental effects on a number of tissues including the heart, bone, brain, and eyes^{120,121}.

1.7c Vision: The effects of VA on vision was first shown thousands of years ago with the treatment of night blindness with animal liver, an organ enriched with VA and its derivatives, but it would not be until the early 1900s that studies distinctly showed the presence of VA in the retina^{2,122}. It was discovered that RAL is the form of VA present in the retina and that this VA

derivative can transition between the *cis*- and *trans*-isoforms within the retina¹²³⁻¹²⁶.

Furthermore, it was during this period in the evolutionary elucidation of the vision cycle that rhodopsin was characterized and its mechanism of synthesis revealed¹²⁷. VA was also found to be critical for the development of the cornea as individuals with diets deficient in VA typically develop xerophthalmia¹²⁸. Over time there were more studies conducted on eye structure and mechanisms required for vision, therefore, the process that we now know as the retinoid visual cycle has been largely revealed. This cycle relies primarily on retinal pigment epithelial (RPE) cells, photoreceptor cells, and Müller cells. RPE is a monolayer of epithelial cells adjacent to the outer region of the photoreceptors, separating the photoreceptor cells from the microvascular network. Therefore, RPE cells assist in the uptake of all-*trans*-ROL from the choroid blood vessels to be converted to 11-*cis*-RAL for the introduction into photoreceptors to combine with opsin to form visual pigment^{129,130}. Rod cells and cone cells are together classified as photoreceptor cells which contain the transmembrane pigment rhodopsin and they face the microvascular network¹⁹. Finally, Müller cells are a type of glial cell that spans across the photoreceptor cells and contains cellular retinaldehyde binding protein (CRALBP) and cellular retinol binding protein (CRBP) that are pertinent to the visual cycle^{19,131,132}.

In the visual cycle all visual pigment will contain opsin that is combined with the chromophore 11-*cis*-RAL to form rhodopsin¹⁹. The opsin portion is used to determine the wavelength that the pigment responds to while the 11-*cis*-RAL will assist in the shape change due to light exposure. In a series of steps termed “bleaching” the activation of rhodopsin by light exposure will isomerize 11-*cis*-RAL to all-*trans*-RAL which will now dissociate from opsin and move into the cytosol of the photoreceptor cells¹⁹. Once in the photoreceptor cells all-*trans*-RAL is converted to 11-*trans*-ROL by RDH8 and RDH12, so that the 11-*trans*-ROL can be

transported to the RPE^{133,134}. Once in the RPE cell, the 11-*trans*-ROL will either be stored or bind to CRBP I and it will be esterified by LRAT to form a RE¹³⁵. The RE will be hydrolyzed by RPE65 to produce 11-*cis*-ROL which will bind to CRABPI, so that it can be oxidized to 11-*cis*-RAL by 11-*cis*-ROL dehydrogenase¹³⁶. This visual cycle is completed by the transport of 11-*cis*-RAL back to the photoreceptor cells to bind with opsin to form rhodopsin¹⁹.

1.7d Immunity, Infection, and Inflammation: The role of VA in immunity has been studied for decades with VA being shown to be a major factor in assisting in host defense against bacterial and viral diseases along with the status of VA having a major impact on the chronic inflammatory response¹³⁷. VA and retinoids play a role in innate immunity, cell-mediated immunity, and humoral-mediated immunity^{138,139}. Like most other micronutrients, a deficiency in VA can increase the risk for infection while supplementation can prevent morbidity and mortality particularly in children^{140,141}. VA, particularly RA, has been shown to up-regulate the production of cytokines favoring the generation of T-helper type 2 (T_H2) cells and down-regulate the T-helper type 1 (T_H1) response while VA deficiency will have the opposite effect¹⁴²⁻¹⁴⁵. The Th2 response is responsible for protecting against non-invasive infections and is required to promote antibody production and maturation as well as for the deactivation of macrophages which are immune cells commonly recruited to sites of inflammation¹⁴⁶. The T_H1 is responsible for protection against intracellular infections and its response leads to the activation of macrophages and production of inflammatory cytokines, thus promoting cytotoxicity¹⁴⁶. RA has been shown to induce the expression of various immune cells including, but not limited to B-cells, T-cells, neutrophils, dendritic cells, and macrophages¹⁴⁷. RA is required for the proper development and maturity of B-cells and subsequently antibody production by assisting in the regulation of B-cell proliferation and differentiation^{147,148}. RA has been shown to repress the

transcription of genes involved in the NF- κ B signaling pathway as well as IL-2, both of which are typically up-regulated during periods of inflammation¹⁴⁹⁻¹⁵¹. RA has also been identified as a suppressor of IFN- γ and TNF- α gene expression which is an example of the RA-mediated Th2 anti-inflammatory response^{145,152}. A deficiency in VA will impair antibody production and promotes a T_H1 inflammatory response by suppressing the production of anti-inflammatory cytokines while increasing the production of pro-inflammatory cytokines^{137,153-155}. Conversely, *in-vitro* data has shown the supplementation of VA will dose-dependently increase the expression of anti-inflammatory cytokines IL-4, IL-5, and IL-13, thus promoting the T_H2 response^{155,156}.

RA also seems to play a role in mucosal immunity as RA has been shown to reduce TGF- β expression in the intestinal epithelial cells, contributing to the intestine's anti-inflammatory responses¹⁵⁷. A deficiency in VA can therefore impair both the humoral and cellular immunity of the intestinal mucosa resulting in susceptibility to infections¹⁵⁸. T-cells, neutrophils, macrophages, and natural killer (NK) cells all have altered responses during periods of VA deficiency and chronic marginal VA status¹⁵⁹⁻¹⁶². It should be noted that certain infections can possibly lead to a deficiency in VA due to decreasing appetite and nutrient intake, possibly causing nutrient malabsorption, nutrient loss through vomiting and diarrhea, and altering typical nutrient utilization^{138,163-165}.

2. Insulin Signaling, Glucose Transporters, and Metabolic Disorders

2.1 Insulin Overview and Signaling Pathway

Insulin is a peptide hormone secreted from the pancreatic β -cells primarily in response to the increase in glucose due to meal consumption. Frederick Banting and John Macleod were

awarded the Nobel Prize for the discovery of insulin in 1923 and it was in 1958 that Frederick Sanger won the Nobel Prize in chemistry for determining the protein structure of insulin – the first protein to have its sequence determined¹⁶⁶. Insulin is first synthesized as a single polypeptide chain referred to as preproinsulin. Preproinsulin contains a 24-residue signal peptide that assists with the delivery of the newly formed protein to the rough endoplasmic reticulum (RER) where the signal peptide is then cleaved to produce proinsulin¹⁶⁷. Within the cisternal space of the lumen in the RER, folding of proinsulin leads to the formation of three disulfide bonds which is required for the transport of proinsulin to the Golgi complex. Here, proinsulin is cleaved by endo- and exo-nucleases to release a fragment referred to as C-peptide and the mature insulin product which is two polypeptide chains, A- and B-chains, which are linked together by two disulfide bonds^{167,168}. This mature insulin product will be packaged within granules inside pancreatic β -cells awaiting for metabolic stimulus for its release. The effects of insulin are most prominent in the liver, adipose tissue, and muscle. Insulin primarily stimulates anabolic functions: (1) insulin will decrease circulating fatty acids by inhibiting the activity of hormone-sensitive lipase which degrades triacylglycerol (TAG) in adipose tissue and it will increase the transport and metabolism of glucose in adipose cells to assist with TAG synthesis; (2) insulin will decrease glycogenolysis and gluconeogenesis in liver; (3) insulin will promote amino acid uptake and protein synthesis; (4) insulin will promote glucose uptake in target cells expressing insulin receptors and promote the formation of glycogen^{169,170}.

As shown in Figure 1.5, the release of insulin from the β -cells is mediated first by the entry of glucose into the β -cell through the glucose transporter 2 (GLUT2)¹⁷¹. Once in the cell, glucose will enter glycolysis leading to a rise in the ATP:ADP ratio which will close the ATP-sensitive potassium channel¹⁷². This will prevent potassium ions (K^+) from leaving the cell and

the buildup of K^+ will lead to depolarization of the cell membrane resulting in opening of voltage-sensitive calcium ions (Ca^{2+}) allowing the Ca^{2+} to enter the cell by facilitated diffusion¹⁷²⁻¹⁷⁵. Once the intracellular concentration of Ca^{2+} rises, the enzyme phospholipase C (PLC) becomes activated. PLC will cleave the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 will bind to receptor proteins found in the ER plasma membrane to assist with Ca^{2+} entry into the cells via IP_3 gated channels. With the significant increases in Ca^{2+} in the β -cells, the insulin stored within the secretory vesicles will be released via exocytosis¹⁷³.

Once insulin binds to insulin receptors on target cells (myocytes and adipocytes), there is a signaling cascade that follows to change the metabolism of the target cell. Insulin will bind to the extracellular α -subunit of the insulin receptor which leads to a conformation change in the receptor leading to the activation of a kinase domain in the intracellular β -subunit^{176,177}. This kinase can now phosphorylate tyrosine residues on the C-terminus of the receptor as well as in the insulin receptor substrate (IRS)¹⁷⁸. There are six known IRS's with IRS-1 proposed as the major IRS in skeletal muscle and IRS-2 as the main IRS in liver¹⁷⁹. IRS can now bind to and activate phosphatidylinositol 3-kinase (PI3K) which is recruited to the plasma membrane and will convert PIP_2 and ATP into PIP_3 and ADP. The increases in PIP_3 will lead to the recruitment of Akt (also referred to as protein kinase B, PKB) by interacting with the pleckstrin homology domain of Akt¹⁸⁰. PIP_3 also recruits PIP_3 -bound phosphoinositide-dependent protein kinase-1 (PDK1) to the plasma membrane which will phosphorylate and activate Akt¹⁸¹. PIP_3 has also been shown to recruit and activate the mammalian target of rapamycin complex-2 (mTORC2) by a mechanism yet to be fully established. mTORC2, like PDK1 will phosphorylate Akt which inactivates glycogen synthase kinase (GSK) by phosphorylating the α - and β -subunit

of the enzyme^{182,183}. By inhibiting GSK, glycogen synthase phosphatase (GSP) can activate glycogen synthase (GS)¹⁸¹. This allows GS to continue producing glycogen and Akt will allow for vesicle fusion, resulting in the translocation of a specific GLUT (GLUT4 in the case of muscle and adipose tissue) to the plasma membrane for glucose entry into the cell¹⁸⁴.

2.2 Glucose Transporters

It was in 1948 that the concept of glucose transporters were first hypothesized due to the obvious requirement of glucose transport across lipid bilayers¹⁸⁵. It would not be revealed until 30 years later that a specific protein embedded in the plasma membrane of erythrocytes was responsible for mediating the glucose uptake^{186,187}. Since this discovery there have been 14 members of the GLUT protein family identified in humans, all of which are encoded by the soluble carrier family 2 (SLC2) genes^{188,189}. The GLUT family has been categorized into three classes based on their sequence similarity: 1) Class 1 includes GLUT1-4, GLUT14; 2) Class 2 includes GLUT5, GLUT7, GLUT9, and GLUT11; 3) Class 3 includes GLUT6, GLUT8, GLUT10, GLUT12, and GLUT13¹⁸⁸. All of the GLUTs characterized to date have been shown to contain roughly 500 amino acid residues arranged in a 12 membrane-spanning helical structure that allows both its amino and carboxyl terminals to be positioned in the cytoplasm^{188,190}. Furthermore, the GLUTs helical structure contains seven conserved glycine residues, two conserved tryptophan residues, and two conserved tyrosine residues¹⁸⁸. A substrate binding site will located on the outside of the cell, but a binding site can also be inside the cell if the transporter functions bidirectionally¹⁸⁹. Once glucose or a related hexose binds to the substrate binding site a conformational change in the GLUT occurs allowing glucose to be transported and released on the other side of the membrane¹⁹⁰. GLUTs 1-4 have been the most

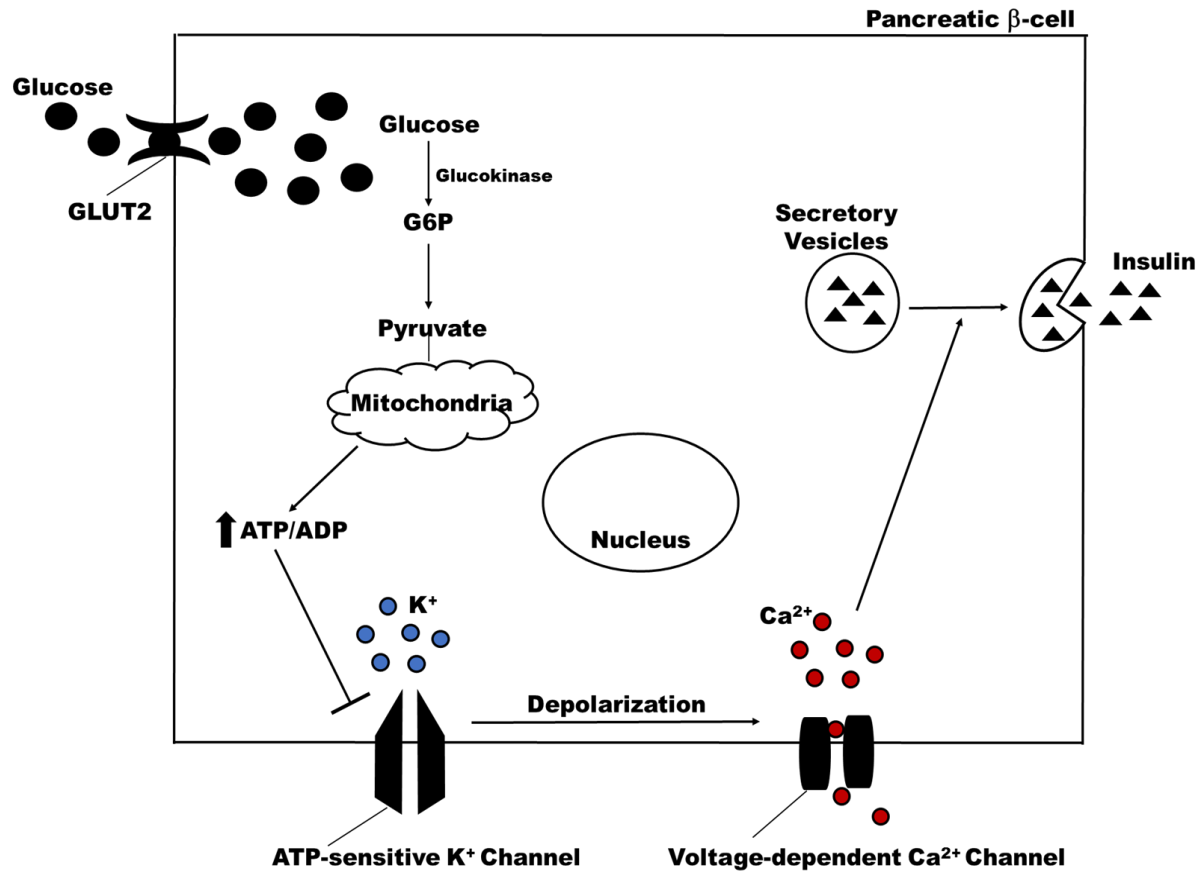


Figure 1.5 Schematic depiction of glucose stimulated insulin secretion within pancreatic β -cells. See section 2.1 for description. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; G6P, glucose-6-phosphate; GLUT2, glucose transporter type 2.

studied and characterized, therefore, the following sections contain a brief summary on their distribution and physiological importance as well as a summary on the known actions of GLUT6.

2.2a GLUT1: Glucose transporter 1 (GLUT1) is a 492 amino acid residue protein. encoded by the SLC2A1 gene in humans¹⁹¹. GLUT1 functions as a high affinity glucose transporter expressed at high levels in erythrocytes and endothelial cells in fetal tissue along with most other tissues through adulthood to assist with the transport of glucose across tissues with endothelial and epithelial barriers including the blood-brain barrier¹⁹²⁻¹⁹⁴. GLUT1 is the primary GLUT isoform regulating the transfer of glucose from maternal to fetal tissues in humans¹⁹⁵. GLUT1 is known to be expressed at higher levels during states of reduced glucose and expression in membranes is decreased with high levels of glucose, thus to maintain a low-level basal glucose uptake¹⁹². In addition to glucose, GLUT1 has been shown to transfer substrates such as galactose, glucosamine, and mannose as well as vitamin C in the reduced ascorbate state¹⁹⁶. Due to the brain's dependence on glucose for fuel, a disruption in proper GLUT1 regulation and impaired glucose transport to the brain can have dire consequences. A mutation in the SLC2A1 gene can lead to an autosomal dominant diseases termed GLUT1 deficiency syndrome (GDS) which can result in developmental delay, ataxia, dystonia, and neurobehavioral symptoms¹⁹⁷. The current therapy for GDS is replacing an individual's typical diet with a ketogenic diet to supply alternative fuel sources¹⁹⁷. Within skeletal muscle, GLUT1 is thought to be primarily responsible for the uptake of glucose during basal (non-insulin dependent) conditions¹⁹⁸. GLUT1 mRNA expression levels have also been shown to be increased during prolonged treatment of muscle cells with insulin¹⁹⁹. In a study in which skeletal muscle biopsies

(vastus lateralis) was obtained from either lean, nondiabetic obese, or subjects with T2DM, it was shown that GLUT1 protein expression levels were significantly decreased in T2DM²⁰⁰.

2.2b GLUT2: Glucose transporter 2 (GLUT2) is a 524 amino acid residue protein encoded by the gene SLC2A2 and is expressed by kidney and small intestinal epithelial cells, astrocytes, hepatocytes, and pancreatic β -cells^{192,201}. Besides being a glucose transporter, GLUT2 is also known to transport fructose, galactose, and mannose all with low affinity, but will transport glucosamine with high affinity^{202,203}. The bidirectional function of GLUT2 is of utmost importance in hepatocytes during the fed and fasting state by regulating the uptake of glucose for glycolysis, but also for the release of glucose during gluconeogenesis¹⁹². Periods of high glucose concentrations will stimulate the activation and translocation of GLUT2 to allow for the entry of glucose into the pancreatic β -cells to stimulate the production and release of insulin¹⁹². Diabetic rodent models have shown that the presence of high glucose, but low insulin levels lead to increased GLUT2 expression levels in the intestine and liver, however, these same conditions lead to decreased GLUT2 mRNA levels in the pancreas²⁰⁴⁻²⁰⁶. Mutations in the SLC2A2 gene is known to cause an autosomal recessive disorder called Fanconi-Bickel syndrome which is characterized by hepatomegaly, short stature, and renal dysfunction resulting in aminoaciduria and glucosuria²⁰⁷.

2.2c GLUT3: Glucose transporter 3 (GLUT3) is a 496 amino acid residue protein encoded by the gene SLC2A3²⁰⁸. GLUT3 is a high-affinity transporter expressed primarily in neurons, but has also been shown to be expressed in the testis, placenta, and skeletal muscle^{192,209,210}. GLUT3 is similar to GLUT1 as it is also often regarded as a facilitator of constitutive glucose uptake as well as it will transport galactose, mannose, and reduced ascorbate^{192,211}. Although GLUT1 and GLUT3 are both expressed in brain tissue, GLUT3 is the

primary mediator of glucose uptake in neurons²¹². GLUT3 has also been shown to be of importance in embryonic development and work along with GLUT1 in transporting glucose from the placenta to the fetus^{213,214}. GLUT3 has also been implicated in playing a key role in glucose uptake during fetal skeletal muscle development as well during adult skeletal muscle fiber regeneration^{215,216}. GLUT3 mRNA and protein levels have been shown to be increased in rat myoblasts during the fusion stage of differentiation, however, GLUT3 mRNA and protein levels were decreased once the cells reached the state of myotube contraction²¹⁶. In a study utilizing small interfering RNA (siRNA) to target GLUT3 showed that the knockdown of GLUT3 expression in L6 muscle cells significantly reduced glucose uptake²¹⁷. This same study demonstrated that GLUT3 protein expression was increased by insulin-like growth factor-1 (IGF-1) in L6 cells²¹⁷. The treatment of L6 cells with insulin for 18 hours increased GLUT3 and GLUT1, but not GLUT4 protein expression levels²¹⁸.

2.2d GLUT4: Glucose transporter 4 (GLUT4) is a 509 amino acid residue protein found in adipose tissue, skeletal muscle, and cardiac muscle¹⁹¹. It is encoded by the SLC2A4 gene in humans²¹⁹. Besides the transport of glucose, GLUT4 will also transport glucosamine and dehydroascorbic acid (DHA) across the plasma membrane^{189,220}. GLUT4 is often referred to as the insulin-stimulated glucose transporter as its expression and translocation is stimulated by the binding of insulin to its receptor in target cells²²¹. Since GLUT1 has been shown to assist with skeletal muscle glucose uptake during a basal state, GLUT1 will reside in the plasma membrane during this period with GLUT4 residing within the intracellular membrane compartment of the cell^{222,223}. Due to its widespread tissue distribution and role in maintaining whole body glucose homeostasis, GLUT4 may be the most studied membrane transporter. Exercise has been shown to enhance skeletal muscle insulin sensitivity by increasing the transcriptional expression of

SLC2A4 resulting in enhanced glucose uptake^{224,225}. Conditions that correlate with insulin resistance such as obesity and T2DM have been linked to impaired GLUT4 regulation²²⁶. GLUT4 gene expression has been shown to be decreased during diabetes and this is regulated in a tissue-specific manner with the expression decreasing more rapidly in adipose tissue than in skeletal muscle^{227,228}. However, the exact mechanisms leading to the GLUT4 impairment remain unknown due to the complex signaling events mediated by insulin as well as other potential mediators that can alter GLUT4 regulation. It has been shown that GLUT4 is expressed predominately within type 1 skeletal muscle fibers and the expression of GLUT4 within these fibers is significantly reduced in subjects with T2DM^{229,230}. Mice with muscle-specific knockout of GLUT4 displayed signs of insulin resistance such as decreased glucose uptake, however, muscle glycogen content was increased²³¹.

2.2e GLUT6: Glucose transporter 6 (GLUT6) is a 507 amino acid residue protein encoded by the SLC2A6 gene in humans which was formerly referred to as GLUT9²³². Limited research has been performed on the characterization of GLUT6. GLUT6 has been shown to be expressed within the spleen, brain, and leukocytes as well as in muscle and adipose tissue^{189,233,234}. The primary physiological substrate of GLUT6 has not been clearly elucidated yet nor has the kinetics of its transport and translocation activities. In rat adipose tissue, GLUT6 was shown to not be stimulated by insulin for the translocation to the plasma membrane²³³. GLUT6 is thought to be maintained intracellularly by an N-terminal dileucine motif²³³. No stimuli are known to promote the translocation of GLUT6 to the plasma membrane of cells.

2.3 Insulin Resistance

2.3a Insulin Resistance Overview: It was in 1960 that the insulin radioimmunoassay was developed which helped pave the way for understanding the effects of insulin on glucose homeostasis under diverse conditions^{235,236}. With insulin playing such a vital role in participating in the regulation of macronutrient metabolism, any disturbance in insulin signaling can have detrimental outcomes. In individuals with metabolic stresses such as those that are overweight and obese, the insulin released in response to meal consumption may only have suboptimal responses when compared to healthy, lean individuals. The exact mechanisms leading to insulin resistance are not fully elucidated, but research has pointed to defects in the activation of the insulin receptor and downstream proteins, or issues with proper GLUT function and translocation²³⁷. Furthermore, impairment of insulin signaling has been postulated to occur due to systemic inflammation and dysregulated lipid metabolism – both characteristics of obesity as well as diabetes²³⁸. It is also important to note that insulin resistance has been shown to exist during other disease states such as HIV and rheumatoid arthritis^{239,240}. Tissues such as muscle and adipose are those primarily affected by defects in insulin activity due to these tissues being insulin dependent in regards for proper glucose transport.

2.3b Insulin Resistance in Muscle: Due to its mass, muscle is considered the major tissue for the insulin-mediated glucose uptake, any disturbance in the insulin signaling pathway or GLUT4 translocation can have dire consequences. Insulin sensitivity has been shown to be impaired in muscle tissue during a state of obesity due to the increased presence of pro-inflammatory cytokines²⁴¹. *In-vitro* studies have shown that inflammatory cytokines are capable of inducing insulin resistance in muscle cells^{242,243}. Similar results have been shown *in-vivo* by the infusion of cytokines into humans with the test subjects displaying decreases in glucose

uptake and glycogen synthesis²⁴⁴. This impairment in glucose uptake and glycogen synthesis due to insulin resistance appears to be largely mediated by reduced intracellular glucose absorption due to either decreased synthesis of GLUT4 or impairment in insulin:GLUT4 signaling²⁴⁵.

2.3c Insulin Resistance in Adipose Tissue: Adipose tissue accounts for about 10% of whole body glucose uptake²⁴⁶. Within adipose tissue, insulin resistance is often associated with the accumulation of activated macrophages and pro-inflammatory cytokines as well as impairment of GLUT4 translocation^{238,246}. The elevation in pro-inflammatory cytokines are associated with attenuating the insulin signaling pathway within tissue^{247,248}. For example, when adipocytes were treated with TNF- α , the cells displayed decreases in insulin signaling along with decreases in glucose uptake^{249,250}. Overnutrition can promote such cytokine production by maintaining a constant supply of nutrient metabolites such as FFA's that can stimulate the synthesis of cytokines within immune cells²⁴⁸. Furthermore, having dysregulation of nutrient metabolism as well as hormonal balance can predispose individuals to the development of obesity²⁵¹. Similar to skeletal muscle, GLUT4 translocation has also been shown to be impaired in adipocytes of diabetic and insulin resistant individuals²⁴⁶. A state of insulin resistance will also impair insulin signaling itself in adipocytes via decreased expression of IRS-1 as well as PI3K and Akt²⁴⁶.

2.4 Metabolic Disorders

2.4a Obesity: The pathogenesis of obesity has been shown to be multifactorial with genetic, environmental, and nutritional factors all contributing to the development of the disease²⁵¹. The early onset of obesity has been shown to be partially attributed to the dysregulation appetite-regulating hormones such as leptin²⁵². It was first identified that obesity

was a state of inflammation when obese mouse tissue contained significantly higher levels of TNF- α in adipose tissue compared to the tissue of lean controls²⁵³. Furthermore, common pathways involved in inflammatory signaling such as inhibitor of kappa kinase (IKK) and c-jun N-terminal kinase (JNK) have been shown to have increased levels of activation in obese tissues compared to tissues of lean controls^{254,255}. Therefore, an additional feature of the inflammatory state of obesity is the infiltration of immune cells such as macrophages that will secrete pro-inflammatory cytokines²⁵⁶. Even though there is an obvious connection between elevated nutrition intake and obesity, the exact mechanisms in which overnutrition leads to obesity and other metabolic disorders are not fully understood. It is reasonable to believe that the accumulation of specific nutrients resulting from overnutrition can lead to the infiltration and activation of immune cells in certain tissues.

Body mass index (BMI) is a relative measure of body fat in a person, which is calculated via dividing a person's weight by the square of the person's height. The reference ranges for BMI are as follows: 1) Underweight $\leq 18.4 \text{ kg/m}^2$; 2) Normal weight $18.5 - 24.9 \text{ kg/m}^2$; 3) Overweight $25 - 29.9 \text{ kg/m}^2$; 4) Obese $30 - 34.9 \text{ kg/m}^2$; 5) Severely obese $35 - 39.9 \text{ kg/m}^2$; 6) Morbid obese $\geq 40 \text{ kg/m}^2$ ²⁵⁷. The presence of obesity leads to an inflammatory state and has been associated with the development of other diseases such as heart disease, certain cancers, sleep disorders, arthritis, and type 2 diabetes mellitus (T2DM)²⁵⁷⁻²⁵⁹. The presence of these obesity-related diseases are subsequently linked to reduced life expectancy and death. Since excessive nutrient intake and lack of physical activity are two of the driving forces leading to obesity, caloric restriction and exercising are common natural treatments for obesity²⁶⁰. Individuals may also choose to use drugs and/or supplements that support weight lost or may

choose surgery that will reduce stomach volume and/or bowel length to reduce nutrient absorption, but obesity surgery can result in adverse side effects²⁶¹⁻²⁶³.

2.4b Diabetes Mellitus: The condition known as diabetes is one that has been on a staggering rise especially within the past 20 years. Collectively, diabetes is a term referring to a state of high blood glucose levels over a prolonged period that can lead to multiple complications if left untreated. With insulin being the primary hormone responsible for the regulation of glucose uptake in skeletal muscle and adipose tissue, any disturbance in its secretion or interaction with its receptors are signs that a form of diabetes is present. There are three main types of diabetes: 1) Type 1 Diabetes Mellitus (T1DM) which stems from autoimmune destruction of the pancreatic β -cells that results in absolute insulin deficiency and predominately begins during adolescents even though individuals up to 30 years old can develop the disease, 2) T2DM is characterized by hyperglycemia, hyperinsulinemia, accumulation of TAG in cells, and a reduced ability to oxidize lipids for their removal, 3) Gestational diabetes which has similar characteristics to T2DM, but occurs during pregnancy and typically ceases after²⁶⁴. Typical symptoms of diabetes includes polydipsia, polyphagia, and polyuria²⁶⁵. Even though T1DM and T2DM differ in regards to development they both can result in a variety of pathological conditions including nephropathy, neuropathy, and myopathy. The muscles of adolescents that are diabetic can experience irreversible damage due to the development of the disease occurring during the major growth period²⁶⁶. Patients will be diagnosed with diabetes if they meet at least one of the following criteria: 1) Fasting plasma glucose level ≥ 7.0 mmol/l (126 mg/dl); 2) Plasma glucose ≥ 11.1 mmol/l (200 mg/dl) two hours following a 75 g oral glucose consumption; 3) Symptoms associated with diabetes and a casual plasma glucose ≥ 11.1 mmol/l (200 mg/dl); 4) Glycated hemoglobin (HbA1C) $\geq 6.5\%$ ^{267,268}.

T1DM accounts for roughly 10% of all diabetic cases, but the prevalence has continued to increase for the last quarter century²⁶⁹. T1DM is known as an autoimmune-mediated disease resulting from an infiltration of immune cells such as macrophages and T_H cells that will secrete pro-inflammatory cytokines at high concentrations for long durations resulting in decreases in β -cell function, eventually leading to cell death²⁷⁰⁻²⁷². Individuals with T1DM often have symptoms as hyperglycemia, ketoacidosis, and hypoglycemia²⁶⁵. T2DM currently accounts for roughly 90% of all diabetic cases in the world and it has been projected that the condition known as T2DM will affect 8% of the worldwide population by 2030²⁷³. The American Diabetes Association (ADA) suggests that the current Western lifestyle encompasses a diet that is high in dietary fat and a sedentary activity level which could part explain the T2DM epidemic²⁶⁴. Therefore, like obesity, T2DM is considered a multifactorial disease with genetic, environmental, and lifestyle factors all contributing to its manifestation. T2DM is characterized by the presence of insulin resistance that is often accompanied by reduced insulin secretion, thought to also be due to inflammation and β -cell death²⁷⁴. T2DM shares many of the symptoms with T1DM, but while those with T1DM will require insulin injections for the rest of their lives, it is strongly believed that through dietary and lifestyle intervention that T2DM can be reversed due to a reduction in overweight and obesity^{258,275}.

2.5 VA and Pancreatic β -cell Function

The islets of Langerhans in the pancreas contain the β -cells which are essential for the regulation of glucose due to their secretion of the anabolic hormone, insulin¹⁶⁹. Studies have linked VA and β -cell function by showing that in islets collected from rats fed a VAD diet displayed impaired glucose-stimulated insulin secretion and decreases in β -cell mass^{276,277}.

However, when VA was re-established in the diet this impairment was reversed, suggesting that retinoids assist in regulating insulin secretion²⁷⁶. Furthermore, high levels of retinoid-associated proteins such as TTR, RBP, CRBPI, and CRABPI have been found within pancreatic islets²⁷⁸. In rats subjected to streptozotocin (STZ)-induced diabetes development, treatment with retinyl palmitate alleviated the diabetes development by supporting the prevention of β -cell death²⁷⁹. The treatment of rat insulinoma RINm5F cells as well as pancreatic islets collected from both adult and fetal rats with all-*trans*-RA led to increases in glucokinase (GCK) gene expression and increases in insulin secretion at both high and low glucose concentrations^{280,281}. This increase in insulin secretion can be at least partially attributed to the increase in proinsulin mRNA levels in RINm5F cells and insulin mRNA levels in isolated human islets with all-*trans*-RA treatment^{280,282}. All-*trans*-RA treatment in INS-1 cells was associated with increases in GLUT2 gene expression which is essential for glucose entry into the β -cells²⁸³. Conversely, the treatment of mice with 9-*cis*-RA led to glucose intolerance and attenuated glucose-stimulated insulin secretion during periods of high glucose^{284,285}. Furthermore, treatment of rodent islets with 9-*cis*-RA is associated with decreases in GCK and GLUT2 activity²⁸⁴. Retinoid nuclear receptors are thought to be associated with the regulation in β -cell insulin secretion by RA due to the identification and characterization of a RARE sequence in the human insulin gene promoter²⁸². Therefore, it is plausible that all-*trans*-RA and RAR are assisting in glucose homeostasis under normal conditions, but during periods of hyperinsulinemia 9-*cis*-RA and RXR assist with preventing excessive insulin secretion.

3. Skeletal Muscle

3.1 Skeletal Muscle Physiology

3.1a Skeletal Muscle Structure and Contraction: Skeletal muscle refers to a number of muscle fibers that are joined together by connective tissue. Each muscle fiber is a single, cylindrical muscle cell that is formed by the fusion of multiple mononucleated and undifferentiated cells termed myoblasts (Figure 1.6). The differentiation of myoblasts into myotubes is a highly regulated process involving the expression of distinct families of genes including MRFs and myocyte enhancer factors (MEFs)²⁸⁶. The differentiation of the myoblasts are typically complete by birth and continue to grow in size into adulthood with no new fibers actually formed from myoblasts²⁸⁶. Instead, if new fibers are required to be formed following exercise or injury, then it is from another undifferentiated cell type known as muscle satellite cells, which like myoblasts will undergo differentiation²⁸⁷⁻²⁸⁹. However, this cell type alone will not restore severely damaged muscle to full strength, but it is through the coupling of the satellite cell proliferation and hypertrophy of the remaining muscle tissue to compensate for loss of muscle tissue²⁹⁰.

Skeletal muscle is assembled as a striated pattern involving numerous thick and thin filaments in the cytoplasm that form myofibrils. The fibers are assembled in a repeating pattern of light and dark bands due to the presence of the thick and thin filaments. The thick filaments are composed mainly of the protein myosin and the thin filaments are primarily composed of actin, but also contains the proteins troponin and tropomyosin^{291,292}. It is the collection of these proteins along with other accessory factors such as calcium that allows for muscle contraction through the activation of cross-bridges²⁹³. The process of skeletal muscle contraction is termed

the “sliding filament theory” and it was first described in the early 1950s²⁹⁴⁻²⁹⁶. There are numerous steps involved in the contraction of skeletal muscle: 1) An action potential originating in the CNS will propagate down an axon terminal in a motor neuron; 2) Once the action potential reaches the neuromuscular junction it triggers the influx of calcium into the axon terminals; 3) The calcium influx will trigger the release of acetylcholine (ACh) from the axon terminals; 4) ACh will diffuse from axon terminals and bind to nicotinic ACh receptors on the neuromuscular motor end plate which will stimulate an influx of Na^+ to depolarize the membrane and produce another action potential; 5) The action potential will move along the transverse tubules (T-tubules) in the fiber and depolarizes the dihydropyridine (DHP) receptors to open the ryanodine receptor channels to stimulate the release of Ca^{2+} from the lateral sacs of the sarcoplasmic reticulum; 6) Ca^{2+} will bind to troponin on the actin thin filaments which will initiate a conformational change in troponin to release the inhibitory subunit of troponin that is bound to tropomyosin, allowing tropomyosin to move away from and expose the myosin (cross-bridge) binding site on actin; 7) An energized myosin on the thick filament can now bind to actin which will stimulate the release of ADP and inorganic phosphate from myosin resulting in the “power stroke” motion; 8) ATP will then bind to myosin, breaking the link between actin and myosin, thus separating the cross-bridge from actin; 9) ATP bound to myosin will be hydrolyzed to energize the myosin cross-bridge by moving it into the “cocked” position; 10) Cross-bridges repeat steps 7-9 to produce the “sliding” movement of thin filaments past thick filaments, this will continue as long as ATP is available and Ca^{2+} is bound to troponin; 11) Cytosolic levels of Ca^{2+} will decrease as Ca^{2+} -ATPase activity transports Ca^{2+} back into the sarcoplasmic reticulum, therefore the removal of Ca^{2+} from troponin will allow tropomyosin to block the binding sites again and the myosin cross-bridge ceases allowing the muscle fiber to relax²⁹⁴⁻²⁹⁸. This is similar

to periods of rest to where cytoplasmic calcium concentrations are low in muscle and myosin cross-bridges are not bound to actin. ATP has three primary roles in the skeletal muscle contraction process: 1) Provides energy through its hydrolysis for cross-bridge movement; 2) Its binding to myosin will break the link between myosin and actin allowing the cycle to repeat; 3) The hydrolysis of ATP by Ca^{2+} -ATPase in the sarcoplasmic reticulum provides energy for calcium ion transport into the reticulum to lower the cytosolic calcium levels and allowing the contraction to end and the muscle to relax^{298,299}.

3.1b Skeletal Muscle Regeneration: Damaged skeletal muscle tissue must be repaired in a process that requires a specific cell-type referred to as satellite cells. This regeneration will begin simultaneously with the inflammatory response that is occurring due to the damaged muscle fiber (Figure 1.6)³⁰⁰. This process is typically considered to take place in four distinct stages: 1) Activation of satellite cells; 2) Proliferation of myoblasts; 3) Differentiation of cells; 4) Return to quiescence³⁰¹. The activation of the satellite cells occurs when the cells enter the cell cycle with this process primarily studied by measuring changes in morphology and protein markers³⁰². A quiescent satellite cell will contain only a few organelles with a limited amount of cytoplasm whereas an activated satellite cell will contain more enlarged organelles and much more cytoplasm^{302,303}. The binding of hepatocyte growth factor receptor (HGFR) with its ligand, hepatocyte growth factor (HGF), is typically viewed as an interaction marking the activation of satellite cells^{303,304}. The second stage of regeneration is marked by the proliferation of myoblasts which is linked to the increased expression of muscle regulatory factors including MyoD and myf5^{305,306}. The proliferating myoblasts will then withdraw from the cell cycle and start fusing with each other as well as with existing fibers to form new fibers in the process of differentiation³⁰⁷. Differentiation is typically marked by the increased expression of myogenic

regulatory and enhancer factors myogenin, MEF2, and MRF4 as well as by the increases in muscle tissue creatine kinase (CK) activity³⁰⁷⁻³¹⁰. The events that occur after differentiation it is still under debate as it is not totally clear if some of the satellite cells that became activated re-enter into quiescence or if the pool of satellite cells is re-populated by daughter cells. What is better established is the events taking place to mark the end of differentiation such as the increased expression of differentiation-repressing proteins including myostatin and other TGF- β family members³¹¹. The TGF- β members are known to have a negative role on myoblast differentiation by increasing MyoD degradation, suppressing the function of MEF2, and decreasing the expression of myogenin as well as myosin heavy chain (MHC)³¹²⁻³¹⁴. Together, the interplay between differentiation activation and repression factors allows for the proper regeneration and repair of skeletal muscle.

3.1c Types of Skeletal Muscle Fibers: Skeletal muscle fibers are distinguished by their maximal rate of cross-bridge cycling and by which pathway they use for generating energy (ATP) (Table 1.1). Fibers that contain myosin will differ in the maximal rate in which they split ATP such that fibers containing myosin with high ATPase activity are considered to be fast fibers, also called type II fibers, whereas fibers containing myosin with low ATPase activity are termed slow or type I fibers³¹⁵. The second method used for classifying skeletal muscle fibers is based on their process for generating ATP³¹⁵. Oxidative fibers contain a high number of mitochondria, therefore, they have a high capacity for oxidative phosphorylation. Oxidative fibers are also referred to as red muscle fibers due to the high levels of myoglobin which give the fibers a dark red color as well as an adequate supply of oxygen³¹⁵. A second fiber type, termed glycolytic fibers, are much lower in mitochondria number, but contain high concentrations of

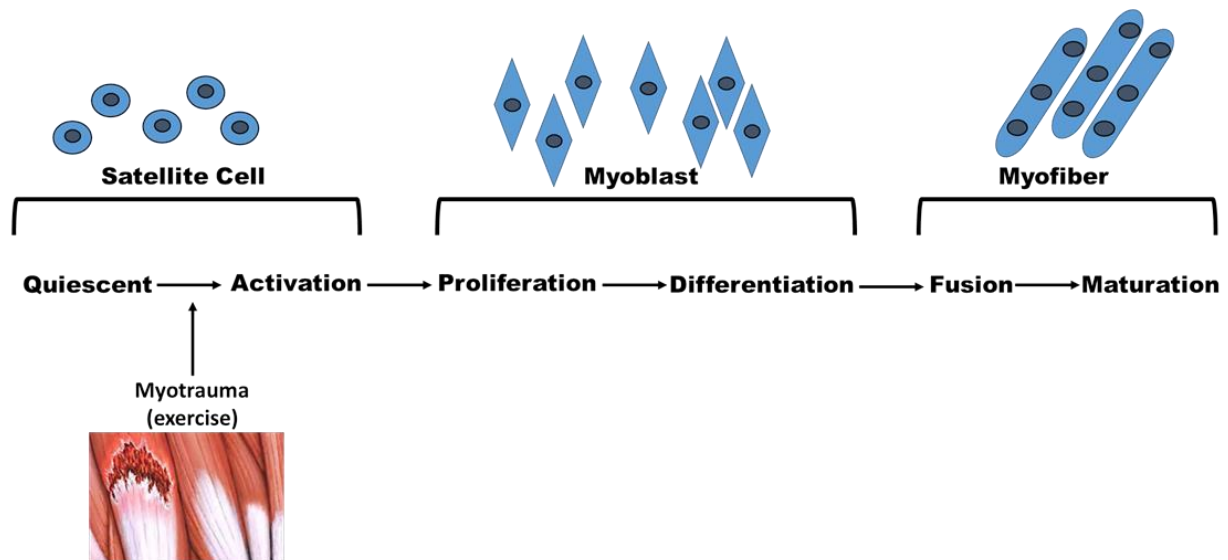


Figure 1.6 Schematic depiction of skeletal muscle regeneration and differentiation process.

See section 3.1 for description.

glycolytic enzymes as well as large stores of glycogen³¹⁵. Unlike oxidative fibers which have an abundance of myoglobin, glycolytic fibers have limited amounts of myoglobin and oxygen, therefore, these fibers are also referred to as white muscle fibers. Three types of skeletal muscle fibers have been established based on their maximal rate of cross-bridge cycling and energy processing: 1) Slow-oxidative fibers (Type I) – these fibers have low ATPase activity with high oxidative capacity; 2) Fast-oxidative-glycolytic fibers (Type IIa) – have high ATPase activity with high oxidative capacity and intermediate glycolytic capacity; 3) Fast-glycolytic fibers (Type IIb) – have high ATPase activity with high glycolytic capacity³¹⁵. These three types of fibers also differ in their capacity to resist fatigue as Type IIb fibers fatigue rapidly while Type I fibers are very resistant to fatigue and Type IIa have an intermediate capacity to resist fatigue. Type I fibers will be the first recruited during weak contractions, followed by Type IIa and finally Type IIb for strong contractions^{316,317}. Of course, exercise will have an impact on fiber type by altering muscle strength and susceptibility to fatigue. During short bouts of high-intensity exercise the muscle fiber's diameter will increase due to increased synthesis of actin and myosin, thus resulting in increased strength³¹⁸⁻³²¹. For long bouts of low-intensity exercise the muscle fiber's mitochondrial content will increase leading to improved endurance^{321,322}.

3.1d Skeletal Muscle Energy Metabolism and Substrate Utilization: Due to skeletal muscle being a tissue that rapidly goes from a state of rest to contractility, the breakdown of ATP must be a highly-organized process. For a fiber to maintain a state of contractility or is going through a cycle of contractility such as during exercise, then ATP must be produced as rapidly as it is hydrolyzed. This is going to require an adequate supply of macronutrients and micronutrients to assist with the formation of ATP. There are a number of ways that ATP can be produced in skeletal muscle: 1) Oxidative phosphorylation of ADP in the mitochondria of the

muscle cell; 2) Substrate-level phosphorylation of ADP in the cytosol of the muscle cell; 3) Phosphorylation of ADP to creatine phosphate (CP). During the beginning of a contraction, CP provides a readily available source of phosphate to assist in the formation of ATP in a reversible reaction catalyzed by CK³²³. The production of ATP from CP is a restricted reaction due to the limited supply of CP in the cell. Therefore, after the first few seconds of contractility another source must be used to support the production of ATP. For the first ten or so minutes of moderate level of muscular activity the ATP that is produced is primarily by oxidative phosphorylation^{324,325}. Stored muscle glycogen has been shown to be the primary source of glucose for fueling oxidative phosphorylation³²⁶. As muscular activity continues for the next thirty minutes or so, it is blood glucose and fatty acids that contribute to ATP production through glycolysis and oxidative phosphorylation^{327,328}. An activity beyond this point is going to rely more heavily on fatty acids for ATP production as the muscle's glucose utilization will decrease without dietary replenishment^{327,328}. The glucose used for glycolysis in muscle cells are obtained from two sources – muscle glycogen stores and blood glucose. With increases in muscular activity intensity there is a shift in ATP production from aerobic glycolysis to anaerobic glycolysis which subsequently results in lactic acid production via lactate dehydrogenase (LDH)^{329,330}. Following intense muscular activity, individuals will breathe heavily due to the fact that there is going to be an increased demand for oxygen to metabolize the lactic acid that has accumulated and return blood oxygen levels back to pre-activity levels^{331,332}.

Muscular fatigue can result from a variety of factors depending on the type of activity performed. With short bouts of high-intensity muscular activity the onset of fatigue has been attributed primarily to three mechanisms: 1) Lactic acid accumulation in muscle; 2) Cross-bridge cycling inhibition; 3) Action potential conduction failure. With the buildup of lactic acid

resulting in excessive hydrogen ion levels, there are alterations in muscle protein activity, including actin and myosin as well as those involved in muscle cell differentiation. The accumulation of ADP and inorganic phosphate can also be problematic by slowing the rate of cross-bridge attachment from actin, therefore, decreasing the overall rate of cross-bridge cycling. Sarcoplasmic reticulum calcium release can be prevented if muscle action potential fail to be conducted along the T-tubules of the muscle fiber from the accumulation of potassium ions. This increased volume of potassium ions can disturb the repolarization/depolarization equilibrium leading to inactivation of the sodium channels, therefore, failing to produce the action potential to stimulate cross-bridge cycling. With long bouts of low-intensity muscular activity it is the depletion of fuel substrates that seem to play the primary role in inducing muscular fatigue. Decreases in muscle glycogen, low blood glucose levels, and dehydration are all factors implicated in causing fatigue in low-intensity activity.

3.2 Diabetic Myopathy

3.2a Diabetic Myopathy Overview: Diabetic myopathy is a condition that is typically overlooked, probably due to the numerous other conditions associated with diabetes, but the decrease in skeletal muscle is detrimental to physical well-being. It is known that skeletal muscle is the body's largest site for glucose uptake and utilization, therefore a decrease in muscle cell number and function can negatively impact whole body glucose homeostasis. In T1DM mice it has been shown that the disease can result in reduced skeletal muscle mass, poor muscle capillarization, and reduced muscle regeneration³³³⁻³³⁵. Typically, intracellular lipids can serve as an available energy source during physical activity, but with a high dietary fat intake and elevated levels of plasma fatty acids the fat accumulation is associated with the development of

Table 1.1 Skeletal Muscle Fiber Classification and Characterization

Characteristic	Type I Fibers (slow-oxidative)	Type IIa Fibers (fast-oxidative)	Type IIb Fibers (fast-glycolytic)
Myosin-ATPase Activity	Low	High	High
Contraction Rate	Slow	Fast	Fast
Fatigue Resistance	High	Intermediate	Low
Activity Type Promoting Fiber Activation	Aerobic (marathon running)	Aerobic/ Long-term Anaerobic (400 m race)	Anaerobic (powerlifting)
Fiber Force Production	Low	High	Very High
Mitochondrial Number and Oxidative Phosphorylation Capacity	High	High	Low
Capillary Density	High	Intermediate	Low
Myoglobin Content	High	High	Low
Fiber Color	Red	Red	White
Glycogen Content	Low	Intermediate	High
Major Storage Fuel	TAG	CP, Glycogen	CP, Glycogen
Fiber Diameter	Small	Intermediate	Large

Abbreviations: TAG, triacylglycerol; CP, creatine phosphate.

insulin resistance. This can be specifically harmful in prediabetic individuals suffering from decreased insulin secretion and reduced mitochondrial function as the addition of insulin resistance would now class the individuals as being type 2 diabetic. Obesity, which is a common characteristic of T2DM is associated with impaired mitochondrial function and enhanced mitochondrial damage due to the presence of reactive oxygen species (ROS). Intracellular fatty acids are prone to peroxidation and these lipid peroxides can induce oxidative damage to cells including muscle cells. Insulin resistance indicates that there is a reduced state of responsiveness of tissues including skeletal muscle to the circulating insulin, thus without insulin binding to its receptors on muscle cells then glucose cannot enter the cell to provide energy in the form of ATP. The coupling of the insulin resistance with the increases in reactive oxygen species can be damaging to muscle tissue in type 2 diabetic individuals.

3.2b Factors Contributing to Diabetic Myopathy:

3.2b-1 Oxidative Stress and Mitochondrial Dysfunction: The presence of oxidative stress is evident in both T1DM and T2DM as it has been directly correlated to elevated glucose concentrations³³⁶. It is common for diabetic individuals to suffer from alterations in muscle metabolism including having a decreased inner myofibril mitochondrial content as well as abnormal lipid deposition in the muscle cells leading to the muscle becoming obstinate as it will not readily switch between carbohydrate and fat oxidation in response to insulin like it typically would in a non-diabetic individual³³⁷⁻³³⁹. This can lead to a variety of functional impairments including a decline in muscular strength and eventually atrophy. This muscular damage is most likely due to ROS decreasing the expression of important myogenic factors required for muscle proliferation, differentiation, and regeneration. Oxidative stress has been shown to impair glucose uptake in muscles and decreases insulin secretion from the pancreatic β -

cells^{340,341}. Oxidative stress can result from either an excessive oxidative load or from inadequate balance of nutrients favoring pro-oxidant actions where there is an imbalance between pro-oxidant reactions and the presence of anti-oxidants. The increase in oxidative stress can lead to the production of harmful substances termed free radicals that can either be of oxygen origin (superoxide, hydrogen peroxide, hydroxyl radical) or of nitrogen origin (peroxynitrite and nitrogen dioxide). The reduced presence of anti-oxidants subsequent with the decrease in anti-oxidant enzyme activity is correlated with the severity of insulin resistance as well as the reduced ability of muscle cells to oxidize lipids^{342,343}. This increased oxidative stress can also lead to the production of pro-inflammatory cytokines that can lead to dysregulation in muscle metabolism. These cytokines, which are also referred to adipokines or myokines, can alter fuel usage by either up-regulating or down-regulating hormones such as insulin. A number of cytokines such as interleukin-1 β (IL-1 β), TNF- α , and IFN- γ have been implicated in the autoimmune process that leads to the destruction of the pancreatic β -cells and subsequent diagnosis of T1DM³⁴⁴.

T2DM is often associated with obesity which on its own is becoming a global epidemic. In obese diabetic individuals the lipids from the diet can excessively accumulate in skeletal muscle and can alter insulin signaling as well as muscle maintenance and regeneration. Having uncontrolled diabetes and subsequent poor glucose control can be detrimental to the fundamental role of muscle and its cells as it has been shown that by exposing skeletal muscle cells to high levels of glucose that the muscle cells will actually differentiate into adipocytes³⁴⁵. Fatty acids that are obtained from the diet are oxidized in tissues such as skeletal muscle to be further metabolized and used for metabolic processes, but an increase in muscle lipid content is associated with a reduction in muscle lipid oxidation, thus promoting lipid deposits which is often identifiable by increases in intramyocellular fat and triglycerides levels³⁴⁶. Therefore, the

increases in muscle cell lipid content seen in obesity can impair fatty acid oxidation itself and play a role in the development of insulin resistance. In obese diabetic individuals that suffer from insulin resistance the fatty acid metabolism in skeletal muscle seems to shift from oxidation to esterification and/or peroxidation. The ability of the muscle to select the proper fuels for metabolism is altered during insulin resistance as the ability to shift between fatty acid and carbohydrate oxidation is lost³⁴⁷. The increased concentration of lipid in muscle cells have been associated with mitochondrial respiratory chain defects, particularly decreases in the expression of various genes involved in oxidative phosphorylation, thus decreased production of ATP³⁴⁸. Furthermore, the structural integrity of the mitochondria of the cells itself can be damaged due to the increased presence of ROS during diabetes³⁴⁹.

3.2b-2 Inflammation: The concept of inflammation in skeletal muscle is multifaceted as inflammation typically occurs after an injury, but it can also be activated upon stress such as that discussed in section 3.2b-1 with oxidative/lipid-induced stress. The process of inflammation has to coordinate with myogenic differentiation to achieve muscle regeneration. After a muscle has endured some sort of injury or stress there is recruitment of pro-inflammatory macrophages termed M1 macrophages that will remove debris and promote muscle cell proliferation. As diabetes progresses so does a multitude of pro-inflammatory factors that contributes to the detrimental effects diabetes has on muscle and many of these factors can be secreted by immune cells such as the M1 macrophages. One such inflammatory protein is TNF- α and it is known not only to be associated with diabetes development and progression, but it has also been found to alter glucose uptake in muscle cells as well as promote insulin resistance^{350,351}. High levels of TNF- α exist in visceral adipose tissue in obese individuals, but diabetic individuals have high levels circulating in plasma as well as in skeletal muscle^{241,352}.

TNF- α is thought to promote cells entering the cell cycle for proliferation instead of differentiation, meaning that they simply replicate instead of forming muscle fibers³⁵³. TNF- α is also a potent inducer of mitochondrial ROS which can have detrimental effects on the mitochondrial membrane leading to cell death and an attraction of inflammatory cells to promote inflammation³⁵⁴. During the later stages of muscle repair and regeneration there are anti-inflammatory cytokines termed TGF- β and IL-10 that are being secreted from M2 macrophages (anti-inflammatory macrophages) that promote muscle differentiation instead of proliferation^{355,356}.

IL-6 is a cytokine that seems to have more controversy surrounding it. It has been shown that IL-6 can alter insulin signaling in hepatocytes and adipocytes leading to impaired glucose uptake by GLUT4 thus promoting increases in blood glucose levels³⁵⁷. Even though IL-6 is often seen as a pro-inflammatory cytokine it can also have anti-inflammatory functions which can lead to the confusion in affecting glucose uptake and muscle metabolism. It does seem that in most studies demonstrating negative effects of IL-6 on insulin signaling is in adipocytes or hepatocytes, whereas the positive effects of IL-6 on insulin signaling seems to occur in muscle cells³⁵⁷⁻³⁵⁹. One can easily see that the interaction between skeletal muscle cells and immune cells have to be finely orchestrated in order for proper muscle regeneration to occur, so if you take a state of chronic inflammation such as during the early stages of T1DM or because of obesity then there is constant increased levels of circulating pro-inflammatory cytokines such as TNF- α that will have distinct roles in muscle metabolism, but the end result is typically the same – atrophy²⁴⁸.

3.3 Physical Activity

Physical activity (PA) continues to receive an increasing amount of publicity as one of the primary lifestyle factors that can prevent and reverse the effects of T2DM, but also significantly decrease the various detrimental effects that T1DM has on the body. The US Physicians Health Study reported that substantial decreases in the relative risk of T2DM can be achieved with lifelong regular physical activity³⁶⁰. Numerous studies have indicated that PA can exert beneficial effects such as increasing glucose uptake in muscle, increasing fatty acid oxidation, increasing insulin sensitivity, and at least partially restore normal metabolic control^{361,362}. GLUT4 translocation has been shown to be increased in response to acute bouts of exercise while chronic exercise training will increase GLUT4 expression³⁶³. Exercise training will also enhance glucose transport by increasing signaling activity at the IRS protein level as well as by increasing PI3K expression³⁶¹.

It was as recent as 1990 that the American College of Sports Medicine (ACSM) first published guidelines for recommendation of resistance training which is particularly important for diabetic individuals that suffer from diabetic induced myopathy. The current PA recommendations for diabetic individuals according to both ACSM and ADA are 1) 150 minutes or more of moderate-level activity a week and 2) perform resistance training at least 2 times a week on non-consecutive days³⁶⁴. Aerobic training is of utmost importance for diabetic individuals since this population is susceptible to a number of other conditions including nephropathy, neuropathy, and cardiovascular disease, therefore, one would expect that with aerobic training there would be increased blood flow, capillarization, and oxygen delivery which would decrease the presence of such conditions. Resistance training has also been shown to

improve glycemic control in patients with T2DM as well as decrease their risk for cardiovascular disease³⁶⁵.

3.4 VA and Muscle

Limited research has been performed on the effects of VA in the form of RA on macronutrient metabolism in skeletal muscle. Most of the literature regarding RA and skeletal muscle cells is in regards to differentiation of muscle cells. It has been shown that RA as well as insulin can induce differentiation of skeletal muscle myoblasts to form myotubes^{98,366}. In regards to protein metabolism, rats that are deficient in VA display decreased rates of protein synthesis with concomitant increases in proteolysis rate, but when VA is present at toxic levels only for acute period's proteolysis is also increased, thus favoring the development of myopathy in VA deficient and toxic states³⁶⁷⁻³⁶⁹. Intracellular FA's are prone to peroxidation and these lipid peroxides can induce oxidative damage to cells including muscle cells³⁷⁰. RA has been shown to increase the expression of genes regulating fatty acid oxidation and thermogenesis in mouse skeletal muscle, thereby demonstrating a possible anti-inflammatory mechanism of RA in skeletal muscle³⁷¹. In regards to carbohydrate metabolism, the treatment of undifferentiated mouse myoblasts with RA led to an increase in glucose uptake in which the authors postulated was through AMP activated protein kinase (AMPK)³⁷². Therefore, it is obvious that more studies need to be conducted in determining the mechanisms regulating macronutrient metabolism in differentiated skeletal muscle cells and the products produced by the actions of these nutrients.

3.5 Commonly Used *In-Vitro* Myogenic Model Systems

3.5a Rat L6 Cells: L6 cells are immortal primary culture cells isolated from the thigh muscle of newborn rats³⁷³. These myoblasts were shown to divide two-three times before fusing

together into multinucleated muscle fibers and that under the appropriate maintenance conditions, these cells can be cultured for months at a time as well as kept in a frozen state without losing their differentiation potentialities and viability³⁷³. In addition to studying differentiation, this cell model system has been shown to be useful in studying skeletal muscle glucose metabolism. L6 myotubes have been used to successfully investigate muscle GLUT regulation in response to a variety of treatments including insulin, retinoids, and inositol derivatives³⁷⁴⁻³⁷⁶. In response to insulin, these cells have been shown to exhibit a greater degree of glucose uptake and GLUT4 translocation than other *in-vitro* murine skeletal muscle model systems^{377,378}. This suggests that L6 myotubes may be the most efficient *in-vitro* model for studying muscle glucose metabolism.

3.5b Rat L8 Cells: Rat L8 cells like L6 cells are also isolated from the thigh muscle of newborn rats³⁷⁹. This cell line has been used for the investigation of myoblast differentiation, glucose metabolism, and insulin signaling mechanisms³⁸⁰⁻³⁸⁴. GLUT1 has been shown to be the primary glucose transporter in this myoblast cell line, therefore, most of the research on L8 glucose regulation has been to investigate the mechanisms involving the GLUT1 translocation and activity^{382,383}. One such mechanism to be elucidated in the regulation of glucose transport in L8 myoblasts is the role of hexose 6-phosphate in mediating the subcellular redistribution of GLUT1³⁸⁵.

3.5c Mouse C2C12 Cells: The immortal adherent C2C12 cell line is a subclone of the established C2 myoblast cell line isolated from the thigh muscle of a two month old female C3H mice^{386,387}. These cells are known to differentiate quickly forming contractile myotubes expressing muscle proteins, therefore, these cells have been used to study agents that can affect the differentiation process as well as skeletal muscle glucose metabolism and insulin

signaling^{100,314,388}. For example, these cells have been used to study the mechanisms regulating GLUT4 transcription and regulation³⁸⁹. Interestingly, the treatment of C2C12 cells with bone morphogenetic protein-2 (BMP-2), which is a member of the TGF- β family of proteins, will convert the cell line from having a myoblastic fate to an osteoblastic cell³⁹⁰.

4. Glycogen Metabolism and Regulation

4.1 Glycogen Overview

The ability to acquire and store energy is essential for an organism to face environmental challenges such as the lack of readily available nutrients. Glycogen, the highly branched polysaccharide, serves as the primary storage form of glucose³⁹¹. The liver and skeletal muscle are the major storage sites of glycogen in human body. As shown in Figure 1.7, glycogen in the liver and skeletal muscle is used differently³⁹². The role of glycogen within the skeletal muscle is to provide glucose for energy production while the primary role of glycogen in the liver is to supply glucose in the blood for the use in other tissues³⁹². During the fed-state, the entry of dietary nutrients into the blood leads to the secretion of the anabolic hormone, insulin, from the pancreatic β -cells³⁹². The activation of insulin signaling pathway increases the activities of enzymes involved in glycogenesis including GS, and in turn, enhance glycogenesis in hepatocytes and muscle cells^{393,394}. During a fasting state, the entry of dietary nutrients into blood stops, and the pancreas reduces insulin and increases glucagon secretion, a process that reduces glycogenesis and enhances glycogenolysis³⁹². GS and glycogen phosphorylase (GP) are primary enzymes for glycogenesis and glycogenolysis, respectively³⁹⁵. The hepatic glycogenolysis provides a source of glucose to be released into the blood³⁹⁵. Unlike the liver, muscle lacks glucose-6-phosphatase activity which results in the use of glucose inside muscle cells³⁹⁶.

The conversion of glucose to glycogen is an ordered process that has been studied extensively. UTP-glucose-1-phosphate uridylyltransferase catalyzes the conversion of glucose-1-phosphate to uridine diphosphate (UDP)-glucose in the expenditure of uridine triphosphate (UTP)³⁹⁷. At the core of the glycogen molecule is a protein homodimer known as glycogenin. Glycogenin has two tyrosine residues that will accept glucose moiety from UDP-glucose³⁹³. Therefore, glycogenin serves two roles in the process of glycogen synthesis: 1) the anchoring core molecule for glycogen formation, and 2) the initiating enzyme to begin the growing glycogen chain. UDP released from the reaction is recycled for the reformation of UTP. Glucose molecules are first linked by $\alpha(1\rightarrow4)$ glycosidic bonds until the formation of a branch³⁹³. After roughly eight to ten glucose units have been linked via $\alpha(1\rightarrow4)$ glycosidic linkage, the enzyme GS takes over the elongation process. After the glycogen molecule grows in length, the glycogen branching enzyme will catalyze the transfer of a terminal fragment of six to ten glucose moieties from a non-reducing end to the C-6 hydroxyl group of a glucose moiety closer to the interior of the glycogen molecule, forming an $\alpha(1\rightarrow6)$ glycosidic bond^{397,398}. With the increase of branching points in glycogen particles, it allows more non-reducing end for hydrolysis by glycogen phosphorylase (GP) at the same time and increases particle's solubility, which helps more efficient glucose storage³⁹⁸.

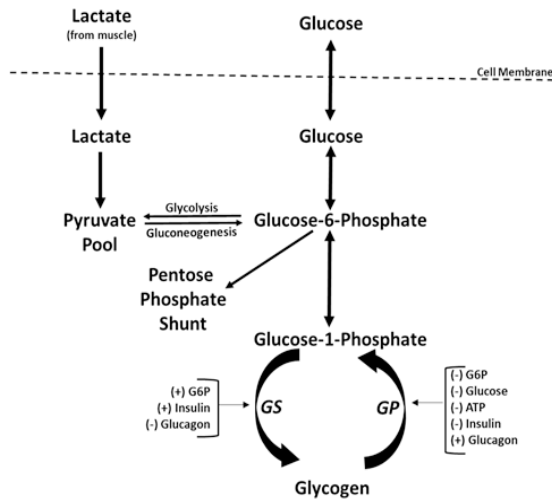
As shown in Figure 1.7, the activities of GS and GP are regulated covalently and allosterically. The activities of GS and GP are regulated in the liver and skeletal muscle covalently through phosphorylation and through allosteric effectors such as intracellular glucose-6-phosphate (G6P) and ATP levels^{394,399}. G6P can either be used to enter the glycolytic pathway or converted to glucose-1-phosphate (G1P) by phosphoglucomutase³⁹⁷. Intracellular increases in G6P will assist in the activation of GS and inactivation of GP, therefore, favoring glycogen

synthesis³⁹⁷. During periods of elevated energy (ATP) levels, GP will remain dephosphorylated in favor of glycogen formation⁴⁰⁰. Within skeletal muscle, when adenosine monophosphate (AMP) levels rise due to factors such as exercise, GP is phosphorylated to promote the formation of G1P, which can then enter the glycolytic pathway⁴⁰⁰.

Glycogenesis and glycogenolysis are regulated by catabolic and anabolic hormones. As shown in Figure 1.8, the anabolic hormone insulin initiates its action after binding to the insulin receptor, a process that starts an intracellular signaling cascade. The binding of insulin and its receptor brings closer of the two insulin receptor β -subunits, which is a tyrosine kinase, and leads to auto-phosphorylation of tyrosine residues on each other⁴⁰¹. Subsequently, activated β -subunits phosphorylate IRS proteins, with IRS-1 being the primary IRS in skeletal muscle, which recruits and activates PI3K⁴⁰¹. Activated PI3K catalyzes the conversion of the membrane-associated PIP2 and ATP into PIP3 and ADP. The increases in PIP3 leads to the recruitment of Akt, a serine/threonine protein kinase, by interacting with its pleckstrin homology domain¹⁸⁰. PIP3 also recruits PDK1 to the plasma membrane which phosphorylates Akt at Thr308^{181,402}. mTORC2 will phosphorylate Akt at Ser473 leading to its activation⁴⁰³. In muscle cells, the insulin stimulation leads to an increase in amount of GLUT4 on the cell membrane, which is translocated from intracellular storage vesicles²²⁴. Akt will inactivate glycogen synthase kinase-3 (GSK-3) by phosphorylating specific sites on the enzyme^{182,183}. By inhibiting GSK-3, glycogen synthase phosphatase (GSP) can activate GS by dephosphorylation^{181,404}. The insulin signaling cascade is also associated with the activation of protein phosphatase 1 (PP1) which will lead to the dephosphorylation and activation of GS while inactivating GP, favoring glycogen formation⁴⁰⁵.

Adrenaline, also referred to as epinephrine, and glucagon are catabolic hormones that

A. Liver Glucose Metabolism



B. Skeletal Muscle Glucose Metabolism

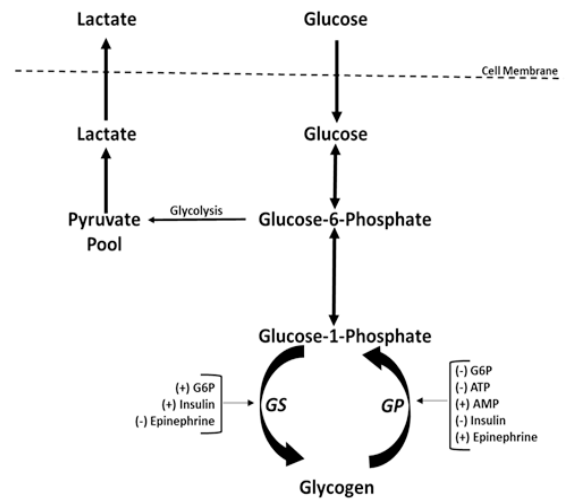


Figure 1.7 Differential regulation of glycogen metabolism within the (A) liver and (B) skeletal muscle. See section 4.1 for description. The hormones or metabolites that positively or negatively affect the activities of Glycogen Synthase (GS) or Glycogen Phosphorylase (GP) are indicated as (+) and (-), respectively. Abbreviations: G6P, Glucose 6-Phosphate; ATP, Adenosine Triphosphate.

stimulate an intracellular signaling cascade to promote glycogenolysis as shown in Figure 1.8. Upon their secretion, glucagon binds to G-protein coupled receptors (GPCRs) located on the surface of liver cells, whereas, adrenaline will bind to GPCRs on the surface of liver and muscle cells. The binding results in a conformation change that leads to the $G_{s\alpha}$ subunit of the stimulated G protein complex to exchange guanine diphosphate (GDP) for guanine triphosphate (GTP) and $G_{s\alpha}$ is released from the heterotrimeric complex⁴⁰⁶. The activated $G_{s\alpha}$ will activate adenylyl cyclase, which, in turn, catalyzes the conversion of ATP into cyclic adenosine monophosphate (cAMP). The increase in cAMP level will activate protein kinase A (PKA)⁴⁰⁶. PKA is a complex that encompasses two regulatory subunits and two catalytic subunits. Once cAMP binds to the regulatory subunits, the complex dissociates, allowing the catalytic subunits of PKA to interact with and phosphorylate specific residues on a number of proteins⁴⁰⁷. Phosphorylase kinase and GS are two such proteins. The phosphorylation and activation of phosphorylase kinase by PKA will lead to the activation of GP, which results in glycogen breakdown⁴⁰⁸. PKA will also inhibit the acetylation of GP, which decreases PP1's ability to bind to, dephosphorylate, and inactivate GP⁴⁰⁹. The phosphorylation of GS by PKA will result in reducing its activity, thus abrogating glycogen synthesis⁴¹⁰.

4.2 Recent Progresses on Factors Contributing to Skeletal Muscle Glycogen Synthase Regulation

As insulin regulates the activities of enzymes for glycogenesis and glycogenolysis, glycogen metabolism is altered in diabetes⁴¹¹. There are numerous other conditions that can arise from or be associated with irregularities in skeletal muscle glycogen regulation⁴¹². The key events in the synthesis of glycogen have been well characterized, and factors affecting muscle

glycogen synthesis have drawn attention for the past decade. The focus of this review is to identify factors that can potentially promote or abrogate the activities of GS as demonstrated by scholarly articles published within the past 10 years.

Studies were identified by searching the PubMed electronic database from June 2015 to February 2016. The search terms included were: skeletal muscle, glycogen, glycogen synthase. Time constraints were set on the date of publication with January 2005-February 2016 being eligible for inclusion. Only articles published in English were reviewed. Identified abstracts were screened for duplicates. Abstracts identified were reviewed and full articles for abstracts meeting criteria were retrieved and further evaluated.

Only original research studies reporting the influence of a manipulation that either decreases or increases in GS gene expression, protein level, and/or GS activity in which statistical significance was reported, were considered for inclusion. Both *in-vivo* and *in-vitro* study designs were eligible for inclusion. There was no restriction on type of subjects involved in *in-vivo* studies to be included in this review. There was no restriction on length of experimental manipulation to be included in the review.

For the included studies, general study characteristics (author, year of publication, study design, comparison groups, and testing duration), characteristics of study population (for humans – age, gender, disease state; for animals - species, strain, disease state), experimental manipulation (exercise, diet, compound treatment), and study outcomes (regulation of GS gene expression, protein levels, and/or activity) were extracted from the included studies. Matthew Goff independently extracted data from each study. Discrepancies regarding data extraction in studies were resolved by discussion with Dr. Guoxun Chen.

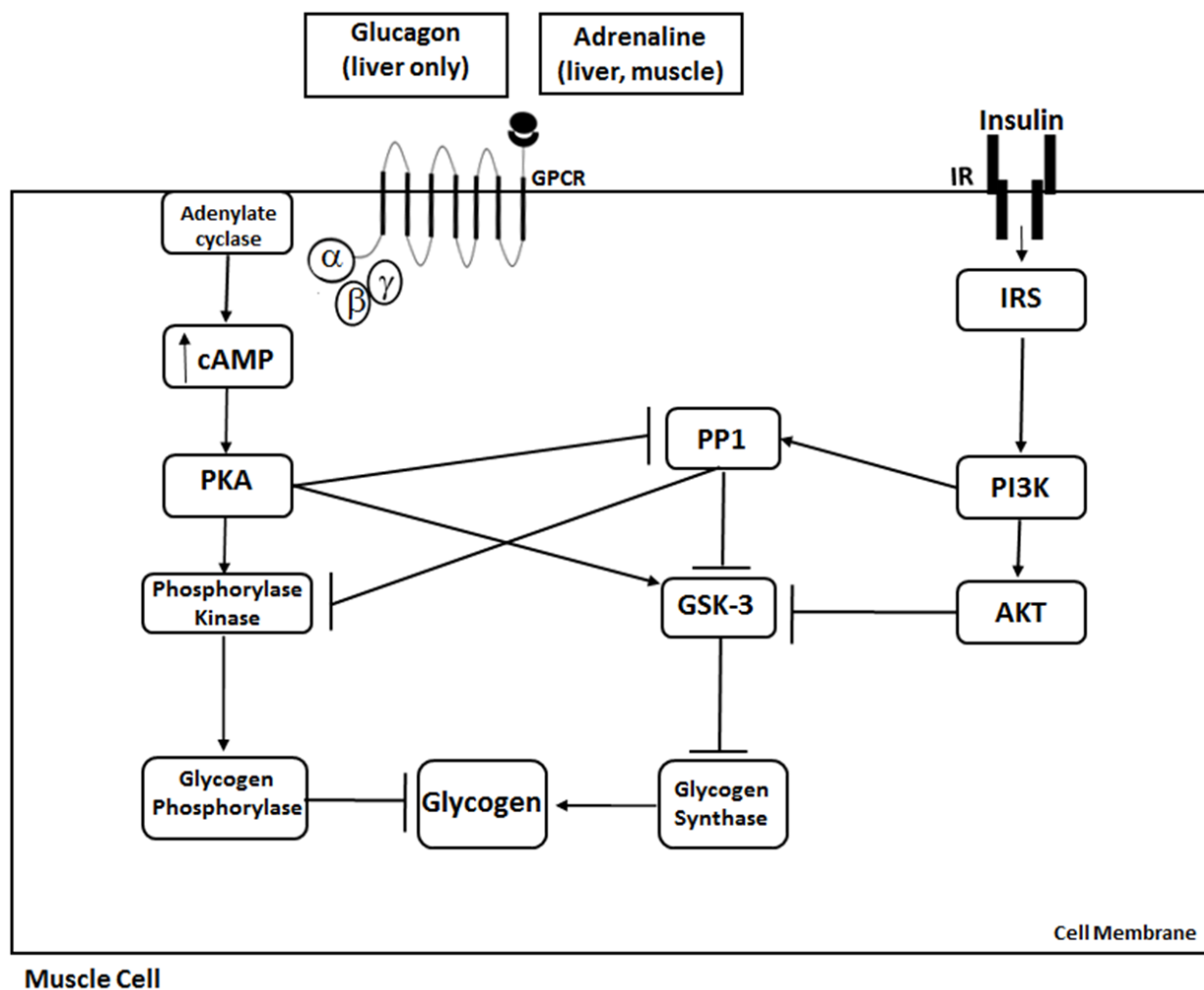


Figure 1.8 Hormonal regulation of glycogen metabolism within skeletal muscle. See section 4.1 for description. Abbreviations: cAMP, cyclic adenosine monophosphate; GPCR, g-protein coupled receptor; GSK-3, glycogen synthase kinase-3; IR, insulin receptor; IRS, insulin receptor substrate; PKA, protein kinase A; PP1, protein phosphatase 1; PI3K, phosphatidylinositol 3-kinase.

All extracted studies were divided into two categories based upon the study outcomes: promotion or abrogation of GS regulation. All identified studies had to report on at least one of the following outcomes: promotion or abrogation of GS gene expression, protein level, and/or activity. Studies that reported only on upstream or downstream factors of GS, but did not specifically report significant findings on GS were excluded from this review.

4.2a Factors Promoting GS Regulation: Factors leading to the positive regulation of GS has been of much interest within the past decade in both human (Table 1.2) and animal (Table 1.3) model systems.

4.2a-1 Behavior and Physical Activity: As shown in Table 1.2, a study investigating the effects of a 6-month weight loss and aerobic activity program in elderly insulin-resistant men found that glucose utilization in them improved⁴¹³. This is most likely due to the reported increase in insulin-induced GS activity⁴¹³. Similar results were seen in rat skeletal muscle, in which insulin and muscle contraction have an additive effect on induction of GS activity that correlated to decreases in GS phosphorylation⁴¹⁴. It has been shown that exercise-induced activation of muscle GS in obesity and T2DM is associated with dephosphorylation of GS at sites Ser7, Ser10, Ser640, and Ser644⁴¹⁵. These results were further confirmed in another study using obese individuals displaying normal glucose tolerance and T2DM subjects with their GS fractional activity (GS activity in the presence of G6P divided by total GS activity) being increased and GS phosphorylation decreased post-exercise⁴¹⁶. In obese individuals with T2DM and obese non-diabetic individuals, a 10-week endurance exercise regime increased protein content of Akt, Tre-2/USP6, BUB2, Cdc16 domain family, member 4 (TBC1D4), AMPK, GS, and GLUT4 in their vastus lateralis muscle⁴¹⁷. Furthermore, in 12 elderly male subjects who completed an 8-week exercise training, GS and glycogen content were increased in their vastus

lateralis muscle⁴¹⁸. The dephosphorylation of GS at Ser7/10/640 was enhanced after the exercise intervention⁴¹⁸. Another group who investigated the effects of endurance training on glucose metabolism in skeletal muscle found that in middle-aged males who regularly perform endurance physical activity had significantly higher GS protein levels than that of healthy sedentary controls⁴¹⁹. Post-menopausal obese women with impaired glucose tolerance, who performed either a 6-month period of caloric restriction (subtract 500 calories daily from their typical diet) or caloric restriction + aerobic exercise (3x/week for 45min), those who performed both exercise and caloric restriction had greater GS activity than those with only the caloric restriction⁴²⁰. Collectively, these results indicate that repetitive muscular contraction may be beneficial in obese, insulin-resistant, and elderly individuals in regards to the regulation of glucose.

To understand the mechanisms for exercise-induced increases in muscle glycogen, the effects of exercise training on glycogen regulatory proteins in female Sprague Dawley rats performing voluntary wheel running for 1, 4, or 7 weeks were investigated⁴²¹. It was found that rat muscle glycogen and GS did not change after 1 week of training, but increased significantly after 4 and 7 weeks. Consistent with the GS activity, one of its primary activators, PP1 activity was also significantly increased after 4 and 7 weeks of training⁴²¹. Exercise is also associated with a phenomenon known as glycogen overcompensation which prepares the body for bouts of strenuous exercise. When the tibialis anterior of rabbits were stimulated for 24 hours to mimic exercise, glycogen level increased and remained at least 50% greater than the basal level for 6 hours after stimulation⁴²². The increase in glycogen correlated with a decrease in GP activity and an increase in the GS activity due to its dephosphorylation⁴²².

4.2a-2 Components in Signal Transduction Pathways: Mice with the muscle-specific knockout of GLUT4 have been shown to have increased GS activity and decreased GP

activity following an overnight fasting (basal state), whereas contraction-induced glycogen breakdown remains normal²³¹. The GLUT4 knockout mice display reductions in glucose transport that are associated with reductions in the IRS-1/Akt-PI3K signaling pathway. However, the increases in GS during basal state in these mice are correlated to increases in the activity of PP1 and protein levels of hexokinase II, muscle-specific regulatory subunit (R_{GL}), and protein targeting to glycogen (PTG) compared to wild-type mice²³¹. Therefore, the catalytic activity of PP1 of mice in fasting state seems to dominate over the actions of GSK-3 effects, allowing for the increase in muscle glycogen content.

Of the AMPK heterotrimeric complex, the $\gamma 3$ subunit is specifically expressed in skeletal muscle⁴²³. Transgenic mice with skeletal muscle-specific overexpressing the $\gamma 3$ AMPK subunit had increased glycogen content and GS activity, and decreased GP activity⁴²³. Another group who developed a murine model of chronic skeletal muscle AMPK activation, found a selective increase in skeletal muscle glycogen content⁴²⁴. This increase in glycogen content was attributed to the catalytic α -subunit of AMPK⁴²⁴.

A study aimed to elucidate the role of the GSK-3 α in insulin resistance of human skeletal muscle cells from nondiabetic subjects found that the reduction of GSK-3 α expression resulted in an increase of the insulin-induced glucose uptake and GS fractional velocity. However, the same effects were not witnessed in the basal state⁴²⁵.

In a study investigating insulin-stimulated GS activity in human skeletal muscle, the increase in GS activity was positively associated with phosphorylation of Akt at Thr308 site and total Akt expression while both proteins were shown to be negatively associated with NH₂-terminal GS phosphorylation⁴²⁶. The authors reported no association with the activation of GS

with the phosphorylation of Akt at Ser473 site or with IRS-1-PI3K signaling⁴²⁶. In cultured human skeletal muscle cells, it was shown that the knockdown of GSK-3 β using siRNA had no effect on total GS activity. However, GS phosphorylation was decreased due to the absence of GSK-3 β leading to increase in GS fractional velocity at the basal as well as insulin-stimulated states⁴²⁷.

Pompe disease, also known as glycogen storage disease (GSD) type II, is caused by deficiency of lysosomal acid α -glucosidase (GAA). Muscle lysates collected from GAA^{-/-} mouse strains had elevated GS, glycogenin, hexokinase, and G6P, and decreased GP activity in the absence of AMP⁴²⁸. It has been shown that recombinant human GAA (rhGAA) can improve the clinical outcomes of Pompe disease with variable results⁴²⁹. The co-administration of rapamycin (mTORC1 inhibitor) with rhGAA in a GAA knockout mouse model led to reduction of glycogen content in muscle more than rhGAA or rapamycin alone, suggesting inhibition of mTORC1 signaling can provide outcome benefits in certain GSDs⁴²⁹.

4.2a-3 Cytokines: Interleukin-6 (IL-6) is a cytokine that has both anti- and pro-inflammatory actions depending on the tissue affected⁴³⁰. A group who used primary human skeletal muscle cells to investigate the role of IL-6 in muscle cell differentiation and glucose metabolism found that chronic exposure of the cells to IL-6 (25 ng/mL) resulted in an increase in glucose uptake and incorporation into glycogen which was associated with increased gene expression levels of GLUT4 and GS⁴³¹. The effect that IL-6 had on glucose metabolism was attributed to the regulation of the PI3-kinase pathway since the PI3-kinase inhibitor LY294002 suppressed the aforementioned IL-6 effects⁴³¹.

Another cytokine that has an impact on glucose metabolism and insulin resistance is adiponectin. Plasma adiponectin has been found to be significantly reduced in T2DM subjects compared to obese and lean subjects⁴³². In these same subjects, plasma adiponectin level was positively associated with insulin-stimulated glucose disposal and oxidation, and stimulation of skeletal muscle GS activity, but negatively associated with lipid oxidation after adjustment for body fat⁴³².

A protein known as skeletal muscle and kidney-enriched inositol phosphatase (SKIP) is a phosphatase that converts PI(3,4,5)P3 to PI(3,4)P2, which negatively regulates the insulin-induced glycogen synthesis in skeletal muscle⁴³³. It has been shown that overexpression of the wild-type SKIP gene in differentiating mouse C2C12 skeletal muscle cells leads to decreases in the insulin-stimulated glycogen synthesis. However, using siRNA that targeted the SKIP gene allowed insulin to induce the phosphorylation of Akt and GSK-3 β with the subsequent dephosphorylation and activation of GS⁴³³. Obviously, the SKIP gene could be of much research interest in discovering possible therapeutic interventions for GSDs.

The molecular actions of oxidant stress on insulin signaling and glucose transport have been studied in isolated soleus muscle of lean Zucker rats. It was found that oxidant stress (100 mU/mL glucose oxidase) alone led to significant increases in basal glucose transport, glycogen synthesis, and GS activity concomitant with increases in the phosphorylation of insulin receptor, Akt at Ser473, and GSK-3 β at Ser9⁴³⁴. However, oxidant stress had negative effects on the insulin-stimulated glucose transport and glycogen synthesis⁴³⁴.

The effects of transcription factor E3 (TFE3) was examined in transgenic mice who selectively expressed TFE3 in their skeletal muscle to better understand its effects on skeletal muscle glucose regulation⁴³⁵. The glycogen content in skeletal muscle of TFE3 transgenic mice

was more than twofold higher than that of wild-type mice, and this effect was associated with an upregulation of the mRNA expression levels of GLUT4, hexokinase II, and GS. Interestingly, these transgenic mice also displayed enhanced insulin sensitivity and exercise endurance capacity⁴³⁵.

4.2a-4 Nutritional Factors: When mice were fed an experimental diet containing whey protein hydrolysates (WPH), a mixture of whey amino acids (WAAs), or casein for 5 weeks, the gastrocnemius muscle glycogen level was significantly higher in the WPH group than in the WAA or casein group⁴³⁶. Furthermore, the WPH group exhibited significantly higher total GS protein level and lower phosphorylated GS level than the WAA or control group had⁴³⁶.

A study using rat L6 skeletal muscle cells found that linoleate (1 mM) can increase GS protein in insoluble, but not cytosolic or membrane fractions during both the basal and insulin-stimulated state⁴³⁷. This effect was associated with an increase of GS protein in insoluble fractions of L6 cells, but not in cytosolic or membrane fractions⁴³⁷. Studies have shown that the plant derived oleanolic acid (OA) can have antidiabetic effects^{438,439}. One study investigated the effects of OA in skeletal muscle of streptozotocin-induced diabetic male Sprague Dawley rats⁴⁴⁰. Following treatment with insulin (4 IU/kg), OA (80 mg/kg), or the combination of OA + insulin for 14 days, GS activity was significantly increased in diabetic rats receiving both OA and insulin compared with non-diabetic and diabetic controls⁴⁴⁰.

4.2a-5 Pharmaceutical Reagents: Women with polycystic ovary syndrome (PCOS) typically display reduced insulin-mediated glycogen synthesis in skeletal muscle⁴⁴¹. After a 16-week treatment with pioglitazone (30 mg/day), patients with PCOS displayed improved insulin-stimulated glucose metabolism and GS activity. This action was attributed to restoration of insulin's ability to dephosphorylate GS⁴⁴¹.

4.2b Factors Abrogating GS Regulation: Studies performed in the past decade have elucidated a number of factors that regulate GS negatively in both human (Table 1.4) and animal (Table 1.5) model systems.

4.2b-1 Genetic Defects: It has been reported that a frameshift mutation in PPP1R3A, the gene encoding R_{GL} resulted in decreased skeletal muscle GS activity with concomitant increases in GP activity in mice⁴⁴². Humans expressing this variant have low basal and postprandial muscle glycogen content⁴⁴². Pompe disease is a rare condition characterized by abnormal glycogen regulation leading to myopathy^{443,444}. In an effort to find ways to decrease skeletal muscle glycogen accumulation seen with Pompe disease, a group of researchers discovered that a phosphorodiamidate morpholino oligonucleotide (PMO) designed to invoke exon skipping in the transcript for muscle specific GS led to a dose-dependent decrease in GS transcripts in the quadriceps of mice⁴⁴⁵. This resulted in the reductions of skeletal muscle GS level and activity in the mice⁴⁴⁵.

4.2b-2 Components in Signal Transduction Pathways:

4.2b-2a AMPK Pathway: In isolated rat muscle treated for 40 minutes with the AMPK activator 5-aminoimidazole-4-carboxamide-1- β -D-ribonucleoside (AICAR, 2 mM) and insulin (1 μ M), insulin-stimulated GS activity was decreased whereas lactate release was markedly increased⁴⁴⁶. Similar results were seen in another study in which rat soleus muscle was treated for 30 minutes with AICAR (2 mM) before insulin (100 nM) was added to the samples for 45, 60, and 75 minutes⁴⁴⁷. AICAR treatment significantly suppressed insulin-stimulated glycogen synthesis which correlated with the increase in the phosphorylation of

Table 1.2 Factors Positively Impacting GS Regulation in Studies Using Human Materials

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
Physical Activity/Muscle Contraction	Obese subjects with or without T2DM (examined vastus lateralis muscle)	On a cycle ergometer for 60 minutes at an intensity (~70% VO _{2max}) followed with 1-3 hour of recovery period	↑ GS activity ↓ GS phosphorylation at Ser7/10/640/644	Exercise increases substrate affinity and decreases GSK-3β activity.	415
	Lean and obese subjects with normal glucose tolerance, obese subjects with T2DM (examined vastus lateralis muscle)	On a cycle ergometer for 40 minutes at an intensity (~70% VO _{2max})	↓ Glycogen content and GS phosphorylation at Ser7/641/645/649/653/657 ↑ GS activity	Exercise increases GS affinity for UDP-glucose and decreases GS phosphorylation at Ser7/641/645/649/653/657.	416
	Obese subjects with or without T2DM (examined vastus lateralis muscle)	On cycle ergometer with four to five sessions of 20 to 35 min per week at an average intensity (~65% VO _{2max}) – 10 weeks	↑ GS activity and GS protein level	Exercise can increase TBC1D4 signaling and influence key insulin-signaling protein levels.	417
	Healthy, elderly male subjects (examined vastus lateralis muscle)	On cycle ergometers twice per week, strength and mobility training once per week and a 5 km walk once a week. – 8 weeks	↑ Glycogen content and GS protein level ↓ GS phosphorylation at Ser7/10/640/644	Exercise enhances glucose uptake and decreases GS (Ser7/10/640) phosphorylation.	418
	Middle-aged sedentary or regularly active subjects (examined vastus lateralis muscle)	Male subjects who performed endurance training Duration – 4 to 7 days between screening day and experimental (biopsy) day	↑ GS protein level	Mechanism not described in detail, however, having a high VO _{2max} may influence insulin sensitivity in muscle.	419

Table 1.2 Continued

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
Calorie Restriction + Physical Activity	Older, overweight/obese insulin-resistant subjects (examined vastus lateralis muscle)	Aerobic exercise training (treadmill at 50-70% $\text{VO}_{2\text{max}}$ for 20 to 50 minutes) + weight loss (restrict intake by 500 calories/day). - 6 months	↑ GS activity (vs. baseline)	Calorie restriction + physical activity may enhance whole-body insulin sensitivity.	413
	Healthy, overweight, or obese post-menopausal women with normal or impaired glucose tolerance (examined vastus lateralis muscle)	Women exercised at >85% heart rate reserve for 45 minutes, 3x per week and/or were instructed to reduce their caloric intake by 500 kcal/day. - 6 months	↑ GS activity (in women with impaired glucose tolerance who exercised and restricted calories vs. restricted calories alone)	Calorie restriction + physical activity may enhance whole-body insulin sensitivity.	420
GSK-3 α Repression	Human skeletal muscle cells isolated from nondiabetic subjects (biopsy samples of the vastus lateralis)	Antisense oligo against GSK-3 α were used for 6 hours to knockdown expression in differentiating cells. Insulin (33 nM) for 30 minutes.	↑ insulin-stimulated GS fractional velocity	GSK-3 α repression may enhance insulin actions while overexpression can lead to insulin signaling impairment.	425
GSK-3 β Repression	Human skeletal muscle cells obtained from nondiabetic subjects (biopsy samples of the vastus lateralis)	Transfection of cultured Muscle cells with siRNA against GSK-3 β for 4 hours. Insulin (33 nM) for 30 minutes.	↑ basal and insulin-stimulated GS fractional velocity	GSK-3 β expression can affect GS activity in both basal and insulin-stimulated state, with GSK-3 β repression allowing for activation of GS.	427

Table 1.2 Continued

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
IL-6	Human skeletal muscle cells obtained from nondiabetic subjects (biopsy samples of rectus abdominus)	IL-6 (5, 25, or 100 ng/ml). treatment for 20 minutes, 3 hours, or 8 days	↑ Glycogen synthesis (IL-6 $\geq$ 5 ng/mL) ↑ GS mRNA expression (IL-6 25 ng/mL, 8 days)	IL-6 increases glycogen synthesis via a PI3-kinase-dependent mechanism.	431
Adiponectin	Lean subjects, obese subjects with or without T2DM (examined vastus lateralis muscle)	Euglycemic-hyperinsulinemic clamp performed for 4 hours	↑ insulin-stimulated GS fractional velocity (positive association with plasma adiponectin levels)	Plasma adiponectin may improve the ability to switch from lipid to glucose oxidation, and enhance muscle insulin sensitivity.	432
Pioglitazone	Obese women with PCOS (examined vastus lateralis muscle)	Euglycemic-hyperinsulinemic clamp performed for 3 hours. Treatment with either pioglitazone (30 mg) or placebo once daily – 16 weeks	↑ insulin -stimulated GS activity and dephosphorylation of GS at Ser7/640/644	Pioglitazone enhances insulin sensitivity and GS activity by promoting insulin-mediated dephosphorylation of GS at Ser7/640/644.	441

Abbreviations: GS, glycogen synthase; GSK, glycogen synthase kinase; IL-6, interleukin-6; mRNA, messenger ribonucleic acid; PCOS, polycystic ovary syndrome; T2DM, type 2 diabetes mellitus; TBC1D4, Tre-2/USP6, BUB2, Cdc16 domain family, member 4; UDP, uridine diphosphate; ↑ = significant increases due to factor/manipulation; ↓ = significant decreases due to factor/manipulation.

Table 1.3 Factors Positively Impacting GS Regulation in Animal Studies

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
Physical Activity/ Muscle Contraction	Male Wistar rats (examined epitrochlearis muscle)	Muscles were kept rested or contracted electrically while incubating with or without insulin (10 mU/mL) for 30 minutes	↑ insulin-stimulated GS activity ↓ GS phosphorylation at Ser645/649/653/657	Muscle contraction can increase AMPK α phosphorylation at Thr172 and decrease GS phosphorylation at Ser645/649/653/657.	414
	Female Sprague Dawley rats (examined triceps and epitrochlearis muscles)	Two groups- rats in the untrained group were housed individually in plastic cages and rats in the trained group were housed in wheel cages. Trained either for 1 week, 4 weeks, or 7 weeks.	↑ Glycogen content after 4 and 7 weeks (triceps) ↑ GS activity after 4 and 7 weeks (triceps)	Physical activity may enhance insulin sensitivity and PP1 activity.	421
	Female white New Zealand rabbits (examined tibialis anterior muscle)	Electrostimulations to mimic contraction were applied for 1 hour or 24 hours. After stimulation, animals were allowed to recover for 0, 1, 6, 12, or 24 hours.	↑ Glycogen content during a 6-hour recovery phase after a 24-hour stimulation ↑ GS activity ↓ GP activity and GS (Ser640) phosphorylation	Prolonged muscle contraction can increase GS activity by decreasing GS phosphorylation at Ser640 while decreasing GP activity.	422
GLUT4 Repression	Wild-type and muscle-specific GLUT4-knockout mice. Parent strains - 129SV and C57Bl/6J mice (examined soleus, gastrocnemius, and tibialis anterior muscles)	Transgenic mice with muscle-specific GLUT4 knockout. Electrical stimulation to mimic contraction. Mice were studied at 4 to 12 months of age.	↑ Glycogen content ↑ GS activity (tibialis anterior and gastrocnemius) ↓ GS phosphorylation at Ser641 (gastrocnemius) ↓ GP activity (tibialis anterior and gastrocnemius)	Decreased muscle glucose transport was associated with decreasing GS phosphorylation at Ser641 and GP activity.	231

Table 1.3 Continued

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
AMPK Overexpression	Wild-type and transgenic mice with AMPK γ 3 or AMPK γ 3 R225Q mutant overexpressions Parent strain – FVB mice (examined EDL, soleus, gastrocnemius, and tibialis anterior muscles)	Mice were compared at 8 to 10 weeks of age.	↑ Glycogen content (both transgenic models; EDL, gastrocnemius, and tibialis anterior) ↑ GS activity (both transgenic models in presence of G6P; gastrocnemius) ↓ GP activity (wild-type AMPK γ 3 mice; gastrocnemius)	Mechanism not described in detail, however, postulated that AMPK γ could alter the function of the AMPK β subunit's glycogen-binding domain in stimulating the activity of the glycogen debranching enzyme.	423
	Wild-type and AMPK overexpressing mice (examined EDL, soleus, quadriceps, gastrocnemius, and tibialis anterior muscles)	Mice were compared at 9 to 12 weeks of age.	↑ Glycogen content (quadriceps, gastrocnemius, and tibialis anterior) ↑ GS activity (gastrocnemius)	With AMPK γ overexpression, there was reduction of G6P content which may result from increased G6P utilization and glycogen synthesis.	424
SKIP Repression	Mouse C2C12 muscle cells	Transfection of cultured C2C12 cells with siRNA against SKIP gene for 48 hours. Insulin (10 and 100 nM) for 30 minutes.	↑ insulin-stimulated glycogen synthesis and dephosphorylation of GS at Ser641	SKIP negatively regulates the insulin-signaling pathway. Repression of SKIP allows for insulin-stimulated glycogen synthesis.	433
Oxidant Stress	Female lean Zucker rats at 9–10 weeks of age (examined soleus muscle)	Isolated muscle incubated for 2 hours in the absence or presence of 100 mU/mL glucose oxidase without or with 5 mU/ml insulin	↑ basal glycogen synthesis and GS activity	Oxidant stress increased phosphorylation of insulin receptor, Akt at Ser473, and GSK-3 β at Ser9.	434

Table 1.3 Continued

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
Whey Protein Hydrolysates	Male ddY mice (examined gastrocnemius muscle)	Mice fed 1 of 3 test diets for 5 weeks using AIN-93 protocol: control diet, control + 39.550 g/kg of whey amino acids, control + 50.00 g/kg of whey protein hydrolysates.	↑ Glycogen content (whey protein hydrolysates > whey amino acids > control) ↑ GS mRNA expression and GS protein level (whey protein hydrolysates) ↓ GS phosphorylation at Ser641 (whey protein hydrolysates)	Consumption of whey protein decreases GS phosphorylation at Ser641 and could decrease 6-phosphofructokinase (regulatory glycolytic enzyme) activity to increase GS and glycogen content.	436
Linoleate	Rat L6 skeletal muscle cells	Differentiating myotubes pre-treated for 16 hours with 1 mM linoleate in the absence or presence of insulin (100 nM) for 1 hour.	↑ basal and insulin -stimulated GS protein level (insoluble fraction)	GS was sequestered from activation by insulin treatment due to lipid treatment.	437
OA	Control and STZ-induced diabetic male Sprague-Dawley rats (examined hindquarter muscle)	Rats were treated with insulin (4 IU/kg), OA (80 mg/kg), or the combination of OA + insulin in acute (60 minutes) and sub-chronic (14 days) studies.	↑ GS activity after insulin + OA in diabetic group	OA enhances insulin signaling pathway including phosphorylation of Akt at Ser473	440

Abbreviations: AMPK, 5'-AMP-activated protein kinase; EDL, extensor digitorum longus; GP, glycogen phosphorylase; GS,

glycogen synthase; G6P, Glucose-6-phosphate; mRNA, messenger ribonucleic acid; OA, oleanolic acid; PP1, protein phosphatase 1;

SKIP, skeletal muscle and kidney-enriched inositol phosphatase; STZ, streptozotocin; TFE3, transcription factor E3; ↑ = significant

increases due to factor/manipulation; ↓ = significant decreases due to factor/manipulation.

GS⁴⁴⁷. The AICAR treatment in this study also led to a significant increase in lactate production in rat soleus muscles⁴⁴⁷.

Another study investigated the mechanism by which AMPK activation regulates muscle glycogen levels used AICAR, insulin, and adrenaline treatments on the extensor digitorum longus (EDL) muscle of C57BL/6 mice⁴⁴⁸. After the treatment for 40 minutes, AICAR (2 mM) resulted in a modest but significant decrease in GS activity while no change in GS or GP phosphorylation was detected. However, treatment with adrenaline (10 μ M) led to a strong increase in phosphorylation of GS at Ser8 and Ser11, and in phosphorylation of GP at Ser15 which correlated with a decrease in GS activity⁴⁴⁸. This same study measured the effect of AICAR on GS activity in EDL muscle isolated from transgenic mice with mutant AMPK α 2 (kinase dead, K45R mutation) or wild-type mice. In unstimulated muscle, the GS activity in AMPK α 2 mutant mice was significantly higher than wild-type littermates. This was correlated with a decrease in phosphorylation of GS at sites Ser8 and Ser641. Furthermore, AICAR treatment failed to reduce GS activity in AMPK α 2 mutant mice whereas wild-type mice had a significant decrease⁴⁴⁸. It has also been shown in primary human muscle cell cultures that a 5-hour insulin treatment (300 nM) led to impairment in GS dephosphorylation at Ser640/644 as well as GS fractional activity⁴⁴⁹. However, treatment with AICAR (2 mM) restored the glycogen synthesis and GS activation in the 5-hour insulin-treated cells⁴⁴⁹.

4.2b-2b cAMP Pathway: In a study that investigated the effect of adrenaline on insulin-mediated regulation of glucose metabolism in human tibialis anterior muscle found that in the presence of adrenaline (infusion rate of 0.05 μ g/kg/min for 240 minutes), insulin could not activate GS or dephosphorylate GS at Ser641⁴⁵⁰. The day after adrenaline infusion, an increase in plasma lactate concentration was associated with a decrease in

muscle glycogen content⁴⁵⁰. In the soleus muscle of male Wistar rats, it was shown that the insulin-stimulated GSK-3 β Ser9 phosphorylation was accompanied by decreases in GS phosphorylation as expected⁴⁵¹. However, adrenaline (10 μ M)-stimulated GSK-3 β Ser9 phosphorylation was associated with an increase in GS phosphorylation⁴⁵¹.

The major skeletal muscle glycogen targeting subunit (G_M) of PP1 has also been investigated in regards to control by various hormones⁴⁵². $G_M^{-/-}$ mice had increased phosphorylation of GP at Ser14 and GS at Ser7, Ser640, and Ser644 along with a decrease in insulin-stimulated GS activity compared to $G_M^{+/+}$ mice⁴⁵². This indicates that the G_M subunit of PP1 complex is the major phosphatase complex that leads to the dephosphorylation of these sites *in-vivo*. Adrenaline (0.5 mg/g) treatment for only 15 minutes led to a significant increase in GP activity while decreasing GS activity in control mice⁴⁵². However, the same treatment conditions yielded no significant changes in GP or GS activity in $G_M^{-/-}$ mice, suggesting that the G_M subunit of PP1 is required for adrenaline's actions⁴⁵².

4.2b-2c Akt Pathway: Individuals with T2DM display lower GS activity and increased GS phosphorylation during the recovery period post-exercise, which implies these individuals have impaired response to exercise⁴¹⁵. Compared to non-diabetic controls, patients with T2DM undergoing an euglycemic-hyperinsulinemic clamp displayed an impairment of the insulin-stimulated glucose disposal and glucose oxidation during euglycemia⁴⁵³. This effect was associated with impairment of insulin-stimulated GS activity and decrease of GS dephosphorylation with reduction of Akt phosphorylation at Thr308 and Ser473. During hyperglycemia, the insulin-stimulated glucose disposal and oxidation normalized, however, the dephosphorylation of GS as well as phosphorylation of Akt at Thr308 and Ser473 remained impaired⁴⁵³. Women with PCOS have the risk of developing insulin resistance in skeletal muscle.

Compared to women without PCOS, women with PCOS display the reduction of insulin-stimulated GS activity and dephosphorylation of GS⁴⁴¹.

4.2b-3 Aging: Aging also seems to play a role in altering GS level and glycogen accumulation. It has been shown that the older rats (24 months) displayed reduced GS and GP activity, lower muscle GS protein levels, and increased phosphorylation of GS in soleus muscle than young mice (6 months)⁴⁵⁴. Along with aging, extended bed rest occurs. One group who investigated the mechanisms behind physical inactivity-induced insulin resistance in the skeletal muscle (vastus lateralis) of 12 healthy male subjects after 7 days of bed rest found that the ability of insulin to activate GS was reduced⁴⁵⁵. They found that phosphorylation of GS at Ser7 and Ser10 increased following bed rest⁴⁵⁵. Interestingly, in rat soleus muscle during the basal state, oxidant stress (100 mU/mL glucose oxidase) increased GS activity. However, GSK-3 β remained active in the presence of both oxidant stress and insulin⁴³⁴. The presence of oxidant stress also significantly inhibits the insulin-induced glucose transport and glycogen synthesis⁴³⁴.

4.2b-4 Hormones: In human muscle cells treated with or without 25 mM glucose for 24 hours in combination with angiotensin (1 μ M), glucose deprivation plus angiotensin increased levels of ERK $\frac{1}{2}$ phosphorylation and GSK-3 β (Ser9) phosphorylation⁴⁵⁶. However, only those treated with high glucose and angiotensin displayed an increase in GS phosphorylation at Ser640/641 which correlated with a decrease in GS activity⁴⁵⁶.

Glucocorticoids such as dexamethasone have been known to have an impact on glucose and fat metabolism, which could lead to alterations such as insulin resistance and dyslipidemia^{457,458}. In the red and white gastrocnemius muscle of rats, the administration of dexamethasone (1 mg/rat/day) increased glycogen concentration while reducing GS activity in

both muscle fiber types⁴⁵⁹. Rat treated with dexamethasone and sucrose had decreased muscle glycogen content compared with the dexamethasone alone group. However, this effect was also associated with a decrease in GS activity similar to that seen in animals treated with the dexamethasone alone⁴⁵⁹.

4.2c Perspectives: Although the glycogen synthesis pathway has been studied extensively, there seems to be more to be investigated. For example, GSK-3 is known as a major regulator of GS activation through phosphorylation. Using GSK-3 knock-in mice with GSK-3 α and - β genes being replaced with mutant forms (GSK-3 $\alpha/\beta^{S21A/S21A/S9A/S9A}$), it has been shown that the insulin-mediated inhibition of GSK-3 is not a rate-limiting step in skeletal muscle glycogen synthesis⁴⁶⁰. Even though insulin failed to promote the activation of GS in these GSK-3 α/β mutant mice, the glycogen content within the skeletal muscle of these mice were similar to their wild-type controls⁴⁶⁰.

One determinant of GS activity is the existing amount of glycogen within the muscle as well as muscle fiber type^{461,462}. As demonstrated in rodents, a low glycogen content was associated with decrease in GS phosphorylation and a high glycogen content was associated with decrease in GS activation⁴⁶³⁻⁴⁶⁵. One group has shown that epinephrine stimulates glycogenolysis in Wistar rat skeletal muscles with normal and high, but not low, glycogen content. The glycogenolysis actually being associated with decreases in GS phosphorylation and increases in GS activity⁴⁶⁶. This demonstrates an association between GS activation and glycogen content in this condition.

Physical activity has also been shown to affect glycogen content in rodents with the phosphorylation of GS being reduced 30 minutes' post-exercise, but returning to baseline at 90 minutes' post-exercise⁴⁶⁷. As described previously, physical activity can result in the

Table 1.4 Factors Negatively Impacting GS Regulation in Studies Using Human Materials

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
Pro-longed Insulin Treatment	Human skeletal muscle cells	Insulin (300 nM) – 5 hours	↑ Glycogen content ↓ GS activity and glycogen synthesis rate	Pro-longed insulin treatment could result in a negative feedback mechanism on insulin signaling pathway	449
Adrenaline	Male and female healthy subjects (examined tibialis anterior muscle)	Adrenaline (0.05 µg/kg/minute) or saline infusion for 240 minutes. Euglycaemic-hyperinsulinemic clamp for 120-240 minutes	↓ insulin-stimulated glycogen synthesis, GS activity, and dephosphorylation of GS at Ser641	Adrenaline blocks insulin-mediated GS activation most likely due to preventing dephosphorylation of GS at Ser641.	450
T2DM	Obese male subjects with or without T2DM (examined vastus lateralis muscle)	Exercise on a cycle ergometer for 60 minutes at an intensity (~70% VO _{2max}) followed with 1-3 hour of recovery period	↓ GS activity ↑ GS P-Ser7/10 during recovery period (diabetic vs. obese)	Muscle GS phosphorylation regulation in T2DM is impaired in response to recovery from exercise.	415
	Obese subjects with or without T2DM, and lean non-diabetic subjects (examined vastus lateralis muscle)	Following overnight fast, lean and obese controls underwent a euglycemic-hyperinsulinemic clamp (4-hour) with tracer glucose and combined with indirect calorimetry; diabetic patients were clamped twice with either a euglycemic or an isoglycemic- hyperinsulinemic clamp (fixing glucose concentrations to each individual's spontaneous fasting glucose concentration) separated by 4–6 weeks	↓ insulin-stimulated GS activity during euglycemia ↓ insulin-stimulated dephosphorylation of GS at Ser7/10 during euglycemia and hyperglycemia	T2DM impairs insulin-stimulated glycogen synthesis involving Akt phosphorylation at Ser473 and Thr308, and dephosphorylation of GS at Ser7/10.	453
PCOS	Obese women with or without PCOS (examined vastus lateralis muscle)	Euglycemic-hyperinsulinemic clamp performed for 3 hours.	↓ insulin-stimulated GS activity and dephosphorylation of GS at Ser7/10 (PCOS vs. control)	PCOS decreased the insulin-mediated dephosphorylation of GS at Ser7/10.	441

Table 1.4 Continued

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
Physical Inactivity	Healthy male subjects (examined vastus lateralis muscle)	Bed rest (all transport of subjects took place in wheelchairs) – 7 days	↓ GS activity ↑ GS phosphorylation at Ser7/10	Physical inactivity/bed rest impairs muscle insulin signaling and dephosphorylation of GS at Ser7/10.	455
High glucose + Angiotensin	Human skeletal muscle Cells	Muscle cells were incubated with or without 25 mM glucose for 24 hours. Angiotensin treatment (1 μ M)– 20 minutes	↓ GS activity ↑ GS phosphorylation at Ser640/641	Angiotensin reduced ERK $\frac{1}{2}$ phosphorylation at Thr202/Tyr204 and impaired dephosphorylation of GS at Ser640/641.	456

Abbreviations: BMI, body mass index; ERK, extracellular regulated signal kinase; GS, glycogen synthase; GSK, glycogen synthase

kinase; T2DM, type 2 diabetes mellitus; ↑ = significant increases due to factor/manipulation; ↓ = significant decreases due to

factor/manipulation.

Table 1.5 Factors Negatively Impacting GS Regulation in Animal Studies

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
PPP1R3A Mutation	Wild-type and mutant (premature stop mutation in PPP1R3A) mice Parent strain – Chimeric mice cross-bed with C57BL/6J mice (examined muscle not specified)	Tissues collected during ad libitum. Age used not described.	↓ Glycogen content and GS activity ↑ GP activity	The reduction in GS activity was most likely due to the inability of PP1 to colocalize with GS and to bind to glycogen.	442
GS-PMO	C57BL/6 + GAA ^{-/-} mice (examined quadriceps muscle)	GS-PMO administered at 15 or 30 mg/kg bodyweight by tail vein injection once every 2 weeks for a total of 12 weeks, muscle isolated 2 weeks after stopping treatment	↓ Glycogen content, GS activity, GS mRNA expression, and GS protein level	GS-PMO forces exon skipping in the processing of <i>Gys1</i> mRNA, leading to a premature termination codon.	445
AICAR	Male Sprague-Dawley Rats (examined epitrochlearis muscles)	AICAR (2 mM) and insulin (1 µM)– 40 minutes	↓ basal and insulin-stimulated GS activity	AICAR treatment favors an increase in glycolytic flux instead of glycogen synthesis.	446
	Male Albino-Wistar rats (examined soleus and epitrochlearis muscles)	AICAR (2 mM) and insulin (100 nM) – 45, 60, and 75 minutes (preincubated with AICAR 30 minutes before insulin added)	↓ insulin-stimulated glycogen synthesis and glycogen content (soleus muscle) ↑ GS phosphorylation at Ser641 (45 minutes, soleus muscle)	AICAR treatment led to a time-dependent reduction in Akt phosphorylation at Thr308 and activation of GSK-3α/β.	447

Table 1.5 Continued

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
Adrenaline	C57BL/6 J mice (examined EDL muscle)	Adrenaline (10 μ M) – 40 minutes	↓ GS activity ↑ GS phosphorylation at Ser8/11, GP activity, and GP phosphorylation at Ser15	Adrenaline impairs dephosphorylation of GS at Ser8/11 while promoting GP phosphorylation at Ser15.	448
	Male Wistar rats (examined soleus muscle)	Adrenaline (10 μ M)– 30 minutes	↑ GS (Ser645/649/653/657) phosphorylation	Adrenaline impairs dephosphorylation of GS at Ser645/649/653/657 most likely through PKA signaling pathway, which may also lead to decreases in PP1 activity.	451
	Wild-type and null G_M allele (deletion encompassing 1 st exon encoding PP1) mice Parent strain: C57BL/6 mice (examined hind limb muscles)	Adrenaline (0.5 mg/g) for 15 minutes, insulin (150 mU/g) for 20 minutes, or saline intraperitoneal injections	↓ GS activity ↑ GP activity	Adrenaline's effects may be attributed to decrease in PP1 activity and/or increase in phosphorylase kinase activity.	452
Age	Male Fischer 344 rats (examined soleus and tibialis anterior muscles)	Old (24 months) rats compared to young (6 months) rats maintained ad libitum or calorie restricted	↓ GS and GP activity and GS protein levels (soleus) ↑ GS phosphorylation at Ser640 (soleus)	Aging negatively affects GS and GP activity in soleus muscle, which may be associated with impairment of dephosphorylation of GS at Ser640.	454
Oxidant Stress	Female lean Zucker rats (examined soleus muscle)	Isolated muscle from 9-10 week old rats incubated for 2 hours in the absence or presence of 100 mU/mL glucose oxidase, without or with 5 mU/ml insulin.	↓ insulin-stimulated GS activity and glycogen synthesis	Oxidant stress was found to be associated with decreased phosphorylation of insulin receptor, Akt at Ser473, and GSK-3 β at Ser9.	434

Table 1.5 Continued

Factor/ Manipulation	Participants/Model System	Treatment/Manipulation; Duration	Findings	Proposed Mechanisms	Citation
G _M Null Allele	Wild-type and null G _M allele (deletion encompassing 1 st exon encoding PP1) mice Parent strain – C57BL/6 mice (examined hind limb muscles)	Adrenaline (0.5 mg/g) for 15 minutes, insulin (150 mU/g) for 20 minutes, or saline via intraperitoneal injection	↓ insulin-stimulated GS activity (null G _M mice) ↑ GS phosphorylation at Ser7/640/644 and GP phosphorylation at Ser14 (null G _M mice)	Decreased PP1 activity allows for GS and GP phosphorylation which may also be attributed to increases in phosphorylase kinase activity.	452
Dexamethasone	Male Sprague–Dawley rats (examined gastrocnemius muscle)	4 groups: saline, 10% sucrose drinking solution, dexamethasone (1 mg), and dexamethasone + sucrose – 7 days.	↑ Glycogen content (dexamethasone alone) ↓ GS activity (dexamethasone and dexamethasone + sucrose)	Dexamethasone may regulate GSK-3 and/or CAMKII activity.	459

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside; AMPK, 5'-AMP-activated protein kinase; CAMKII,

calmodulin kinase II; EDL, extensor digitorum longus; GP, glycogen phosphorylase; GS, glycogen synthase; mRNA, messenger

ribonucleic acid; PKA, protein kinase A; PMO, phosphorodiamidate morpholino oligonucleotide; PP1, protein phosphatase 1; ↑ =

significant increases due to factor/manipulation; ↓ = significant decreases due to factor/manipulation.

enhancement of GS activity. On the other hand, it is also well known that during vigorous physical activity, endurance or strength training, the skeletal muscle relies a great deal on the breakdown of glycogen, as well as fat, to provide adequate energy⁴⁶⁸. Besides depletion of muscle glycogen stores, skeletal muscle protein degradation will also occur during such physical activity⁴⁶⁹. The depletion of glycogen will result in the increase of proteolysis to get amino acids and β -oxidation of fatty acid from muscle fat, and reduction of pyruvate oxidation⁴⁷⁰. Therefore, nutritional strategies should be implemented during the post-recovery period to maximize skeletal muscle glycogen and protein synthesis. Besides for the importance of replenishing endogenous substrate stores, optimal nutritional intake can assist in facilitating the repair of damaged muscle⁴⁷¹. For athletes, nutrition can play a key role in determining the adequate period of time between competitions for complete muscle recovery. Similarly, pre-physical activity nutrition can have a major impact on metabolism and activity during performance⁴⁷². Further research is needed to optimize pre- and post-physical activity nutritional strategies involving the investigation of the regulation of key enzymes in glycogenesis such as GS, GP, and GSK-3.

From a pharmaceutical standpoint, having a greater understanding of the regulation of glycogen synthesis could be beneficial to a number of individuals suffering from a variety of diseases. With the millions of insulin-resistant men and women, the development of glycogen regulating enzyme activators/inhibitors could help alleviate the formation of other conditions such as myopathy. GP inhibitors, for instance, have been receiving attention for being possible therapeutic agents for diabetes and cancer⁴⁷³. The regulation of GSK-3 is also a topic of interest among many due to GSK-3 regulation being connected to a number of conditions such as Alzheimer's disease, cancer, diabetes, and general muscle wasting^{474,475}. Therefore, GSK-3 inhibition may provide therapeutic effects to a broad range of conditions.

5. Conclusion

There is much still being discovered regarding glucose metabolism in skeletal muscle. VA as well as insulin play key roles in the regulation of macronutrient metabolism in humans. With the majority of the physiological functions of VA being exerted through the actions of RA, having a greater understanding of how RA works within different organs and tissues can lead to possible therapeutic advancements. With skeletal muscle accounting for the largest site of glucose usage we believe that further investigations into how a fat-soluble vitamin such as VA and its derivative (RA) can affect this process. This requires research in: (1) the interactions between RA and insulin in regulating skeletal muscle glucose usage; (2) the role of VA (RA) in macronutrient metabolism during periods of metabolic stress (e.g. hyperinsulinemia) due to conditions such as obesity and T2DM. By having a greater understanding of how a natural compound such as VA can influence macronutrient metabolism, especially during disease states, possible therapeutic strategies can be developed to combat the development and reverse the detrimental outcomes of such metabolic diseases.

CHAPTER II. RA AND INSULIN PROMOTE GLUCOSE USAGE IN L6 SKELETAL MUSCLE CELLS

1. Introduction

The skeletal muscle as a whole is the largest organ of the body by mass, for this reason it has the biggest capacity for glucose utilization⁴⁷⁶. The regulation of skeletal muscle glucose metabolism can affect whole body glucose homeostasis significantly. Skeletal muscle metabolism can be regulated by a number of factors including hormonal and nutritional stimuli as well as exercise and microvasculature structure⁴⁷⁷⁻⁴⁷⁹. Metabolic disorders such as obesity and type 2 diabetes mellitus (T2DM) are often associated with disturbances in the regulation of skeletal muscle glucose metabolism⁴⁸⁰. Even though the pathogenesis of metabolic syndrome, obesity, and T2DM have not been fully elucidated, insulin resistance has been established as a key risk factor⁴⁸⁰. In healthy individuals, insulin is released postprandially and will promote glucose usage in hepatic, adipose, and skeletal muscle cells for either storage or further metabolism¹⁷⁰. Therefore, any dysregulation in skeletal muscle's response to insulin's actions can negatively impact whole body glucose metabolism⁴⁸¹. Insulin sensitivity has been shown to be impaired in skeletal muscle during a state of obesity due to factors such as an increase in pro-inflammatory cytokines as well reactive oxygen species²⁴¹. Impairment in glucose uptake and glycogen synthesis in skeletal muscle due to insulin resistance has also been shown to be associated with reduced intracellular glucose absorption due to impairment of insulin-induced GLUT4 translocation²⁴⁵.

Vitamin A (VA, also referred to as retinol, ROL) is an essential lipophilic micronutrient that is implicated in the regulation of glucose, protein, and lipid metabolism^{482,483}. There are two stepwise cytosolic enzymatic reactions that convert ROL into retinoic acid (RA, all-*trans* RA or 9-*cis* RA). RA can then enter the nucleus and modulates gene expression through the activation of retinoid acid receptors (RAR) and retinoid-X-receptors (RXR)^{50,484,485}. RAR and RXR share a

common ligand, 9-*cis*-RA, whereas all-*trans*-RA only binds to RAR⁶¹. While 9-*cis*-RA binds to both RAR and RXR, this ligand seems to be only produced endogenously and there is no proof that all-*trans*-RA goes through isomerization to form 9-*cis*-RA⁶². The majority of the literature investigating the effects of RA on skeletal muscle focus on its ability to be an inducer of muscle cell differentiation by increasing the expression of key myogenic regulatory factors as well as inhibitor of anti-myogenic factors^{98,100}. It has been shown that RA can promote insulin-stimulated glucose uptake and increase GLUT4 mRNA levels in L6 skeletal muscle cells³⁷⁶. However, limited research has been performed on the effects of VA and its metabolites on glucose metabolism in skeletal muscle.

The aim of this study was to investigate the effects of ROL, retinal (RAL) and RA on L6 skeletal muscle cell glucose utilization. Effects of RA or insulin on L6 skeletal muscle cells have been investigated previously^{374,376}. We demonstrate that RA and insulin, cooperatively promote glucose utilization in L6 cells. Furthermore, we demonstrate that the RA-mediated effect is mimicked by an RAR agonist, suggesting that RA promotes glucose utilization within skeletal muscle by activation of the RAR signaling pathway, but not through induction of GLUT4 expression.

2. Materials & Methods

2.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM) and penicillin/streptomycin were purchased from Mediatech (Manassas, VA, U.S.A.). Fetal bovine serum (FBS) was purchased from Life Technologies (Grand Island, NY, U.S.A.). Horse serum (HS) was purchased from Hyclone Laboratories (Logan, UT, U.S.A.). For glucose measurement, Glucose Liquicolor kit

was purchased from StanBio Laboratory (Boerne, TX, U.S.A.). T0101317 (T1317) was from J-Star Research (South Plainfield, NJ, U.S.A.). LG268 was synthesized in the core facility at the University of Texas Southwestern Medical Center at Dallas. All-*trans* retinoic acid, all-*trans* retinol, all-*trans* retinal, TTNPB, and insulin were purchased from Sigma (St. Louis, MO, U.S.A.). BCA protein assay kit and ECL Western Blotting Substrate were purchased from Pierce (Rockford, IL, U.S.A.). Immobilon®-PSQ PVDF membrane was purchased from EMD Millipore (Billerica, MA, U.S.A.). Polyvinylpyrrolidone (PVP) was purchased from Amresco (Solon, OH, U.S.A.). For immunoblotting, GLUT1 (#1340) and GLUT4 (#1346) were purchased from Chemicon Industries (Temecula, CA, U.S.A.). β -Actin (#4970), and goat anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.).

2.2 L6 rat skeletal muscle cell culture and differentiation

Rat L6 cells were incubated in 60 mm dishes at 37°C and 5% CO₂. For subculture, L6 cells were maintained in DMEM with 4.5 g/L (25 mM) glucose supplemented with 10% FBS + 1% penicillin/streptomycin. When cells reached ~100% confluence, medium was changed to DMEM containing 25 mM glucose with 2% HS + 1% penicillin/streptomycin to induce differentiation. In the glucose dosage experiment, when cells reached ~100% confluence, medium was changed to DMEM containing 5 mM, 15 mM, or 25 mM glucose with 2% HS + 1% penicillin/streptomycin.

2.3 Glucose & pH Measurements

Once L6 cells reached ~100% confluence, DMEM medium with 10% FBS was switched to DMEM + 2% HS containing the indicated reagents as described in figure legends. Following the initial day of treatment (day 0), the medium from each 60 mm dish was collected and replaced with a fresh medium containing the same reagents every 2 days. The collected medium

was used for glucose and pH measurements. The pH measurement was conducted immediately using a pH meter (Oakton Instruments, Vernon Hills, IL, U.S.A.) after the medium was collected into 15 mL plastic tubes, and sealed with rubber corks. For glucose measurements, 500 μ L of collected medium was distributed into 1.5 mL Eppendorf tubes. The samples were centrifuged at 12,000 x g for 5 minutes to remove cell debris. The medium was diluted 10-fold with deionized H₂O and the glucose amount in the medium was measured using the StanBio Glucose Liquicolor kit. Briefly, in separate 1.5 mL Eppendorf tubes, 500 μ L of glucose reagent was added to each tube and incubated for 5 minutes at 37°C. Following the incubation period, one glucose reagent containing tube received 5 μ L deionized H₂O to serve as blank control, one tube received 5 μ L of glucose standard, and the remaining tubes received 5 μ L of diluted medium. The samples were incubated for 5 minutes at 37°C and then loaded in a clear 96-well plate for analysis on Glomax® Multi+Detection System (Promega, Madison, WI, U.S.A.) at a wavelength of 500 nm. Values were derived by comparing the absorbance of the unknown (u) with that of the standard (s): $\text{Glucose (mg/dL)} = ((A_u \times 10)/A_s) \times 100$. For the pH and glucose measurements, DMEM containing 2% HS alone served as control for comparison to respective treatment conditions. The pH level of the control group was arbitrarily set as 100% with data expressed as percentage change of pH. The glucose level of the control group was arbitrarily set as 100% with data expressed as percentage of relative glucose levels.

2.4 Protein Isolation and Immunoblotting

Whole cell lysate was prepared by washing L6 cells once with ice-cold PBS and lysing the cells in 400 μ L ice cold whole-cell lysis buffer and subsequently scraped into chilled 1.5 mL centrifugation tubes. The lysates were vortexed vigorously and placed on ice for at least 20 minutes. Lysates were then centrifuged at 12,000 x g for 15 minutes at 4°C and supernatant

collected into clean 1.5 mL Eppendorf tubes and stored at -80°C. Protein was quantified by Pierce BCA protein assay kit using manufacturer recommendations and analyzed using Glomax® Multi+Detection System. Total cell protein (40 µg) was then separated on an 8% SDS-PAGE gel and transferred to Immobilon®-PSQ PVDF membrane³⁹. Membranes were blocked using 1% PVP in 1x TBST for 1 hour at room temperature and then probed with specific antibodies diluted in 1% PVP in 1x TBST overnight at 4°C⁴⁸⁶. After that, the membranes were washed three times for 5 minutes each with 1x TBST and then incubated 1-2 hours with goat anti-rabbit IgG conjugated with horseradish peroxidase (1/2000 dilution in 1% PVP in 1x TBST). Following secondary incubation, three more 5 minute washes with 1x TBST were carried out followed by visual detection using ECL Western Blotting Substrate on ChemiDoc Software System (BIO-RAD Laboratories, Hercules, CA, U.S.A.). Quantification analysis was performed using Image Lab version 2.0.1 software (BIO-RAD Laboratories, Hercules, CA, U.S.A.). The density of a protein band was determined by subtracting the background in an area of the same size immediately in front of protein band. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis.

2.5 Statistical Analysis

All experiments have been conducted in triplicate. One-way ANOVA with LSD post-hoc statistical analysis and Students t-test was performed using SPSS version 22 statistical software (IBM, Armonk, NY, U.S.A.). Data were presented as means $\pm$ S.E.M. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Changes of pH and glucose levels in media of L6 cells treated with ROL, RAL, or RA

L6 cells at confluence were treated with 1 μ M ROL, RAL, or RA for 4 to 14 days. After 4 days of treatment with RA, both medium pH (Figure 2.1A) and glucose (Figure 2.1B) levels were significantly decreased compared to that of the control group. This trend with RA continued out to day 14. On the other hand, treatment with RAL did not start producing significant reductions in medium pH (Figure 2.1A) and glucose levels (Figure 2.1B) until day 10 of treatment. Treatment of L6 cells with ROL did not yield significant changes in medium pH and glucose levels at any day measured (Figure 2.1A-B).

3.2 Changes of pH and glucose levels in media of L6 cells treated with increasing doses of insulin in the absence or presence of 1 μ M RA

Next, we performed an insulin dose curve (0.1 - 100 nM) in the absence or presence of 1 μ M RA as shown in Figure 2.2. This allowed us to determine whether insulin was capable of altering medium pH and glucose levels similar to that seen with RA, and whether there is a synergy between RA and insulin. Starting at day 2, insulin (1 – 100 nM) was capable of significantly reducing medium pH (Figure 2.2A) and glucose (Figure 2.2B) levels compared to control. Insulin (1 – 100 nM) with the addition of RA (1 μ M) at day 2 resulted in significant reductions in medium pH (Figure 2.2A) and glucose levels (Figure 2.2B) compared to RA alone. After 4 days of treatment, insulin (10 nM) + RA led to significant reduction in medium pH (Figure 2.2C) and glucose levels (Figure 2.2D) compared with the control, RA, and 10 nM insulin groups. Similar results were observed after the treatment for 6 days with 10 nM insulin +

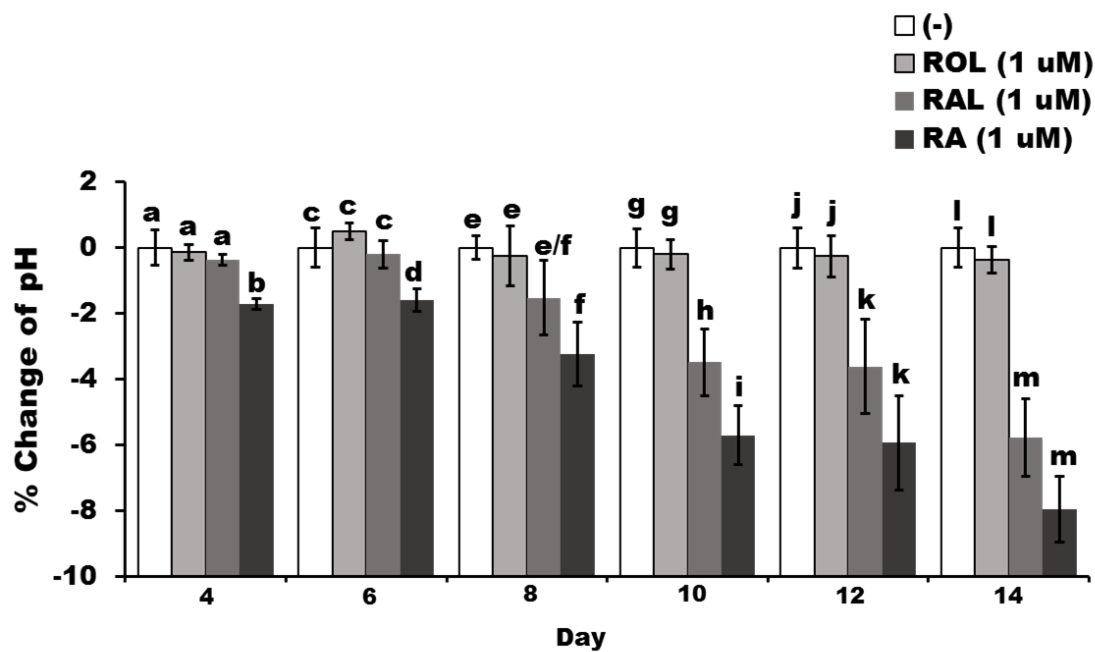
RA maintaining significant reductions in medium pH (Figure 2.2E) and glucose levels (Figure 2.2F). However, after 6 days of treatment with 1 nM insulin, this dose no longer produced significant reductions in medium pH (Figure 2.2E) and glucose levels (Figure 2.2F) compared to control, whereas 10 nM insulin did. Therefore, the remaining experiments were performed using insulin at a concentration of 10 nM.

3.3 Changes of pH and glucose levels in media of L6 cells treated with increasing doses of RA in the absence or presence of 10 nM insulin

To determine that appropriate dose of RA to use for further experimentation, an RA dose curve (0.3 – 10 μ M) was performed with or without insulin (10 nM). As shown in Figure 2.3, after 2 days of treatment, RA alone did not lead to significant reductions in medium pH (Figure 2.3A) or glucose levels (Figure 2.3B) at any dose tested. However, treatment with 10 μ M RA + insulin led to significant reductions in medium pH (Figure 2.3A) and glucose levels (Figure 2.3B) compared to control, insulin, and 10 μ M RA after 2 days. After 4 days of treatment, RA (1 – 10 μ M) caused significant reductions in medium glucose levels (Figure 2.3D). After 4 and 6 days of treatment, L6 cells treated with insulin + RA (1 – 10 μ M) significantly reduced pH (Figures 2.3C and 2.3E) and glucose levels (Figures 2.3D and 2.3F) compared to control, insulin, and RA (1 – 10 μ M). RA at 1 μ M had significantly reduced both medium pH (Figure 2.3E) and glucose levels (Figure 2.3F) after 6 days of treatment, with 3 and 10 μ M RA having no additional effect. Therefore, the remaining experiments were performed using RA at a concentration of 1 μ M.

Figure 2.1. Changes of pH and glucose levels in media of L6 cells treated with ROL, RAL, or RA. L6 cells were maintained in 60 mm dishes until ~100 confluence and were subjected to differentiation with 2% HS alone (control), 2% HS + ROL (1 μ M), 2% HS + RAL (1 μ M), or 2% HS+ RA (1 μ M). The medium was collected from each dish and replaced every 2 days with its respective treatment condition. The collected medium was pipetted into 15 mL tubes sealed with rubber corks for pH measurements using a standard pH meter and into 1.5 mL Eppendorf tubes for glucose measurements using the StanBio Glucose Liquicolor kit. A. Changes of medium pH after the indicated treatments for 4, 6, 8, 10, 12, and 14 days. B. Changes of medium glucose after the indicated treatments for 4, 6, 8, 10, 12, and 14 days. The pH level of the control group was arbitrarily set as 100% with data expressed as percentage change of pH. The glucose level of the control group was arbitrarily set as 100% with data expressed as percentage. All p-values ≤ 0.05 ; for (A) a>b, c>d, e>f, g>h>i, j>k, l>m; for (B) a'>b', c'>d', e'>f', g'>h', i'>j'>k', l'>m'>n'. One-way ANOVA statistical analysis was performed using SPSS software. Data is presented as means $\pm$ S.E.M (n=3).

A



B

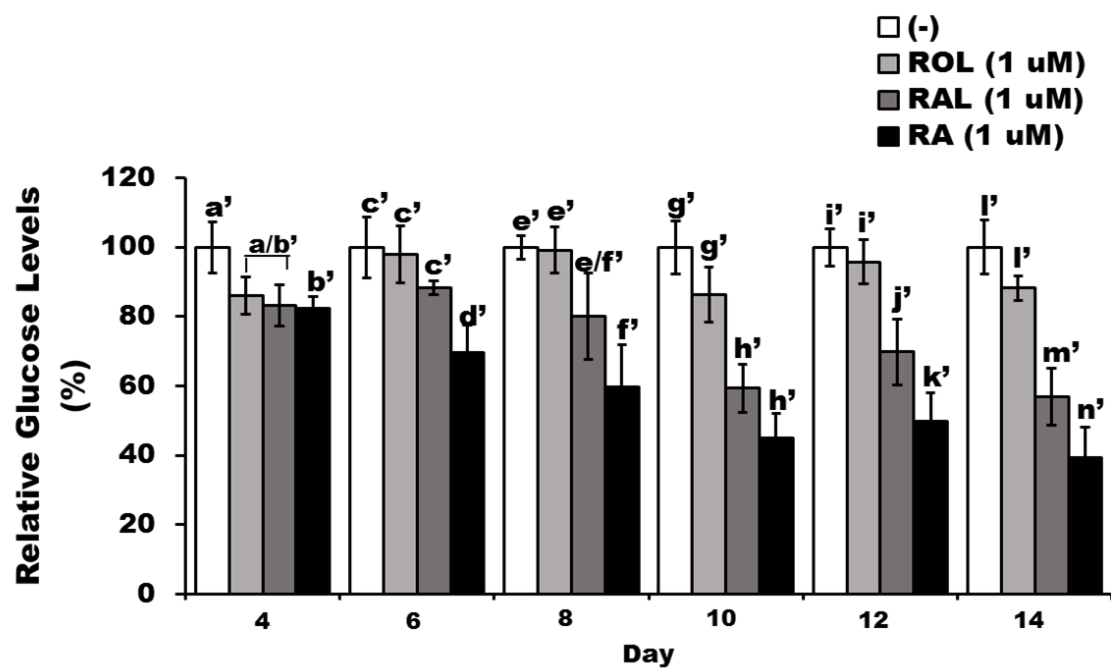


Figure 2.1

Figure 2.2. Changes of pH and glucose levels in media of L6 cells treated with increasing doses of insulin and 1 μ M RA. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), 2% HS + RA (1 μ M), 2% HS + insulin (0.1-100 nM), or 2% HS + RA + insulin. The medium was collected from each dish and replaced every 2 days with its respective treatment condition. The collected medium was pipetted into 15 mL tubes sealed with rubber corks for pH measurements using a standard pH meter and into 1.5 mL Eppendorf tubes for glucose measurements using the StanBio Glucose Liquicolor kit. A, C, and E. Changes of medium pH after the indicated treatments for 2, 4, and 6 days, respectively. B, D, and F. Changes of medium glucose after the indicated treatments for 2, 4, and 6 days, respectively. The pH level of the control group was arbitrarily set as 100% with data expressed as percentage change of pH. The glucose level of the control group was arbitrarily set as 100% with data expressed as percentage. All p-values ≤ 0.05 ; for (A) $a > b > c$, $a' > b' > c'$; for (B) $a > b > c$, $a/b > c$, $a' > b' > c'$, $a' > b/c'$; for (C) $a > b > c > d$, $a' > b' > c' > d'$; for (D) $a > b > c$, $a' > b' > c'$, $a/b' > c'$; for (E) $a > b$, $a' > b' > c'$; for (F) $a > b$, $a' > b'$, using one-way ANOVA. * for comparing those that received RA to those that did not receive RA using Students t-test statistical analysis with SPSS software. Data is presented as means $\pm$ S.E.M (n=3).

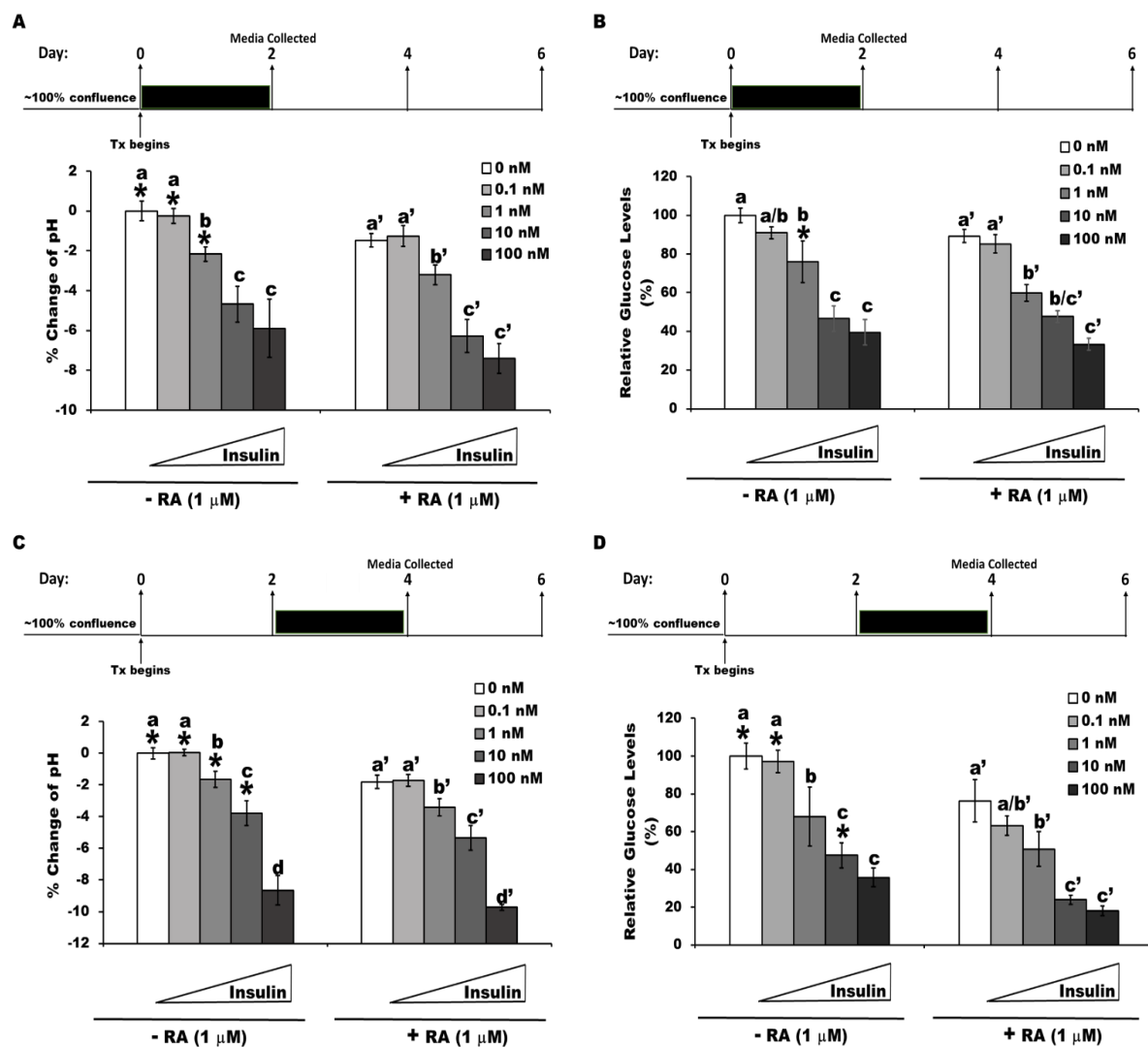


Figure 2.2

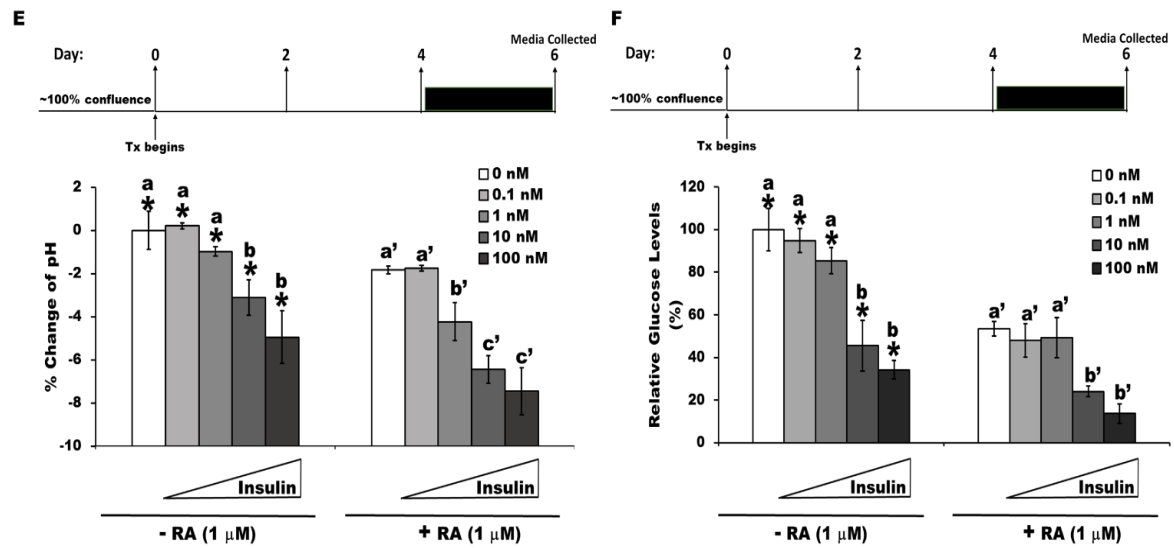


Figure 2.2 Continued

Figure 2.3. Changes of pH and glucose levels in medium of L6 cells treated with increasing doses of RA and 10 nM insulin. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), 2% HS + insulin (10 nM), 2% HS + RA (0.3-10 μ M), or 2% HS + insulin + RA. The medium was collected from each dish and replaced every 2 days with its respective treatment condition. The collected medium was pipetted into 15 mL tubes sealed with rubber corks for pH measurements using a standard pH meter and into 1.5 mL Eppendorf tubes for glucose measurements using the StanBio Glucose Liquicolor kit. A, C, and E. Changes of medium pH after the indicated treatments for 2, 4, and 6 days, respectively. B, D, and F. Changes of medium glucose after the indicated treatments for 2, 4, and 6 days, respectively. The pH level of the control group was arbitrarily set as 100% with data expressed as percentage change of pH. The glucose level of the control group was arbitrarily set as 100% with data expressed as percentage. All p-values ≤ 0.05 ; for (A) $a' > b' > c'$; for (B) $a' > b'$; for (C) $a' > b' > c'$, $a' > b/c'$; for (D) $a > b$, $a' > b'$; for (E) $a > b$, $a' > b'$; for (F) $a > b > c$, $a' > b'$, using one-way ANOVA. * for comparing those that received insulin to those that did not receive insulin using Students t-test statistical analysis with SPSS software. Data is presented as means $\pm$ S.E.M (n=3

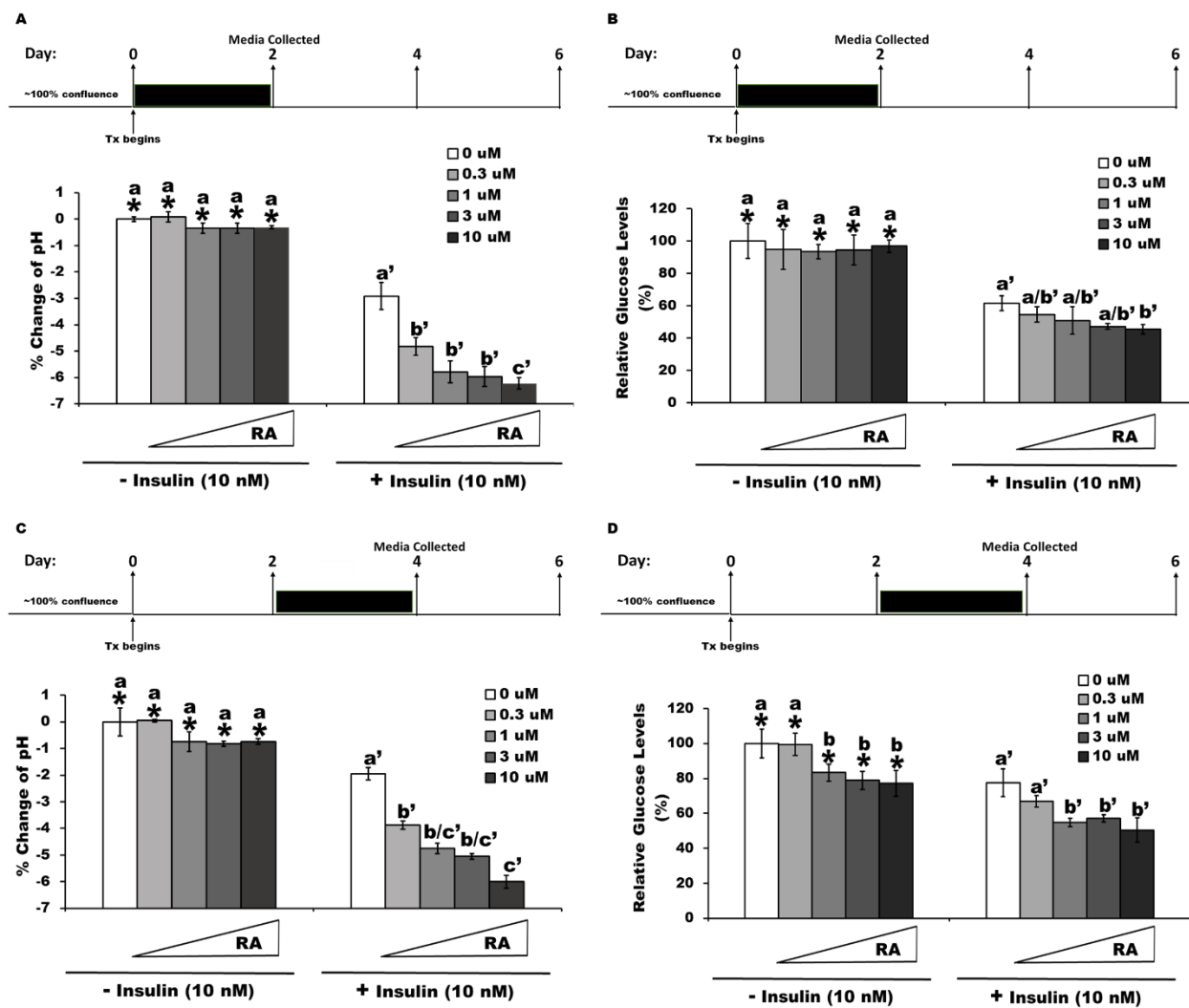


Figure 2.3

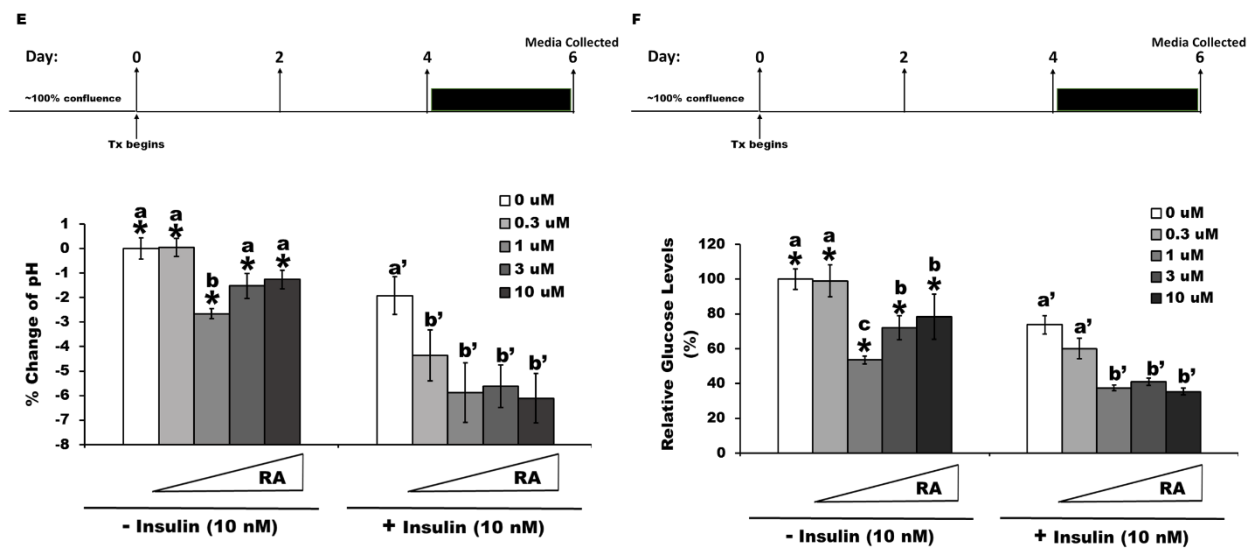


Figure 2.3 Continued

3.4 Changes of pH and glucose levels in medium of L6 cells treated with 1 μ M RA, LG268, TTNPB, or T1317 with or without 10 nM insulin

To determine if the RA-mediated effect on medium pH and glucose changes in L6 cells was due to the activation of specific nuclear receptors, the effects of 1 μ M of LG268 (RXR agonist), TTNPB (RAR agonist), or T1317 (LXR agonist) were tested. As shown in Figure 2.4, after 4 days of treatment, both RA and TTNPB led to significant reductions in medium pH and glucose levels compared to the control group. After 4 days of treatment, RA + insulin significantly reduced medium pH (Figure 2.4C) and glucose levels (Figure 2.4D) compared to the control, insulin, and RA alone. Similar results were also seen with TTNPB + insulin after 4 and 6 days of treatment (Figure 2.4E-F). Treatment of L6 cells with LG268 and T1317 produced similar results to that seen with the control group (Figure 2.4). Likewise, the addition of insulin to LG268 and T1317 produced similar results to that seen with insulin (Figure 2.4). Therefore, we conclude that the RA-mediated effect on medium pH and glucose changes in L6 cells was due to the activation of RARs.

3.5 Medium pH and glucose levels in L6 cells treated with 1 μ M each RA or TTNPB with or without 10 nM insulin at increasing glucose concentrations

To investigate whether the RA and insulin-mediated effects on medium pH and glucose changes were dependent on glucose availability and concentration, L6 cells were treated with increasing concentrations (5, 15, and 25 mM) of glucose for 2, 4 and 6 days. As shown in Figure 2.5, after 2 days of treatment, the L6 cells with 15 and 25 mM glucose displayed significant reductions in medium pH and glucose levels in those treated with insulin, RA + insulin, and TTNPB + insulin (Figure 2.5C-F). However, this effect was most likely due to insulin's actions

Figure 2.4. Changes of pH and glucose levels in media of L6 cells treated with 1 μ M each RA, LG268, TTNPB, or T1317 with or without 10 nM insulin. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), 2% HS + insulin (10 nM), 2% HS + RA (1 μ M), 2% HS + RA + insulin. 2% HS + LG268 (1 μ M), 2% HS + LG268 + insulin, 2% HS + TTNPB (1 μ M), 2% HS + TTNPB + insulin, 2% HS + T1317 (1 μ M), 2% HS + T1317 + insulin. The medium was collected from each dish and replaced every 2 days with its respective treatment condition. The collected medium was pipetted into 15 mL tubes sealed with rubber corks for pH measurements using a standard pH meter and into 1.5 mL Eppendorf tubes for glucose measurements using the StanBio Glucose Liquicolor kit. A, C, and E. Changes of medium pH after the indicated treatments for 2, 4, and 6 days, respectively. B, D, and F. Changes of medium glucose after the indicated treatments for 2, 4, and 6 days, respectively. The pH level of the control group was arbitrarily set as 100% with data expressed as percentage change of pH. The glucose level of the control group was arbitrarily set as 100% with data expressed as percentage. All p-values ≤ 0.05 ; for (A) $a > b$; for (B) $a > b$; for (C) $a > b > c$; for (D) $a > b > c$, $a > b/c$; for (E) $a > b > c$; for (F) $a > b > c$. One-way ANOVA statistical analysis was performed using SPSS software. Data is presented as means $\pm$ S.E.M (n=3).

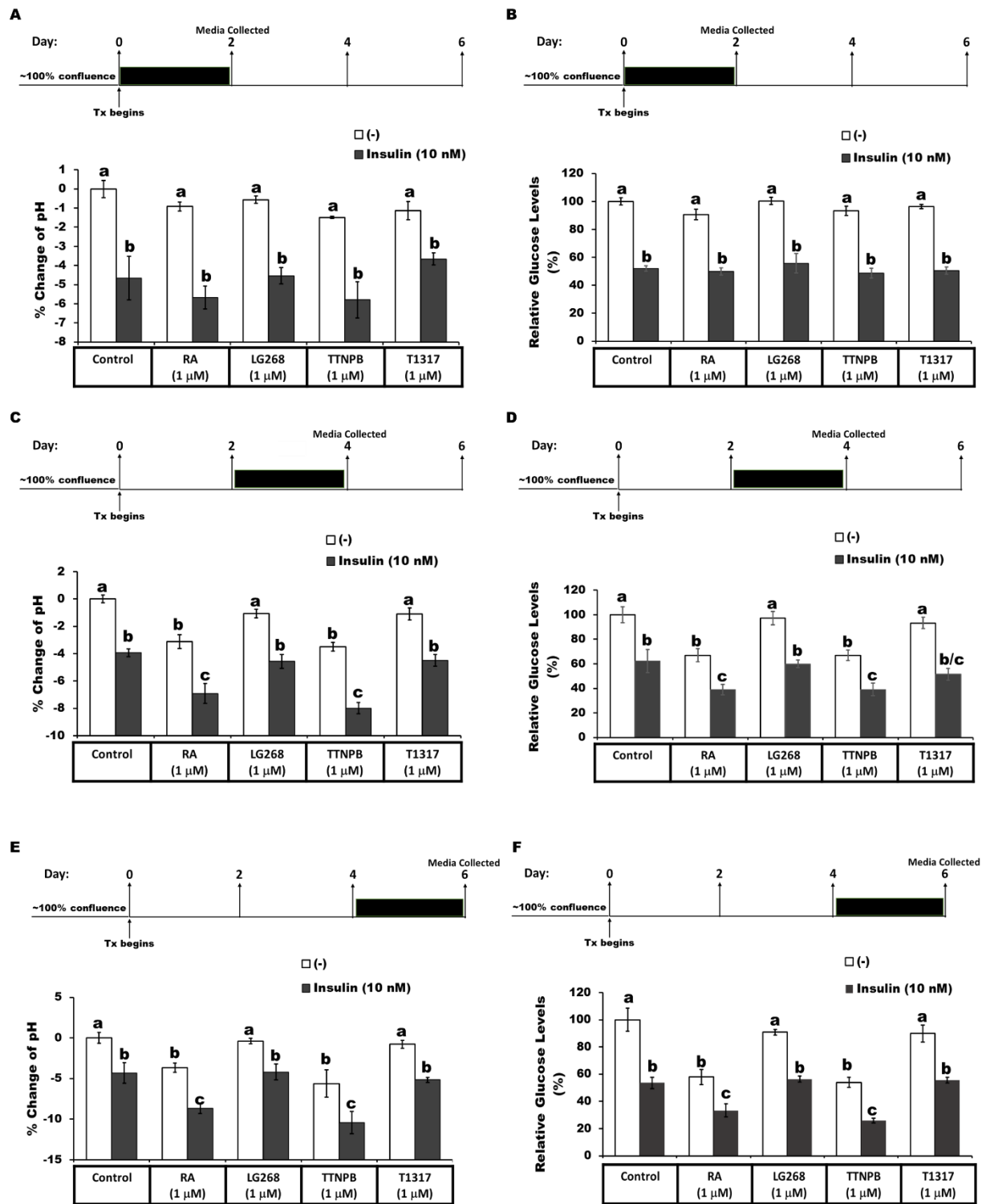


Figure 2.4

as RA or TTNPB alone did not have significant effect compared to the control (Figure 2.5C-F). After 4 days of treatment, L6 cells treated with insulin, RA, and TTNPB led to significant reductions in medium pH and glucose levels compared to the control at both 15 and 25 mM glucose (Figure 2.6C-F). The medium containing 25 mM glucose displayed significant reductions in medium pH (Figure 2.6E) and glucose levels (Figure 2.6F) in RA + insulin compared with control, insulin, and RA after 4 days of treatment. Similar results were seen with TTNPB + insulin (Figure 2.6E-F). The medium containing 15 mM glucose only displayed significant reductions in glucose levels (Figure 2.6D) in RA + insulin and TTNPB + insulin groups compared to the control and their respective counterpart (RA and TTNPB), but not insulin after 4 days of treatment. After treatment for 6 days, both medium containing 15 and 25 mM glucose displayed significant reductions in medium pH and glucose levels in RA + insulin group compared to the control, insulin, and RA alone (Figure 2.7C-F). Similar results were seen with the treatment of TTNPB + insulin (Figure 2.7C-F). No significant changes were observed in medium containing 5 mM glucose (Figures 2.5-7). This suggests that the RA, TTNPB, and insulin-mediated effects on medium pH and glucose changes depend on glucose availability and concentration.

3.6 Decreases of GLUT1 and GLUT4 protein expression levels in L6 cells treated with RA + Insulin for 6 days

With GLUT1 being known as the glucose transporter utilized in skeletal muscle cells under basal conditions and GLUT4 being known as the insulin-stimulated glucose transporter that will translocate from vesicles to the plasma membrane in skeletal muscle cells for glucose uptake in response to insulin stimulation, we measured whether there was any change of GLUT1 and GLUT4 expression levels in L6 cells treated with RA and insulin. Surprisingly, the protein

Figure 2.5. Medium pH and glucose levels in L6 cells following 2 days of treatment with 1 μ M each RA or TTNPB with or without 10 nM insulin at increasing glucose concentrations.

Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), 2% HS + insulin (10 nM), 2% HS + RA (1 μ M), 2% HS + RA + insulin, 2% HS + TTNPB (1 μ M), 2% HS + TTNPB + insulin. The treatments were made in medium containing either 5, 15, or 25 mM glucose. The medium was collected from each dish and replaced every 2 days with its respective treatment condition. The collected medium was pipetted into 15 mL tubes sealed with rubber corks for pH measurements using a standard pH meter and into 1.5 mL Eppendorf tubes for glucose measurements using the StanBio Glucose Liquicolor kit. A, C, and E. Changes of medium pH after the indicated treatments for 2 days. B, D, and F. Changes of medium glucose after the indicated treatments for 2 days. The pH level of the control group was arbitrarily set as 100% with data expressed as percentage change of pH. The glucose level of the control group was arbitrarily set as 100% with data expressed as percentage. All p-values ≤ 0.05 ; for (C-F) $a > b$. One-way ANOVA statistical analysis was performed using SPSS software. Data is presented as means $\pm$ S.E.M (n=3).

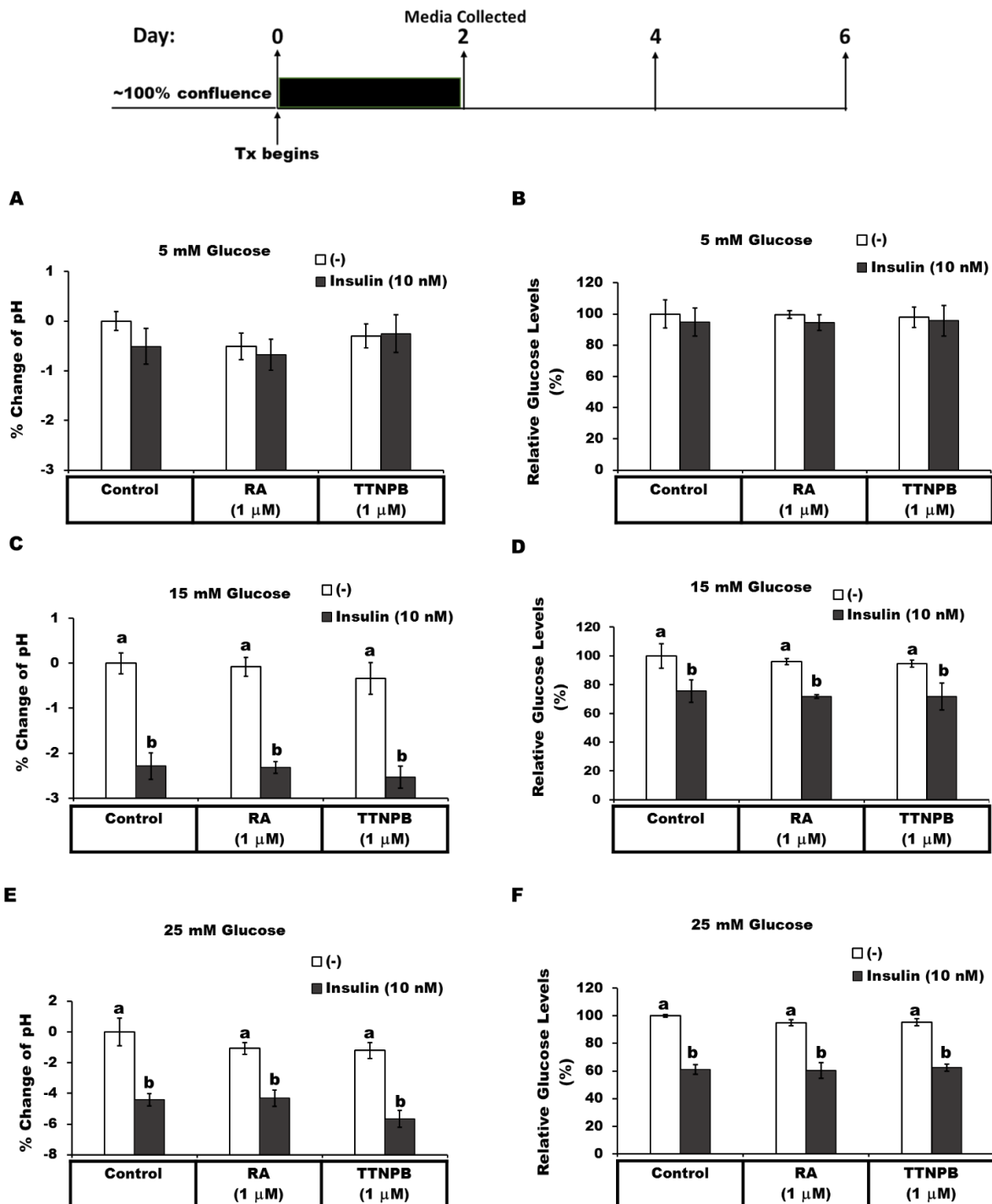


Figure 2.5

Figure 2.6. Medium pH and glucose levels in L6 cells following 4 days of treatment with 1 μ M each RA or TTNPB with or without 10 nM insulin at increasing glucose concentrations.

Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), 2% HS + insulin (10 nM), 2% HS + RA (1 μ M), 2% HS + RA + insulin, 2% HS + TTNPB (1 μ M), 2% HS + TTNPB + insulin. The treatments were made in medium containing either 5, 15, or 25 mM glucose. The medium was collected from each dish and replaced every 2 days with its respective treatment condition. The collected medium was pipetted into 15 mL tubes sealed with rubber corks for pH measurements using a standard pH meter and into 1.5 mL Eppendorf tubes for glucose measurements using the StanBio Glucose Liquicolor kit. A, C, and E. Changes of medium pH after the indicated treatments for 4 days. B, D, and F. Changes of medium glucose after the indicated treatments for 4 days. The pH level of the control group was arbitrarily set as 100% with data expressed as percentage change of pH. The glucose level of the control group was arbitrarily set as 100% with data expressed as percentage. All p-values $\leq$ 0.05; for (C) $a > b > c$, $a > b/c$; for (D) $a > b > c$, $a > b/c$; for (E) $a > b > c > d$; for (F) $a > b > c$. One-way ANOVA statistical analysis was performed using SPSS software. Data is presented as means $\pm$ S.E.M (n=3).

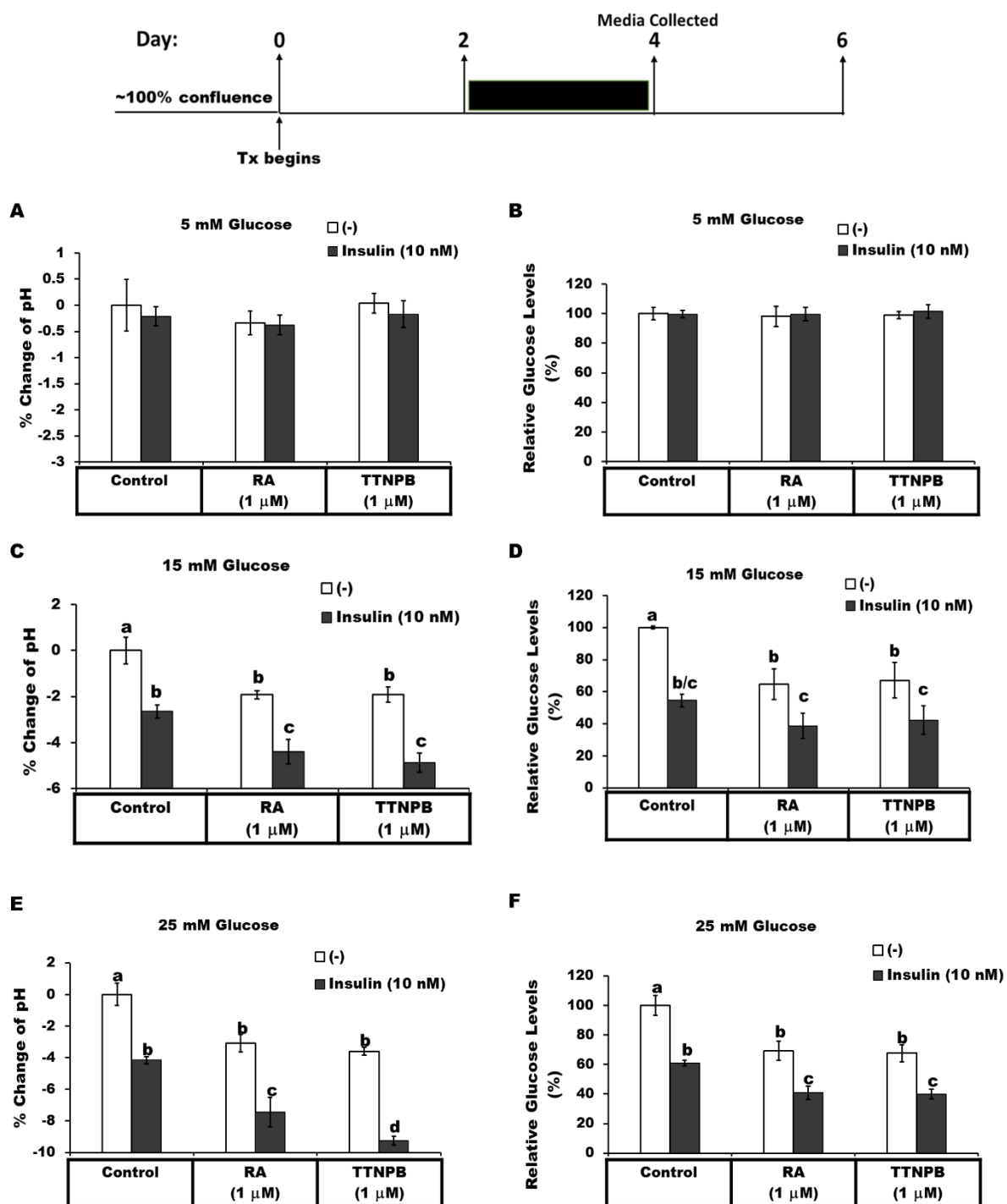


Figure 2.6

Figure 2.7. Medium pH and glucose levels in L6 cells following 6 days of treatment with 1 μ M each RA or TTNPB with or without 10 nM insulin at increasing glucose concentrations.

Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), 2% HS + insulin (10 nM), 2% HS + RA (1 μ M), 2% HS + RA + insulin, 2% HS + TTNPB (1 μ M), 2% HS + TTNPB + insulin. The treatments were made in medium containing either 5, 15, or 25 mM glucose. The medium was collected from each dish and replaced every 2 days with its respective treatment condition. The collected medium was pipetted into 15 mL tubes sealed with rubber corks for pH measurements using a standard pH meter and into 1.5 mL Eppendorf tubes for glucose measurements using the StanBio Glucose Liquicolor kit. A, C, and E. Changes of medium pH after the indicated treatments for 6 days. B, D, and F. Changes of medium glucose after the indicated treatments for 6 days. The pH level of the control group was arbitrarily set as 100% with data expressed as percentage change of pH. The glucose level of the control group was arbitrarily set as 100% with data expressed as percentage. All p-values $\leq$ 0.05; for (C-F) $a > b > c$. One-way ANOVA statistical analysis was performed using SPSS software. Data is presented as means $\pm$ S.E.M (n=3).

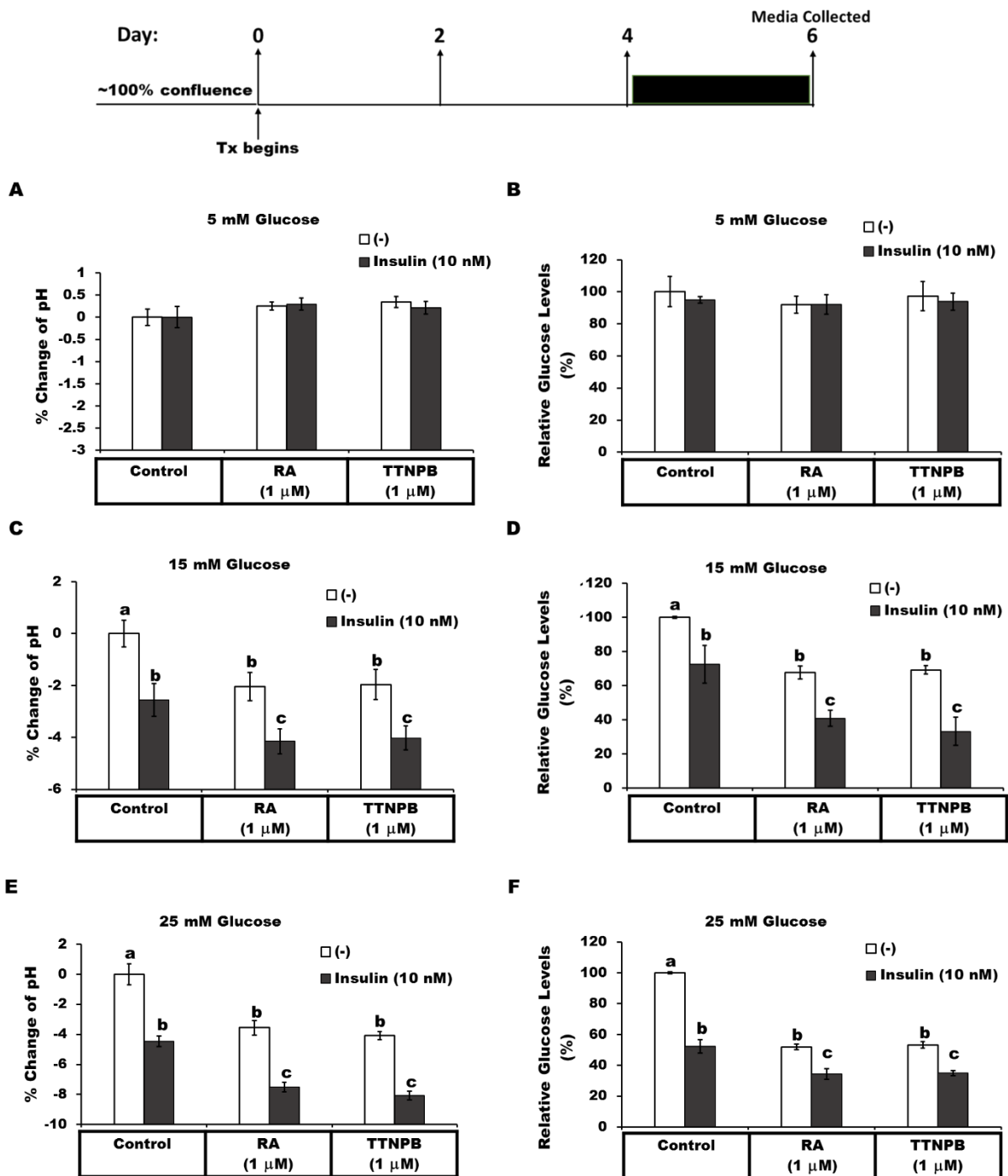


Figure 2.7

expression levels of GLUT1 and GLUT4 were significantly decreased in L6 cells treated with insulin in comparison with the control group. RA alone did not change GLUT1 and GLUT4 expression levels. However, the treatment of L6 cells with RA + insulin caused further reduction of GLUT1 and GLUT4 protein levels compared to control, insulin, and RA (Figure 2.8). This demonstrates that RA and insulin work together to reduce the expression levels of GLUT1 and GLUT4 in L6 cells.

4. Discussion

With the skeletal muscle playing a pivotal role in whole body glucose homeostasis, our study was aimed to understand how skeletal muscle cells respond to nutritional and hormonal stimuli. Recently, the insulin-stimulated glucose uptake via GLUT translocation has been a topic researched extensively^{487,488}. Factors such as nutrient availability, contraction, and growth factors can influence the insulin-stimulated glucose uptake and disposal in skeletal muscle⁴⁸⁹. Although studies have shown that RA plays a role in favoring muscle cell differentiation from myocyte to myotube, there are a limited number of studies investigating the effects of RA and other retinoids on skeletal muscle glucose utilization^{482,490,491}.

The changes in medium pH levels within this study was reciprocated with changes in medium glucose levels. Therefore, we believe that the decreases seen in medium glucose levels are due to increased glucose utilization within the muscle cells and the changes in medium pH are indicative of increased cell metabolism. In Figure 2.1, the changes of medium pH and glucose levels in L6 cell treated with the retinoids mimicked what you may expect with VA metabolism. RAL led to significant reductions in medium pH and glucose levels after 10 days, whereas ROL did not produce any significant differences (Figure 2.1). With the conversion of

ROL to RAL to RA, we show that the RA treated L6 cells display significant reductions in medium pH (Figure 2.1A) and glucose levels (Figure 2.1B) as early as 4 days after treatment began. The effects seen with RA remained throughout the 14-day experiment time frame with the RA treated L6 cells having a gradual decrease in medium pH and glucose levels. After 10 days of treatment with RA, there was ~50% less medium glucose compared to control and this remained through day 14 (Figure 2.1). These results indicate that RA provides the biological activity within skeletal muscle as it does in many other tissues^{26,251}.

Since insulin promotes glucose uptake and utilization in skeletal muscle, we performed an insulin dose curve (Figure 2.2)⁴⁹². The dose curve was performed to determine if insulin elicited the same response seen with the retinoids and to determine a dose that would maintain significant decreases in medium pH and glucose levels without compromising cell integrity. For this reason, 10 nM insulin was chosen for the remaining experiments due to its ability to maintain significant reductions in medium pH and glucose levels after day 2, 4, and 6 of treatment (Figure 2.2). Similarly, an RA dose curve was performed to determine the concentration closest to physiological relevance that would maintain significant reduction in medium pH and glucose levels over a 6-day period (Figure 2.3). It was evident after 4 days of treatment that 0.3 μ M RA alone had no effect on medium pH and glucose levels, however, 1 μ M RA caused significant reductions in both medium pH and glucose level, with 3 and 10 μ M RA having no additional effect (Figure 2.3). This results suggest that a relative high amount of RA is needed to achieve the effect. So far, we do not have an explanation to this phenomenon. Whether a low affinity receptor of RA or relatively rapid elimination of RA in L6 cells remains to be determined.

Figure 2.8. Decreases of GLUT1 and GLUT4 protein levels in L6 cells treated with insulin and RA. A. A representative blot of protein levels of GLUT1, GLUT4, and β -actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 μ M), and RA + insulin for 6 days. B-C. Quantification analysis of GLUT1 and GLUT4, respectively. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS (control), insulin (10 nM), RA (1 μ M), and RA + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 6 of treatment, whole cell lysate was collected as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis with control group set as 1. All p-values ≤ 0.05 ; * indicates statistical significance. One-way ANOVA statistical analysis was performed using SPSS software.

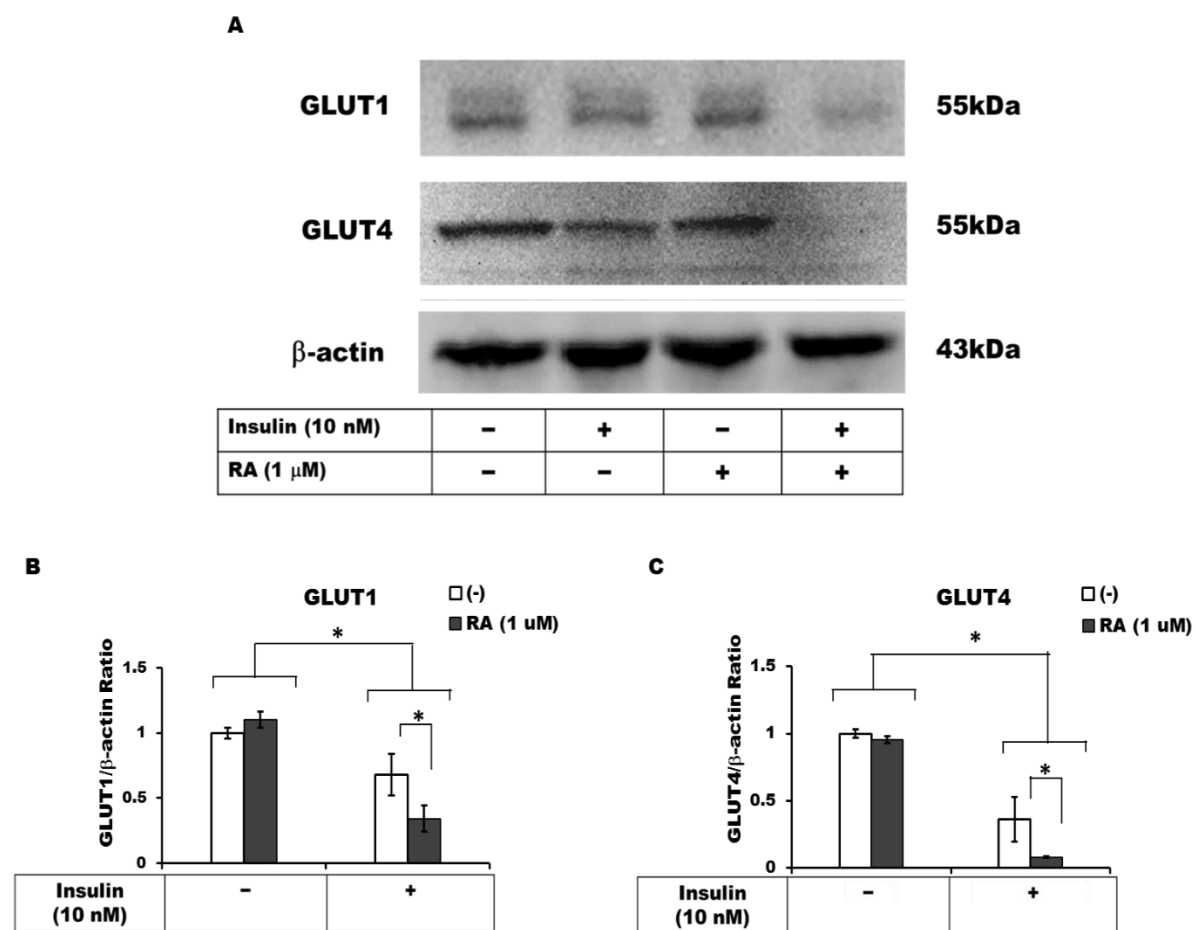


Figure 2.8

Retinoids mediate their actions through the binding of specific nuclear receptors within target cells^{493,494}. Although RARs and RXRs are the classical nuclear receptors known to be regulated by RA, other nuclear receptors such as liver-X-receptors (LXR) have been shown to be activated by RA in various cell types⁴⁹⁵⁻⁴⁹⁷. Therefore, we treated L6 cells with 1 μ M of LG268 (RXR agonist), TTNPB (RAR agonist), and T1317 (LXR agonist) to determine if the RA-mediated effect on medium pH and glucose changes were due to the activation of a specific nuclear receptor signaling pathway (Figure 2.4). LG268 and T1317 had no effect on medium pH and glucose levels on any day measured (Figure 2.4). However, TTNPB mimicked the responses seen with RA alone and with insulin, suggesting that the RA-mediated effect is by activation of the RAR nuclear receptor (Figure 2.4).

To confirm that the measurements in medium pH and glucose levels were actually due to changes in available glucose in the medium, we performed a glucose dose curve experiment in which the medium contained either 5, 15, or 25 mM glucose (Figures 2.5-7). L6 cells treated with 5 mM glucose did not elicit a response from insulin, RA, or TTNPB on any day. However, the L6 cells containing 15 and 25 mM glucose started displaying reductions in medium pH and glucose levels after 2 days of treatment and continued through day 6 (Figure 2.5-7). This suggests that the changes seen are due to the muscle cells taking in extracellular glucose into the cells for either storage or metabolism, thus resulting in decreases in remaining medium glucose levels.

GLUT4 is the glucose transporter commonly referred to as the insulin-stimulated glucose transporter in skeletal muscle whereas GLUT1 is thought to regulate skeletal muscle glucose uptake during basal conditions^{199,498}. Therefore, it is interesting to find that following 6 days of treatment, insulin alone and RA + insulin promoted reductions in both GLUT1 and GLUT4

(Figure 2.8). This could imply that L6 cells respond to pro-longed insulin and RA treatment with a homeostatic mechanism to decrease glucose uptake by decreasing GLUT1 and GLUT4 protein levels. As far as we know, this is the first time that this phenomenon is reported. The underlying mechanism deserves to be investigated.

Collectively, our results demonstrate that when L6 cells are treated with both RA and insulin, there is an elevation of glucose utilization. The L6 cells were cultured in the presence of 2% house serum, indicating that they had been induced to differentiation and kept differentiation media all the time. Currently, we have not determined whether L6 cells treated with insulin + RA are more differentiated than those treated with 2% HS, which are already in differentiation condition. An interesting project for future research might be whether to an increase in L6 cell metabolism is influenced by the state of its differentiation. Nevertheless, we believe that the regeneration and differentiation of skeletal muscle is a dynamic process and this study gives insight into the metabolism taking place. Furthermore, we demonstrate that the RA-mediated effect on L6 cells glucose utilization is in part due to the activation of the RAR signaling pathway and is not dependent on GLUT4 under these treatment conditions.

5. Conclusion

By investigating the cross-talk between retinoids and insulin within skeletal muscle cells, we can have a greater grasp on how this tissue regulates its glucose metabolism. If RA can work cooperatively with insulin in regulating skeletal muscle glucose metabolism then this could ultimately lead to the generation of new and improved therapies for insulin resistance and diabetes.

CHAPTER III. REGULATION OF GLYCOGEN
SYNTHASE AND GLUCOSE TRANSPORTERS BY
VITAMIN A AND INSULIN IN L6 CELLS AND RAT
SKELETAL MUSCLE TISSUE

1. Introduction

Skeletal muscle mass accounts for ~40% of the total body mass, therefore, it plays a major role in energy balance and nutrient metabolism⁴⁹⁹. Disorders that are associated with insulin resistance or insensitivity such as obesity and type 2 diabetes mellitus (T2DM) can negatively impact skeletal muscle glucose metabolism⁵⁰⁰. During the postprandial state, insulin is secreted from pancreatic β -cells and will inhibit hepatic glucose output while promoting glucose uptake in the skeletal muscle⁴⁸⁷. The skeletal muscle cells can also take in glucose in a non-insulin mediated manner by muscular contractions performed during exercise⁵⁰¹. By having a greater understanding of the glucose uptake and utilization pathways within skeletal muscle, we can determine the importance of diet and exercise on glucose homeostasis.

The actions of insulin are mediated through an intracellular signaling cascade. The binding of insulin to its receptor on target cells leads to the phosphorylation of insulin receptor substrate (IRS) proteins, with IRS-1 being the primary IRS in skeletal muscle, which recruits and activates phosphatidylinositol 3-kinase (PI3K)⁴⁰¹. Activated PI3K catalyzes the conversion of the membrane-associated phosphatidylinositol-4,5-bisphosphate (PIP2) and ATP into phosphatidylinositol-3,4,5-triphosphate (PIP3) and ADP. The increases in PIP3 leads to the recruitment of Akt, a serine/threonine protein kinase, by interacting with its pleckstrin homology domain¹⁸⁰. PIP3 also recruits PDK1 to the plasma membrane which phosphorylates Akt at Thr308^{181,402}. Mammalian target of rapamycin complex-2 (mTORC2) will phosphorylate Akt at Ser473 leading to its activation⁴⁰³. In muscle cells, the insulin stimulation leads to an increase in amount of GLUT4 on the cell membrane, which is translocated from intracellular storage vesicles to facilitate the uptake of glucose^{502,503}. Glucose can then be metabolized through glycolysis for energy production or it can be converted to glycogen for storage^{487,504}.

Glycogen is a highly branched polysaccharide that serves as the primary storage form of glucose³⁹¹. The liver and skeletal muscle are the major storage sites of glycogen in human body. The role of glycogen within the skeletal muscle is to provide glucose for energy production while in the liver the primary goal of glycogen is to supply glucose in the blood for the use in other tissues³⁹². The activation of insulin signaling pathway increases the activities of enzymes involved in glycogenesis including glycogen synthase (GS), and in turn, enhance glycogenesis in hepatocytes and muscle cells^{393,394}. The insulin-mediated activation of Akt will inactivate glycogen synthase kinase-3 (GSK-3) by phosphorylating specific sites on the enzyme^{182,183}. By inhibiting GSK-3, glycogen synthase phosphatase (GSP) can activate GS by dephosphorylation^{181,404}. The insulin signaling cascade will also lead to the activation of protein phosphatase 1 (PP1) which can dephosphorylate and activate GS, thus promoting glycogen formation⁴⁰⁵.

Vitamin A (VA, i.e. retinol, ROL) is an essential micronutrient that is implicated in the regulation of macronutrient metabolism⁴⁸². For biological activity, ROL proceeds through two stepwise cytosolic enzymatic reactions that first forms retinal (RAL) and then retinoic acid (RA, *all-trans* RA or *9-cis* RA)⁴⁹³. RA can then enter the nucleus of target cells and modules gene expression through the activation of specific nuclear receptors termed retinoid acid receptors (RAR) and retinoid-X-receptors (RXR)^{50,484}. RAR and RXR share a common ligand in *9-cis*-RA whereas *all-trans*-RA only binds to RAR⁶¹. Both of these classes of nuclear receptors will bind to a retinoic acid response element (RARE) that is located in the promoter of various genes. The RARE sequence consists of two consensus half sites (AGGTGA) that are separated by nucleotides that arrange the site as either palindromic, inverted palindromic, or direct repeats⁷⁵. Once the ligand binds to its respective receptor a conformational change occurs that allows the

receptor to interact with transcriptional cofactors that can alter the regulation of their target genes^{64,494}. While RA has been shown to play a role in carbohydrate, protein, and lipid metabolism in tissues such as the liver and adipose, the regulation of nutrient metabolism by RA in skeletal muscle is less clear^{482,497}. It has been shown in rat L6 skeletal muscle cells that RA can promote insulin-stimulated glucose uptake and increase GLUT4 mRNA levels³⁷⁶. Although there are studies investigating the effects of RA on skeletal muscle, most of these studies focus on muscle cell differentiation, therefore, the effects of RA on skeletal muscle glucose utilization are largely unknown.

In the previous chapter, we have shown that RA synergized with insulin to induce the glucose utilization in L6 cells. This induction was not due to the elevation of expression levels of GLUT1 or GLUT4 in the treated cells. The aim of this study was to further investigate the effects of VA in the form of RA on skeletal muscle cell glucose utilization and explore the underlying mechanism of this induction *in-vitro* and *in-vivo*. For this purpose, we used L6 skeletal muscle cells which have been previously shown to be responsive to both RA and insulin^{374,376}. We demonstrate that the treatment of RA and insulin, synergistically promote GS protein level, a process through the activation of RAR. These *in-vitro* results were confirmed with an *in-vivo* model of VA deficient (VAD) and sufficient (VAS) diabetic rats. The VAS rats that received insulin injection displayed significantly higher levels of GS protein expression that correlated with significantly lower levels of GS phosphorylation. Furthermore, L6 cells treated with RA + insulin display significantly higher amounts of glucose release to indicate increases in stored glycogen amounts. In addition, the treatment of RA + insulin in L6 cells altered the expression levels of GLUT3 and GLUT6, indicating that other transport mechanisms may play a role in the

uptake of glucose under these treatment conditions.

2. Materials & Methods

2.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM) and penicillin/streptomycin were purchased from Mediatech (Manassas, VA, U.S.A.). Fetal bovine serum (FBS) was purchased from Life Technologies (Grand Island, NY, U.S.A.). Horse serum (HS) was purchased from Hyclone Laboratories (Logan, UT, U.S.A.). For RNA extraction, RNA STAT-60™ was purchased from Tel-Test (Friendswood, TX, U.S.A.). DNA-free™ kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, U.S.A.). For cDNA synthesis, Verso cDNA synthesis kit was purchased from Thermo Scientific (Pittsburg, PA, U.S.A.). BCA protein assay kit and ECL Western Blotting Substrate were purchased from Pierce (Rockford, IL, U.S.A.). Immobilon®-PSQ PVDF membrane was purchased from EMD Millipore (Billerica, MA, U.S.A.). Polyvinylpyrrolidone (PVP) was purchased from Amresco (Solon, OH, U.S.A.). LG268 was synthesized in the core facility at the University of Texas Southwestern Medical Center at Dallas. Potassium hydroxide, isopropanol, all-*trans* retinoic acid, all-*trans* retinol, all-*trans* retinal, TTNPB, insulin, and diethylpyrocarbonate (DEPC) were purchased from Sigma (St. Louis, MO, U.S.A.). Perchloric acid was purchased from Alfa Aesar (Ward Hill, MA, U.S.A.). Amyloglucosidase was purchased from Carolina Biological Supply (Burlington, NC, U.S.A.). For immunoblotting, Total AKT (#9272), PO₄-AKT Ser473 (#9271) and Thr308 (#9275), glycogen synthase (#3886), PO₄-glycogen synthase Ser641 (#3891), GSK-3 α (#4337), PO₄-GSK-3 α Ser21 (#9316), GSK-3 β (#9315), PO₄-GSK3 β Ser9 (#9323), GAPDH (#5174), lactate dehydrogenase (#3582), pyruvate dehydrogenase (#3205), pyruvate kinase M1/2 (#3190), IRS-1 (#3407), IRS-2 (#4502), β -Actin (#4970), and goat anti-rabbit IgG (#7074) were

purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). GLUT1 (#1340) and GLUT4 (#1346) were purchased from Chemicon Industries (Temecula, CA, U.S.A.). GLUT3 (#38121) and GLUT6 (#43594) were purchased from One World Lab (San Diego, CA, U.S.A.). IR- β subunit (#06-492) was purchased Upstate Biotechnology, Inc. (Lake Placid, NY, U.S.A.).

2.2 L6 rat skeletal muscle cell culture and differentiation

Rat L6 cells were incubated in 60 mm dishes at 37°C and 5% CO₂. For subculture, L6 cells were maintained in DMEM with 4.5 g/L (25 mM) glucose supplemented with 10% FBS + 1% penicillin/streptomycin. When cells reached ~100% confluence, medium was changed to DMEM containing 25 mM glucose with 2% HS + 1% penicillin/streptomycin to induce differentiation.

2.3 Animal Experiment

The skeletal muscle tissue samples were collected in another study, which will be submitted for publication elsewhere (manuscript in submission). In brief, male Zucker lean (ZL) rats at weaning (3 weeks old) were fed a VAS diet (#5755, TestDiet) or an isocaloric VAD diet (#5822, TestDiet) diet. Rats were fasted for 24 hours, and received a single injection of 65 mg/kg BM streptozotocin (STZ) via the tail vein and allowed to have their respective diet ad libitum. The successful induction of STZ-induced diabetes was confirmed when plasma glucose level was higher than 250 mg/dL. Then the VAS or VAD rats with STZ-induced diabetes were divided into 2 groups. They were treated with saline (control, 100 μ L, intramuscular injection, VAS-C or VAD-C), or insulin (0.5 IU/100 g BM, intramuscular injection, VAS-INS or VAD-INS). Rats were fed ad libitum for 3-4 hours and then euthanized for tissue sample collection. The

procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee at Knoxville (Protocol numbers 1256 and 1899).

2.4 Muscle Sample Preparation

About 100 mg of frozen gastrocnemius tissues was homogenized for 1-2 minutes at 28,000 RPM using a Omni Tissue Master 240 (Omni International, Kennesaw, GA, U.S.A.) in 1 mL ice-cold protein lysis buffer (1% Triton X-100, 10% glycerol, 1% IGEPAL CA-630, 50 mM Hepes, 100 mM NaF, 1 mM sodium molybdate, 1 mM sodium β -glycerophosphate, 5 mM sodium orthovanadate, 1.9 mg/mL aprotinin, 5 mg/ml leupeptin, 1 mM benzamide, 2.5 mM PMSF, pH 8.0). The lysates were transferred to cold 1.5 mL Eppendorf tubes and kept on ice at all times. The samples were vortexed for 10 seconds and maintained on ice for at least 20 minutes with vortexing every 5 minutes. After that lysates were centrifuged at 13,000 RPM for 15 minutes at 4°C. The supernatant was collected into clean 1.5 mL Eppendorf tubes and stored at -80°C before being used. Protein content of the lysate was quantified using Pierce BCA protein assay kit according to the manufacturer's instruction and analyzed on Glomax® Multi+Detection System (Promega, Madison, WI, U.S.A.).

2.5 RNA Isolation and cDNA synthesis

The methods for total RNA isolation and cDNA synthesis were described elsewhere⁵⁰⁵. Briefly, 1 mL RNA STAT-60™ was added to one 60 mm dish with L6 cells. The RNA STAT-60™ reagent was pipetted up and down quickly to homogenize the cells and transferred to a 1.5 mL tube. After that, 200 μ L of chloroform was added to each tube and the mixture was shaken vigorously for 15 seconds. Following a 3-minute incubation period at room temperature, samples were centrifuged at $12,000 \times g$ at 4°C for 15 minutes. Roughly 500 μ L of aqueous phase was

transferred to a new 1.5 mL tube, and 500 μ L of isopropanol was added to precipitate out the RNA. Following a 10-minute incubation at room temperature, samples were centrifuged at $12,000 \times g$ for 10 minutes at 4°C . The supernatant was discarded without disturbing the pellet and 1 mL of 70% ethanol in DEPC-treated water was added. Samples were centrifuged at $7,500 \times g$ for 5 minutes at 4°C . The supernatant was discarded without disturbing the pellet and the pellet was air dried for 3 minutes. The pellet (RNA) was dissolved in 88 μ L of DEPC water for 30 minutes at room temperature. The contaminated DNA in RNA samples was removed using the DNA-freeTM kit. Following the 30-minute incubation period, 10x DNase I buffer (0.1 volume) and 4 units of DNase in 2 μ L were added to the RNA samples. Following gentle mixing, the samples were incubated at 37°C for 30 minutes. After that, 10 μ L DNase Inactivation reagent (0.1 volume) was added and mixed well. Mixture was incubated at room temperature for 2 minutes and then centrifuged at $10,000 \times g$ at 4°C for 2 minutes. The supernatant ($\sim 100 \mu\text{L}$ DNA-free RNA) was transferred to a new 1.5 mL tube and quantified using NanoDrop ND-1000 spectrophotometer and software (version 3.1.1, NanoDrop Technologies Inc., Wilmington, DE, U.S.A.). First strand cDNA was synthesized using cDNA synthesis kit. The reaction was carried out in a total volume of 100 μ L, containing 2 μg of DNA-free RNA, 50 mM Tris-HCl, 75 mM KCl, 10 mM DTT, 8.5 mM magnesium chloride, 0.5 mM of each dNTP (dATP, dCTP, dTTP, and dGTP), 2.5 mM random hexamer primers, 40 units of RNase inhibitor, 125 units of Multiscribe reverse transcriptase. The conditions are 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. The newly synthesized cDNA was stored at -20°C before being analyzed.

2.6 Real-time PCR analysis

The gene expression level was determined by real-time PCR using SYBR Green. Each SYBR Green based real-time PCR reaction contains, in a final volume of 14 μ L, cDNA from 14 ng of reverse transcribed total RNA, 2.33 pmol primers, and 7 μ L of 2x SYBR Green PCR Master Mix. Triplicate PCR reactions were carried out in 96-well plates using 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.). The conditions are 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The data was normalized to the mRNA level of the control gene β -actin. The data were presented fold induction using $\Delta\Delta C_t$ methods with the control treatment group set as 1. See Table 3.1 in Appendix for primer sequences.

2.7 Protein Isolation and Immunoblotting

Whole cell lysate was prepared by washing L6 cells once with ice-cold PBS and lysing the cells in 400 μ L ice cold whole-cell lysis buffer and subsequently scraped into chilled 1.5 mL centrifugation tubes. The lysates were vortexed vigorously and placed on ice for at least 20 minutes. Lysates were then centrifuged at 12,000 x g for 15 minutes at 4°C and supernatant collected into clean 1.5 mL Eppendorf tubes and stored at -80°C. Protein was quantified by Pierce BCA protein assay kit using manufacturer recommendations and analyzed using Glomax® Multi+Detection System. Total protein (40 μ g) was then separated on an 8% SDS-PAGE gel and transferred to Immobilon®-PSQ PVDF membrane³⁹. Membranes were blocked using 1% PVP in 1x TBST for 1 hour at room temperature and then probed with specific antibodies diluted in 1% PVP in 1x TBST overnight at 4°C⁴⁸⁶. After that, the membranes were washed three times for 5 minutes each with 1x TBST and then incubated 1-2 hours with goat anti-rabbit IgG conjugated with horseradish peroxidase (1/2000 dilution in 1% PVP in 1x

TBST). Following secondary incubation, three more 5 minute washes with 1x TBST were carried out followed by visual detection using ECL Western Blotting Substrate on ChemiDoc Software System (BIO-RAD Laboratories, Hercules, CA, U.S.A.). Quantification analysis was performed using Image Lab version 2.0.1 software (BIO-RAD Laboratories, Hercules, CA, U.S.A.). The density of a protein band was determined by subtracting the background in an area of the same size immediately in front of protein band. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis.

2.8 Glucose Release Assay for the Measurement of Glycogen Content

For the measurement of cellular glycogen content, we adapted the glucose release method developed to determine the tissue glycogen content⁵⁰⁶. Following the 6-days treatment in differentiation medium containing the indicated reagents as described in the figure legends, L6 cells were washed twice with 2 mL of ice cold PBS and PBS was immediately removed. After that, 400 μ L ice cold PBS was added to each dish and L6 cells were scraped into chilled 1.5 mL Eppendorf tubes. Samples were centrifuged at $12,000 \times g$ for 12 minutes at 4°C. The supernatant was discarded without disturbing the pellet and the samples were placed on ice. For glycogen precipitation, 35 μ L of ice cold perchloric acid (0.6 N) was added to each sample followed by pipetting up and down to mix contents. For neutralization of perchloric acid, 5 μ L of potassium hydroxide (5.4 N) was added and samples were mixed by pipetting up and down. 35 μ L of amyloglucosidase (300 AGU/mL) was then added to each sample followed by pipetting to mix each sample. The samples were then incubated for 2 hours at 40°C with occasional mixing. Following incubation, the samples were centrifuged at $12,000 \times g$ for 5 minutes at 4°C. The glucose amount released was measured using the StanBio Glucose Liquicolor kit. Briefly, in individual 1.5 mL Eppendorf tubes, 500 μ L of glucose reagent was added to each tube and

incubated for 5 minutes at 37°C. Following the incubation period, one glucose reagent containing tube received 5 µL of blank control (35 µL perchloric acid + 5 µL potassium hydroxide + 35 µL amyloglucosidase), one tube received 5 µL of glucose standard, and the remaining tubes received 5 µL of each mixed sample. The samples were incubated for 5 minutes at 37°C and then loaded in a clear 96-well plate for analysis on Glomax® Multi+Detection System at 500 nm wavelength. Values were derived by comparing the absorbance of the unknown (u) with that of the standard (s): $\text{Glucose (mg/dL)} = ((A_u \times 10)/A_s) \times 100$. L6 cells treated with 2% HS alone served as control for comparison to respective treatment conditions. Data was normalized to total protein and presented as both fold induction with the control group being set as 1 and total glucose released in mg glucose/mg total protein.

2.10 Statistical Analysis

All experiments have been conducted in triplicate. One-way ANOVA with LSD post-hoc statistical analysis was performed using SPSS version 22 statistical software (IBM, Armonk, NY, U.S.A.). Data were presented as means $\pm$ S.E.M. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Expression levels of key proteins in insulin signaling pathway in L6 cells treated with RA, LG268, or TTNPB without or with insulin

To understand the mechanism of enhanced glucose usage in L6 cells treated with RA and insulin, L6 cells were treated with 1 µM of RA, LG268 (RXR agonist) and TTNPB (RAR agonist) with or without 10 nM insulin for 6 days with change of the same fresh medium every

two days. After 6 days of treatment with RA, IRS-1 protein levels were significantly increased compared to control, insulin, and RA + insulin (Figure 3.1A and 3.1C). Similar results were also seen with TTNPB. IRS-2 protein levels were significantly increased with RA and RA + insulin compared to control and insulin alone, however, no significant changes were seen with TTNPB (Figure 3.1A and 3.1D). Phosphorylation of Akt at Thr308 was significantly increased in L6 cells with all groups treated with insulin compared to control (Figure 3.1A and 3.1E). RA + insulin led to significant further increase in Akt (Thr308) phosphorylation compared to control, insulin, and RA (Figure 3.1A and 3.1E). Interestingly, TTNPB treatment led to significant increases in phosphorylation of Akt (Thr308) compared to control, and TTNPB + insulin significantly increased phosphorylation at this site compared to control and insulin (Figure 3.1A and 3.1E). LG268 + insulin also displayed increased phosphorylation of Akt (Thr308) compared to control, insulin, and LG268 (Figure 3.1A and 3.1E).

3.2 Expression levels of key proteins in glycogen metabolic pathway in L6 cells treated with RA, LG268, or TTNPB without or with insulin

The phosphorylation of GSK-3 α at site Ser21 was increased with RA + insulin, TTNPB, and TTNPB + insulin compared to control (Figure 3.2A-B). Similar results were seen with the phosphorylation of GSK-3 β at site Ser9 with the addition of RA also significantly increasing phosphorylation at this site (Figure 3.2A and 3.2D). The phosphorylation of GS at site Ser641 was significantly reduced by the treatment of insulin, RA, RA + insulin, LG268 + insulin, and TTNPB + insulin compared to control (Figure 3.2A and 3.2F). Total GS protein levels were significantly increased by insulin, RA + insulin, and TTNPB + insulin compared to control (Figure 3.2A and 3.2G). RA + insulin led to significant further increase in GS protein levels compared to RA alone and insulin (Figure 3.2A and 3.2G). TTNPB + insulin led to significant

Figure 3.1. Abundance levels of key proteins in insulin signaling pathway in L6 cells treated with RA, LG268, or TTNPB in the absence or presence of insulin for 6 days. A. A representative blot of protein levels of total Akt, PO4-Akt Ser473 and Thr308, IRS-1, IRS-2, IR- β subunit and β -actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin for 6 days. B-G. Quantification analysis of IR- β , IRS-1, IRS-2, PO4-Akt Thr308, PO4-Akt Ser473, and total Akt, respectively. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 6 of treatment, whole cell lysate was collected as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis with control group set as 1. All p-values ≤ 0.05 ; for (C) $a > b > c$; for (D) $a > b$; for (E) $a > b > c$, $a > b/c$. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

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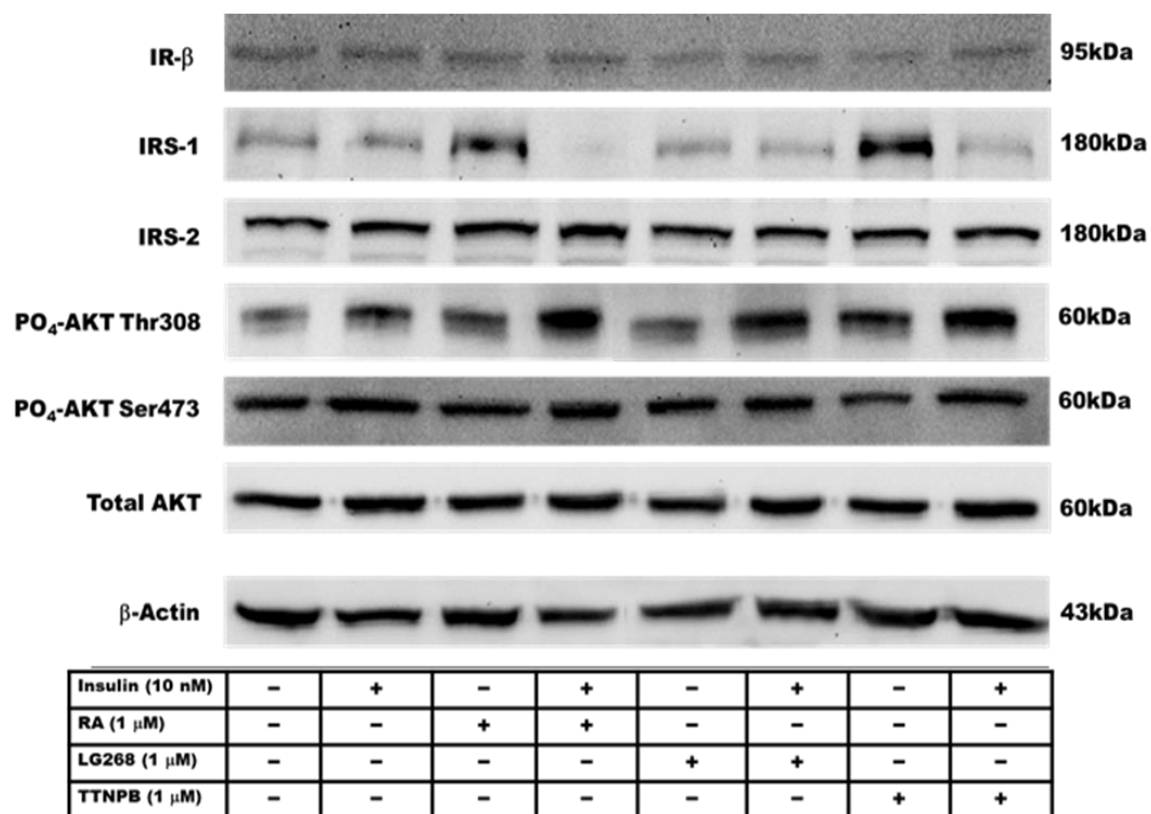


Figure 3.1

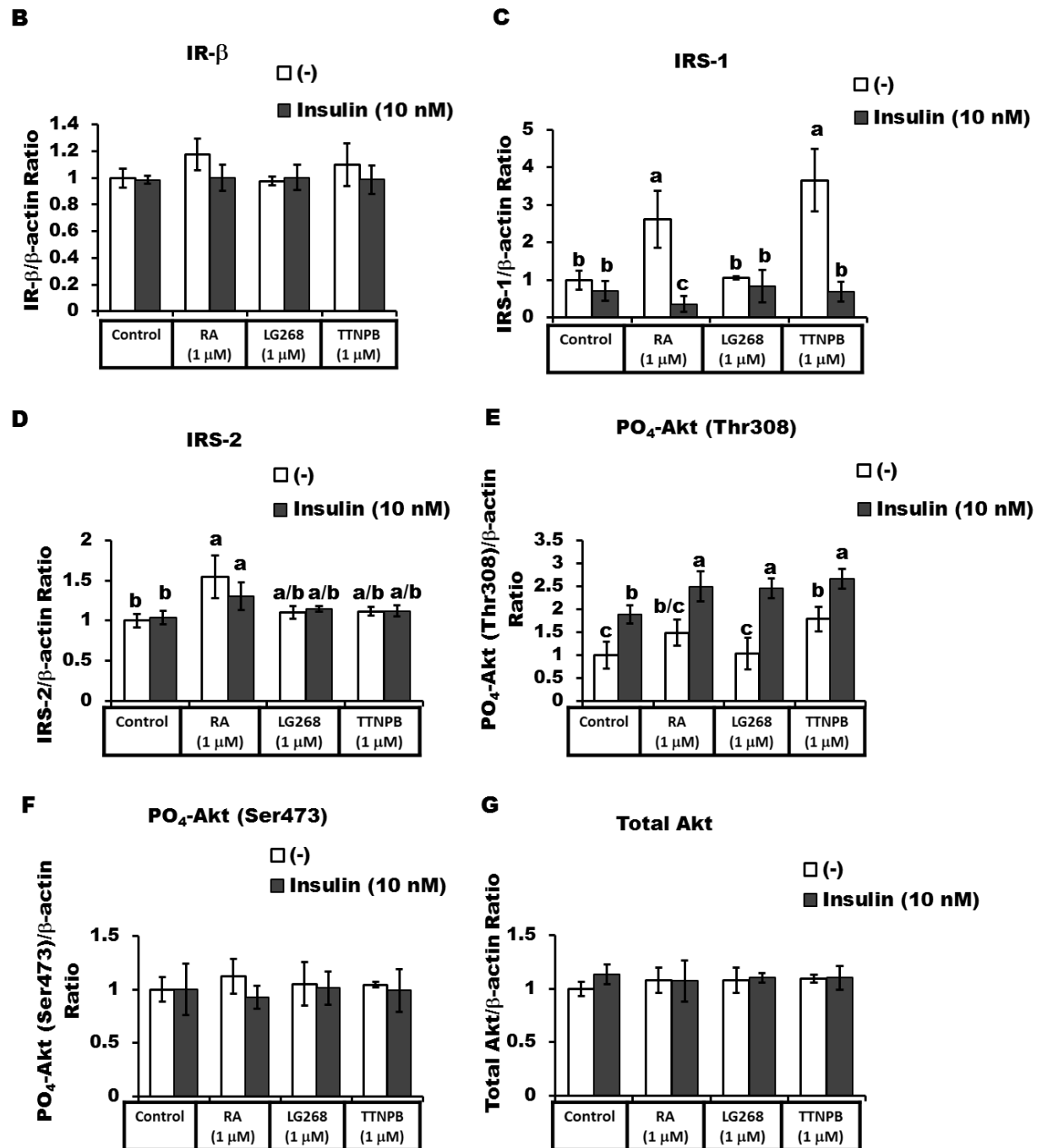


Figure 3.1 Continued

Figure 3.2. Abundance levels of key proteins in glycogen signaling pathway in L6 cells treated with RA, LG268, or TTNPB in the absence or presence of insulin for 6 days. A. A representative blot of protein levels of GS, PO4-GS Ser641, GSK-3 α , PO4-GSK-3 α Ser21, GSK-3 β , PO4-GSK-3 β Ser9 and β -actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin for 6 days. B-G. Quantification analysis of PO4-GSK-3 α Ser21, GSK-3 α , PO4-GSK-3 β Ser9, GSK-3 β , PO4-GS Ser641, and GS, respectively. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 6 of treatment, whole cell lysate was collected as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis with control group set as 1. All p-values ≤ 0.05 ; for (B) a>b>c, a/b>c; for (D) a>b>c, a/b>c; for (F) a>b>c, a>b/c; for (G) a>b>c>d, a>b>c/d. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

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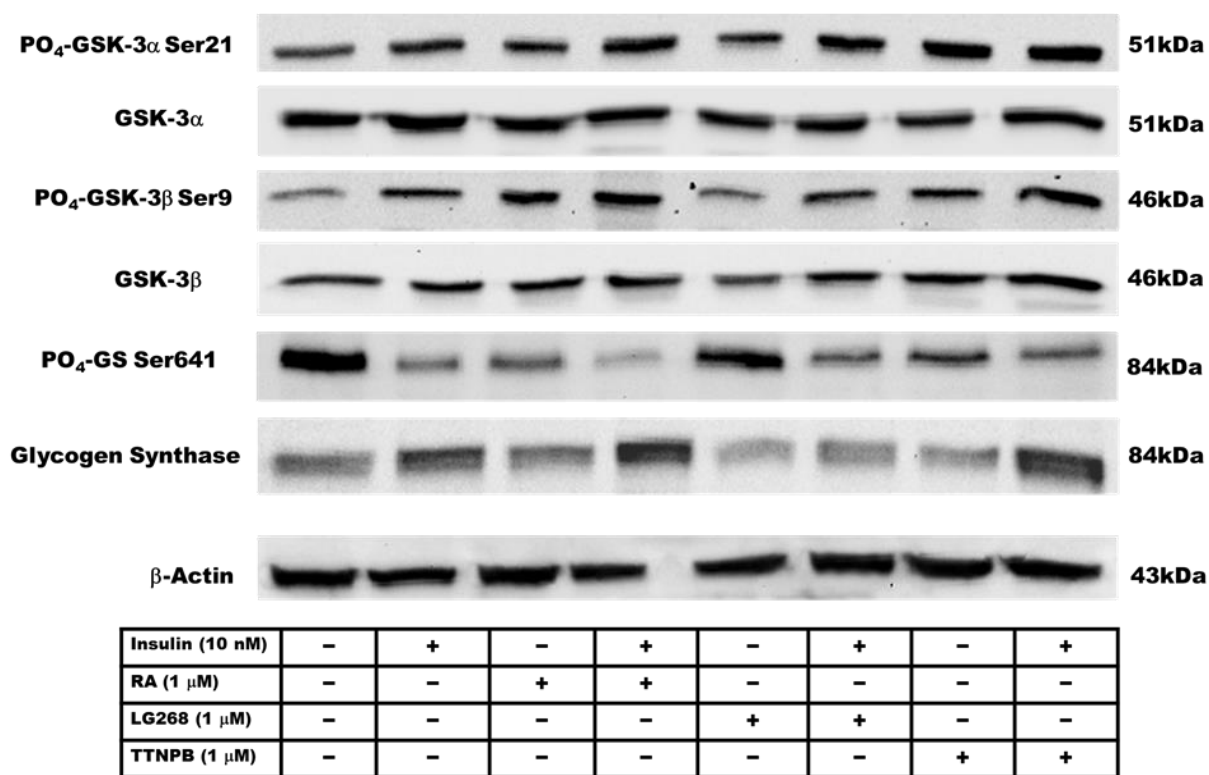


Figure 3.2

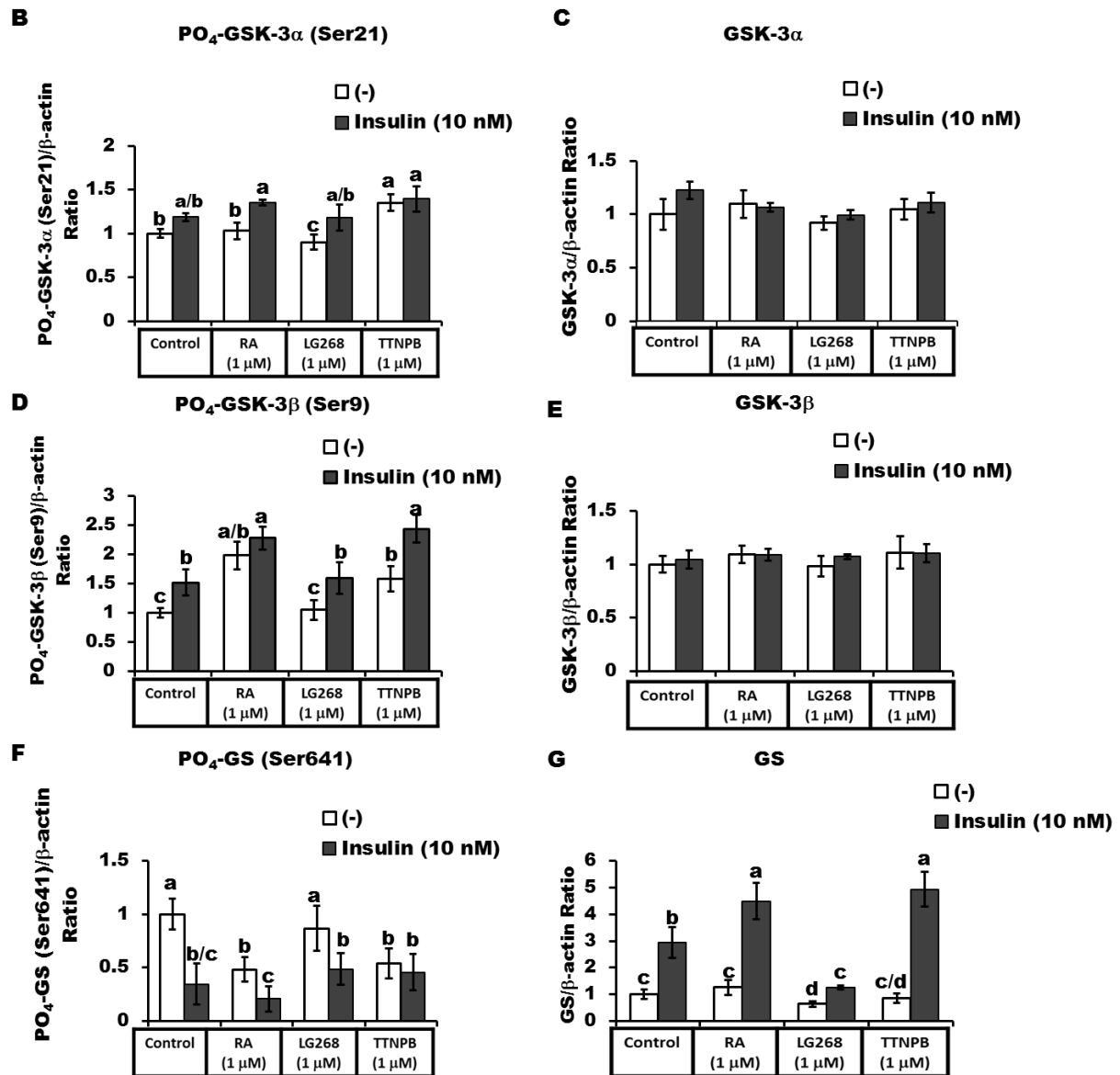


Figure 3.2 Continued

increases in GS protein levels compared to TTNPB and insulin (Figure 3.2A and 3.2G).

3.3 Expression levels of key proteins in glucose usage pathway in L6 cells treated with RA, LG268, or TTNPB without or with insulin

Following 6 days of treatment with either RA, TTNPB, or LG268 with or without insulin, no significant changes were seen in the protein levels of GAPDH, LDH, PDH, or pyruvate kinase (Figure 3.3).

3.4 Treatment of insulin enhances *Gys1* gene mRNA expression in L6 cells

The treatments of insulin, RA + insulin, LG268 + insulin, and TTNPB + insulin in L6 cells led to significant increases in *Gys1* gene expression compared to control (Figure 3.4A). None of the ligands + insulin produced significant differences in *Gys1* gene expression compared to insulin (Figure 3.4A). RA led to significant reduction in *Ldha* gene expression compared to insulin (Figure 3.4B). None of the treatments produced significant differences in *Slc2a4* gene expression levels (Figure 3.4C). Only TTNPB + insulin produced a significant increase in *Ppargc1a* gene expression levels compared to control (Figure 3.4D). *Ppargc1b* gene expression levels were increased by insulin, RA + insulin, and TTNPB + insulin compared to control (Figure 3.4E). Similar to that seen with GS, this increase in *Ppargc1b* gene expression levels is driven by the actions of insulin.

3.5 Glycogen content in L6 cells treated with RA, LG268, or TTNPB in the absence or presence of insulin for 6 days

Following treatment of L6 cells with 1 μ M RA, LG268, or TTNPB in the absence or presence of 10 nM insulin, cellular glycogen was measured using glucose release assay (Figure 3.5). Insulin alone led to significant increases of glycogen in L6 cells as shown in both fold

Figure 3.3. Abundance levels of key proteins in glucose usage pathway in L6 cells treated with RA, LG268, or TTNPB in the absence or presence of insulin for 6 days. A. A representative blot of protein levels of GAPDH, lactate dehydrogenase (LDH), pyruvate dehydrogenase (PDH), pyruvate kinase mutase (PKM $\frac{1}{2}$) and β -actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin for 6 days. B-E. Quantification analysis of GAPDH, PKM $\frac{1}{2}$, LDH, and PDH, respectively. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 6 of treatment, whole cell lysate was collected as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis with control group set as 1. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

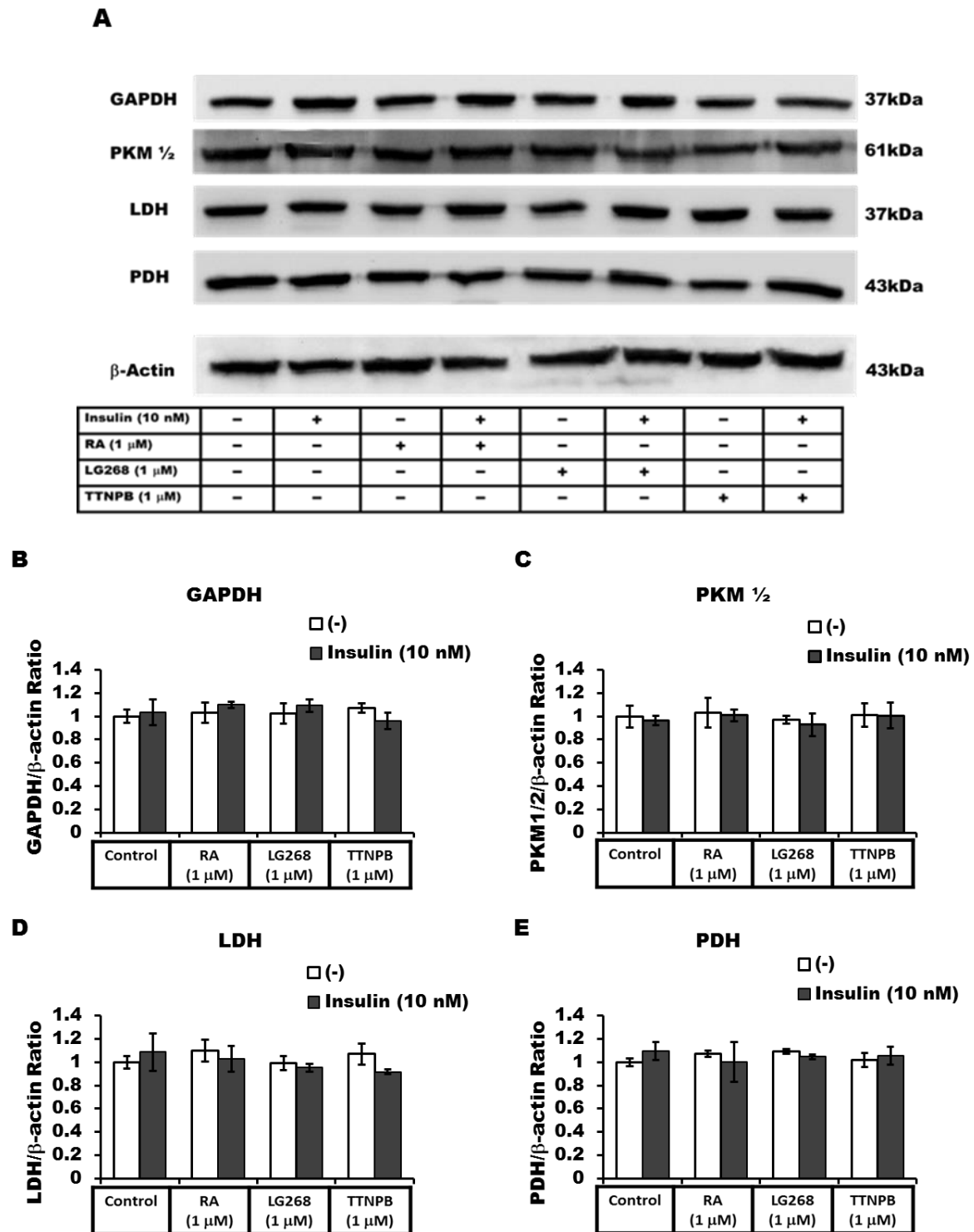


Figure 3.3

Figure 3.4. Treatment with insulin enhances *Gys1* gene expression in L6 cells. A-E.

Expression levels of *Gys1* (A) *Ldha* (B), *Slc2a4* (C), *Ppargc1a* (D) and *Ppargc1b* (E) transcripts. L6 cells in 60 mm dishes were incubated in medium containing 2% HS alone (control) or 2% HS with insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), or TTNPB + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 6 of treatment, RNA isolation and cDNA synthesis was performed as described in Materials & Methods. Real-time PCR analysis was performed using Applied Biosystems 7300 Real-Time PCR System. The data were normalized to the mRNA level of the control gene β -actin. The data were presented fold induction using $\Delta\Delta C_t$ methods with the control treatment group set as 1. All p-values ≤ 0.05 ; for (A) a>b; for (B) a>b; for (D) a>b; for (E) a>b>c, a/b>b/c. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

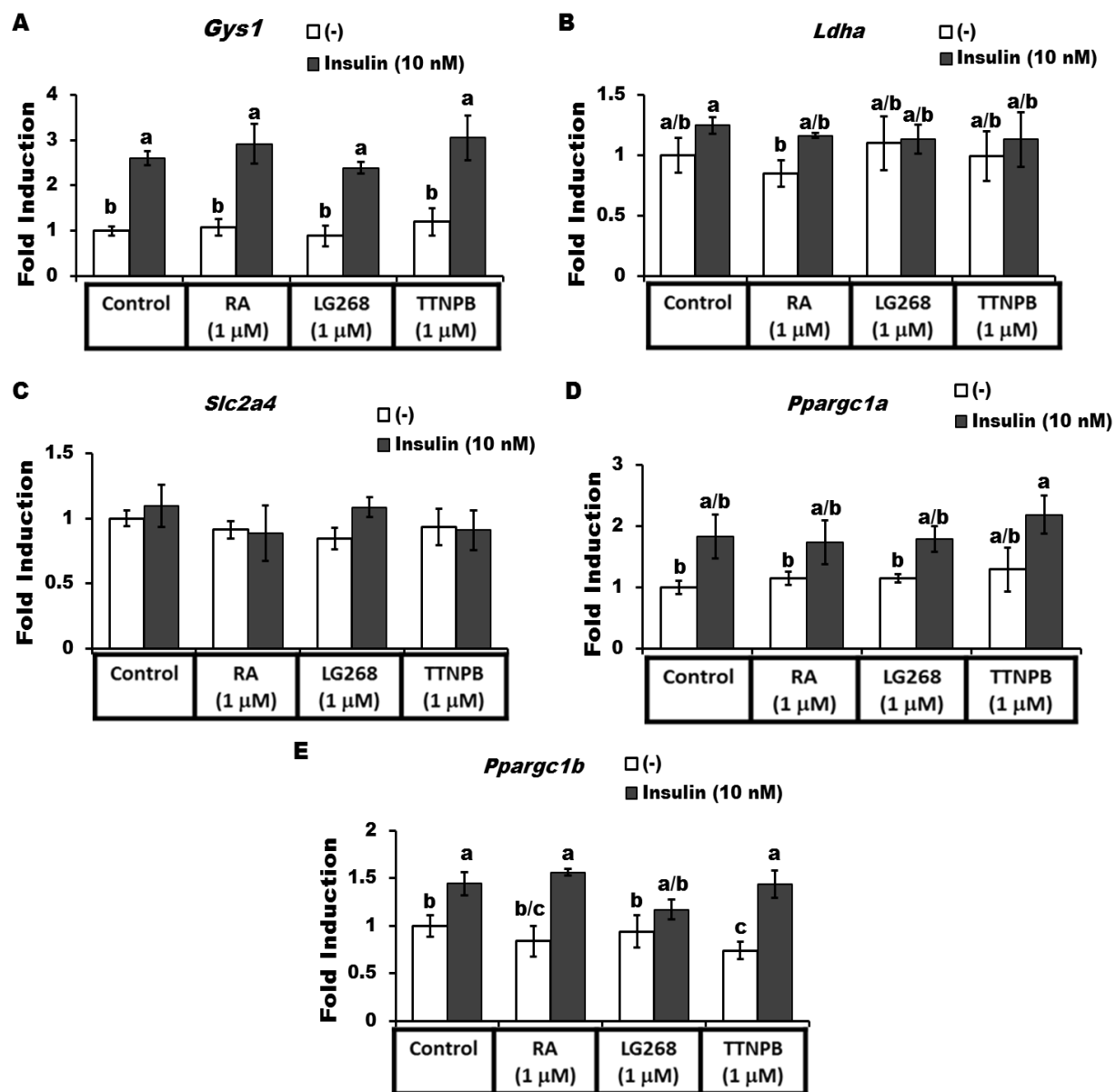


Figure 3.4

induction and glucose release. The addition of RA + insulin or TTNPB + insulin produced significantly higher levels of glycogen compared to insulin alone group (Figure 3.5). These results demonstrated that insulin alone induced glycogen content, and insulin + RA or TTNPB further increased the glycogen content in L6 cells after 6-days treatment, supporting the western blot data of elevated GS protein expression and GSK phosphorylation. All these indicated that the elevated glucose usage in L6 cells treated with insulin, RA and insulin + RA is through an increase of GS expression and reduction of its phosphorylation, which promotes glycogen synthesis. Interestingly, the effects seen with insulin were dramatically decreased with the addition of LG268 (Figure 3.5).

3.6 Expression levels of key proteins in insulin signaling pathway in gastrocnemius muscle of VAD and VAS STZ-induced diabetic rats treated with saline or insulin

The protein levels of IRS-1 were significantly increased in the VAD-C rats compared to the other groups (Figure 3.6A and 3.6C). However, the VAS-INS rats did have significantly higher IRS-1 levels compared to VAS-C and VAD-INS rats (Figure 3.6A and 3.6C). Both the VAS-C and VAS-INS groups had significantly higher phosphorylation of Akt at site Thr308 compared to both VAD groups (Figure 3.6A and 3.6E). The phosphorylation of Akt at site Ser473 was significantly lower in the VAD-C group compared to the other 3 groups (Figure 3.6A and 3.6F).

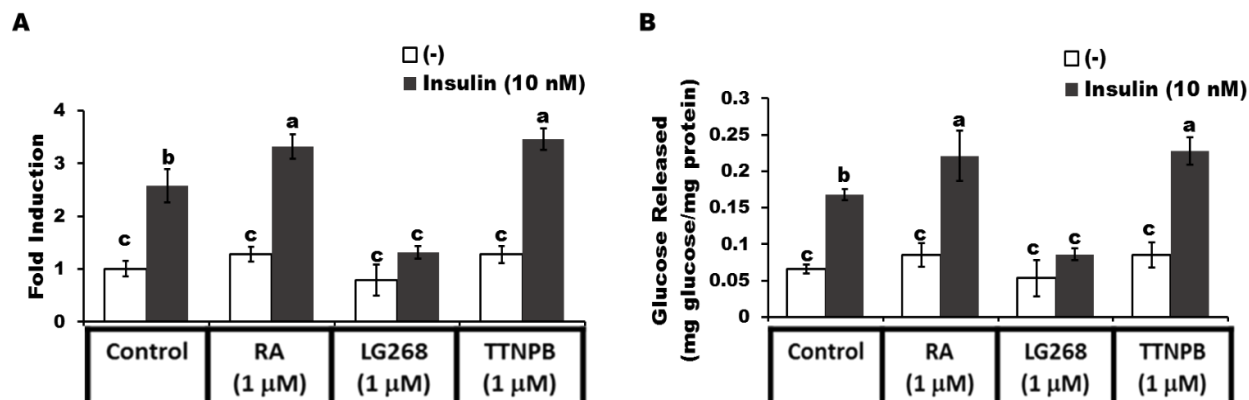


Figure 3.5. Increased glycogen content expressed as fold induction (A) and glucose release (B) in L6 cells treated with insulin, RA + insulin, and TTNPB + insulin. A. Representative of glucose released presented as fold induction. B. Representative of total glucose released in mg glucose/mg total protein. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS alone (control), insulin (10 nM), RA (1 μM), RA + insulin, LG268 (1 μM), LG268 + insulin, TTNPB (1 μM), or TTNPB + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 6 of differentiation, glycogen measurement in the form of glucose released was measured as described in Materials & Methods. The glucose amount released was measured using the StanBio Glucose Liquicolor kit and analyzed Glomax® Multi+Detection System. Data presented as either (A) fold induction with control group set as 1 or (B) total glucose released in mg glucose per mg total protein. All p-values ≤ 0.05; for (A-B) a>b>c. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means ± S.E.M (n=3).

Figure 3.6. Abundance levels of key proteins in insulin signaling pathway in gastrocnemius muscle of VAD and VAS STZ-induced diabetic rats treated with saline or insulin. A. Protein levels of total Akt, PO4-Akt Ser473 and Thr308, IRS-1, IRS-2, IR- β subunit and β -actin in VAD or VAS STZ-diabetic ZL rats treated with saline or insulin. B-G. Quantification analysis of IR- β , IRS-1, IRS-2, PO4-Akt Thr308, PO4-Akt Ser473, and total Akt, respectively. Male ZL rats at weaning were fed a VAS diet or an isocaloric VAD diet. Rats were fasted for 24 hours, and received a single injection of STZ via the tail vein and allowed to have their respective diet ad libitum. Then the VAS or VAD rats with STZ-induced diabetes were treated with saline (control, 100 μ L, intramuscular injection, VAS-C or VAD-C) or insulin (0.5 IU/100 g BM, intramuscular injection, VAS-INS or VAD-INS). Rats were fed ad libitum for 3-4 hours and then euthanized for tissue sample collection. Muscle tissue protein isolation and western blotting procedure was performed as described in Materials and Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis by averaging each group with the VAD-C group arbitrarily set as 1 for control. All p-values ≤ 0.05 ; * indicates statistical significance. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

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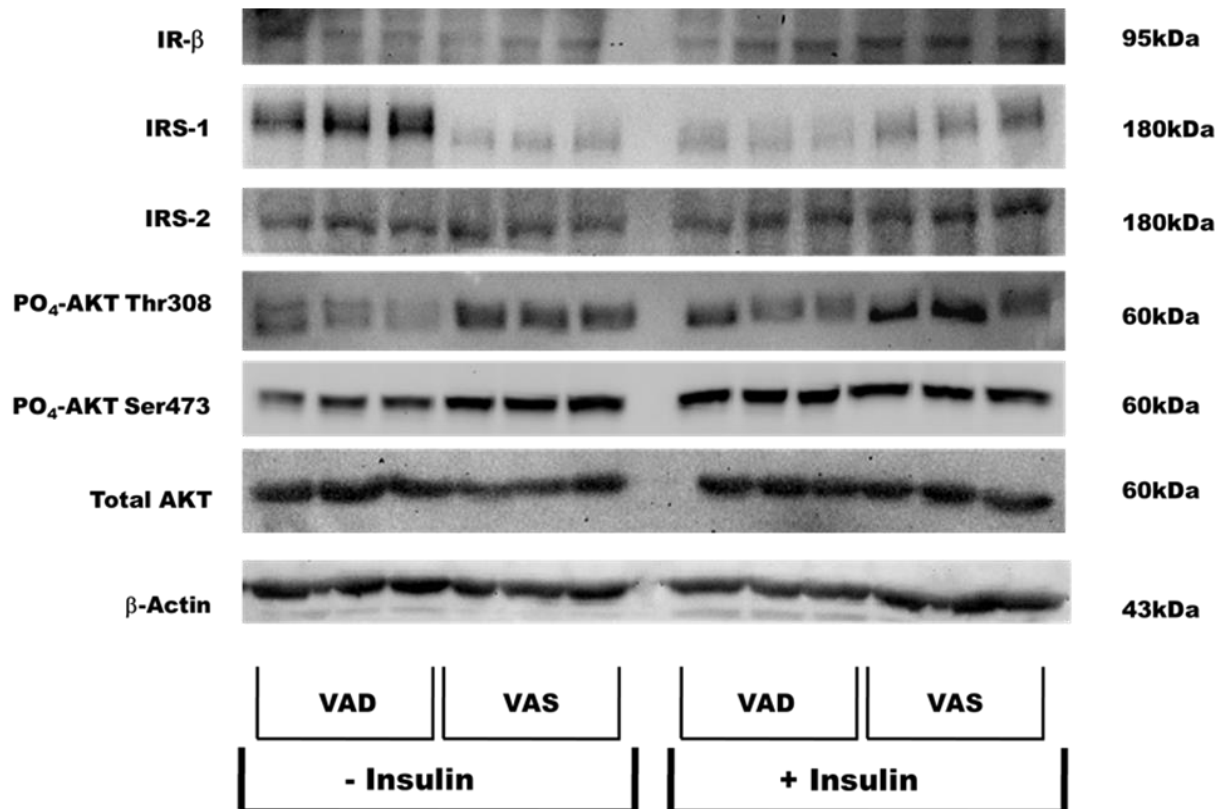


Figure 3.6

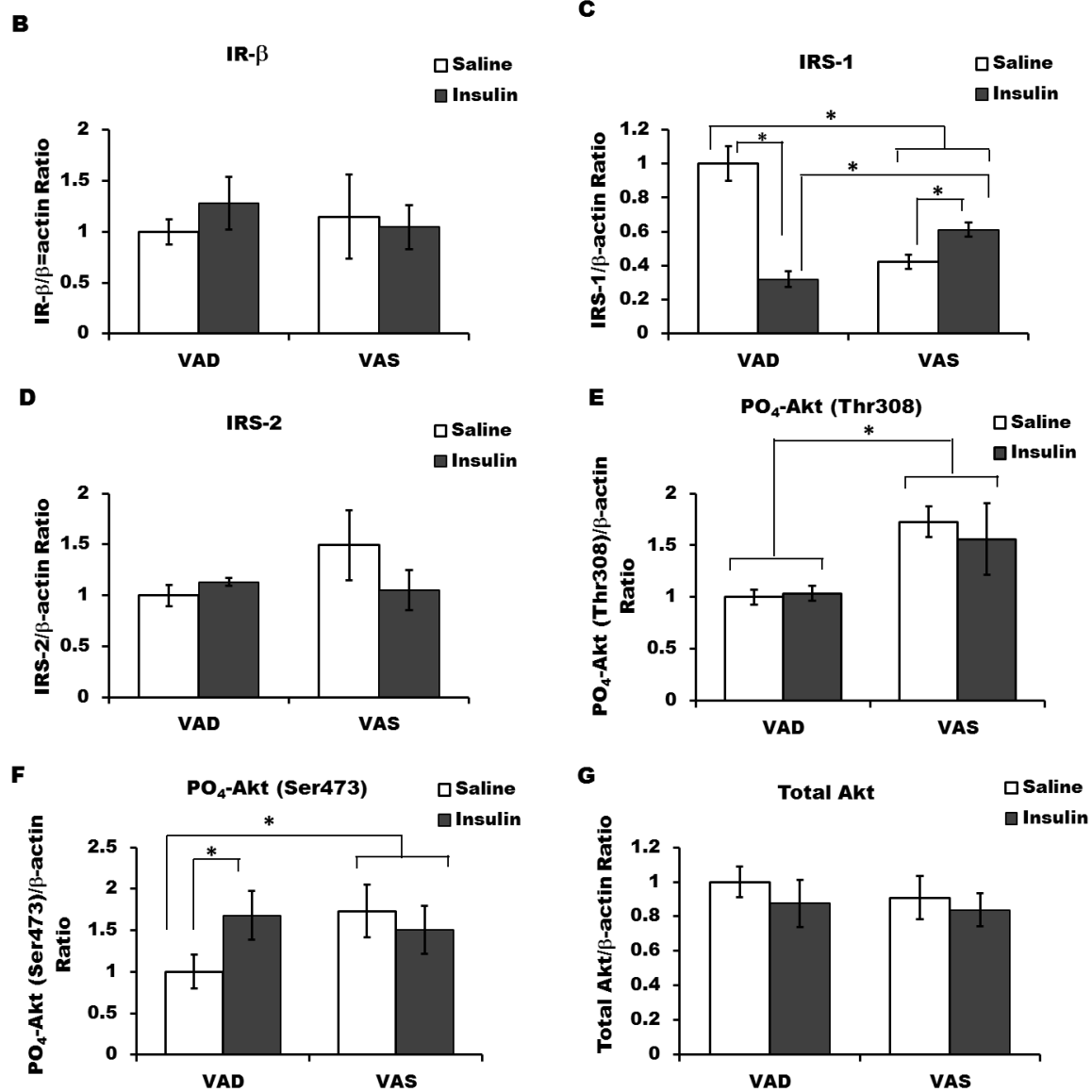


Figure 3.6 Continued

3.7 Expression levels of key proteins in glycogen signaling pathway in gastrocnemius muscle of VAD and VAS STZ-induced diabetic rats treated with saline or insulin

The phosphorylation of GSK-3 α at site Ser21 was significantly increased in the VAD-INS group compared to the saline controls (Figure 3.7A-B). However, total GSK-3 α protein levels were significantly lower in both VAD-INS and VAS-INS than that in VAD-C and VAS-C groups, respectively. The phosphorylation of GSK-3 β at site Ser9 was significantly increased in the VAS-INS group compared to the other 3 groups (Figure 3.7A and 3.7D). Total GSK-3 β protein levels were significantly lower in the VAS-INS group compared to the VAD-INS group (Figure 3.7A and 3.7E). The phosphorylation of GS at site Ser641 was significantly higher in the VAD-C group compared to the other 3 groups (Figure 3.7A and 3.7F). The VAS-INS group displayed significantly lower phosphorylation of GS than the other groups (Figure 3.7A and 3.7F). The VAS-INS group did display significantly higher total GS protein levels compared to the other 3 groups (Figure 3.7A and 3.7G).

3.8 Expression levels of key proteins in glucose uptake and usage pathway in gastrocnemius muscle of VAD and VAS STZ-induced diabetic rats treated with saline or insulin

GLUT1 protein levels was significantly higher in the saline treated animals compared to those who received insulin (Figure 3.8A-B). However, GLUT4 protein levels were significantly increased in the animals that received insulin (VAD-INS and VAS-INS) compared to those who received saline (Figure 3.8A and 3.8C). The protein levels of GAPDH and PDH did not display significant differences between the groups (Figure 3.8A, 3.8D and 3.8G). Pyruvate kinase was

Figure 3.7. Abundance levels of key proteins in glycogen signaling pathway in gastrocnemius muscle in VAD and VAS STZ-induced diabetic rats treated with saline or insulin. A. Protein levels of GS, PO4-GS Ser641, GSK-3 α , PO4-GSK-3 α Ser21, GSK-3 β , PO4-GSK3 β Ser9 and β -actin in VAD or VAS STZ-diabetic ZL rats treated with saline or insulin. B-G. Quantification analysis of PO4-GSK-3 α Ser21, GSK-3 α , PO4-GSK-3 β Ser9, GSK-3 β , PO4-GS Ser641, and GS, respectively. Male ZL rats at weaning were fed a VAS diet or an isocaloric VAD diet. Rats were fasted for 24 hours, and received a single injection of STZ via the tail vein and allowed to have their respective diet ad libitum. Then the VAS or VAD rats with STZ-induced diabetes were treated with saline (control, 100 μ L, intramuscular injection, VAS-C or VAD-C) or insulin (0.5 IU/100 g BM, intramuscular injection, VAS-INS or VAD-INS). Rats were fed ad libitum for 3-4 hours and then euthanized for tissue sample collection. Muscle tissue protein isolation and western blotting procedure was performed as described in Materials and Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis by averaging each group with the VAD-C group arbitrarily set as 1 for control. All p-values ≤ 0.05 ; * indicates statistical significance. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

A

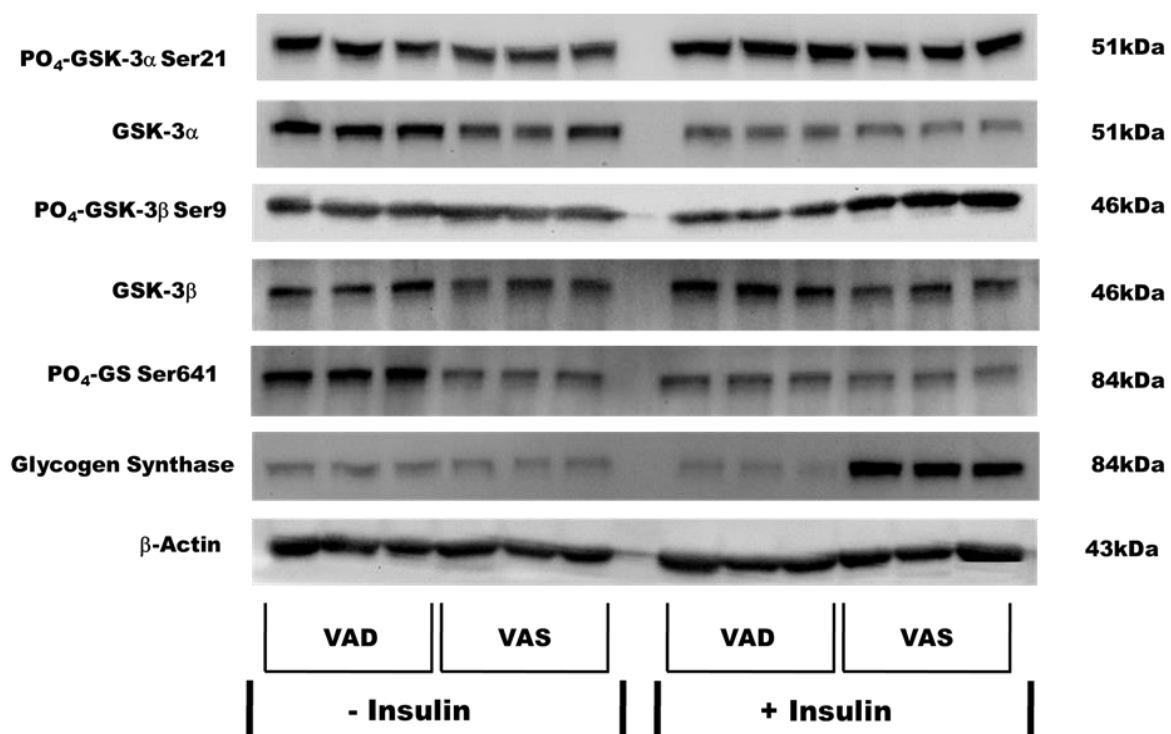


Figure 3.7

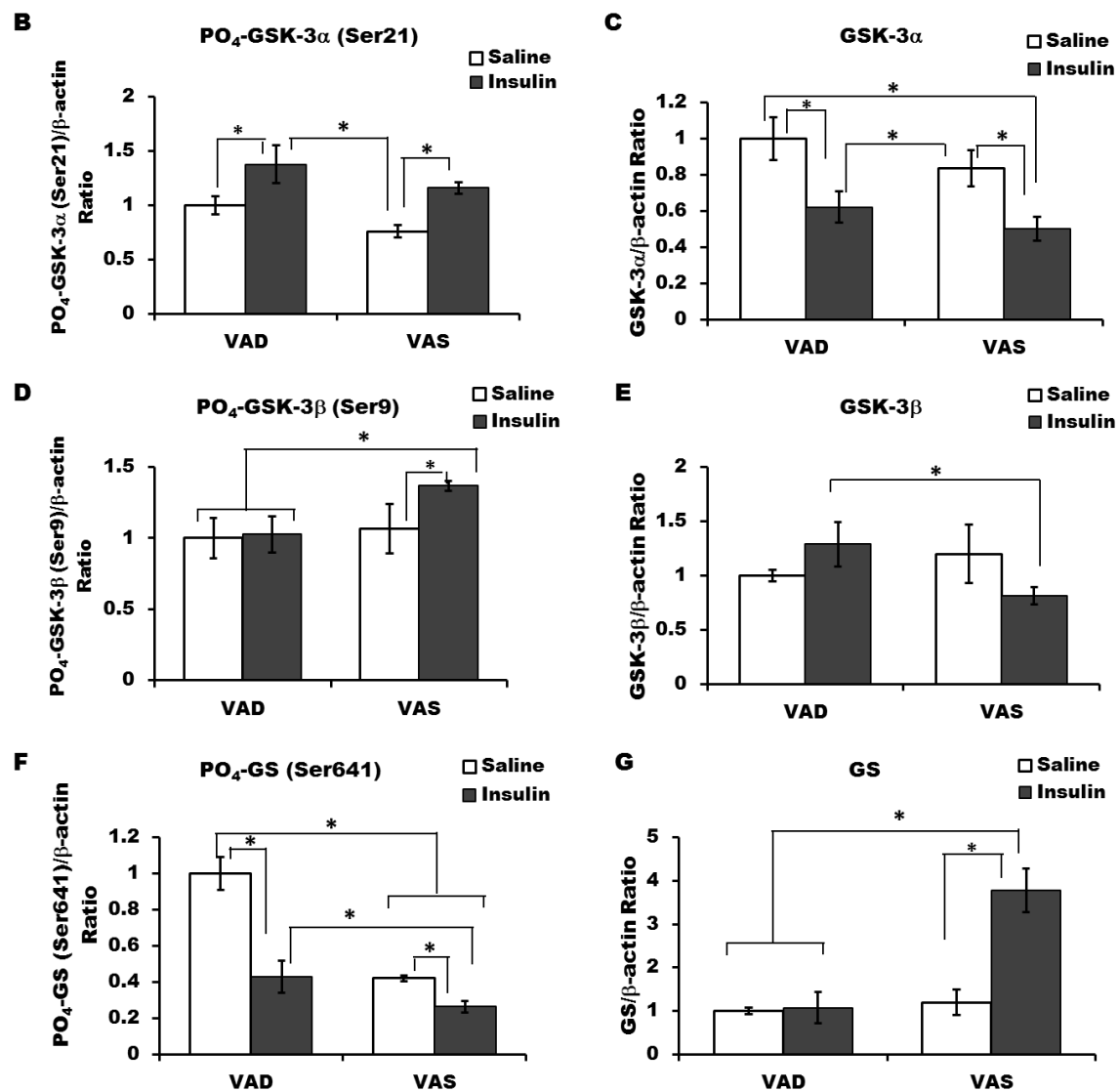


Figure 3.7 Continued

significantly higher in the VAD-C group compared to the other 3 groups (Figure 3.8A and 3.8E). VAS-INS group also displayed higher pyruvate kinase protein levels compared to VAD-INS group (Figure 3.8A and 3.8E). The protein levels of LDH was significantly higher in the VAD groups compared to the VAS groups (Figure 3.8A and 3.8F).

3.9 GLUT1 and GLUT4 protein expression levels in L6 cells treated with RA, LG268, and TTNPB in the presence or absence of insulin for only one hour

To determine if the L6 cells display variations in GLUT1 and GLUT4 protein levels under acute treatment conditions, the cells were treated with 1 μ M each RA, LG268, and TTNPB with and without insulin (10 nM) for one hour following differentiation. GLUT4 protein expression was significantly increased in all conditions in which insulin was used (Figure 3.9A-C). However, GLUT1 protein expression was significantly reduced in all conditions in which insulin was used (Figure 3.9A and 3.9B). The addition of the ligands did not affect GLUT1 protein expression, however, TTNPB led to significant reductions in GLUT4 protein expression compared to control and TTNPB + insulin led to significant reductions compared to insulin (Figure 3.9).

3.10 Changes in GLUT1, GLUT3, GLUT4 and GLUT6 levels in L6 cells treated with RA, LG268, or TTNPB in the presence or absence of insulin for 6 days

A major role of insulin in muscle glucose metabolism is to induce GLUT4 translocation, therefore, we measured whether there was any change of GLUT1 and GLUT4 expression levels in L6 cells treated with RA, LG268, and TTNPB in the absence or presence of insulin for 6 days.

Figure 3.8. Abundance levels of key proteins in glucose uptake and usage pathway in gastrocnemius muscle in VAD and VAS STZ-induced diabetic rats treated with saline or insulin. A. Protein levels of GLUT1, GLUT4, GAPDH, lactate dehydrogenase (LDH), pyruvate dehydrogenase (PDH), pyruvate kinase mutase (PKM $\frac{1}{2}$), and β -actin in VAD or VAS STZ-diabetic ZL rats treated with saline or insulin. B-G. Quantification analysis of GLUT1, GLUT4, GAPDH, PKM $\frac{1}{2}$, LDH, and PDH, respectively. Male ZL rats at weaning were fed a VAS diet or an isocaloric VAD diet. Rats were fasted for 24 hours, and received a single injection of STZ via the tail vein and allowed to have their respective diet ad libitum. Then the VAS or VAD rats with STZ-induced diabetes were treated with saline (control, 100 μ L, intramuscular injection, VAS-C or VAD-C) or insulin (0.5 IU/100 g BM, intramuscular injection, VAS-INS or VAD-INS). Rats were fed ad libitum for 3-4 hours and then euthanized for tissue sample collection. Muscle tissue protein isolation and western blotting procedure was performed as described in Materials and Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis by averaging each group with the VAD-C group arbitrarily set as 1 for control. All p-values ≤ 0.05 ; * indicates statistical significance. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

A

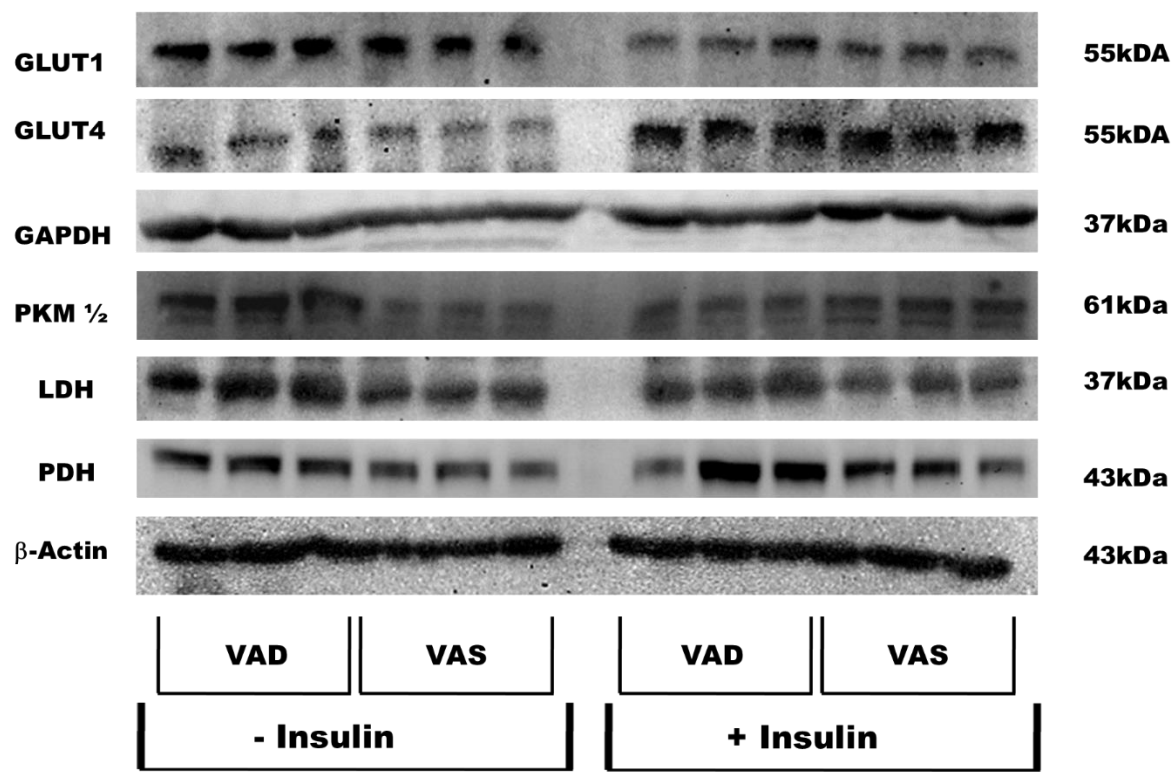


Figure 3.8

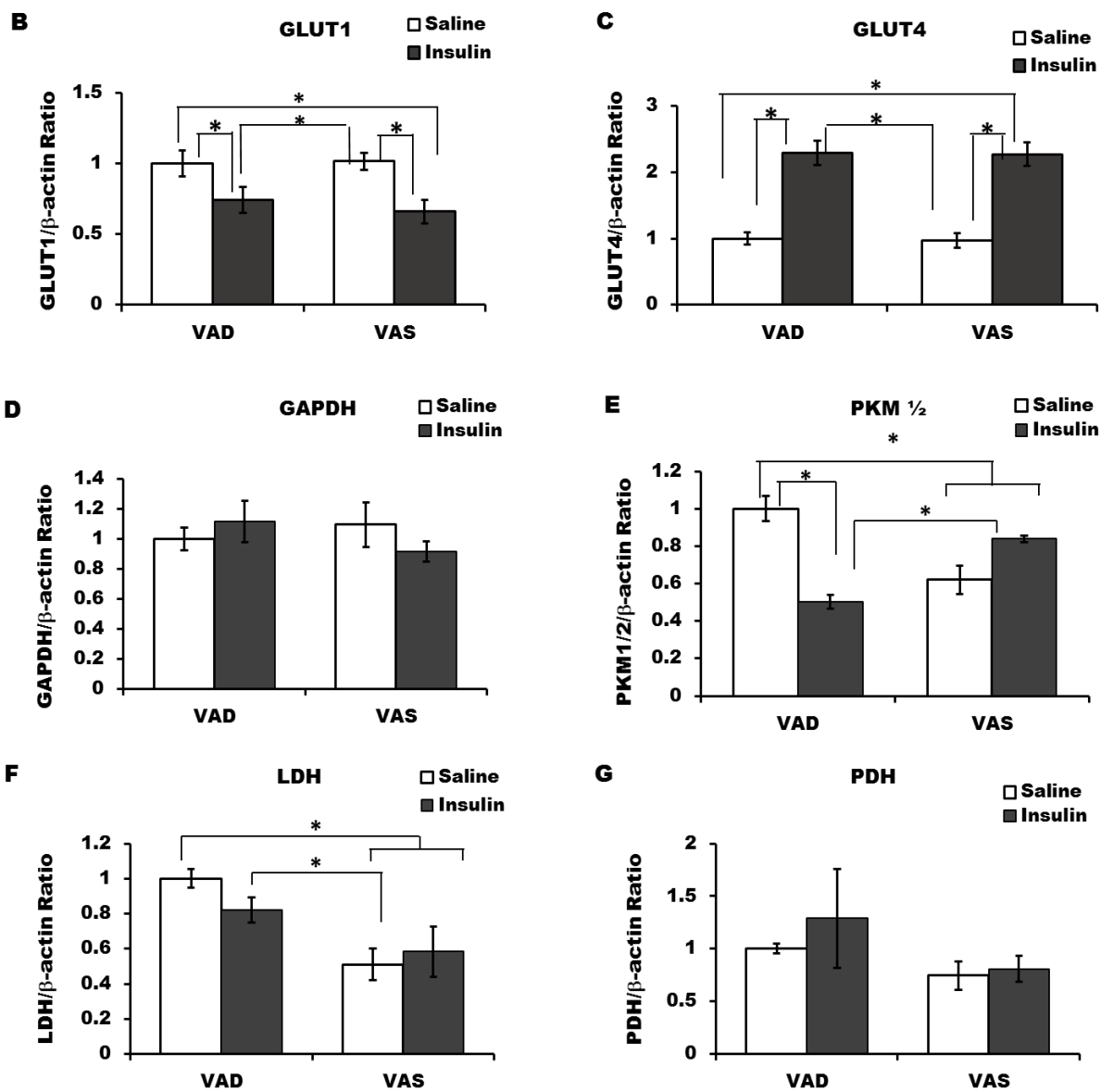
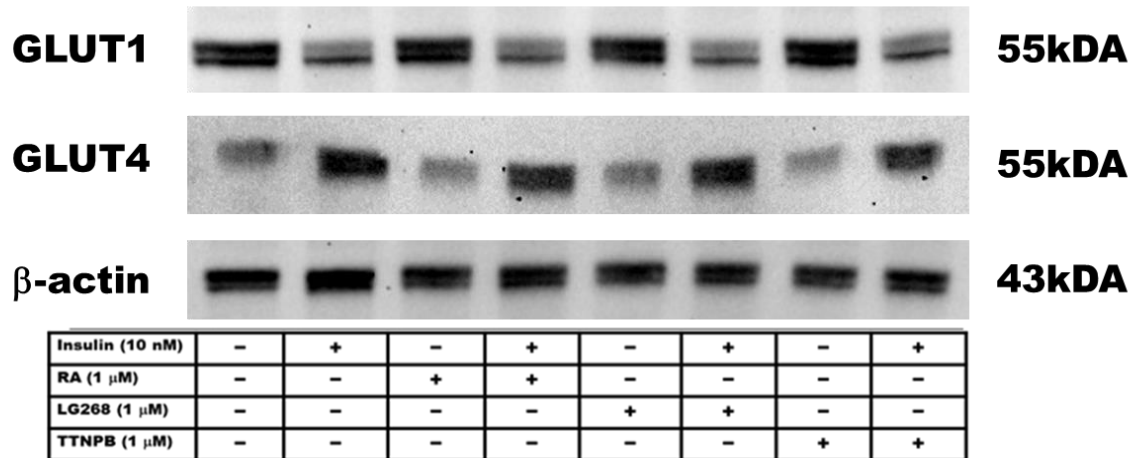


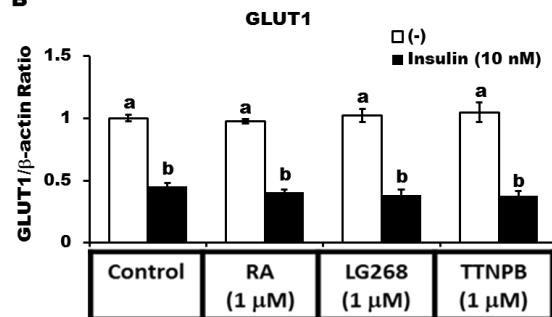
Figure 3.8 Continued

Figure 3.9. GLUT1 and GLUT4 protein levels in L6 cells following one-hour treatment of RA, LG268, or TTNPB in the presence or absence of insulin. A. A representative blot of protein levels of GLUT1, GLUT4, and β -actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin for one hour. B-C. Quantification analysis of GLUT1 and GLUT4, respectively. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS for 6 days. Following differentiation, L6 cells were treated with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin for 1 hour. Following treatment, whole cell lysate was collected as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis with control group set as 1. All p-values ≤ 0.05 ; for (B) $a > b$; for (C) $a > b > c > d$, $a/b > c/d$. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

A



B



C

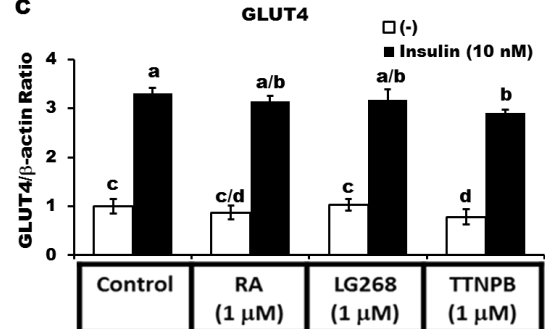


Figure 3.9

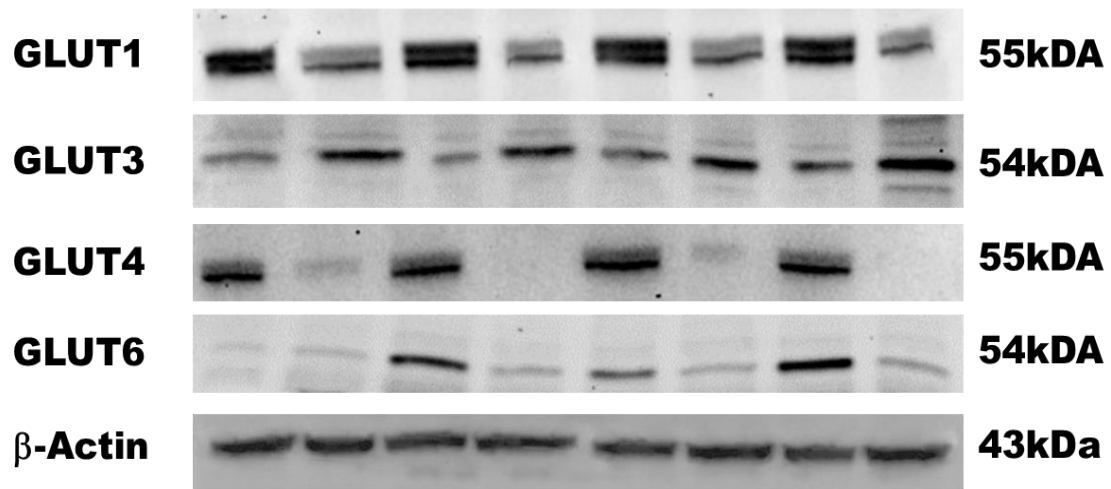
To our surprise and similar to the results shown in Chapter II, the protein expression levels of GLUT1 and GLUT4 were significantly decreased in L6 cells treated with insulin in comparison with the control group (Figure 3.10A-C). None of the ligands alone led to changes in GLUT1 and GLUT4 expression levels. Interestingly, RA + insulin and TTNPB + insulin caused further reduction of GLUT1 and GLUT4 expressions compared to control, insulin, RA, and TTNPB (Figure 3.10A-C), demonstrating the synergistic effect of RA and insulin as well as the possible signaling pathway mediating RA's effects. We also measure the expression levels of GLUT2, 3, 5, 6 and 8 in the same cell samples. GLUT3 protein expression was markedly increased in L6 cells treated with TTNPB + insulin (Figure 3.10A and 3.10D). Insulin alone, RA + insulin and LG268 + insulin significantly increased GLUT3 protein expression compared to control, RA, LG268, and TTNPB (Figure 3.10A and 3.10D). Interestingly, L6 cells treated with RA alone displayed significant reductions in GLUT3 protein levels compared to all treatment conditions (Figure 3.10A and 3.10D). GLUT6 protein expression levels were markedly enhanced with the treatment of L6 cells with RA and TTNPB (Figure 3.10A and 3.10E). RA + insulin, LG268 + insulin, and TTNPB + insulin all significantly increased GLUT6 protein expression compared to control (Figure 3.10A and 3.10E). We could not detect the protein levels of GLUT2, 5 and 8 in the samples.

4. Discussion

Due to skeletal muscle glucose metabolism playing a pivotal role in whole body glucose homeostasis, the aim of our study was to investigate possible mechanisms in which skeletal muscle cells respond to nutritional and hormonal stimuli. It has been thought for a while that the postprandial rise of plasma insulin stimulates glucose uptake in skeletal muscle via GLUT4 translocation, which has been a topic researched extensively as well as the beneficial effects of

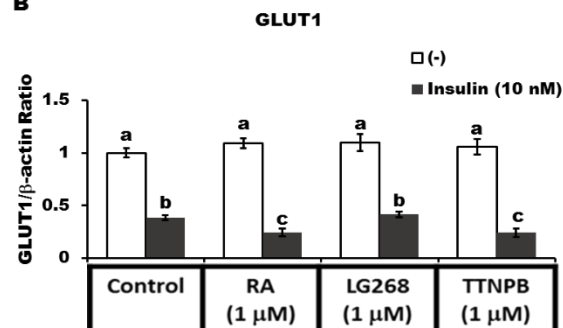
Figure 3.10. Changes in glucose transporter protein levels in L6 cells treated with RA, LG268, or TTNPB in the presence or absence of insulin. A. A representative blot of protein levels of GLUT1, GLUT3, GLUT4, GLUT6 and β -actin in L6 cells treated with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin for 6 days. B-E. Quantification analysis of GLUT1, GLUT4, GLUT3, and GLUT6, respectively. Cells were maintained in 60 mm dishes and were subjected to differentiation with 2% HS (control), insulin (10 nM), RA (1 μ M), RA + insulin, LG268 (1 μ M), LG268 + insulin, TTNPB (1 μ M), TTNPB + insulin. The medium was replaced every 2 days with its respective treatment condition. On day 6 of treatment, whole cell lysate was collected as described in Materials & Methods. Blots were visually detected using ECL Western Blotting Substrate on ChemiDoc Software System. The ratio of the densities of specified proteins to β -Actin in the same treatment group was calculated and used for statistical analysis with control group set as 1. All p-values ≤ 0.05 ; for (B) $a > b$; for (C) $a > b > c$; for (D) $a > b > c > d$; for (E) $a > b > c > d > e$, $c/d > d/e$. One-way ANOVA statistical analysis was performed using SPSS software. Data are presented as means $\pm$ S.E.M (n=3).

A

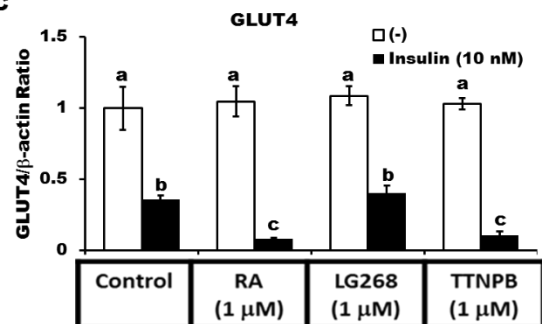


Insulin (10 nM)	-	+	-	+	-	+	-	+
RA (1 μM)	-	-	+	+	-	-	-	-
LG268 (1 μM)	-	-	-	-	+	+	-	-
TTNPB (1 μM)	-	-	-	-	-	-	+	+

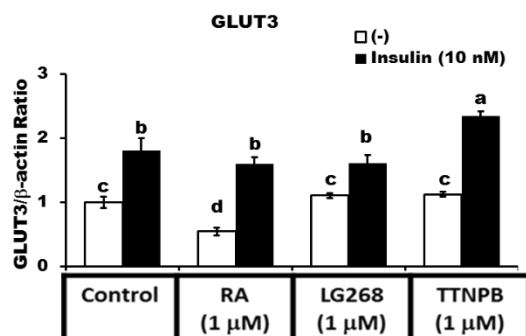
B



C



D



E

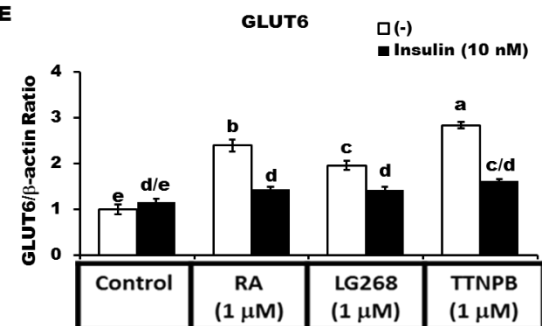


Figure 3.10

physical exercise on skeletal muscle glucose regulation^{224,487,488,507}. There are a multitude of factors that can influence skeletal muscle glucose metabolism such as skeletal muscle fiber type, level of physical exercise, nutrient availability, and hormonal factors, all of which can positively or negatively regulate glucose uptake and utilization in skeletal muscle^{489,508,509}. Within skeletal muscle, studies investigating the effects of VA and its derivatives on glucose regulation have been limited^{376,482}. Due to the obesogenic/high-fat diet society, it is important to have a greater understanding of the mechanisms in which a fat-soluble vitamin such as VA and its derivatives have on skeletal muscle glucose regulation.

The anabolic hormone insulin initiates its action after binding to the insulin receptor, a process that starts an intracellular signaling cascade³⁶². The binding of insulin and its receptor will lead to the phosphorylation and activation of IRS proteins, with IRS-1 often viewed as the primary IRS in skeletal muscle⁴⁰¹. However, we show in Figure 3.1 that RA and TTNPB can actually increase IRS-1 protein levels following 6 days of treatment whereas the addition of insulin to RA led to significant decreases in IRS-1 (Figure 3.1). This could be due to a homeostatic mechanisms initiated by the L6 muscle cells in response to insulin treatment for 6 days. The same effect was not reciprocated in the animal experiment in which STZ-induced diabetic VAD-C and VAS-INS rats had significantly higher levels of IRS-1 compared to the other groups (Figure 3.6). However, the phosphorylation of Akt at Thr308 was increased significantly by the ligands + insulin in L6 cells and in the VAS-C and VAS-INS experiment (Figures 3.1 and 3.6). No significant changes were seen in the phosphorylation Akt at Ser473 in L6 cells. However, phosphorylation at this site was significantly increased in VAS-C, VAD-INS, and VAS-INS rats compared to VAD-C (Figures 3.1 and 3.6). One reason is the different length of treatment time, 6 days for L6 cells and 3-4 hours for diabetic rats. Nevertheless, the results

shown in this study suggest that the presence of VA and its derivatives within skeletal muscle can assist in the activation of Akt upon insulin stimulation.

Within skeletal muscle cells, the insulin-stimulated activation of Akt will lead to inactivation glycogen synthase kinase-3 (GSK-3) by phosphorylating specific sites on the enzyme such as site Ser21 on GSK-3 α and Ser9 on GSK-3 β ^{182,183}. Through this inhibition of GSK-3, GS can be activated^{181,404}. In L6 cells we find that RA + insulin, TTNPB alone, and TTNPB + insulin are capable of increasing the phosphorylation of GSK-3 α (Ser21) compared to the control group (Figure 3.2A-B). A similar effect was seen with the phosphorylation of GSK-3 β (Ser9) in L6 cells with the addition of RA alone capable of significantly increasing phosphorylation (Figure 3.2A and 3.2D). In the VAD diabetic rats, those receiving insulin had significantly higher levels of GSK-3 α phosphorylation compared to those receiving saline (Figure 3.7A-B). Interestingly, the insulin-treated VAD and VAS diabetic rats also displayed significantly lower levels of total GSK-3 α than the saline-treated rats (Figure 3.7A and 3.7C). This suggests that insulin may not only promote the inactivation of GSK-3 α , but also decreases total GSK-3 α levels. We also found that only the VAS-INS group displayed significantly higher levels of GSK-3 β phosphorylation with concomitant decreases in total GSK-3 β protein levels compared to VAD-INS (Figure 3.7A, 3.7D-E), suggesting the critical role of VA status in the GSK-3 β protein expression.

In L6 cells, the phosphorylation of GS at Ser641 was significantly reduced in all conditions in which insulin was used and of the ligands, only RA alone was also capable of significantly reducing GS phosphorylation (Figure 3.2A and 3.2F). The reduced phosphorylation of GS correlated with increases in total GS protein levels with the L6 cells treated with RA +

insulin and TTNPB + insulin having a synergistic effect (Figure 3.2A and 3.2F-G). However, *Gys1* gene expression in L6 cells seems to be driven primarily by the effects of insulin (Figure 3.4A). Interestingly, the L6 cells receiving both LG268 and insulin displayed total GS levels similar to that seen with control (Figure 3.2A and 3.2G). The VAD-C rats displayed significantly higher phosphorylation of GS than the other groups while the VAS-INS displayed even lower levels of phosphorylation than the VAD-INS and VAS-C rats (Figure 3.7A and 3.7F). Again, this significant reduction in GS phosphorylation correlated with significant increases in total GS protein levels in the VAS-INS rats (Figure 3.7A and 3.7F-G). Due to the variations in both the total proteins and their phosphorylated forms under the treatment conditions, the ratio of phosphorylated protein to the respective total protein was not chosen as the presentation format. We decided to use the ratios of those proteins to β -actin as the presentation format as the protein abundance levels of β -actin appeared to be unchanged by the treatment conditions. These results suggest that in the presence of VA and insulin, the activity of GSK-3 is reduced, thus promoting GS protein abundance. Another study using the soleus muscle of 24-month old rats found that an increase in the phosphorylation of GS corresponded with decreases in total GS protein levels⁴⁵⁴. Therefore, it is possible that the phosphorylation state of GS might have altered its stability and in turn impact total GS protein levels. The *in-vitro* GS data was confirmed in measurement of glycogen content as glucose release measures. RA + insulin and TTNPB + insulin both led to significant increases in the amount of glucose released in the L6 cells following six days of treatment (Figure 3.5). These results indicate that under these conditions that there is an increase in glucose storage in the form of glycogen.

Besides being used for glycogen formation, glucose that enters skeletal muscle can be used for energy production and can also be converted to lactate^{510,511}. In the L6 cells we show

that the protein levels GAPDH, PDH, LDH, and pyruvate kinase remain unchanged by all treatment conditions (Figure 3.3). Only the L6 cells treated with RA displayed significantly lower *Ldha* gene expression levels compared to those treated with insulin alone (Figure 3.4B). The VAD and VAS diabetic rats did not display any significant differences in GAPDH or PDH (Figure 3.8A-B and 3.8E). However, the VAD-C group displayed significantly higher levels of pyruvate kinase and LDH compared to the VAS groups, thus further indicating that presence of VA promotes the shuttling of glucose towards the formation of glycogen (Figure 3.8A and 3.8C-D).

The peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator-1alpha (PGC-1 α) and PGC-1 β are nuclear encoded transcriptional coactivators that have been shown to play a role in skeletal muscle metabolism and function⁵¹²⁻⁵¹⁴. We show in L6 cells, that the expression levels of the gene encoding PGC-1 α (*Ppargc1a*) are only induced by TTNPB + insulin after 6 days of treatment (Figure 3.4D). However, the expression levels of the gene encoding PGC-1 β (*Ppargc1b*) are increased by insulin, RA + insulin, and TTNPB + insulin, suggesting that insulin is driving the expression of PGC-1 β in L6 muscle cells (Figure 3.4E).

GLUT4 is the glucose transporter commonly referred to as the insulin-stimulated glucose transporter in skeletal muscle whereas GLUT1 is thought to regulate skeletal muscle glucose uptake during basal conditions^{199,498}. Both GLUT1 and GLUT4 protein levels have been shown to be decreased in the skeletal muscle of individuals with T2DM^{200,515}. Even though GLUT4 is the insulin-responsive glucose transporter, its gene expression levels in the L6 cells also did not display any significant changes by any of the long-term (6 days) treatment conditions (Figure 3.4C). It was interesting to find that following 6 days of treatment, RA + insulin and TTNPB + insulin promoted significant reductions in both GLUT4 and GLUT1 protein levels compared to

all other treatment conditions (Figure 3.10). Insulin alone and LG268 + insulin also produced significant reductions compared to control, RA, LG268, and TTNPB (Figure 3.10). This could imply that L6 cells respond to pro-longed insulin and RA treatment with a homeostatic mechanism to decrease glucose uptake by decreasing GLUT4 and GLUT1 protein levels. We demonstrated here that the expression levels of IR- β in L6 cells were not different among treatment groups. These results indicated that our total cell lysate included membrane fraction. It is not possible that the decreases in GLUT1 and GLUT4 protein levels could be the loss of the plasma membrane fraction during whole-cell lysate collection. We found that GLUT3 and GLUT6 protein levels can also be regulated by RA and insulin within the L6 cells. GLUT3 protein expression levels were significantly increased with all conditions in which insulin was used, but showed the greatest increase with TTNPB + insulin (Figure 3.10). GLUT6, on the other hand, was found to be markedly increased by RA and TTNPB (Figure 3.10). As far as we know, this is the first time that this phenomenon is reported. While limited research has been conducted on the kinetics and potential stimuli of GLUT6, it has been shown previously in L6 cells that GLUT3 protein expression can be increased using triiodothyronine as well as by insulin-like growth factor-1 (IGF-1)^{217,516,517}. The underlying mechanism regulating the glucose transport under these conditions deserves to be investigated in more detail. Short-term treatment of L6 cells with insulin for one hour produced significant increases in GLUT4 protein levels (Figure 3.9). This indicates that the L6 cells are indeed responsive to insulin and that the regulation of GLUT4 may be time dependent.

With the significant changes seen in the protein expression levels of key enzymes in the glycogen synthesis pathway in both *in-vitro* and *in-vivo* coupled with no changes seen in the protein expression levels of GAPDH, LDH, PDH, or pyruvate kinase in the L6 cells while the

VAD-C rats displayed significant increases in LDH and pyruvate kinase protein levels, our results demonstrate that when skeletal muscle is exposed to both VA and insulin, they work cooperatively to regulate glucose metabolism through increase of GS expression, which may have enhanced the glucose utilization via further metabolism to storage. This can be of importance in diverting glucose from lactic acid production to glycogen formation in individuals that suffer from complications associated with diabetes such as myopathy and/or neuropathy. Furthermore, we demonstrate that the RAR signaling pathway is in part how RA-mediate its effects on L6 cells glucose utilization and GS regulation (Figure 3.11). We propose that through the binding of RA with RARs, it will work synergistically with insulin signaling to favor the formation of glycogen by inhibiting the activity of GSKs via their phosphorylation, thus enhancing glycogen synthase to promote glycogen formation. In a study that utilized mice with muscle-specific knockout of GLUT4, the mice displayed significant decreases in IRS-1/Akt signaling while having increases in GS activity and glycogen content under basal (non-insulin dependent) conditions²³¹. The results shown here indicate that the mechanisms in which VA and insulin elicit their effects are complicated and require more study.

5. Conclusion

Due to the regulation of skeletal muscle glucose metabolism being able to have an effect on whole body glucose homeostasis, it is important to understand the mechanisms in which hormonal and nutritional stimuli interact within skeletal muscle. Here, we demonstrate that VA and RA can interact with insulin to promote GS protein abundance within skeletal muscle *in-vivo* and *in-vitro*. These findings could lead to the development of new and improved therapies for diseases with altered glucose metabolism.

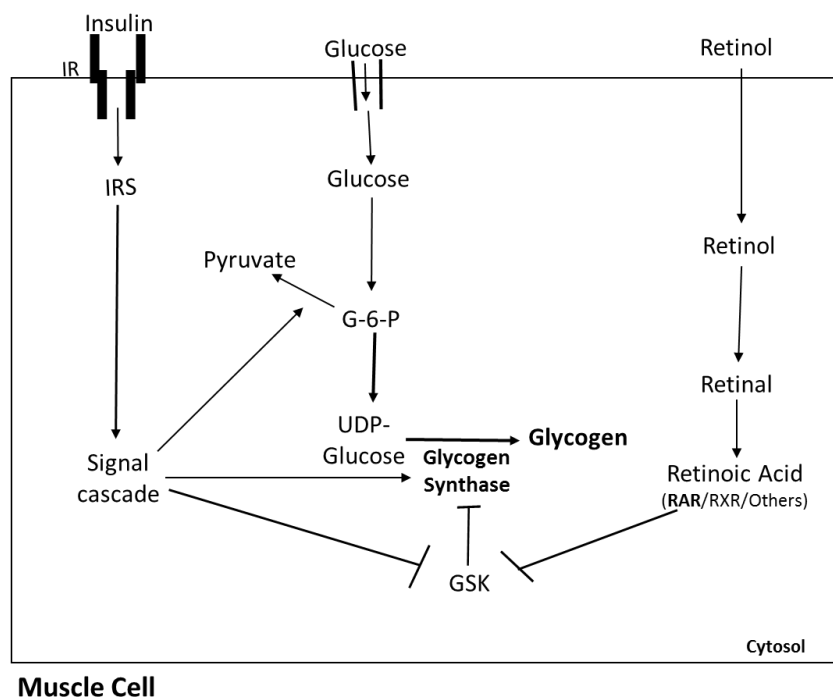


Figure 3.11. Schematic representation of proposed mechanisms involved in the long-term presence of RA and insulin effecting glucose utilization in L6 muscle cells. Upon insulin binding to the insulin receptor there is activation of IRS proteins leading to a signaling cascade. Insulin signaling is involved in the formation of glycogen by inhibiting GSK and by stimulating the activity of GS. We propose that through the binding of RA with RAR, it will work synergistically with insulin signaling to favor the formation of glycogen by inhibiting GSK, thus enhancing glycogen synthase. Abbreviations: G-6-P, glucose-6-phosphate; GS, glycogen synthase; GSK, glycogen synthase kinase; IR, insulin receptor; IRS, insulin receptor substrate; RAR, retinoic acid receptor; RXR, retinoid-X-receptor; UDP, uridine diphosphate.

CHAPTER IV. CONCLUSIONS AND FUTURE DIRECTIONS

1. Conclusion

In this dissertation, we have shown that vitamin A (VA) derivative, retinoic acid (RA), can synergize with insulin to induce the expression of glycogen synthase (GS) in skeletal muscle in both *in-vitro* and *in-vivo* models systems. Specifically, we used rat L6 skeletal muscle cells and skeletal muscle from VA deficient (VAD) and VA sufficient (VAS) STZ-induced diabetic rats that received either saline (VAD-C and VAS-C) or insulin (VAD-INS and VAS-INS) injections. The research results shown in this dissertation probe the mechanisms underlying two study questions: (1) How does RA alone and in conjunction with insulin affect glucose metabolism in L6 skeletal muscle cells? (2) What are the glucose-derived end product(s) associated with the combination of VA and insulin in skeletal muscle cells? The answers to these two questions are delineated in Chapters II and III, which can provide insights into the mechanisms for the development of skeletal muscle insulin resistance and metabolic diseases as well as possible therapeutic interventions.

In Chapter II, we investigated the effects of retinoids including RA, retinal (RAL), and retinol (ROL) on rat L6 skeletal muscle cell glucose usage, and obtained the following results. First, the changes in medium pH and glucose levels with the retinoids mimicked what you may expect with the accelerated or enhanced glucose metabolism. ROL did not produce any significant changes in medium pH and glucose levels. However, RAL led to significant reductions in medium pH and glucose levels after 10 days after treatment began, suggesting possible conversion of RAL to RA. The RA treated L6 cells display significant reductions in medium pH and glucose levels as early as 4 days after treatment began. This indicates that RA exerts the biological activity within skeletal muscle. Secondly, when used in combination, RA and insulin had a synergistic effect in reducing medium pH and glucose levels. Third, the

retinoic acid receptor (RAR) specific agonist TTNPB mimicked the responses seen with RA alone and with insulin, suggesting that the RA-mediated effect is by activation of the RAR nuclear receptor. Fourth, L6 cells incubated in media with increasing concentrations of glucose (15 and 25 mM vs. 5 mM glucose) started displaying reductions in medium pH and glucose levels after 2 days of treatment and this trend continued through day 6. These results suggest that the reductions in medium pH and glucose levels are due to the muscle cells taking in the extracellular glucose for either storage or further metabolism. Fifth, following 6 days of treatment, insulin alone and RA + insulin promoted reductions in both GLUT1 and GLUT4 protein levels. These results could be due to a homeostatic mechanism within these cells in response to pro-longed insulin treatment. It seems that the presence of RA decreased the insulin-mediated reduction of GLUT4 even further to almost disappearance. Collectively, these results demonstrate that there is an elevation of glucose utilization in rat L6 skeletal muscle cells when treated with both RA and insulin, with the RA-mediated effect being due to the activation of the RAR signaling pathway.

In Chapter III, we investigated the potential mechanism of the RA and insulin-mediated enhancement of glucose usage through investigation of the effects of VA and insulin on key proteins involved in insulin signaling, glucose usage, and glycogen metabolism in both L6 skeletal muscle cells and in the gastrocnemius muscle of VAS and VAD STZ-induced diabetic rats. First, the phosphorylation of Akt at Thr308 was increased significantly by the ligands + insulin in L6 cells and in the gastrocnemius muscle of VAS-diabetic rats treated with and without insulin. The levels of Akt phosphorylation at Ser473 were significantly increased in VAS-C, VAD-INS, and VAS-INS rats compared to VAD-C. However, no change of Akt phosphorylation at Ser 473 was observed in L6 cells with any of the treatment conditions. This

discrepancy may be due to the different length of treatment time, 6 days for L6 cells versus 3-4 hours for rats. Secondly, we found that the presence of VA and insulin can have a profound effect on the regulation of key proteins involved in glycogen signaling. Specifically, L6 cells treated with RA + insulin, TTNPB alone, and TTNPB + insulin displayed significant increases in the phosphorylation of GSK-3 α (Ser21) compared to the control group with a similar effect seen in the phosphorylation of GSK-3 β (Ser9) with the addition of RA alone capable of significantly increasing phosphorylation. Both, the VAD-INS and VAS-INS diabetic rats displayed significantly higher levels of glycogen synthase kinase-3 α (GSK-3 α) phosphorylation concomitantly with significantly lower levels of total GSK-3 α compared to the saline-treated rats. These results suggest that insulin may promote both the inactivation of GSK-3 α as well as in decreases total GSK-3 α protein levels. The VAS-INS group displayed significantly higher levels of GSK-3 β phosphorylation with concomitant decreases in total GSK-3 β protein levels compared to that of VAD-INS, which suggests that VA status is of importance in order for insulin to mediate the same effects seen in GSK-3 β protein expression as with GSK-3 α . In L6 cells, the treatment of RA + insulin and TTNPB + insulin had a synergistic effect leading to significant increases in total glycogen synthase (GS) protein levels that correlated with significant reductions in the phosphorylation of GS at Ser641. Similarly, rats in the VAS-INS group displayed significantly lower levels of GS phosphorylation compared to the VAD-C, VAD-INS, and VAS-C groups which correlated with significant increases in total GS protein levels.

We also show in Chapter III that proteins in the glucose usage pathway, including lactate dehydrogenase (LDH) and pyruvate kinase, remain unchanged by all treatment conditions in L6 cells. However, the VAD-C group displayed significantly higher levels of pyruvate kinase and

LDH compared to the VAS groups, further suggesting that presence of VA promotes the shuttling of glucose towards the formation of glycogen. Finally, RA + insulin and TTNPB + insulin had a synergistic effect in enhancing glycogen content in L6 skeletal muscle cells compared to all other treatment conditions. These results further support the western blot data indicating that in skeletal muscle VA and insulin work cooperatively to regulate glucose metabolism through the regulation of proteins involved in glycogen synthesis to promote glucose storage in the form of glycogen. Furthermore, we demonstrate that the RAR signaling pathway is in part how RA-mediate its effects on L6 cells glucose utilization and GS regulation. We also have shown that this effect is not dependent upon the enhanced protein expression of GLUT4 with GLUT4 protein levels being decreased by the treatments of RA + insulin and TTNPB + insulin in L6 cells. However, GLUT3 protein levels were significantly increased by insulin, RA + insulin, LG268 + insulin, and TTNPB + insulin with TTNPB + insulin having the greatest effect. GLUT6 protein levels were significantly higher in L6 cells treated with RA, LG268, and TTNPB with TTNPB having the greatest effect. Therefore, these results suggest that multiple glucose transport systems could be working cooperatively for glucose uptake under these treatment conditions.

2. Future Directions

Even though the results presented in this dissertation have provided ample evidence that VA and insulin work cooperatively in skeletal muscle to enhance glucose utilization, future research questions (see Figure 4.1) can be extracted from the findings to help extend our understandings of the development of muscle-related insulin resistance, myopathies, and various metabolic diseases.

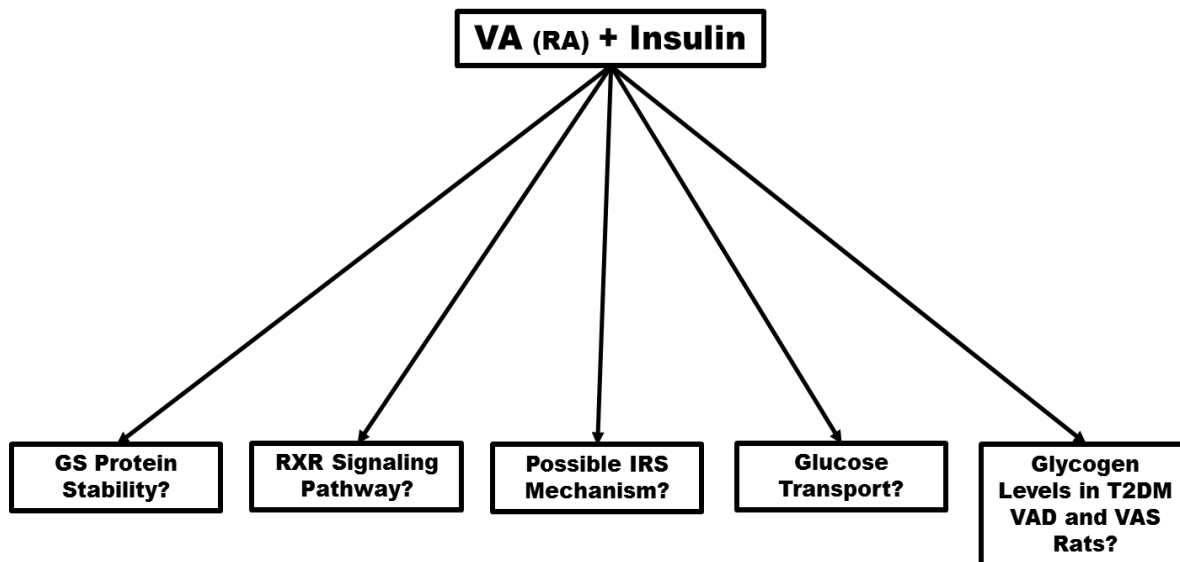


Figure 4.1 Proposed future experiments to be performed. See Ch.4 section 2 for description.

Abbreviations: GS, glycogen synthase; IRS, insulin receptor substrate; RA, retinoic acid; RXR, retinoid-X-receptor; T2DM, type 2 diabetes mellitus; VA, vitamin A; VAD, vitamin A deficient; VAS, vitamin A sufficient.

2.1 Do RA and insulin work cooperatively to enhance GS protein stability in rat L6 skeletal muscle cells?

In this dissertation, we show that GS is regulated at the protein level by RA and insulin in L6 cells, however, *Gys1* gene expression is driven primarily by the actions of insulin. This increased expression level of protein could be due to an increase in protein stability due to the treatment with RA and insulin. Therefore, it would be worth performing protein stability experiments such as a cycloheximide chase assay and western blotting to determine if the combination of RA and insulin enhance GS protein stability through increasing translation rates. If an increase in translation is not the case, then RA and insulin could be mediating their effects through lowering degradation rates, therefore, experiments could be performed that focused on measuring protein degradation.

2.2 What role does retinoid-X-receptor (RXR) play in rat L6 skeletal muscle cell glucose utilization?

In Chapters II, we have shown that the RXR agonist LG268 alone and in combination with insulin had no effect on glucose utilization in the L6 cells in regards to changes in medium pH and glucose levels compared to control and insulin alone. In Chapter III we show that LG268 actually abrogates the effects of insulin on total GS protein levels in L6 cells and this effect was also seen in the glycogen measurements. These results suggest that treatment of L6 cells with LG268 does not favor glycogen formation, therefore, other pathways that could use glucose such as fatty acid synthesis for example, should be investigated in more detail.

2.3 How does insulin mediate its effect on rat L6 skeletal muscle cell glucose utilization if the expression level of insulin receptor substrate-1 (IRS-1) is decreased?

One interesting finding in Chapter III was that IRS-1 protein levels were significantly reduced in L6 cells treated with RA + insulin, whereas RA alone and TTNPB alone led to significant increases in IRS-1 protein levels. We believe that this could be a homeostatic mechanism to pro-longed insulin treatment in which the cell starts limiting its typical response to insulin by decreasing IRS-1 protein levels. However, as shown in Chapter two, insulin in part is mediating the utilization of glucose within the L6 cells. In Chapter three, we also show no change in IRS-2 protein levels with insulin treatment alone in the L6 cells, however, RA and RA + insulin led to significant increases in IRS-2 protein levels. Therefore, it is worth investigating if other IRS's or membrane associated signaling proteins are contributing to the insulin-mediated effect. Since RA is capable of increasing IRS-1 and IRS-2 protein levels, it would also be interesting to determine the mechanism of this induction. Therefore, protein stability assay and reporter gene assay may be used to explore the potential mechanisms. It remains to be determined whether there are RARE sequences located within the promoters of IRS-1 and IRS-2 genes, and if so, which sequences are required for this RA-mediated effect.

2.4 How is glucose transported into the L6 muscle cell with pro-longed treatment of insulin and RA?

Similar to what was seen with IRS-1, GLUT1 and GLUT4 protein levels were significantly reduced in response to RA + insulin as well as insulin alone following six days of treatment in the L6 cells. Again, we believe that this is a homeostatic mechanism initiated by the

L6 cells to limit glucose uptake. It has been shown in mice that muscle-specific knockout of GLUT4 led to significant increases in skeletal muscle GS activity and glycogen content while significantly decreasing muscle glucose uptake²³¹. These results agree with those presented in this dissertation, therefore, there may be another glucose transporting system that also plays a role in skeletal muscle glucose uptake. We show in this dissertation that both GLUT3 and GLUT6 can possibly play a role in the glucose uptake and usage in L6 cells when RA and insulin are used for pro-longed periods, however, we could not detect GLUT2, GLUT5, or GLUT8. Western blot assays can be performed on more of the known glucose transporters (GLUTs) to determine if any has higher protein expression with the treatment conditions used in this dissertation. Also, it has been shown that vitamin C can be transported into cells by a sodium vitamin C dependent (SVCT) transporter as well as GLUTs such as GLUT1, GLUT3, and GLUT4, therefore, it is possible that glucose is using a nontraditional GLUT under these treatment conditions²²⁰.

2.5 Does VA status and insulin promote glycogen storage in the skeletal muscle of rats with type 2 diabetes mellitus (T2DM)?

The prolonged insulin treatment in the *in-vitro* model system in this dissertation resembled that more of a T2DM state, while the *in-vivo* model system was the type 1 diabetes mellitus (T1DM) state. Therefore, further studies in our lab can be performed using VAD and VAS rats still receiving saline or insulin injections, but with diabetes induced to resemble that of T2DM to determine if these animals display similar results in regards to GS phosphorylation and total GS protein levels. Furthermore, glycogen content within the animals can also be directly measured. It would also be interesting to use both lean and obese animal model systems to

determine if adiposity influences the effects seen with VA status and insulin on skeletal muscle glucose utilization and metabolism.

3. Summary

The work presented in this dissertation lays the foundation and provides evidence that there are possible benefits in studying the role of VA in skeletal muscle glucose metabolism. There is much still being discovered regarding glucose metabolism in skeletal muscle. More in-depth studies performed both *in-vitro* and *in-vivo* are needed to grasp a full understanding of the regulation of skeletal muscle glucose metabolism and glycogen homeostasis. With a greater understanding, possible therapeutic benefits and intervention points could arise for individuals suffering from a variety of metabolic diseases.

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APPENDIX

Table 3.1 List of real-time PCR primers used in this dissertation

Protein	Gene Symbol	Direction	Sequence
Glycogen Synthase (GS)	<i>Gys1</i>	Forward Reverse	5' TTTTCCTTGGTCCAGTGTCC 3' 3' CGGTGGGCTCACTTTTGTAT 5'
Lactate Dehydrogenase (LDH)	<i>Ldha</i>	Forward Reverse	5' CCGTTACCTGATGGGAGAAA 3' 3' ACGTTCACACCACTCCACAC 5'
Glucose Transporter Type 4 (GLUT4)	<i>Slc2a4</i>	Forward Reverse	5' TCAGGCATCAATGCTGTTTTCTAC 3' 3' TCTACTAAGAGCACCGAGACCAAC 5'
Peroxisome Proliferator-Activated Receptor Gamma, Coactivator-1 Alpha (PGC-1 α)	<i>Ppargc1a</i>	Forward Reverse	5' CGCAACATGCTCAAGCCA 3' 3' TTAGGCCTGCAGTTCCAGAGAG 5'
Peroxisome Proliferator-Activated Receptor Gamma, Coactivator-1 Beta (PGC-1 β)	<i>Ppargc1b</i>	Forward Reverse	5' AGAAGCTCCTCCTGGCCACATC 3' 3' TGTACAAGGCCGCTCTGAGAC 5'

VITA

Matthew Ray Goff was born in Pikeville, KY on June 24th, 1986 to Dorbie and Donna Goff. He received his elementary education at Millard Elementary school and his middle school education at Millard Jr. High school in Millard, KY. He obtained his high school diploma in 2005 from East Ridge High school in Lick Creek, KY. He obtained his Associates of Science degree in 2008 from Pellissippi State Technical Community College in Knoxville, TN. He then pursued a dual Bachelors degree in Exercise Science and Nutrition at the University of Tennessee-Knoxville, and obtained those degrees in 2011 with a cumulative GPA of 3.93. He was accepted to the PhD program in the department of Nutrition at UTK and began his graduate student career in the Fall of 2011 under the tutelage of Dr. James Jason Collier to investigate the cellular mechanisms involved in the pathogenesis of Type 1 Diabetes Mellitus. In the Fall of 2013 he began working under the instruction of Dr. Guoxun Chen in which he began his investigation of the effects of retinoids on muscle metabolism. During his time as a graduate student at UTK, Matthew served as a graduate teaching assistant for Nutrition 311 (Physiological Chemistry), Nutrition 313 (Vitamins and Minerals), and Nutrition 100 (Introductory Nutrition). Also, during his time as a graduate student Matthew married Jordan Lynne Pierson on May 12th, 2012 and two years later Jordan gave birth to a beautiful baby girl, Audrey Grace Goff, on July 8th, 2014. Matthew defended his dissertation on July 14th, 2016.