University of Alberta

Effects of α-tocopherol supplementation on dexamethasone-induced insulin resistance

by

Deon Brendan Williams

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

Fall 2010
Edmonton, Alberta

Examining Committee

David C. Wright, Agricultural, Food and Nutritional Science

Catherine C. Field, Agricultural, Food and Nutritional Science

Normand Boulé, Physical Education and Recreation

Abstract

This study aimed to examine potential mechanisms for glucocorticoid (GC)-induced decreases in glucose clearance, and to determine if a reduction in oxidative stress load via dietary pre-treatment with an antioxidant-rich diet has a positive net effect on glucose tolerance following a sub-chronic treatment with the GC analogue dexamethasone (DEX). Rats fed a diet supplemented with 700IU of α-tocopherol for two weeks had improved glucose clearance after five days of DEX-treatment relative to unsupplemented rats as well as decreased markers of oxidative stress. Following an intraperitoneal bolus of insulin, phosphorylation of AMP activated protein kinase (AMPK) was preserved in the supplemented groups despite no significant differences in upstream insulin signalling cascade intermediates between DEX-treated groups. This was corroborated by a similar increase ($p<0.05$) in phosphorylation of the downstream AMPK substrate acetyl CoA carboxylase. This study demonstrated that α-tocopherol supplementation can attenuate GC-induced decreases in glucose clearance in an AMPK-dependent manner.

Acknowledgements

Since the moment I arrived at the University of Alberta, my education has not stopped. For this, I'd like to extend my deep thanks to my supervisor, Dr. David Wright, for his amazing (and probably demanding) job of keeping me on the right track and for teaching me everything that I needed to know to tackle the numerous academic challenges that arose over my time in the lab. I know he's always had my best interests at heart, helping me figure out the rest of my academic and career goals through numerous reference letters and conversations.

I also need to thank the rest of my committee members for their patience and advice. To Dr. Catherine Field, thank you not only for your project assistance, but also for your guidance and immense support of my future goals. To Dr. Normand Boulé, your zen-like personality is a gift in an otherwise fast-paced academic experience, so thank you for helping make this experience an enjoyable one.

In case I didn't make it known during the course of my time here, I owe a huge thank you to Susan Goruk, Paige Sorochan, Abha Dunichand-Hoedl, Dr. Bruce Frier, Lindsey Sutherland and the rest of the Wright lab (past and present) for their assistance, teaching and patience in the lab. Whether it's in or out of the lab, I have to thank my friends that I've made here. You've all been amazing in your own way, and without the support, energy and enthusiasm that I've received from you, lab life and graduate school wouldn't have been the same. Being surrounded by great people makes for great experiences, and this has not been an exception.

Finally I'd like to give my thanks to both the Alberta Diabetes Foundation and the Muttart Diabetes Research and Training Centre for their incredible funding support which helped this project through.

Table of Contents

List of Tables and Figures

List of Abbreviations

ACC	Acetyl-CoA carboxylase
AICAR	Aminoimidazole carboxamide ribonucleotide
AMPK	AMP activated protein kinase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
CAMK	Calcium/calmodulin-dependent protein kinase
CCAC	Canadian Council on Animal Care
CV	Coefficient of variance
DEX	Dexamethasone
DTNB	5,5’-dithiobis-2-nitrobenzoic acid
ECL	Enhanced chemiluminescence
EDL	Extensor digitorum longus
ESR	Electron spin resonance
GAP	GTPase-activating protein
GC	Glucocorticoid
GPD	Guanosine diphosphate
GLUT	Glucose transporter
GPx	Glutathione peroxidase
Grb-2	Growth factor receptor-bound protein-2
GSH	Glutathione
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate

HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High performance liquid chromatography
IKK	Inhibitor of κB kinase
ISI	Insulin sensitivity index
i.p	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IRAP	Insulin receptor aminopeptidase
IRS	Insulin receptor substrate
IU	International units
JNK	c-Jun N-terminal kinase
LSD	Least significant difference
MAPK	Mitogen activated protein kinase
MPA	Metaphosphoric acid
M2VP	1-methyl-2-vinyl-pyridium trifluoromethane sulfonate
mTOR	Mammalian target of rapamycin
NAPDH	Nicotinamide adenine dinucleotide phosphate
O-GlcNac	O-linked N-acetylglucosamine monosaccharides
PBS	Phosphate-buffered saline
PDK-1	Phosphoinositide dependent protein kinase
PH	Pleckstrin homology
PI3K	Phosphoinositide-3 kinase
PIKfyve	Phosphoinositide kinase, FYVE finger containing protein
PIP-2	Phosphoinositol-4,5-bisphophosphate
PIP-3	Phosphoinositol-3,4,5-triphosphate

PKB	Protein kinase B
PKC	Protein kinase C
PTB	Protein tyrosine binding
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHP	SH-2 domain-containing protein-tyrosine phosphatase
siRNA	small interfering ribonucleic acid
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TBC1D1	TBC1 domain family, member 1
TBST	Tris buffered saline/0.1% Tween 20
w/v	weight per volume
w/w	weight per weight

1.0 Objectives and Hypotheses

The goal of this project was to determine how glucocorticoids induce insulin resistance and what role the well characterized antioxidant vitamin E (α-tocopherol) may have in attenuating or mitigating these effects in an *in vivo* model. This was tested by means of three key objectives.

Primary Objective: To determine the effects of dietary antioxidant (α-tocopherol) supplementation on reducing a dexamethasone-induced decrease in insulin sensitivity in a rodent model.

Secondary Objective: To determine if dietary antioxidants can decrease whole-body or tissue-specific levels of reactive oxygen species and/or associated biomarkers of reactive oxygen species.

Tertiary Objective: To identify specific insulin signalling intermediates are involved in a diminished insulin signalling response in dexamethasone treated animals, and if these changes are prevented by α-tocopherol.

In this project it was hypothesized that:

i) α-tocopherol supplementation would attenuate a dexamethasone-induced decrease in glucose clearance

ii) α-tocopherol supplementation would decrease levels of oxidative stress markers or attenuate an increase in these markers as a result of dexamethasone exposure, and

iii) dexamethasone would result in a decrease in active phosphorylation of several key insulin signalling cascade intermediates and that this would be prevented by α-tocopherol.

2.0 Literature Review

2.1 Glucose metabolism and homeostasis

Glucose is an energy-rich fuel source and as such, holds a unique and important position in mammalian metabolism. Stored as an intracellular polymer in the form of glycogen, cells are able to maintain substantial reserves of individual glucose molecules for aerobic or anaerobic production of ATP when energy demands increase (Nelson & Cox, 2005). However, despite having an important role in providing cellular energy, circulating glucose levels must be tightly regulated in order to maintain proper metabolic function and avoid numerous associated pathologies. Normal plasma glucose concentration is maintained between 4.7 and 6.0 mM during fasting conditions (Ferrannini *et al.*, 2005) through an interplay between insulin secretion, insulin utilization and subsequent glucose clearance. By comparison, postprandial blood glucose has been shown to rise to as high as 16 mM (Dalla Man *et al.*, 2005). Given that sustained hyperglycemia has been associated with metabolic complications, glucose clearance can play a significant role in maintaining whole body homeostasis. Glucose clearance is essentially mediated by its absorption into peripheral tissues such as muscle and adipose, albeit by varying degrees. When focusing on the development of insulin resistance, mechanisms behind glucose clearance become a critical area of understanding.

Several tissues are known to assist in the disposal of blood glucose. Skeletal muscle is largely viewed as the primary depot, accounting for

approximately 80 percent of glucose clearance under euglycemic hyperinsulinemic conditions (Thiebaud *et al.*, 1982). For this reason, skeletal muscle has been a main focus for understanding whole body insulin resistance.

More recently, adipose tissue has been shown to be involved in glucose metabolism and homeostasis and as such, a basis for insulin resistance. Virtanen and colleagues (2003) deduced that average total adipose tissue is responsible for approximately 22 to 26 percent of glucose clearance. Additionally, increased mobilization of fatty acids from fat stores, prompted by a myriad of factors, can lead to insulin resistance in otherwise insulin-sensitive tissues (Lewis *et al.*, 2002). In a similar fashion, liver plays a significant role in managing circulating glucose. The liver is responsible for contributing 70 to 80 percent of systemic glucose release (Meyer *et al.*, 1998) and therefore, under normal conditions is tightly regulated by hormones such as insulin. Ferrannini and others (1988) were able to show that in diabetic patients, postabsorptive glucose output was significantly higher than in non-diabetic patients, alluding to the significant effects deregulated hepatic glucose metabolism may have. While hepatic glucose uptake may be as high as nearly 40 percent following a glucose load by hepatic vein (Ludvik *et al.*, 1997), skeletal muscle is the primary peripheral tissue and as such is the focus of this project.

2.2 Glucose and the insulin signalling cascade

The insulin signalling cascade is both a highly regulated and complex orchestration of key kinases and intermediates. The intricate nature of the pathway allows for various entry points for a metabolic perturbation to deregulate the insulin-stimulated uptake of glucose. Currently, the vast majority of literature focuses on a few key intermediates, notably for their central roles in metabolism and in particular, their large number of regulatory sites. These molecules include insulin receptor substrate (IRS), Akt, and Akt substrate at 160 (AS160).

2.2.1 Insulin receptor substrates

Under normal physiological conditions, insulin secreted from the pancreas will dock to the insulin receptor on the cell surface. The insulin receptor is a tyrosine-specific protein kinase consisting of two identical α-chains on the exterior of the plasma membrane and two β-subunits extending their carboxy-terminus into the cytosol. More generally, the α-subunits provide for the insulin specific domain while the β-subunits are responsible for kinase activity. Upon insulin docking to the insulin receptor, the tyrosine-specific kinase activity of the β-subunits is activated. Each of the α/β monomers effectively phosphorylates three critical tyrosine sites on the partner β-subunit in the dimer. A key outcome from autophosphorylation of the β-subunits is the exposure of the active site of the enzyme which will in turn allow for the phosphorylation of tyrosine residues on downstream proteins in the cascade (Nelson & Cox, 2005).

One such protein group of interest are the insulin receptor substrates (IRS). There at least six known substrates found with varying tissue-specific distribution and as some groups have suggested, varying functions (Fritsche *et al.*, 2008). Members of the IRS family share a similar general protein structure, including an N-terminus pleckstrin homology (PH) domain which confers its ability to bind to phophatidylinositol lipids within membranes. In addition to this domain, they contain a protein tyrosine binding (PTB) domain that allows for the interaction of IRS with the phosphorylated insulin receptor.

Contrasting the highly conserved nature of the N-terminus motifs, the C-terminus of IRS have variably-expressed tyrosine residues that when phosphorylated, effectively translate the activation of the insulin receptor to downstream adaptor molecules such as growth factor receptor bound protein (Grb)-2 and the p85 subunit of phosphoinositide-3 kinase (PI3K), and enzymes like the SH-2 domain-containing protein-tyrosine phosphatase (SHP)-2, that are involved in the multiple mitogenic and metabolic pathways (Taniguchi *et al.*, 2006). The diversity of interacting proteins is a function of the relatively low amino acid sequence homology of approximately 35 percent (Sesti *et al.*, 2001), and likely lends itself to the resulting diversity in IRS substrates and functions. However, despite the multiple effects stemming from IRS interactions, it is important to note that IRSs do not have kinase activity, and are likely regulated in an indirect fashion.

While phosphorylation has important stimulatory roles, it has an equally significant role in inhibition of various substrates including IRS. Certain kinases such as mammalian target of rapamycin (mTOR), c-Jun N-terminal kinase (JNK), protein kinase C (PKC), inhibitor of κB kinase (IKK), glycogen synthase kinase (GSK) and mitogen activated protein kinase (MAPK) have all been shown under various conditions to effectively turn off the insulin stimulation signal when constitutively expressed through inhibitory phosphorylation on specific sites (Fritsche *et al.*, 2008; Herschkovitz *et al.*, 2007). Serine-307 of IRS-1 is one such inhibitory phosphorylation site (Zick, 2005) and is responsible for the PI3K-associated downregulation of insulin signals since it can interfere with the IRS-insulin receptor interaction (Aguirre *et al.*, 2000; Gao *et al.*, 2002).

Conversely, it has been hypothesized that serine phosphorylation is not entirely inhibitory. When serine residues at 302, 318, 325, 789 and 1216 are phosphorylated, there is an improvement in insulin signalling (Fritsche *et al.*, 2008). It has also been shown that early phosphorylation of serine-318 is involved in improved insulin action; however it is also needed in late phases to attenuate insulin action (Weigert *et al.*, 2008). Thus, it appears that specific individual site phosphorylation is not as important as the timing and interdependency of the serine phosphorylation sites.

Another form of IRS-1 regulation is through various forms of post-translational modifications. Postranslational modification of IRS-1 with O-linked N-acetylglucosamine monosaccharides (O-GlcNAc) has been noted in adipose and muscle tissue caused by the increased activity of the hexosamine biosynthetic pathway (Klein *et al.*, 2009). Under hyperglycaemic and hyperlipidemic conditions, the flux through this pathway is increased and thus O-GlcNAc-modication is enhanced during insulin resistant states. The modification can occur on phosphorylation sites and thus prevent normal phosphorylation patterns. Another modification of IRS-1 has been described in muscle tissue. S-nitrosylation via nitric oxide has been implicated in the downregulation of insulin sensitivity. This modification induces the proteosomal downregulation of IRS-1 in skeletal muscle of diabetic rats (Carvalho-Filho *et al.*, 2005; Yasukawa *et al.*, 2005). IRS-1 can also be modified by acetylation, which is the transfer of an acetyl group to a lysine residue. While this usually occurs on histone proteins for the purposes of chromatin regulation, some histone acetyltransferases and histone deactylatses have non-histone substrates, including diabetes related proteins like IRS-1 (Kaiser & James, 2004). In summary, IRS is highly regulated by post translational modifications. These structural changes may have significant impact on the various phosphorylation sites of IRS and thus can play a critical role in the pathogenesis of insulin resistance.

2.2.2 Akt/PKB

Akt, also commonly known as protein kinase B (PKB), has been classified as a series of kinases with homology related to both protein kinase A and protein kinase C, consisting of three similarly structured homologues (Akt1, Akt2 and Akt3) which show specific and different roles *in vivo* (Dummler & Hemmings, 2007). The characterization of Akt and its downstream substrates has been extensively studied as Akt has a committed role in the transduction of the insulin signalling cascade. All isoforms of Akt are inactive and, in general, their activity is regulated through receptor tyrosine pathways. More specifically, upon stimulation by insulin or other growth factors, Akt is activated by its upstream mediators via the phosphoinositide 3 kinase (PI3K) pathway which then permits the subsequent translocation of glucose transporters (Nelson & Cox, 2005).

PI3Ks are a family of intracellular lipid kinases with a key function of phosphorylating phosphatidylinositols and phosphoinositides. There are three known classes of PI3Ks, separated on the basis of their substrate specificity and protein structure. More specific to the insulin signalling cascade are the Class 1 PI3Ks which are lipid kinases responsible for the phosphorylation of phosphoinositol-4,5-bisphophosphate (PIP_2) to phosphoinositol-3,4,5-triphosphate (PIP_3) This particular class is further subdivided into Class 1a that responds specifically to growth factor receptor tyrosine kinases. Upon activation, Class 1a PI3K phosphorylates PIP_2 to PIP_3 at the level of the plasma membrane (Nelson & Cox, 2005). Through interactions with the PH domain of Akt and

phosphoinositide dependent protein kinase (PDK)-1, these downstream intermediates are brought to the plasma membrane where PDK-1 will phosphorylate Akt (Liao & Hung, 2010). PDK-1 is generally characterized as a 'master kinase' however it is only one of several possible upstream activators of Akt. Other activators include insulin-like growth factor 1, oxidative stress, and mTOR complex 2 (Matheny & Adamo, 2009).

The phosphorylation of Akt on both serine-473 and threonine-308 sites is a root of activity for downstream metabolic and mitogenic signalling (Jiang *et al.*, 1999; Kohn *et al.*, 1996). The phosphorylation at these specific sites is crucial for normal functioning of the Akt kinase activity, which includes managing glucose uptake. This has been confirmed by the numerous studies using mutant Akt proteins lacking the associated serine and threonine phosphorylation sites, whereby downstream substrate phosphorylation was inhibited (Green *et al.*, 2008; Kitamura *et al.*, 1998; Kuroda *et al.*, 1998). Among the dozens of currently characterized substrates of Akt, Akt substrate at 160kD has recently been shown to have a key role in mediating insulin's effects on glucose homeostasis (Eguez *et al.*, 2005; Ngo *et al.*, 2009; Thong *et al.*, 2007; Treebak *et al.*, 2009)..

2.2.3 Akt substrate at 160

In 2002, Kane and colleagues used a combined immunoprecipitation-mass spectroscopy technique to characterize Akt downstream substrates. This technique

resulted in the discovery of AS160, a Rab GTPase-activating protein (GAP). The AS160 substrate includes two PTB domains, a C-terminus Rab GAP domain and six phosphorylation motifs that can be targeted by Akt and other kinases (Kane *et al.*, 2002) and additionally, a calmodulin binding region positioned N-terminally to the GAP domain (Kane & Lienhard, 2005; Kramer *et al.*, 2007). The Rab protein family consists of monomeric G-proteins and have been shown to be directly involved in vesicular trafficking, including glucose transporter translocation (Zerial & McBride, 2001). Under basal conditions, the GAP activity of AS160 restricts Rab proteins to the inactive GDP-bound form in turn disallowing vesicular translocation (Sano *et al.,* 2003). Upon phosphorylation by upstream kinases on its multiple PTB domains, the GAP activity is inhibited, allowing the active GTP-bound form of Rab proteins to accumulate and relieve the inhibition of internalized vesicles from reaching the plasma membrane. Therefore, as a Rab GAP, AS160 is capable of modulating GLUT-4 vesicular trafficking (Eguez *et al.*, 2005; Ngo *et al.*, 2009; Thong *et al.*, 2007; Treebak *et al.*, 2009).

Numerous functional studies have been performed with respect to AS160 expression and its relationship with upstream insulin signalling intermediates and the subsequent tissue disposal of glucose. In rodent skeletal muscle models, insulin treatments resulted in an increase in AS160 phosphorylation that was inhibited by treatment with wortmannin, a noted PI3K inhibitor, and fully blunted in Akt2 knockout mice (Kramer *et al.*, 2006a). Similarly, the use of small

interfering (si)RNA gene silencing of IRS-1 in human skeletal muscle cells attenuated insulin-stimulated AS160 phosphorylation (Bouzakri *et al.*, 2006). These findings suggest that AS160 is highly regulated by the insulin signalling cascade, however it does not appear that the role of insulin in glucose clearance is exclusive to AS160 phosphorylation. Eguez and colleagues (2005) determined that in an AS160 knockdown model in adipocytes, despite basal glucose transporter motility being upregulated threefold in the knockdown cells, insulin was still able to elicit a fourfold increase in glucose transporter trafficking over basal. Thus, these findings lend to the notion that AS160 is not an obligate intermediate in insulin-stimulated glucose clearance since its ablation does not have the same effect as insulin in wildtype models. Some studies have found that insulin may help regulate intracellular trafficking through other phosphoinositide kinases such as PIKfyve proteins (Ikonomov *et al.*, 2007) and SNAREs (Min *et al.*, 1999). Nonetheless, the complexity of the glucose transporter cellular machinery makes determining pathway specifics difficult to ascertain in traditional models.

2.2.4. Glucose transporter 4

The plasma membranes of cells are impermeable to carboyhydrates and as such, require specific transporters to facilitate their entry into the cell. A broad family of specialized glucose transporters (GLUT) with varying substrate specificities and kinetics work to carry out the task of managing glucose transport

in a tissue-specific distribution relative to their functional role. Glucose transporter 4 (GLUT-4) is one member of this family of GLUTs which exclusively facilitate the uptake of glucose into cells down glucose concentration gradients in response to insulin in tissues such as skeletal muscle (Shepherd & Kahn, 1999). GLUT-4 is thought to be contained in insulin responsive vesicles that are continuously recycled to and from the plasma membrane, with the net effect favouring endocytosis under basal conditions (Dugani & Klip, 2005).

As previously discussed, there is a close functional relationship between AS160 and glucose transporters such as GLUT-4. AS160 associates with GLUT-4 via a direct interaction with insulin-regulated aminopeptidase (IRAP), which locates and promotes hydrolysis of associated GTP-bound Rab proteins, thus limiting Rab activity (Bogan & Kandror, 2010; Peck *et al.*, 2006). Several members of the Rab family of proteins have been found associated with GLUT-4 vesicles (Miinea *et al.*, 2005) and more recently, tissue-specific Rab proteins have been identified (Ishikura *et al.*, 2007; Sano *et al.*, 2007).

2.3 Glucocorticoid excess

Glucocorticoids (GC), including the much discussed steroid hormone cortisol, play a variety of roles in maintaining homeostasis during multiple forms of stress (Munck *et al.*, 1984) and produce a myriad of tissue-specific physiological effects (Sapolsky *et al.*, 2000; Schacke *et al.*, 2002). These effects

range from macronutrient metabolism regulation to immune system regulation and include specific roles in growth, reproduction and apoptosis (Sapolsky *et al.*, 2000). However, under physiological or pharmacological conditions, GC excess, also labelled hypercortisolism and more generally as Cushing's syndrome, can lead to a variety of metabolic perturbations. Currently, studies link GC excess to peripheral insulin resistance. *In vivo* rodent models and human cohorts mimicking GC excess have shown reduced insulin- and growth factor-stimulated hexose uptake in rat skeletal muscle (Dimitriadis *et al.*, 1997; Weinstein *et al.*, 1998) and decreased insulin sensitivity and hyperinsulinemia (Nicod *et al.*, 2003; Pagano *et al.*, 1983; Rizza *et al.*, 1982; Schneiter & Tappy, 1998), respectively.

Cushing's syndrome is a blanket term for hypercortisolism of all origins and has particular significance when understanding the role of GCs, GC excess and perturbations in glucose homeostasis. Glucose intolerance occurs in upwards of 60 percent of those with Cushing's syndrome (Newell-Price *et al.*, 2006). This figure, in conjunction with the current understanding of how GCs provoke hyperglycemia and hyperinsulinemia, provides a rationale for a more in depth study of the relationship between GC exposure, insulin signalling and glucose clearance.

2.3.1 Causes of GC excess

The origins of physiologically induced, or endogenous, GC excess can be separated into corticotropin-dependent and corticotropin-independent causes. Of the corticotropin dependent causes, the vast majority are a result of a pituitary gland adenoma under the label of Cushing's disease or in less frequent cases, ectopic corticotropin release, such as from small cell carcinomas of the lung. Both causes have the final result of increasing corticotropin which in turn elevates cortisol release from the adrenals via the hypothalamus-pituitary-adrenal (HPA) axis. Corticotropin independent causes for GC excess are linked directly to adenomas or carcinomas of the adrenals resulting in increased stimulus to secret cortisol (Newell-Price *et al.*, 2006). It has also been shown that environmental stress, through overstimulation of the HPA axis, can upregulate GC production, leading to similar endpoints (Rosmond, 2003).

As mentioned previously, GCs are responsible for producing a variety of tissue-specific effects. Some of these effects, namely their anti-inflammatory and immunosuppressive characteristics, are exploited pharmacologically to treat a myriad of conditions ranging from neoplasias to chronic inflammatory diseases (van Staa *et al.*, 2000; Walsh *et al.*, 1996). Some of the most commonly administered long-term use pharmacological agents are hydrocortisone, prednisone, and dexamethasone. Moreover, prednisone and dexamethaone have been found to be approximately four and 30 times as potent as hydrocortisone with respect to impairing glucose metabolism (Cantrill *et al.*, 1975; Liapi &

Chrousos, 1992). However, it has widely been shown that GCs like dexamethasone are responsible for a variety of deleterious physiological side effects both in the long and short term (Nicod *et al.*, 2003; Ruzzin *et al.*, 2005; Schneiter & Tappy, 1998). Such changes include increased serum glucose via increased hepatic glucose production and impairments in peripheral insulin sensitivity and secretion, manifesting as hyperglycemia and hyperinsulinemia (Giorgino *et al.*, 1993; Nicod *et al.*, 2003; Rafacho *et al.*, 2009; Ruzzin *et al.*, 2005; Venkatesan *et al.*, 1996).

Several changes and impairments to the insulin signalling cascade have been documented in human, rodent and *in vitro* models under various exposures to GCs. Based on the previously discussed complexity of the insulin signalling cascade, there are several significant entry-points for GC-induced impairment of insulin sensitivity and glucose tolerance that have been amply discussed in literature (Buren *et al.*, 2002; Ewart *et al.*, 1998; Giorgino *et al.*, 1993; Lundgren *et al.*, 2004; Rojas *et al.*, 2003; Saad *et al.*, 1993; Sakoda *et al.*, 2000).

2.3.2 Impaired signal intermediates due to GC

GCs have been purported to affect protein expression and subsequent kinase activity in various signalling intermediates in numerous tissues. Moreover, the determination of which signal cascade intermediates are impaired in tissues following GC excess has been thoroughly investigated. Several *ex vivo* and *in vivo*

studies have reported that the insulin receptor variably increases in rodent skeletal muscle and in Fao hepatocytes (Giorgino *et al.*, 1993; Saad *et al.*, 1993; Saad *et al.*, 1995) With respect to IRS-1 protein content, numerous groups have reported a significant decrease following GC treatments in skeletal muscle (Ewart *et al.*, 1998; Giorgino *et al.*, 1993; Rojas *et al.*, 2003; Saad *et al.*, 1993) and adipocyte cell culture models (Buren *et al.*, 2002; Lundgren *et al.*, 2004; Sakoda *et al.*, 2000; Turnbow *et al.*, 1994). Interestingly, IRS-1 protein content was shown to significantly increase over control tissues in liver (Rojas *et al.*, 2003; Saad *et al.*, 1993) and in a Fao hepatocyte (Saad *et al.*, 1995) models while in 3T3-L1 adipocytes, decreases in IRS-1 were paralleled by a significant increase (approximately 150 percent) in IRS-2 protein content. However despite the lower overall content of IRS-1 in skeletal muscle and adipose, an examination of the phosphorylation stoichiometry suggests that there is be an increase in active phosphorylation per mole of IRS-1 during hypercortisolemia since overall IRS-1 phosphorylation was unchanged or only mildly reduced. Thus, *in vivo* tyrosine phosphorylation of IRS-1 appears to be regulated independent of total IRS-1 protein.

Further downstream of IRS-1, it was determined that basal and insulin-stimulated Akt phosphorylation was drastically reduced by upwards of 50 percent in 3T3-L1 adipocytes (Buren *et al.*, 2002) and rodent skeletal muscle (Buren *et al.*, 2008; Ruzzin *et al.*, 2005), following dexamethasone (DEX) exposure. Akt has long been a suspect of possible perturbation in the insulin signalling cascade

as a result of DEX due to its important role in the regulation of glucose metabolism; however its indirect upstream partner molecule, PI3K has also been implicated. PI3K, as previously mentioned, is directly activated as a function of IRS-1 phosphorlyation and is responsible for the indirect activation of Akt through PDK-1. It has been generally shown that DEX exposure will decrease both total PI3K as well as IRS-1-associated PI3K (Buren *et al.*, 2002; Saad *et al.*, 1993; Sakoda *et al.*, 2000), however some evidence suggests that the decrease may be tissue specific, as shown by the 70 percent increase in IRS-1-associated PI3K in Fao hepatocytes (Saad *et al.*, 1995). Interestingly, some studies have shown that despite a decrease in PI3K expression in muscle or adipose, Akt function remains normal during insulin stimulation (Kruszynska *et al.*, 2002; Nadler *et al.*, 2001).

There are two common characteristics in the majority of literature defining GC effects on glucose homeostasis and the associated decrease in glucose clearance capacity. First, it appears that GCs are directly associated with a decrease in glucose uptake. In rodent models, DEX treatments for two days (Weinstein *et al.*, 1998), five days (Dimitriadis *et al.*, 1997) and 12 days (Ruzzin *et al.*, 2005) resulted in a decrease in glucose clearance in fast twitch muscle of nearly 50 percent. Similarly, in 3T3-L1 adipocytes there was an equal drop in glucose transport of approximately 40 percent (Sakoda *et al.*, 2000), 50 percent (Ngo *et al.*, 2009) and 70 percent (Buren *et al.*, 2002) within a 24 hour exposure to DEX. The second common characteristic is the disconnect between GLUT-4

protein content and subsequent GLUT-4 translocation. Specifically, it was shown that GLUT-4 protein is increased following DEX (Ewart *et al.*, 1998). Thus, while the majority of sources indicate that DEX exposure causes, at a minimum, an increase in GLUT-4 protein, the consensus is that active GLUT-4 at the plasma membrane is significantly decreased (Ngo *et al.*, 2009; Venkatesan *et al.*, 1996; Weinstein *et al.*, 1998). Taken together, GC effects on GLUT-4 recruitment and/or activation may act as a novel point of investigation for how GCs deregulate glucose metabolism, leading to the induction of hyperglycemia and hyperinsulinemia.

Thus, the literature suggests that GC induced insulin resistance is ultimately a result of impaired glucose uptake. Numerous studies aiming to elucidate a GLUT-4-centric mechanism for GC effects on glucose metabolism have come to examine AS160 and its role in glucose clearance. As previously discussed, AS160 is a small G-protein GTPase activating protein (GAP) which is thought to be involved in the translocation of GLUT-4 vesicles to the plasma membrane through the regulation of Rab proteins. Activation of AS160 suppresses its ability to convert GTP to GDP, which allows for the essential activation of GTP-bound Rab to accumulate, thereby allowing for the GLUT-4 translocation required to clear glucose from the plasma (Sano *et al.*, 2003). Phosphorylation of AS160 can occur in several pathways, including insulin-stimulation of Akt (Kramer *et al.*, 2006a), exercise/contraction (Bruss *et al.*, 2005;

Kramer *et al.*, 2006b) and possibly through activation of AMP-activated protein kinase (AMPK) (Kramer *et al.*, 2006a).

2.3.3 AMPK effects with GCs

AMPK is a ubiquitous energy sensing enzyme with significant roles in managing glucose uptake and lipid metabolism in various tissues and has been linked to the onset and development of insulin resistance in both skeletal muscle and liver (Zhou *et al.*, 2009). AMPK is thought to have an important function in managing insulin secretion from pancreatic β-cells (Salt *et al.*, 1998), and in particular in translating muscle contraction stimulus to increase glucose uptake (Mu *et al.*, 2001). There is ample evidence in the literature demonstrating the relationship between AMPK, AS160 phosphorylation and GLUT-4 translocation in mediating glucose clearance from the plasma. In specific terms, several groups have shown how activated AMPK is responsible for a portion of GLUT-4 vesicle translocation to the plasma membrane using AMPK knockout (Gaidhu *et al.*, 2010; Mu *et al.*, 2001) and knockdown (Pehmoller *et al.*, 2009) rodent models. This was previously shown in isolated rat skeletal and cardiac muscle whereby treatment with the AMPK agonists metformin (Zhou *et al.*, 2001) and AICAR (Russell *et al.*, 1999) were associated with an upregulation of AMPK and subsequently improved glucose transport. Interestingly, these studies and others (Fazakerley *et al.*, 2010; Fisher *et al.*, 2002) have shown that activated AMPK effects were independent of, and also additive to, insulin on glucose clearance.

Kramer and colleagues (2006a) elaborated on this using *in vivo* and *in vitro* rodent skeletal muscle models, specifically showing that AICAR-induced AMPK activation was associated with increased AS160 activation and thus increased glucose transport.

It is important to note that while both AMPK and the insulin signalling pathway are shown to regulate glucose uptake, the two mechanisms are distinct in a variety of ways. While insulin relies on the translation of a regulated signal through the PI3K pathway, AMPK is readily acted upon by broader upstream stimuli, such as contraction (Fisher *et al.*, 2002; Mu *et al.*, 2001; Pehmoller *et al.*, 2009). It has been shown in numerous studies that contraction stimulus via AMPK activation increases glucose disposal and that this effect is additive in the presence of insulin, suggesting independent regulation (Kramer *et al.*, 2006a; Musi *et al.*, 2001). Moreover, in a recent study by Treebak and colleagues (2010) it was shown that a serine residue on AS160 (serine-711) can be phosphorylated by AMPK even without insulin. These data give credence to the notion that despite having similar endpoints with respect to glucose clearance, the mechanisms and stimuli behind each differ.

AS160 and its related substrates have been at the forefront of much study in recent years. As more research points to GLUT-4 vesicular translocation as a likely nexus for insulin resistance, the inhibitory roles of AS160 have become interesting points of analysis, in particular in relation to AMPK. Recently, studies

using 3T3-L1 adipocytes and *ex vivo* rodent EDL muscles have led to the conclusion that, not only does overexpression the AS160 paralog TBC1D1 result in inhibited GLUT-4 translocation, AMPK activation or inhibition will readily improve or blunt GLUT-4-derived glucose transport, respectively (Chavez *et al.*, 2008; Peck *et al.*, 2009; Pehmoller *et al.*, 2009).

Given the common downstream pathway substrates between AMPK and GCs, a thorough investigation into their relationship is warranted. However, due to the variable tissue-specific functions of GCs and AMPK, further connections are more difficult to elucidate. For example, recent studies have demonstrated that GC exposure in the form of corticosterone or DEX resulted in upregulated AMPK expression in liver (Christ-Crain *et al.*, 2008; Viana *et al.*, 2006), however similar treatment methods resulted in decreased expression in adipose (Viana *et al.*, 2006) and skeletal muscle (Ruzzin *et al.*, 2005). When examining studies in cardiac muscle, results become more interesting due to the consistent increase in AMPK protein expression following GC exposure (Puthanveetil *et al.*, 2008), despite significantly lower AMPK activity (Christ-Crain *et al.*, 2008). Given the difference found with respect to relative enzyme abundance and enzyme activity in tissues, it is perhaps more important to consider the significance of choosing an appropriate experimental model for determination of GC effects on glucose metabolism. An understanding of GC-induced disturbances in glucose transport and AMPK deregulation, it is tempting to consider what effects GC-antagonist, or

similarly GC byproduct-antagonists may have on mitigating some of the GC-related effects on glucose homeostasis.

2.4. Oxidative Stress

Oxidative stress is generally defined as an imbalance in pro-oxidant and anti-oxidant mechanisms favouring a pro-oxidative system (Sies, 1991; Sies, 1997). There is particular interest in this metabolic state due to its recent links to diseased states, including the pathogenesis of insulin resistance (Archuleta *et al.*, 2009; Houstis *et al.*, 2006; Maddux *et al.*, 2001; Singh *et al.*, 2008; Vinayagamoorthi *et al.*, 2008). Under physiological conditions, the primary groupings of bioactive molecules responsible for the shift to a pro-oxidant state are reactive oxygen species (ROS). ROS are typically generated *in vivo* via 'leaky' mitochondrial electron donors during aerobic metabolism as well as through enzymes during oxidative phosphorylation (Kehrer, 1993). Well known members of this group of ROS include hydrogen peroxide, hydroxyl radical and superoxide anion. It is also possible to increase ROS production through the transition metal-catalyzed autooxidation of glucose (Hunt & Wolff, 1991; Monnier *et al.*, 2006) and protein glycation (Brownlee, 2000; Hunt & Wolff, 1991; Matsuoka *et al.*, 1997) as a result of chronic and/or fluctuating hyperglycemia. These findings are particularly interesting in that while exogenously derived oxidative stress may lead to the development of insulin

resistance, it is also possible that hyperglycemia in the insulin-resistant state can lead to oxidative stress and self-perpetuate the disease progression.

Additionally, elevated plasma free fatty acid levels have been shown to increase ROS generation through a protein kinase C (PKC) dependent pathway in vascular smooth muscle cells (Inoguchi *et al.*, 2000), and in C2C12 skeletal muscle cells (Ragheb *et al.*, 2009). The specific mechanism by which PKC isoforms promote the pathogenesis of insulin resistance has not been thoroughly characterized, however certain findings may shed some light onto the role PKC plays in managing oxidative stress-related pathologies. When PKC was inhibited with compound Gö6976 (Hansen *et al.*, 1999) or a variety of antioxidants and PKC inhibitors (Saberi *et al.*, 2008), hydrogen peroxide-related inhibition of insulin signalling and associated tissue necrosis was minimized. With respect to insulin sensitivity and glucose clearance, PKC has multiple roles. PKC isoforms are either deemed conventional, new or atypical, with atypical PKC being important for insulin-stimulated glucose transport (Farese *et al.*, 2005) and conventional PKC being inhibitory (Kajita *et al.*, 2001). In short, there are several possible interventions and pathways available that can shift the metabolic balance towards oxidative stress and perhaps more potential downstream pathways capable of mediating the associated defects in glucose clearance.

2.4.1 Quantifying oxidative stress

Quantification of oxidative stress in most experimental models is possible by measuring actual reactive species (i.e. free radicals) or by measuring known by-products of oxidative stress in various tissues and/or cell components. Current methodologies used for measuring free radicals in biological matrices include electron spin resonance (ESR) and spin trapping. In this method, a specialized free radical binding molecule, such as a nitrone or nitroso compound, is permitted to interact with unstable free radicals (containing unpaired electrons), ultimately trapping it and creating a more stable radical. By exposing this molecule to a varying magnetic field, magnetic resonance spectra pertaining to their unpaired electron state can be detected and analyzed. Despite the sensitivity of this technique, it is practically limited to chemical matrices due to a predictable interference of water in most biological scenarios as well as its technical requirements (Jackson, 1999; Khan & Swartz, 2002).

Given the complex and laboratory intensive forms of analysis, direct quantification by ESR is neither efficient nor practical in the clinical setting. For these reasons, determining the degree of oxidative stress through direct analysis of susceptible components, such as protein, lipids and nucleic acids, is the more viable option. Moreover, the determination of key oxidative stress by-products may be more biologically and clinically relevant as they are more stable in biological systems and their accrual may be directly linked to disease progression. Some of the more commonly analyzed by-products of oxidative stress include

carbonylation in proteins, malondialdehyde in lipids or base nitration in nucleic acids (Giustarini *et al.*, 2009).

2.4.2 Oxidative stress in disease

In both animal models and cell culture models, exposure to oxidative conditions results in accumulation of oxidized protein (Schuessler & Schilling, 1984). Oxidative stress has been shown to induce certain physiological adaptations such as 4-hydroxynonenal induced-protein damage and degradation in Parkinson's disease (Jenner, 2003), hepatic protein glycation in inflammation-induced liver injury (Kuhla *et al.*, 2010), increased amyloid-beta plaques in Alzheimer's disease (Lin & Beal, 2006; Moreira *et al.*, 2008), and increased proteolysis and degradation of lung parenchymal cells in emphysema and chronic obstructive pulmonary disease (Cantin & Crystal, 1985).

While the examination of the link between oxidative stress and disease is frequent, not all studies are able to draw specific and direct connections to the likelihood of disease outcome, in particular with respect to insulin resistance. For example, fluctuating hyperglycemia in type 2 diabetes has been associated with oxidative stress (Monnier *et al.*, 2006), however this association was not observed in those with type 1 diabetes (Wentholt *et al.*, 2008). Current hypotheses of oxidative stress-induced insulin signalling damage include *i*) oxidative damage and alteration to the insulin receptor or IRS-1 (Archuleta *et al.*, 2009; Hansen *et*

al., 1999), *ii*) increased inhibitory phosphorylation of IRS-1 (Aguirre *et al.*, 2000; Vinayagamoorthi *et al.*, 2008; Zick, 2005), *iii*) oxidative damage or impairment of Akt activation (Tirosh *et al.*, 2001), *iv*) inhibitory S-nitrosylation of IRS and Akt (Yasukawa *et al.*, 2005), *v*) inhibitory mechanisms that are independent of IRS-1 (Hoehn *et al.*, 2008) and *vi*) inhibition of glucose transporter translocation (Henriksen *et al.*, 1997; Rudich *et al.*, 1998). These hypotheses, while not an exhaustive list, parallel past experiments involving chronic GC exposure and impairments in glucose homeostasis.

However, it should be noted that the effects of ROS on insulin sensitivity are likely dose dependent. Higaki and others (2008) demonstrated this with exposure of isolated rodent EDL to low levels of hydrogen peroxide resulting in an increased non-insulin dependent uptake of glucose. Previously, this same result was found in isolated Zucker rat epitrochlearis and soleus muscle (Kim *et al.*, 2006). Furthermore, an overexpression of antioxidant enzymes such as glutathione peroxidase (GPx) was reported to interfere with insulin signalling due to a perturbation in low levels of insulin-sensitizing ROS (McClung *et al.*, 2004; Wang *et al.*, 2008). In a similar study, it was also found that GPx knockout mice had enhanced insulin signalling due to a preserved oxidative inhibition of protein tyrosine phosphatases; enzymes which are known to inhibit insulin-stimulated glucose uptake (Loh *et al.*, 2009). These data suggest a sensitizing effect of ROS at a basal level, although the conclusions are not consistent with a large body of the literature which show that prooxidants such as hydrogen peroxide are able to

nearly abolish insulin-stimulated glucose uptake at micromolar concentrations (Hansen *et al.*, 1999; Maddux *et al.*, 2001).

More recently, it has been suggested that there is a particular role for ROS in the triggering of the insulin resistant state. Houstis and colleagues (2006) noted that in two *in vitro* models of insulin resistance using 3T3-L1 adipocytes, increased ROS were a common defining characteristic. Moreover, cellular treatments causing a perturbation in ROS levels showed varying degrees of success in minimizing the degree of insulin resistance. The use of similar methodologies (ROS induction and ROS-reducing treatments) has yielded comparable results in other adipocyte models (Fukuoka *et al.*, 2010), in H4IIEC3 hepatocytes (Nakamura *et al.*, 2009), isolated mouse skeletal muscle (Dokken *et al.*, 2008; Lastra *et al.*, 2008), and L6 myotubules (Singh *et al.*, 2008; Vinayagamoorthi *et al.*, 2008).

The specific downstream pathways related to ROS that lead to the development of insulin resistance have yet to be elucidated. Several studies point to the activation of JNK as a potential mediator of these effects. Not only has JNK been shown to be activated following oxidative stress (Houstis *et al.*, 2006; Kamata *et al.*, 2005; Lo *et al.*, 1996), but genetic knockout (Hirosumi *et al.*, 2002) and JNK-inhibitory protein infusion studies (Kaneto *et al.*, 2004) led to improved insulin signalling, glucose infusion rate, and basal glucose levels. JNK activation

has been directly shown to phosphorylate IRS-1 on its serine substrates, resulting in IRS-1 inhibition (Aguirre *et al.,* 2000).

2.4.3 GCs and oxidative stress

Prior studies have described chronic GC exposure in detail in terms of metabolic adaptations (Nicod *et al.*, 2003; Ruzzin *et al.*, 2005; Schneiter & Tappy, 1998). Recent attempts to characterize the adaptations have been studied in relation to the effects GCs produce via the glucocorticoid receptor, however a growing body of evidence indicates several effects may be mediated by ROS. These propositions are based in a number of studies that provide a link between GC exposure and ROS production, and others linking antioxidant (thus, ROS quenching) exposure to improved insulin signalling and/or glucose homeostasis.

Iuchi and others (2003) have illustrated the ROS-producing ability of DEX in vascular endothelial cells. In this cell culture model, DEX significantly upregulated hydrogen peroxide production through increased oxidative metabolism via NADPH oxidase and xanthine oxidase activity. Similar results showing increased ROS and decreased ROS-quenching systems following DEX treatment have been reported in lymphoblastic cell cultures (Baker *et al.*, 1996), 3T3-L1 adipocytes (Houstis *et al.,* 2006), and in rat hippocampal cultures (You *et al.*, 2009). DEX exposure has also been able to produce similar results in one rodent model (Orzechowski *et al.,* 2002), however further animal models are

lacking in the literature. Despite a paucity of studies performed using skeletal muscle culture or *in vivo* models, there appears to be a trend in the literature to associate these effects with the activation of the glucocorticoid receptor (Iuchi *et al.*, 2003; Ngo *et al.*, 2009; Schafer *et al.*, 2005; Turnbow *et al.*, 1994; You *et al.*, 2009). These findings are particularly powerful as shown by the amelioration of GC-induced adaptations in glucose homeostasis and oxidative stress by the GR antagonist, RU-486. This suggests that at least some of the effects seen from chronic GC exposure are mediated via the GR and thus, may point to an important point of origin for the attenuation or prevention of GC-induced insulin resistance.

2.4.4 Oxidative stress and antioxidants

Several groups have examined the link between oxidative stress and insulin resistance using dietary and *in vitro* antioxidant models. There have been a number of studies showing the beneficial effects of several known antioxidants when provided in conjunction with oxidative stress-inducing treatments such as hydrogen peroxide, glucose oxidase and specifically via exposure to GCs such as DEX. To date, studies have been conducted to examine the effects of minimizing ROS generation and/or fortifying the antioxidant/oxidant relationship using cell, animal and human models. Primary examples of such biological antioxidants include; superoxide dismutase, vitamin E, vitamin C and glutathione (Brigelius-Flohe, 2009).

2.4.4.1 Vitamin E

The term ‘vitamin E’ is a blanket term referring to eight different isoforms of lipid-soluble antioxidants known for their peroxyl radical scavenging abilities within the lipid membrane. These variants include α-, β-, γ-, and δ-tocopherols and four similarly labelled tocotrienols. These compounds have potent antioxidant capacities as a function of both their isoform-specific chromanol ring and their hydrophobic isoprenoid side chain (Brigelius-Flohe, 2009).

While all forms of vitamin E are bioavailable, α-tocopherol is the isoform that is primarily stored in the body (International Institute of Medicine, 2000). This assessment is generally obtained by the measurement of levels of tocopherols and tocotrienols using HPLC techniques (Abidi, 2000). Thus, despite its counterpart, α-tocotrienol, having higher antioxidant potency, α-tocopherol is the key isoform that is referred to in dietary allowances (International Institute of Medicine, 2000).

Upon an interaction with a peroxyl radical at the cell membrane, tocopherols and tocotrienols become a relatively stable tocopheroxyl or tocotrienoxyl radical, respectively. These radicals are rapidly transferred and eventually quenched by working in conjunction with other cellular antioxidants to neutralize the free radical and recycle the various antioxidant components (Brigelius-Flohe, 2009). Leung and colleagues (1981) determined that vitamin E works catalytically and synergistically with other water- and lipid-soluble

antioxidants via enzymatic and non-enzymatic reactions. Other components of this network include water soluble vitamin C (ascorbate) and thiol antioxidants glutathione and α-lipoic acid. Several studies have been conducted using various durations of vitamin E supplementation to demonstrate an improvement in glucose tolerance in diabetic or insulin resistant patients (Manning *et al.*, 2004; Paolisso *et al.*, 1993a; Paolisso *et al.*, 1993b) however the long term outcomes and mechanisms of these effects are yet to be determined.

2.4.4.2 Glutathione

Glutathione (GSH) refers to a potent tripeptide antioxidant (gamma glutamate-cysteine-glycine) found in a variety of tissues in the millimolar range (Hayes & McLellan, 1999). The strong antioxidant potential of GSH lends itself to the thiol moiety associated with the cysteine residue. Together with water- and lipid-soluble antioxidants such as ascorbate and vitamin E isoforms, GSH and its associated enzyme, glutathione peroxidise, assist in the detoxification of hydrogen peroxide by reducing the transient vitamin E radicals and by terminating the propagation cycle by dimerizing to GSSG, or oxidized glutathione. Oxidized GSH can later be recycled through the NADPH-catalyzed enzymatic reduction by glutathione reductase, thus assisting in the regeneration of the larger antioxidant network (Pastore *et al.*, 2003). Moreover, the ratio of reduced to oxidized glutathione is a reputable marker of general oxidative stress (Sharma *et al.*, 2000a). Common methods for measuring both forms of glutathione include

spectrophotometric, bioluminometric and combinations of HPLC and mass spectroscopy (Pastore *et al.*, 2003).

The relationship between disease pathology and erythrocyte glutathione status has been investigated in observational studies. In patients with diabetes, glutathione status was shown to be approximately 70 to 85 percent that of non-diabetics and was thus associated with higher susceptibility to oxidative cellular damage (Samiec *et al.*, 1998; Vijayalingam *et al.*, 1996; Yoshida *et al.*, 1995, De Mattia *et al.*, 1998). However, when glutathione peroxidase was overexpressed in transgenic mice models, incidence of insulin resistance was significantly higher than controls which can further suggest a role for a basal level of oxidative stress in facilitating the function of insulin at both the level of Akt phosphorlylation in liver (McClung *et al.*, 2004) and pancreatic insulin secretion (Wang *et al.*, 2008).

2.4.5 Antioxidant status in the diseased state

The general content and concentration of various biological antioxidants has been shown to change in the diseased state. Specific to insulin resistance and diabetes, several groups have examined changes in the antioxidant defence system. In particular, decreased levels of superoxide dismutase and glutathione peroxidase were seen in patients with diabetes for more than 5 years (Hartnett *et al.*, 2000) which suggests a link between decreased antioxidant capacity and diabetes-related disease pathology (Uchimura *et al.*, 1999). Similarly, a

significantly low vitamin E status was correlated with an increase in risk of developing diabetes mellitus within 4 years (Salonen *et al.,* 1995). Thus, these studies illustrate that antioxidants may be involved in the development and/or progression of the diabetic state. There have been numerous *in vitro* and *in vivo* studies that have examined the short and long-term effects of vitamin E and other molecules on oxidative stress-induced insulin resistance (Maddux *et al.,* 2001; Vinayagamoorthi *et al.,* 2008; Estrada *et al.,* 1996; Henriksen *et al.,* 1997; Yaworsky *et al.,* 2000; Furukawa *et al.,* 2004; Li *et al.,* 2000; Jacob *et al.,* 1996; Laight *et al.,* 1999; Gokkusu *et al.,* 2001; Vincent *et al.,* 2009).

2.4.5.1 Cell culture studies in insulin resistance

Prior studies using glucose oxidase to produce hydrogen peroxide have resulted in a decrease in insulin stimulated glucose clearance (Maddux *et al.,* 2001; Dokken *et al.,* 2008; Singh *et al.,* 2008). However, a pre-treatment with upwards of 1000 μM α-lipoic acid blunted the hydrogen peroxide induced decrease as measured by 2-deoxyglucose uptake assay. Specifically, hydrogen peroxide-induced oxidative stress in L6 myotubules preceded an increase in serine phosphorylation and a significant decrease in tyrosine phosphorylation of IRS-1. This effect was blunted by an 18 hour *in vitro* exposure to α-lipoic acid (Maddux *et al.,* 2001). Vitamin E, in conjunction with ascorbate and α-lipoic acid, produced similar results by normalizing glucose transport following oxidative exposure (Vinayagamoorthi *et al.,* 2008). A similar pre-treatment protocol restored insulin

stimulated glucose uptake following exposure to hydrogen peroxide as shown through 2-deoxyglucose uptake. These changes were also paralleled by an inhibition of JNK activation. In other groups, similar results were seen with L6 myotubules and 3T3-L1 adipocytes, whereby insulin-stimulated glucose uptake was improved following α-lipoic acid exposure (Estrada *et al.*, 1996). However, treatment with wortmannin, a noted PI3K inhibitor, blunted only a portion of these effects. In accordance with other later studies (Henriksen *et al.*, 1997; Yaworsky *et al.*, 2000) it seems plausible that α-lipoic acid may work primarily independently of the insulin signalling pathway.

2.4.5.2 Animal studies in insulin resistance

The primary goal of animal studies in antioxidant supplementation is to determine what effects antioxidants may have on minimizing ROS load, and subsequently on the development of insulin resistance. However, this task has been difficult to perform and validate due to the broad spectrum of whole-body adaptations known to occur with oxidative stress. On one hand, studies have been performed using rodent models that have an inhibited NAPDH oxidase system in adipose tissue (Furukawa *et al.*, 2004) or overexpress proton-dispersing uncoupling protein 1 in skeletal muscle (Li *et al.*, 2000) to show that minimized mitochondrial ROS generation would protect against obesity-related insulin resistance. However, with respect to the results of Li and others (2000), model-associated tissue pathologies such as muscle wasting and weakness and decreased

muscle performance may be a limitation to the applicability to the normal human population with functional muscle tissue (Han *et al.*, 2004).

On the other hand, studies have looked at antioxidant supplementation to maintain the prooxidant/oxidant ratio within normal parameters. Jacob and colleagues (1996) were able to show that in obese Zucker rats (fa/fa), noted models of insulin resistance and elevated oxidative stress, a 10-day parenteral administration of α-lipoic acid (30 mg/kg bodyweight) could enhance insulin-stimulated glucose uptake in epitrochlearis muscle. In a similar Zucker rat animal model, feeding a vitamin E enriched diet (0.5% w/w) for 4 weeks reversed of elevated epi-prostaglandins, markers of oxidative stress, and significant normalization in fasting plasma insulin (Laight *et al.*, 1999). Similar to the previously mentioned experimentation, the myriad of metabolic effects associated with oxidative stress and various antioxidant treatments create a problem with determining causation. Specifically, these studies are not able to determine if supplementation is able to reduce the ROS load, or if an independent mechanism lowers oxidative stress through the improvement of glucose clearance and insulin sensitivity.

2.4.5.3 Human studies in insulin resistance

Daily dietary supplementation with vitamin E (800 IU), in conjunction with vitamin C and β-carotene resulted in a moderate improvement in measures of insulin sensitivity and overall oxidative stress load (Gokkusu *et al.*, 2001; Vincent

et al., 2009). Consistent with this result, Manning and colleagues (2004) showed a similar finding supplementing 800 to 1200 IU of vitamin E for 3 to 6 months; however improvements in insulin sensitivity were thought to be transient. Specific to this study, vitamin E-associated improvements in insulin sensitivity were not maintained after 3 months despite compliance, leading to the hypothesis that 800 IU/day is above the intestinal saturation point in dietary interventions. Interestingly, daily vitamin E supplementation (400 mg or 440 IU) in normal weight and overweight patients with diabetes reduced oxidative stress, however fasting and postprandial glucose levels were not significantly different (Sharma *et al.*, 2000a; Sharma, *et al.*, 2000b). The lack of change in glucose homeostasis in the latter studies may be due to the relatively shorter duration of supplementation (1 month versus 3 months) and a lower overall dosage of the antioxidant (440 IU versus approximately 800 IU).

2.5 Summary

Understanding how GC excess affects glucose clearance requires consideration of the multiple pathways involved. It has been shown here that not only are GC physiology, oxidative stress and glucose clearance highly interconnected, they involve many steps. However, while the bulk of the literature links glucose clearance and oxidative stress, the mechanisms at work have not been established. As the scope of analysis narrows from broader physiological endpoints such as decreased glucose clearance and insulin resistance to more

specific endpoints like inhibited GLUT-4 translocation, one can find various entry points for elucidating a mechanism for the inhibition of glucose clearance.

GC excess was shown to cause hyperglycemia and hyperinsulinemia associated with decreased glucose disposal and blunted insulin signalling. Furthermore, studies have suggested that GC excess induced oxidative stress through the overproduction of reactive oxygen species, and additionally, that both the prooxidative state could be mitigated by treatment with antioxidants such as α-tocopherol and α-lipoic acid. Despite antioxidant supplementation studies demonstrating improvements in insulin sensitivity in various models, there is a lack of understanding of the connection between a GC-induced insulin resistant model and an antioxidant supplementation with respect to preventing GC-induced inhibition of glucose clearance. Thus, the bulk of this data provides a rational for a more thorough investigation to find possible connections between GC excess and the proven positive effects of α-tocopherol supplementation.

2.6 Literature Cited

Abidi, S. L. (2000). Chromatographic analysis of tocol-derived lipid antioxidants. *Journal of Chromatography.A, 881*(1-2), 197-216.

Aguirre, V., Uchida, T., Yenush, L., Davis, R., & White, M. F. (2000). The c-jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of ser(307). *The Journal of Biological Chemistry, 275*(12), 9047-9054.

Archuleta, T. L., Lemieux, A. M., Saengsirisuwan, V., Teachey, M. K., Lindborg, K. A., Kim, J. S., et al. (2009). Oxidant stress-induced loss of IRS-1 and IRS-2 proteins in rat skeletal muscle: Role of p38 MAPK. *Free Radical Biology & Medicine, 47*(10), 1486-1493.

Baker, A. F., Briehl, M. M., Dorr, R., & Powis, G. (1996). Decreased antioxidant defence and increased oxidant stress during dexamethasone-induced apoptosis: Bcl-2 prevents the loss of antioxidant enzyme activity. *Cell Death and Differentiation, 3*(2), 207-213.

Bogan, J. S., & Kandror, K. V. (2010). Biogenesis and regulation of insulin-responsive vesicles containing GLUT4. *Current Opinion in Cell Biology,*

Bouzakri, K., Zachrisson, A., Al-Khalili, L., Zhang, B. B., Koistinen, H. A., Krook, A., et al. (2006). siRNA-based gene silencing reveals specialized roles of IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism in human skeletal muscle. *Cell Metabolism, 4*(1), 89-96.

Brigelius-Flohe, R. (2009). Vitamin E: The shrew waiting to be tamed. *Free Radical Biology & Medicine*, 46(5), 543-554.

Brownlee, M. (2000). Negative consequences of glycation. *Metabolism: Clinical and Experimental, 49*(2 Suppl 1), 9-13.

Bruss, M. D., Arias, E. B., Lienhard, G. E., & Cartee, G. D. (2005). Increased phosphorylation of akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes, 54*(1), 41-50.

Buren, J., Lai, Y. C., Lundgren, M., Eriksson, J. W., & Jensen, J. (2008). Insulin action and signalling in fat and muscle from dexamethasone-treated rats. *Archives of Biochemistry and Biophysics, 474*(1), 91-101.

Buren, J., Liu, H. X., Jensen, J., & Eriksson, J. W. (2002). Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in

primary cultured rat adipocytes. *European Journal of Endocrinology / European Federation of Endocrine Societies, 146*(3), 419-429.

Cantin, A., & Crystal, R. G. (1985). Oxidants, antioxidants and the pathogenesis of emphysema. *European Journal of Respiratory Diseases.Supplement, 139*, 7-17.

Cantrill, H. L., Waltman, S. R., Palmberg, P. F., Zink, H. A., & Becker, B. (1975). In vitro determination of relative corticosteroid potency. *The Journal of Clinical Endocrinology and Metabolism, 40*(6), 1073-1077.

Carvalho-Filho, M. A., Ueno, M., Hirabara, S. M., Seabra, A. B., Carvalheira, J. B., de Oliveira, M. G., et al. (2005). S-nitrosation of the insulin receptor, insulin receptor substrate 1, and protein kinase B/Akt: A novel mechanism of insulin resistance. *Diabetes, 54*(4), 959-967.

Chavez, J. A., Roach, W. G., Keller, S. R., Lane, W. S., & Lienhard, G. E. (2008). Inhibition of GLUT4 translocation by Tbc1d1, a rab GTPase-activating protein abundant in skeletal muscle, is partially relieved by AMP-activated protein kinase activation. *The Journal of Biological Chemistry, 283*(14), 9187-9195.

Christ-Crain, M., Kola, B., Lolli, F., Fekete, C., Seboek, D., Wittmann, G., et al. (2008). AMP-activated protein kinase mediates glucocorticoid-induced metabolic changes: A novel mechanism in cushing's syndrome. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 22*(6), 1672-1683.

Dalla Man, C., Campioni, M., Polonsky, K. S., Basu, R., Rizza, R. A., Toffolo, G., et al. (2005). Two-hour seven-sample oral glucose tolerance test and meal protocol: Minimal model assessment of beta-cell responsivity and insulin sensitivity in nondiabetic individuals. *Diabetes, 54*(11), 3265-3273.

De Mattia, G., Bravi, M. C., Laurenti, O., Cassone-Faldetta, M., Armiento, A., Ferri, C., et al. (1998). Influence of reduced glutathione infusion on glucose metabolism in patients with non-insulin-dependent diabetes mellitus. Metabolism: Clinical and Experimental, 47(8), 993-997.

Dimitriadis, G., Leighton, B., Parry-Billings, M., Sasson, S., Young, M., Krause, U., et al. (1997). Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle. *The Biochemical Journal, 321 (Pt 3)*(Pt 3), 707-712.

Dokken, B. B., Saengsirisuwan, V., Kim, J. S., Teachey, M. K., & Henriksen, E. J. (2008). Oxidative stress-induced insulin resistance in rat skeletal muscle:

Role of glycogen synthase kinase-3. *American Journal of Physiology.Endocrinology and Metabolism, 294*(3), E615-21.

Dugani, C. B., & Klip, A. (2005). Glucose transporter 4: Cycling, compartments and controversies. *EMBO Reports, 6*(12), 1137-1142.

Dummler, B., & Hemmings, B. A. (2007). Physiological roles of PKB/Akt isoforms in development and disease. *Biochemical Society Transactions, 35*(Pt 2), 231-235.

Eguez, L., Lee, A., Chavez, J. A., Miinea, C. P., Kane, S., Lienhard, G. E., et al. (2005). Full intracellular retention of GLUT4 requires AS160 rab GTPase activating protein. *Cell Metabolism, 2*(4), 263-272.

Estrada, D. E., Ewart, H. S., Tsakiridis, T., Volchuk, A., Ramlal, T., Tritschler, H., et al. (1996). Stimulation of glucose uptake by the natural coenzyme alpha-lipoic acid/thioctic acid: Participation of elements of the insulin signaling pathway. *Diabetes, 45*(12), 1798-1804.

Ewart, H. S., Somwar, R., & Klip, A. (1998). Dexamethasone stimulates the expression of GLUT1 and GLUT4 proteins via different signalling pathways in L6 skeletal muscle cells. *FEBS Letters, 425*(1), 179-183.

Farese, R. V., Sajan, M. P., & Standaert, M. L. (2005). Atypical protein kinase C in insulin action and insulin resistance. *Biochemical Society Transactions, 33*(Pt 2), 350-353.

Fazakerley, D. J., Holman, G. D., Marley, A., James, D. E., Stockli, J., & Coster, A. C. (2010). Kinetic evidence for unique regulation of GLUT4 trafficking by insulin and AMP-activated protein kinase activators in L6 myotubes. *The Journal of Biological Chemistry, 285*(3), 1653-1660.

Ferrannini, E., Gastaldelli, A., Miyazaki, Y., Matsuda, M., Mari, A., & DeFronzo, R. A. (2005). Beta-cell function in subjects spanning the range from normal glucose tolerance to overt diabetes: A new analysis. *The Journal of Clinical Endocrinology and Metabolism, 90*(1), 493-500.

Ferrannini, E., Simonson, D. C., Katz, L. D., Reichard, G.,Jr, Bevilacqua, S., Barrett, E. J., et al. (1988). The disposal of an oral glucose load in patients with non-insulin-dependent diabetes. *Metabolism: Clinical and Experimental, 37*(1), 79-85.

Fisher, J. S., Gao, J., Han, D. H., Holloszy, J. O., & Nolte, L. A. (2002). Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *American Journal of Physiology.Endocrinology and Metabolism, 282*(1), E18-23.

Fritsche, L., Weigert, C., Haring, H. U., & Lehmann, R. (2008). How insulin receptor substrate proteins regulate the metabolic capacity of the liver--implications for health and disease. *Current Medicinal Chemistry, 15*(13), 1316-1329.

Fukuoka, H., Iida, K., Nishizawa, H., Imanaka, M., Takeno, R., Iguchi, G., et al. (2010). IGF-I stimulates reactive oxygen species (ROS) production and inhibits insulin-dependent glucose uptake via ROS in 3T3-L1 adipocytes. *Growth Hormone & IGF Research : Official Journal of the Growth Hormone Research Society and the International IGF Research Society,*

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., et al. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of Clinical Investigation, 114*(12), 1752-1761.

Gaidhu, M. P., Perry, R. L., Noor, F., & Ceddia, R. B. (2010). Disruption of AMPK{alpha}1 signaling prevents AICAR-induced inhibition of AS160/TBC1D4 phosphorylation and glucose uptake in primary rat adipocytes. *Molecular Endocrinology (Baltimore, Md.),*

Gao, Z., Hwang, D., Bataille, F., Lefevre, M., York, D., Quon, M. J., et al. (2002). Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *The Journal of Biological Chemistry, 277*(50), 48115-48121.

Giorgino, F., Almahfouz, A., Goodyear, L. J., & Smith, R. J. (1993). Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle in vivo. *The Journal of Clinical Investigation, 91*(5), 2020-2030.

Giustarini, D., Dalle-Donne, I., Tsikas, D., & Rossi, R. (2009). Oxidative stress and human diseases: Origin, link, measurement, mechanisms, and biomarkers. *Critical Reviews in Clinical Laboratory Sciences, 46*(5-6), 241-281.

Gokkusu, C., Palanduz, S., Ademoglu, E., & Tamer, S. (2001). Oxidant and antioxidant systems in niddm patients: Influence of vitamin E supplementation. *Endocrine Research, 27*(3), 377-386.

Green, C. J., Goransson, O., Kular, G. S., Leslie, N. R., Gray, A., Alessi, D. R., et al. (2008). Use of akt inhibitor and a drug-resistant mutant validates a critical role for protein kinase B/Akt in the insulin-dependent regulation of glucose and system A amino acid uptake. *The Journal of Biological Chemistry, 283*(41), 27653-27667.

Han, D. H., Nolte, L. A., Ju, J. S., Coleman, T., Holloszy, J. O., & Semenkovich, C. F. (2004). UCP-mediated energy depletion in skeletal muscle increases glucose transport despite lipid accumulation and mitochondrial dysfunction. *American Journal of Physiology.Endocrinology and Metabolism, 286*(3), E347-53.

Hansen, L. L., Ikeda, Y., Olsen, G. S., Busch, A. K., & Mosthaf, L. (1999). Insulin signaling is inhibited by micromolar concentrations of H(2)O(2). evidence for a role of H(2)O(2) in tumor necrosis factor alpha-mediated insulin resistance. *The Journal of Biological Chemistry, 274*(35), 25078-25084.

Hartnett, M. E., Stratton, R. D., Browne, R. W., Rosner, B. A., Lanham, R. J., & Armstrong, D. (2000). Serum markers of oxidative stress and severity of diabetic retinopathy. *Diabetes Care, 23*(2), 234-240.

Hayes, J. D., & McLellan, L. I. (1999). Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radical Research, 31*(4), 273-300.

Henriksen, E. J., Jacob, S., Streeper, R. S., Fogt, D. L., Hokama, J. Y., & Tritschler, H. J. (1997). Stimulation by alpha-lipoic acid of glucose transport activity in skeletal muscle of lean and obese zucker rats. *Life Sciences, 61*(8), 805-812.

Herschkovitz, A., Liu, Y. F., Ilan, E., Ronen, D., Boura-Halfon, S., & Zick, Y. (2007). Common inhibitory serine sites phosphorylated by IRS-1 kinases, triggered by insulin and inducers of insulin resistance. *The Journal of Biological Chemistry, 282*(25), 18018-18027.

Higaki, Y., Mikami, T., Fujii, N., Hirshman, M. F., Koyama, K., Seino, T., et al. (2008). Oxidative stress stimulates skeletal muscle glucose uptake through a phosphatidylinositol 3-kinase-dependent pathway. *American Journal of Physiology.Endocrinology and Metabolism, 294*(5), E889-97.

Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., et al. (2002). A central role for JNK in obesity and insulin resistance. *Nature, 420*(6913), 333-336.

Hoehn, K. L., Hohnen-Behrens, C., Cederberg, A., Wu, L. E., Turner, N., Yuasa, T., et al. (2008). IRS1-independent defects define major nodes of insulin resistance. *Cell Metabolism, 7*(5), 421-433.

Houstis, N., Rosen, E. D., & Lander, E. S. (2006). Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature, 440*(7086), 944-948.

Hunt, J. V., & Wolff, S. P. (1991). Oxidative glycation and free radical production: A causal mechanism of diabetic complications. *Free Radical Research Communications, 12-13 Pt 1,* 115-123.

Ikonomov, O. C., Sbrissa, D., Dondapati, R., & Shisheva, A. (2007). ArPIKfyve-PIKfyve interaction and role in insulin-regulated GLUT4 translocation and glucose transport in 3T3-L1 adipocytes. *Experimental Cell Research, 313*(11), 2404-2416.

Inoguchi, T., Li, P., Umeda, F., Yu, H. Y., Kakimoto, M., Imamura, M., et al. (2000). High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes, 49*(11), 1939-1945.

International Institute of Medicine. (2000). Standing committee on the scientific evaluation of dietary reference intake of vitamin C, vitamin E, selenium, and carotonoids.

Ishikura, S., Bilan, P. J., & Klip, A. (2007). Rabs 8A and 14 are targets of the insulin-regulated rab-GAP AS160 regulating GLUT4 traffic in muscle cells. *Biochemical and Biophysical Research Communications, 353*(4), 1074-1079.

Iuchi, T., Akaike, M., Mitsui, T., Ohshima, Y., Shintani, Y., Azuma, H., et al. (2003). Glucocorticoid excess induces superoxide production in vascular endothelial cells and elicits vascular endothelial dysfunction. *Circulation Research, 92*(1), 81-87.

Jackson, M. J. (1999). An overview of methods for assessment of free radical activity in biology. *The Proceedings of the Nutrition Society, 58*(4), 1001-1006.

Jacob, S., Streeper, R. S., Fogt, D. L., Hokama, J. Y., Tritschler, H. J., Dietze, G. J., et al. (1996). The antioxidant alpha-lipoic acid enhances insulin-stimulated glucose metabolism in insulin-resistant rat skeletal muscle. *Diabetes, 45*(8), 1024-1029.

Jenner, P. (2003). Oxidative stress in parkinson's disease. *Annals of Neurology, 53 Suppl 3,* S26-36; discussion S36-8.

Jiang, B. H., Aoki, M., Zheng, J. Z., Li, J., & Vogt, P. K. (1999). Myogenic signaling of phosphatidylinositol 3-kinase requires the serine-threonine kinase Akt/protein kinase B. *Proceedings of the National Academy of Sciences of the United States of America, 96*(5), 2077-2081.

Kaiser, C., & James, S. R. (2004). Acetylation of insulin receptor substrate-1 is permissive for tyrosine phosphorylation. *BMC Biology, 2*, 23.

Kajita, K., Ishizuka, T., Miura, A., Kanoh, Y., Ishizawa, M., Kimura, M., et al. (2001). Glucocorticoid-induced insulin resistance associates with activation of protein kinase C isoforms. *Cellular Signalling, 13*(3), 169-175.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., & Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell, 120*(5), 649-661.

Kane, S., & Lienhard, G. E. (2005). Calmodulin binds to the rab GTPase activating protein required for insulin-stimulated GLUT4 translocation. *Biochemical and Biophysical Research Communications, 335*(1), 175-180.

Kane, S., Sano, H., Liu, S. C., Asara, J. M., Lane, W. S., Garner, C. C., et al. (2002). A method to identify serine kinase substrates. akt phosphorylates a novel adipocyte protein with a rab GTPase-activating protein (GAP) domain. *The Journal of Biological Chemistry, 277*(25), 22115-22118.

Kaneto, H., Nakatani, Y., Miyatsuka, T., Kawamori, D., Matsuoka, T. A., Matsuhisa, M., et al. (2004). Possible novel therapy for diabetes with cell-permeable JNK-inhibitory peptide. *Nature Medicine, 10*(10), 1128-1132.

Kehrer, J. P. (1993). Free radicals as mediators of tissue injury and disease. *Critical Reviews in Toxicology, 23*(1), 21-48.

Khan, N., & Swartz, H. (2002). Measurements in vivo of parameters pertinent to ROS/RNS using EPR spectroscopy. *Molecular and Cellular Biochemistry, 234-235*(1-2), 341-357.

Kim, J. S., Saengsirisuwan, V., Sloniger, J. A., Teachey, M. K., & Henriksen, E. J. (2006). Oxidant stress and skeletal muscle glucose transport: Roles of insulin signaling and p38 MAPK. *Free Radical Biology & Medicine, 41*(5), 818-824.

Kitamura, T., Ogawa, W., Sakaue, H., Hino, Y., Kuroda, S., Takata, M., et al. (1998). Requirement for activation of the serine-threonine kinase akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Molecular and Cellular Biology, 18*(7), 3708-3717.

Klein, A. L., Berkaw, M. N., Buse, M. G., & Ball, L. E. (2009). O-linked N-acetylglucosamine modification of insulin receptor substrate-1 occurs in close proximity to multiple SH2 domain binding motifs. *Molecular & Cellular Proteomics : MCP, 8*(12), 2733-2745.

Kohn, A. D., Summers, S. A., Birnbaum, M. J., & Roth, R. A. (1996). Expression of a constitutively active akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *The Journal of Biological Chemistry, 271*(49), 31372-31378.

Kramer, H. F., Taylor, E. B., Witczak, C. A., Fujii, N., Hirshman, M. F., & Goodyear, L. J. (2007). Calmodulin-binding domain of AS160 regulates contraction- but not insulin-stimulated glucose uptake in skeletal muscle. *Diabetes, 56*(12), 2854-2862.

Kramer, H. F., Witczak, C. A., Fujii, N., Jessen, N., Taylor, E. B., Arnolds, D. E., et al. (2006a). Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. *Diabetes, 55*(7), 2067-2076.

Kramer, H. F., Witczak, C. A., Taylor, E. B., Fujii, N., Hirshman, M. F., & Goodyear, L. J. (2006b). AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *The Journal of Biological Chemistry, 281*(42), 31478-31485.

Kruszynska, Y. T., Worrall, D. S., Ofrecio, J., Frias, J. P., Macaraeg, G., & Olefsky, J. M. (2002). Fatty acid-induced insulin resistance: Decreased muscle PI3K activation but unchanged akt phosphorylation. *The Journal of Clinical Endocrinology and Metabolism, 87*(1), 226-234.

Kuhla, A., Hettwer, C., Menger, M. D., & Vollmar, B. (2010). Oxidative stress-associated rise of hepatic protein glycation increases inflammatory liver injury in uncoupling protein-2 deficient mice. *Laboratory Investigation; a Journal of Technical Methods and Pathology,*

Kuroda, S., Ogawa, W., Kitamura, T., Konishi, H., Kikkawa, U., & Kasuga, M. (1998). Requirement of the serine-threonine kinase akt for heat treatment-induced activation of p70 S6 kinase. *Biochemical and Biophysical Research Communications, 249*(3), 781-785.

Laight, D. W., Desai, K. M., Gopaul, N. K., Anggard, E. E., & Carrier, M. J. (1999). F2-isoprostane evidence of oxidant stress in the insulin resistant, obese zucker rat: Effects of vitamin E. *European Journal of Pharmacology, 377*(1), 89-92.

Lastra, G., Whaley-Connell, A., Manrique, C., Habibi, J., Gutweiler, A. A., Appesh, L., et al. (2008). Low-dose spironolactone reduces reactive oxygen species generation and improves insulin-stimulated glucose transport in skeletal muscle in the TG(mRen2)27 rat. *American Journal of Physiology.Endocrinology and Metabolism, 295*(1), E110-6.

Leung, H. W., Vang, M. J., & Mavis, R. D. (1981). The cooperative interaction between vitamin E and vitamin C in suppression of peroxidation of membrane phospholipids. *Biochimica Et Biophysica Acta, 664*(2), 266-272.

Lewis, G. F., Carpentier, A., Adeli, K., & Giacca, A. (2002). Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocrine Reviews, 23*(2), 201-229.

Li, B., Nolte, L. A., Ju, J. S., Han, D. H., Coleman, T., Holloszy, J. O., et al. (2000). Skeletal muscle respiratory uncoupling prevents diet-induced obesity and insulin resistance in mice. *Nature Medicine, 6*(10), 1115-1120.

Liao, Y., & Hung, M. C. (2010). Physiological regulation of akt activity and stability. *American Journal of Translational Research, 2*(1), 19-42.

Liapi, C., & Chrousos, G. (1992). Glucocorticoids. In S. Jaffe, & J. Aranda (Eds.), *Pediatric pharmacology* (2nd ed.,). Philadephia: WB Saunders Co.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature, 443*(7113), 787-795.

Lo, Y. Y., Wong, J. M., & Cruz, T. F. (1996). Reactive oxygen species mediate cytokine activation of c-jun NH2-terminal kinases. *The Journal of Biological Chemistry, 271*(26), 15703-15707.

Loh, K., Deng, H., Fukushima, A., Cai, X., Boivin, B., Galic, S., et al. (2009). Reactive oxygen species enhance insulin sensitivity. *Cell Metabolism, 10*(4), 260-272.

Ludvik, B., Nolan, J. J., Roberts, A., Baloga, J., Joyce, M., Bell, J. M., et al. (1997). Evidence for decreased splanchnic glucose uptake after oral glucose administration in non-insulin-dependent diabetes mellitus. *The Journal of Clinical Investigation, 100*(9), 2354-2361.

Lundgren, M., Buren, J., Ruge, T., Myrnas, T., & Eriksson, J. W. (2004). Glucocorticoids down-regulate glucose uptake capacity and insulin-signaling proteins in omental but not subcutaneous human adipocytes. *The Journal of Clinical Endocrinology and Metabolism, 89*(6), 2989-2997.

Maddux, B. A., See, W., Lawrence, J. C.,Jr, Goldfine, A. L., Goldfine, I. D., & Evans, J. L. (2001). Protection against oxidative stress-induced insulin resistance in rat L6 muscle cells by mircomolar concentrations of alpha-lipoic acid. *Diabetes, 50*(2), 404-410.

Manning, P. J., Sutherland, W. H., Walker, R. J., Williams, S. M., De Jong, S. A., Ryalls, A. R., et al. (2004). Effect of high-dose vitamin E on insulin

resistance and associated parameters in overweight subjects. *Diabetes Care, 27*(9), 2166-2171.

Matheny, R. W., Jr., & Adamo, M. L. (2009). Current perspectives on akt akt-ivation and akt-ions. *Proceedings of the Society for Experimental Biology and Medicine,*

Matsuoka, T., Kajimoto, Y., Watada, H., Kaneto, H., Kishimoto, M., Umayahara, Y., et al. (1997). Glycation-dependent, reactive oxygen species-mediated suppression of the insulin gene promoter activity in HIT cells. *The Journal of Clinical Investigation, 99*(1), 144-150.

McClung, J. P., Roneker, C. A., Mu, W., Lisk, D. J., Langlais, P., Liu, F., et al. (2004). Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. *Proceedings of the National Academy of Sciences of the United States of America, 101*(24), 8852-8857.

Meyer, C., Dostou, J., Nadkarni, V., & Gerich, J. (1998). Effects of physiological hyperinsulinemia on systemic, renal, and hepatic substrate metabolism. *The American Journal of Physiology, 275*(6 Pt 2), F915-21.

Miinea, C. P., Sano, H., Kane, S., Sano, E., Fukuda, M., Peranen, J., et al. (2005). AS160, the akt substrate regulating GLUT4 translocation, has a functional rab GTPase-activating protein domain. *The Biochemical Journal, 391*(Pt 1), 87-93.

Min, J., Okada, S., Kanzaki, M., Elmendorf, J. S., Coker, K. J., Ceresa, B. P., et al. (1999). Synip: A novel insulin-regulated syntaxin 4-binding protein mediating GLUT4 translocation in adipocytes. *Molecular Cell, 3*(6), 751-760.

Monnier, L., Mas, E., Ginet, C., Michel, F., Villon, L., Cristol, J. P., et al. (2006). Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. *JAMA : The Journal of the American Medical Association, 295*(14), 1681-1687.

Moreira, P. I., Santos, M. S., Oliveira, C. R., Shenk, J. C., Nunomura, A., Smith, M. A., et al. (2008). Alzheimer disease and the role of free radicals in the pathogenesis of the disease. *CNS & Neurological Disorders Drug Targets, 7*(1), 3-10.

Mu, J., Brozinick, J. T.,Jr, Valladares, O., Bucan, M., & Birnbaum, M. J. (2001). A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Molecular Cell, 7*(5), 1085-1094.

Munck, A., Guyre, P. M., & Holbrook, N. J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocrine Reviews, 5*(1), 25-44.

Musi, N., Hayashi, T., Fujii, N., Hirshman, M. F., Witters, L. A., & Goodyear, L. J. (2001). AMP-activated protein kinase activity and glucose uptake in rat skeletal muscle. *American Journal of Physiology.Endocrinology and Metabolism, 280*(5), E677-84.

Nadler, S. T., Stoehr, J. P., Rabaglia, M. E., Schueler, K. L., Birnbaum, M. J., & Attie, A. D. (2001). Normal Akt/PKB with reduced PI3K activation in insulin-resistant mice. *American Journal of Physiology.Endocrinology and Metabolism, 281*(6), E1249-54.

Nakamura, S., Takamura, T., Matsuzawa-Nagata, N., Takayama, H., Misu, H., Noda, H., et al. (2009). Palmitate induces insulin resistance in H4IIEC3 hepatocytes through reactive oxygen species produced by mitochondria. *The Journal of Biological Chemistry, 284*(22), 14809-14818.

Nelson, D., & Cox, M. (2005). *Lehninger principles of biochemistry* (4th edition ed.). New York: W. H. Freeman and Company.

Newell-Price, J., Bertagna, X., Grossman, A. B., & Nieman, L. K. (2006). Cushing's syndrome. *Lancet, 367*(9522), 1605-1617.

Ngo, S., Barry, J. B., Nisbet, J. C., Prins, J. B., & Whitehead, J. P. (2009). Reduced phosphorylation of AS160 contributes to glucocorticoid-mediated inhibition of glucose uptake in human and murine adipocytes. *Molecular and Cellular Endocrinology, 302*(1), 33-40.

Nicod, N., Giusti, V., Besse, C., & Tappy, L. (2003). Metabolic adaptations to dexamethasone-induced insulin resistance in healthy volunteers. *Obesity Research, 11*(5), 625-631.

Orzechowski, A., Ostaszewski, P., Wilczak, J., Jank, M., Bałasińska, B., War, P., et al. (2002). Rats with a glucocorticoid-induced catabolic state show symptoms of oxidative stress and spleen atrophy: The effects of age and recovery. *Journal of Veterinary Medicine Series A, 49*(5), 256-263.

Pagano, G., Cavallo-Perin, P., Cassader, M., Bruno, A., Ozzello, A., Masciola, P., et al. (1983). An in vivo and in vitro study of the mechanism of prednisone-induced insulin resistance in healthy subjects. *The Journal of Clinical Investigation, 72*(5), 1814-1820.

Paolisso, G., D'Amore, A., Galzerano, D., Balbi, V., Giugliano, D., Varricchio, M., et al. (1993). Daily vitamin E supplements improve metabolic control

but not insulin secretion in elderly type II diabetic patients. *Diabetes Care, 16*(11), 1433-1437.

Paolisso, G., D'Amore, A., Giugliano, D., Ceriello, A., Varricchio, M., & D'Onofrio, F. (1993). Pharmacologic doses of vitamin E improve insulin action in healthy subjects and non-insulin-dependent diabetic patients. *The American Journal of Clinical Nutrition, 57*(5), 650-656.

Pastore, A., Federici, G., Bertini, E., & Piemonte, F. (2003). Analysis of glutathione: Implication in redox and detoxification. *Clinica Chimica Acta; International Journal of Clinical Chemistry, 333*(1), 19-39.

Peck, G. R., Chavez, J. A., Roach, W. G., Budnik, B. A., Lane, W. S., Karlsson, H. K., et al. (2009). Insulin-stimulated phosphorylation of the rab GTPase-activating protein TBC1D1 regulates GLUT4 translocation. *The Journal of Biological Chemistry, 284*(44), 30016-30023.

Peck, G. R., Ye, S., Pham, V., Fernando, R. N., Macaulay, S. L., Chai, S. Y., et al. (2006). Interaction of the akt substrate, AS160, with the glucose transporter 4 vesicle marker protein, insulin-regulated aminopeptidase. *Molecular Endocrinology (Baltimore, Md.), 20*(10), 2576-2583.

Pehmoller, C., Treebak, J. T., Birk, J. B., Chen, S., Mackintosh, C., Hardie, D. G., et al. (2009). Genetic disruption of AMPK signaling abolishes both contraction- and insulin-stimulated TBC1D1 phosphorylation and 14-3-3 binding in mouse skeletal muscle. *American Journal of Physiology.Endocrinology and Metabolism, 297*(3), E665-75.

Puthanveetil, P., Wang, F., Kewalramani, G., Kim, M. S., Hosseini-Beheshti, E., Ng, N., et al. (2008). Cardiac glycogen accumulation after dexamethasone is regulated by AMPK. *American Journal of Physiology.Heart and Circulatory Physiology, 295*(4), H1753-62.

Rafacho, A., Cestari, T. M., Taboga, S. R., Boschero, A. C., & Bosqueiro, J. R. (2009). High doses of dexamethasone induce increased beta-cell proliferation in pancreatic rat islets. *American Journal of Physiology.Endocrinology and Metabolism, 296*(4), E681-9.

Ragheb, R., Shanab, G. M., Medhat, A. M., Seoudi, D. M., Adeli, K., & Fantus, I. G. (2009). Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: Evidence for PKC activation and oxidative stress-activated signaling pathways. *Biochemical and Biophysical Research Communications, 389*(2), 211-216.

Rizza, R. A., Mandarino, L. J., & Gerich, J. E. (1982). Cortisol-induced insulin resistance in man: Impaired suppression of glucose production and

stimulation of glucose utilization due to a postreceptor detect of insulin action. *The Journal of Clinical Endocrinology and Metabolism, 54*(1), 131-138.

Rojas, F. A., Hirata, A. E., & Saad, M. J. (2003). Regulation of insulin receptor substrate-2 tyrosine phosphorylation in animal models of insulin resistance. *Endocrine, 21*(2), 115-122.

Rosmond, R. (2003). Stress induced disturbances of the HPA axis: A pathway to type 2 diabetes? *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research, 9*(2), RA35-9.

Rudich, A., Tirosh, A., Potashnik, R., Hemi, R., Kanety, H., & Bashan, N. (1998). Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes, 47*(10), 1562-1569.

Russell, R. R.,3rd, Bergeron, R., Shulman, G. I., & Young, L. H. (1999). Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *The American Journal of Physiology, 277*(2 Pt 2), H643-9.

Ruzzin, J., Wagman, A. S., & Jensen, J. (2005). Glucocorticoid-induced insulin resistance in skeletal muscles: Defects in insulin signalling and the effects of a selective glycogen synthase kinase-3 inhibitor. *Diabetologia, 48*(10), 2119-2130.

Saad, M. J., Folli, F., & Kahn, C. R. (1995). Insulin and dexamethasone regulate insulin receptors, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in fao hepatoma cells. *Endocrinology, 136*(4), 1579-1588.

Saad, M. J., Folli, F., Kahn, J. A., & Kahn, C. R. (1993). Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *The Journal of Clinical Investigation, 92*(4), 2065-2072.

Saberi, B., Shinohara, M., Ybanez, M. D., Hanawa, N., Gaarde, W. A., Kaplowitz, N., et al. (2008). Regulation of H(2)O(2)-induced necrosis by PKC and AMP-activated kinase signaling in primary cultured hepatocytes. *American Journal of Physiology.Cell Physiology, 295*(1), C50-63.

Sakoda, H., Ogihara, T., Anai, M., Funaki, M., Inukai, K., Katagiri, H., et al. (2000). Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. *Diabetes, 49*(10), 1700-1708.

Salonen, J. T., Nyyssonen, K., Tuomainen, T. P., Maenpaa, P. H., Korpela, H., Kaplan, G. A., et al. (1995). Increased risk of non-insulin dependent diabetes mellitus at low plasma vitamin E concentrations: A four year follow up study in men. *BMJ (Clinical Research Ed.), 311*(7013), 1124-1127.

Salt, I. P., Johnson, G., Ashcroft, S. J., & Hardie, D. G. (1998). AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *The Biochemical Journal, 335 (Pt 3)*(Pt 3), 533-539.

Samiec, P. S., Drews-Botsch, C., Flagg, E. W., Kurtz, J. C., Sternberg, P.,Jr, Reed, R. L., et al. (1998). Glutathione in human plasma: Decline in association with aging, age-related macular degeneration, and diabetes. *Free Radical Biology & Medicine, 24*(5), 699-704.

Sano, H., Eguez, L., Teruel, M. N., Fukuda, M., Chuang, T. D., Chavez, J. A., et al. (2007). Rab10, a target of the AS160 rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. *Cell Metabolism, 5*(4), 293-303.

Sano, H., Kane, S., Sano, E., Miinea, C. P., Asara, J. M., Lane, W. S., et al. (2003). Insulin-stimulated phosphorylation of a rab GTPase-activating protein regulates GLUT4 translocation. *The Journal of Biological Chemistry, 278*(17), 14599-14602.

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews, 21*(1), 55-89.

Schacke, H., Docke, W. D., & Asadullah, K. (2002). Mechanisms involved in the side effects of glucocorticoids. *Pharmacology & Therapeutics, 96*(1), 23-43.

Schafer, S. C., Wallerath, T., Closs, E. I., Schmidt, C., Schwarz, P. M., Forstermann, U., et al. (2005). Dexamethasone suppresses eNOS and CAT-1 and induces oxidative stress in mouse resistance arterioles. *AJP - Heart and Circulatory Physiology, 288*(1), H436-444.

Schneiter, P., & Tappy, L. (1998). Kinetics of dexamethasone-induced alterations of glucose metabolism in healthy humans. *The American Journal of Physiology, 275*(5 Pt 1), E806-13.

Schuessler, H., & Schilling, K. (1984). Oxygen effect in the radiolysis of proteins. part 2. bovine serum albumin. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry, and Medicine, 45*(3), 267-281.

Sesti, G., Federici, M., Hribal, M. L., Lauro, D., Sbraccia, P., & Lauro, R. (2001). Defects of the insulin receptor substrate (IRS) system in human metabolic disorders. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 15*(12), 2099-2111.

Sharma, A., Kharb, S., Chugh, S. N., Kakkar, R., & Singh, G. P. (2000a). Effect of glycemic control and vitamin E supplementation on total glutathione content in non-insulin-dependent diabetes mellitus. *Annals of Nutrition & Metabolism, 44*(1), 11-13.

Sharma, A., Kharb, S., Chugh, S. N., Kakkar, R., & Singh, G. P. (2000b). Evaluation of oxidative stress before and after control of glycemia and after vitamin E supplementation in diabetic patients. *Metabolism: Clinical and Experimental, 49*(2), 160-162.

Shepherd, P. R., & Kahn, B. B. (1999). Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *The New England Journal of Medicine, 341*(4), 248-257.

Sies, H. (1991). Oxidative stress: From basic research to clinical application. *The American Journal of Medicine, 91*(3C), 31S-38S.

Sies, H. (1997). Oxidative stress: Oxidants and antioxidants. *Experimental Physiology, 82*(2), 291-295.

Singh, I., Carey, A. L., Watson, N., Febbraio, M. A., & Hawley, J. A. (2008). Oxidative stress-induced insulin resistance in skeletal muscle cells is ameliorated by gamma-tocopherol treatment. *European Journal of Nutrition, 47*(7), 387-392.

Taniguchi, C. M., Emanuelli, B., & Kahn, C. R. (2006). Critical nodes in signalling pathways: Insights into insulin action. *Nature Reviews.Molecular Cell Biology, 7*(2), 85-96.

Thiebaud, D., Jacot, E., DeFronzo, R. A., Maeder, E., Jequier, E., & Felber, J. P. (1982). The effect of graded doses of insulin on total glucose uptake, glucose oxidation, and glucose storage in man. *Diabetes, 31*(11), 957-963.

Thong, F. S., Bilan, P. J., & Klip, A. (2007). The rab GTPase-activating protein AS160 integrates akt, protein kinase C, and AMP-activated protein kinase signals regulating GLUT4 traffic. *Diabetes, 56*(2), 414-423.

Tirosh, A., Rudich, A., Potashnik, R., & Bashan, N. (2001). Oxidative stress impairs insulin but not platelet-derived growth factor signalling in 3T3-L1 adipocytes. *The Biochemical Journal, 355*(Pt 3), 757-763.

Treebak, J. T., Frosig, C., Pehmoller, C., Chen, S., Maarbjerg, S. J., Brandt, N., et al. (2009). Potential role of TBC1D4 in enhanced post-exercise insulin action in human skeletal muscle. *Diabetologia, 52*(5), 891-900.

Treebak, J. T., Taylor, E. B., Witczak, C. A., An, D., Toyoda, T., Koh, H. J., et al. (2010). Identification of a novel phosphorylation site on TBC1D4 regulated by AMP-activated protein kinase in skeletal muscle. *American Journal of Physiology.Cell Physiology, 298*(2), C377-85.

Turnbow, M., Keller, S., Rice, K., & Garner, C. (1994). Dexamethasone down-regulation of insulin receptor substrate-1 in 3T3-L1 adipocytes. *Journal of Biological Chemistry, 269*(4), 2516-2520.

Uchimura, K., Nagasaka, A., Hayashi, R., Makino, M., Nagata, M., Kakizawa, H., et al. (1999). Changes in superoxide dismutase activities and concentrations and myeloperoxidase activities in leukocytes from patients with diabetes mellitus. *Journal of Diabetes and its Complications, 13*(5-6), 264-270.

van Staa, T. P., Leufkens, H. G., Abenhaim, L., Begaud, B., Zhang, B., & Cooper, C. (2000). Use of oral corticosteroids in the united kingdom. *QJM : Monthly Journal of the Association of Physicians, 93*(2), 105-111.

Venkatesan, N., Lim, J., Bouch, C., Marciano, D., & Davidson, M. B. (1996). Dexamethasone-induced impairment in skeletal muscle glucose transport is not reversed by inhibition of free fatty acid oxidation. *Metabolism: Clinical and Experimental, 45*(1), 92-100.

Viana, A. Y. I., Sakoda, H., Anai, M., Fujishiro, M., Ono, H., Kushiyama, A., et al. (2006). Role of hepatic AMPK activation in glucose metabolism and dexamethasone-induced regulation of AMPK expression. *Diabetes Research and Clinical Practice, 73*(2), 135-142.

Vijayalingam, S., Parthiban, A., Shanmugasundaram, K. R., & Mohan, V. (1996). Abnormal antioxidant status in impaired glucose tolerance and non-insulin-dependent diabetes mellitus. *Diabetic Medicine : A Journal of the British Diabetic Association, 13*(8), 715-719.

Vinayagamoorthi, R., Bobby, Z., & Sridhar, M. G. (2008). Antioxidants preserve redox balance and inhibit c-jun-N-terminal kinase pathway while improving insulin signaling in fat-fed rats: Evidence for the role of oxidative stress on IRS-1 serine phosphorylation and insulin resistance. *The Journal of Endocrinology, 197*(2), 287-296.

Vincent, H. K., Bourguignon, C. M., Weltman, A. L., Vincent, K. R., Barrett, E., Innes, K. E., et al. (2009). Effects of antioxidant supplementation on insulin

sensitivity, endothelial adhesion molecules, and oxidative stress in normal-weight and overweight young adults. *Metabolism, 58*(2), 254-262.

Virtanen, K. A., Hallsten, K., Parkkola, R., Janatuinen, T., Lonnqvist, F., Viljanen, T., et al. (2003). Differential effects of rosiglitazone and metformin on adipose tissue distribution and glucose uptake in type 2 diabetic subjects. *Diabetes, 52*(2), 283-290.

Walsh, L. J., Wong, C. A., Pringle, M., & Tattersfield, A. E. (1996). Use of oral corticosteroids in the community and the prevention of secondary osteoporosis: A cross sectional study. *BMJ (Clinical Research Ed.), 313*(7053), 344-346.

Wang, X. D., Vatamaniuk, M. Z., Wang, S. K., Roneker, C. A., Simmons, R. A., & Lei, X. G. (2008). Molecular mechanisms for hyperinsulinaemia induced by overproduction of selenium-dependent glutathione peroxidase-1 in mice. *Diabetologia, 51*(8), 1515-1524.

Weigert, C., Kron, M., Kalbacher, H., Pohl, A. K., Runge, H., Haring, H. U., et al. (2008). Interplay and effects of temporal changes in the phosphorylation state of serine-302, -307, and -318 of insulin receptor substrate-1 on insulin action in skeletal muscle cells. *Molecular Endocrinology (Baltimore, Md.), 22*(12), 2729-2740.

Weinstein, S. P., Wilson, C. M., Pritsker, A., & Cushman, S. W. (1998). Dexamethasone inhibits insulin-stimulated recruitment of GLUT4 to the cell surface in rat skeletal muscle. *Metabolism: Clinical and Experimental, 47*(1), 3-6.

Wentholt, I. M., Kulik, W., Michels, R. P., Hoekstra, J. B., & DeVries, J. H. (2008). Glucose fluctuations and activation of oxidative stress in patients with type 1 diabetes. *Diabetologia, 51*(1), 183-190.

Yasukawa, T., Tokunaga, E., Ota, H., Sugita, H., Martyn, J. A., & Kaneki, M. (2005). S-nitrosylation-dependent inactivation of Akt/protein kinase B in insulin resistance. *The Journal of Biological Chemistry, 280*(9), 7511-7518.

Yaworsky, K., Somwar, R., Ramlal, T., Tritschler, H. J., & Klip, A. (2000). Engagement of the insulin-sensitive pathway in the stimulation of glucose transport by alpha-lipoic acid in 3T3-L1 adipocytes. *Diabetologia, 43*(3), 294-303.

Yoshida, K., Hirokawa, J., Tagami, S., Kawakami, Y., Urata, Y., & Kondo, T. (1995). Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: Regulation of glutathione synthesis and efflux. *Diabetologia, 38*(2), 201-210.

You, J. M., Yun, S. J., Nam, K. N., Kang, C., Won, R., & Lee, E. H. (2009). Mechanism of glucocorticoid-induced oxidative stress in rat hippocampal slice cultures. *Canadian Journal of Physiology and Pharmacology, 87*(6), 440-447.

Zerial, M., & McBride, H. (2001). Rab proteins as membrane organizers. *Nature Reviews.Molecular Cell Biology, 2*(2), 107-117.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., et al. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of Clinical Investigation, 108*(8), 1167-1174.

Zhou, G., Sebhat, I. K., & Zhang, B. B. (2009). AMPK activators--potential therapeutics for metabolic and other diseases. *Acta Physiologica (Oxford, England), 196*(1), 175-190.

Zick, Y. (2005). Ser/Thr phosphorylation of IRS proteins: A molecular basis for insulin resistance. *Science's STKE : Signal Transduction Knowledge Environment, 2005*(268), pe4.

3.0 Effects of α-tocopherol supplementation on dexamethasone-induced insulin resistance

3.1 Introduction

Glucocorticoid (GC) excess has been implicated in producing a variety of effects associated with insulin resistance and glucose tolerance. It has been shown previously that chronic GC exposure can induce hyperglycemia, hyperinsulinemia and increased lipolysis (Nicod *et al.*, 2003; Ruzzin *et al.*, 2005; Schneiter & Tappy, 1998; Tappy *et al.*, 1994). Recent attempts to characterize the metabolic changes have been studied in relation to the effects GCs produce via the glucocorticoid receptor (Iuchi *et al.*, 2003; Ngo *et al.*, 2009; Schafer *et al.*, 2005; Turnbow *et al.*, 1994; You *et al.*, 2009), however a growing body of evidence indicates several effects may be mediated by oxidative stress through the GC-induced production of reactive oxygen species (ROS).

Oxidative stress has recently been linked to various negative health outcomes. With respect to glucose clearance, it has been demonstrated that elevated ROS levels and/or decreased ROS-quenching antioxidant systems can significantly inhibit glucose clearance in several tissue types (Dokken *et al.*, 2008; Fukuoka *et al.*, 2010; Lastra *et al.*, 2008; Nakamura *et al.*, 2009). Furthermore, it was recently shown that treating 3T3-L1 adipocytes with the GC analogue dexamethasone (DEX) stimulates ROS production as well as decreases insulin sensitivity (Houstis *et al.*, 2006). Several groups have hypothesized that through this mechanism there is a cell type-specific loss of various components within the

insulin signalling cascade; notably insulin receptor substrate (IRS)-1 (Archuleta *et al.*, 2009) and Akt (Tirosh *et al.*, 2001)(Tirosh 2001).

Under normal conditions, an interaction of insulin with the insulin receptor will result in the phosphorylation of IRS-1. Through a cascade of kinase intermediates, Akt is subsequently phosphorylated and is then able to assist in the translocation of glucose transporter (GLUT) vesicles to the plasma membrane via the inhibition of its downstream intermediate, Akt substrate at 160 (AS160). AS160 is a thoroughly characterized Rab GTPase activating protein (GAP) that has several known phosphorylation domains (Kane *et al.*, 2002). Moreover, AS160 has been shown to be phosphorylated by the upstream kinase, AMP activated protein kinase (AMPK) (Kramer *et al.*, 2006). The Rab protein family are monomeric G-proteins and are directly involved in vesicular trafficking, including GLUT translocation (Zerial & McBride, 2001). The GAP activity of AS160 restricts Rab proteins to the inactive GDP form and in turn, disallows vesicular translocation (Sano *et al.*, 2003). Upon phosphorylation by upstream kinases on its multiple domains, the GAP activity is inhibited allowing the active GTP-bound form of Rab proteins to accumulate and relieve the inhibition of internalized vesicles, ultimately permitting exocytosis (Eguez *et al.*, 2005; Thong *et al.*, 2007; Treebak *et al.*, 2009).

Given the complex mechanics involved in translating the anabolic signals of insulin, there is mounting interest in strengthening the current understanding of

AS160 as it relates to GLUT vesicular translocation and subsequent glucose clearance. Furthermore, with the additional awareness of ROS-induced protein damage and cellular adaptations, several groups have examined the role that antioxidants may play in mediating GC-, and thus, ROS-induced insulin resistance and glucose intolerance. Whole body antioxidant models using type 2 diabetics (Gokkusu *et al.,* 2001), obese young adults (Vincent *et al.,* 2009) and obese Zucker rats (Laight *et al.,* 1999) as well as *in vitro* antioxidant models (Houstis *et al.,* 2006; Maddux *et al.,* 2001) have been used in experimentation, demonstrating a clear improvement in glucose clearance as well as a blunting of ROS-induced signalling intermediate depletion. Previously it has been shown that α-tocopherol treatment may upregulate AMPK phosphorylation in certain tissues through a protein kinase C dependent pathway (Ricciarelli *et al.,* 1998).

Building on these findings, it was hypothesized that dietary supplementation with α-tocopherol may attenuate a GC induced decrease in glucose clearance and that this effect may be mediated by the protective antioxidant potential of α-tocopherol. Specifically, the goal of this project was to determine how GC excess translates into insulin resistance and what role the antioxidant α-tocopherol may have in attenuating or mitigating these effects following a two-week dietary supplementation model with five days of GC challenge in a rodent model.

3.2 Materials and Methods

3.2.1 Materials

Reagents, molecular weight marker and nitrocellulose membrane for SDS-PAGE were from Bio-Rad (Mississauga, ON). ECL+ was a product of Amersham Pharmacia Biotech (Arlington Heights, IL). Insulin and dexamethasone were purchased from Eli Lilly (Indianapolis, IN) and Sigma-Aldrich (Oakville, ON), respectively. Antibodies against total and/or phosphorylated insulin receptor, IRS-1, Akt, AS160, AMPK and ACC were from Cell Signaling Technology (Beverly, MA). Polyclonal GLUT-4 antibodies were purchased from Millipore (Billerica, MA) and horseradish peroxidase-conjugated donkey anti-rabbit antibodies were acquired from Jackson ImmunoResearch Laboratories (West Grove, PA). Glucose and insulin colorimetric assays were from Biopacific Diagnostics (Biopacific Diagnostics Inc., North Vancouver, BC) and Alpco (Salem, NH), respectively. Assays for oxidized and reduced glutathione were from Percipio Biosciences Inc. (Burlingame, CA) while those for 8-iso-prostaglandin F2α were from Assay Designs (Ann Arbor, MI).

3.2.2 Treatment of animals

All treatment and housing protocols followed the Canadian Council on Animal Care guidelines and were approved by the Animal Use and Welfare Committee at the University of Alberta. Male Wistar rats (Charles River, Wilmington, MA) weighing approximately 250 grams were housed two per cage with a 12 hour light-dark cycle (06:00 to 18:00). Following one week of

acclimatization, rats were randomly divided into three groups and were fed one of two prepared diets as shown below:

***Group 1*:** Control —– rats fed prepared control diet

***Group 2*:** Control diet + DEX —– rats fed prepared control diet and received scheduled subcutaneous DEX injections

***Group 3*:** α-tocopherol + DEX —– rats fed α-tocopherol supplemented diet and received scheduled subcutaneous DEX injections

Groups 1 and 2 were fed a semi-purified powdered control diet (15% fat w/w, 27% protein w/w, 46% carbohydrate w/w). The total fat sources consisted of flaxseed oil, hydrogenated canola oil and sunflower oil, providing a polyunsaturated to saturated fat ratio of 0.5. Group 3 was fed an identical basal diet supplemented with 500 mg/kg each of L-ascorbate (Sigma-Aldrich, Oakville, ON) and dl-all *rac*-alpha-tocopherol (Sigma-Aldrich, Oakville, ON). In addition to the 200 mg/kg of vitamin E in the prepared basal diet, the final concentration of dl-all-*rac*-alpha-tocopherol in the supplemented diet was 700 mg/kg (770 IU/kg). The control diet was supplemented with an equivalent mass of cellulose to maintain nutrient density. All groups were provided with diet and water *ad libitum* for 14 days. On each of the final five days of feeding, Groups 2 and 3 received weight-adjusted subcutaneous DEX injections (0.8 mg/kg body weight) between

09:00 and 11:00, while Group 1 received an equivalent volume of sterile phosphate buffered saline (PBS).

3.2.3 IPGTT

Following the 14-day experimental period, an intraperitoneal (i.p.) glucose tolerance test was performed. After an overnight fast, animals were given an i.p. injection of glucose (2.0 g/kg body weight). Blood was collected in K_2/EDTA-coated tubes at 0, 15, 30, 45, 60 and 120 minutes (t_0-t_5) via tail venipuncture for quantification of plasma glucose and insulin parameters. Whole blood was centrifuged at 13 000 x g for 10 minutes at 4 °C. Plasma was aliquoted and stored at -20 °C until analysis.

3.2.4 Analysis of plasma glucose and insulin

Plasma glucose and insulin concentrations were obtained using a standard glucose oxidase method (Biopacific Diagnostics Inc., North Vancouver, BC) and a rat-specific sandwich ELISA (Alpco, Salem, NH), respectively. Endpoints for both glucose and insulin were measured spectrophotometrically in duplicate using a microplate reader (Molecular Devices, Sunnyvale, CA) as per the assay protocols. A CV of less than 5 percent was accepted. Area under the curve (AUC) was calculated using the trapezoidal method of quantification:

Eq. 1 $\mathrm{AUC} = \sum \frac{1}{2}(\mathrm{C_{n+1}} + \mathrm{C_n}) \times (\mathrm{t_{n+1}} - \mathrm{t_n})$,

where C is the glucose or insulin concentration at the various time points, t_n. AUCs for glucose and insulin were analyzed independently for treatment effects. In addition to AUC calculations, general insulin sensitivity was calculated using the homeostatic model assessment of insulin resistance (HOMA-IR) using the method of Matthews *et al.* (1985) as shown in Equation 2, and the Insulin Sensitivity Index (ISI) as estimated for the rat by Vera and others (2002) and Matsuda and DeFronzo (1999) for whole body insulin sensitivity following a glucose challenge, calculated using Equation 3.

Eq. 2 $\text{HOMA-IR} = \frac{G_{fasting} \times I_{fasting}}{22.5}$

Eq. 3 $\text{ISI} = \frac{100}{\sqrt{(G_{fasting} \times I_{fasting} \times G_{mean} \times I_{mean})}}$

3.2.5 Insulin stimulation

At the end of a separate 14-day experimental period and overnight fast, Groups 1, 2 and 3 were further subdivided to receive either a 10 U/kg bodyweight i.p. injection of insulin or an equivalent volume of sterile PBS as has been used in previous studies (Giorgino *et al.,* 1993). Rats were anaesthetized with i.p. sodium pentobarbital (5 mg/100 g body weight) and received the appropriate insulin or

PBS injection. After a 10 minute interval, tricep and soleus muscles, retroperitoneal and epididymal adipose tissue and liver were quickly and carefully dissected and immediately clamp frozen to the temperature of liquid nitrogen. Samples were stored at -80 °C until analysis.

3.2.6 SDS-PAGE and Western blotting

Clamp frozen muscle, liver and adipose samples were homogenized in 10, 15, and 2 volumes, respectively, of ice-cold cell lysis buffer (Invitrogen, Burlington, ON) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich, Oakville, ON), phenylmethylsulfonyl fluoride and sodium fluoride. Homogenized samples were briefly sonicated before being centrifuged at 2500 x g for 15 minutes at 4 °C. For muscle and liver, the supernatants were collected and aliquoted and protein concentration was determined in triplicate using the BCA method (Smith *et al.*, 1985). For this assay, a CV of less than 5 percent was accepted. Adipose homogenates were quickly chilled with liquid nitrogen to facilitate removal of the infranatant, which was also aliquoted and analyzed for protein concentration. Protein homogenates were standardized in Laemmli buffer and were heated at 95 °C for 5 minutes. Total protein content and/or relative phosphorylation status was determined for insulin receptor-beta, IRS-1, Akt, AS160, AMPK, ACC and GLUT-4 via SDS-PAGE as has been previously described (Wright *et al.*, 2007; Williams *et al.*,2009). In short, proteins were separated on 6.25% (IRS-1, AS160 and ACC) 7.5% (Insulin receptor) or 10%

(Akt, AMPK and GLUT-4) polyacrylamide gels. Transfer of proteins to nitrocellulose membrane was performed for approximately 90 minutes (Akt and GLUT-4) or 180 minutes (IRS-1, AS160, AMPK, ACC) at 200 mA per tank via wet electrophoretic transfer. Membranes were blocked with 5% (w/v) non-fat dry milk diluted in tris-buffered saline-0.1% Tween 20 (TBST-5% milk) for 1 hour at room temperature with gentle agitation. Blocked membranes were incubated overnight in either TBST-5% milk or 5% BSA (w/v) diluted in TBST containing the appropriate primary antibody at 4°C with gentle agitation. After overnight incubation, blots were briefly washed in TBST and were subsequently incubated in TBST-1% (w/v) non-fat dry milk containing a HRP-conjugated secondary antibody for 1 hour at room temperature with gentle agitation. Bands were visualized using ECL+ and captured using a Typhoon Imaging system (GE Health Care. Piscataway, NJ). Relative band intensity was determined with ImageQuant software (GE Health Care, Piscataway, NJ).

3.2.7 GSH/GSSG determination

Total reduced and oxidized GSH was determined by measuring the proportional production of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm for 3 minutes (10 second intervals) using a microplate reader, with some modifications to the assay manufacturer's protocol. Clamp frozen tricep was ground by mortar and pestle over dry ice. Fifty milligrams of powdered tissue was then combined with either 20 μL of 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) in HCl and 80 μL of 5% (w/v) metaphosphoric acid (MPA) for

oxidized GSH or 100 μL of 5% MPA for reduced GSH. Samples were homogenized via sonicator for 45 seconds. The homogenates were then treated according to assay protocol for determination of total oxidized and reduced GSH. Values were standardized to respective protein concentrations acquired by the BCA method.

3.2.8 8-iso-prostaglandin F2*α* determination

8-iso-prostaglandin F2α (8-iso-PGF) levels in skeletal muscle were quantified using a competitive enzyme immunoassay. Clamp-frozen tricep homogenate from previous protein analysis was standardized to 800 μg protein/μL with cell lysis buffer and treated as per the assay manufacturer's instructions. Samples were read on a microplate reader at 405 nm and concentrations calculated using Softmax computer software (Molecular Devices, Sunnyvale, CA).

3.2.9 Statistical analysis

Data were presented as means plus/minus standard error. Group comparisons of all insulin-stimulated parameters were made using two-way ANOVA followed by Fisher LSD post-hoc test. A one-way ANOVA was performed for Group comparisons of total protein and oxidative stress and plasma parameters. HOMA-IR values were transformed logarithmically prior to statistical analysis to ensure normal distribution. Statistical significance was accepted at $p<0.05$.

3.3 Results

3.3.1 Body weight and food intake with DEX and Vitamin E treatment

While body weights gains in Groups 2 and 3 were significantly lower than Group 1 following DEX treatments, weights did not differ between Group 2 and Group 3 (Table 3.1A). Similarly, food intakes were shown to decrease at a similar rate in Group 2 and 3 after DEX treatments began, but were both significantly different from Group 1 (Table 3.1B).

3.3.2 Plasma glucose and insulin parameters

In order to compare diet and/or DEX exposure on glucose tolerance between the three groups, an IPGTT was performed and glucose and insulin plasma levels were quantified. As represented by the AUC, glucose clearance was significantly decreased following DEX treatment as shown by the greater glucose area under the curve (AUC) in Groups 2 and 3 relative to Group 1 controls. Both Groups 2 and 3 were significantly different from control Group 1 ($p<0.001$), while Groups 2 was significantly higher than Group 3 ($p<0.05$) (Figure 3.1A). Insulin, however, showed contrasting results from glucose AUC. Specifically, Groups 2 and 3 were significantly different from Group 1, however they were not different from each other (Figure 3.1B).

To obtain a measure of insulin sensitivity, two indices were calculated. First, the HOMA-IR was determined using fasting glucose and insulin levels in the plasma, with a higher HOMA score representing lower insulin sensitivity. As

shown in Table 3.1, HOMA was significantly lower in Group 1 controls. Moreover, HOMA in Group 2 was higher than that of Group 3. Second, the ISI was calculated to give an additional level of analysis by incorporating average glucose and insulin levels over the course of the IPGTT, with a lower ISI being inversely proportional to insulin sensitivity. These calculations revealed somewhat divergent results, specifically showing that following a glucose load (2 g glucose/kg bodyweight), insulin sensitivity was not significantly different between both DEX groups (Group 2 and 3), despite being significantly lower than Group 1 (Table 3.2).

3.3.3 Markers of oxidative stress

Reduced (GSH) and oxidized (GSSG) glutathione as well as 8-iso-PG were measured in clamp frozen tricep following the experimental protocol to obtain a relative representation of oxidative stress and antioxidant potential. The overall GSH/GSSG ratio was then calculated as a partial measure of antioxidant system potential; however there were no intergroup differences (Figure 3.2A). GSH levels in both DEX-treated groups (Groups 2 and 3) decreased significantly to 77 and 79 percent that of Group 1 ($p<0.01$) (Figure 3.2B). The production of 8-iso-PG was significantly blunted in Group 3 relative to Groups 1 and 2 (Figure 3.2C).

3.3.4 Dexamethasone decreases Akt phosphorylation

Following the 14 day experimental period, relative expression of various insulin signalling intermediates, with or without insulin stimulation, was determined in tricep, soleus, liver and retroperitoneal adipose tissue. While Akt phosphorylation on serine-473 was preserved across all three treatment groups in tricep muscle, there was a significant drop to approximately 48 percent (Group 2) and 54 percent (Group 3) that of the control group (Figure 3.3A). At the threonine-308 activation site, this drop was more pronounced to approximately 25 percent of control phosphorylation (Figure 3.3B). There were no significant differences between levels of basal levels of phosphorylation at either activation site. Relative phosphorylation status of threonine-308 in soleus muscle followed a similar trend and was decreased to approximately 50 percent (Figure 3.3D), while showing no difference in basal phosphorylation levels. Insulin stimulated serine-473 phosphorylation in soleus was not significantly different between any groups (Figure 3.3C). A similar decrease by approximately 40 percent was seen in both liver (Figure 3.4A) and retroperitoneal adipose tissue (Figure 3.4B).

3.3.5 Dexamethasone decreases IRS-1 protein and increases GLUT-4

In both Groups 2 and 3, an approximate 60 percent decrease in total IRS-1 protein from tricep was observed, suggesting a DEX affect on IRS-1 expression or post-translational modifications Figure 3.5A). GLUT-4 protein expression across all three groups showed divergent results with Group 2 showing a more than

twofold increase in GLUT-4 protein and Group 3, a decrease of 50 percent, relatively to control Group 1 (Figure 3.5B).

3.3.6 Vitamin E supplementation restores AS160 phosphorylation

AS160, a substrate of Akt, was measured as evidence points to this substrate as a converging point in the recruitment of GLUT-4 to the plasma membrane and subsequent plasma glucose clearance. In tricep, differences in basal phosphorylated expression of AS160 were not significant between groups. However, while insulin-stimulated increases in phospho-AS160 over basal were not significant in Group 1, Group 3 showed a significant 81 percent increase in phosphorylation relative to its basal levels. Conversely, Group 2 showed no such change in phospho-AS160 following insulin stimulation, remaining at the baseline. When comparing the effect of insulin on AS160 phosphorylation between both DEX groups, a significant 2 fold increase is seen in Group 3 over that of Group 2 (Figure 3.6).

3.3.7 Vitamin E supplementation restores AMPK and ACC phosphorylation

Immunoblotting for phophorylated AMPK illustrated a significant DEX-related effect, specifically showing reduced phosphorylation of AMPK in Group 2 relative to controls. Of note, while Group 2 was significantly different from Group 1, there was no significant difference between Group 3 and Group 1

(Figure 3.7A). To ascertain a Vitamin E effect, the phosphorylation of ACC, a downstream substrate of AMPK was measured by immunoblot. These data confirmed what was seen in the AMPK data, showing a 40 percent decrease in Group 2, relative to Group 1, while no such decrease was observed in the α-tocopherol group (Figure 3.7B).

3.3.8 No change in total IR, Akt or AS160

Treatment with DEX had no statistically significant impact on protein expression of the insulin receptor, total Akt or total AS160 across any group (Figure 3.8).

Table 3.1A: Daily mean body weight gains before i.p. saline (Group 1), DEX treatment (Group 2) or DEX treatment with α-tocopherol supplementation (Group 3) and after treatment in male Wistar rats.

	Mean daily body weight gain (g)	
Group ID	**Before Treatment**	**After Treatment**
Group 1	7.4 ± 1.3	6.8 ± 0.5[a]
Group 2	7.5 ± 1.4	-10.6 ± 0.5[b]
Group 3	7.6 ± 1.1	-9.9 ± 0.6[b]

Data are presented as mean ± SEM for 10 animals per group.
Letters correspond to a significant difference between groups (p<0.05)

Table 3.1B: Mean daily food intake before i.p. saline (Group 1), DEX treatment (Group 2) or DEX treatment with α-tocopherol supplementation (Group 3) and after treatment in male Wistar rats.

	Mean daily food intake (g)	
Group ID	**Before Treatment**	**After Treatment**
Group 1	29.69 ± 0.5	29.55 ± 0.1[a]
Group 2	29.02 ± 0.5	18.95 ± 1.5[b]
Group 3	28.98 ± 0.4	19.60 ± 1.0[b]

Data are presented as mean ± SEM for 10 animals per group.
Letters correspond to a significant difference between groups (p<0.05)

Table 3.2: Determination of insulin sensitivity index (ISI) and homeostatic model assessment of insulin resistance (HOMA)-IR using plasma glucose and insulin parameters from an IPGTT (120 minutes) in male Wistar rats. ISI and HOMA-IR were calculated using Equations 2 and 3 in Materials and Methods.

Group ID	Glucose (mmol/L)		Insulin (ng/mL)		Insulin Sensitivity Index (ISI)	HOMA-IR
	Mean fasting glucose	Mean IPGTT glucose	Mean fasting insulin	Mean IPGTT insulin		
Group 1	6.28 ± 0.23^{a}	10.19 ± 0.50^{a}	0.706 ± 0.10^{a}	1.51 ± 0.19^{a}	2.30 ± 0.18^{a}	1.29 ± 0.09^{a}
Group 2	13.70 ± 1.03^{b}	19.10 ± 0.64^{b}	7.64 ± 0.90^{b}	7.69 ± 0.60^{b}	-0.21 ± 0.05^{b}	4.49 ± 0.08^{b}
Group 3	10.37 ± 1.28^{c}	15.11 ± 1.36^{c}	6.32 ± 1.0^{b}	6.76 ± 0.71^{b}	0.31 ± 0.23^{b}	3.86 ± 0.26^{c}

Data presented as mean ± SEM for 10, 9 and 8 animals for Groups 1, 2 and 3, respectively.

Letters correspond to significant differences between groups ($p<0.05$)

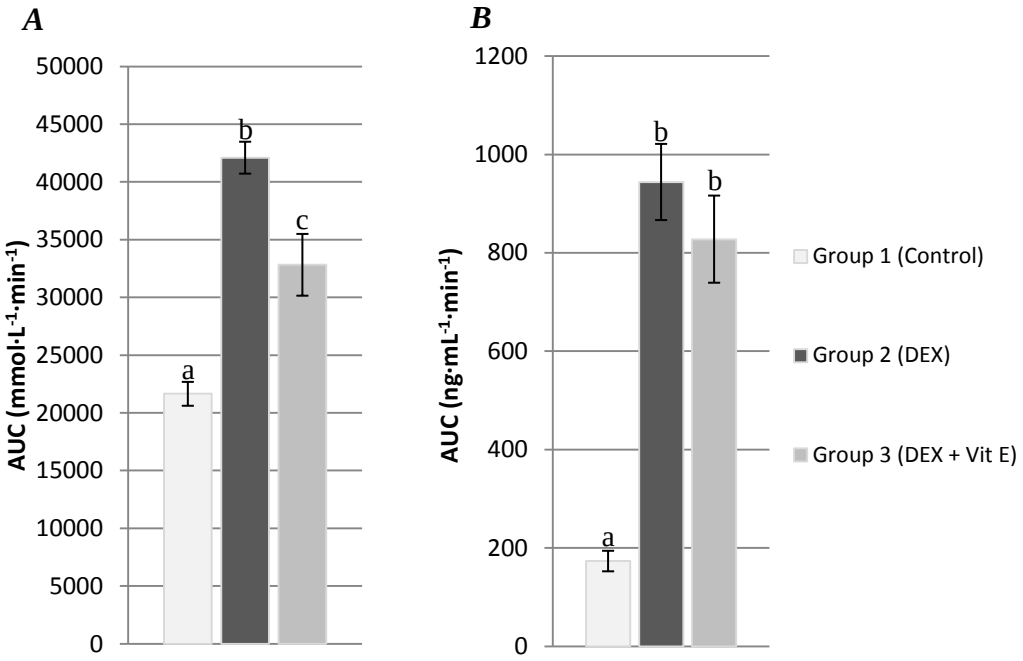

Figure 3.1: Plasma glucose (A) and insulin (B) area under the curve (AUC) following a 120 minute IPGTT in male Wistar rats following i.p. saline (Group 1) or DEX (Groups 2 and 3) treatments. Data are presented as mean ± SEM for 10 animals per group. Letters indicate significant differences between groups (p<0.05).

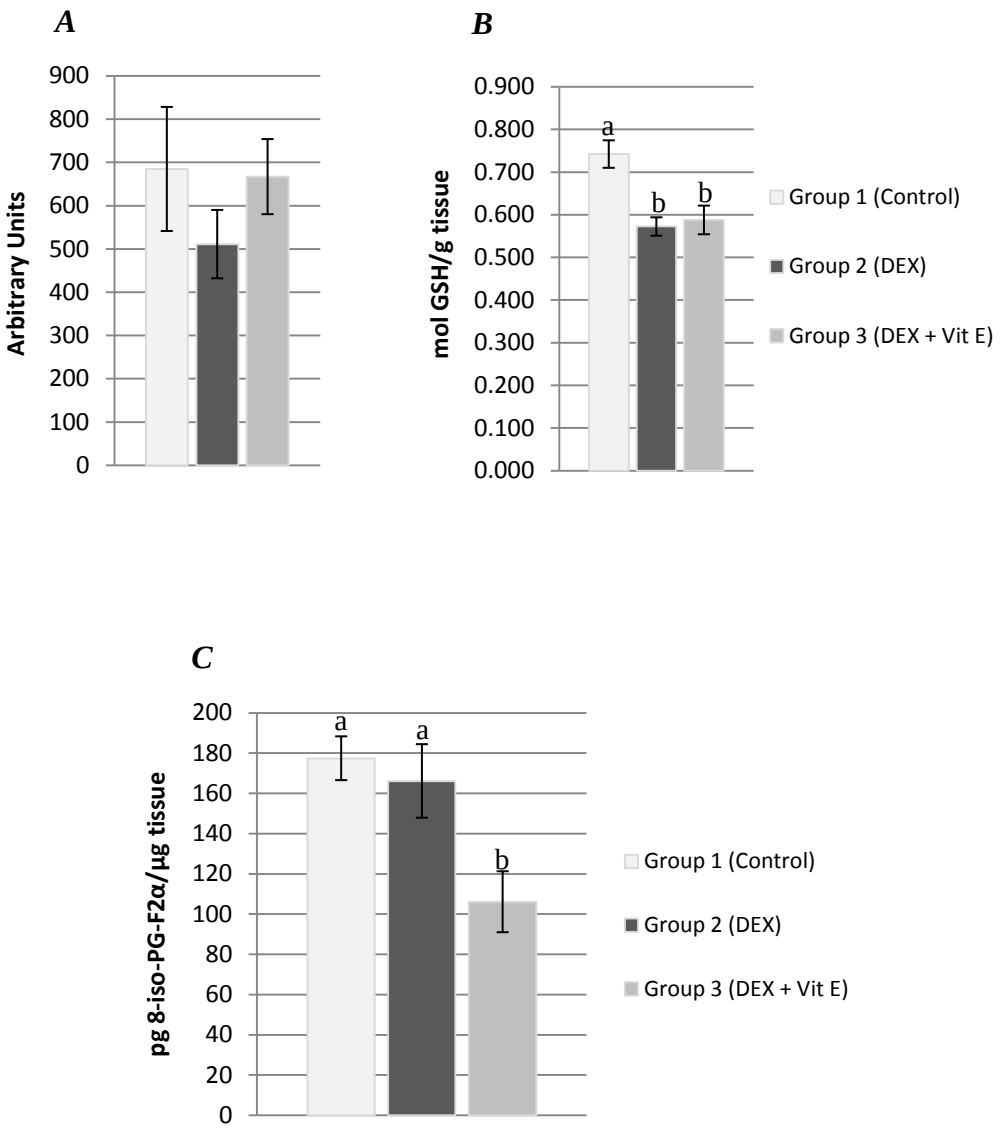

Figure 3.2: GSH/GSSG ratio (A), Total GSH (B) and 8-iso-PG-F2α (C) determination from tricep muscle taken from male Wistar rats following i.p. saline (Group 1) or DEX (Groups 2 and 3) treatments. Data are presented as mean ± SEM for 10 animals per group. Letters correspond to significant differences between groups (p<0.05).

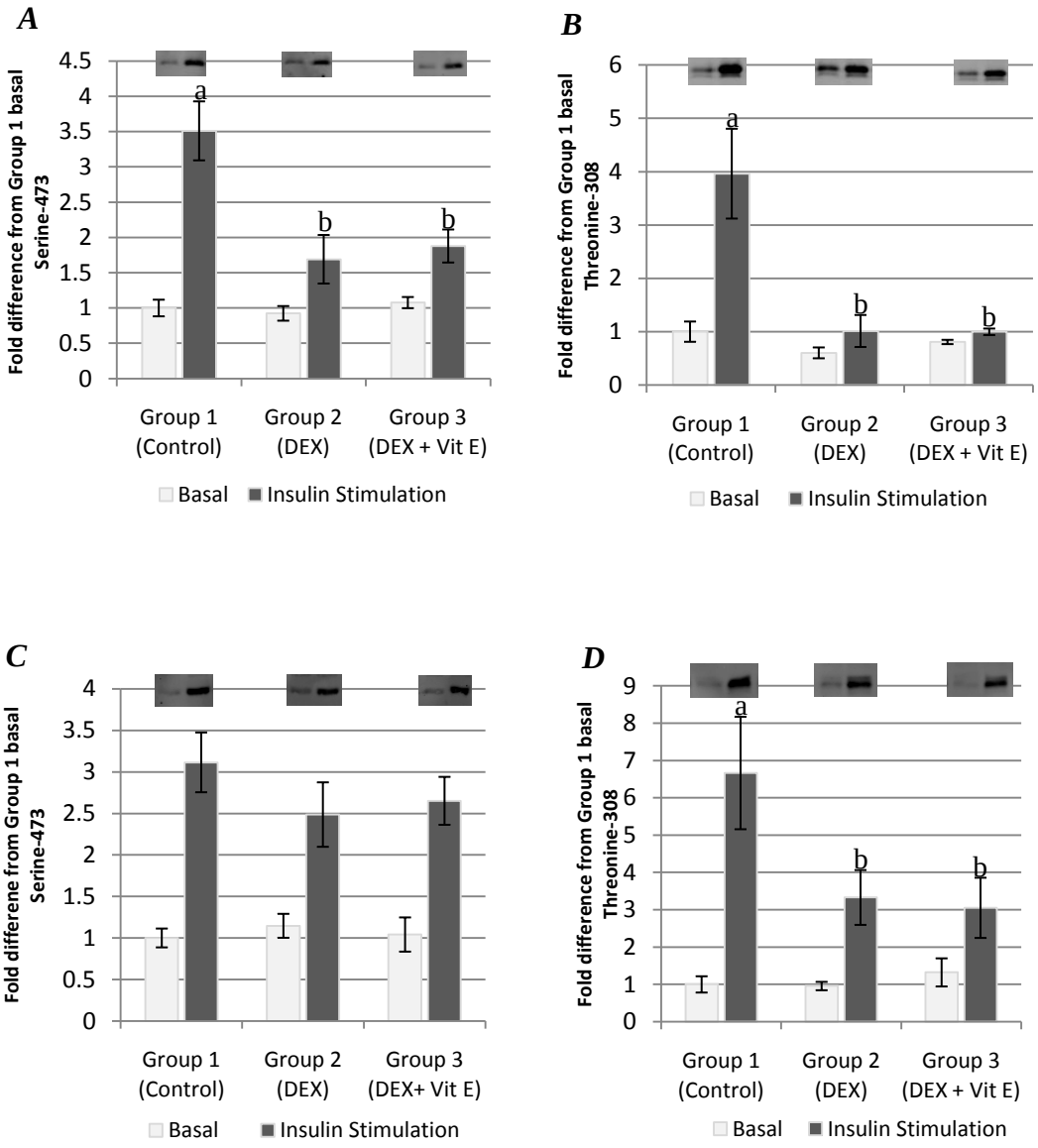

Figure 3.3: Basal and insulin stimulated phosphorylated Akt at serine-473 and threonine-308 in tricep (A and B) and soleus muscle (C and D) following i.p. saline (Group 1) or DEX (Groups 2 and 3) treatments. Data are presented as mean ± SEM with 5 animals per group. Different letters indicate significant differences between insulin stimulated groups (p<0.05).

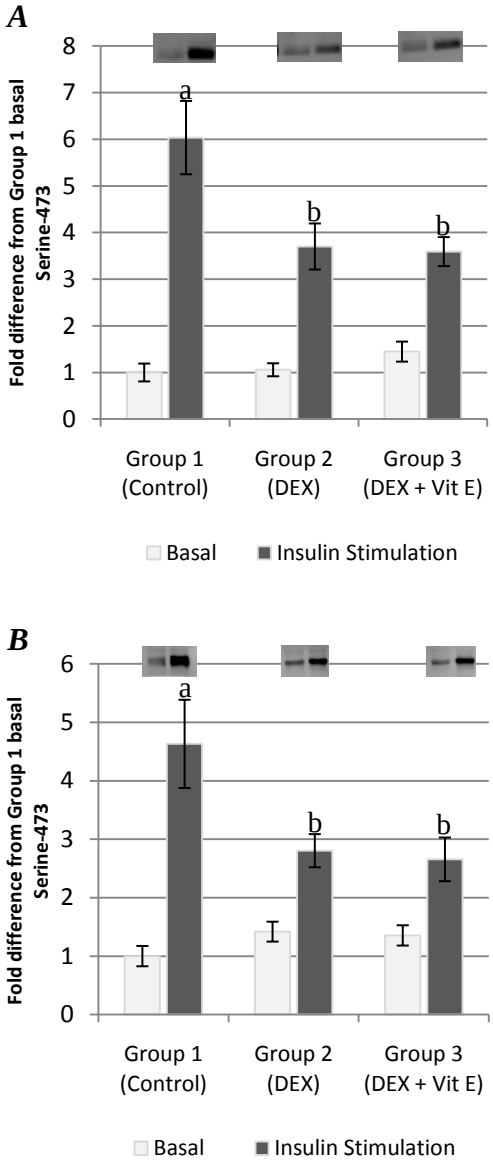

Figure 3.4: Basal and insulin stimulated phosphorylation of Akt at serine-473 in liver (A) and retroperitoneal adipose (B) following i.p. saline (Group 1) or DEX (Groups 2 and 3) treatments.. Data are presented as mean ± SEM with 5 animals per group. Letters indicate significant differences between insulin stimulation groups ($p<0.05$).

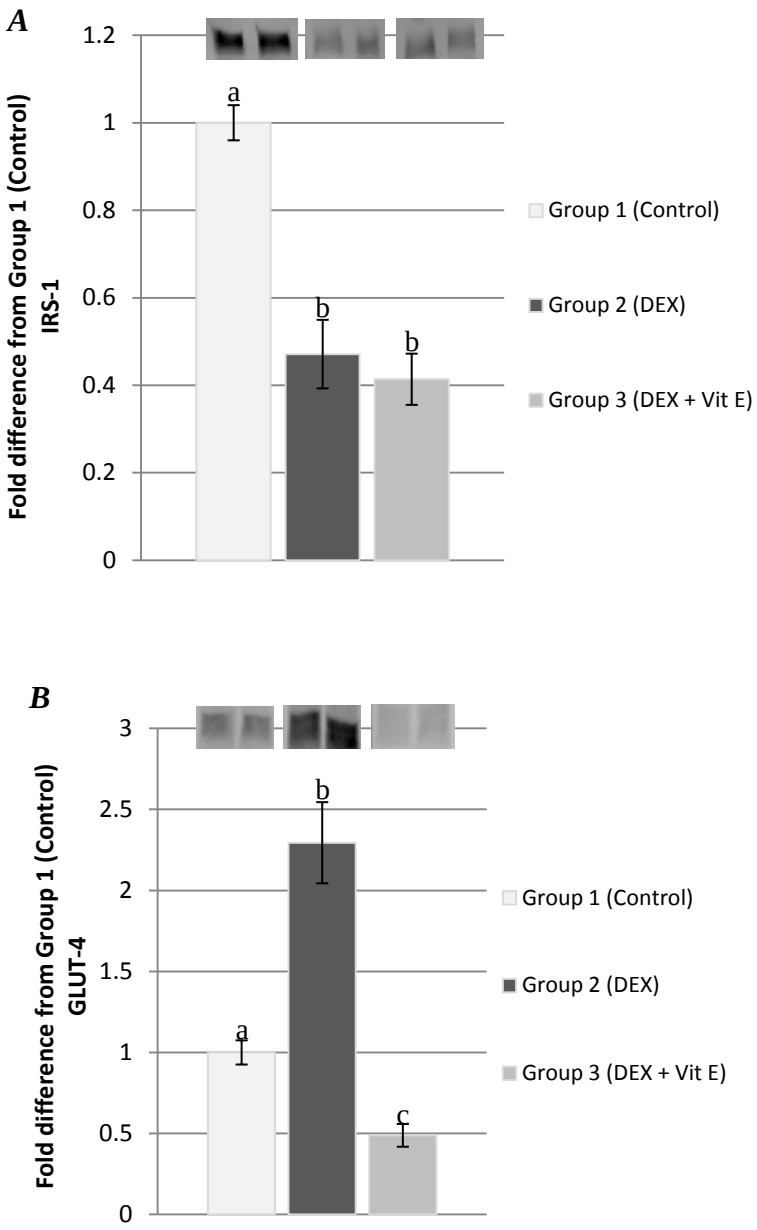

Figure 3.5: Total IRS-1 (A) and GLUT-4 (B) protein in tricep muscle of male Wistar rats following dietary intervention. Data are presented as mean ± SEM with 10 animals per group. Letters indicated significant differences between groups (p<0.05).

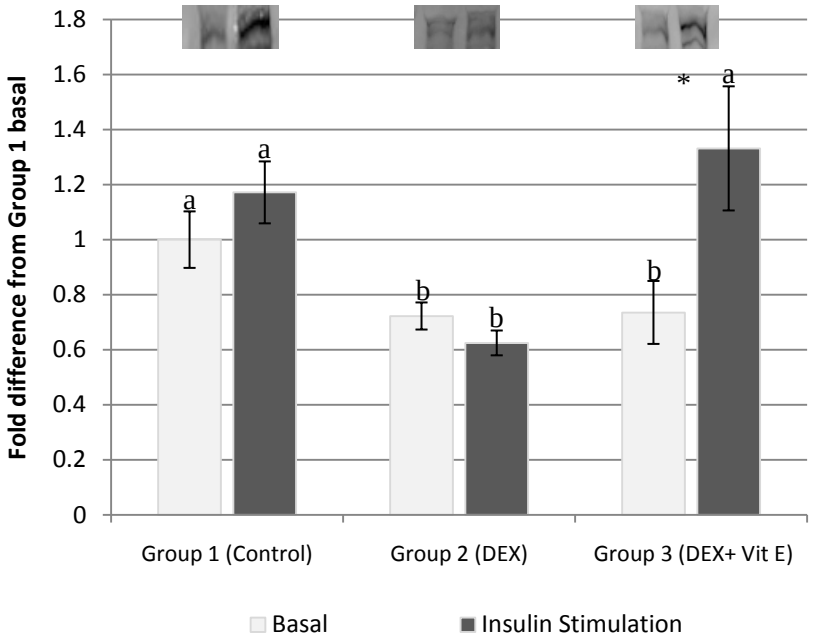

Figure 3.6: Basal and insulin stimulated phosphorylation of AS160 in tricep of male Wistar rats following i.p. saline (Group 1) or DEX (Groups 2 and 3) treatments. Data are presented as mean ± SEM with 5 animals per group. Letters indicate significant differences between respective groups while asterisk represents significance within a Group (p<0.05).

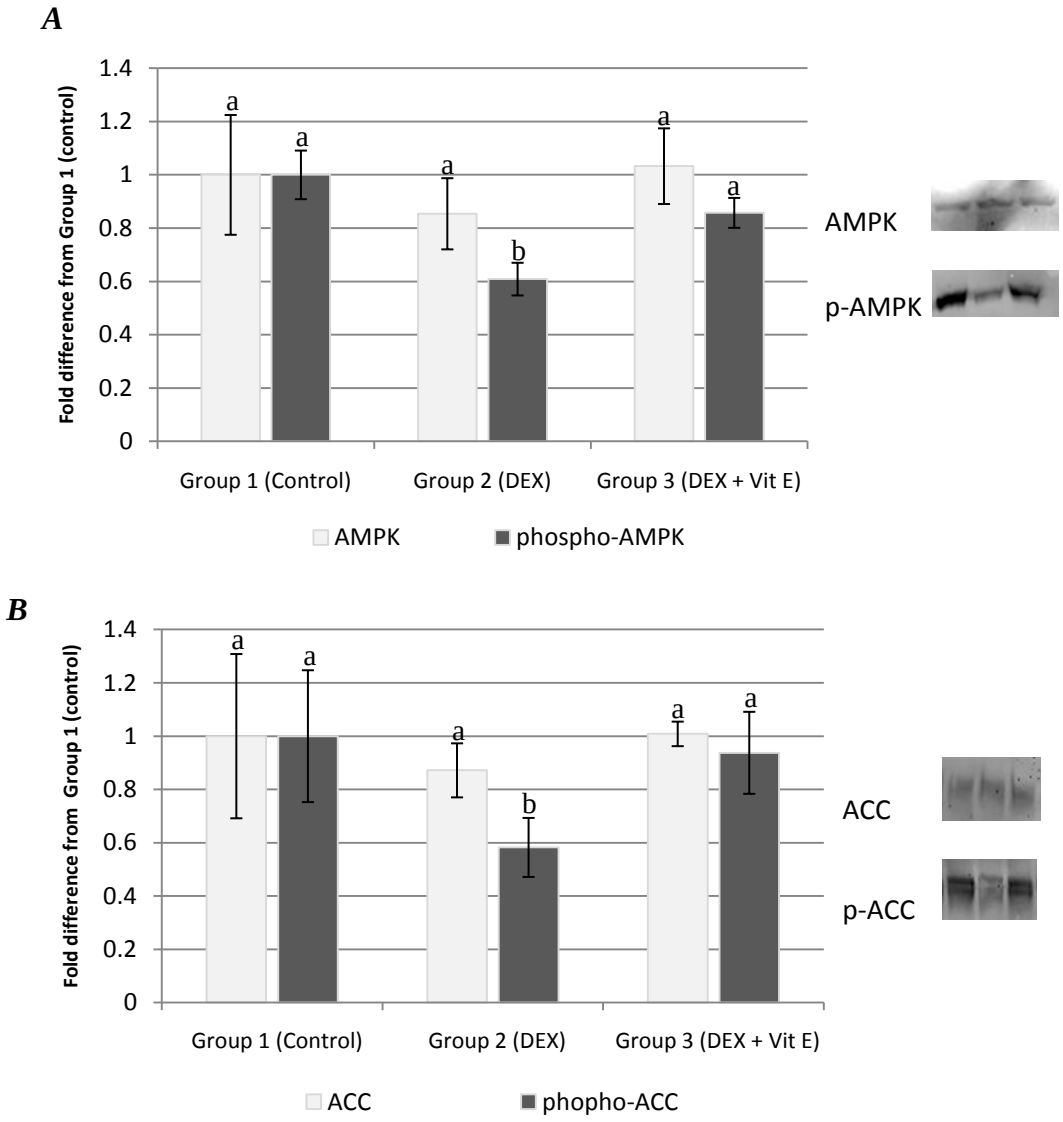

Figure 3.7: Total and phosphorylated AMPK (A) and ACC (B) in tricep muscle of male Wistar rats following ditary intervention. Data are presented as mean ± SEM with 10 animals per group. Letters indicated significant differences between phosphorylated groups (p<0.05).

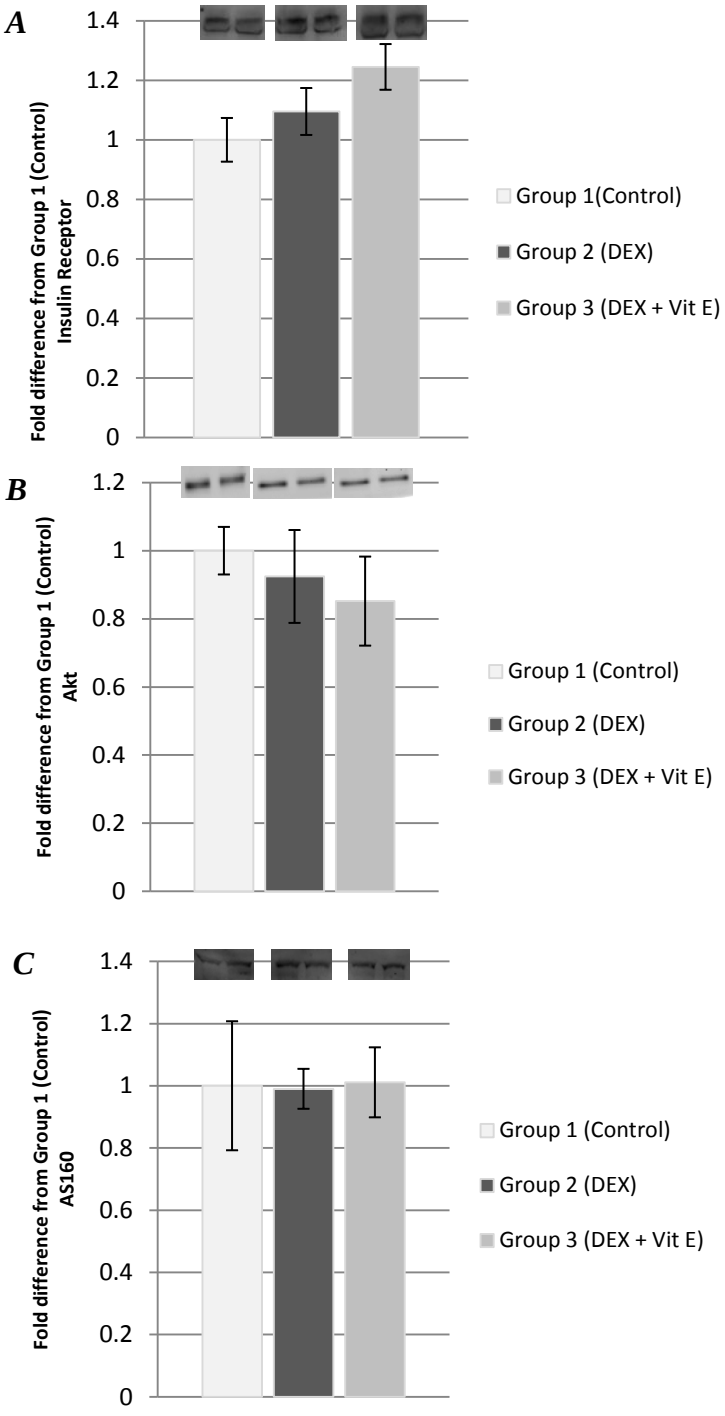

Figure 3.8: Total insulin receptor (A), Akt (B) and AS160 (C) in tricep of male Wistar rats following dietary intervention. Data are presented as mean ± SEM with 10 animals per group.

3.4 Summary of Results

This project aimed to determine if dexamethasone (DEX) exposure can result in the development of insulin resistance through oxidative stress-related mechanisms and if α-tocopherol is able to attenuate these pathologies by shifting the antioxidant balance to a less oxidative state.

Primary Objective: To determine the effects of dietary antioxidant (α-tocopherol) supplementation on a DEX-induced decrease in insulin sensitivity in a rodent model.

The results obtained from an IPGTT indicated that α-tocopherol is able to attenuate DEX-related inhibition of glucose clearance in an insulin-independent manner. Furthermore, it was determined that between both DEX-treated groups, fasting insulin sensitivity was higher in the α-tocopherol supplemented group.

Secondary Objective: To determine if dietary antioxidants can decrease whole-body or tissue-specific levels of reactive oxygen species and/or associated biomarkers of reactive oxygen species.

It was hypothesized that DEX would increase markers of oxidative stress and that α-tocopherol supplementation would mitigate this. It was determined that while markers of lipid peroxidation were not increased in the DEX-only group of animals, the supplemented group showed significantly lower levels. Moreover, total GSH was reduced in both

groups relative to controls while GSH ratios were not significantly different between all groups.

Tertiary Objective: To determine what specific insulin signalling intermediates are involved in a diminished insulin signalling response, resulting in insulin resistance.

The data from this experiment point to α-tocopherol–induced increases in AMPK phosphorylation. There were no significant differences between DEX-treated groups to indicate an improvement in glucose clearance due to improved insulin signalling. Thus, it was concluded that AMPK may be responsible for increased glucose clearance in the α-tocopherol supplemented animals.

3.5 Discussion

Glucocorticoid (GC) excess can have significant detrimental effect on whole body glucose homeostasis. This project hypothesized that subchronic treatment with the glucocorticoid dexamethasone (DEX) would result in insulin resistance through an oxidative stress-related mechanisms and that this outcome could be prevented by a diet supplemented with vitamin E (α-tocopherol), a well known cellular antioxidant, in conjunction with vitamin C (ascorbate). Three general research objectives were developed to assess the relationship between oxidative stress, antioxidant load and insulin sensitivity. First, to determine what effects α-tocopherol has on insulin sensitivity following a sub-chronic treatment with DEX; second, to determine if dietary antioxidants can decrease levels of oxidative stress. The third objective was to elucidate what insulin signalling pathway intermediates were being perturbed or modified with DEX and/or α-tocopherol treatment.

In this study, it was confirmed that treatment with DEX results in rapid onset of insulin resistance and that pre-treatment with an α-tocopherol rich diet can attenuate this outcome. Furthermore, it was shown that the improvements seen in glucose clearance with dietary α-tocopherol supplementation may in fact be independent of the insulin signalling pathway and are likely mediated by changes to phosphorylation in AS160 through AMPK. Together, these data provide a potential mechanism for how DEX, a commonly prescribed GC agonist,

may mediate its effects on glucose clearance and insulin sensitivity and how α-tocopherol is able to attenuate these effects.

Consistent with other findings (Amaral *et al.*, 2010; Giozzet *et al.*, 2008), it was shown that sub-chronic DEX exposure results in a decrease in whole body glucose clearance as shown via IPGTT. This was confirmed by the calculation of AUCs, indicating that plasma glucose levels in both DEX-treated groups were significantly elevated above those of control animals (Group 1). Moreover, while the Group 3 glucose AUC was significantly higher than Group 1, it was intermediate between Group 1 and that of Group 2. Insulin AUC levels were not as clear and showed that both DEX-treated groups were significantly higher than that of controls. This is consistent with the majority of literature (Giorgino *et al.*, 1993; Nicod *et al.*, 2003; Rafacho *et al.*, 2009; Ruzzin *et al.*, 2005; Venkatesan *et al.*, 1996), which has found that DEX, a significantly more potent GC than the naturally occurring cortisol GC, leads to hyperglycemia and hyperinsulinemia. Furthermore, HOMA-IR was calculated to determine fasting insulin sensitivity and ISI was determined to characterize insulin sensitivity over the course of the IPGTT. Interestingly, fasting insulin levels in Groups 2 and 3 were divergent, with the α-tocopherol supplemented Group 3 having an intermediate value between controls and Group 2, despite showing no such difference in the ISI analysis. Thus, these findings indicate that α-tocopherol supplementation can ameliorate glucose clearance, but it does so without affecting insulin levels.

It was hypothesized that α-tocopherol, a potent lipid soluble antioxidant, may mediate its positive effects by increasing tissue antioxidant potential and thus minimizing tissue specific oxidative damage. GC excess has long been associated with oxidative stress-induced lipid peroxidation in skeletal muscle of rats (Ohtsuka *et al.*, 1998; Orzechowski *et al.*, 2002; Pereira *et al.*, 1999) in addition to insulin resistance. Thus it was hypothesized that pre-treatment with α-tocopherol would minimize these effects and consequently reduce the observed degree of insulin resistance. The treatment methodology used in the current set of studies did not entirely support this hypothesis. On one hand, 8-iso-prostaglandin F2α, a marker of lipid peroxidation, was not elevated in tricep muscle following the DEX treatment. Alternatively, the group that received the α-tocopherol supplemented diet had significantly reduced markers of lipid peroxidation. Furthermore, an analysis of total gluthathione (GSH) and the ratio of oxidized and reduced GSH did not support the hypothesis. Specifically, the overall GSH ratio was not significantly different between any of the groups, nor was there a significant difference between Groups 2 and 3. Therefore, it can be surmised that in the experimentation model used, increased insulin resistance due to DEX and improvements seen as a result of α-tocopherol supplementation were neither a function of a direct oxidative stress nor improved antioxidant potential, respectively.

Given the convincing data supporting the role of GC-induced insulin resistance in skeletal muscle (Dimitriadis *et al.*, 1997; Ruzzin *et al.*, 2005;

Weinstein *et al.*, 1998), it was logical to track the degree of insulin signalling following an intraperitoneal bolus of insulin in addition to total protein content of relevant insulin cascade intermediates. The observed depletion of IRS-1 total protein in DEX-treated animals was similar to what has been found in *in vitro* models (Buren *et al.*, 2002; Giorgino *et al.*, 1993; Saad *et al.*, 1993). Consistent results were also seen in GLUT-4 protein expression in tricep muscle. While total GLUT-4 protein was significantly increased over controls in Group 2 animals by magnitudes seen in previous studies (Ewart *et al.*, 1998; Haber & Weinstein, 1992), there was a significant decrease in total GLUT-4 protein in the Group 3 animals relative to controls. This specific result may be mediated by a relative decrease in GLUT-4 protein expression due to muscle atrophy (Op 't Eijnde *et al.*, 2001; Wu *et al.*, 2010). More importantly, in relation to the Group 2 GLUT-4 levels, it indicates that a diet effect of α-tocopherol was observed. The initial results of insulin-stimulated Akt phosphorylation in skeletal muscle (Buren *et al.*, 2008; Ruzzin *et al.*, 2005) and adipose tissue (Buren *et al.*, 2002) were consistent with previous studies using *in vivo* animal models. Briefly, despite a significant DEX-associated decrease in insulin-stimulated Akt phosphorylation relative to the control group, there were no significant differences between the two DEX-treated groups. Akt functions as a major nexus for translating upstream kinase signals to elicit GLUT-4 vesicular translocation, thus it is likely that while DEX treatments may decrease the stimulatory effect of insulin with respect to glucose clearance, improved glucose clearance in Group 3 relative to Group 2 is by an Akt-independent mechanism, mediated by the α-tocopherol-supplemented diet. For

this reason, it was logical to assess the degree of AS160 phosphorylation as it may be activated by a variety of other regulators such as AMPK (Gaidhu *et al.*, 2010; Mu *et al.*, 2001; Pehmoller *et al.*, 2009) and CaMK (Kramer *et al.*, 2007). Perhaps more importantly, since there was no significant difference in Akt stimulation in either DEX-treated group, an assessment of any potential changes in a downstream, common substrate would provide further insight into differences seen in glucose clearance between the two DEX-treated groups.

Phosphorylated AS160 was significantly increased in Group 3 group over the Group 2 animals in the insulin stimulated state. Interestingly, the basal levels of phosphorylation across all groups were not significantly different from each other. This is a curious finding in that the supporting data suggest that α-tocopherol is working independently of insulin sensitivity as shown by the lack of intergroup differences in upstream kinase activity. Therefore, it is tempting to hypothesize that an additional upstream mediator of AS160 phosphorylation, such as AMPK, is upregulated in the α-tocopherol group and functions to increase the insulin stimulated response in an additive fashion. In this study, phosphorylated AMPK levels were shown to be significantly lower in Group 2 animals but were similar to control values in Group 3 animals. This finding was corroborated by the similar changes observed in ACC phosphorylation; a downstream substrate of AMPK. If AMPK is driving the observed changes in this circumstance, one may expect to see significantly higher basal phosphorylation between the DEX-treated groups, however, this was not observed. Alternatively, it was shown in rodent

epitrochlearis muscle that activation of AMPK can potentiate insulin action (Fisher *et al.,* 2002). Building on this, another hypothesis is that given an α-tocopherol-induced upregulation of AMPK phosphorylation and equal insulin plasma levels, an increase in AS160 and subsequent glucose clearance would occur in muscle following insulin-stimulation by intraperitoneal injection.

From results in smooth muscle (Ganz & Seftel, 2000) and liver (Saberi *et al.*, 2008) it is more likely that AMPK upregulation mediates the α-tocopherol-associated attenuation of insulin resistance from GC exposure. Although these studies were not conducted in skeletal muscle, the data suggest that there may be a role that α-tocopherol plays in mediating the observed improvements in glucose clearance as a function of PKC inhibition. In a study by Cortright and others (2000), inhibition and activation of PKC in human skeletal muscle strips resulted in a twofold increase and a 40 percent decrease in glucose uptake, respectively. Furthermore, when PKC was inhibited by antisense oligonucleotides in rodent models, hepatic glucose output was blunted and insulin stimulated glucose metabolism in white adipose tissue was improved (Samuel *et al.,* 2007). These findings are of particular interest when the association with AMPK is examined. Briefly, it was shown that in primary hepatocytes, inhibition of PKC with antioxidants and broad-spectrum inhibitors was directly associated with an increase in AMPK phosphorylation (Saberi *et al.,* 2008). Interestingly, several studies have confirmed that in a variety of tissues, α-tocopherol treatment can inhibit various isoforms of PKC (Ganz & Seftel, 2000; Ricciarelli *et al.,* 1998;

Venugopal *et al.*, 2002). Furthermore, a study by Azzi and colleagues (2000) showed that α-tocopherol is capable of increasing diacylglycerol (DAG) kinase, thereby decreasing DAG, which functions as an allosteric activator of PKC. While it is known that different families of PKC isoforms have varying roles in mediating insulin sensitivity (Farese *et al.*, 2005; Kajita *et al.*, 2001), together, these results suggest the possibility that α-tocopherol is able to downregulated an inhibitory isoform of PKC, and in turn upregulate the phosphorylation of AMPK.

Because of the noted difficulty in translating *in vitro* results and tissue-specific findings to whole body situations, it is difficult to make the implication that this pathway is at work in the current model. However, these connections do provide a rationale for future experimentation with respect to GC induced inhibition of glucose clearance. The current study was limited in three key areas. First, it was not able to confirm what specific roles supplementation had on plasma membrane incorporation of α-tocopherol, or more importantly, what intermediates were specifically being acted upon by α-tocopherol. Moreover, whether the supplementation of ascorbate had protective effects on α-tocopherol status was not determined. Second, it was assumed based on the changes in upstream GLUT-4 recruitment intermediates that GLUT-4 membrane translocation was affected, however a membrane fraction of translocated GLUT-4 was not obtained to confirm this. Finally, it is well established that certain compounds with antioxidant potential also have noted physiological effect independent of managing oxidative stress. For example, it was found that α-

tocopherol is involved in regulating white blood cell recruitment in an isoform-specific manner (Berdnikovs *et al.*, 2009) and more generally, it is able to regulate the metabolism of various pharmaceutical xenobiotics, including DEX, via hepatic cytochrome P450 enzymes (Brigelius-Flohe, 2007; Murray, 2006). The current study was able to determine a whole body effect of the interaction between α-tocopherol supplementation and DEX, however in order to determine specifics, further detailed studies are required.

To show that tocopherol supplementation resulted in increased plasma membrane incorporation of α-tocopherol would provide both a confirmation of diet-treatment as well as an indication of the whole body effects of α-tocopherol supplementation in conjunction with other antioxidants. Specifically, α-tocopherol plasma membrane incorporation as determined via HPLC would act as an added measure of cellular antioxidant capacity (Iuliano *et al.*, 2003) and would thus give some insight into whole body changes seen in this study, such as whether or not membrane antioxidant capacity was a major factor. Alternatively, as suggested by other groups, determining tissue levels of α-tocopherol would lend to understanding the role of the non-antioxidant roles that α-tocopherol plays in managing gene expression and other cellular functions (Azzi *et al.*, 2000; Brigelius-Flohe, 2009).

Since GLUT-4 ultimately acts as the final step in glucose clearance in skeletal muscle, measuring translocated GLUT-4 protein at the plasma membrane

would be ideal. However, technical laboratory requirements inhibited such analysis of *ex vivo* muscle samples due to tissue viability during experimentation. In subsequent studies, measuring GLUT-4 in a plasma protein fraction should be done using an on-site C2C12 or L6 myocyte model to ensure tissue viability.

Finally, in an effort to address the multitude of effects shown by α-tocopherol and various other antioxidants, a dose-response analysis of various compounds with respect to DEX in a myocyte cell culture model would provide some insight into what role these compounds play in managing a DEX-induced decrease in glucose clearance. On one hand, this type of study may provide more evidence that perhaps a non-antioxidant mechanism of α-tocopherol is at work (such as DAG metabolism), and on the other, it may indicate that there is a hormetic (U-shaped) dose response curve that was missed or simply not optimized in the current study.

In summary, these data support a potential mechanism for GC-mediated insulin resistance and moreover, a role for α-tocopherol supplementation in mitigating these effects. This study has shown that dietary supplementation with α-tocopherol can improve fasting insulin sensitivity in cases of GC-induced loss of insulin sensitivity. Additionally, despite improvements in one marker of oxidative stress as a function of α-tocopherol, it is possible that GC such as DEX may not mediate their insulin desensitizing effects through increases in oxidative stress, but rather through insulin-independent mechanisms. In short, while DEX

treatment may induce insulin resistance through an insulin-dependent pathway, it has been shown that α-tocopherol can attenuate these effects through an insulin-independent pathway via AMPK.

3.7 Literature Cited

Amaral, S. L., de Andrade Louzada, J. C., Dionisio, T. J., Viscelli, B. A., Dionisio, E. J., Bosqueiro, J. R., et al. (2010). Opposite effects of exercise and dexamethasone on skeletal muscle glucose uptake: Role of AMPK and CaMKII. *The FASEB Journal, 24* (MeetingAbstracts), 806.19.

Archuleta, T. L., Lemieux, A. M., Saengsirisuwan, V., Teachey, M. K., Lindborg, K. A., Kim, J. S., et al. (2009). Oxidant stress-induced loss of IRS-1 and IRS-2 proteins in rat skeletal muscle: Role of p38 MAPK. *Free Radical Biology & Medicine, 47*(10), 1486-1493.

Azzi, A., Breyer, I., Feher, M., Pastori, M., Ricciarelli, R., Spycher, S., et al. (2000). Specific cellular responses to alpha-tocopherol. *The Journal of Nutrition, 130*(7), 1649-1652.

Berdnikovs, S., Abdala-Valencia, H., McCary, C., Somand, M., Cole, R., Garcia, A., et al. (2009). Isoforms of vitamin E have opposing immunoregulatory functions during inflammation by regulating leukocyte recruitment. *Journal of Immunology (Baltimore, Md.: 1950), 182*(7), 4395-4405.

Brigelius-Flohe, R. (2007). Adverse effects of vitamin E by induction of drug metabolism. *Genes & Nutrition, 2*(3), 249-256.

Brigelius-Flohe, R. (2009). Vitamin E: The shrew waiting to be tamed. *Free Radical Biology & Medicine, 46*(5), 543-554.

Buren, J., Lai, Y. C., Lundgren, M., Eriksson, J. W., & Jensen, J. (2008). Insulin action and signalling in fat and muscle from dexamethasone-treated rats. *Archives of Biochemistry and Biophysics, 474*(1), 91-101.

Buren, J., Liu, H. X., Jensen, J., & Eriksson, J. W. (2002). Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes. *European Journal of Endocrinology / European Federation of Endocrine Societies, 146*(3), 419-429.

Cortright, R. N., Azevedo, J. L.,Jr, Zhou, Q., Sinha, M., Pories, W. J., Itani, S. I., et al. (2000). Protein kinase C modulates insulin action in human skeletal muscle. *American Journal of Physiology.Endocrinology and Metabolism, 278*(3), E553-62.

Dimitriadis, G., Leighton, B., Parry-Billings, M., Sasson, S., Young, M., Krause, U., et al. (1997). Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle. *The Biochemical Journal, 321 (Pt 3)*(Pt 3), 707-712.

Dokken, B. B., Saengsirisuwan, V., Kim, J. S., Teachey, M. K., & Henriksen, E. J. (2008). Oxidative stress-induced insulin resistance in rat skeletal muscle: Role of glycogen synthase kinase-3. *American Journal of Physiology.Endocrinology and Metabolism, 294*(3), E615-21.

Eguez, L., Lee, A., Chavez, J. A., Miinea, C. P., Kane, S., Lienhard, G. E., et al. (2005). Full intracellular retention of GLUT4 requires AS160 rab GTPase activating protein. *Cell Metabolism, 2*(4), 263-272.

Ewart, H. S., Somwar, R., & Klip, A. (1998). Dexamethasone stimulates the expression of GLUT1 and GLUT4 proteins via different signalling pathways in L6 skeletal muscle cells. *FEBS Letters, 425*(1), 179-183.

Farese, R. V., Sajan, M. P., & Standaert, M. L. (2005). Atypical protein kinase C in insulin action and insulin resistance. *Biochemical Society Transactions, 33*(Pt 2), 350-353.

Fisher, J. S., Gao, J., Han, D. H., Holloszy, J. O., & Nolte, L. A. (2002). Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *American Journal of Physiology.Endocrinology and Metabolism, 282*(1), E18-23.

Fukuoka, H., Iida, K., Nishizawa, H., Imanaka, M., Takeno, R., Iguchi, G., et al. (2010). IGF-I stimulates reactive oxygen species (ROS) production and inhibits insulin-dependent glucose uptake via ROS in 3T3-L1 adipocytes. *Growth Hormone & IGF Research : Official Journal of the Growth Hormone Research Society and the International IGF Research Society,*

Gaidhu, M. P., Perry, R. L., Noor, F., & Ceddia, R. B. (2010). Disruption of AMPK{alpha}1 signaling prevents AICAR-induced inhibition of AS160/TBC1D4 phosphorylation and glucose uptake in primary rat adipocytes. *Molecular Endocrinology (Baltimore, Md.),*

Ganz, M. B., & Seftel, A. (2000). Glucose-induced changes in protein kinase C and nitric oxide are prevented by vitamin E. *American Journal of Physiology.Endocrinology and Metabolism, 278*(1), E146-52.

Giorgino, F., Almahfouz, A., Goodyear, L. J., & Smith, R. J. (1993). Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle in vivo. *The Journal of Clinical Investigation, 91*(5), 2020-2030.

Giozzet, V. A., Rafacho, A., Boschero, A. C., Carneiro, E. M., & Bosqueiro, J. R. (2008). Dexamethasone treatment in vivo counteracts the functional pancreatic islet alterations caused by malnourishment in rats. *Metabolism: Clinical and Experimental, 57*(5), 617-624.

Gokkusu, C., Palanduz, S., Ademoglu, E., & Tamer, S. (2001). Oxidant and antioxidant systems in niddm patients: Influence of vitamin E supplementation. *Endocrine Research, 27*(3), 377-386.

Haber, R. S., & Weinstein, S. P. (1992). Role of glucose transporters in glucocorticoid-induced insulin resistance. GLUT4 isoform in rat skeletal muscle is not decreased by dexamethasone. *Diabetes, 41*(6), 728-735.

Houstis, N., Rosen, E. D., & Lander, E. S. (2006). Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature, 440*(7086), 944-948.

Iuchi, T., Akaike, M., Mitsui, T., Ohshima, Y., Shintani, Y., Azuma, H., et al. (2003). Glucocorticoid excess induces superoxide production in vascular endothelial cells and elicits vascular endothelial dysfunction. *Circulation Research, 92*(1), 81-87.

Iuliano, L., Micheletta, F., Natoli, S., Ginanni Corradini, S., Iappelli, M., Elisei, W., et al. (2003). Measurement of oxysterols and alpha-tocopherol in plasma and tissue samples as indices of oxidant stress status. *Analytical Biochemistry, 312*(2), 217-223.

Kajita, K., Ishizuka, T., Miura, A., Kanoh, Y., Ishizawa, M., Kimura, M., et al. (2001). Glucocorticoid-induced insulin resistance associates with activation of protein kinase C isoforms. *Cellular Signalling, 13*(3), 169-175.

Kane, S., Sano, H., Liu, S. C., Asara, J. M., Lane, W. S., Garner, C. C., et al. (2002). A method to identify serine kinase substrates. akt phosphorylates a novel adipocyte protein with a rab GTPase-activating protein (GAP) domain. *The Journal of Biological Chemistry, 277*(25), 22115-22118.

Kramer, H. F., Taylor, E. B., Witczak, C. A., Fujii, N., Hirshman, M. F., & Goodyear, L. J. (2007). Calmodulin-binding domain of AS160 regulates contraction- but not insulin-stimulated glucose uptake in skeletal muscle. *Diabetes, 56*(12), 2854-2862.

Kramer, H. F., Witczak, C. A., Taylor, E. B., Fujii, N., Hirshman, M. F., & Goodyear, L. J. (2006b). AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *The Journal of Biological Chemistry, 281*(42), 31478-31485.

Laight, D. W., Desai, K. M., Gopaul, N. K., Anggard, E. E., & Carrier, M. J. (1999). F2-isoprostane evidence of oxidant stress in the insulin resistant, obese zucker rat: Effects of vitamin E. *European Journal of Pharmacology, 377*(1), 89-92.

Lastra, G., Whaley-Connell, A., Manrique, C., Habibi, J., Gutweiler, A. A., Appesh, L., et al. (2008). Low-dose spironolactone reduces reactive oxygen species generation and improves insulin-stimulated glucose transport in skeletal muscle in the TG(mRen2)27 rat. *American Journal of Physiology.Endocrinology and Metabolism, 295*(1), E110-6.

Maddux, B. A., See, W., Lawrence, J. C.,Jr, Goldfine, A. L., Goldfine, I. D., & Evans, J. L. (2001). Protection against oxidative stress-induced insulin resistance in rat L6 muscle cells by mircomolar concentrations of alpha-lipoic acid. *Diabetes, 50*(2), 404-410.

Matsuda, M., & DeFronzo, R. A. (1999). Insulin sensitivity indices obtained from oral glucose tolerance testing: Comparison with the euglycemic insulin clamp. *Diabetes Care, 22*(9), 1462-1470.

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., & Turner, R. C. (1985). Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia, 28*(7), 412-419.

Mu, J., Brozinick, J. T.,Jr, Valladares, O., Bucan, M., & Birnbaum, M. J. (2001). A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Molecular Cell, 7*(5), 1085-1094.

Murray, M. (2006). Altered CYP expression and function in response to dietary factors: Potential roles in disease pathogenesis. *Current Drug Metabolism, 7*(1), 67-81.

Nakamura, S., Takamura, T., Matsuzawa-Nagata, N., Takayama, H., Misu, H., Noda, H., et al. (2009). Palmitate induces insulin resistance in H4IIEC3 hepatocytes through reactive oxygen species produced by mitochondria. *The Journal of Biological Chemistry, 284*(22), 14809-14818.

Ngo, S., Barry, J. B., Nisbet, J. C., Prins, J. B., & Whitehead, J. P. (2009). Reduced phosphorylation of AS160 contributes to glucocorticoid-mediated inhibition of glucose uptake in human and murine adipocytes. *Molecular and Cellular Endocrinology, 302*(1), 33-40.

Nicod, N., Giusti, V., Besse, C., & Tappy, L. (2003). Metabolic adaptations to dexamethasone-induced insulin resistance in healthy volunteers. *Obesity Research, 11*(5), 625-631.

Ohtsuka, A., Kojima, H., Ohtani, T., & Hayashi, K. (1998). Vitamin E reduces glucocorticoid-induced oxidative stress in rat skeletal muscle. *Journal of Nutritional Science and Vitaminology, 44*(6), 779-786.

Op 't Eijnde, B., Urso, B., Richter, E. A., Greenhaff, P. L., & Hespel, P. (2001). Effect of oral creatine supplementation on human muscle GLUT4 protein content after immobilization. *Diabetes, 50*(1), 18-23.

Orzechowski, A., Ostaszewski, P., Wilczak, J., Jank, M., Bałasińska, B., War, P., et al. (2002). Rats with a glucocorticoid-induced catabolic state show symptoms of oxidative stress and spleen atrophy: The effects of age and recovery. *Journal of Veterinary Medicine Series A, 49*(5), 256-263.

Pehmoller, C., Treebak, J. T., Birk, J. B., Chen, S., Mackintosh, C., Hardie, D. G., et al. (2009). Genetic disruption of AMPK signaling abolishes both contraction- and insulin-stimulated TBC1D1 phosphorylation and 14-3-3 binding in mouse skeletal muscle. *American Journal of Physiology.Endocrinology and Metabolism, 297*(3), E665-75.

Pereira, B., Bechara, E. J., Mendonca, J. R., & Curi, R. (1999). Superoxide dismutase, catalase and glutathione peroxidase activities in the lymphoid organs and skeletal muscles of rats treated with dexamethasone. *Cell Biochemistry and Function, 17*(1), 15-19.

Rafacho, A., Cestari, T. M., Taboga, S. R., Boschero, A. C., & Bosqueiro, J. R. (2009). High doses of dexamethasone induce increased beta-cell proliferation in pancreatic rat islets. *American Journal of Physiology.Endocrinology and Metabolism, 296*(4), E681-9.

Ricciarelli, R., Tasinato, A., Clement, S., Ozer, N. K., Boscoboinik, D., & Azzi, A. (1998). Alpha-tocopherol specifically inactivates cellular protein kinase C alpha by changing its phosphorylation state. *The Biochemical Journal, 334 (Pt 1)*(Pt 1), 243-249.

Ruzzin, J., Wagman, A. S., & Jensen, J. (2005). Glucocorticoid-induced insulin resistance in skeletal muscles: Defects in insulin signalling and the effects of a selective glycogen synthase kinase-3 inhibitor. *Diabetologia, 48*(10), 2119-2130.

Saad, M. J., Folli, F., Kahn, J. A., & Kahn, C. R. (1993). Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *The Journal of Clinical Investigation, 92*(4), 2065-2072.

Saberi, B., Shinohara, M., Ybanez, M. D., Hanawa, N., Gaarde, W. A., Kaplowitz, N., et al. (2008). Regulation of H(2)O(2)-induced necrosis by PKC and AMP-activated kinase signaling in primary cultured hepatocytes. *American Journal of Physiology.Cell Physiology, 295*(1), C50-63.

Samuel, V. T., Liu, Z. X., Wang, A., Beddow, S. A., Geisler, J. G., Kahn, M., et al. (2007). Inhibition of protein kinase cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. *The Journal of Clinical Investigation, 117*(3), 739-745.

Sano, H., Kane, S., Sano, E., Miinea, C. P., Asara, J. M., Lane, W. S., et al. (2003). Insulin-stimulated phosphorylation of a rab GTPase-activating protein regulates GLUT4 translocation. The Journal of Biological Chemistry, 278(17), 14599-14602.

Schafer, S. C., Wallerath, T., Closs, E. I., Schmidt, C., Schwarz, P. M., Forstermann, U., et al. (2005). Dexamethasone suppresses eNOS and CAT-1 and induces oxidative stress in mouse resistance arterioles. *AJP - Heart and Circulatory Physiology, 288*(1), H436-444.

Schneiter, P., & Tappy, L. (1998). Kinetics of dexamethasone-induced alterations of glucose metabolism in healthy humans. *The American Journal of Physiology, 275*(5 Pt 1), E806-13.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*(1), 76-85.

Tappy, L., Randin, D., Vollenweider, P., Vollenweider, L., Paquot, N., Scherrer, U., et al. (1994). Mechanisms of dexamethasone-induced insulin resistance in healthy humans. *The Journal of Clinical Endocrinology and Metabolism, 79*(4), 1063-1069.

Thong, F. S., Bilan, P. J., & Klip, A. (2007). The rab GTPase-activating protein AS160 integrates akt, protein kinase C, and AMP-activated protein kinase signals regulating GLUT4 traffic. *Diabetes, 56*(2), 414-423.

Tirosh, A., Rudich, A., Potashnik, R., & Bashan, N. (2001). Oxidative stress impairs insulin but not platelet-derived growth factor signalling in 3T3-L1 adipocytes. *The Biochemical Journal, 355*(Pt 3), 757-763.

Treebak, J. T., Frosig, C., Pehmoller, C., Chen, S., Maarbjerg, S. J., Brandt, N., et al. (2009). Potential role of TBC1D4 in enhanced post-exercise insulin action in human skeletal muscle. *Diabetologia, 52*(5), 891-900.

Turnbow, M., Keller, S., Rice, K., & Garner, C. (1994). Dexamethasone down-regulation of insulin receptor substrate-1 in 3T3-L1 adipocytes. *Journal of Biological Chemistry, 269*(4), 2516-2520.

Venkatesan, N., Lim, J., Bouch, C., Marciano, D., & Davidson, M. B. (1996). Dexamethasone-induced impairment in skeletal muscle glucose transport is

not reversed by inhibition of free fatty acid oxidation. *Metabolism: Clinical and Experimental, 45*(1), 92-100.

Venugopal, S. K., Devaraj, S., Yang, T., & Jialal, I. (2002). Alpha-tocopherol decreases superoxide anion release in human monocytes under hyperglycemic conditions via inhibition of protein kinase C-alpha. *Diabetes, 51*(10), 3049-3054.

Vera, E. R., Battell, M. L., Bhanot, S., & McNeill, J. H. (2002). Effects of age and anesthetic on plasma glucose and insulin levels and insulin sensitivity in spontaneously hypertensive and wistar rats. *Canadian Journal of Physiology and Pharmacology, 80*(10), 962-970.

Vincent, H. K., Bourguignon, C. M., Weltman, A. L., Vincent, K. R., Barrett, E., Innes, K. E., et al. (2009). Effects of antioxidant supplementation on insulin sensitivity, endothelial adhesion molecules, and oxidative stress in normal-weight and overweight young adults. *Metabolism, 58*(2), 254-262.

Weinstein, S. P., Wilson, C. M., Pritsker, A., & Cushman, S. W. (1998). Dexamethasone inhibits insulin-stimulated recruitment of GLUT4 to the cell surface in rat skeletal muscle. *Metabolism: Clinical and Experimental, 47*(1), 3-6.

Williams, D. B., Sutherland, L. N., Bomhof, M. R., Basaraba, S. A., Thrush, A. B., Dyck, D. J., et al. (2009). Muscle-specific differences in the response of mitochondrial proteins to beta-GPA feeding: An evaluation of potential mechanisms. *American Journal of Physiology.Endocrinology and Metabolism, 296*(6), E1400-8.

Wright, D. C., Han, D. H., Garcia-Roves, P. M., Geiger, P. C., Jones, T. E., & Holloszy, J. O. (2007). Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression. *The Journal of Biological Chemistry, 282*(1), 194-199.

Wu, Y., Zhao, W., Zhao, J., Zhang, Y., Qin, W., Pan, J., et al. (2010). REDD1 is a major target of testosterone action in preventing dexamethasone-induced muscle loss. *Endocrinology, 151*(3), 1050-1059.

You, J. M., Yun, S. J., Nam, K. N., Kang, C., Won, R., & Lee, E. H. (2009). Mechanism of glucocorticoid-induced oxidative stress in rat hippocampal slice cultures. *Canadian Journal of Physiology and Pharmacology, 87*(6), 440-447.

Zerial, M., & McBride, H. (2001). Rab proteins as membrane organizers. *Nature Reviews.Molecular Cell Biology, 2*(2), 107-117.

4.0 Appendix

4.1 Control and supplemented diet formulation and nutrient composition

Component	Control Diet	Supplemented Diet
	g/kg	
Casein	317.6	317.6
L-Methionine	2.94	2.94
Dextrose, monohydrate	275.417	275.417
Corn Starch	264.0	264.0
Cellulose	59.82	58.82
Mineral Mix, Bemhart-Tomarelli	59.82	59.82
Sodium Selenite (0.0445% in sucrose)	0.353	0.353
Manganese Sulfate, monohydrate	0.28	0.28
Vitamin Mix, A.O.A.C.	11.8	11.8
Inositol	7.35	7.35
Choline Chloride	1.62	1.62
Flax oil	8.04	8.04
Sunflower oil	59.04	59.04
Hydrogenated canola	82.92	82.92
dl-all-*rac*-α-tocopherol	-	0.500
L-ascorbate	-	0.500
Nutrient	**% of calories**	
Fat	28	
Carbohydrate	45	
Protein	27	

Modified from Harlan Teklad Custom Research Diets (Madison, WI); TD.06206 85% Basal Diet