Methylglyoxal and Glyoxalase 1 Metabolism in Skeletal Muscle:

Effect of Type 2 Diabetes and Exercise

BY

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THESIS

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DEDICATION PAGE

To my wife, Hannah: Your love and support over the years have been instrumental throughout this entire process. You helped make this possible and I am forever grateful.

To my Mom and Dad: You raised me to always do my best and to work my hardest. You taught me to be my own person and cultured my ingenuity. All my prior, current and future accomplishments are due to that loving and nurturing environment in which you raised all your children.

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AE	Aerobic exercise training
AGE	Advanced glycation endproducts
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AKR1B1	Aldose reductase (family 1, member 1)
AKT	serine/threonine kinase Akt, also known as protein kinase B or PKB
AMP	Adenosine monophosphate
AMPK	5’ amp activated protein kinase
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CVD	Cardiovascular disease
DAG	Diacylglycerol
DEXA	Dual energy X-ray absorptiometry (DXA)
DHAP	Dihydroxy-acetone-phosphate
ERK1/2	Extracellular signal regulated protein kinases 1 and 2
FFA	Free fatty acids
G3P	Glyceraldehyde-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDR	Glucose disposal rate determined from the hyperinsulinemic-euglycemic clamp
GLO1	Glyoxalase-1
GLO2	Glyoxalase-2
GLUT4	Glucose transporter type 4
GS	Glycogen synthase
GSH	Glutathione

ABBREVIATIONS (continued)

HbA1c	Hemoglobin A1c
HESP	Hesperitin
HSKMC	Primary human skeletal muscle cell line
IRS1	Insulin receptor substrate 1
kDa	kiloDaltons (molecular weight)
Keap1	Kelch-like ECH-Associating protein 1
LHC	Lean healthy control group
MG	Methylglyoxal
MG-BSA	MG-modified bovine serum albumin
NOGD	Non-oxidative glucose disposal
NRF2	Nuclear factor-erythroid 2 p45 subunit-related factor 2
OB-IR	Obese, insulin resistant individuals
pACC	Phosphorylated acetyl-CoA carboxylase
PI3K	Phosphoinositide 3-kinase
RAGE	Receptor for advanced glycation endproducts
RBC	Red blood cells
ROS	Reactive oxygen species
T2DM	Type II Diabetes Mellitus
TPI	Triose phosphate isomerase
tRES	trans-resveratrol

SUMMARY

Type 2 diabetes (T2DM) results in chronic hyperglycemia (5), which damages tissues in large part from the excessive generation of the highly reactive, α-dicarbonyl, methylglyoxal (MG). MG modifies proteins, which then accumulate in the plasma and tissues of individuals with T2DM. The primary detoxification of MG occurs through the enzyme, glyoxalase-1 (GLO1). However, in T2DM, GLO1 is reduced in insulin-independent tissues and MG can accumulate and modify proteins, a situation known as 'dicarbonyl stress.' Dicarbonyl stress and the MG-GLO1 axis have not been characterized in skeletal muscle, but recent cell culture and pre-clinical evidence implicates a role in the development of skeletal muscle insulin resistance, the primary defect in T2DM. Given the well-defined role of aerobic exercise on improving of skeletal muscle insulin sensitivity and recent gene microarray data showing improvements in skeletal muscle GLO1 protein, it is logical that aerobic exercise may exert beneficial effects on skeletal muscle by improving GLO1 protein expression and reducing dicarbonyl stress.

Therefore, we investigated the effects of T2DM and exercise on the MG-GLO1 axis in skeletal muscle utilizing a clinical-translational approach involving human subjects and cell culture studies. First, a cross-sectional analysis was performed to characterize the MG-GLO1 axis in lean, healthy control (LHC) subjects compared to individuals with T2DM by performing a hyperinsulinemic-euglycemic clamp with skeletal muscle biopsies. The effect of exercise was investigated by implementing a 12-week aerobic exercise intervention in a separate group of obese, insulin resistant adults. Finally, we utilized an *in vitro* model to investigate skeletal muscle specific pathways of MG-modification of proteins and elucidate potential GLO1 gain-of-function strategies (T2DM therapeutics (metformin) and exercise stimulation).

The skeletal muscle of individuals with T2DM showed reduced GLO1 protein expression ($p<0.05$) which was associated with measures of obesity (BMI and percent body fat, $p<0.05$) and insulin resistance (HOMA-IR and glucose disposal rate, $p<0.05$). During the clamp, MG-modified proteins increased in the T2DM group only ($p<0.05$), implicating a differential effect of insulin-stimulation between LHC and T2DM. In concert, primary GLO1 regulatory proteins were altered in T2DM (NRF2 and Keap1, $p<0.05$) compared to LHC both at baseline and during the clamp. The completion of a 12wk aerobic exercise

intervention rescued skeletal muscle GLO1 protein expression in obese, insulin resistant adults ($p<0.05$) to levels comparable to LHC. Cell culture studies revealed that: hyperglycemia *per se* increases MG-modifications in human skeletal muscle cells, MG alters the activity of specific skeletal muscle metabolic regulatory proteins, and metformin (first line medication for T2DM) reduces MG-modification of proteins and increases GLO1 protein expression.

Collectively, our data indicate that the MG-GLO1 axis is dysregulated in the skeletal muscle of T2DM and that aerobic exercise and metformin may play a protective role acting through this axis. Further investigation is warranted to understand the therapeutic potential of targeting the MG- GLO1 axis in skeletal muscle.

CHAPTER I: INTRODUCTION

Background

Despite decades of coordinated efforts, T2DM remains a serious public health issue. In the United States alone, over 29 million adults are suffering from T2DM and ~86 million are pre-diabetic and without intervention, may develop overt T2DM as well (6). T2DM is characterized by chronic hyperglycemia secondary to peripheral (primarily skeletal muscle) insulin resistance (7). Although the etiology and pathology of T2DM and its complications remain multifactorial, an emerging area of research is the highly reactive glycolytic byproduct, methylglyoxal (MG)**.** MG is a dicarbonyl that modifies or 'glycates' proteins by covalent cross-linking, which alters susceptibility to proteolysis, causes structural distortion and loss of side chain charge (8, 9). MG forms as a spontaneous byproduct of glycolysis, although dysfunction of the glycolytic enzymes triose phosphate isomerase (TPI) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can also increase MG production. The excess accumulation of MG, termed 'dicarbonyl stress,' forms glycation adducts on proteins and generates advanced glycation endproducts (AGE). The most prevalent MG-directed AGE is the hydroimidizalone, MG-H1, caused by a direct MG attack on arginine residues. This MG-modification has the potential to disrupt protein function, as arginine residues are commonly located in sites common to protein-protein interfaces, enzyme-substrate contact or protein-DNA assembly. Proteins that become modified by MG and become dysfunctional are known as the 'dicarbonyl proteome'

The biological natural defense against dicarbonyl stress is the glyoxalase enzymatic defense system, composed of 2 enzymes: glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2). Together, GLO1 and GLO2 convert the highly reactive MG moiety into a more stable D-lactate molecule. GLO1 is a ubiquitously expressed cytosolic protein that catalyzes the conversion of MG first to an s-lactoylglutathione intermediate and ultimately to D-lactate via an additional reaction catalyzed by GLO2. This two-step detoxification of MG is extremely efficient, converting greater than 99% of MG to D-lactate under normal physiologic conditions. Also, as transient increases in MG and oxidative stress have been shown to increase GLO1 protein expression, thus imparting further protection from MG-directed protein modifications (10). A

working model of cellularMG-GLO1 axis regulation is presented in **Figure 1**. However, GLO1 expression and thus the ability to detoxify MG decreases with T2DM in both animal models (11-13) and humans (14). The resultant MG accumulation is believed to contribute to complications associated with diabetes such as

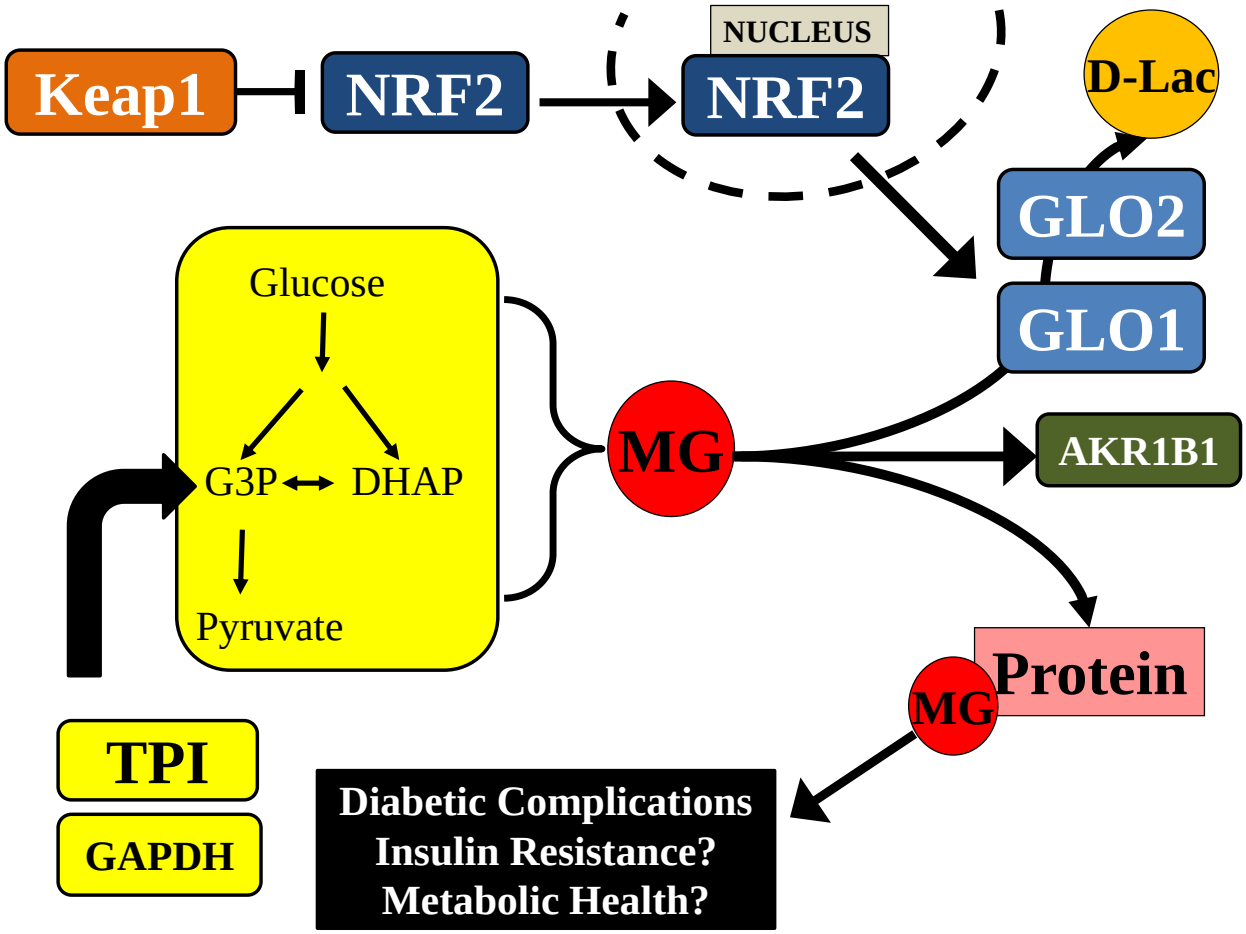

Figure 1. Working model of the MG-GLO1 axis
MG is produced as a spontaneous byproduct of glycolysis and excessive production can occur from dysregulation of TPI and/or GAPDH from a backlog of triose phosphate intermediate buildup (G3P and DHAP). In T2DM, MG accumulates and modifies proteins, which contribute to diabetic complications like nephropathy, neuropathy and retinopathy. In healthy physiology, MG is efficiently detoxified to D-Lac by the glyoxalase system comprised of GLO1 and GLO2. Protein expression of GLO1 is transcriptionally regulated by the NRF2-Keap1 system. AKR1B1 also detoxifies MG, but at lower rates compared to GLO1-GLO2. MG, methylglyoxal; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone-phosphate; T2DM. type II diabetes mellitus; D-Lac, D-lactate; GLO1 & 2, glyoxalase 1 & 2; NRF2, nuclear factor-erythroid 2 p45 subunit-related factor 2; Keap1, kelch-like ECH-associating protein 1; AKR1B1, aldose reductase, family 1, member 1.

nephropathy (15), neuropathy (16) and retinopathy (17). Further, one additional enzyme, aldo keto reductase (AKR1B1), also has the ability to detoxify MG to a less harmful byproduct, but plays a minor role compared to GLO1.

Although the focus of MG and GLO1 research has been directed at insulin-independent tissues, recent evidence suggests a dysregulation of the MG-GLO1 axis extends to the metabolic skeletal muscle tissue. Dicarbonyl stress has been linked to important skeletal muscle pathways including the disruption of insulin signaling in L6 skeletal muscle cells (18) and potentiation of oxidative stress in MG-treated rats (19). In addition, rat skeletal muscle GLO1 activity is reduced in response to oxidative stress and likely contributes to increased mitochondrial damage (20). Further, a major skeletal muscle regulatory protein, 5' amp activated protein kinase (AMPK), has structural characteristics implicating a susceptibility to MG-modification and potential metabolic dysregulation (4). Together, these data suggest dysregulation of the MG-GLO1 axis may affect skeletal muscle health.

Given the importance of skeletal muscle tissue on whole body metabolic health, elucidating metabolic consequences of MG-modified proteins may have implications for metabolic diseases and give rise to innovative therapeutics. Aerobic exercise (AE) training is already established as a countermeasure for skeletal muscle insulin resistance (21, 22), eliciting beneficial effects on whole body glucose metabolism. Pre-clinical evidence provides mechanistic support that AE may stimulate GLO1 protein through transcriptional regulation by the NRF2-Keap1 system (10). Additionally, gene microarray data shows skeletal muscle *GLO1 mRNA* increases with chronic aerobic exercise training (23). Furthermore, individuals at risk for developing T2DM (obese, insulin resistant individuals) can reduce the risk of progression to T2DM by over 50% with a lifestyle intervention that focuses on reducing calorie intake and increasing exercise (6). Despite the well-documented benefits of exercise, daily activity recommendations are often not followed leaving the onus of clinical support on medications. Specifically, Metformin is a first-line medication for treating T2DM and has recently been implicated as a scavenger of MG due to its biguanidine structure (24). The glucose lowering effect of metformin is well known, but metformin may play an additional role in preventing complications of diabetes through preventing AGE formation (25, 26).

Understanding the therapeutic potential of MG biology and the regulation of GLO1 protein expression and activity in human skeletal muscle will enhance our progression to the prevention and treatment of insulin resistance and T2DM.

Therefore, the primary goal of this research will be to characterize the skeletal muscle MG-GLO1 axis in the context of T2DM and aerobic exercise and to investigate muscle specific mechanisms of MG-GLO1 axis regulation and MG-directed protein modifications.

Specific Aims and Hypotheses

The central hypothesis of our research is that the T2DM condition disrupts the MG-GLO1 axis in skeletal muscle, conferring susceptibility to dicarbonyl stress, which disrupts important metabolic pathways, and that aerobic exercise may play a protective role through enhancing GLO1 protein expression and enzymatic activity. We sought to investigate this central hypothesis by investigating the following specific aims:

Specific Aim 1: Determine the effects of T2DM and whole body insulin stimulation on MG-modified proteins, GLO1 protein expression, GLO1 enzymatic activity and additional proteins regulating the MG-GLO1 axis in human skeletal muscle tissue.

Hypothesis 1: MG-modified proteins will be increased and GLO1 protein expression and enzymatic activity will be reduced in the skeletal muscle of individuals with T2DM compared to a lean, healthy control group (LHC).

Hypothesis 2: Proteins regulating the MG-GLO1 axis will be differentially effected by whole body insulin stimulation in the skeletal muscle of individuals with T2DM compared to LHC.

Specific Aim 2: Determine the effects of an aerobic exercise training intervention on MG-modified proteins, GLO1 protein expression and GLO1 enzymatic activity in the skeletal muscle tissue of obese, insulin resistant adults.

Hypothesis: An intensive lifestyle intervention involving 12-weeks of aerobic exercise training will attenuate MG-modified proteins while increasing GLO1 protein expression and GLO1 enzymatic activity in skeletal muscle tissue of obese, insulin resistant adults.

Specific Aim 3: Identify mechanisms that contribute to dicarbonyl stress and specific MG-modifications of proteins critical to skeletal muscle metabolism; and elucidate potential GLO1 gain-of-function strategies using an in vitro approach.

Hypothesis 1: Hyperglycemia will increase MG-modification of proteins in human skeletal muscle cells (HSKMCs).

Hypothesis 2: Dicarbonyl stress imposed by increasing the MG concentration in cell culture media will specifically modify AMPK, reduce AMPK activity and inhibit the stimulatory effect of AICAR (a potent AMPK activator) in HSKMCs.

Hypothesis 3: Metformin (the most commonly prescribed medication for T2DM) will elicit a protective effect on HSKMCs by preventing MG-induced modification of proteins and increasing GLO1 protein expression.

CHAPTER II: LITERATURE REVIEWED

Introduction

The prevalence of type 2 diabetes is a well-defined public health concern (27) and places a tremendous burden placed on health care systems (28). Chronic hyperglycemia is the hallmark of T2DM and damages cells and tissues from a multitude of both established and still to be defined pathways. One proposed mechanism of hyperglycemia-associated damage is through elevated levels of advanced glycation endproducts (29-31). The majority of intracellular AGE production occurs spontaneously with nutrient metabolism (32, 33) from the highly reactive α-dicarbonyl, methylglyoxal (34). MG-directed AGEs have been shown to disrupt important metabolic pathways related to insulin signaling (18, 35) and induce oxidative stress (19, 36). MG overproduction, termed 'dicarbonyl stress,' occurs during metabolic diseases, such as T2DM and metabolic syndrome (37-39) and is directly related to a reduced function of the enzyme, glyoxylase-1 (14, 40). GLO1 detoxifies MG as its primary cellular function, preventing MG-directed protein modification and subsequent AGE formation (10). Hyperglycemia-associated dysregulation of the MG-GLO1 axis is well documented in insulin-independent tissues and contributes to debilitating diabetic complications such as nephropathy, neuropathy and retinopathy, while *in vitro* and pre-clinical models suggest a role for MG-GLO1 axis dysregulation in skeletal muscle tissue as well. In addition, aerobic exercise, a well-known countermeasure to skeletal muscle insulin resistance, may stimulate GLO1 production (23), which has been shown to be protective to dicarbonyl stress in cell culture and animal models. Despite the importance of maintaining healthy skeletal muscle metabolism in preventing insulin resistance and the progression towards T2DM, MG-GLO1 axis regulation has yet to be well investigated in human skeletal muscle and its potential role in the both the etiology and pathology of insulin resistance and T2DM remains intriguing.

Importance of Skeletal Muscle in Whole Body Metabolism

Skeletal muscles are one of the most physically and metabolically important tissues in the body. Skeletal muscle makes up ~40% of total body weight in humans and contains roughly two thirds of total

proteins in the body. The mechanical function of skeletal muscle cannot be overlooked, as its primary physiologic role is to convert chemical energy to mechanical energy, maintaining whole body posture and providing a means of locomotion. But skeletal muscle also plays a vital role in whole body metabolism and is the largest metabolic organ contributing to ~20% of basal energy expenditure (41). Skeletal muscle has a profound ability to metabolize both lipids (oxidative phosphorylation) and glucose (anaerobic and aerobic glycolysis) and is the primary reservoir for amino acids. Importantly, skeletal muscle has an abundance of both mitochondria and glycolytic enzymes providing the machinery to shift its primary substrate utilization depending on availability and energy requirements. The majority of this cellular energy is obtained from mitochondrial ATP production within the muscle cells. The importance of skeletal muscle nutrient metabolism is highlighted by considering the disparate difference in muscle energy requirements during rest and during exercise. Using the example of a 70 kg man, with a basal energy expenditure of 2000 kcal/day (42), skeletal muscle would require ~400 kcal/day (calculated as 20% of basal energy requirements (41)) or ~17 kcal per hour. However, if that same man was in the midst of running a marathon at Olympic speed, skeletal muscle requirements may raise higher than 2000 kcal for the two-hour bout alone. This would require the generation and utilization of ~1000 kcal/hour, over 50 fold higher than at resting! Furthermore, the ability to regulate protein synthesis and breakdown to not only store, but also selectively release amino acids for use by other tissues for the production of organ specific proteins, hormones or even glucose is imperative to whole body metabolic health and functionality. The profound metabolic flexibility of healthy skeletal muscle is controlled by internal cell signaling events and hormonal direction, the latter of which is primarily regulated by insulin.

Skeletal Muscle Insulin Action

Insulin is an important regulatory hormone, released by the ß-cells of the pancreas in response to increases in blood glucose and amino acids, which occur after meals. Insulin action on metabolic tissue like the skeletal muscle, adipose tissue and the liver work in concert to control whole body nutrient equilibrium and fuel utilization or storage. Some primary functions of insulin on skeletal muscle carbohydrate

metabolism include increasing glucose transport into the skeletal muscle, increasing the rate of glycolysis stimulating glycogen synthesis and inhibiting glycogen breakdown (43-45). In the fasted state, plasma insulin concentrations in healthy individuals are *ca.* 50 pM, but upon mixed meal ingestion, rise to *ca.* 300 pM at 30 minutes and follow a steady decent back to baseline levels by *ca.* 180 minutes after the meal (46). During this 3-4 hour postprandial hyperinsulinemia, insulin acts on the liver to inhibit endogenous glucose production (47) and increase peripheral (skeletal muscle and adipose) tissue glucose uptake (48). Maximal peripheral stimulation occurs at higher doses (*ca.*700 pM insulin), which is blunted in insulin resistant individuals (48). It should be noted that impaired suppression of endogenous glucose production occurs at plasma insulin levels <300pM in insulin resistant individuals and those with T2DM

Skeletal muscle primarily utilizes fat as an energy substrate during the fasting (postabsorptive) state, but glucose during fed (postprandial) states. Control of this substrate utilization between fed and fasting states is directed by insulin action and termed 'metabolic flexibility' (49). During the fasting state, insulin levels are low, which promotes adipocyte lipolysis and availability of circulating free fatty acids (FFA) for skeletal muscle use while preserving blood glucose for utilization by the brain (50). After a carbohydrate or mixed macronutrient meal, elevations in blood glucose trigger insulin secretion by the beta cells of the pancreas. This post-prandial hyperinsulinemia reduces hepatic glucose output, blunts adipocyte lipolysis and simultaneously signals the skeletal muscle to increase glucose uptake and utilization (51). However, if insulin action on the muscle is inhibited, a common characteristic in the skeletal muscle of individuals with T2DM, fuel switching is impaired. Kelley et al (52) first defined metabolic inflexibility as the inability to switch fuel sources depending on nutrient availability. This group demonstrated a clear inability in skeletal muscle to shift fuel sources between the fasted and 'fed' or insulin stimulated state of the hyperinsulinemic-euglycemic clamp by measuring gas exchange across the leg. Lean individuals quickly shifted from fat metabolism in the fasted state to glucose metabolism in when both glucose and insulin were provided, mimicking a postprandial state. However, the individuals with T2DM were not capable of the same response as they continued to oxidize fat despite the presence of hyperinsulinemia and a variable rate glucose infusion to maintain euglycemia at 90 mg/dl. Understanding the control of fuel

utilization processes and regulation within the skeletal muscle is paramount to a better knowledge of T2DM etiology and pathology.

For glucose to be utilized within skeletal muscle, two processes must initially be activated: glucose transport into the cell and glucose phosphorylation to glucose-6-phosphate. The control of glucose metabolism is attributed to these two primary mechanisms, which are discussed in the context of insulin resistance below. However, in healthy physiology, glucose is rapidly phosphorylated upon entry into the cell, implicating glucose delivery as the main regulator of glucose metabolism. During exercise, when both muscle blood flow and glucose transport mechanisms are enhanced, the rate of glucose phosphorylation has the potential to be limiting (53). Inside the cell, phosphorylated glucose (glucose-6-phosphate) has two primary fates: further processing into glycogen for storage or immediate utilization for energy through glycolysis. Glycolysis has the unique ability to generate energy in either aerobic or anaerobic conditions. In resting muscle, aerobic glycolysis predominates as oxygen is available for electron transport and ATP generation, and will be the focus herein.

Direct action of insulin on skeletal muscle insulin receptors and auto-phosphorylation of insulin receptor substrate-1 (IRS1) initiates a signaling cascade involving a central component of phosphoinositide 3-kinase (PI3K) and serine/threonine kinase Akt (Akt) activation that stimulates glucose transporter type 4 (GLUT4) translocation to the cell membrane resulting in a ~3-5 fold increase in glucose uptake (54, 55). Although other metabolic tissues like the liver and adipose are effected by insulin, skeletal muscle is responsible for ~80% of whole body insulin stimulated glucose uptake (56). This glucose flux into skeletal muscle has two primary routes of disposal: oxidative glycolysis or glycogen synthesis. A schematic overview of the glycolytic pathway converting glucose into pyruvate is provided in **Figure 2**. Specific enzymes pertinent to this dissertation are highlighted (triose phosphate isomerase, TPI; glyceraldehyde-3-phosphate dehydrogenase, GAPDH) and are discussed below. Glycogen synthesis is primarily controlled by glycogen synthase (GS), which is activated by dephosphorylation in response to insulin signaling, allowing glucose to be efficiently stored in skeletal muscle for later use. Under basal conditions, glucose oxidation through glycolysis and glycogen synthesis equally dispose glucose, however, under normal

insulin stimulation, glycogen synthesis is increased more than glycolysis, contributing ~70% of skeletal muscle glucose disposal (55). Dysregulation of these primary glucose disposal pathways is implicated in the development of skeletal muscle insulin resistance and progression to T2DM (45).

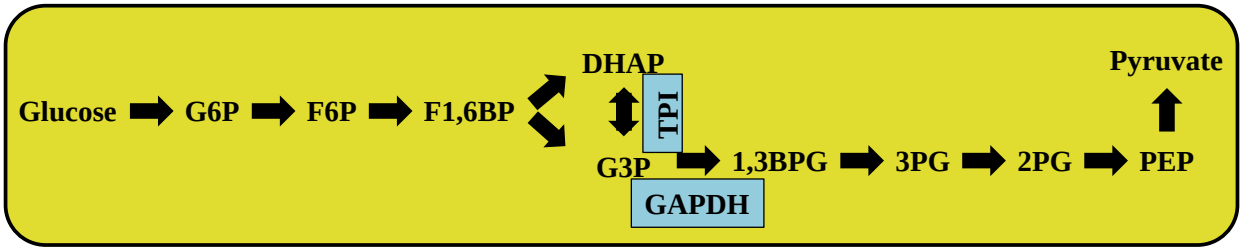

Figure 2. Glycolysis
Classical 10-step sequence of reactions, also known as the Embden-Meyerhof pathway, that converts one six-carbon glucose molecule into two three-carbon pyruvate molecules. The enzymes TPI and GAPDH have been highlighted for their role in processing the three-carbon intermediates G3P and DHAP (discussed below). The overall reaction under aerobic and anaerobic conditions is provided in APPENDIX B. P; phosphate; BP, bis-phosphate; G6P, glucose-6-P; F6P, fructose-6-P; F1,6BP, fructose 1,6,-bis-P; G3P, glyceraldehyde-3-P; DHAP, dihydroxyacetone-P; 1,3,BPG, 1,3 bis-PG; PEP, phosphoenolpyruvate.

Skeletal Muscle Insulin Resistance and Development of T2DM

Skeletal muscle insulin resistance is characterized by a blunted uptake of glucose from the blood and reduced oxidative (glycolysis) and non-oxidative (glycogen synthesis) disposal in response to insulin stimulation. This impairment in skeletal muscle glucose metabolism occurs prior to the onset of T2DM and presents even prior to chronic and postprandial hyperglycemia (57). The body's innate response to skeletal muscle insulin resistance is increased production and secretion of insulin by the pancreatic beta cells. By this means, fasting and postprandial hyperglycemia are prevented with appropriate increases in insulin levels. However, eventually the beta cells become overstressed, unable to continually increase insulin output at a level that offsets the progression of insulin resistance, and begin to fail. Fasting and postprandial hyperglycemia presents alongside unabated hepatic glucose output and blunted skeletal muscle glucose

uptake, leading to overt T2DM. This progression of increased insulin output by the beta cells paralleled with progression of insulin resistance at the liver and skeletal muscle resulting in beta cell failure and overt T2DM is referred to as the natural history of T2DM (58).

Counter regulatory hormones play a role as well, as hyperglucagonemia present in individuals with T2DM contributes to a reduced insulin:glucagon ratio along with increased hepatic glucose output and fasting plasma glucose levels (59). Towards this purpose, somatostatin-esque or incretin analog therapy like exenatide (60) lowers blood glucose secondary to inhibition of glucagon secretion (61). However, the primary defect in T2DM remains peripheral tissue insulin resistance leading to an overworking of the ß-cells to produce insulin, ultimately leading to ß-cell failure and dependence on exogenous insulin administration.

There is much discrepancy as to the effect of sex on insulin sensitivity and some have suggested that sex differences exist related to insulin action on skeletal muscle with men being more insulin sensitive than women (62). However, this has not been shown to occur at the insulin concentrations used in the hyperinsulinemic-euglycemic clamp procedure (described in 'Methods') (63) when glucose disposal rates are normalized to metabolic tissue, commonly lean body mass. Interestingly, skeletal muscle specific glucose uptake using positron emission tomography is increased in women compared to men (64), which conversely suggests greater insulin sensitivity in the skeletal muscle of women. More work needs to be done to determine if a true and significant difference exists between men and women on insulin sensitivity. At this time, human research should aim at using a mix of men and women in investigations related to skeletal muscle insulin sensitivity.

Although multiple etiologies of T2DM exist, including ß-cell dysfunction, dysregulated fatty acid metabolism, and hepatic insulin resistance (65), the primary defect remains skeletal muscle insulin resistance (66, 67), often as a consequence of a sedentary lifestyle combined with excessive kilocalorie consumption (68). The exemplary research by DeFronzo et al. utilizing the gold-standard hyperinsulinemic-euglycemic clamp technique has unequivocally shown that individuals with T2DM have a marked reduction of ~50% or greater in insulin stimulated glucose disposal compared to healthy individuals (69). The

deficiencies in skeletal muscle glucose metabolism, such as glycolysis and glycogen synthesis, are mirrored by dysregulation of the molecular insulin signaling cascade as well, whereby insulin receptor phosphorylation, IRS1 phosphorylation and PI3K activity are all markedly reduced in response to insulin (70) in insulin resistant skeletal muscle. Clearly, glucose transport into skeletal muscle cells is reduced in the insulin resistant state. Regardless, a large quantity of glucose enters skeletal muscle in the postprandial state, even in insulin resistant or individuals with T2DM, as glucose disposal rates during the clamp, although reduced compared to healthy, insulin sensitive individuals, remain at ~3 mg of glucose/ kg of bodyweight/ minute and are attributed primarily to skeletal muscle uptake. Upon entering the cell, glucose must be processed (phosphorylated) to avoid gradient-induced reverse diffusion. The routes of disposal of glucose once it enters skeletal muscle are of high research interest due to the inherent toxicity of unprocessed glucose and rely primarily on regulation of glycolysis.

Classical glycolytic regulation

In the context of methylglyoxal metabolism, a dysregulation of the glycolytic pathway leads to a buildup of 3-carbon intermediates and subsequent generation of methylglyoxal. However, in healthy physiology, the glycolytic 3-carbon intermediates are transient and their concentrations remain unchanged even in a state of increased glycolytic flux. The proposed mechanisms surrounding 3-carbon intermediate buildup and methylglyoxal generation involve regulation of glycolytic enzymes (GAPDH and TPI) that are not classically considered role players in the control of glucose metabolism. Given the well-documented insulin-mediated regulation of three critical control points (hexokinase, phosphofructokinase, pyruvate kinase) in glycolysis, this classical or textbook regulation of glycolysis will be reviewed in the context of insulin resistance and T2DM below. The maintenance of this classical regulation is imperative to cell health and function, and persists even in the context of insulin resistance and T2DM. Proper control of the glycolytic pathway imparts onto skeletal muscle, the unique ability to shift energy utilization depending on the needs of the cell. Take for instance, skeletal muscle at rest and during contractions. Contraction is an energy consuming process, and to compensate for this immense energy need, the skeletal muscle is able to

not only increase substrate (glucose uptake) but also optimize glucose oxidation for energy utilization. Three primary regulatory points exist in the 10-step process of glycolysis: hexokinase, phosphofructokinase, and pyruvate kinase (71).

Hexokinase

Hexokinase initiates the first step of glycolysis, phosphorylating glucose inside the cytosol to glucose-6-phosphate (G6P). This phosphorylation charges the glucose molecule with the addition of a negatively charged phosphate group, which prevents gradient mediated dissociation out of the cell. The conversation of glucose to G6P is irreversible, in a sense 'locking' glucose inside the skeletal muscle tissue. Secondarily, the phosphorylation step destabilizes the glucose structure and increases the affinity with phosphoglucose isomerase, increasing the rate of reaction and moving the glucose molecule through the glycolytic pathway. Importantly, the hexokinase enzyme in skeletal muscle (hexokinase II) has a low Km, meaning it is maximally stimulated at the majority of intracellular glucose concentrations whereas the liver form of hexokinase (hexokinase IV, or glucokinase) has a high Km, which is primarily activated at high glucose concentrations. Hexokinase II plays an important role in glucose transport, and is regulated via feedback inhibition of its primary product, G6P. Therefore, if hexokinase II protein levels are reduced or if G6P levels rise, hexokinase II activity is inhibited reducing the glucose gradient across the cell, and slows glucose transport independent of non-limiting GLUT4 concentration on the membrane (72-74). Insulin has been shown to regulate hexokinase II expression in skeletal muscle (75, 76). In the context of T2DM, physiologic insulin stimulation induced by the hyperinsulinemic-euglycemic clamp (40mU insulin) increased hexokinase II mRNA in skeletal muscle of lean, but not T2DM individuals. Interestingly, the same trial involved a 240mU insulin clamp, which equally stimulated skeletal muscle hexokinase II in both lean and T2DM individuals (77). The molecular mechanisms controlling the non-uniform response to insulin is not fully defined, and further work investigating hexokinase regulation in response to mixed meals may provide a better understanding of the pathophysiology of glycolytic dysregulation in the skeletal muscle of T2DM individuals. Whether accentuated postprandial hyperinsulinemia, which occurs during the

natural history of T2DM prior to beta cell failure, is able to compensate for the reduced hexokinase response to insulin stimulation has yet to be established. Furthermore, it may be more important to consider hexokinase enzymatic activity compared to gene or protein expression, as exercise as a potential therapeutic increased hexokinase II mRNA but did not affect activity in obese and T2DM individuals (78). Given the diverse metabolic roles of hexokinase II in skeletal muscle and its intense feedback regulation (79), it seems unlikely that physiologic reduction of this enzyme in skeletal muscle precedes the diabetic condition. The current literature suggests that postprandial compensatory hyperinsulinemia, secondary to peripheral insulin resistance, may result in similar hexokinase II stimulation in skeletal muscle of individuals with T2DM. Further, others have described increases in glycolytic enzyme protein expression and activity including hexokinase II in individuals with T2DM (80, 81). The backlog of 3-carbon glycolytic intermediates that occurs in skeletal muscle across the natural history of T2DM is not clearly defined, but is not likely a result of hexokinase II dysregulation.

Phosphofructokinase-1

Phosphofructokinase-1 (PFK-1) catalyzes the irreversible phosphorylation of fructose-6-phosphate to fructose-1,6,bisphosphate, which is arguably the most important regulatory step in glycolysis because this is the rate limiting reaction. It is regulated by two primary means: cellular energy status and allosteric regulation by fructose-2,6,bisphosphate (F2,6-BP) . Cellular energy status is 'sensed' by PFK-1 through ATP, citrate and AMP levels. High levels of ATP and citrate collaborate to allosterically inhibit PFK-1, signaling an energy rich cell and a reduced need for glycolysis. Conversely, increased concentrations of AMP represent low energy status and the need for increased rates of glycolysis to provide for the cell energy needs. PFK-1 regulation via F2,6-BP provides a mechanism of insulin mediated regulation whereby an increased insulin to glucagon ratio (as would occur in the postprandial state) increases F2,6-BP concentrations. F2,6-BP in turn not only increases the affinity of PFK-1 for F-6-P (its substrate) but removes ATP-mediated inhibition (71). Although the insulin-mediated regulation of glycolysis in this manner implicates a role for PFK-1 in skeletal muscle insulin resistance, the functionality of this enzyme may be

of too much importance that dysregulation would be catastrophic to cell function and health. The multiple regulatory mechanisms likely remain functional throughout the natural history of T2DM and skeletal muscle PFK-1 mRNA, protein expression and activity have been documented to remain normal in insulin resistance and T2DM (82, 83), while others have proposed an increase in PFK-1 in individuals with T2DM (81). Further, active and sedentary individuals maintain similar PFK-1 expression and activity, while exercise intervention have no consensus effect (83). The combination of the profound importance of PFK-1 functionality to cell health and the available literature suggest PFK-1 regulation is unlikely to play a role in the 3-carbon intermediate buildup witnessed in insulin resistance and T2DM.

Pyruvate Kinase

The final textbook glycolytic regulatory enzyme is pyruvate kinase (PK) which catalyzes the final step of glycolysis – the conversion of phosphoenolpyruvate (PEP) to pyruvate. PK falls under both allosteric and hormonal regulation. Allosteric regulation of PK occurs as a feedforward activation mechanism by F-1,6-BP and as a feedback inhibition mechanism by ATP. Because the rate limiting step of glycolysis is PFK-1 (discussed above), it is logical for the product of the rate limiting reaction to positively regulate the final step in the process. In opposing fashion, ATP, which inhibits PFK-1 as well, also inhibits PK. Hormonal regulation again occurs through insulin, where increased insulin concentrations dephosphorylate PK, causing it to increase activity (dephosphorylated PK is the active form) (71). Given that PK regulates the only control point in glycolysis that occurs downstream of the 3-carbon intermediate generation, it stands to reason that reduced function of this step may cause a backlog of 3-carobn intermediates, as they will be continually produced, but unable to process fully through. A rare autosomal recessive disorder known as pyruvate kinase deficiency provides unique insight into the potential role of PK in effecting the buildup of 3-carbon glycolytic intermediates, which are known contributors to MG generation. PK deficiency occurs in red blood cells and PK activity is reduced to ~5-20% of normal levels, causing a buildup of 3-carbon glycolytic intermediates (although this has not been directly attributed to increases in MG production). Additionally, NRF2 (a master cellular redox sensor and GLO1 transcription factor;

discussed below) has been shown to decrease pyruvate kinase protein expression (84). This may potentially cause a backup of the normal glycolytic flux (discussed below) and contribute to a buildup of glucose-triose intermediates (85) as triose phosphates are increased in diabetes (86).

However, despite this mechanistic potential, proteomic analysis of skeletal muscle has yet to reveal a clear dysregulation of PK in insulin resistant or T2DM skeletal muscle (87, 88). Much has yet to be elucidated involving the classic glycolytic regulation in insulin resistance and T2DM as it relates to the buildup of 3-carbon glycolytic intermediates and subsequent methylglyoxal generation. Given the current evidence, it is more likely that alternate mechanisms are contributing to the dysregulation of glycolytic flux in skeletal muscle with insulin resistance.

Glycolytic Flux

In general metabolism, 'flux' through a biological pathway refers to the rate of substrates entering the pathway, through any intermediate or metabolite transitions, and finally to the end products of the pathway. For glycolysis, this would encompass all events from glucose entry through the 10-step glycolytic process (described above) to the end products of pyruvate. During healthy skeletal muscle physiology, glucose flux through glycolysis is tightly regulated by glucose transport (GLUT4), glucose phosphorylation (hexokinase II), and subsequent processing (PFK-1, PK and glycogen synthase). With glycolysis being comprised of a 10-step, enzyme regulation, understanding which enzymes provide the greatest control strength will highlight which enzymes are most regulated. Control strength of an enzyme in a multi-enzyme pathway is defined as the change in system flux normalized to the change in a particular enzyme activity (89). In this context, HK, PFK and PK are considered regulatory while the other 7 enzymes in glycolysis are generally considered non-regulatory and are assumed to catalyze near-equilibrium reactions.

Dysregulation of Nuanced Glycolytic Enzymes

Regulation of glycolysis in healthy physiology has been extensively investigated and the classic control points (above; HK, PFK, PK) are known to be regulated by insulin, cellular energy status and

glycolytic intermediates and metabolites. However, in the insulin resistant condition, dysregulation of these primary steps have been unable to fully explain the buildup of 3-carbon glycolytic intermediates in diabetes (86) and subsequent MG accumulation. Other potential role players include dysregulation of other glycolytic enzymes (TPI and GAPDH) and mitochondrial dysfunction resulting in increased oxidative stress and altered redox status in insulin resistant skeletal muscle.

Triose Phosphate Isomerase

Triose Phosphate Isomerase catalyzes the conversion of DHAP into G3P (interconversion of 3-carbon intermediates; step 5 of glycolysis described above). This reaction is very rapid and also reversible, meaning a buildup of either 3-carbon intermediate will undergo TPI catalyzed interconversion to reach equilibrium. This conversion reaction is an oxidation-reduction reaction to transform ketones from DHAP into an aldose on G3P, essentially shifting a hydrogen atom from carbon-1 to carbon-3. The active site of TPI is composed of alpha-beta barrels with catalytic residues to perform acid base reactions. Glu165 acts as a base while His95 acts as an acid. This reaction forms an enediol intermediate, which in itself, is unstable and can spontaneously generate methylglyoxal. However, stable and efficient TPI processing maximizes the interconversion of DHAP to G3P, minimizing non-enzymatic reactions like methylglyoxal generation. In healthy cell metabolism, G3P continues through glycolysis and TPI interconversion of glycolytic intermediates is a necessary but non-regulatory point in glycolysis.

However, interesting insight is gained from studying the rare autosomal recessive disorder, triose phosphate isomerase deficiency. Rabbani et al. embarked on a unique case study investigation in a Hungarian family that not only had members with the disease, but a pair of identical twins as well (90), quantifying multiple measures related to MG, GLO1 and oxidative stress. Disease onset presents prior to 2 years old and most individuals die prior to age 6. The disease causes a dramatic reduction in TPI activity (>95% reduction) while DHAP is elevated to ~50 fold compared to healthy individuals (at least in the red blood cells – the tissue analyzed in the case study above. In line with Rabbani et al.'s initial hypothesis, the increase in DHAP was accompanied by increases in MG, MG-H1 and d-lactate. This is intuitive because

DHAP spontaneously converts into MG, so increased concentration of DHAP should shift the equilibrium towards greater production of MG as well. Further, MG-H1 describes that this MG buildup continues on to modify proteins, which get broken down into MG-H1 adducts (further described below). Finally, increases in D-lactate represent the product of the primary detoxification process by the glyoxalase system (discussed further below). Interesting GLO1 was increased in the red blood cells of the effected individuals; however, it was unable to compensate for increases in MG production verifying that in physiologic context, the production rates of MG can override the potential detoxification capabilities of GLO1. The study also characterized a stark increase in protein oxidation and nitrosylation damage. Whether these phenomenon were due to increases in MG directed damage, or occurs in concert with other physiologic issues, has yet to be determined.

Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) converts G3P into 1,3-bisphosphoglycerate. As a dehydrogenase, GAPDH catalyzes the transfer of a hydride group between molecules. This reaction requires NAD+ as a coenzyme, generating a reduced NADH. This is an important energy generating step from the glycolytic process. GAPDH has 2 important catalytic residues, Cys149 and His176. The sulfahydroxyl group of Cys149 creates a nucleophilic attack on the carbonyl group of G3P, forming a tetrahedral molecule, hemithioacetal. Next, His179 provides electrons for reduction to a thioester intermediate, reducing NAD+ to NADH. Finally, free phosphate attacks the thioester intermediate, forming 1,3-BPG and regenerating the Cys149 and His176 charges on GAPDH. The importance of the thiol at the active site (Cys149) is evidenced by binding by molecules that can affect its activity. GAPDH can be modified by glucose metabolites, aldehydes and carbonyl compounds (91-93)– modifications which have to potential to inhibit GAPDH enzyme activity(94). These modifications are particularly important in the context of diabetes as chronic hyperglycemia increases glucose and glucose metabolites such as mentioned previously. Additionally, GAPDH activity is reduced in response to oxidation (95-97), again relevant to diabetes because of the increased amount of oxidative stress.

The importance of the potential reduction in GAPDH activity is particularly relevant to the accumulation of triose phosphate intermediates because GAPDH regulates the process of G3P continuing through glycolysis. Reduced activity of GAPDH without concomitant reductions in glycolytic flux have the potential to cause a backlog of 3-carbon glycolytic intermediates. The collection of data suggest that GAPDH is susceptible to reduced enzyme activity in the context of the diabetic milieu, and this reduction is known to increase not only triose phosphate intermediates, but is also related to increased MG production (98). Further, others have shown MG per se can modify GAPDH and reduce its function, generating a futile cycles of increasing MG formation (99).

Glucotoxicity in Skeletal Muscle

During normal skeletal muscle metabolism, glucose efficiently enters glycolysis and is utilized immediately to supply the muscles energy needs or it is stored as glycogen for later use. However, in the context of hyperglycemia, glucose and glycolytic intermediates build up (especially early glycolytic intermediates from steps 1-5 of glycolysis discussed above) (100). The cause of this glycolytic backlog is not fully understood, although it may involve a redox imbalance between NAD+ and NADH. This increase in glycolytic intermediates without the compensatory flux through glycolysis causes a shift in the flux of glucose through alternate pathways such as the polyol pathway and the hexosamine pathway while concomitantly increasing reactive glycolytic byproducts like MG (101).

Mitochondria regulation of glycolytic flux

It is possible that ischemia as a common complication of diabetes is known to trigger anaerobic glycolysis through mechanisms involving HIF1α (102). This increase in anaerobic glycolysis has been documented to cause a greater shift towards MG generation from glycolytic flux (103). Similar to the Warburg effect in cancer cells, this dysfunction of mitochondria may cause a shift towards anaerobic glycolysis despite oxygen presence also (104). This is further evidenced by observations describing

increased in glycolytic enzymes but a reduction in oxidative enzymes in the skeletal muscle of individuals with T2DM.

Another route of mitochondrial dysfunction is through hyperglycemia induced glycolytic flux. In healthy cells, glucose is metabolized through the tricarboxylic acid cycle (TCA) generating NADH, an electron donor. Electrons are then sent through mitochondrial electron transport chain proteins beginning with mitochondrial complex I. The electron transfer is used to pump protons across the mitochondrial membrane and generate a voltage gradient, which drives the synthesis of ATP and such, supplies the cell with energy. However, in the diabetic condition, too much NADH is produced due to increased glycolytic flux and electrons become stalled in the electron transport chain as their rate of input into the electron transport chain exceeds the rate of complete transfer through ATP synthase to molecular oxygen (2 electrons at a time, which generates H2O). Instead, transfer occurs at coenzyme Q, which generates 1 electron at a time to oxygen, instead generating superoxide, a potentially damaging oxidative species. This process and stark comparison between the healthy and diabetic condition was eloquently highlighted by Michael Brownlee in his 2004 Banting Lecture (105). Further, this mitochondrial mediated ROS production has been shown to inhibit GAPDH function, and increase triose phosphate intermediate rate of production through glycolysis and finally increase the rate of MG formation. This series of events may independently or collectively play a major role in the development of diabetic complications and should continue to be a area of focused research for the prevention and management of insulin resistance, T2DM and T2DM related complications.

Potential contribution of dietary fructose

Dietary fructose in the western diet can be substantial giving the high content of sucrose (a disaccharide composed of a 1:1 ratio of the monosaccharides glucose and fructose) or high-fructose corn syrups found in soft drinks. Accordingly, diets high in fructose have been related to increased incidences of T2DM and obesity, at least in the United States (106). However, unlike glucose, fructose transport into cells is through GLUT5, which is not heavily expressed in skeletal muscle. It is however, highly expressed

in the liver and contributes to increases in circulating free fatty acids and cholesterol (107). Additionally, liver fructose metabolism allows entry into glycolysis as G3P or DHAP, which avoids the rate-limiting control point from phosphofructokinase. Therefore, the negative health effects of dietary fructose may persist and affect skeletal muscle insulin sensitivity and glucose metabolism indirectly, but the effects of dietary fructose at physiologically relevant levels are not currently attributed directly to skeletal muscle fructose flux and glycolytic metabolism.

Advanced Glycation Endproduct Formation

Many molecular factors contribute to the etiology of skeletal muscle insulin resistance including elevated circulating and intramuscular lipids, inflammatory cytokines and mitochondrial dysfunction among others, but an emerging area of research involves the accumulation of advanced glycation endproducts (AGEs). AGEs are modified proteins, lipids or DNA molecules produced as spontaneous byproducts of glucose during normal metabolism. These modifications affect the structural and functional integrity of the macromolecules and can cause them to become dysfunctional and potentially damaging to cell metabolism (9). Furthermore, these structural changes to proteins modifies their susceptibility to proteolysis (8). AGEs are a heterogeneous group of modified proteins that have been studied in various respects since their discovery in 1912 by Louis Maillard. The spontaneous reaction of glucose with amino acid residues is now known as the Maillard reaction, but was originally described as non-enzymatic browning (108). This reaction has been highly utilized by the food industry to improve the appearance, taste and texture of foodstuffs and is exemplified by the brown and crisp texture of a grilled steak or toasted bread (109). The formation of AGEs on food products occurs at an acerbated nature due to the high temperature of cooking. Although the Maillard reaction and formation of AGEs are important for the food industry, it is important to note that AGEs are functionally glycotoxins *in vivo* and have been shown to disrupt important metabolic pathways related to insulin signaling (18, 35) and potentiation of oxidative stress (19, 36). Furthermore, they are implicated in the pathogenesis of several metabolic and age-related diseases including T2DM (29-31). Accordingly, AGEs have been reported to be higher in diabetic subjects

compared to non-diabetic counterparts (29, 110). Accumulation of AGEs in older adults is associated with poor grip strength (111), slow walking speed (112), reduced muscle strength (113), and reduced performance in a battery of muscle function tests (114).

AGEs do appear *in* vivo from dietary consumption and AGE content has been measured and compiled in >500 food products (115, 116). AGE assimilation from the diet is an important consideration as ~10% of ingested AGEs appear in the plasma (116-118). High AGE diets are associated with increased risk for CVD (119, 120), whereas reducing the content of AGEs in the diet by minimizing grilled, charred and processed food stuffs has proven beneficial for improving health markers for diabetes (116, 120) and kidney disease (121, 122) and have even been linked to improving insulin resistance (123, 124) and lipid profile (117). From this information, modifying the AGE content of foods by utilizing cooking techniques like stewing or steaming compared to broiling or frying will reduce the total AGE content in the diet and may play an important role in the clinical care setting (125).

Despite the clear contribution of the diet to the whole-body AGE pool, the majority of AGE production occurs endogenously during nutrient metabolism and aging (32, 33) and is accelerated with oxidative stress and hyperglycemia indicative of the diabetic condition. The seminal work by Monnier et al at Case Western Reserve University was the first to provide substantial evidence of the Maillard reaction *in vivo* (126). The initial investigations into the Maillard reaction products *in vivo* were conducted in extremely long-lived proteins with minimal proteolytic turnover. The exemplary model in Monnier's work was the lens crystallines in the eye. Turnover of these proteins are essentially zero, as their lifespan equals the cells themselves. As such, advanced glycation of these proteins is substantial, builds up, and is directly related to their lifespan. The findings of this research led to a novel hypothesis of aging: 'Toward a Maillard Reaction Theory of Aging' (127).

The application of AGE accumulation extends beyond the aging field, as AGE formation is accelerated with hyperglycemia and oxidative stress indicative of the diabetic milieu. Of primary concern to the hyperglycemic condition is an acceleration of AGE formation through the Maillard reaction. The initial step in this reaction is reversible and generates a Schiff base intermediate from the condensation of

the carbonyl group of a reducing sugar (such as glucose or a glucose metabolite) with a free amino acid residue on a protein **(Figure 3)**. This process occurs rather quickly and accelerates with the heat of cooking or *in vivo* conditions like hyperglycemia and increased oxidative stress. Further rearrangement of the Schiff base generates a more stable protein moiety known as an Amadori product (127), yet this structure is still reversible. Amadori product rearrangement generally occurs over several weeks and is most apparent in long-lived proteins that are readily exposed to glucose. Examples of these are long-lived proteins in the glycolytic red blood cells, or extracellular matrix proteins in the basement membrane, or collagenous proteins in the skeletal muscle extracellular matrix. The most common clinical application of this protein glycation is the use of HbA1c to monitor long-term glucose control. HbA1c is an Amadori product created from the reaction of circulating glucose with hemoglobin and, importantly, increases linearly with glucose concentration in the blood (128). Through this example, it is clear how hyperglycemia accelerates the

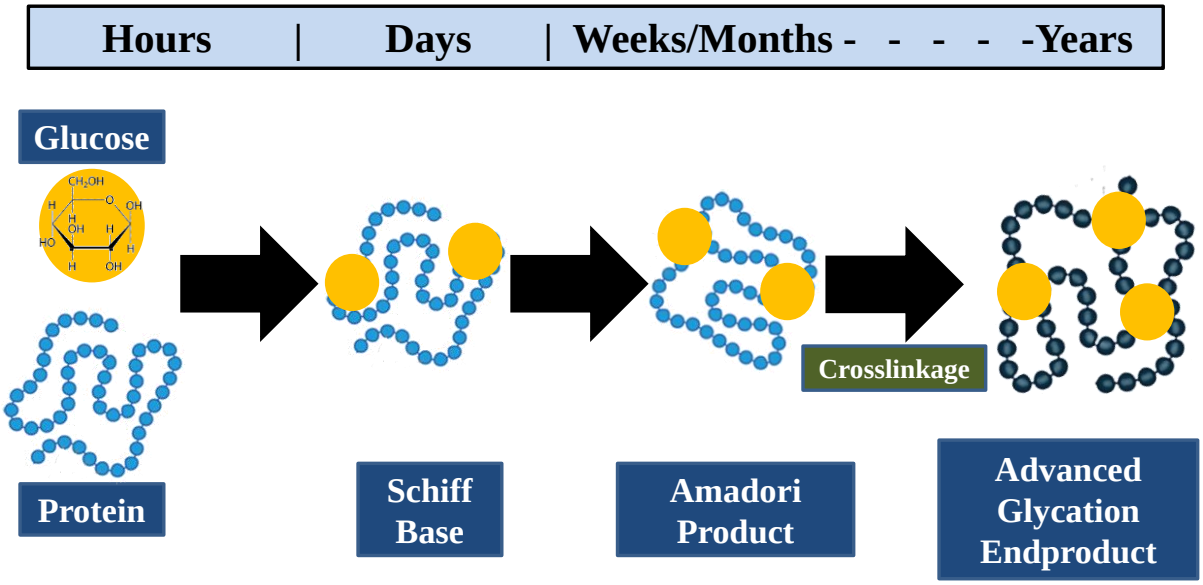

Figure 3. Formation of advanced glycation endproducts
The time course generation of an advanced glycation endproduct through the Maillard reaction involves several distinct intermediates. The process begins with the spontaneous reaction between a glucose molecule and an amino acid residue on a protein molecule generating a Schiff base intermediate. Further rearrangement forms a more stable, yet still reversible Amadori product. After covalent rearrangement and formation of crosslinks, a final irreversible advanced glycation endproduct is formed. Adopted and modified from (1).

Maillard reaction, as increasing glucose concentrations shift the reaction of glucose and protein substrates towards Schiff base formation and Amadori rearrangement. Final covalent rearrangement of the Amadori product, combined with crosslink formation, produces a fully formed, irreversible, AGE. It is the accumulation of these final AGE products that are increased in circulation of aging and diseased animals and humans. A schematic representation of the Maillard reaction *in vivo* and the generation of advanced glycation endproducts is presented above (1).

Many unique AGE moieties have been identified, having been produced from the Maillard reaction or direct modification of proteins by AGE precursors coming from oxidative stress or lipid peroxidation. These collective AGEs have three primary routes that cause cellular damage (105). The first is modification of extracellular matrix proteins (37) which can alter cell signaling processes and cause dysfunction (129). AGEs can cause damage to blood vessels by damaging vessel walls in both large and medium size arteries contributing to the hardening of vessel walls. This results in accelerated atherosclerosis and increased risk for cardiovascular disease. Similarly, peripheral arteries are effected as well, contributing to peripheral vascular disease. This is characterized by decreased blood flow, particularly to the leg, and associated pain. Another is the effect of circulating AGEs binding to their cell surface receptor, the receptor for advanced glycation endproducts (RAGE), which initiates the production of oxidative stress, inflammation and further induction of AGE formation (130). However, our interest in the etiology and pathology of skeletal muscle insulin resistance involves the third route of AGE damage, which is disruption of intracellular signaling processes. Importantly, the primary intracellular process that generates AGEs is via direct modification of proteins by the highly reactive glycolytic byproduct, methylglyoxal.

Methylglyoxal and Dicarbonyl Stress

A Brief History of Methylglyoxal Research

Methylglyoxal research has undergone immense changes from its early chemical interest in the 1800s hundreds, toward an investigation into being a primary role player in glycolysis and culminating in a range of research areas surrounding cancer, protein modifications and metabolism today. A collection and

timeline of this research was eloquently generated by Miklos Kalapos and is summarized below (131). Interest in methylglyoxal dates back to the late 1800s, but this research was aimed at the chemical characteristics of MG and MG reactions outside of *in vivo* physiology. Discovery of an enzymatic conversion by the glyoxalase system (discussed below) of MG into D-lactate sparked interest, particularly because of the ubiquitous nature of the glyoxalase system in all cells and because full elucidation of glycolysis or the Embden-Meyerhof pathway had not been completed. It was maintained that MG was an integral part of glycolysis until the Embden-Meyerhof pathway was developed in the 1930s. During this time, the following key experiments led to the dismissal of MG as part of glycolysis. First, no enzymatic generation of MG was identified and was determined to be a spontaneous byproduct of glycolytic intermediates. Second, increasing substrates from MG's conversion to lactate was unable to enhance total glycolytic activity in skeletal muscle extracts and third, the discovery that tissues readily utilized L-lactate but not the byproduct of MG metabolism, D-lactate. After the dismissal of MG as a glycolytic intermediate and apparent resident role as a mere byproduct of metabolism in living cells, research interest in MG was lost until a resurgence in the 1950s and 1960's arose from a potential role of MG in cancer therapy, postulated by French and Freedlander. This spurred Albert Szent-Gyorgyi to investigate MG and the glyoxalase system as regulatory growth factors in a retine/promine theory of cell division. Although this theory has since been dismissed, it generated a thrust of research on MG and GLO1 in cancer biology. Current research directed at MG and GLO1 in human biology has been largely pioneered by Vincent Monnier and Paul Thornalley. The emphasis of recent MG and GLO1 research has been consequences of aging and diseases, however, novel data produced by our lab and others suggests a novel role in contributing to the development of metabolic diseases.

Physiology of Methylglyoxal and Dicarbonyl Stress

MG is a reactive α-dicarbonyl who accumulation leads to modification of protein, DNA and lipid molecules, culminating in cellular dysfunction. Despite a dramatically reduced concentration in the plasma of MG (~100nM) compared the glucose, MG has an astoundingly higher reactivity than glucose (~10,000-

50,000 fold higher (132). This implicates MG-directed AGE generation occurs *in vivo* with transient MG fluxes (133). The accumulation of MG-directed protein and DNA modification is termed 'dicarbonyl stress' and is apparent in both aging and T2DM (134). Although other dicarbonyls exist, such as glyoxal and 3-deocyglcusosone, MG has gained the most attention as MG-modified proteins are increased in the plasma and tissues of individuals with diabetes and has been linked to vascular complications (37). In healthy individuals, MG is present in the plasma at nanomolar concentrations (~50-100nM), but micromolar concentrations within the cells (1-4μM) (135). However, plasma MG in individuals with T2DM is increased ~4 fold (37) and has been estimated to breach 300μM intracellularly when dicarbonyl stress is excessive. Understanding the formation and metabolism of MG is imperative to understanding its role in T2DM.

Endogenous and Exogenous Sources of Methylglyoxal

Sources of MG originate from exogenous ingestion (food or drink) and endogenous production through degradation of glycated proteins of the Maillard reaction, oxidation of aminoacetone from amino acid catabolism, lipid peroxidation and spontaneous degradation of triosephosphates. Total MG flux totals ~3mmol per day. Of this, ~99% is formed from endogenous means, leaving MG contribution from the diet miniscule. Total MG content in most foods or drinks are quite low (136) combined with minimal intestinal absorption (137) evidences the minute dietary contribution to the total MG pool of <`1%. Endogenous degradation of Maillard reaction products is estimated to contribute ~10% of total MG *in vivo* whereas oxidation of aminoacetone is estimated to contribute ~3% of total MG exposure. Under normal physiologic conditions, lipid peroxidation and ketone oxidations are miniscule, but may play a role in pathological conditions like diabetic ketoacidosis (138). The remaining >85% of MG formation *in vivo* is produced spontaneously from the 3-carbon intermediates dihydroxy-acetone-phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) of glycolysis. As discussed above, skeletal muscle is a highly metabolic tissue and a primary site of glycolysis. For these reasons, our focus on MG generation revolves around spontaneous triosephosphate metabolism in skeletal muscle tissue.

Spontaneous MG formation occurs at a constant rate of ~0.05-0.1% of glycolytic flux and occurs in equal proportion from DHAP and G3P (139). During physiologic situations where DHAP and G3P are increased such as increased rates of anaerobic glycolysis or increased glucose metabolism associated with hyperglycemia and diabetes (86), MG generation from this system may be increased. Although MG is highly reactive with protein residues such as lysine and arginine, its diffusive limit stretches as far as 2-3 cm *in vivo* and it has been shown to maintain the capability to cross cell membranes. This implicates that MG overproduction or dicarbonyl stress within tissues may have the potential to damage not only the cell at the site of generation but transverse the cell membrane and modify proteins in nearby tissues or the circulation. However, the contribution of various tissues to the circulating MG pool has yet to be elucidated and requires technically intense methodology, likely requiring radio labeled isotopes.

Enzymatic Production of Methylglyoxal

An enzymatic route to MG production has been defined in biological systems, but is currently only well-described in prokaryotic organisms (140). The proposed function of this enzyme is as a 'glycolytic bypass' allowing cells to accommodate increased glucose flux. Without this enzymatic mechanism, lower organisms would be severely susceptible to osmotic pressure caused by increased glucose uptake that surpasses glucose disposal mechanisms. This glucose-induced osmotic damage is physiologically relevant for complications of diabetes secondary to hyperglycemia, particularly in the Schwann cells and pericytes. Under this situation, excessive glucose load is shunted towards the polyol pathway, increasing sorbitol concentration and inducing osmotic cell swelling and potential cell lysis. This process contributes to neuropathy and retinopathy. However, it should be noted that methylglyoxal synthase has been proposed to be present in mammalian cells. Although some researchers have claimed to identify methylglyoxal synthase in goat liver (141), their methodology utilized an enzymatic reaction of goal liver homogenate to DHAP. Unintended DHAP conversion to MG is now a known confounding factor to proper MG measurement, and may have led to improper verification of an enzyme-induced generation of MG in the goal liver homogenate. Additionally, they did not measure MG directly, by methods now well defined by

Thornalley et al and, even with these crude assay methods, produced ~25% of what would be expected to be produced from an enzymatic production of MG (142). Another study proposed to isolate MG synthase from rat liver (143) yet again, the technical methodology to perform the proper measurement of MG production was not well defined until Thornalley et al published their standard operating procedures in 2014 (135). To this date, nearly 3 decades after this first claim of the existence of methylglyoxal synthase in mammalian tissue, identification of methylglyoxal synthase has yet to be shown in mammalian systems utilizing newer and more accurate protein isolation and MG measurement and identification technology, including the methods of Thornalley indicating the need for accuracy using LC-MS/MS. The literature is ultimately lacking on the existence of MG synthase in mammalian tissue, and further research is needed before a ubiquitous conclusion can be made. Protein modification by MG is presented in **Figure 4.**

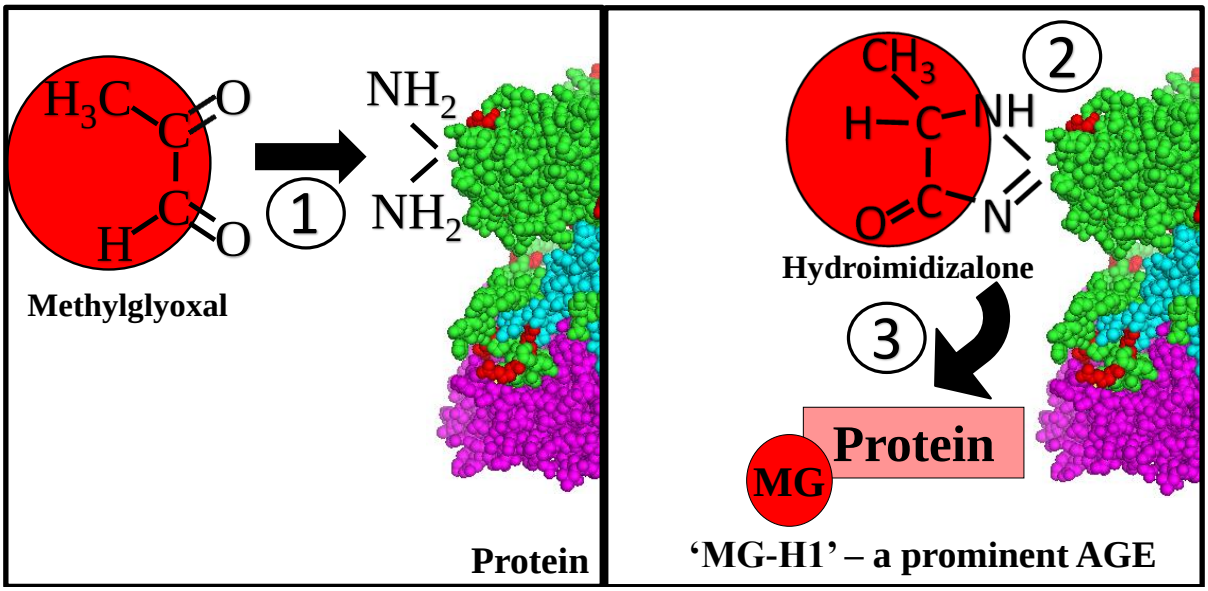

Figure 4. Methylglyoxal modification of proteins
1) Methylglyoxal is a highly reactive α-oxoaldehyde that is particularly attracted to arginine residues on proteins. The nitrogen atoms in the R' group side chain of an arginine residue has been highlighted. 2) Through a series of electron transfers and bond rearrangements, MG covalently modifies the arginine residue forming a hydroimidizalone adduct (MG-H1). 3) MG-modified proteins accumulate in cells and are related to many disease conditions, including diabetes. MG-modified proteins are eventually broken down through intracellular proteolytic processes, releasing MG-H1 adducts into circulation. MG, methylglyoxal; MG-H1, hydroimidizalone. Adopted and modified from (3).

Biochemical effects of MG-modifications on proteins

Methylglyoxal is also known as pyruvaldehyde, pyruvaic aldehyde, 2-oxopropanal, 2-ketoproprion-aldehyde or acetyl-formaldehyde. It is a 3-carbon, highly reactive α-oxoaldehyde and has 2 reactive carbon oxygen double bonds that undergo chemical reactions of both aldehyde and ketone groups. Excessive MG produces dicarbonyl stress, glycating proteins DNA and lipids. MG-modifications of proteins are of primary concern as MG reacts with proteins generating hydroimidazolone adducts, the most prevalent of which is MG-H1 (discussed below). MG-modification can induce a loss of side chain charge on proteins, potentially altering their structure and function. MG-modified proteins may also be signaled for receptor mediated endocytosis and degradation (144). Proteins on which MG-modification imposes functional impairment are defined as the 'dicarbonyl proteome' (145). Chemical modifications by MG directed at arginine residues on proteins are depicted above was adopted from (3).

Intracellular MG concentrations are estimated to range between 1-4μM and others have shown that incorporation of MG into cultured cells is ~3% of the media concentration of L6 rat muscle cells (18) and rat aortic smooth muscle cells (146). It is particularly important to highlight the differences between protein glycation by glucose (a 6-carbon molecule resulting in a fructosamine adduct formation) and protein glycation by methylglyoxal (a 3-carbon molecule resulting in hydroimidazlone, MG-HA adduct formation). Glycation by glucose is directed towards lysine residues, which only moderately enriched in protein functional sites. The resultant fructosamine formation from glucose does not modify the proteins 'healthy' charge or electrostatic characteristics. Compare this to MG modifications directed toward arginine residues, which are highly concentrated at protein functional sites and the formation of MG-H1 adducts which confer a loss of protein charge and inhibition of electrostatic interactions. Furthermore, fructosamine residues are enzymatically reversed while MG-H1 maintains slow dynamic reversibility (147).

Urinary excretion of MG-H1

Urinary excretion of damaged proteins is often used to interpret fluxes in oxidative stress and protein damage and has potential as diagnostic markers. When proteins are damaged by oxidation, nitration or MG, they eventually undergo intracellular proteolysis. The proteolytically digested proteins are reduced to their damaged adducts and released into circulation for whole body removal. In the case of MG-modified proteins, this primarily results in MG-H1 adducts appearing in plasma. MG-H1 adducts are filtered in the kidneys and finally excreted in the urine. Current research implicates MG-H1 is primarily generated intracellularly and appears into plasma and urine by this manner. When a bilateral nephrectomy was performed in a rat model (preventing renal clearance of plasma MG-H1), an increase in MG-H1 free adducts was observed without an increase in MG-H1 residues on plasma proteins (148, 149). These results indicated that plasma clearance into urine is primarily due to MG-H1 free adduct removal (150). This further implicates that proteins containing MG-H1 adducts are proteolytically digested intracellularly and the MG-H1 free adduct is returned to plasma for urinary excretion.

Clinical relevance of MG-H1 production and excretion dynamics

Understanding MG-H1 production and excretion dynamics is clinically relevant because plasma MG-H1 increases with declining kidney function. However, understanding whether changes in plasma MG-H1 is occurring from increased intracellular production or reduced excretion by the kidney may help better understand the cause and potential effects of dicarbonyl stress in multiple chronic diseases. For example, if MG-H1 plasma build-up is determined to occur from reduced kidney function (normalization to glomerular filtration rate), then increased dicarbonyl stress may not be occurring intracellularly. Rather, plasma increases in MG-H1 in this situation is occurring from the reduced ability for the kidneys to filter MG-H1 adducts from the plasma subsequently reduced excretion in the urine. However, if MG-H1 appearance cannot be explained by reduced kidney filtration and urine excretion, then it is likely that intracellular dicarbonyl stress in large tissues is contributing to this plasma increase and therefore, therapeutic routes aimed at reducing dicarbonyl stress may be beneficial.

Methylglyoxal-derived Advanced Glycation Endproducts

Although free MG can be measured *in vitro* and in human plasma and tissues via LC-MS/MS, another quantification of cellular dicarbonyl stress is the measurement of MG-modification of proteins or MG-directed AGEs. The irreversible binding of MG to proteins is largely directed and arginine residues forming hydroimidazolone modifications. The most abundant hydroimidazolone adduct in physiologic systems is MG-H1 and is estimated to occur on ~1-5% of proteins, which may become more prevalent in aging or metabolic disease (34, 151). Other hydroimidazolone isoforms are MG-H2 and MG-H3, while other MG-directed AGEs involve lysine modifications like carboxyethyllysine (CEL) and MG-derived lysine dimer cross-link (MOLD), but the total concentration of all these minor MG-directed modifications are still less than MG-H1 alone. MG-H1 adducts are particularly deleterious to protein function due to the high probability of arginine residues at the functional sites of proteins (152). Intuitively, MG-H1 adduct formation is associated with increases in free MG generation. However, the previously discussed rates of MG generation (~3mmol/day) is not accounted for by bodily excretion of MG-H1, which is ~10μmol per day. At these disparate rates of production compared to excretion, MG-H1 accumulation would be astounding. However, MG-H1 is estimated to occur on ~0.1% of all available protein residues in normal physiology. It is worthwhile to highlight the lack of physiologic mechanisms to 'de-glycate' MG-H1 adducts on proteins. The half-life of MG-H1 adducts is ~12 days, therefore, with reductions in MG concentrations, a slow de-glycation will spontaneously occur at this rate (153, 154).

Still, a large discrepancy between quantified MG flux and lack of substantial MG-modified proteins in healthy cells exists. This phenomenon is explained by the efficient metabolism (>99%) of intracellular MG to the stable metabolic byproduct, D-lactate by glyoxalase enzymatic defense system. Therefore, dicarbonyl stress and subsequent MG-H1 accumulation is less dependent on just rates of MG formation in normal physiology, rather, the primary mechanism dictating MG accumulation and subsequent protein modification is likely regulation of the glyoxalase enzymatic defense system.

The Glyoxalase Enzymatic Defense System

Physiologic Role of the Glyoxalase Enzymatic Defense System

The biological natural defense against dicarbonyl stress is the glyoxalase enzymatic defense system (**Figure 5)**. It is composed of 2 enzymes and requires a catalytic amount of reduced glutathione (GSH) and catalyzes the metabolism of MG to D-lactate. The enzymes and cofactors for the glyoxalase enzymatic defense system is expressed in all cells. Glyoxalase 1 is the rate limiting enzyme in this MG detoxification pathway and catalyzes the first step in the glutathione-dependent sequestration of MG (155) at an efficiency of >99% (2). MG first reacts with GSH, generating a hemithioacetal adduct and through enzymatic conversion through GLO1, in the generation of a S-lactoylglutathione intermediate. The second step of the reaction follows by conversion of S-lactoylglutathione to d-lactate by the second enzyme in the pathway,

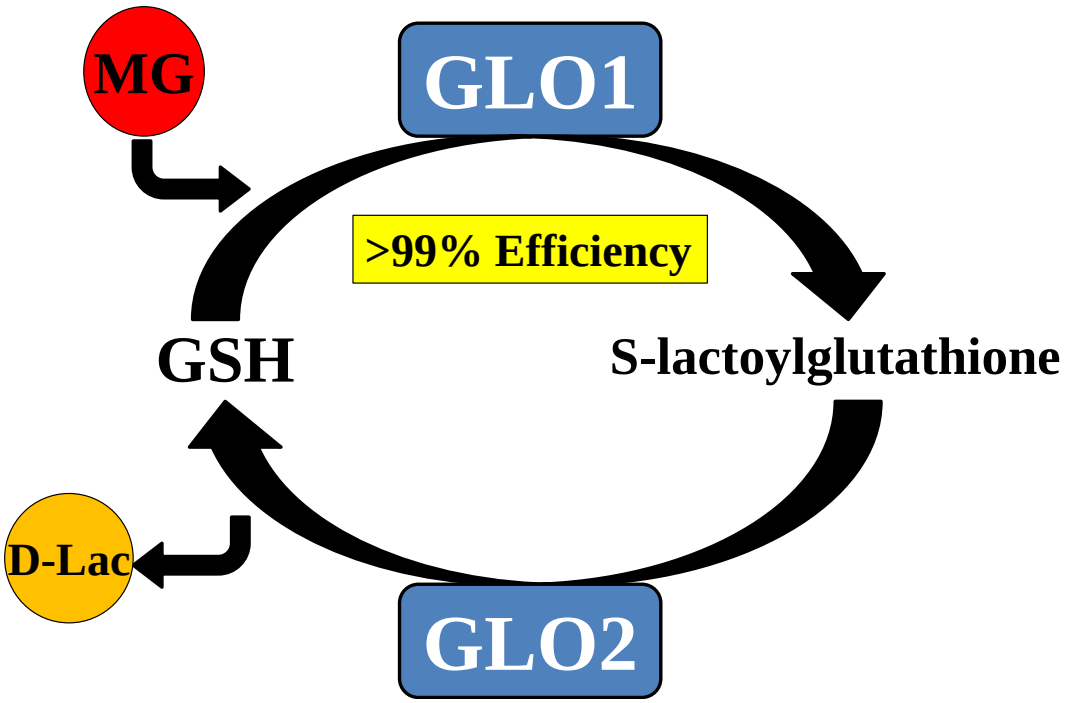

Figure 5. Methylglyoxal metabolism by the glyoxalase system
The primary role of the glyoxalase system is the enzymatic detoxification of MG to the stable d-lactate. MG, methylglyoxal; GLO1, glyoxalase-1; GLO2, glyoxalase-2; GSH, reduced glutathione; D-Lac, d-lactate. Adopted and modified from (2).

Glyoxalase 2 (GLO2). Additionally, GLO2 regenerates GSH from S-lactoylglutathione, which was utilized prior by the GLO1 reaction, depicted above was adopted from Thornalley et al. (2).

By these means, the glyoxalase enzymatic defense system detoxifies MG to D-lactate without the consumption of reduced glutathione, and prevents any potentially aberrant reductions in this important intracellular antioxidant and concomitant increase in cellular oxidative stress. However, in the reverse situation, if cellular oxidative stress is excessive and reduced glutathione become depleted, GLO1 activity is reduced. Evidence from *in situ* experiments suggests this GLO1 reduction is directly proportional to the loss of reduced glutathione (151).

The importance of GLO1 in human physiology in preventing dicarbonyl stress is evidenced by its ubiquitously expressed nature, as it is present in the cytosol of all cells, and its abundance, remaining in the top 13% of human proteins and being present in a concentration of ~0.2µg of GLO1 per 1 milligram of human tissue (156). Additionally, a rare mutation in human *GLO1* gene that produces a non-functional GLO1 protein is embryonically lethal and others have suggested this is due to an inability to prevent dicarbonyl stress (157). Gene deletion of GLO1 is also embryonically lethal in mice and humans (158).

Molecular Characteristics of GLO1

Human GLO1 functions in tissue as a dimer resulting from a gene that codes for two individual subunits, which can be either GLO1-A or GLO1-B. The GLO1 dimer is therefore an allozyme, produced from the combination of either similar GLO1 subunit and can be composed as GLO1-A|GLO1-A, GLO1-A|GLO1-B or GLO1-B|GLO1-B. All dimer variants maintain many similar molecular characteristics: the molecular mass is 44 kilo Daltons (kDa) (33), the isoelectric point p*I* values are 4.8-5.1, although they maintain unique charge densities and molecular shapes. Each individual subunit contains a single zinc ion in humans. Interestingly, despite a sequence homology of ~42%, GLO1 in *E. coli*. is a nickel metalloenzyme as opposed to zinc, although this difference has no defined physiologic consequence. The final GLO1 protein contains 184 amino acids and has several known post-translational modifications. During standard post-translational protein processing, the N-terminal Methionine is removed and the N-terminal Alanine is

blocked. Four possible phosphorylation sites have been identified but their role or lack thereof in the function of GLO1 activity is not well described. GSH can be a limiting factor for GLO1 activity and oxidative stress mediated GSH oxidation has been shown to cause increased MG formation *in vitro* (159). The activity of GLO1 is assayed by measuring the initial rate of the S-lactoylglutathione intermediate of the glyoxalase system by spectrophotometric quantification at 240nm after supplying the MG-GSH hemithioacetal reactant to tissue or cell homogenate.

Characteristics of the Human GLO1 Gene

The GLO1 dimer allozymes result from variations in a single diallelic genetic locus. In heritance of GLO1-A and GLO1-B genes exists in standard codominant fashion. The GLO1 gene presents on chromosome 6 in humans. Population genetic studies have shown that GLO1-A allelic frequencies are expressed most in Alaskan natives with stepwise reductions in GLO1-A allele frequency in Europe and South America, reduced further in Africa, the Middle East populations and India, and are expressed lowest in East Asian populations. This stepwise decrease follows an interesting pattern from the geographic Northwest via a graded reduction stretching to the Southeastern hemisphere (160). Population differences in functional GLO1 protein from this intriguing genetic GLO1 pattern has not been investigated – likely because phenotypically, all GLO1 allozymes have similar physiologic activity. The final GLO1 product of these allelic differences result only in an amino acid switch from alanine in GLO1-A to glutamine in GLO1-B (161). The human GLO1 gene is ~27k base pairs long, with ~12k base pairs comprising the coding sequence. Five exons separated by four introns have been described (162). A 982 base pair promoter region contains both an insulin response element, a metal response element and a glucocorticoid response element. Additional regulatory elements have been identified including both NFkB and AP-1. These characteristic regulatory elements in the GLO1 gene implicate a potential regulatory effect of both insulin and oxidative stress, which are particularly important to hyperglycemia and the diabetic condition. Interestingly, the GLO1 gene has been linked to a high carbohydrate, high kilocalorie diet in a congenic mouse model (163). Although certainly much work needs to be done in humans and specifically skeletal muscle tissue to

determine any potential role of diet on GLO1 gene expression, this mouse model provides incentive to investigate GLO1 in the context of the high carbohydrate, hypercaloric diets consistent with the Western diet and high risk of T2DM development.

RAGE signaling and GLO1

Several profound researchers in the glyoxalase field have implicated RAGE signaling as a mechanism for reducing GLO1 protein expression, postulating that RAGE-mediated signaling through NFkB is inhibiting GLO1 transcription-translation processes (164). However, the collection of evidence is suggestive, but far from conclusive. An investigation on GLO1 protein was performed in the glomeruli in mice bred as a model of type 1 diabetes and more specifically, diabetic nephropathy (165). These mice (OVE26) overexpress calmodulin in pancreatic beta cells and present with hyperglycemia and develop complications of nephropathy similar to clinical T1DM. When a RAGE knockout was superimposed on this OVE26 model, the glomeruli showed reduced levels of MG and increased protein and mRNA expression of GLO1. This data led to the conclusion that RAGE signaling inhibits GLO1 expression, despite the lack of *in vitro* models of direct RAGE activation on GLO1 mRNA or protein. Although the RAGE-GLO1 association in this model is clear, many confounding factors including the transgenic overexpression of calmodulin, morphological damage consistent with nephropathy, and reduced globular filtration rate prevent a concrete relationship between RAGE signaling and GLO1 expression. Further research should investigate *in vivo* and *in vitro* approaches utilizing RAGE agonists in multiple tissues to verify a RAGE-mediated signaling effect on GLO1 protein expression. Another cited paper for RAGE's hypothesized role in GLO1 regulation involved analysis of the brains of Alzheimer's Disease (AD) patients (166). The brains of AD patients present with increased RAGE expression and AGE accumulation and Kuhla et al. revealed reduced GLO1 protein expression and activity as well. The most compelling research implicating a RAGE-dependent regulation of GLO1 was published as an abstract and referenced an administration of sRAGE in cultured neurons of mice increased GLO1 expression while incubating with RAGE-ligands reduced GLO1.

A lack of full publication of this methodology and results prevents a true understanding of the relationship of RAGE signaling and GLO1 protein regulation.

In bronchial epithelial cells, NFkB has been shown to increase GLO1 protein expression and mRNA transcription in a dose dependent manner, however this was concomitant with ROS mediated reduction in GLO1 activity, potentially due to a limiting factor of reduced glutathione (which was likely oxidized with increasing ROS generation) (167). Still, work unrelated to GLO1 has shown NFkB can inhibit a primary transcription factor for GLO1, NRF2 (168).

Although some data implicate a relationship between RAGE signaling and GLO1 regulation, the current gaps in the literature prevent a mechanistic understanding of the relationship. RAGE is proposed to elicit many effects through increasing perpetual NFkB signaling. Through this lens, the NFkB response element as an intrinsic characteristic of the GLO1 gene along with dose-dependent increases in GLO1 protein expression with NFkB incubation *in vitro,* suggests RAGE signaling through NFkB would increase, rather than decrease, GLO1 protein expression. Clearly, this warrants further investigation, especially for the context of skeletal muscle.

Role of GLO1 in Regulating Physiologic Dicarbonyl Stress

In metabolically fit muscle cells, transient increases in MG and oxidative stress trigger GLO1 protein expression resulting in an efficient detoxification of MG (10). However, in metabolically compromised muscle, the reduced efficiency of the glyoxalase system can be detrimental, as MG will accumulate when its generation surpasses the capacity of GLO1 detoxification. This increase in intracellular MG may contribute to a host of deleterious effects including damage to mitochondria (20) and mitochondrial DNA (169) concomitant with increases in mitochondrial-mediated ROS production (170) and inflammation related to the accumulation of AGEs (171, 172). There is evidence to suggest GLO1 expression and activity is a major factor in mediating MG stress. Accordingly, dicarbonyl stress is prevented *in vitro* through up-regulation of GLO1 (10, 173, 174). Recently, GLO1 has emerged as a potential target for preventing metabolic disorders. Importantly, GLO1 overexpression has been linked to increased

lifespan in *P. anserina* (175) and *C. elegans* by preventing MG-induced mitochondrial damage (176, 177). Studies in mice demonstrate that GLO1 overexpression is protective to dicarbonyl stress (170). In a mouse model utilizing administration of a high fat diet, GLO1 was reduced in abdominal skeletal muscle (178). These studies provide reason to investigate GLO1 in human skeletal muscle, but the picture is far from complete. The protective role of GLO1's ability to restrain MG-mediated damage has potential in maintaining metabolic homeostasis in skeletal muscle and further investigation of this role in insulin resistance and T2DM is warranted.

TABLE 1. STUDIES INVESTIGATING GLO1 IN HUMAN SKELETAL MUSCLE

Publication	Measurement	Population	Intervention	Timepoint	Outcome
Nirmal & Pearson 1975 (179)	GLO1 activity	Muscular dystrophy (MD) patients (n=24), controls (n=6)	n/a	Baseline	GLO1 activity reduced in skeletal muscle of MD
Radom-aizik et al 2005(23)	Gene microarray	Elderly, sedentary men (n=6)	12 wk aerobic exercise (AE) training	Pre- and post-AE intervention	Gene expression increased after AE
Hussey et al 2013(180)	HPLC-MS/MS	T2DM (n=6), age and BMI-matched controls (n=6)	4 wk, 5d/wk, cycling, only in T2DM	Pre- and post-exercise intervention	No effect of T2DM, decreased post exercise in T2DM

Although GLO1 has been evidenced to be highly expressed in skeletal muscle tissue in humans (181), current data on GLO1 protein expression in the context skeletal muscle physiology as it pertains to

disease, insulin resistance or exercise is lacking. Given the recent advances in the role of dicarbonyl stress in insulin resistance, T2DM and a variety of other disorders, characterizing GLO1 in healthy and diseased physiology is imperative to gaining a better understanding of human skeletal muscle physiology.

The reduced GLO1 in the Hussey et al study, which is not in agreement with our primary hypothesis that GLO1 is reduced in T2DM skeletal muscle but increased with exercise training, may stem from either their subject population or training modality. The T2DM population they studied had not likely progressed to ß-cell failure as their fasting insulin levels were ~80pM indicating they are progressing along the natural history of T2DM. It remains to be seen how the skeletal muscle GLO1 protein expression changes across the glucose tolerance continuum, and Hussey et al results further evidence the need for research in this area. Additionally, other studies have used a single exercise modality of aerobic exercise training, where the Hussey et al study utilized both moderate intensity cycling for 60 minutes 3 days per week, while the other 2 exercise sessions involved high intensity interval training. Although they did not perform these exercises in separate groups, it may be possible that different exercise stimuli have various effects on GLO1 protein expression in skeletal muscle. As more well controlled exercise studies on skeletal muscle GLO1 enter this literature, we will be better able to answer this question. Finally, major limitations to the study include the low subject size (n-6 each) and the lack of control during the exercise training. Therefore, it cannot be discounted that the control group may have seen the same reduction in GLO1 after training. The gene microarray study by Radom-Aizik et al provides incentive to investigate GLO1 protein expression as a therapeutic measure, as they observed significant increases in GLO1 gene expression in elderly individuals, a population that is known to have reduced GLO1 expression in insulin independent tissues. However, gene microarrays in healthy, well trained populations have not shown differences in GLO1 gene expression between untrained, resistance trained, and endurance trained individuals (182). However, it is well known that metabolic disease and exercise have profound roles on global expression of glycolytic proteins in skeletal muscle (183-185). Given the major role of GLO1 in MG detoxification, an unavoidable glycolytic byproduct, it is reasonable to consider GLO1 changes in any situations where glycolytic proteins are known to change (insulin resistance, T2DM and exercise). Despite this, some proteomic profiling methods have

not shown differences in skeletal muscle GLO1 protein expression in non-obese T2DM skeletal muscle (88) or non-T2DM obese skeletal muscle (87). The global proteomic approaches applied in (87, 88) are helpful in identifying novel indicators of each respective pathogensis, but do not observe changes to acute stimuli like insulin, glucose or exercise. This also suggests that multiple physiologic factors regulate GLO1 protein expression and further analysis using appropriate control groups will help elucidate the primary physiologic factors effecting skeletal muscle GLO1 expression. Further, many glycolytic enzymes are multifunctional and it is therefore difficult to interpret direct relevance with insulin resistance (88) *per se*. More studies using a combination of clinical evidence and mechanistic cell culture data will help better elucidate the role and regulation of GLO1 in human skeletal muscle.

Role and Characteristics of Glyoxalase 2

Glyoxalase-2 (GLO2) is the second enzyme in the glyoxalase systems that catalyzes the final reaction from the s-lactoylglutathione intermediate to d-lactate and importantly regenerating the GSH consumed from the GLO1 reaction. GLO2 has 2 isoforms, a 29 kilodalton isoform present in the cytosol and a 34 kilodalton mitochondrial isoform. Although GLO1 catalyzes the rate limiting reaction in the detoxification of GLO1, GLO2 deficiency is toxic. Without the GLO2 catalyzed reaction, the s-lactoylglutatione builds up, but more importantly, the glutathione oxidized from the GLO1 reaction does not get regenerated. This depletion of reduced glutathione not only reduces the cells innate antioxidant defense, but results in reduced GLO1 activity and subsequent MG accumulation and dicarbonyl stress (186). Therefore, GLO1 remains the highest priority for obtaining a better understanding of MG biology, but GLO2 plays an important role and should not be overlooked in glyoxalase research.

D-lactate: the primary product of the glyoxalase system

In healthy physiology d-lactate is maintained at low plasma concentrations (187). Under the construct that individuals with T2DM have increased dicarbonyl stress, it stands to reason that concomitant increases in plasma and urine d-lactate (the primary product of MG detoxification) would be observed as

well. In agreement, several studies have observed this phenomenon (188-190) and further interest exists in the contribution of d-lactate to diabetic acidosis (191, 192). D-lactate represents not only a marker increased MG detoxification by the glyoxalase system, but may also have independent effects in T2DM biology.

Aldose reductase

Aldose reductase (AKR) catalyzes a reaction of glucose reduction to sorbitol as a function of the polyol pathway. The most prominent form in the skeletal muscle is AKR1B1. This is very relevant for many insulin-independent tissues, as increased flux through the polyol pathway leads to sorbitol increases and osmotic stress on the cell while concomitantly disrupting the NAD+/NADH balance, via sorbitol conversion to fructose and the reduction of NAD+. Since aldose reductases are the rate limiting step in this mechanism, aldose reductase inhibitors have seen some success in preventing hyperglycemia-induced diabetic complications (193). In skeletal muscle, this is less of an issue, as the Km of AKR1B1 with glucose is much greater than the Km of Hexokinase II with glucose. As such, glucose flux into healthy skeletal muscle cells is directed toward glycolysis and the polyol pathway is considered negligible. However, increased glucose flux through this pathway can occur with hyperglycemia and oxidative stress, characteristic of conditions in the skeletal muscle of individuals with T2DM.

AKR1B1 plays an additional role by catalyzing the reduction and essential detoxification of methylglyoxal (194). AKR1B1 converts MG to hydroxyacetone and D-lactaldehyde at a 19:1 ratio, utilizing NADPH as a reducing agent in the process (194). Unlike its relatively high Km for glucose, AKR1B1 have high intrinsic catalytic efficiencies for MG (195). AKR1B1 along with being the most predominant form in skeletal muscle, is the most active of all the AKR isozymes at metabolizing MG. In physiologic conditions, AKR1B1 maintains <100 fold reduced enzymatic activity *in situ* for MG compared to GLO1 (194, 196, 197) and a 10-40 fold reduced activity in *in vitro* in metabolic tissue. However, when GLO1 activity is reduced, AKR1B1 plays a significant role in MG detoxification and plays a protective role in human umbilical vein endothelial cells and rat heart (198). A unique feature of AKR1B1 is it becomes overactive in response to oxidation. It contains a cysteine at amino acid 298 that further stabilizes the

enzyme when oxidized, implicating AKR1B1's increased role in the polyol pathway with increased oxidative stress.

NRF2-Keap1 Axis in GLO1 Regulation

Nuclear factor-erythroid 2 p45 subunit-related factor 2 (NRF2) is a transcription factor that promotes expression of genes with 1 or more antioxidant-response elements (ARE), coordinating control of genes related to cell survival and protection from oxidative stress (199). NRF2 is acutely activated by oxidative stress (199) and induces an immediate response in ARE expressing genes as a protective measure. NRF2 has been investigated in the context of T2DM and insulin resistance due to the chronic low grade inflammation that persists in the disease pathology. Studies in NRF2-KO mice have been performed using high fat diet feeding to induce weight gain and insulin resistance for short (1-4 weeks; (200)) medium (3 month; (201)) and long (6 month; (202)) duration, collectively indicating that NRF2-KO mice are moderately protected from high fat diet induced obesity and insulin resistance (203). Although, overexpression of NRF2 using the NRF2 activator CDDO-imidazolide in mice also protected them from diet induced obesity and insulin resistance (204). Data from murine models highlight the potential role of NRF2 regulation in insulin resistance and diabetes. However, to date no human studies have defined the role of NRF2 particularly as it relates to skeletal muscle insulin resistance and T2DM. Clearly, further research is needed to understand the role of NRF2 in T2DM and skeletal muscle insulin resistance.

Murine and *in vitro* models do provide evidence that NRF2 may play a role in exercise-mediated skeletal muscle adaptations. Incubation of L6 myotubes with Ca^{2+} (an *in vitro* exercise mimetic) increases expression of NRF2 through via transcriptional or post-translational control by peroxisome proliferator-activated receptor coactivator-1 PGC1α, implicating exercise stimulus as an independent activator of NRF2. Murine models show NRF2 protein in skeletal muscle is elevated after an acute bout of aerobic exercise. Importantly, GLO1 is governed under transcriptional control by NRF2 and this regulation provides a mechanistic link between exercise-induced increases in GLO1 protein expression through NRF2 signaling. NRF2 promotes basal and inducible expression of GLO1 by binding to an ARE in the *GLO1* gene (10).

Given the importance of GLO1 in reducing dicarbonyl and oxidative stress, this supports a potential role of NRF2-mediated GLO1 expression as a protective response to cellular stress. NRF2 gene expression is constitutively active, but its cytosolic protein is regulated via Kelch-like ECH-Associating protein 1 (Keap1), which binds NRF2 and signals its degradation via the ubiquitin-proteasome system. Therefore, NRF2 protein levels accumulate primarily when negative regulation by Keap1 is inhibited. Keap1 is susceptible to oxidative damage and is transiently degraded with cellular stress or ROS increases, which in turn promotes NRF2 accumulation, translocation into the nucleus, and promotes transcriptional activity of AREs and GLO1 genes. A schematic representation of the Keap1-NRF2-GLO1 axis is described in **Figure 6.**

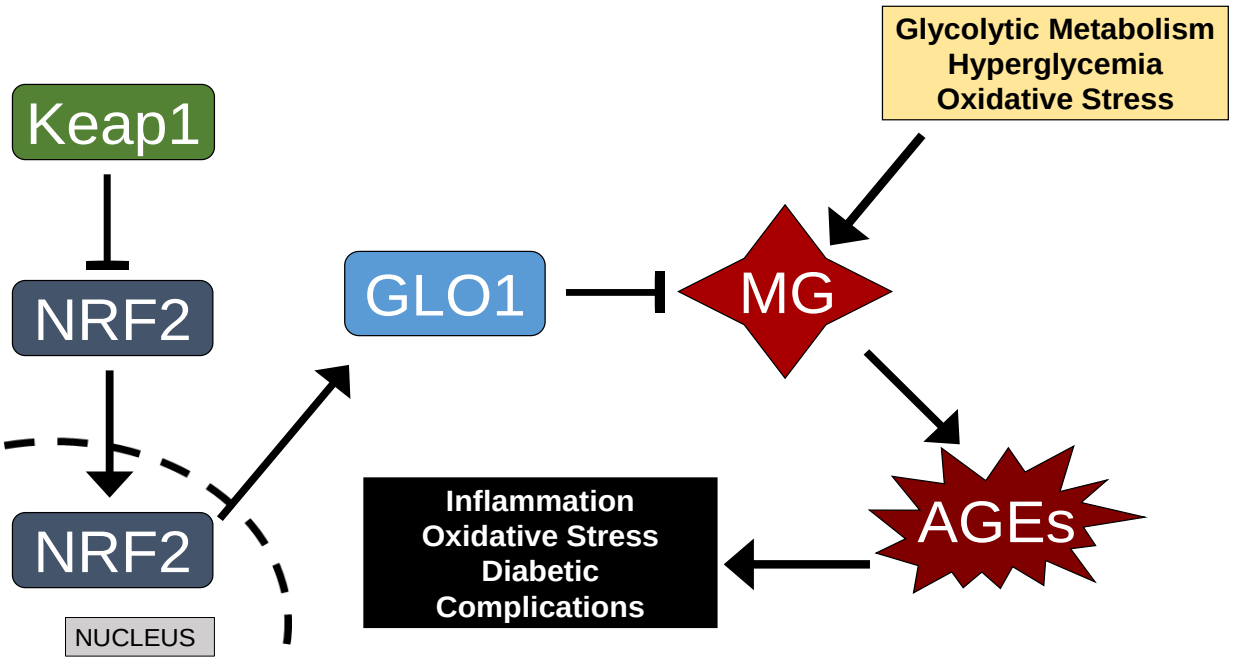

Figure 6. NRF2-Keap1 pathway and the regulation of the MG-GLO1 axis
GLO1 detoxifies MG independent of the source of MG generation thus reducing AGE accumulation. GLO1 is under transcriptional control by the NRF2-Keap1 system. NRF2 is constitutively produced into the cytosol before translating to the nucleus where it increases GLO1 mRNA production. Keap1 regulates NRF2 by binding NRF2 in the cytosol (prior to NRF2 nuclear translocation), signals NRF2 for degradation by the ubiquitin-proteasome system and thus prevents NRF2 directed gene transcription. MG, methylglyoxal; AGEs, advanced glycation endproducts; GLO1, glyoxalase-1; NRF2, nuclear factor-erythroid 2 p45 subunit-related factor 2; Keap1, Kelch-like ECH-Associating protein 1.

Skeletal Muscle GLO1 and Insulin Sensitivity

Plasma MG levels are elevated in diabetic patients who are characterized by skeletal muscle insulin resistance and reduced oxidative capacity (205). We have already described how increased MG stress on the muscle can impart disruption of insulin signaling and reduce oxidative capacity. The literature suggests a potential role for GLO1 in attenuating the dicarbonyl stress associated with MG overproduction. However, in compromised muscle, reduced efficiency of the glyoxalase system can cause dicarbonyl stress as MG will accumulate when its generation surpasses the capacity of GLO1 detoxification. Evidence from *in vitro* studies implicates loss of GLO1 function as a primary cause for increased MG accumulation. Understanding the mechanisms controlling GLO1 expression and activity may prove beneficial in understanding the process of aging in skeletal muscle.

Research with the GLO1-specific inhibitor, *Statil*, shows concomitant MG accumulation (206-208) which has been shown to increase inflammation in skeletal muscle (13). Van Obberghen et al (209, 210) showed that brief exposure of L6 muscle cells to MG inhibited insulin-stimulated phosphorylation of P13K and ERK1/2. Furthermore, they determined that the impaired signaling was independent of MG-generated ROS and likely due to direct binding of MG to IRS-1, preventing its auto-phosphorylation thereby inhibiting insulin signaling conferring insulin resistance.

Overexpression of GLO1 in diabetic rats reduces endothelial dysfunction in blood vessels, attenuates renal impairment in the kidneys (172), reduces AGE accumulation in diabetic retina (17), attenuates hyperglycemia-induced increases in AGE and RAGE (receptor for advanced glycation end products), and lipid peroxidation in skeletal muscle (170). Taken together GLO1 overexpression reduces hyperglycemia-induced MG damage, AGE accumulation and reactive oxygen species (ROS) in skeletal muscle, which is in agreement to what is seen in other tissue and animal models (211) (170). In contrast, Gawlowski et al (212, 213) recorded *increased* glucose uptake in siRNA GLO1 knockdown L6 myoblasts after 48 hours of hyperglycemic conditions. They found this to be specifically linked to altered GLUT4 trafficking mediated by MG accumulation. The discrepancy between these studies is likely due in part to

the length of hyperglycemia. Transient increases in oxidative stress (H_2O_2), known to be caused by chronic hyperglycemia, have been shown to increase glucose uptake and stimulate GLUT4 translocation in muscle cells (214, 215). The increased glucose uptake in this study may have been an artifact of the oxidative stress resulting from the long period of hyperglycemic conditions rather than from the MG accumulation resulting from GLO1 knockdown. Additionally, regulation of the NRF2-Keap1 axis has been implicated in diabetes (216, 217), yet it is unknown whether they play a role in maintaining GLO1 and MG-directed protein modification in the skeletal muscle of individuals with insulin resistance or T2DM. Collectively, increasing GLO1 expression may play a protective role when MG generation becomes exacerbated. Further clarification of GLO1's role in metabolically compromised cells and elucidation of its regulatory proteins and their stimuli may lead to better avenues for T2DM treatment/prevention. Furthermore MG directly inhibits insulin action by direct covalent modification of the insulin molecule (218).

Therapeutic Strategies for the Prevention of Dicarbonyl Stress

Common treatment options for T2DM include sulfonylureas which are a class of anti-diabetic drugs that stimulate insulin secretion from the ß-cells, acting as insulin secretagogs (219). Thiazolidinediones or TZDs are a class of anti-diabetic drugs that improve insulin sensitivity through increasing activation of PPARgamma, which promotes glucose uptake and reduced FFA output into circulation from adipocytes (220). However, neither of these medications target the MG-GLO1 axis. Given the effectiveness of GLO1 inducer therapy on normalizing glucose homeostasis in obese insulin resistant adults, utilizing treatment strategies that target the MG-GLO1 axis may provide additional benefit. These potential therapeutics involve antioxidants, MG-scavengers, and GLO1 inducers.

Although, MG-directed protein modification is associated with increases in oxidative stress and inflammation, dicarbonyl stress exists *per se*. Therefore, antioxidants are ineffective strategies to counter dicarbonyl stress (151). Rather, therapeutic potential lies in preventing the formation of MG or increasing its detoxification, either by GLO1 or MG-scavenging drugs. A primary means to combat MG formation has been increasing glucose flux through the pentose phosphate pathway, preventing triose phosphate

accumulation from glycolytic flux and subsequent spontaneous MG generation. Although this has proven effective in non-insulin dependent tissues in individuals with T2DM (148, 221), it is unlikely this glucose shunt will be able to counteract the tremendous quantity of glucose that skeletal muscle is exposed to. For a highly metabolic tissue like skeletal muscle, utilizing therapies designed at increasing glucose utilization through glycogen synthesis or increasing combustion of glucose via optimizing mitochondrial function may be more appropriate.

Alternatively, MG scavengers have been utilized to varying degrees of success. Aminoguanidine has been successful, especially in cell culture, but *in vivo* usage is associated with toxicity limiting its application in human clinical trials. Metformin is the most commonly prescribed medication for T2DM and plays multiple roles in MG regulation. It is a known scavenger of MG *per se*, but its primary role may be reducing glucose levels thus preventing MG generation in the first place. Importantly for skeletal muscle, Metformin stimulates AMPK and provide a mechanism to prevent accumulation of glycolytic intermediates.

A final therapeutic path for preventing dicarbonyl stress is through inducing GLO1 protein expression and activity. Conceptually, this is more promising than MG scavengers as GLO1 is efficient at metabolizing massive quantities of MG, while MG scavengers generally need to be increased in a 1:1 molecular proportion to MG, which in some tissues like skeletal muscle, would require super physiologic concentrations of the scavengers. Recent human trials have shown the effectiveness of trans-resveratrol and hesperetin (tRES/HESP) finding increased GLO1 protein expression and activity concomitant with reductions in plasma MG and MG-directed protein modification (222). The proposed mechanisms of action of tRES/HESP is through activation of NRF2 (223). Further, tRES/HESP activate NRF2 likely through increases in AMPK and SIRT1 activity (224). The recent work has achieved effective pharmacologic targeting of the NRF2-GLO1-MG axis in human plasma. Whether this work extends to intracellular dicarbonyl metabolism has yet to be established.

Effects of Exercise on Skeletal Muscle GLO1

Chronic aerobic exercise has been shown to have robust benefits on insulin sensitivity, glucose disposal, glycogen content, fatty acid oxidation and metabolic flexibility (225-232). Both acute (233, 234) and chronic aerobic exercise (226, 235, 236) provide these benefits in which are driven by adaptations in skeletal muscle (237, 238). At this time, the signaling mechanisms that underlie these metabolic improvements are not well understood, but may involve regulation of GLO1. Gene microarray data show skeletal muscle *GLO1 mRNA* increases with aerobic exercise in old, healthy, sedentary men (23). GLO1 protein expression increases in response to oxidative stress and exercise causes an acute increase in intracellular oxidative stress in skeletal muscle. It is reasonable to infer that *GLO1* gene is upregulated to account for increased oxidative stress of exercise (239). Similarly, GLO1 protein expression is increased in response to aerobic exercise through NRF2 signaling (10, 173, 174). Furthermore, chronic aerobic exercise increases GLO1 protein expression in the skeletal muscle of middle aged patients with T2DM (180). We propose that exercise enhances GLO1 activity, which increases the rate of MG detoxification yielding reduced AGE accumulation within skeletal muscle. This could lead to both improved insulin signaling and mitochondrial oxidation through reduced MG stress. We intend to further investigate this possibility in skeletal muscle.

The Skeletal Muscle Dicarbonyl Proteome

The dicarbonyl proteome describes proteins modified by MG particularly at functionally relevant sites. To date, many proteins have been identified including circulating proteins like albumin and hemoglobin, mitochondrial proteins, extracellular matrix proteins, transcription factors and cytosolic and membrane proteins (133, 145, 240). These proteins that are part of the dicarbonyl proteome are linked to metabolic dysfunction by compromising the interaction of dicarbonyl proteome proteins with other proteins or substrates. Many of the dicarbonyl proteome proteins described above are increased in diabetes, aging, and other chronic disorders (241, 242). Elucidating particular proteins that have the potential to become modified by MG and further lose their natural protein function will offer insight into 1) potential therapeutic

mechanisms for combatting dicarbonyl stress and 2) potential new diagnostic markers for multiple chronic and metabolic disease progressions, including T2DM. Two skeletal muscle proteins that exhibit high potential to be a part of the dicarbonyl proteome include mitochondrial complex-1 and AMPK.

Mitochondrial Complex-1

Mitochondria are the primary site of ATP synthesis within oxidative tissue. They accept electrons from reducing equivalents produced primarily through glycolysis or ß-oxidation of fatty acids. Mitochondrial complex 1 is the first protein complex in the mitochondrial electron transport chain, which transfers electrons while generating a proton motive force, membrane gradient, and eventual production of ATP through ATP synthase and final electron transfer to molecular oxygen. Others have shown that dysregulation of the mitochondrial complex proteins and electron transport lead to increases in mitochondrial ROS generation and cellular oxidative stress. Further, Proteomic analysis of insulin resistant skeletal muscle revealed reduced mitochondrial enzymes (80). A proposed route of mitochondrial dysfunction is an imbalance between reducing equivalents through mitochondrial complex 1 with the full electron transfer route to molecular oxygen. Others have suggested inhibition of mitochondrial complex 1 by methylglyoxal modification which causes an inhibition of mitochondrial respiration (243, 244). Given the established role of mitochondrial complex 1 as a contributor and effector of the diabetic milieu, recent data implicating its potential modification by MG reveals mitochondrial complex 1 as a potential role player in the skeletal muscle dicarbonyl proteome. This becomes compounded by the pivotal role mitochondrial complex 1 plays in the highly metabolic skeletal muscle, where shifting fuel sources is of utmost importance.

AMPK & γ subunit

Adenosine 5'-monophosphate (AMP-activated protein kinase (AMPK) is a key metabolic regulator and has been heavily investigated in diabetes and metabolic disease research (245). AMPK is known to have a variety of cell energy and stress stimuli, making its potential role in the dicarbonyl proteome

intriguing. AMPK activity in skeletal muscle increases fatty acid oxidation through phosphorylation of acetyl-CoA carboxylase. This metabolic control may play a crucial role in skeletal muscle flexibility, which is well known to be reduced in T2DM. This implicates a potential dysfunction of AMPK signaling in T2DM skeletal muscle. Further, AMPK increases cellular NAD+ levels, if this capability is reduced, it may also contribute to the redox imbalance observed in T2DM skeletal muscle as well. On the opposite end, activation of AMPK through exercise or metformin (the first line diabetic medication) improves skeletal muscle glucose uptake through insulin independent means and also improves mitochondrial proteins. Both these areas are primary mechanisms of T2DM exercise and drug therapy. It therefore stands to reason that investigating AMPK in T2DM skeletal muscle in the context of dicarbonyl stress and the dicarbonyl proteome may offer additional explanation to known mechanisms in diabetic skeletal muscle and potentially provide a novel skeletal muscle specific member of the dicarbonyl proteome.

An intimate knowledge of the structure and function of AMPK is required to better understand the potential role of dicarbonyl stress and AMPK function. AMPK is composed of an α-, ß-, and γ-subunit. Each subunit has multiple isoforms including α1 or α2, ß1 or ß2 or γ1, γ2 or γ3 allowing for 12 possible combinations. To our interest in skeletal muscle, human quadriceps muscle (the site of tissue acquisition via biopsy procedures) is majorly α2, ß2 and γ3 AMPK (246) although three are present with varying levels of expression depending on exercise (247). The α-subunit contains the catalytic site conferring kinase activity and has a phosphorylation site on Thr172 that increases enzyme activity. The ß-subunit is considered regulatory and acts primarily to stabilize the α and γ subunits. The γ-subunit is the primary site of AMP sensing whereby two binding sites exist for AMP to bind. This area consists of arginine rich Bateman domains. The arginine residues extending into the AMP binding domain on the γ subunit, combined with the propensity for MG to modify arginine residues has led others to hypothesize AMPK modification by MG is likely and potentially damaging to enzyme function (4) – represented in **Figure 7**. Additionally, exercise at intensities above ~50%VO2max have been shown to activate AMPK activity in human quadriceps (248). These data together suggest a potential role from AMPK in MG biology as it pertains to T2DM pathology, prevention and treatment and warrants investigation.

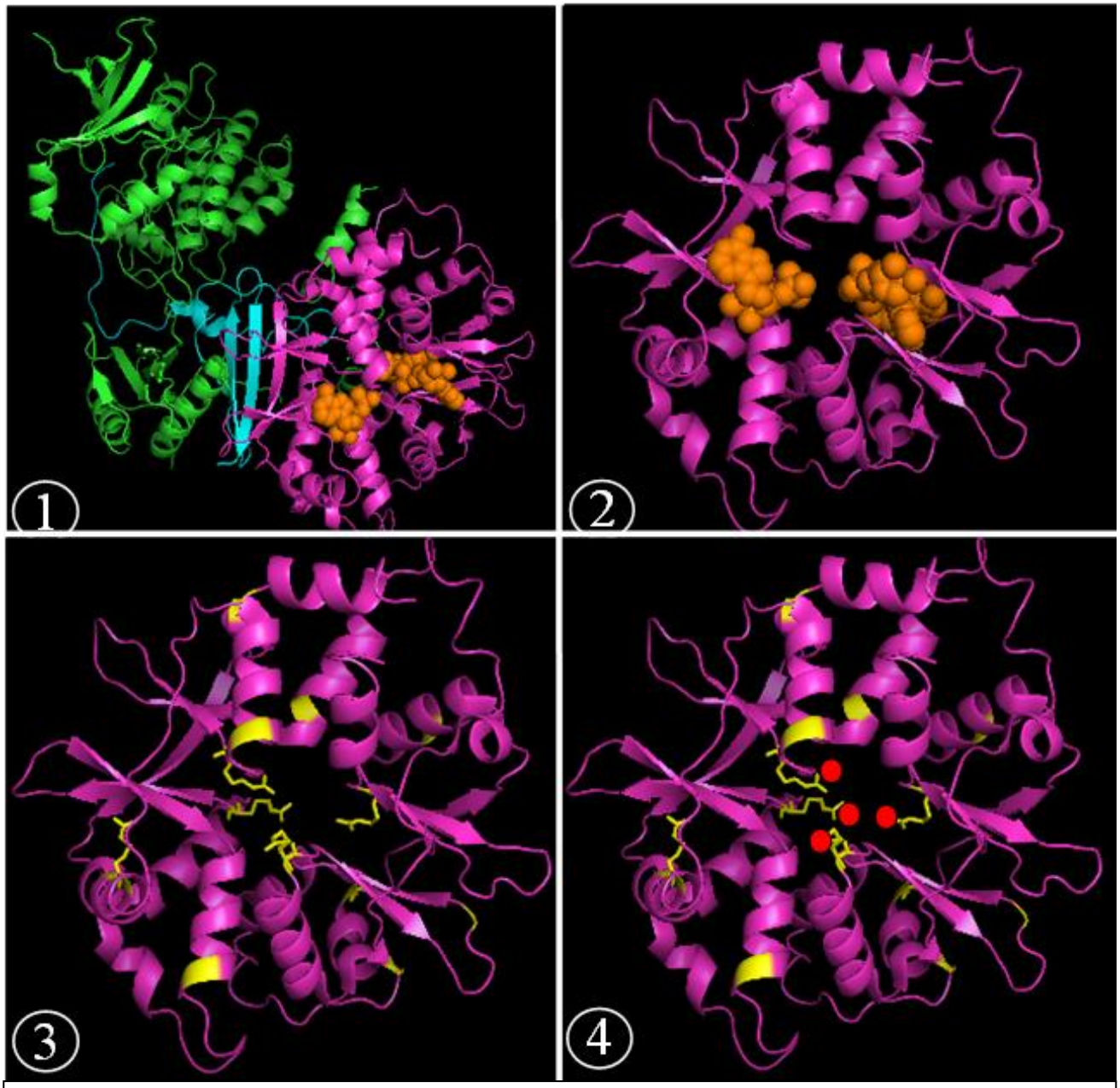

Figure 7. AMPK and potential susceptibility to dicarbonyl stress
1) Three-dimensional visualization of AMPK and AMP. The alpha (green), beta (blue) and gamma (purple) subunits are presented in their quaternary structure. AMP molecules (orange) are bound in the gamma regulatory subunit binding pocket. 2) The AMPK gamma subunit has been isolated and rotated to show the AMP binding pocket. 3) The AMP molecules have been removed and all the arginine residues have been highlighted (yellow). Note the extension of arginine side chains into the AMP binding pocket. 4) Methylglyoxal (red) has been crudely represented (not generated within PyMOL software) in locations suggested to be theoretical hot spots of MG attack on arginine residues by Gugliucci et al. (4). These images were generated by JTM using PyMOL molecular visualization system (Schrodinger LLC, Tokyo, Japan).

Summary

It is important to first highlight the duality of glucose – it is an essential nutrient proving energy and sustenance for human life, yet excessive amounts are extremely detrimental to multiple tissues (101, 193). The damaging cellular effects of acutely or chronically elevated levels of glucose, termed 'glucotoxicity,' are present in T2DM and manifest through a variety of molecular pathways. One of the most prominent of which, is the production of the potent glycolytic by-product, MG and subsequent accumulation of MG-directed protein modification and AGE formation (249). MG-directed protein modification is effectively minimized by the efficient detoxification of MG by the glyoxalase enzymatic defense system, regulated primarily by GLO1 protein expression and activity. A summary of physiologic contributors to MG generation align with glyoxalase system detoxification is presented in **Figure 8**. When GLO1 is compromised or MG production becomes excessive, MG accumulates, creates 'dicarbonyl stress' and damages proteins. MG-directed protein modification is a known contributor to hyperglycemia-induced diabetic complications in a variety of tissues including microvascular complications such as diabetic nephropathy, neuropathy and retinopathy and macrovascular complications such as cardiovascular disease and manifests with reductions in GLO1 protein expression while experimental models of GLO1 overexpression are protective. Recent, work in cell culture and animal models suggests MG-directed protein modification may additionally play a role in the primary metabolic defect in hyperglycemia and T2DM, skeletal muscle insulin resistance. Our research intends to extend the role of MG-directed protein modification and GLO1 regulation from being a mere consequence of T2DM to establishing its function as a contributor to skeletal muscle insulin resistance *per se*, the initial metabolic defect in progression to T2DM. Further, we intend to produce a skeletal muscle specific mechanism of dicarbonyl stress induced insulin resistance and establish the protective role of aerobic exercise training. This research will have a significant impact on the understanding of hyperglycemia induced insulin resistance and the natural history of T2DM.

The astounding healthcare impact of T2DM in the United States is indisputable. T2DM prevalence has breached 9% of the population, remains the 7th leading cause of death and costs nearly $250 billion to

care for annually (28, 250). T2DM is a multi-organ metabolic disorder characterized by chronic hyperglycemia secondary to insulin resistance. The importance of skeletal muscle health cannot be understated, as skeletal muscle tissue is the major metabolic organ accounting for >33% total body mass (251) and >40% total body protein (252). It performs locomotor functions, is a major storage site for glycogen, and is the primary amino acid reservoir in the body. Important to our application, skeletal muscle is estimated to be responsible for ~80% of insulin stimulated glucose disposal (56) and plays a major role in oxidative metabolism of glucose due to its abundance of mitochondria. Skeletal muscle insulin resistance reduces postprandial glucose uptake, prolonging hyperglycemia and exposing non-insulin dependent tissues to potentially damaging levels of glucose. Not surprisingly, skeletal muscle insulin resistance is implicated in the pathogenesis of many diseases like Alzheimer's, T2DM and sarcopenia (14, 132, 155, 156). Understanding the molecular mechanisms responsible for the development of skeletal muscle insulin resistance will help in developing better therapeutic strategies for the prevention and treatment of T2DM and hyperglycemia-mediated complications. Mechanisms related to MG formation is presented in **Figure 8.**

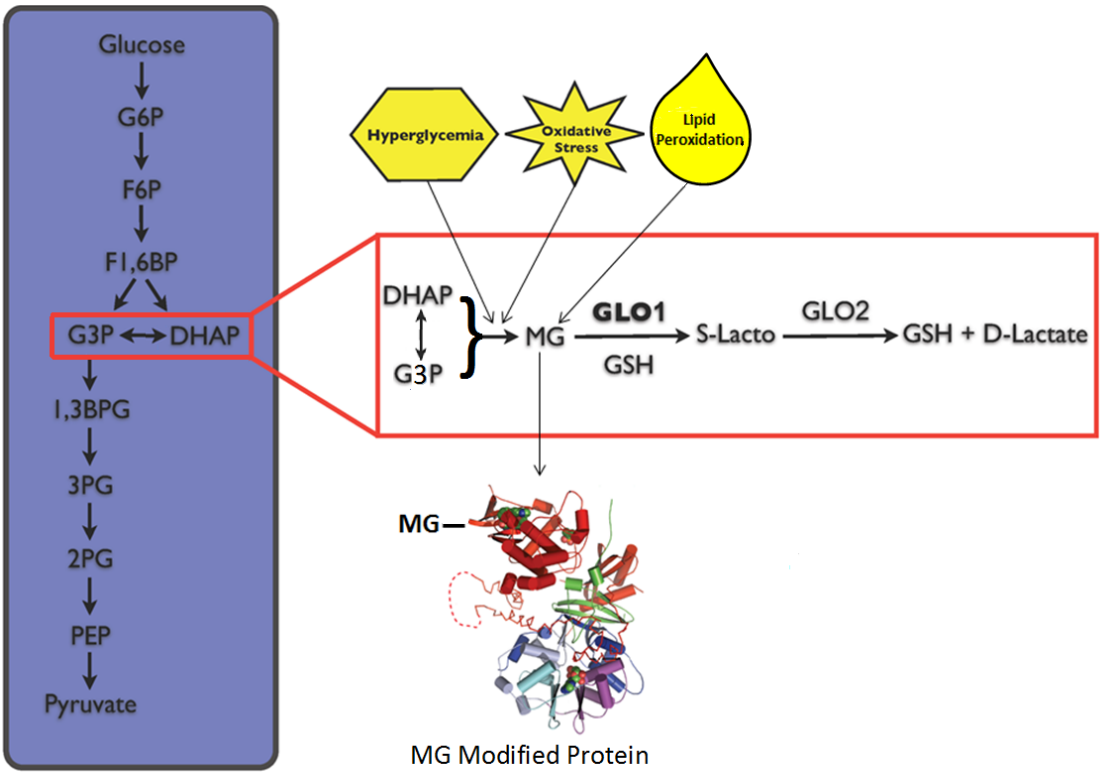

Figure 8. Physiologic contributors to methylglyoxal metabolism

The generation of MG from physiologic systems occurs as a spontaneous byproduct of glycolysis (highlighted in blue above). Under conditions intrinsic to the diabetic condition like hyperglycemia, oxidative stress and increased lipid peroxidation, MG generation accelerates. The enzymatic detoxification of MG to the intermediate s-lactoylglutathione and finally to the stable d-lactate occurs through GLO1 and GLO2 respectively while utilizing a catalytic amount of reduced glutathione. MG, methylglyoxal; G3P and DHAP, three-carbon glycolytic intermediates – glyceraldehyde-3-phosphate and dihydroxyacetone phosphate; GLO1, glyoxalase-1; GLO2, glyoxalase-2; GSH, reduced glutathione; S-lacto, s-lactoylglutathione.

CHAPTER III: METHODS

Overview

This research utilizes a clinical-translational approach involving both human studies and *in vitro* mechanistic studies. The clinical portion encompasses two separate human studies utilizing skeletal muscle biopsies to investigate the MG-GLO1 axis through: 1) a cross-sectional comparison between healthy and individuals with T2DM and 2) a clinical trial involving a 12-week aerobic exercise training intervention. The mechanistic portion will utilize a human skeletal muscle cell (HSKMC) line to: 1) elucidate the role of primary factors indicative of the diabetic condition to dicarbonyl stress in skeletal muscle cells, 2) expand the skeletal muscle dicarbonyl proteome and 3) identify skeletal muscle specific therapeutic strategies to combat dicarbonyl stress.

Methods Common to Aim 1 and Aim 2

Anthropometrics and Body Composition

Prior to all study visits, subjects arrived after a ~12 hour overnight fast. Height and body weight were measured by standard procedures (226). Body composition, including measures of lean body mass, body fat and bone mineral density was assessed by dual energy X-ray absorptiometry (model iDXA; Lunar, Madison, WI).

Indirect Calorimetry

Upon arrival after an overnight fast, subjects were returned to basal resting metabolism by lying supine in a dark room for 15 minutes. Then, expired air was continuously sampled for 20 minutes with the use of an automated system (Viasys, Franklin Lakes NJ; Parvo Medics, Sandy, UT) under a ventilated hood to determine basal metabolic rate (BMR) and substrate utilization (carbohydrate oxidation, COX) via the dilution method (253, 254). This process was repeated during the insulin stimulated state of the hyperinsulinemic-euglycemic clamp (described below) and changes in substrate utilization ($RER_{insulin} - RER_{basal}$) were used as an indicator of metabolic flexibility (calculations provided in **Appendix B**).

Hyperinsulinemic-Euglycemic Clamp

Hyperinsulinemic-euglycemic clamps were used to induce hyperinsulinemia comparative to postprandial levels, increase glucose flux into skeletal muscle and characterize whole body insulin sensitivity. The gold standard hyperinsulinemic (40 $mU/m^2/min$)-euglycemic (5mM) clamp procedure **(Figure 9)** was performed as previously detailed (226, 232, 255-257). To begin, subjects rested supine with head-of-bed position ~30 degrees. Then, a polyethylene catheter was inserted into an antecubital vein for infusion of insulin and glucose. Next, a second catheter was inserted retrograde into a warmed dorsal hand vein for sampling of arterialized venous blood. Once the catheters were placed, baseline blood samples were obtained for analysis of glucose and insulin. Then a basal state skeletal muscle biopsy (see below) was completed. The clamp commenced with a primed-continuous infusion (40 $mU \bullet m^{-2} \bullet min^{-1}$) of human insulin maintained for 120 min. Blood glucose was measured every 5 min via YSI glucose-lactate analyzer (YSI 2300; STAT Plus, Yellow Springs, OH) and was clamped at 90 mg/dl by use of a variable glucose infusion (20% dextrose) calculated according to the method of DeFronzo (258). Blood samples were collected (5 ml) every 15 minutes for analysis of plasma glucose and insulin concentrations. Glucose disposal rates (GDR; mg/kg/min) were calculated as the mean rate obtained during insulin-stimulated conditions using Steele's single-pool model of glucose kinetics as previously described (259). Insulin sensitivity is reported as 'M/I' and calculated from GDR normalized to steady state insulin concentrations during the final 30 min of the clamp procedure. Non-oxidative glucose disposal (NOGD; an indicator of glycogen synthesis) was calculated in the last 30 minutes of the insulin clamp from GDR minus total carbohydrate oxidation as estimated by indirect calorimetry (described above).

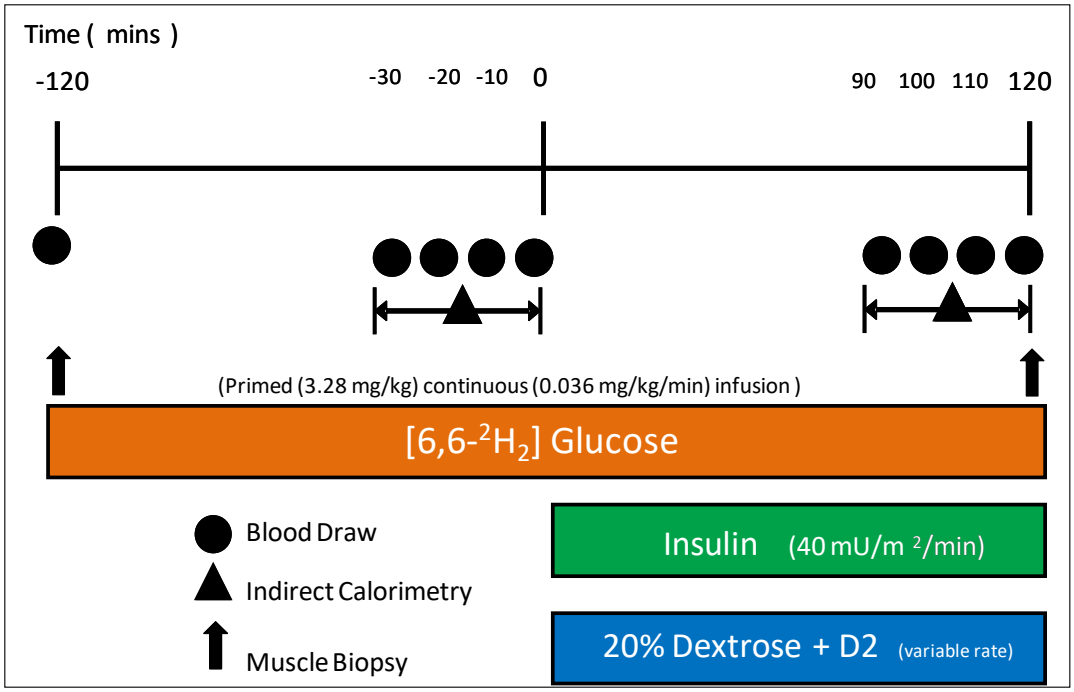

Figure 9. Hyperinsulinemic-euglycemic clamp timeline
The clamp procedure described pictorially above involves a primed continuous infusion of doubly labeled glucose (6,6-$^2H_2$) for determination of endogenous glucose production. At time point 0 minutes, a continuous infusion of insulin is administered to mimic postprandial hyperinsulinemia, while a variable rate infusion of glucose (20% dextrose) maintains euglycemia. Skeletal muscle biopsies are performed at baseline (-120 min) and during the metabolic condition (120 min) of the clamp procedure. Blood draws for downstream applications are obtained as described. Additionally, blood is sampled every 5 minutes and used to adjust the glucose infusion rate to maintain euglycemia.

Oral Glucose Tolerance Test

On a separate day, subjects arrived after an overnight fast (~12 hours) for the administration of a 75-gram glucose tolerance test (OGTT). Following baseline blood draws for the assessment of fasting glucose and insulin levels, a 75-gram glucose drink was provided to the subject and consumed within 10 minutes. Blood samples were collected at 30, 60, 90, 120, and 180 minutes for plasma glucose and insulin analysis. Measured glucose and insulin from the OGTT were used for calculations of fasting and dynamic measures of insulin sensitivity (fasting plasma glucose, FPG; homeostatic model assessment – insulin

resistance, HOMA-IR; Matsuda Index; calculations for measures of insulin sensitivity are provided in **Appendix, Metabolic Equations** (260). Results from the OGTT was used as verification of T2DM status according to ADA guidelines, where 120 minute OGTT glucose >200mg/dl is indicative of the T2DM condition.

Skeletal muscle biopsy

Skeletal muscle biopsies (~200mg) of the *vastus lateralis* were obtained under local anesthetic (Lidocaine HCl 1%), (261) using a 5 mm Bergstrom cannula with suction (262, 263) during the baseline (0 min) and insulin-stimulated (120 min) periods of the hyperinsulinemic-euglycemic clamp procedure (257). Infusion of steady state insulin and glucose was not terminated until completion of the muscle biopsy procedure. Muscle tissue was blotted, trimmed of adipose and connective tissue, and immediately flash frozen in liquid nitrogen and subsequently stored at -80°C until future processing and analysis. For mRNA analysis, an aliquot of muscle tissue (~50mg) was placed into RNAlater (AMBION, Inc., Austin, TX) solution instead of liquid nitrogen, immediately transported to a 4° C refrigerator for 24 hours and then transferred to -20°C storage until processing and mRNA analysis.

Immunoblotting

Approximately 10-15 mg (wet weight) of frozen muscle tissue was homogenized by ceramic beads (lysing matrix D; FastPrep®-24 homogenizer, MP Biomedicals, Santa Ana, CA) in 20 volumes of ice cold buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% Triton, and 1 µg/ml leupeptin (Cell Signaling Technology, Beverly, MA) with an added protease and phosphatase inhibitor cocktail (MS-SAFE, Sigma Aldrich, St. Louis, MO). Total protein concentration was determined via BCA Protein Assay (Pierce Biotechnology, Rockford, IL). Proteins (10-20 µg) were separated via 10% SDS-PAGE and transferred to either PVDF or nitrocellulose membranes followed by blocking with 3-5% non-fat dry milk or BSA in TBST. Conditions were optimized individually for each protein of interest. Immunoblotting

proceeded with the antibodies as follows: GLO1 (Santa Cruz Biotechnologies, Santa Cruz, CA), MG (Cell Biolabs, San Deigo, CA), NRF2 (Abcam, Cambridge, MA), Keap1 (NeoScientific, Cambridge MA), TPI (Santa Cruz), GLO2 (A11; Santa Cruz), AKR1B1 (GeneTex, Irvine, CA) and/or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (D16H1; Cell Signaling Technology)/β-Actin (612657; BD Biosciences, San Jose, CA). Secondary antibodies (LI-COR Biosciences, Lincoln, NE) were selected in a species-specific manner according to manufacturer's instructions. Protein expression was visualized via near infrared fluorescence imaging (Odyssey Clx, LI-COR Biosciences), quantified via Image Studio and normalized to GAPDH or ß-Actin protein expression as appropriate. MG-modified proteins were quantified using western bands between 15-260 kDa (encompassing the range of the molecular weight ladder, Chameleon Duo 90-0000, LI-COR Biosciences).

GLO1 Enzymatic Activity

GLO1 enzymatic activity (QuantiChrom Glyoxalase I Assay Kit, BioAssay Systems, Hayward, CA) was determined via conversion of S-lactoylglutathione (Δ in molar extinction coefficient: $\Delta\varepsilon 240=3.37$ mM/cm) after incubating skeletal muscle homogenates (10 µg) with MG and reduced glutathione, where 1 unit of GLO1 forms 1 µ mole of S-lactoylglutathione per minute at pH 6.6 and 25°C (264). Optical density of the samples was quantified via photospectrometry at 240nm and data are expressed as U/L of s-lactoylglutathione.

Protein Carbonyl Assay

Protein carbonyls are the most common form of protein modification by glycation, oxidative stress, or other oxidative by-products and consist of carbonyl derivatives of arginine, but also lysine threonine and proline residues. Protein carbonyl content of skeletal muscle was analyzed by a commercially available kit (Oxiselect, Cell Biolabs) according to manufacturer's instructions. Briefly, skeletal muscle homogenate was diluted to 10µg/ml and loaded into a pre-coated 96-well plate for 2 hours at 37° C. Following manufacturer's instructions, carbonyl content was quantified by derivatization (of all protein carbonyls

present within the skeletal muscle homogenate) with DNP hydrazone, followed by an anti-DNP primary antibody, a HRP conjugated secondary antibody, and microplate quantification of absorbance at 450nm. Comparison to a standard curve of oxidized-BSA allows for estimation of carbonyl content reported as nmol protein carbonyls/mg total protein.

Blood Analyses

Plasma insulin concentration was quantified via a commercial radio-immunologic assay or commercial ELISA. A complete blood panel was obtained after samples were analyzed by PCL Alverno (Chicago, IL).

Methods Specific to Aim 1

Study Design & Subject Selection

We designed this cross-sectional study to obtain as much information as possible about the MG-GLO1 axis and its regulation in skeletal muscle. Our intent was to characterize a population of young, lean healthy controls (LHC) – representing an optimal state of health – and directly contrast these observations to T2DM subjects. Comparisons to a LHC group allow reference to optimal physiology, thus highlighting the importance of the disease phenotype. Participants were recruited from the Chicago, IL metropolitan area. All subjects were screened via health history, medical exam, resting EKG and fasting blood chemistry in the Clinical Research Center of the University of Illinois at Chicago. Individuals were excluded if they used nicotine, had undergone greater than 2-kg weight change in the last six months, or had evidence of hematological, renal, hepatic, or cardiovascular disease. LHCs were excluded if results of OGTT indicated impaired fasting glucose or impaired glucose tolerance. T2DM subjects self-reported diabetes duration of 4±1 years (174). All studies were approved by the Institutional Review Board of the University of Illinois at Chicago and performed in accordance with the Declaration of Helsinki.

Metabolic Control

All subjects were instructed to maintain their regular dietary eating habits and completed 3-day diet records (2 weekdays and one weekend day). Subjects were asked to refrain from physical activity outside of their normal activities of daily living, consumption of alcoholic beverages and consumption of foods and beverages containing caffeine for 48 h prior to the metabolic testing day. On the day preceding metabolic testing, subjects were counseled to consume ~55% of calories as carbohydrate in order to meet a goal of 250 g (257). At ~ 6:00pm on the evening prior to metabolic testing, participants were provided a balanced metabolic meal (55% carbohydrate, 35% fat, 10% protein: based on their estimated individual nutrient requirements and habitual physical activity levels) (265, 266) to stabilize muscle and liver glycogen stores. After meal consumption, participants fasted overnight for a period of 10-12 hrs. All participants were asked to withhold medications and supplements the morning of metabolic testing that were known to influence primary outcome variables. Female participants who were pre-menopausal with regular menstrual cycles, were studied in the follicular phase.

Statistics

Statistical analysis was performed using PRISM (GraphPad Software, La Jolla, CA). Baseline differences in variables of interest were compared using an independent samples t-test. A two-way ((Group (LHC vs T2DM) x Condition (Basal vs Insulin)) ANOVA with repeated measures for 'condition' was used to investigate protein expression, carbonyl stress and enzymatic activity. Pearson's correlation coefficient was used to investigate relationships among GLO1 protein expression and markers of metabolic health. Linear regression models were developed using SPSS (SPSS Inc., Chicago, IL). Significance was set at $p<0.05$. Data are presented as mean ± SEM.

Methods Specific to Aim 2

Study Design & Subject Selection

A cross sectional investigation of 20 older, obese, insulin resistant adults and 6 lean healthy controls was conducted on subjects recruited as part of two separate studies performed at the Learner Research Institute of the Cleveland Clinic (Cleveland, OH). An outline of the study description is provided in **Figure 10.** Medical screening excluded individuals with heart, kidney, liver, thyroid, intestinal, and pulmonary diseases, or those taking medications known to affect our outcome variables. The studies were approved by the Cleveland Clinic Institutional Review Board, and all subjects provided informed written consent in accordance with guidelines for the protection of human subjects. All subjects underwent a 2-day screening period to ensure study eligibility where they received a health and physical examination, underwent a resting 12-lead ECG, performed an exercise stress test, were administered an OGTT and provided blood samples for blood chemistry. Upon verification of eligibility, all subjects underwent a 3 day pre-testing period involving an inpatient stay which included a DEXA scan, VO_2max test, indirect calorimetry, OGTT, hyperinsulinemic-euglycemic clamp procedure and a skeletal muscle biopsy. OB-IR subjects then entered a 12-week intense lifestyle intervention that involved an isocaloric diet and aerobic exercise training. Finally, OB-IR subjects completed a 3-day post-testing period, where all pre-testing procedures were repeated.

For our analysis, LHC subjects were specifically selected from the original study cohorts according to 1) availability of muscle tissue and 2) age <40 years. We chose this specific cohort of young, lean, healthy

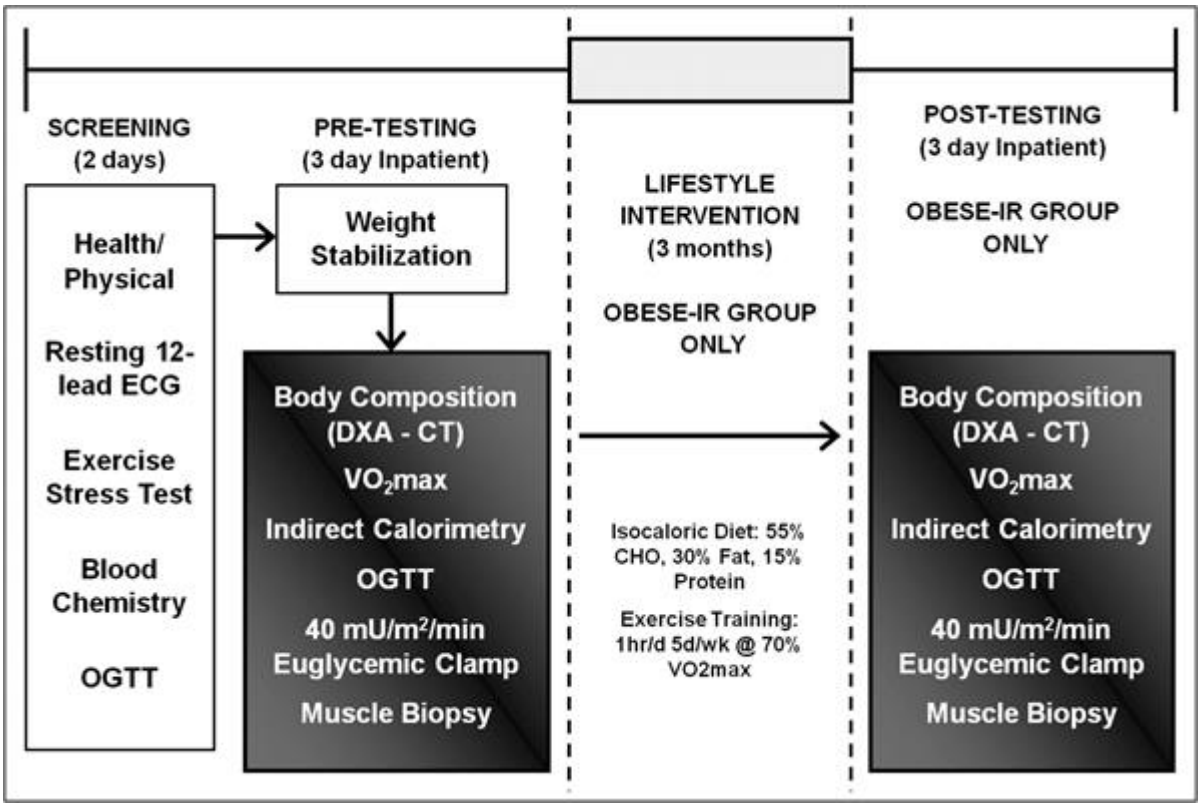

Figure 10. Study design for specific aim 2
The study design for specific aim 2 involves a 12-week intense lifestyle intervention in obese, insulin resistant adults to investigate the effect of the intervention on measures of insulin sensitivity, aerobic fitness and MG-GLO1 axis proteins. This human intervention trial used stringent control procedures involving a 2-day screening procedure, 3-day inpatient pre- and post- testing procedures (which allowed full control of subject diet and physical activity for the purpose of metabolic testing), a 12-week fully supervised exercise protocol and full diet provision during the intervention period as well. Measures of insulin sensitivity were obtained from fasting, OGTT and hyperinsulinemic-euglycemic conditions. A muscle biopsy was obtained at basal and insulin-stimulated conditions of the clamp procedure before and after the lifestyle intervention. A maximal exercise test (VO_2max) was used to assess cardiovascular fitness as well as adjust exercise intensities throughout the 12-week intervention to maintain training intensity at 70% VO_2max. OBESE-IR, obese, insulin resistant participants; OGTT, oral glucose tolerance test; DXA-CT, Dual energy X-ray absorptiometry – computed tomography; VO_2max, velocity of oxygen consumption during a maximal exercise test.

controls because we believe it best represents optimal skeletal muscle health. OB-IR subjects were selected from the original study cohorts according to availability of muscle tissue and completion of a 12-week lifestyle intervention (described below).

Inpatient Control Period

Baseline and post-intervention metabolic assessments were conducted over a 3-day inpatient stay in the Clinical Research Unit of the Cleveland Clinic. Subjects were provided weight-maintenance meals designed by a research dietitian (resting metabolic rate x 1.2 activity factor; 55% CHO, 30% fat, 15% protein). Following the intervention in OB-IR participants, testing procedures were performed approximately 16–18 hours after the final exercise bout.

Measures of Maximal Aerobic Capacity

Aerobic fitness (VO_2max) and maximum heart rate (HRmax) were determined during an incremental graded treadmill exercise test (modified Bruce protocol) via metabolic cart (267). This procedure was repeated every 4 weeks throughout the duration of the lifestyle intervention, and exercise intensity was adjusted to maintain work rate goals (detailed below).

Lifestyle Intervention

Obese, insulin-resistant subjects underwent an intense lifestyle intervention consisting of an isocaloric diet as previously described (226) and 60-minutes of fully supervised aerobic exercise training (AE) 5 days/week for 12 weeks. AE consisted of treadmill walking and/or cycle ergometry at ~85% of maximum heart rate (HRmax) as determined during the baseline aerobic fitness test (described above). Heart rate monitors were worn during each AE session to ensure individualized target HRs were achieved. Additionally, AE sessions incorporated a warm-up and cool-down period that included a series of stretching exercises (231).VO_2max and HRmax were re-assessed every 4 weeks during the intervention to account for aerobic fitness improvements. Baseline measures were repeated in OB-IR after completion of the 12-week lifestyle intervention. All meals and fluids were provided to the participants throughout the intervention. A registered dietitian nutritionist generated isocaloric diets according to baseline calculations (resting metabolic rate x 1.2 activity factor). Weight loss during the interventions was therefore attributed to exercise

energy expenditure, rather than dietary caloric restriction. Dietary compliance was determined by daily food weights, and nutrient analysis of the diet was performed using Nutritionist Pro software (Axxya systems, Stafford, TX) (235, 268).

GLO1 mRNA

Unlike many proteins, GLO1 mRNA strongly correlates with GLO1 protein expression in a murine model (269). Therefore, to enrich our proteomics approach, we analyzed GLO1 mRNA in the skeletal muscle of OB-IR before and after a 12wk aerobic exercise training intervention, although tissue availability limited sample size (n=5). RNA was extracted from muscle tissue samples aliquoted for RNA analysis (described above) using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RT-PCR was performed with SYBR Green QPCR (Agilent Technologies, Santa Clara, CA) according to manufacturer's instructions. PCR reaction for sequencing was performed with Forward: 5'-CAA GATCCTGATGGCTACTGG-3' and Reverse: 5'-CAGTCCTAGATGCTTCTGGTATG-3'.

Statistics

Statistical analysis was performed using PRISM (GraphPad Software). Baseline differences between groups (LHC vs OB-IR) were compared using an unpaired Student's t-test. Differences before and after the lifestyle intervention (OB-IR Pre vs Post) were compared using a paired Student's t-test. A Pearson correlation was performed to investigate relationships among variables of interest. Significance was set at $p<0.05$. Data are presented as mean ± SEM.

Methods Specific to Aim 3

Human Skeletal Muscle Cell Culture (HSKMCs)

The HSKMC line was obtained from Lonza (Walkersville, MD). Cells were seeded into standard 6-well plates or 100mm dishes at a density of 3,500 cells/cm^2 and maintained in skeletal muscle growth medium (SKGM; Lonza) supplemented with 0.1% human epidermal growth factor (hEGF), 1% fetuin, 1%

bovine serum albumin (BSA), 0.1 dexamethasone,1% insulin and 0.1% gentamycin/amphotericin B. Cells were kept in a humid atmosphere at 37°C and 5% CO2 during all stages of the experimental process. When cells reached >95% confluence, growth medium was replaced with differentiation medium (DMEM-F12 supplemented with 2% horse serum and 1% antibiotic). Differentiation medium was refreshed every other day for 3 to 5 days until the multinucleated myotubes were observed throughout the culture. Following differentiation, the cells were returned to growth media for experimental procedures.

Experimental Conditions

We sought to investigate characteristics of the diabetic condition in HSKMCs by supplementing basal media with insulin, glucose and methylglyoxal as described below:

- To mimic postprandial hyperinsulinemia, HSKMC media was supplemented with insulin to a final concentration of 300pM for 0, 30 and 180 minutes.
- To mimic post-prandial hyperglycemia, HSKMC media was supplemented with glucose to a final concentration of 15mM for 0 or 24 hours. To mimic hyperglycemia associated with uncontrolled T2DM, HSKMC media was supplemented with glucose to a final concentration of 30mM for 0, 1 and 5 days.
- To mimic dicarbonyl stress, HSKMC media was supplemented with MG to a final concentration of 0, 1, 5, 10, 100, 250 or 500 μM for 4 hours. Intracellular MG concentrations are estimated to range between 1-4μM and others have shown that incorporation of MG into cultured cells is ~3% of the media concentration L6 rat muscle cells (18) and rat aortic smooth muscle cells (146). Therefore, we developed a range of dicarbonyl stress that mimics healthy through pathologic dicarbonyl stress conditions.
- To test the interference of dicarbonyl stress on AMPK activity, another set of experiments were conducted where HSKMCs were treated with 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR; 2mM, 1 hour), an AMPK stimulant that is a functional AMP mimetic (ZMP) and stimulates AMPK activity through allosteric

activation at the γ-subunit. Treatments consisted of AICAR alone or AICAR after 4 hours of MG treatment as described above. We selected the AICAR stimulus (2mM, 1 hour) because it has been shown to effectively stimulate AMPK activity (as measured by the surrogate marker, pACC) in skeletal muscle cells (270, 271).

- To investigate potential therapeutic mechanisms, HSKMCs were incubated with metformin (MetF) at 5 and 10mM for 24 hours alone or in combination with our models of dicarbonyl stress. Metformin was selected as the therapeutic target of interest due to its biguanidine structure which have high affinity for MG and confers MG scavenging properties (26). Additionally, metformin imparts additional protective effects on insulin-independent tissues beyond what can be explained by its glucose lowering effect in T2DM patients. This phenomenon is suggested to be in part due to protection against MG-mediated protein damage (272).

After the completion of experimental conditions, cells were harvested by the cell scraping method into ice cold cell lysis buffer as described in **Appendix A**. Basal media will be used as a control in all conditions.

Immunoprecipitation of AMPKγ

To assess MG-modification of the AMPKγ subunit, HSKMC media was supplemented with MG to a final concentration of 0, 10, 50, 100, or 500 μM for 4 hours. AMPKγ proteins were immunoprecipitated from 50μg of HSKMC proteins using Dynabeads Protein G (Invitrogen Life Technologies, NY) and anti-AMPKγ antibodies (Cell Signaling). Immunoprecipitated AMPKγ was separated via 10% SDS-PAGE and immunoblotted for MG-modifications (Anti-MG, Cell Biolabs).

AMPK Activity

AMPK activity was analyzed using the phosphorylation status of acetyl CoA-carboxylase (pACC; a downstream target of AMPK) as a surrogate measure. ACC will be assayed via immunoblotting as

described above using a pACC antibody (pACC-ser79, Cell Signaling). This phosphorylation at serine79 occurs from AMPK (273). Additionally, although two isoforms of pACC are recognized by this antibody (α and ß) only the ß isoform (280 kDa) was quantified because it is the major isoform present in skeletal muscle (274).

Statistical Analysis

A 1-way ANOVA across experimental conditions was used to analyze effects of HSKMC treatments. Tukey's honestly significant difference (HSD) post hoc test was used to compare means between experimental conditions when appropriate. All data are expressed as mean ± SEM. A p-value of <0.05 was used to determine statistical significance.

CHAPTER IV: RESULTS

The results of this dissertation investigating the MG-GLO1 axis in human skeletal muscle and the effects of T2DM and exercise are presented according to each specific aim. A working model describing the protein targets surrounding MG and GLO1 metabolism in skeletal muscle is provided in **Figure 1** (page 12).

Results Pertaining to Specific Aim 1

Subject Characteristics

Baseline Characteristics & Metabolic Data

Baseline characteristics of the study population are available in **Table 2**. A young, lean healthy control group (LHC) was selected to represent a model of good health. Of particular interest, T2DM subjects were older, maintained a higher BMI and had higher percentage of body fat than LHC (all $p<0.0001$). Additionally, T2DM subjects were insulin resistant according to fasting (HOMA-IR), glucose stimulated (2hr-OGTT glucose) and metabolic (GDR) measures LHC ($p<0.0001$).

The Effects of T2DM and Whole Body Insulin Stimulation on Skeletal Muscle GLO1 and MG-Modified Proteins

Glyoxalase System Protein Expression & Enzymatic Activity

Skeletal muscle GLO1 protein expression was markedly reduced (-78.8%; $p<0.001$) in T2DM compared to LHC **(Figure 11)**. There was no effect of insulin stimulation on GLO1 protein expression. Since the T2DM group was significantly older and more obese than the LHC group and because chronologic age and obesity have previously been associated with reductions in GLO1 protein expression, we generated a linear regression model to investigate the effect of T2DM status on GLO1 protein expression controlling for age and BMI. The linear regression model is presented in **Figure 12**. Model 1 used basal GLO1 protein expression (AU) as the dependent variable and age and BMI as independent variables. Expectedly, this model revealed a strong R-value of 0.697. However, the addition of group (T2DM) to the model (model 2)

profoundly increased the R-value to 0.845, an increase of ~.15 compared to just age and BMI as the only predictors. Further, the effect of group (T2DM) in Model 2 was significant (p=0.013) while age and BMI were not significant, indicating T2DM as a strong predictor of reduced GLO1 protein expression in our participants. Skeletal muscle GLO1 protein inversely correlated with BMI **(Figure 13A)**, body fat percentage **(Figure 13B)**, and HOMA-IR **(Figure 13C)**, the fasting measure of insulin resistance, while GLO1 positively correlated with skeletal muscle insulin sensitivity (M/I) measured by the hyperinsulinemic-euglycemic clamp **(Figure 13D)**. GLO1 activity was not different between Group or Condition (**Figure 14**).

MG-modified proteins

We utilized immunoblotting similar to the methods reported by others (275) to asses MG-modification of cellular proteins with minor modifications for human skeletal muscle tissue. Interestingly, according to this method of dicarbonyl stress quantification, MG-modified proteins are increased in T2DM skeletal muscle with insulin stimulation during the clamp (**Figure 15**; $p<0.001$). These results implicate MG stress may occur acutely in T2DM skeletal muscle with insulin stimulation or increased glycolytic flux and underlie the basal differences. Additionally, we performed a methodological validation to verify our western blotting procedure accurately measures differences in MG-modified proteins, as we did not utilize the gold standard for MG-H1 quantification, HPLC tandem mass spectrometry as defined by (276). To validate our western blotting quantification procedure, a titration curve of MG-modified bovine serum albumin (MG-BSA; a stable protein modified with MG to a known concentration) is presented in **Figure 16.** The data describe a strong, linear correlation between expected and quantified values, demonstrating the accuracy of our technique.

Carbonyl Stress

A global carbonyl stress assay was performed to assess oxidative and carbonyl modifications of skeletal muscle proteins. This analysis includes MG-modification of proteins, but also quantifies oxidative

damage, which provides an indicator of overall cellular oxidative stress. Skeletal muscle of T2DM had increased levels of carbonyl stress (**Figure 17A**; p=0.0257) which inversely correlated with basal GLO1 protein expression (**Figure 17B**; r^2=0.2671, p=0.0485). Similar to GLO1 protein expression, carbonyl stress was not effected by insulin stimulation during the clamp.

Regulation of GLO1 Protein Expression

NRF2 & Keap1 Protein Expression

To investigate if proteins regulating GLO1 expression were altered in T2DM skeletal muscle in the basal and metabolic state of the hyperinsulinemic-euglycemic clamp, we performed western blot analysis as previously described for NRF2 and Keap1. In agreement with reduced GLO1 protein expression in T2DM, NRF2 was 31.2% lower in T2DM compared to LHC (**Figure 18**; p<0.05). There was also an interaction effect of Group (LHC vs T2DM) x Condition (Basal vs Insulin) (p=0.0086) where skeletal muscle NRF2 decreased in LHC, but increased in T2DM with insulin stimulation. Additionally, Bonferroni's post hoc comparison revealed a significant effect of insulin in LHC (p<0.05), but not in T2DM. In agreement with reduced GLO1 and NRF2 protein expression in T2DM skeletal muscle, Keap1 protein was >2 fold higher in T2DM compared to LHC (**Figure 19**; p=0.006). There was also a significant interaction effect of Group (LHC vs T2DM) x Condition (Basal vs Insulin) on Keap1 protein expression where LHC increased, but T2DM decreased with hyperinsulinemia. (*, p=0.0068). Additionally, Bonferroni post hoc comparison revealed a significant effect of insulin only in T2DM (#, p<0.05).

Alternate Pathways Regulating Dicarbonyl Stress

TPI & GAPDH Protein Expression

To investigate potential mechanisms of MG generation, we quantified the glycolytic enzymes, TPI and GAPDH protein expression in skeletal muscle at basal and insulin stimulated states of the hyperinsulinemic-euglycemic clamp. Dysregulation of these proteins causes triose phosphate intermediates to accumulate and increases MG generation in the context of T2DM *in vitro* models. There was no effect

of Group or Condition on TPI (**Figure 20**) or GAPDH (**Figure 21**) normalized to ß-Actin. This additionally validated our use of GAPDH as a proper loading control in our T2DM samples.

GLO2 and AKR1B1 Protein Expression

To investigate potential mechanisms regulating MG detoxification we quantified GLO2 and AKR1B1 protein expression in skeletal muscle at basal and insulin stimulated states of the hyperinsulinemic-euglycemic clamp. Dysregulation of GLO2 protein may cause a backup of the glyoxalase enzymatic defense system through preventing GLO2 mediated GSH regeneration which has been shown *in situ* to inhibit GLO1 activity and thereby allowing MG to accumulate. There was no effect of Group or Condition on GLO2 protein expression (**Figure 22**). AKR1B1 plays a minor role in MG detoxification, but increases MG detoxification when GLO1 is reduced in preclinical models. There was no effect of Group or Condition on AKR1B1 protein expression (**Figure 23**).

TABLE II. SUBJECT CHARACTERISTICS.

	LHC	T2DM
n (M, F)	10 (4,6)	5 (2,3)
Age, years	27 ± 1	56 ± 5*
BMI, kg/m^2	22.3 ± 1.1	32.3 ± 1.6*
Body fat, %	24.1 ± 1.8	40.7 ± 3.0*
Fat-free mass, kg	48.8 ± 3.1	59.4 ± 4.0*
Total Cholesterol, mg/dL	154 ± 7	151 ± 6
HDL Cholesterol, mg/dL	60 ± 5	52 ± 4
LDL Cholesterol, mg/dL	80 ± 11	81 ± 7
Triglycerides, mg/dL	75 ± 7	86 ± 14
Fasting glucose, mg/dL	90 ± 4	151 ± 24*
HbA1c, %	5.3 ± 0.1	7.7 ± 1.0*
Fasting insulin, µU/mL	5.6 ± 0.6	7.8 ± 1.3
HOMA-IR, AU	1.0 ± 0.1	2.2 ± 0.4*
2-hr OGTT Glucose, mg/dL	101 ± 4	262 ± 60*
OGTT glucose iAUC, mg/dL/2hr	2884 ± 306	11682 ± 1059
OGTT insulin AUC, µU/ml/2hr	3748 ± 307	2676 ± 948
Matsuda Index, AU	8.3 ± 1.0	4.5 ± 0.7
GDR, mg/kg/min	7.1 ± 0.5	2.7 ± 0.5*
Clamp insulin, µU/mL	5.26 ± 0.95	7.29 ± 1.43
M/I, mg/kg/min/µU.ml	0.11 ± 0.01	0.03 ± 0.01*

LHC, Lean Healthy Control subjects; T2DM, individuals with Type 2 Diabetes Mellitus; BMI, Body Mass Index; HOMA-IR, homeostatic model assessment – insulin resistance; OGTT, 75g oral glucose tolerance test; GDR, Glucose Disposal Rate as calculated from the hyperinsulinemic-euglycemic clamp; M/I, clamp-derived glucose disposal rate normalized to prevailing insulin concentration; *, different from LHC, $p<0.05$. Data represent mean ± SEM.

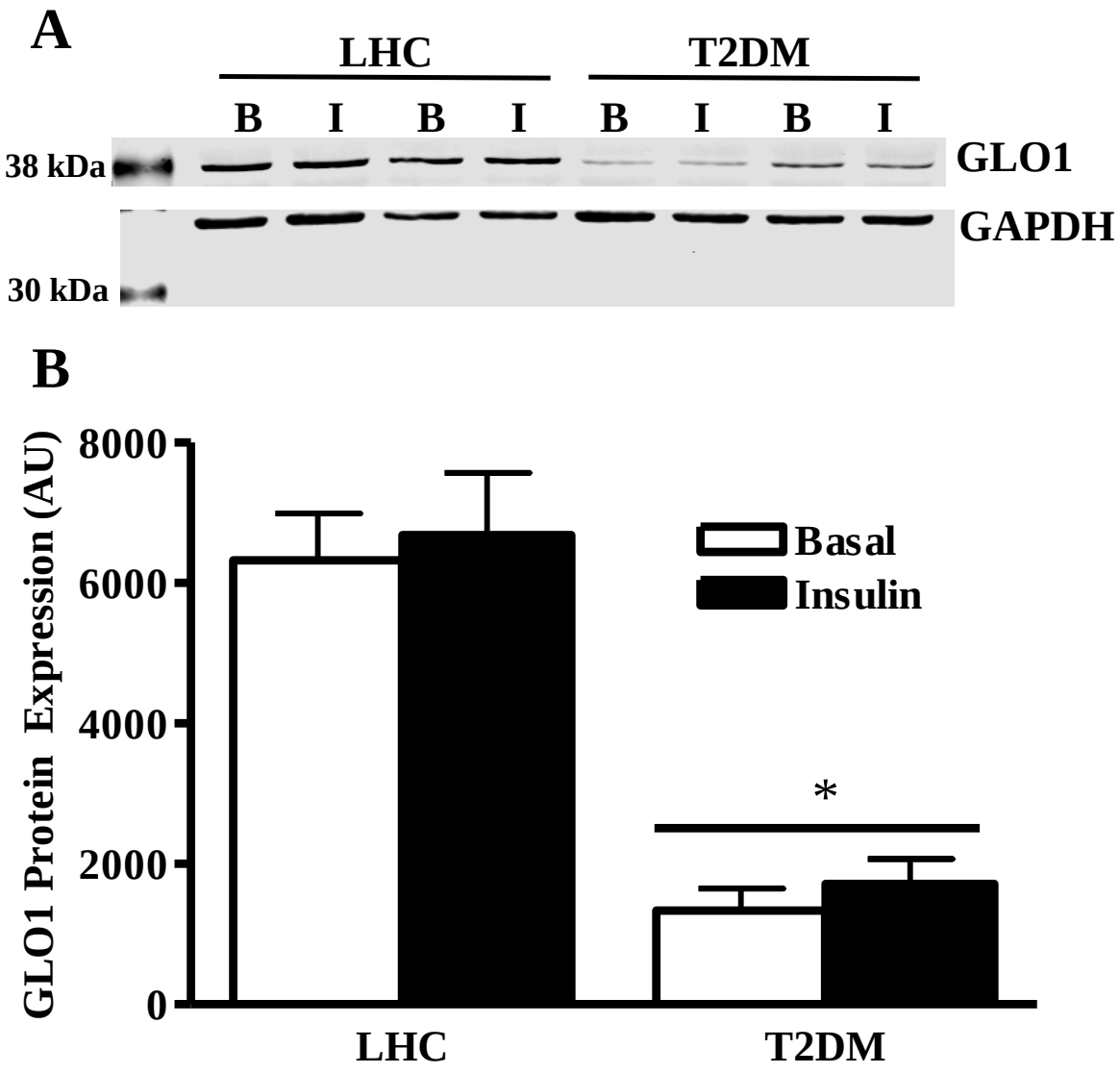

Figure 11. Skeletal Muscle GLO1 Protein Expression.
(A) Representative western blot image. GAPDH used as loading control. (B) GLO1 protein expression is reduced in skeletal muscle of T2DM during basal and insulin stimulated states of the hyperinsulinemic-euglycemic clamp. LHC, n=10; T2DM, n=5; *, Significant effect of Group, p=0.0003. LHC, Lean Healthy Control subjects; T2DM, individuals with Type II Diabetes; B, Basal, t=0 min; I, Hyperinsulinemia, t=120 min.

Variables Entered/Removed

Model	Variable Entered	Variables Removed	Method
1	BMI, Age	n/a	Enter
2	Group	n/a	Enter

Dependent variable: Basal GLO1 protein expression

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	0.697[a]	0.486	0.401	2308.741
2	0.845[b]	0.714	0.636	1799.385

a. Predictors: (constant), BMI, Age

b. Predictors: (constant), BMI, Age, Group

Coefficients

Model		Unstandardized B	Coefficients Std. Error	Standardized Coefficients Beta	t	Significance
1	(Constant)	12967.6	3149.8		4.117	0.001
	Age	-45.482	66.177	-0.239	-0.687	0.505
	BMI	-258.372	182.799	-0.491	-1.413	0.183
2	(Constant)	11336.242	2516.056		4.506	0.001
	Age	93.816	69.832	0.492	1.343	0.206
	BMI	9.591	168.816	0.018	0.057	0.956
	Group	-7780.140	2629.371	-1.273	-2.959	0.013

Dependent variable: Basal GLO1 protein expression

Figure 12. Linear regression model of GLO1 protein expression

Linear regression to determine the effect of T2DM (group) on our primary outcome measure (skeletal muscle GLO1 protein expression). A 2-step model to investigate the effect of T2DM on GLO1 protein expression while controlling for BMI and Age revealed that T2DM was a significant predictor of GLO1 protein expression (p=0.013).

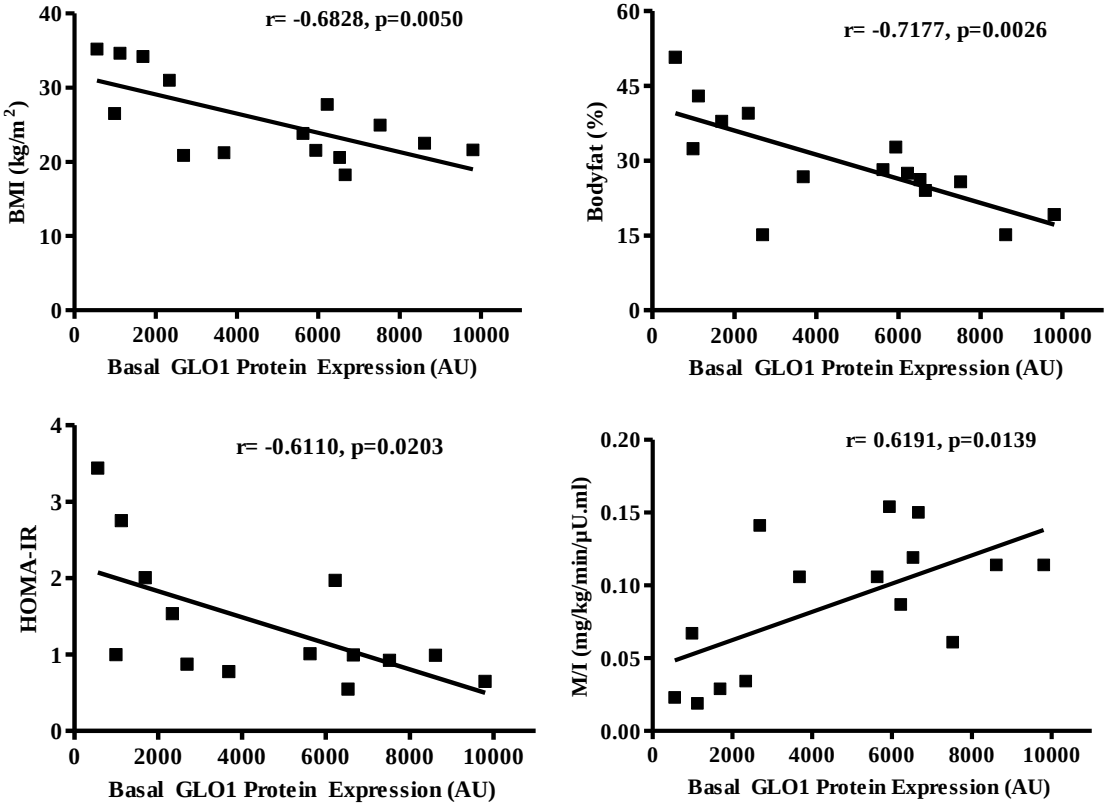

Figure 13. Baseline GLO1 protein correlations.
A significant negative correlation was observed between skeletal muscle GLO1 protein and BMI (A), body fat percentage (B) and fasting indices of insulin resistance (HOMA-IR; C). Additionally, a positive correlation was observed between basal GLO1 protein expression and peripheral insulin sensitivity determined by hyperinsulinemic-euglycemic clamp (M/I; D).

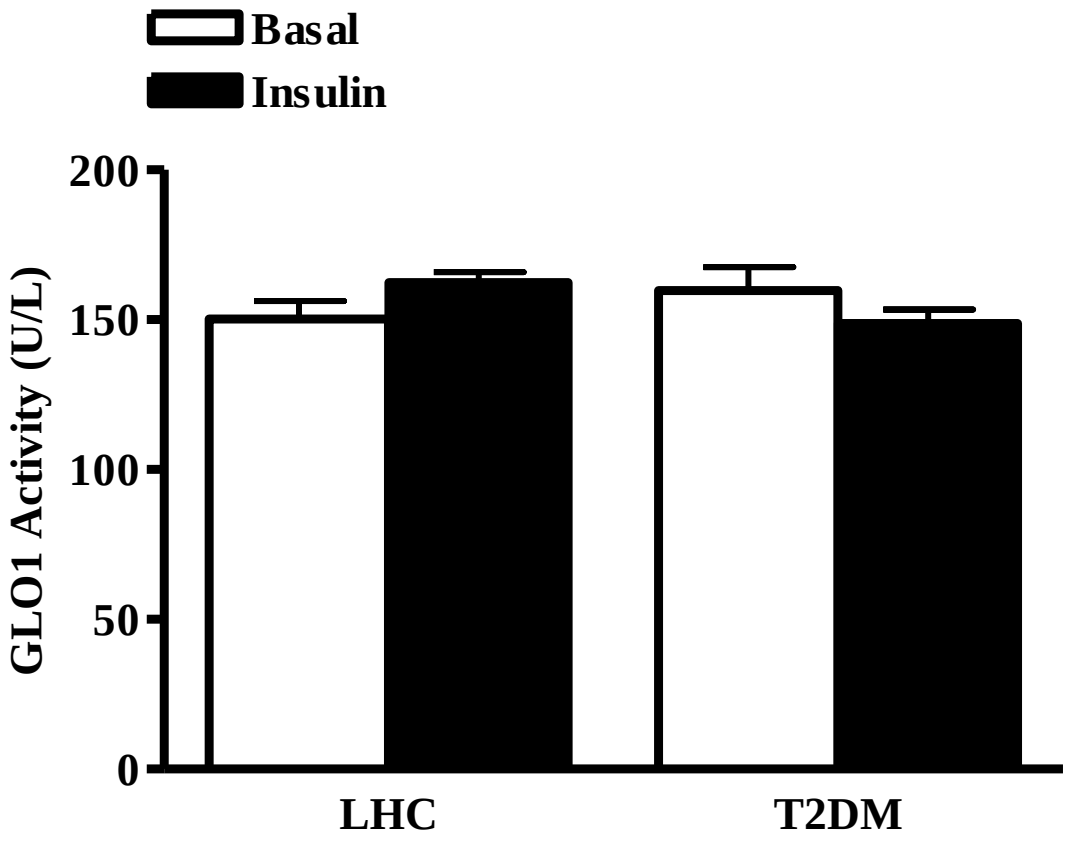

Figure 14. Skeletal Muscle GLO1 Activity.
Skeletal muscle protein (15µg) from LHC (n=10) and T2DM (n=5) participants in basal and insulin stimulated condition of the hyperinsulinemic-euglycemic clamp were assayed for GLO1 activity. GLO1 activity was not different between groups or conditions; $p>0.05$.

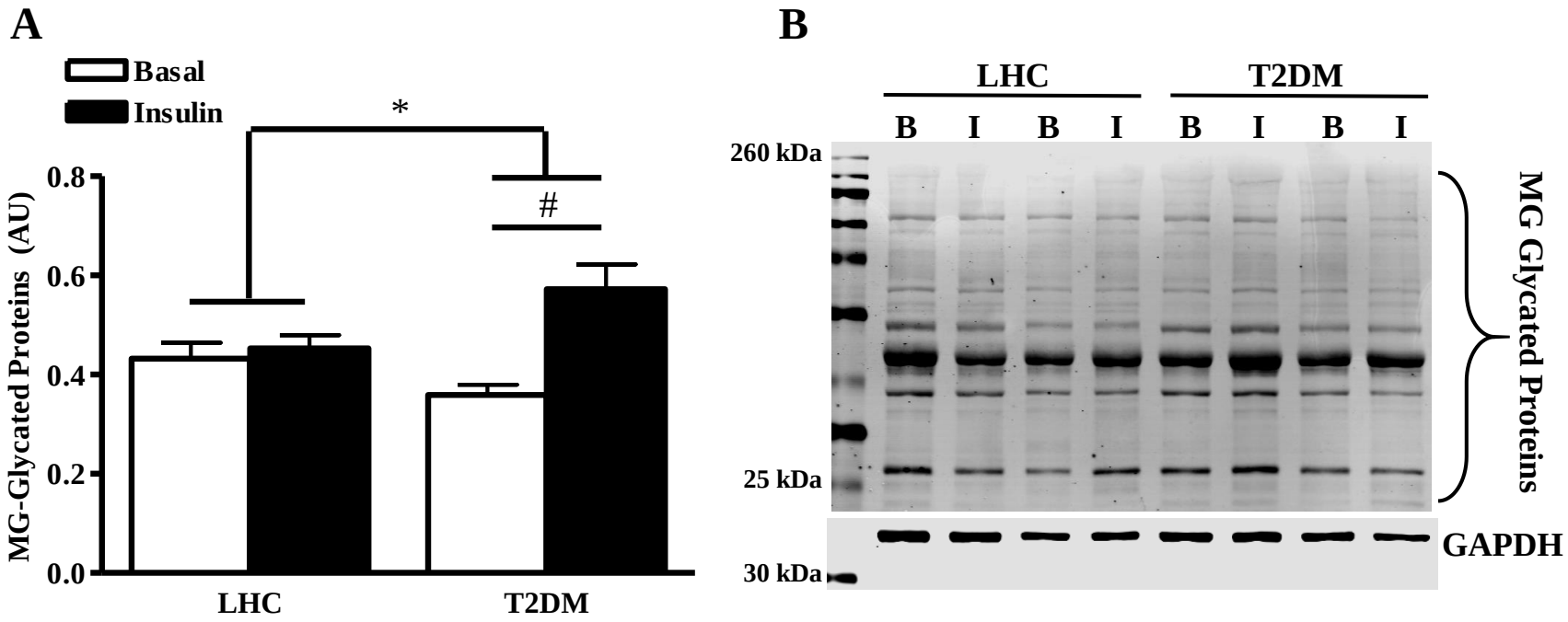

Figure 15. Skeletal Muscle MG-modified Proteins.
(A) Representative western blot image. (B) MG-modified proteins are increased in T2DM with insulin stimulation during a hyperinsulinemic-euglycemic clamp. LHC, n=10; T2DM, n=5; *, p<0.001. LHC, Lean Healthy Control subjects; T2DM, individuals with Type II Diabetes; B, Basal, t=0 min; I, Hyperinsulinemia, t=120 min.

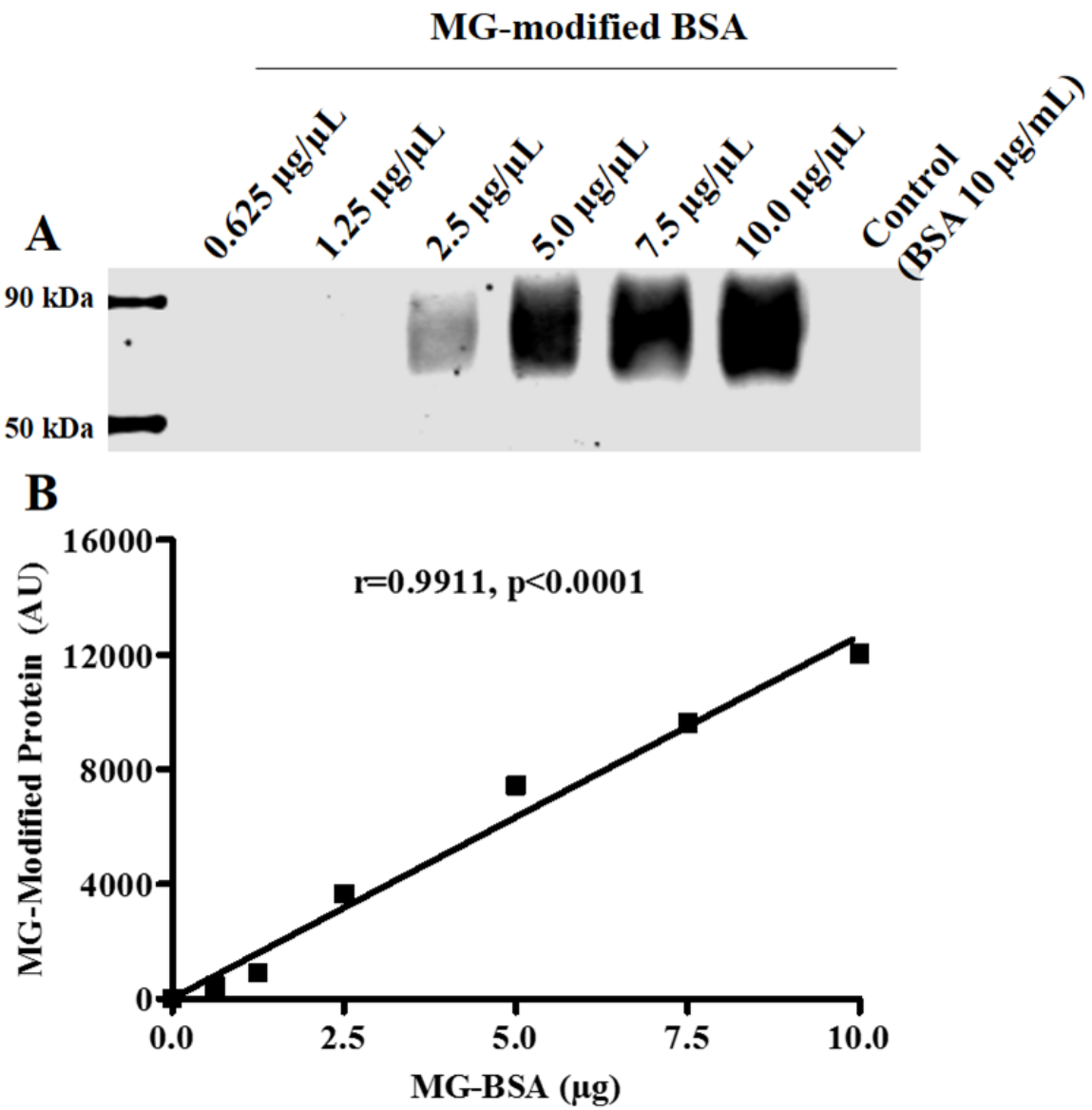

Figure 16. Validation of Quantification Technique for MG-modified Proteins.
(A) Representative western blot image. (B) MG-modified proteins quantified by our methods utilizing an Anti-MG monoclonal antibody and LI-COR immunofluorescent secondary antibody (fully described in Methods) are increased in a strong linear relationship with the loaded quantities of the MG-modified BSA standard.

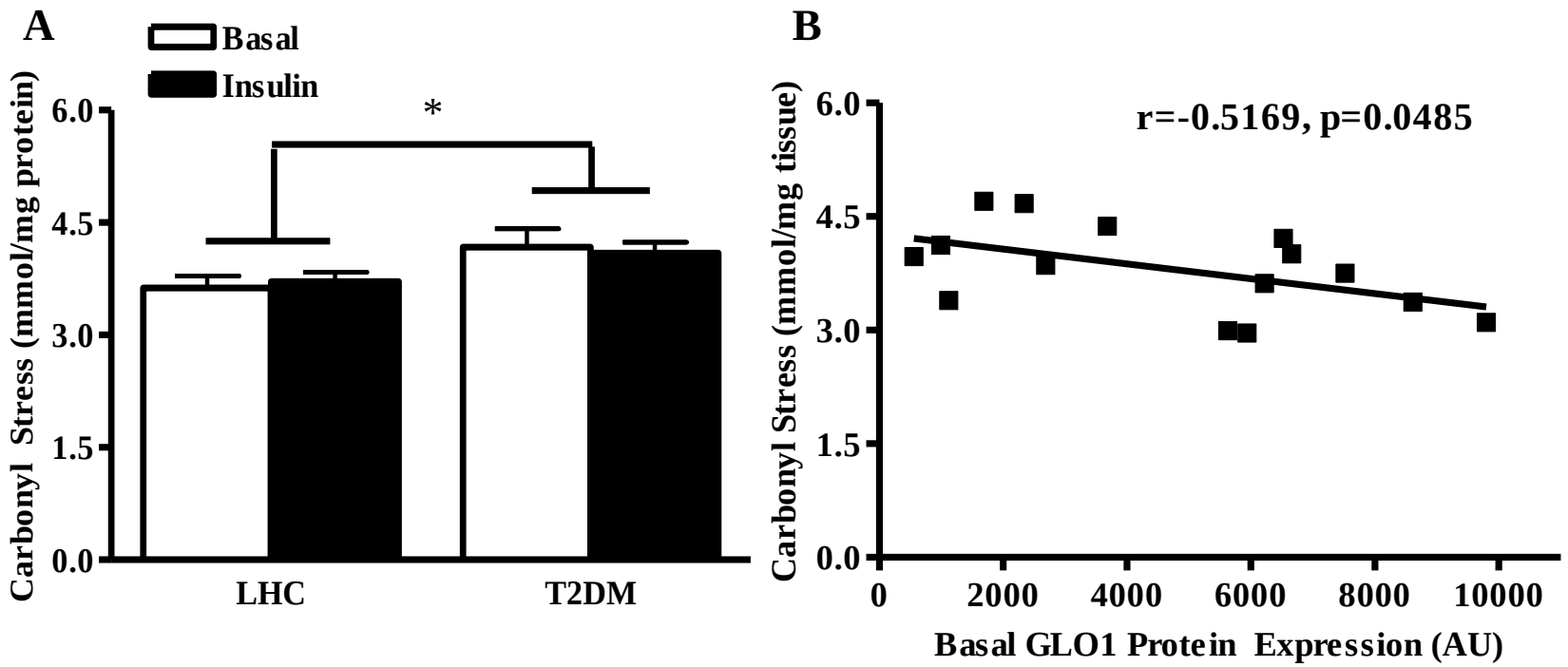

Figure 17. Skeletal Muscle Carbonyl Stress.
(A) Carbonyl stress was elevated in the skeletal muscle of T2DM; Group effect, *p*=0.0257 (B) An inverse correlation was observed between basal carbonyl stress and basal GLO1 protein expression (r=-0.5169, p=0.0485).

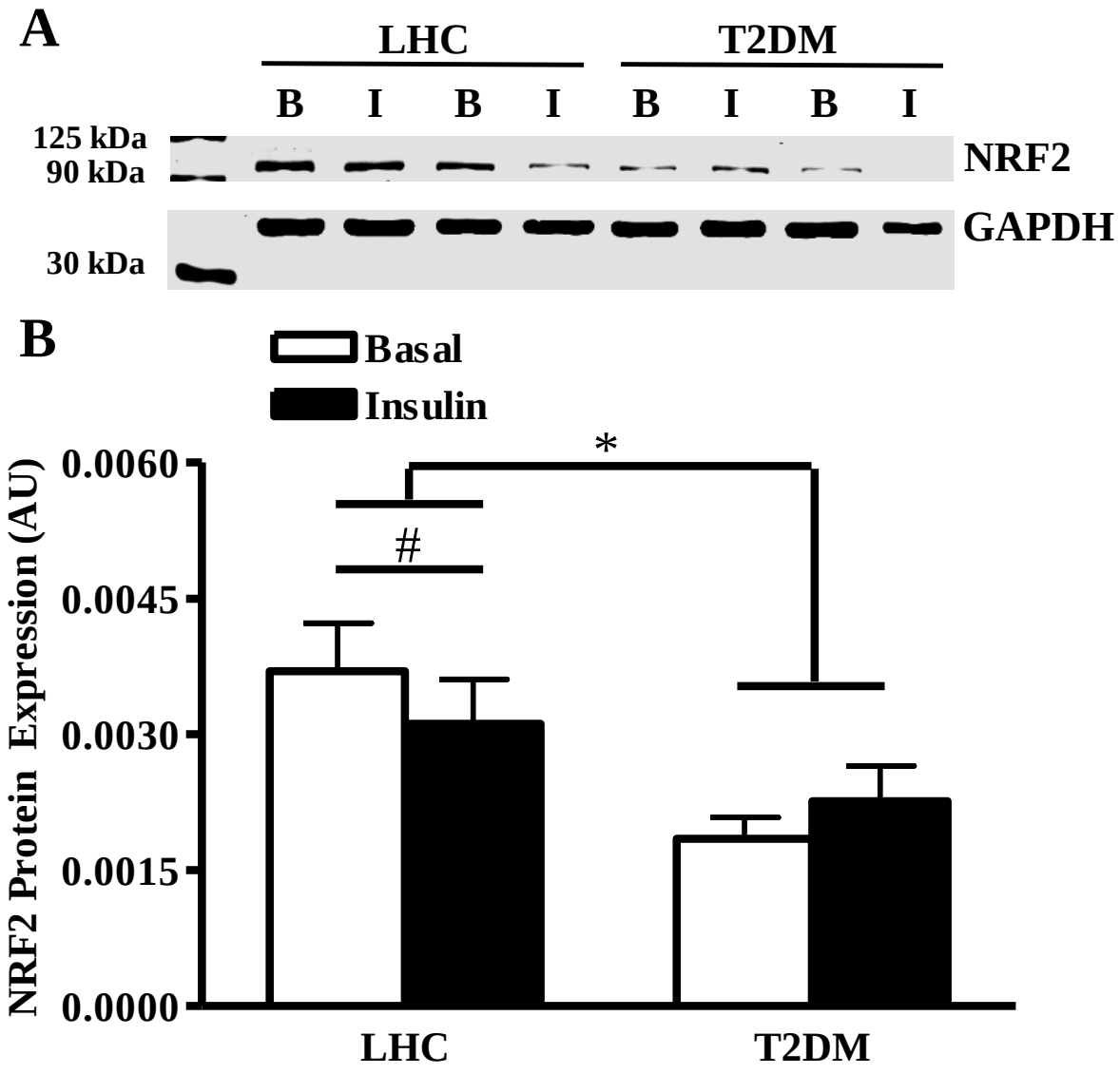

Figure 18. Skeletal Muscle NRF2 Protein Expression.
(A) Representative western blot image. (B) There was a significant interaction effect of Condition and Group on NRF2 protein expression where LHC decreased, but T2DM increased with hyperinsulinemia. LHC, n=10; T2DM, n=5; *, p=0.0086. Additionally, Bonferroni's post hoc comparison revealed a significant effect of insulin in LHC. #, p<0.05 LHC, Lean Healthy Control subjects; T2DM, individuals with Type II Diabetes; B, Basal, t=0 min; I, Hyperinsulinemia, t=120 min.

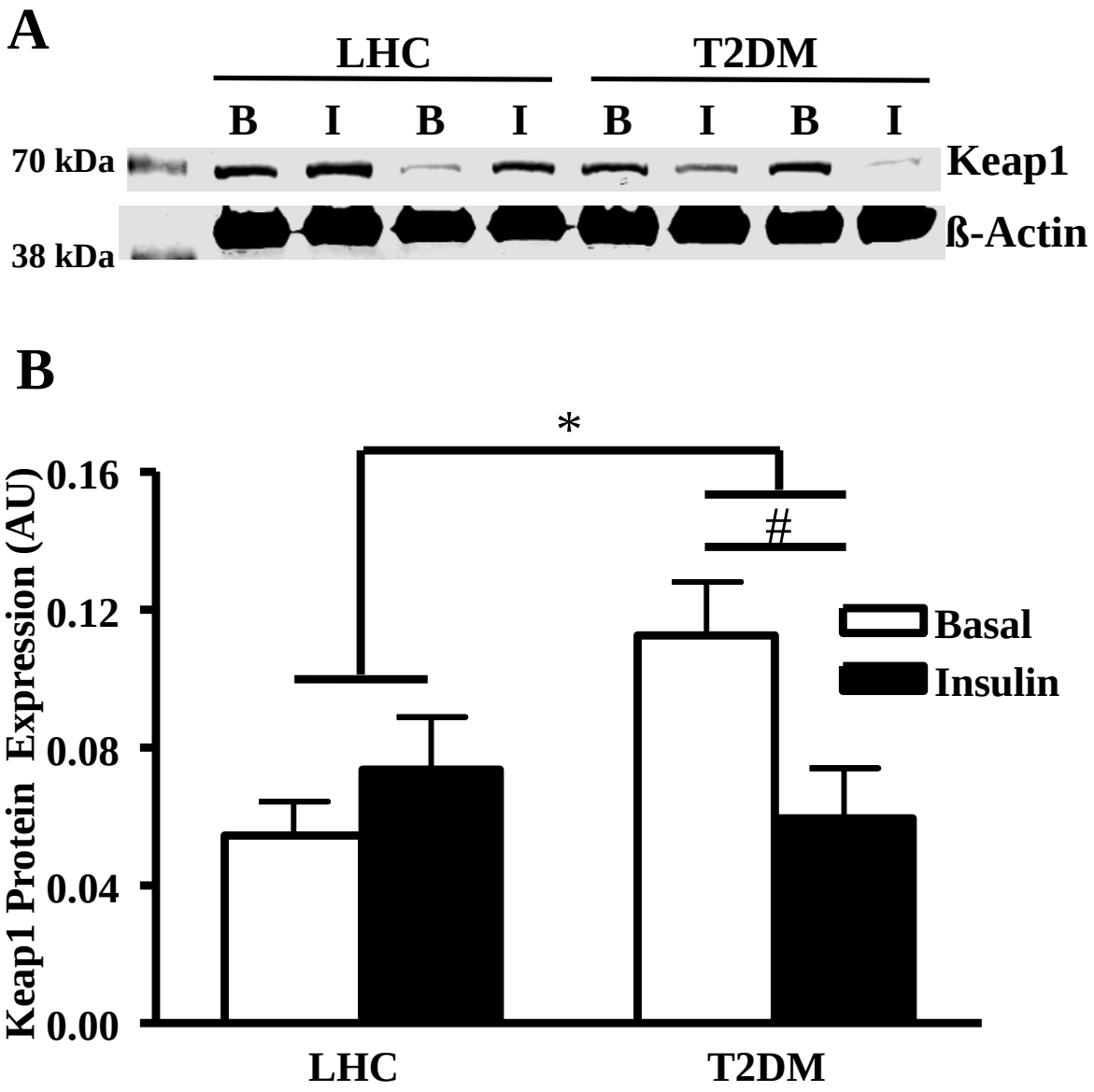

Figure 19. Skeletal Muscle Keap1 Protein Expression.
(A) Representative western blot image. (B) There was a significant interaction effect of Condition and Group on Keap1 protein expression where LHC increased, but T2DM decreased with hyperinsulinemia. LHC, n=10; T2DM, n=5; *, p=0.0068. Additionally, Bonferroni post hoc comparison revealed a significant effect of insulin in T2DM. #, p<0.05 LHC, Lean Healthy Control subjects; T2DM, individuals with Type II Diabetes; B, Basal, t=0 min; I, Hyperinsulinemia, t=120 min.

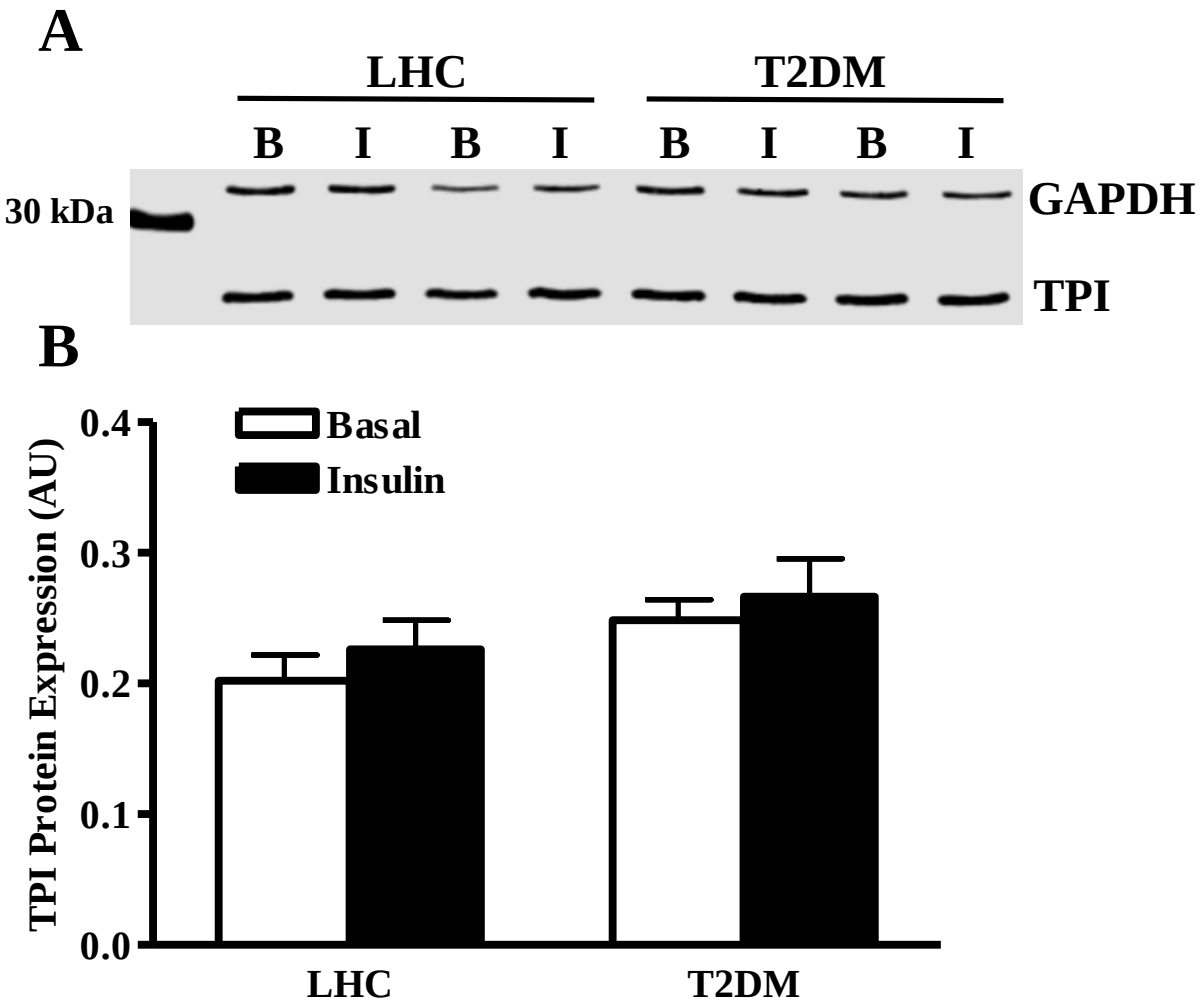

Figure 20. Skeletal Muscle TPI Protein Expression.
(A) Representative western blot images. (B) There was no effect of Condition or Group on TPI protein expression. However, a trend for increased TPI was observed with insulin stimulation (Condition), p= 0.0638. LHC, n=10; T2DM, n=5 TPI, Triose Phosphate Isomerase; LHC, Lean Healthy Control subjects; T2DM, individuals with Type II Diabetes; B, Basal, t=0 min; I, Hyperinsulinemia, t=120 min.

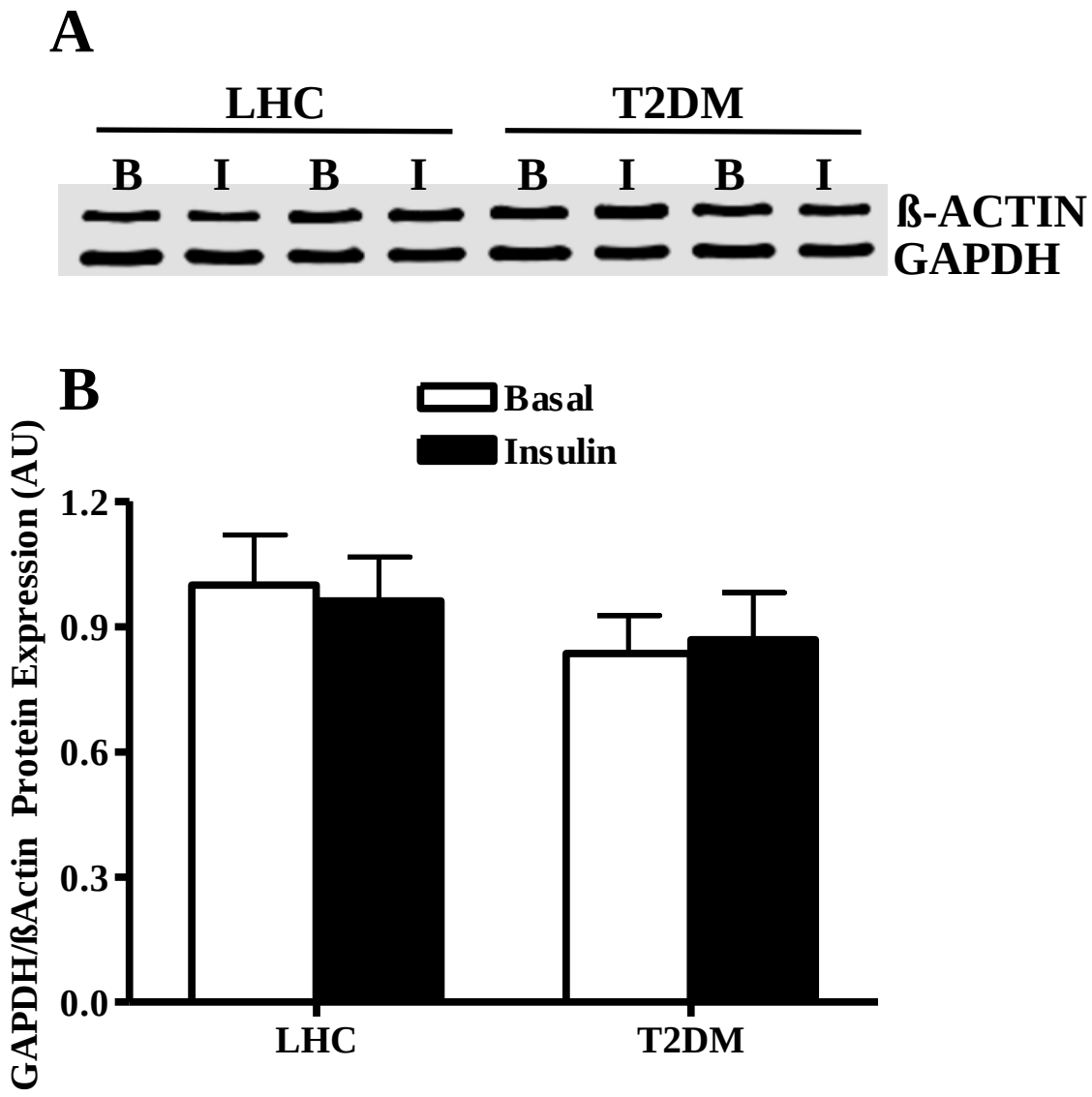

Figure 21. Skeletal Muscle GAPDH & ß-Actin Protein Expression.
(A) Representative western blot image. (B) No effect of either group or condition was observed. P>>0.05. Lean Healthy Control subjects; T2DM, individuals with Type II Diabetes; B, Basal, t=0 min; I, Hyperinsulinemia, t=120 min.

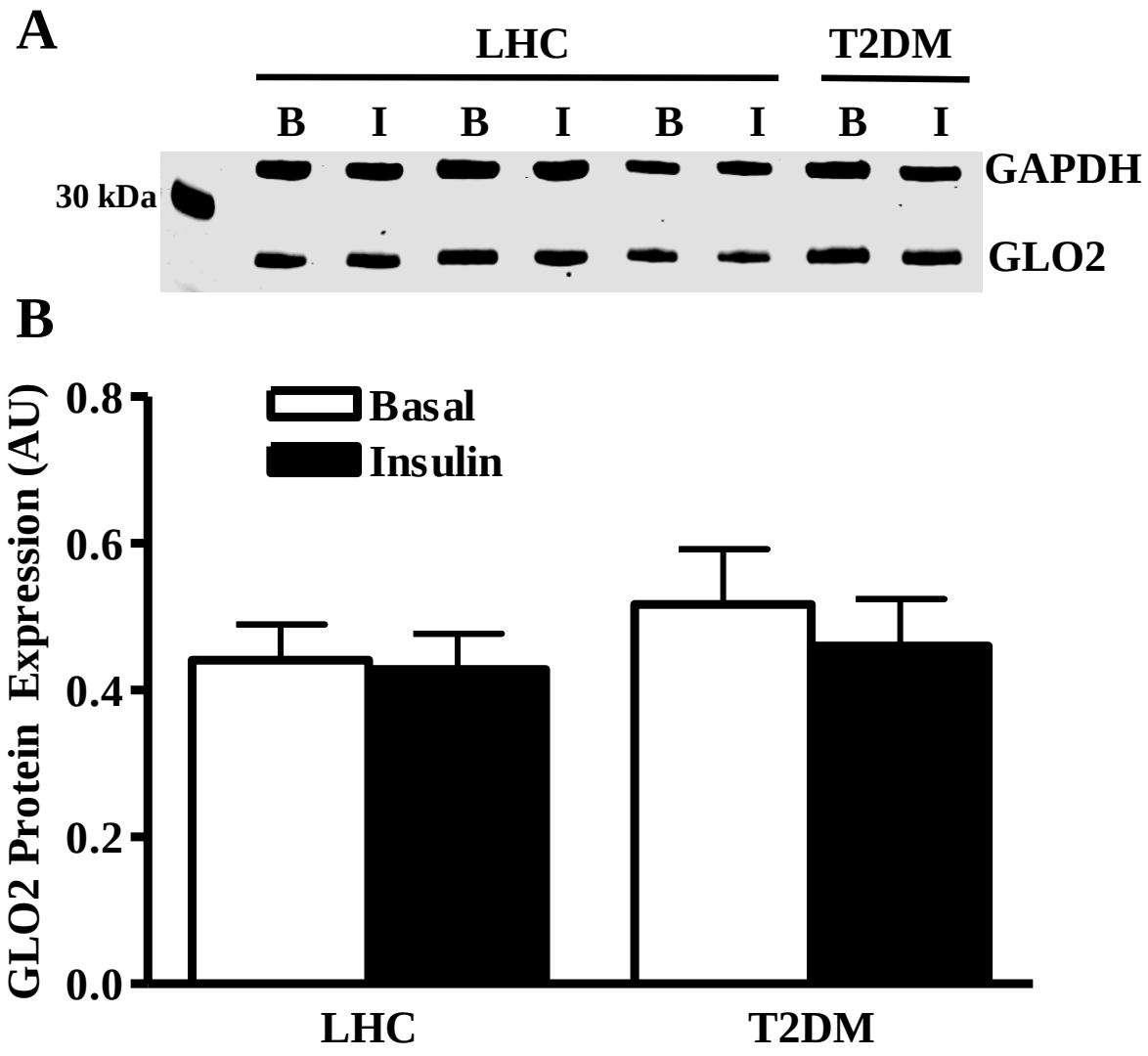

Figure 22. Skeletal Muscle GLO2 Protein Expression.
(A) Representative western blot image. (B) GLO2 protein expression was not different between groups or conditions; $p>0.05$. GAPDH was used as a loading control. LHC, Lean Healthy Control subjects; T2DM, individuals with Type II Diabetes; B, Basal, t=0 min; I, Hyperinsulinemia, t=120 min.

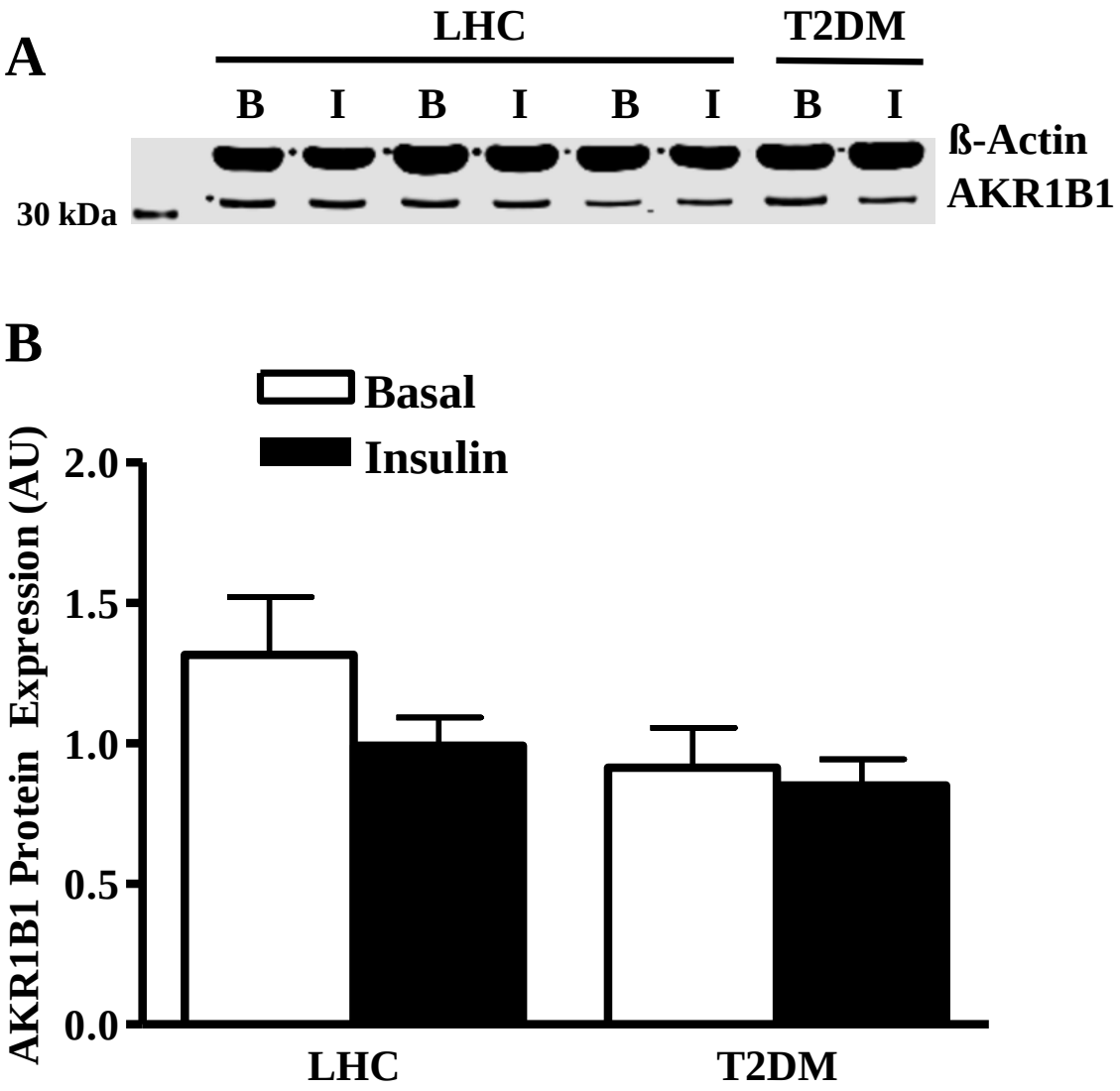

Figure 23. Skeletal Muscle AKR1B1 Protein Expression.
(A) Representative western blot image. (B) AKR1B1 protein expression was not different between groups or conditions; $p>0.05$. ß-Actin was used as a loading control. LHC, Lean Healthy Control subjects; T2DM, individuals with Type II Diabetes; B, Basal, t=0 min; I, Hyperinsulinemia, t=120 min.

Results Pertaining to Specific Aim 2:

Subjects Characteristics

Baseline Characteristics

A large cohort of individuals involved in previous investigations have been presented previously. However, to date, no data has been presented on GLO1. The baseline characteristics of this unique study population are available in **Table 3**. A young, lean healthy control (LHC) group was used as a measure of optimal health. In comparison, OB-IR subjects were older (+34 yrs, weighed more and maintained a higher BMI and percentage of body fat than LHC (all $p<0.0001$). Additionally, OB-IR subjects were insulin resistant (GDR) and less aerobically (VO_2max) fit compared to LHC (both $p<0.0001$).

Effects of the Lifestyle Intervention

After a 12-week lifestyle intervention consisting of an isocaloric diet and supervised aerobic exercise (60 min/day, 5d/week, 12-week at 65% VO2max), OB-IR participants improved markers of physical, metabolic and cardiovascular health **(Table 3)**. Beneficial changes included robust reductions in weight (-9.9 ± 1.3 kg), BMI (-3.3 ± 0.4 kg/m^2) and percent body fat (4.8 ± 0.8 %) with increases in GDR (1.9 ± 0.2 mg/kg/min), non-oxidative glucose disposal (NOGD) and VO_2max (6.3 ± 0.9 ml/kg/min) (all $p<0.0001$).

Glyoxalase System Outcome Measures

GLO1 Protein and Gene Expression

GLO1 protein expression was reduced (Δ = -0.202 ± 0.112 AU; $p=0.0456$) in OB-IR compared to LHC **(Figure 24)**. GLO1 protein expression was rescued in OB-IR after aerobic exercise training ($p<0.05$) to levels comparable to LHC (**Figure 24**, $p>0.05$). Although sample size was limited due to tissue availability, GLO1 mRNA measured in a subset of OB-IR improved after the lifestyle intervention (n=5, p=0.003) **(Figure 25)**. These results agree with the directional change in GLO1 protein and suggest GLO1 protein expression is mediated by GLO1 gene expression, similar to the results of others.

Due to tissue sample availability, GLO1 activity was measured in a subset of samples (n=10) only at baseline to obtain a comparison between LHC and OB-IR. Despite reduced sample size, GLO1 activity was significantly lower (Δ= -399 ± 40 U/L; p=0.0006) in OB-IR compared to LHC **(Figure 26)**. Correlation analysis was performed on variables statistically different between groups at baseline. GLO1 protein expression modestly correlated with BMR (n=18, p=0.0434) displaying a potential relationship between GLO1 and nutrient flux (*data not presented*). GLO1 activity showed a strong negative correlation with chronologic age (n=10, p=0.0044) **(Figure 27A)** and body fat percent (n=9, p=0.0107) **(Figure 27B)**. In contrast, GLO1 activity showed a strong positive correlation with clamp-derived GDR (n=9, p=0.0018) **(Figure 27C)** and VO_2max (n=8, p=0.0118) **(Figure 27D)**.

MG-modified proteins

Since increased GLO1 has been shown to alleviate dicarbonyl stress *in vitro*, we wanted to investigate MG-directed protein modifications in available muscle tissue. In a subset of LHC (n=4), OB-IR (n=5) and OB-IR-Post (n=5), total MG-modified proteins were not different between groups (p>0.05) **(Figure 28)**. Given our limited sample size, we performed a post-hoc power calculation (continuous endpoint, two independent samples) with α=0.05 that revealed our data only had a 9.4% power. To inform us about potential future studies we performed a sample size calculation (continuous endpoint, two independent samples) where α=0.05 and ß=0.2 which revealed 144 subjects (72 per group) would be sufficient to detect a significant difference if our sample means remained the same.

Pre-training hyperglycemia correlation

Given that pre-training hyperglycemia has been shown to blunt skeletal muscle improvements to exercise training (277), we performed correlational analysis in OB-IR subjects who improved GLO1 protein expression by 10% or greater (GLO1 responders; highest 2 tertiles) to investigate the effect of pre-training hyperglycemia on GLO1 protein responses to aerobic exercise training. Pre-training hyperglycemia

(displayed as iAUC OGTT) predicted improvements in GLO1 protein expression in response to 12 weeks of aerobic exercise training in OB-IR subjects (**Figure 29;** n=14, $p<0.05$).

TABLE III. SUBJECT CHARACTERISTICS & EFFECTS OF LIFESTYLE INTERVENTION

	LHC	OB-IR	OB-IR Post
n (M, F)	6 (2,4)	20 (10,10)	-
Age, years	31 ± 3	65 ± 1*	-
Weight, kg	62 ± 5	99 ± 3*	89.1 ± 3†
BMI, kg/m^2	22.8 ± 1.1	34.2 ± 0.7*	30.9 ± 0.7†
Bodyfat (%)	27.7 ± 2.8	43.6 ± 1.5*	39.8 ± 2.2†
GDR, mg/kg/min	5.7 ± 0.8	2.7 ± 0.2*	4.6 ± 0.3†
VO_2max, ml/kg/min	44.7 ± 0.3	22.0 ± 0.8*	28.3 ± 1.5†
COX	-	1.2 ± 0.1	1.1 ± 0.1
NOGD	-	1.2 ± 0.3	2.7 ± 0.3†
BMR	-	0.86 ± 0.01	0.83 ± 0.01

Data represent mean ± SEM.

LHC, Lean Healthy Control subjects; OB-IR, Older, Obese Insulin Resistant subjects; OB-IR Post; Older, Obese Insulin Resistant subjects after a 12wk intense aerobic exercise intervention; BMI, Body Mass Index; GDR, Glucose Disposal Rate as calculated from the hyperinsulinemic-euglycemic clamp; VO_2max, maximal oxygen consumption during maximal aerobic exercise; COX, Carbohydrate Oxidation; NOGD, Non-Oxidative Glucose Disposal; BMR, Basal Metabolic Rate. *, significant difference from LHC; †, significant effect of 12wk lifestyle intervention in OB-IR.

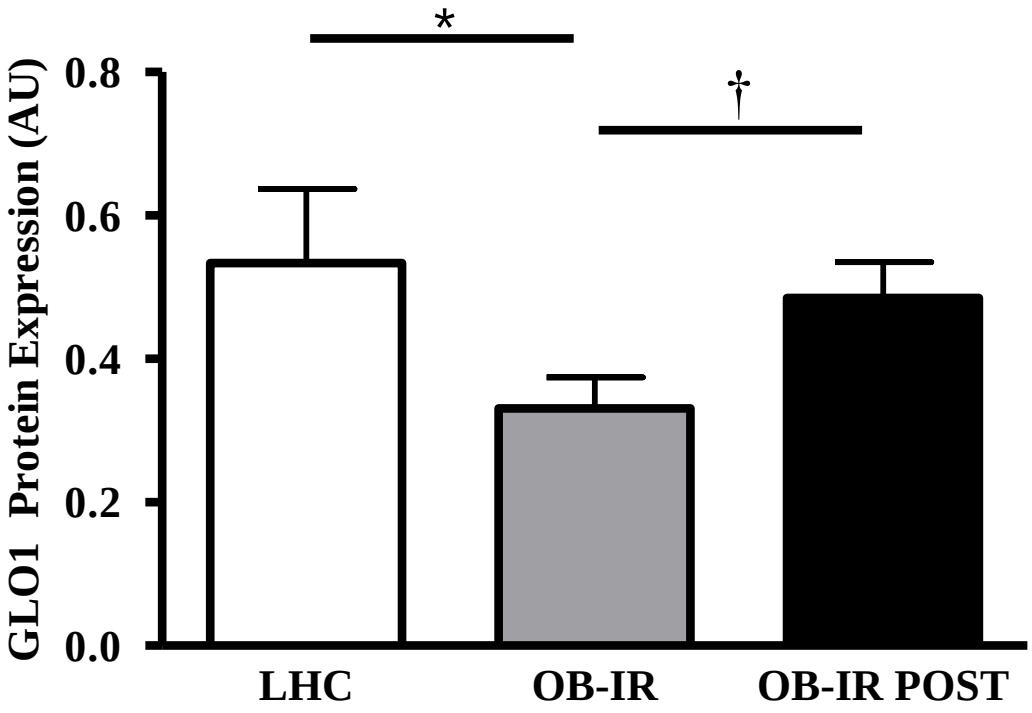

Figure 24. Skeletal muscle GLO1 protein expression
GLO1 protein expression was lower in OB-IR compared to LHC at baseline (*, $p<0.05$), but increased after aerobic exercise training (†, significant effect of 12wk lifestyle intervention in OB-IR) (1way ANOVA, $p=0.0236$). LHC, Lean Healthy Control subjects; OB-IR, Obese, Insulin Resistant subjects; OB-IR POST, OB-IR subjects after 12 weeks of aerobic exercise training.

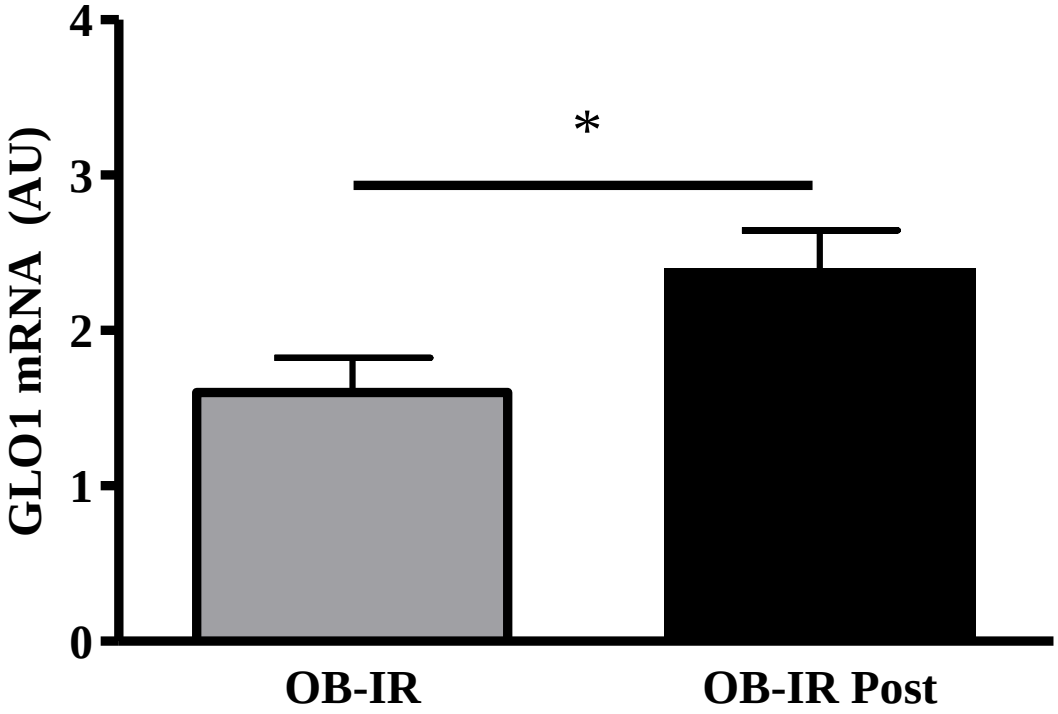

Figure 25. GLO1 gene expression.
Due to sample availability, 5 OB-IR subjects were used to assess GLO1 gene expression before and after 12 weeks of aerobic exercise training. Skeletal muscle GLO1 gene expression increased after training in agreement with increases in GLO1 protein expression. *, p=0.003

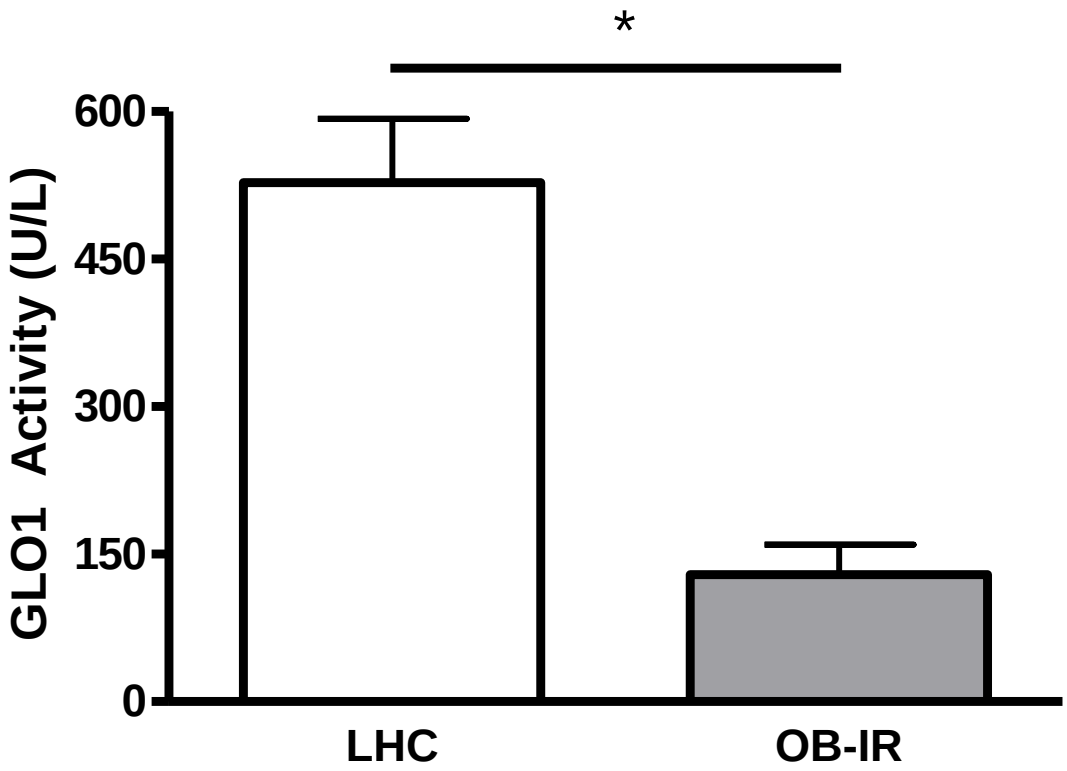

Figure 26. Baseline GLO1 enzymatic activity.
Due to limited sample availability, skeletal muscle homogenates from 5 LHC and 5 OB-IR subjects were assessed for GLO1 enzymatic activity using a commercially available GLO1 activity assay kit. OB-IR display greatly reduced GLO1 activity in skeletal muscle compared to LHC.; n=10; *, $p<0.001$. These results are in agreement with reductions in GLO1 protein expression. GLO1, glyoxalase-1; OB-IR, older, obese, insulin resistant adults; LHC, young, lean healthy controls.

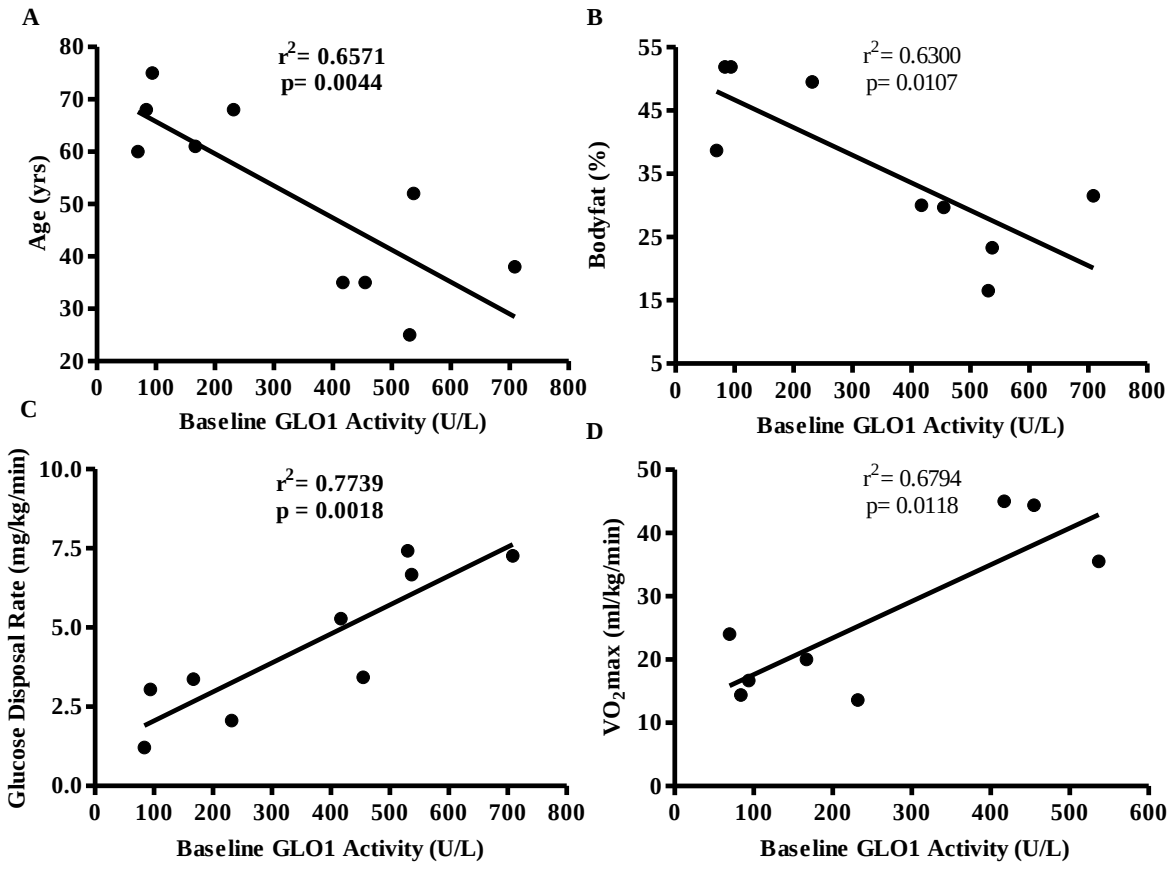

Figure 27. GLO1 Enzymatic Activity Correlations.
GLO1 activity was negatively correlated with both chronologic age (A) and percentage body fat (B). GLO1 activity was positively correlated with both clamp-derived GDR (C) and VO_2max (D).

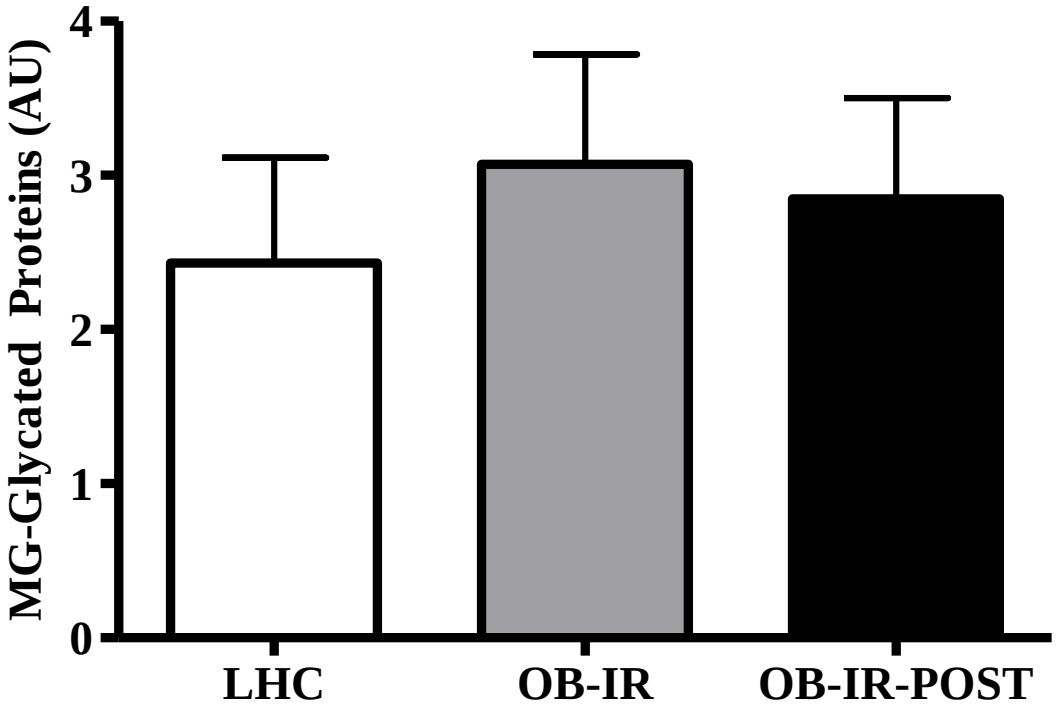

Figure 28. MG-modified Proteins.
Due to limited sample availability, MG-modified protein were assessed in a small cohort of LHC (n=4) and OBIR (n=5) subjects. MG-modified protein expression did not change between groups or after the aerobic exercise intervention, $p>0.05$.

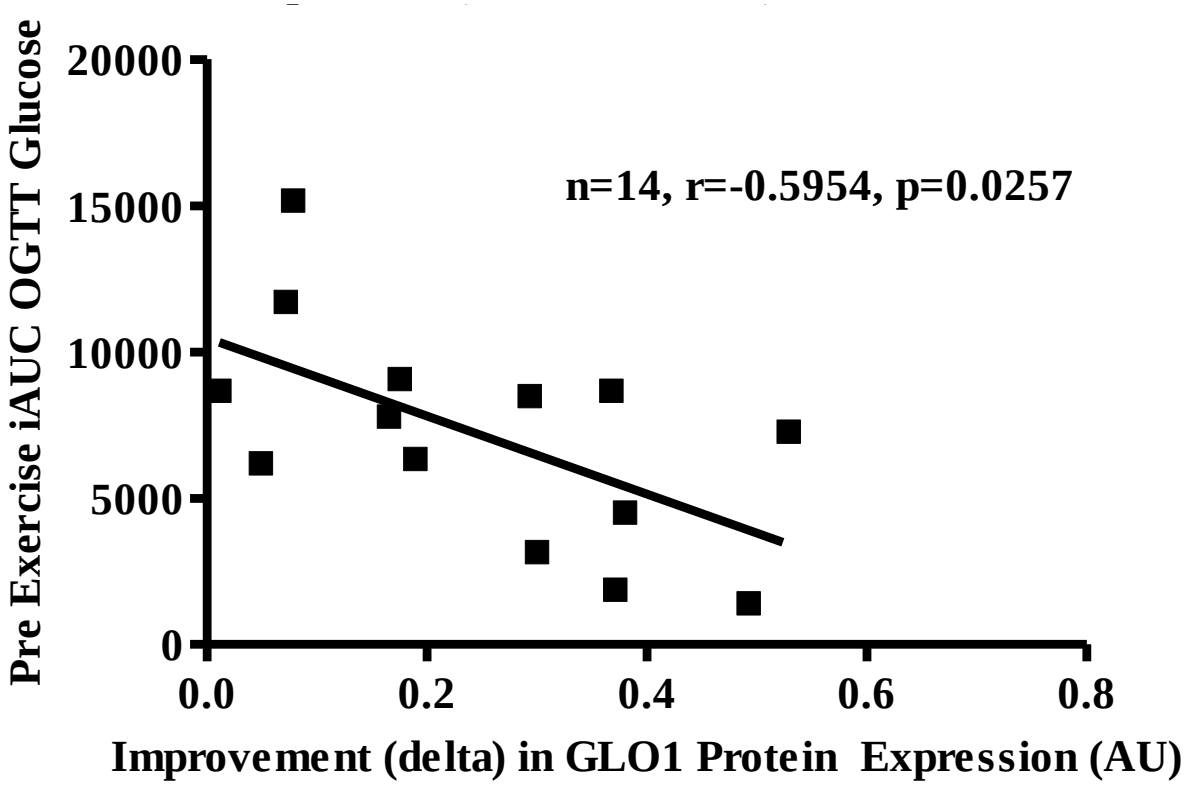

Figure 29. Pre-training Hyperglycemia Reduces GLO1 Protein Responses to Aerobic Exercise. Pre-training hyperglycemia (displayed as iAUC OGTT) predicted improvements in GLO1 protein expression in response to 12 weeks of aerobic exercise training in OB-IR subjects.

Results Pertaining to Specific Aim 3:

Effects of the Diabetic Milieu on the MG-GLO1 Axis

To investigate the potential effect of the diabetic milieu on the MG-GLO1 axis, we supplemented HSKMCs with hyperinsulinemia and hyperglycemia. The hyperinsulinemia model utilized a 300pM incubation over the course of 3 hours. We selected a 300pM dose because it mimics the time course of postprandial insulin concentrations *in vivo* and is similar to the concentrations during the hyperinsulinemic-euglycemic clamp procedure (described in "Methods Specific to Aims 1 and 2"). Hyperinsulinemia (300pM) increased GLO1 protein expression at 30 minutes, but returned toward control levels by 180 minutes ($p>0.05$) **(Figure 30)**, whereas MG-modified proteins were unchanged.

The hyperglycemia experiment utilized a healthy fasting model (5 mM glucose, *ca.* 90mg/dl, comparable to postabsorptive glucose concentrations in healthy individuals), a postprandial glycemia model (15mM glucose, *ca.* 270 mg/dl, comparable to postprandial glucose concentrations in healthy individuals), and an uncontrolled T2DM model (30mM glucose, *ca.* 540 mg/dl, comparable to postprandial glucose concentrations in severely uncontrolled T2DM. Incubations were maintained for 24 hours. However, both MG-modified proteins **(Figure 31)** and GLO1 protein expression (*data not presented*) remained unchanged at any dose. To exert a more robust glucose insult similar to the methods of others (278), we extended our T2DM model (30mM glucose) for 5 days. Hyperglycemia (30mM) had no effect on MG-modified proteins after 1 day, but caused a 50±18% ($p<0.035$) increase in MG-modified proteins after 5 days **(Figure 32)**. GLO1 was unchanged at both 1 and 5 days of hyperglycemia (*data not presented*).

Effect of Dicarbonyl Stress on AMPK

To first develop an experimental model of dicarbonyl stress, we performed a titration curve of MG-supplemented media (0-500μM for 4 hours; described in 'Methods Specific to Aim 3'). We developed our model on the basis that ~3% of MG supplemented into cell culture media appears on intracellular proteins (18) and that the intracellular concentration of MG is ~1-4 μM in healthy cells, but has been estimated to exceed 10μM under dicarbonyl stress. Our model revealed a stepwise increase in

intracellular MG-modified proteins, becoming significant at 500 µM (*data not presented*) and verified that GLO1 protein expression was unchanged at all concentrations (*data not presented*, p>0.05). We therefore utilized a range of MG concentrations in the media to best characterize the effects of multiple levels of dicarbonyl stress.

To investigate whether MG modifies AMPKγ, we first used our verified models of dicarbonyl stress (0, 5, 50, 100, or 500µM MG for 4 hours) to increase MG-modification of intracellular proteins. We then performed an immunoprecipitation of AMPKγ (IP:AMPKγ) followed by immunoblotting for MG-modifications (IB:MG). IP:AMPKγ followed by IB:MG revealed that experimental dicarbonyl stress did not increase MG-modifications of AMPKγ at any MG concentration (**Figure 33**).

To better characterize the effect of dicarbonyl stress on AMPK function, we quantified AMPK activity using the surrogate downstream phosphorylation target, ACCß. Interestingly, ACCβ phosphorylation increased in stepwise fashion with increasing MG concentrations, becoming significant at 10µM (141±4%, p=0.0066), 50 µM (202±9%, p=0.0007) and peaking at 100µM (342±27%, p=0.0010) (**Figure 34**). Interestingly, pre-incubating HSKMCs with low dose MG (10µM) was additive to AICAR stimulated AMPK activity as the combination increased pACCß expression by 24±4% compared to AICAR alone (p=0.011) (**Figure 35)**. However, pre-incubating cells with MG at high doses indicative of physiologic dicarbonyl stress, showed no additional effect or an abolished pACC expression (**Figure 36;** 500µM, p=ns compared to Control).

Therapeutic Strategies Targeted at the MG-GLO1 Axis in Skeletal Muscle

We have shown that direct MG incubation of HSKMCs increases MG modified proteins, becoming significant at high doses (500 µM MG – see above). To investigate the therapeutic effect of metformin on skeletal muscle dicarbonyl stress, we performed a titration curve involving MG alone, MG + 5 mM metformin and MG + 10 mM metformin for 24 hours. Metformin attenuated MG-induced protein modifications in a dose dependent fashion **(Figure 37)**. We are also the first to report a significant increase in GLO1 protein in response to 24-hours metformin treatment (**Figure 38**, 5mM MetF).

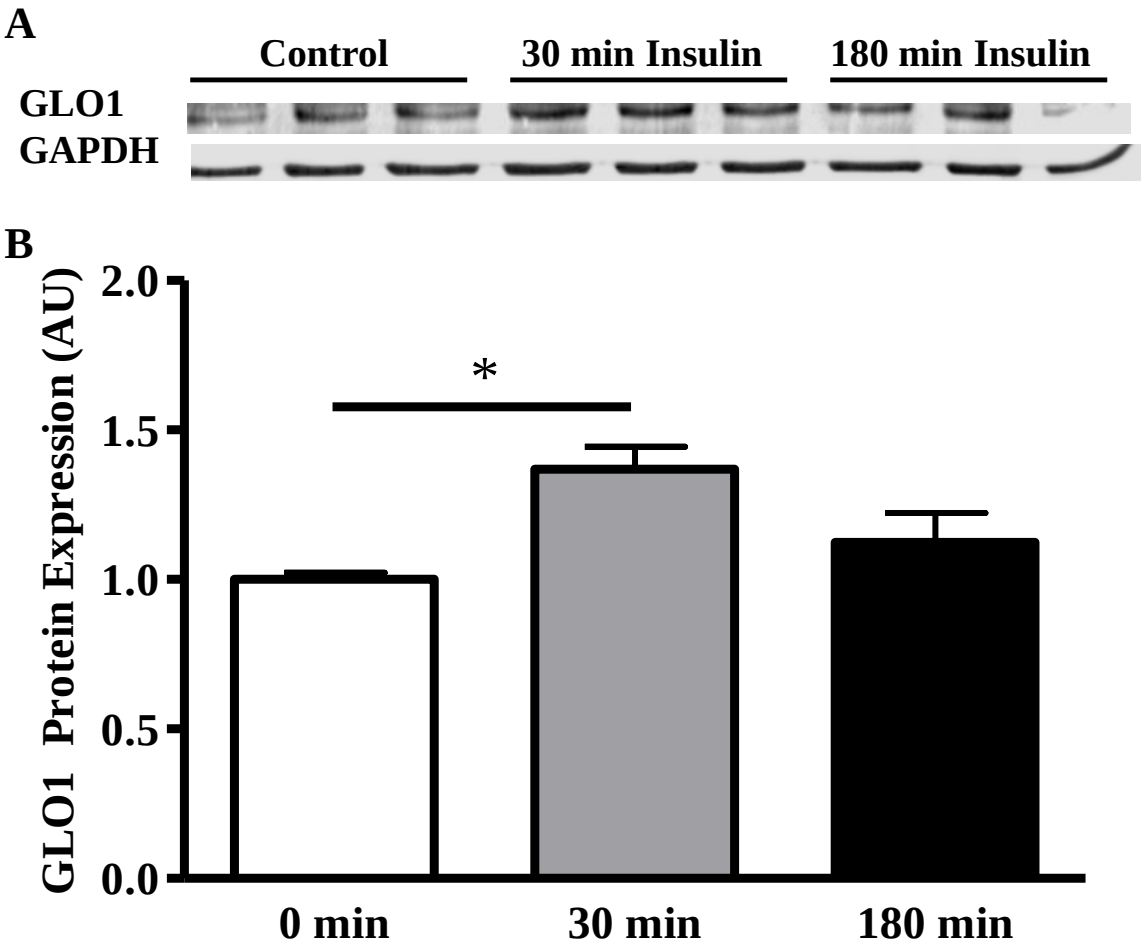

Figure 30. GLO1 protein expression with insulin treatment in human skeletal muscle cells.
(A) Representative western blot images. (B) Human skeletal muscle myotubes were incubated with 300pM insulin or H2O (control) for 0 (n=3), 30 (n=3) and 180 (n=3) minutes. GLO1 protein expression increased 34% above control; *, p= 0.01. Insulin, 300pM insulin treatment; GLO1, glyoxalase-1; GAPDH, loading control.

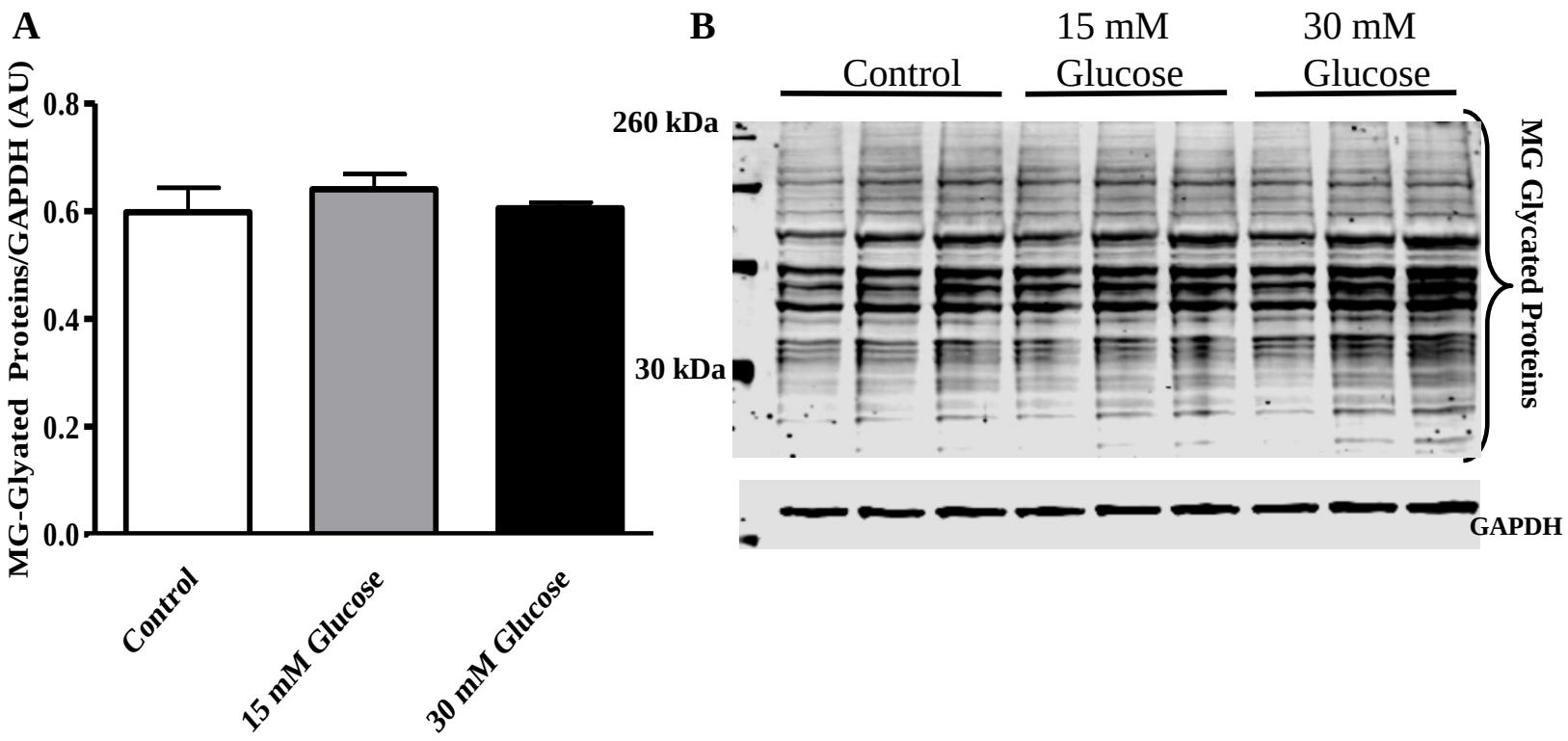

Figure 31. Twenty-four hours of hyperglycemia in human skeletal muscle cells has no effect on MG-modified proteins
(A) HSKMCs were exposed to 24 hours of hyperglycemia. Three glucose concentrations representing a euglycemic model (5mM), postprandial model (15mM), or an uncontrolled T2DM model (30mM) were used, but no change in MG-modified proteins were observed through western blot analysis; n=3 for each condition; $p>0.05$. (B) Representative western blot image. MG, methylglyoxal; GAPDH, loading control.

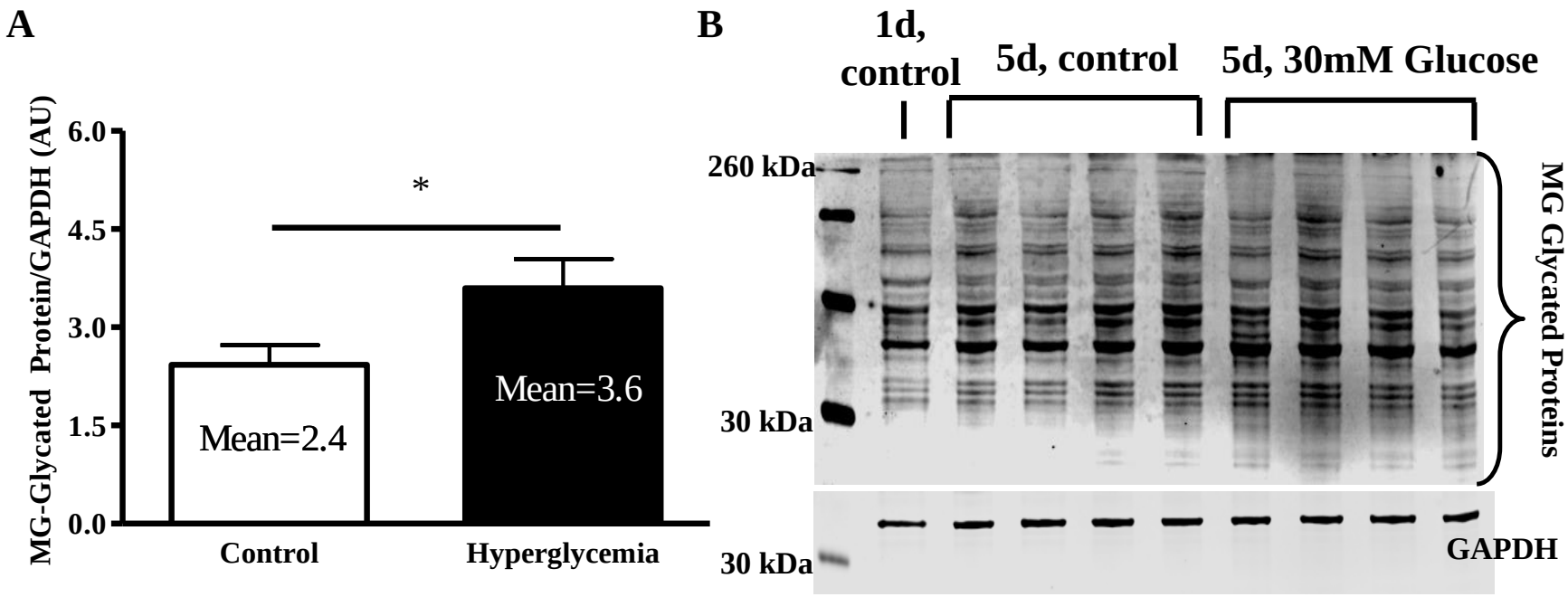

Figure 32. Five days of hyperglycemia in human skeletal muscle cells increases MG-modified proteins
(A) HSKMCs were exposed to basal media or a glucose supplemented model of hyperglycemia (30mM glucose). A time point control (1 day normal media) was plotted to determine if the incubation time had an independent effect on MG-modified proteins. MG-modified proteins, quantified via western blotting, were increased by 50% in the hyperglycemic model compared to control cells.; n=4 for each condition; *, p=0.0352. (B) Representative western blot image. MG, methylglyoxal; GAPDH, loading control.

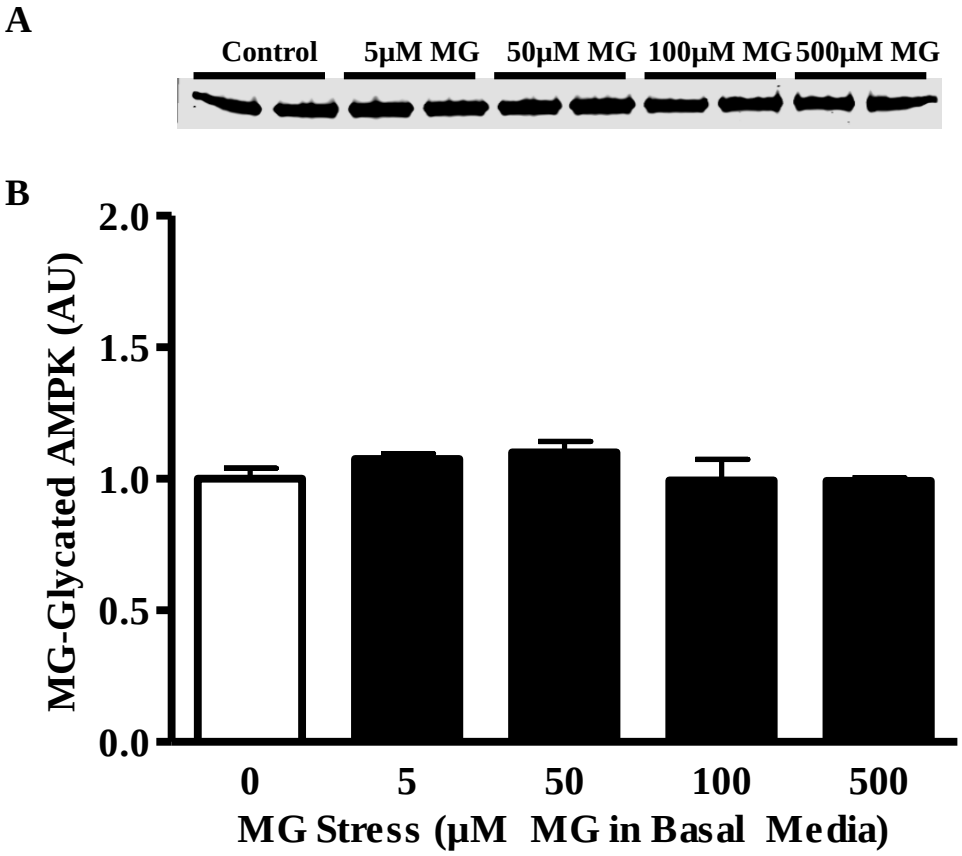

Figure 33. AMPK modification by MG remains unchanged with experimental dicarbonyl stress
(A) Representative western blot image. (B) Human skeletal muscle cells were incubated with 0 (control), 5, 50, 100, or 500 µM MG for 4 hours. AMPKγ was immunoprecipitated and western blotting was subsequently performed for MG-modified proteins. MG-modification of the AMPKγ subunit was unchanged across all conditions, $p>0.05$. MG, methylglyoxal.

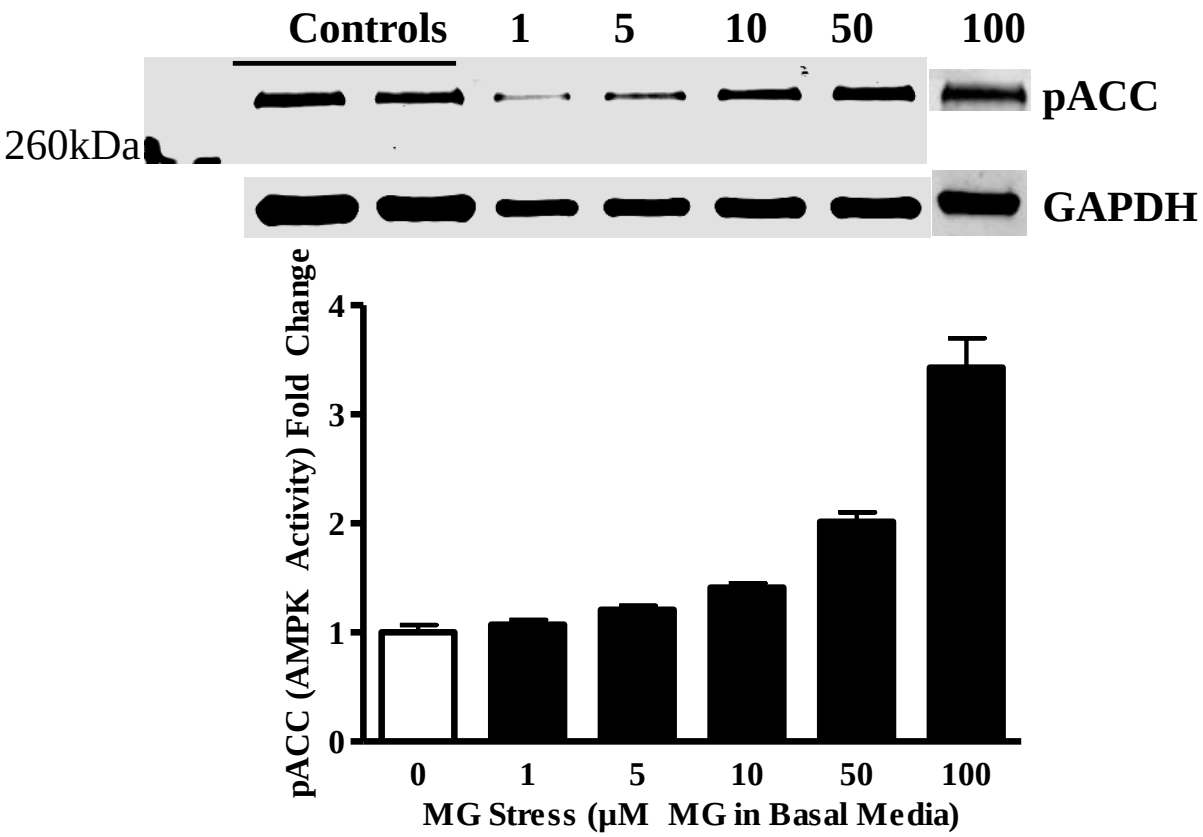

Figure 34. Dicarbonyl stress *per se* increases pACC expression in HSKMCs
(A) Representative western blot image. (B) HSKMCs were exposed to increasing concentrations of MG for 4 hours. MG showed independent effects on pACC (a marker of AMPK activity). Phosphorylation of ACC increased in near linear fashion with increasing MG media concentration. N=3 each; pACC, phosphorylated-acetyl-CoA carboxylase, surrogate marker of AMPK activity; HSKMCs, human skeletal muscle cells; MG, methylglyoxal.

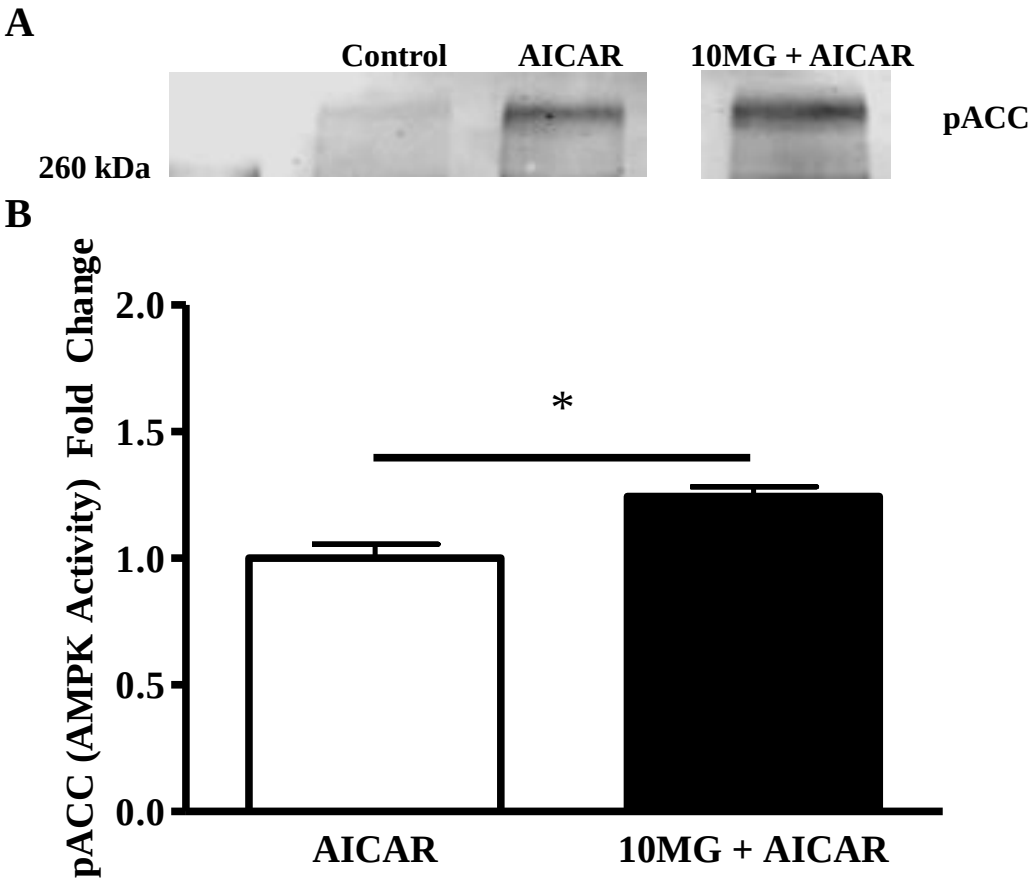

Figure 35. Low dose MG (10 μM) and AICAR have an additive effect on pACC
(A) Representative western blot image. (B) HSKMCs were stimulated with AICAR alone or AICAR with 10μM MG. The addition of MG to the cell culture medium had a synergistic effect with AICAR, significantly increasing the phosphorylation status of ACC (a surrogate of AMPK activity); n=3 each; *, $p<0.05$. AICAR, AMPK stimulus; MG, methylglyoxal; ACC, acetyl-CoA carboxylase.

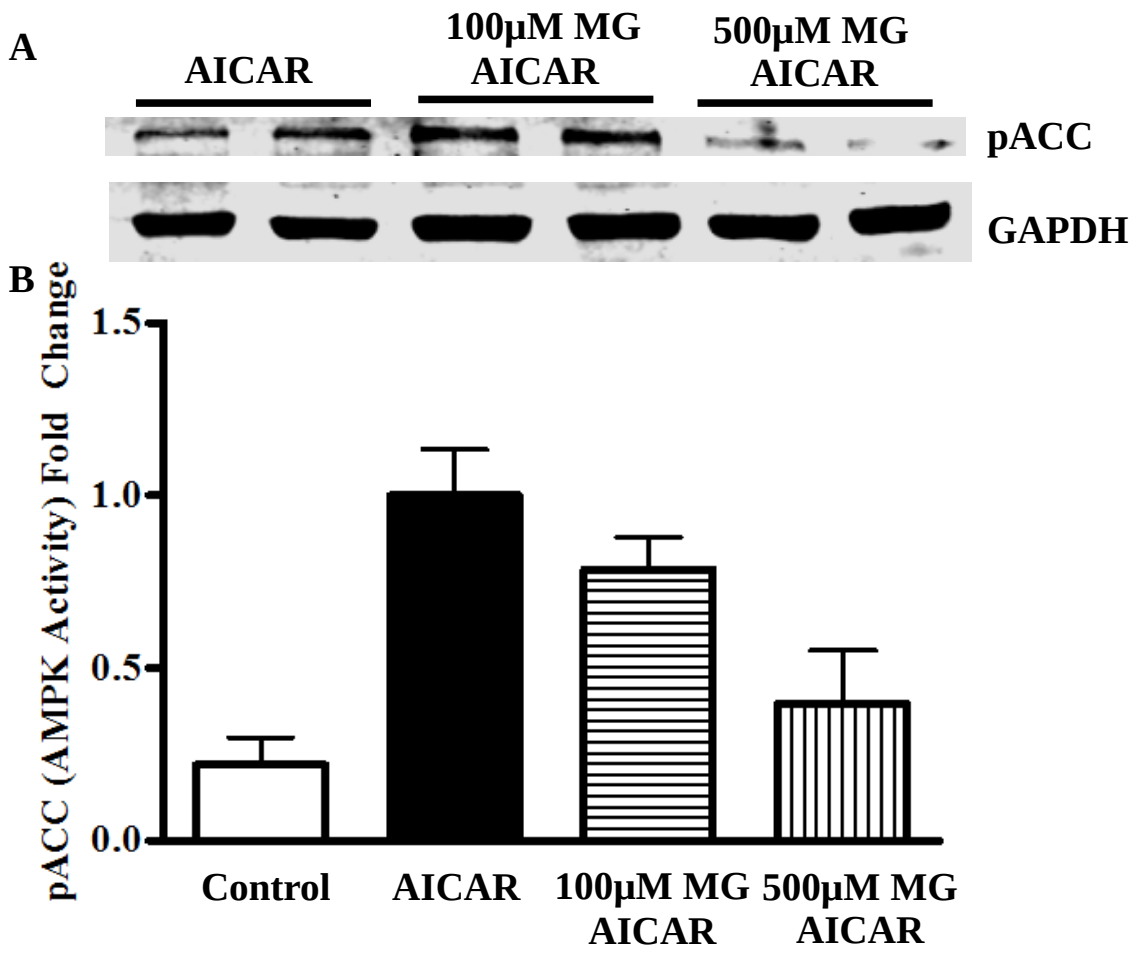

Figure 36. High dose MG inhibits AMPK activity in HSKMCs
(A) Representative western blot image. (B) HSKMCs were incubated with AICAR or a high dose of MG (100 or 500 µM; a condition of experimental dicarbonyl stress). 100 µM MG reduced the AMPK stimulatory effect of AICAR (determined by pACC status, a surrogate marker for AMPK activity). 500 µM MG nearly abolished the stimulatory effect of AICAR, N/S different from control. AICAR, AMPK stimulus; MG, methylglyoxal; ACC, acetyl-CoA carboxylase.

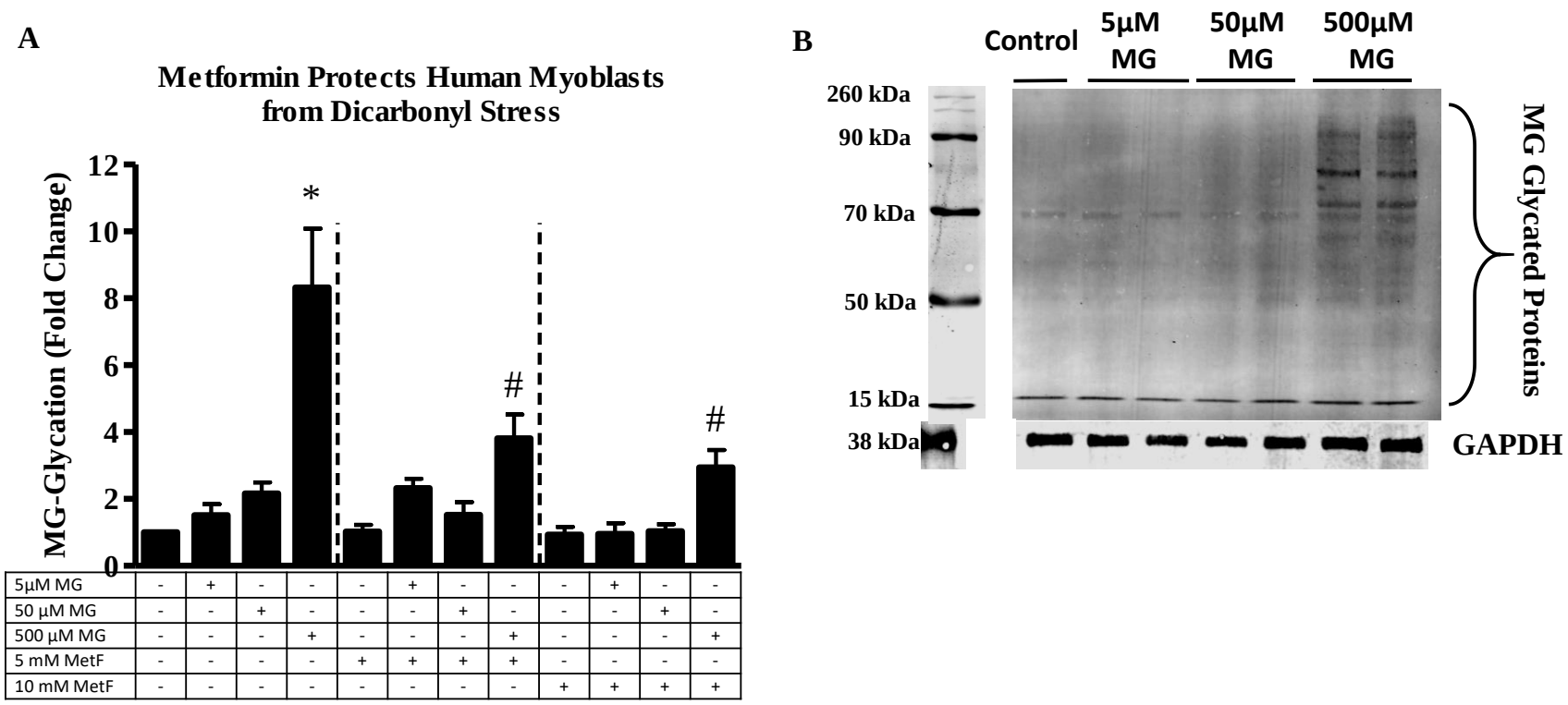

Figure 37. Metformin Protects Human Skeletal Muscle Cells from Dicarbonyl Stress.
(A) Human skeletal muscle myotubes were incubated with either MG, MetF, or MG + MetF for 24 hours. MG treatment alone expectedly increased MG glycated proteins compared to control *, $p<0.001$. Co-incubation with MetF significantly reduced MG-glycation of proteins compared to 500 μM MG alone #, $p<0.001$. (B) Representative western blot images. MG, methylglyoxal; MetF, metformin; GAPDH, loading control.

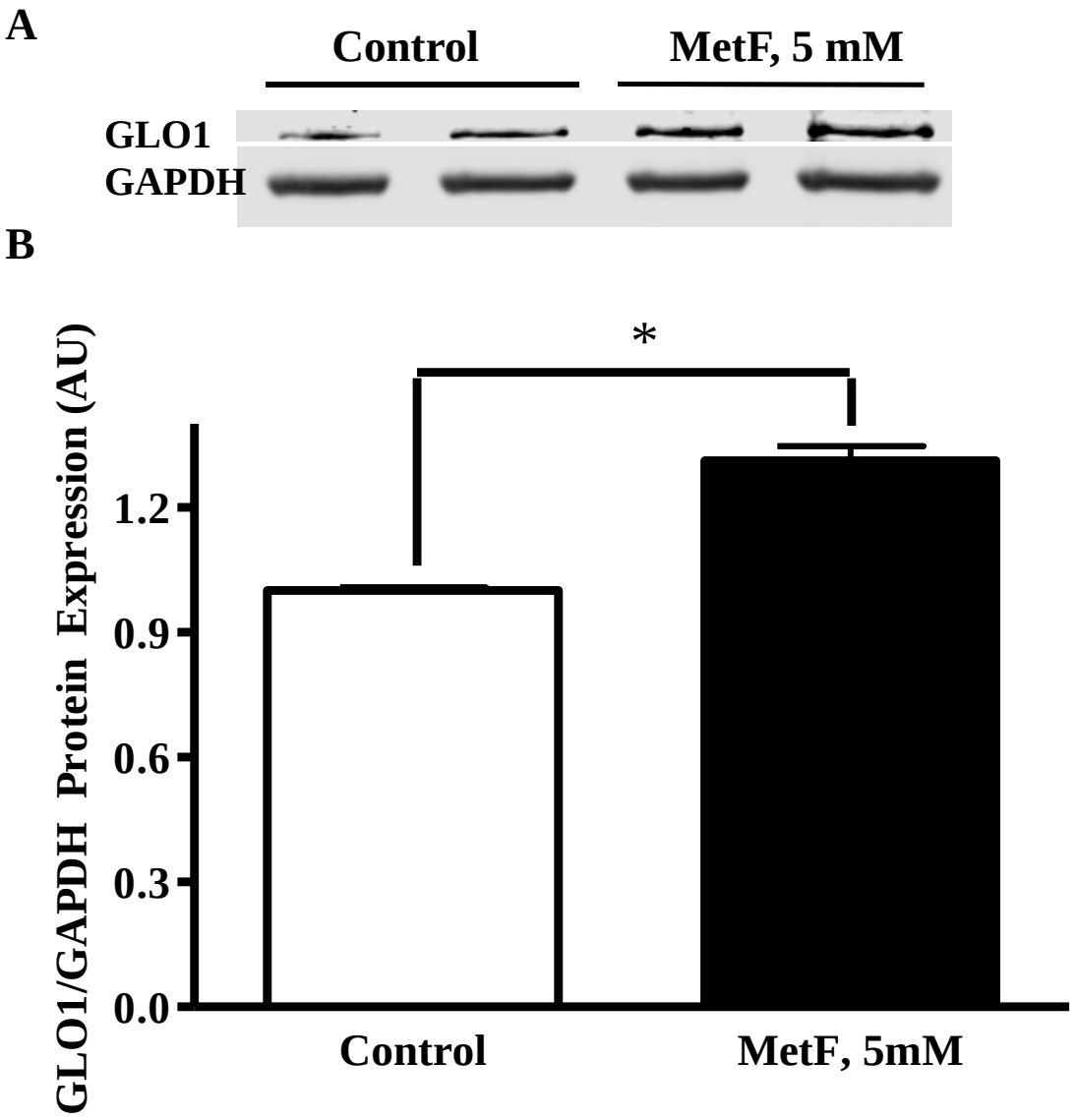

Figure 38. GLO1 Protein Expression is Increased with Metformin in Human Skeletal Muscle Cells. (A) Representative western blot images. (B) Human skeletal muscle myotubes were incubated with 5 mM Metformin (n=4) or H2O (control, n=4) for 24 hours. GLO1 protein expression increased 31% above control; *, p= 0.0001. MetF, metformin treatment; GAPDH, loading control.

CHAPTER V: DISCUSSION

We report here that GLO1 protein expression is markedly reduced in the skeletal muscle of individuals with T2DM compared to healthy control subjects while also demonstrating moderate to strong correlations with anthropometric (BMI, body fat percentage), fasting (HOMA-IR) and metabolic (M/I) indicators of T2DM. Previous research has demonstrated a reduction in GLO1 protein expression in insulin-independent tissues in the context of T2DM, however, to our knowledge, this is the first report of reduced GLO1 protein expression in highly metabolic tissue. This is particularly exciting given the recent findings by Xue et al. (222) that GLO1 inducer therapy improved glycemic control in insulin resistant individuals. In this lens, it is likely that GLO1 inducer therapy effected metabolic tissue (skeletal muscle, adipose or liver) which are known to play a role in whole body glucose homeostasis. Furthermore, our correlations between skeletal muscle GLO1 protein expression with multiple indices of insulin resistance provide strong incentive to investigate potential therapeutic mechanisms of skeletal muscle GLO1 protein.

The primary cellular function of GLO1 is the detoxification of MG. MG has been shown to effect insulin signaling and redox homeostasis *in vitro* and in murine models (18, 19). Therefore, to investigate the effects of reduced GLO1 protein expression in the skeletal muscle of individuals with T2DM, we quantified measures of MG-directed protein modifications. First, we used a global carbonyl assay, which quantifies oxidative protein modification as well as MG-adducts and is used as a general marker of cellular stress. We not only report increases in basal levels carbonyl stress in T2DM skeletal muscle compared to the LHC group, but also an inverse correlation with basal GLO1 protein expression. This relationship suggests that the T2DM skeletal muscle that has comparatively less GLO1 protein expression than LHC skeletal muscle is more susceptible to oxidative and MG-directed cell stress. To attain an indicator of MG-specific protein modifications, we performed immunoblotting analysis of MG-modified proteins in skeletal muscle of LHC and T2DM individuals in the basal and insulin-stimulated conditions of the hyperinsulinemic-euglycemic clamp procedure. This analysis showed an increase in MG-modified proteins only in the T2DM group in the insulin stimulated state and the 2-way repeated measure ANOVA revealed a significant interaction effect of Group (T2DM) x Condition (insulin-stimulated state). Given the profound

reduction in GLO1 protein expression in the skeletal muscle of individuals with T2DM, it is plausible that the increase in MG production caused by the increase in skeletal muscle glycolytic flux induced by the clamp acutely overwhelmed the MG-detoxification capacity of the skeletal muscle glyoxalase system in T2DM individuals. Although further research is required to understand the implications of acute increases in MG-modified proteins in relation to insulin resistance and T2DM, evidence from *in vitro* and animal models (18, 19) indicate a potential for MG to play a causative role in the development of skeletal muscle insulin resistance. To our knowledge, this is the first report of a dysregulation of the MG-GLO1 axis in T2DM skeletal muscle. Within the perspective of available literature, these results highlight the need for additional research to determine if therapeutic enhancement of GLO1 protein expression is protective to dicarbonyl stress in skeletal muscle *in vivo.* The potential benefit this may confer to the prevention and treatment T2DM condition is yet to be determined.

In an effort to determine potential mechanisms to explain the reduction of GLO1 protein expression in T2DM skeletal muscle, we analyzed two primary GLO1 regulatory proteins, NRF2 and Keap1, at basal and insulin states of the clamp. Both NRF2 and Keap1 protein expression were different between LHC and T2DM in the basal and insulin-stimulated states of the clamp, implicating dynamic differential regulation of these proteins in skeletal muscle of individuals with T2DM compared to the LHC group. These basal results showing reduced NRF2 and increased Keap1 protein expression in T2DM skeletal muscle are intuitive because NRF2 is a well-documented transcription factor for GLO1 protein expression (10) while Keap1 negatively regulates NRF2 by signaling constant NRF2 degradation by the ubiquitin-proteosomal system. Due to the known regulation of the Keap1-NRF2 system (discussed further in Chapter II), these basal results are in agreement with reductions in GLO1 protein expression and may potentially underlie the differences observed between LHC and T2DM individuals.

The differential regulation of the Keap1-NRF2 system with insulin stimulation adds additional depth to the hypothesis that T2DM skeletal muscle is susceptible to oxidative and dicarbonyl stress. Recall, the totality of our data describe an insulin-stimulated increase in MG-modified proteins in T2DM skeletal muscle, but not LHC skeletal muscle. It is likely this results from excessive production of MG combined

with reduced GLO1 detoxification capacity. In line with these data, T2DM skeletal muscle displayed a marked reduction in Keap1 protein in the insulin stimulated state. Following this paradigm, these results again align as Keap1 is rapidly degraded in response to cellular stress. This leads to the transient increases in NRF2, which was again significantly observed in our population. Importantly, this transient increase in NRF2 did not result in increased GLO1 protein expression. Although NRF2 is a positive regulator of GLO1, others have shown that NRF2 in concert with inflammation likely downregulates GLO1 expression (168). Alternative regulation of NRF2 activity in concert with oxidative stress include phosphorylation by protein kinases or epigenetic factors like regulatory microRNAs (279). Another potential explanation for increased NRF2 protein expression without resultant increases in GLO1 protein expression could be that the presence of NFkB is inhibitory to GLO1 gene transcription as the GLO1 gene has an NFkB response element. Although our primary focus was NRF2 signaling related to GLO1 protein expression, our data that NRF2 is reduced at basal levels in T2DM skeletal muscle is a novel finding. The literature supports a potential mechanistic explanation as insulin stimulated activation of the PI3K/Akt pathway has been previously shown to regulate NRF2-dependent pathways (280). Given that T2DM skeletal muscle presents with insulin resistance and a known dysregulation of PI3K/Akt signaling, our data is in strong agreement with other preclinical models (281). Future research should continue to investigate the role NRF2/Keap1 regulation on skeletal muscle GLO1 protein regulation, but also on its independent potential role in insulin resistance pathology (282). Although, not classically considered cross-validation, the consistency of our immunoblotting results across the entire MG-GLO1-NRF2-Keap1 axis, agreement with known regulatory molecular mechanisms, and alignment with available literature provides cogency to the dysregulation of this system in T2DM skeletal muscle.

Due to limitations in available tissue, we were unable to investigate additional markers of inflammation and oxidative stress for this study. However, we examined alternate pathways of MG production and detoxification to better characterize the MG-GLO1 axis in T2DM skeletal muscle. Alternate pathways of MG generation include reductions in the glycolytic enzymes GAPDH and TPI causing a backlog of glycolysis in T2DM, generating an accumulation of the triose phosphate intermediates, G3P and

DHAP. Increases in G3P and DHAP lead to greater spontaneous conversion into MG. However, no differences in GAPDH or TPI protein expression were observed between LHC and T2DM. It is possible that our small cohorts were underpowered to realize any difference in protein expression. However, it is more likely that performing enzymatic activity assays for GAPDH and TPI would have been more appropriate to determine the potential role of these protein. Both GAPDH and TPI are susceptible to oxidative modifications which reduced their enzymatic activity without effecting total protein expression. This would comply with the observed increase in insulin stimulated MG-modified proteins in T2DM skeletal muscle because increased oxidative stress is known to increase MG-directed protein modification (29, 283). Our limited tissue availability prevented further investigation of additional indicators of oxidative stress, but future research aimed at understanding the role of GAPDH and TPI in T2DM should utilize this approach. An alternate pathway of MG detoxification occurs via the aldoketo reductase, AKR1B1. In healthy physiology, AKR1B1 plays a minor role of MG detoxification, as GLO1 has a much higher affinity for MG. However, in conditions when GLO1 is insufficient to prevent accumulation of MG, such as observed in our T2DM group, AKR1B1 compensates with an increase in MG detoxification capacity.

As a whole, these results are in agreement with previous *in vitro* (103, 284-286) and human (16, 287, 288) studies showing reductions in GLO1 are associated with increased dicarbonyl stress. Additionally, investigations in non-skeletal muscle tissue have shown reductions in GLO1 protein and increased MG-modified proteins with obesity and hyperglycemia (222, 289). Furthermore, increased glycation of skeletal muscle proteins has previously been shown to correlate with measures of muscle function (114).

Our final proteomic analysis investigated GLO1 enzymatic activity. Given the dramatic reduction in GLO1 protein expression in T2DM skeletal muscle, we expected GLO1 enzymatic activity to be reduced as well. However, contrary to our hypothesis, GLO1 enzymatic activity was not different in LHC compared to T2DM. It is possible that technical issues prevented optimal quantification of GLO1 activity. The assay involves a time-sensitive incubation of skeletal muscle homogenate with MG-GSH, the substrate for GLO1 catalyze conversion into s-lactoylglutathione, which is subsequently estimated via photospectrometry and

used as a surrogate measure of GLO1 enzymatic activity. Data was not available describing the incubation time or amount of skeletal muscle tissue required for optimal assay performance. Although we performed a titration curve utilizing both LHC and T2DM tissue in an attempt to determine optimal assay conditions, we may have used excess skeletal muscle tissue (15 µg). In this situation, the substrate concentration may have been a limiting factor, causing a fatal flaw in the assumptions required for accurate quantification of GLO1 activity and inhibiting our ability to realize true differences between LHC and T2DM. Alternatively, it may be that skeletal muscle GLO1 enzymatic activity is upregulated to account for the loss of total enzyme content, thereby preventing MG-directed protein modification. This hypothesis would agree with the similar levels of basal MG-directed glycation observed in LHC and T2DM and prior work by Thornalley et al. showing increased GLO1 activity in the red blood cells of individuals with T2DM. However, given the increase in MG-directed protein modification that occurred only in the T2DM skeletal muscle, this would imply the existence of a ceiling for increases in GLO1 enzymatic activity. In this context, acute hyperglycemic insults to skeletal muscle may induce increases of dicarbonyl stress that are readily detoxified by GLO1 in LHC due to the high GLO1 protein content, but cannot be fully compensated for in T2DM, despite increased GLO1 enzymatic activity. This phenomenon has been similarly observed in red blood cells of T2DM (290) and additionally in immune cells of individuals with diabetes .

Together, these results suggest MG-directed protein modification is increased due to acute bouts of dicarbonyl stress induced from intracellular hyperglycemia. In agreement with other experiment models of diabetes, no increase in MG-modified proteins was observed in skeletal muscle of mice (38). Due to limitations in our proteomic approach, we cannot dismiss alternative mechanisms of MG production and detoxification. Still, we showed TPI protein expression, GAPDH protein expression, and AKR1B1 protein expression remain unchanged between LHC and T2DM skeletal muscle suggesting that the acute MG-directed protein insults are primarily determined by skeletal muscle GLO1 protein content.

Our use of the hyperinsulinemic-euglycemic clamp provided additional insight into the acute regulation of the GLO1 regulatory proteins, NRF2 and Keap1, and acute MG-glycation of proteins. Two-way repeated measure ANOVA revealed a differential response at 120 minutes of the clamp in LHC and

T2DM skeletal muscle. The clamp not only induces physiologic insulin stimulation, but also increases glycolytic flux into skeletal muscle. It is possible that alterations in the acute responses to skeletal muscle metabolic stimuli underlie the basal differences observed between LHC and T2DM skeletal muscle. In agreement, peripheral insulin resistance indicative of the diabetic condition is known to contribute to aberrant signaling through multiple pathways including, mTOR and REDD1 (257). Our results in combination with GLO1 inducer therapy studies provide evidence of a potential therapeutic role. Future investigations should utilize the combination of the clamp technique in T2DM individuals before and after GLO1 inducer therapy. Determining the best GLO1 inducer will be a high priority issue as most function through NRF2, but not all NRF2 activators are GLO1 inducers (291).

However, these results should be interpreted with our study limitations in mind. The sample size of our T2DM group was rather modest (n=5) however, despite this, the 2-way repeated measures ANOVA is a stringent statistical test and confers confidence in the results. Still, these subject numbers may not meet the statistical assumptions of a 2-way ANOVA, although our data was normally distributed. Future studies using larger, well matched cohorts would be prudent before strict conclusions are made on GLO1 biology in the context of T2DM and exercise. Our interpretation of MG-directed protein modification is also limited by our methodology, which utilized immunoblotting as semi-quantitative measures of MG-directed protein modification. It has been reported that targeted mass spectrometry is the most robust method to quantify MG-directed modification of proteins (2, 276, 292). Although we agree, available tissue and equipment limited our assay capabilities. Similar to others, we used immunoblotting (17, 289, 293) to quantify MG-modified proteins and supplemented this with a titration curve of MG-BSA to verify the accuracy of our immunoblotting procedure. Also, these western blotting results were cross-validated in a chemical assay for total carbonyl protein modifications. Furthermore, the immunoblot determined MG-modified proteins were in agreement with our NRF2 and Keap1 protein data in response to insulin stimulation during the clamp, while the carbonyl stress assay agrees with our basal GLO1 protein data with an inverse correlation. Another limitation involves the lack of age and BMI matched groups. Both chronologic age and BMI have been documented to effect GLO1 protein expression in insulin-independent tissues. Given our aim was to

characterize the MG-GLO1 axis in T2DM, we utilized a younger, leaner LHC group that was representative of a state of optimal health for comparison. Expectedly, the T2DM was significantly older and more obese, limiting our ability to isolate the effect of T2DM per se. additionally, we and others have previously shown that obesity and chronologic age may have independent effects on GLO1. To address these issues, we generated a linear regression model where the dependent variable is basal GLO1 protein expression and the independent variable was T2DM, while controlling for both age and BMI. Importantly, the effect of T2DM was significant ($p=0.013$) while age and BMI were not independently significant. These results provide additional evidence that the observed reductions in skeletal muscle GLO1 protein expression are primarily due to the effect of T2DM in our model. Despite these limitations, we have characterized the MG-GLO1 axis in T2DM skeletal muscle by comparison to a lean, healthy control group and provide proof of concept for larger studies to be performed with the additional control for age and BMI.

After demonstrating a dysregulation of the MG-GLO1 axis in T2DM, we sought to investigate the therapeutic potential of aerobic exercise. This study utilized an obese, insulin resistant group because these individuals are on the characteristic path towards developing T2DM, but have not yet crossed clinical thresholds for diagnosis. It is well described that a lifestyle intervention involving aerobic exercise training in this pre-diabetic condition improves insulin sensitivity and prevent progression towards T2DM. This represents an optimal cohort to investigate skeletal muscle adaptations elicited by aerobic exercise training. We report that GLO1 protein expression is markedly reduced in the skeletal muscle of obese, insulin resistant individuals compared to a lean, healthy control group and that administration of a 12-week intense lifestyle intervention involving supervised aerobic exercise training improved markers of cardiovascular and metabolic health while increasing both skeletal muscle GLO1 protein expression and mRNA. With limited sample availability, we probed a small cohort of LHC and OB-IR participants for skeletal muscle GLO1 activity, which correlated with markers of obesity and metabolic health. Finally, similar to MG-modified proteins, which showed no changes at baseline between LHC and OBIR and no response to the aerobic exercise intervention.

We performed this clinical trial to investigate the potential beneficial effects of aerobic exercise training on skeletal muscle MG and GLO1 biology. The basal state reduction in GLO1 protein expression in OBIR was 62% of our reference LHC group, and although our T2DM group reported in specific aim 1 was compared to a different LHC cohort, had 22% GLO1 expression. This stepwise reduction in skeletal muscle GLO1 protein expression where LHC > OB-IR > T2DM represents a continuous reduction of GLO1 protein across the glucose tolerance continuum. Larger studies involving both fasting and metabolic markers of insulin resistance are warranted to reveal whether the stepwise reduction in GLO1 protein with increasing insulin resistance observed in our preliminary trials persist in a larger cohort. Additionally, future studies investigating GLO1 protein expression in skeletal muscle should carefully consider chronological age and aerobic fitness. The correlations with GLO1 activity observed in our small cohort of LHC and OBIR indicate that these may both be important factors in highly metabolic tissue, like skeletal muscle. However, our GLO1 activity results were quantitatively different between our first investigation between LHC and T2DM compared to our second investigation into the effects of exercise. In our second study, the GLO1 activity levels were ~5 fold higher in LHC individuals compared to the LHC individuals in the first trial. Although this could simple be explained by innate differences in the LHC cohorts, we did not have the appropriate measure to compare the two based on exercise status. With the large differences observed, it is rather likely that technical or methodological limitations to the GLO1 activity assay caused the differences. Given this likelihood, it remained prudent to not compare the GLO1 activity results between trials (essentially conceding to high interassay variability). Future studies should aim to discern the repeatability of the GLO1 activity assay between various cohorts and utilize larger subject groups to provide more conclusive evidence as to the effect of T2DM and exercise training on skeletal muscle GLO1 activity. A final note on the GLO1 activity variability, skeletal muscle tissue is a highly diverse and concentrated collection of proteins involved in nutrient metabolism. It is possible that the many proteins present in skeletal muscle tissue prevents the proper quantification of GLO1 activity through currently available chemical kits. Perhaps purification of skeletal muscle homogenate prior to performing the GLO1 activity assay would help alleviate these concerns and increase reproducibility. In conclusion, our results have

created the framework for a role of GLO1 in insulin resistance and T2DM. Larger studies that adequately match subjects by both chronologic age, BMI and aerobic fitness while utilizing more quantitative protein measures like targeted LC-MS/MS should be performed.

Our investigation into MG-directed protein modification showed no difference between LHC and OB-IR adults and no effect of exercise. Due to limited sample size (LHC, n=4; OB-IR, n=5) these results should be interpreted with caution. Additionally, we utilized western blot quantification of MG-modification of all skeletal muscle proteins, and therefore were unable to identify changes in the dicarbonyl proteome. For example, if MG-modifications are increased on a metabolically inert or structural protein, it may play little role in insulin resistance and skeletal muscle metabolism. However, if MG-modifications are directed at proteins governing skeletal muscle nutrient metabolism, MG-modifications may be detrimental to cellular health. Future studies should aim to expand the dicarbonyl proteome through targeted mass spectrometry approaches designed for identifying hot spots of MG modifications on important metabolic proteins. It has been suggested that mitochondrial complex 1 and AMPK are susceptible to MG-modification and may play an important role in dicarbonyl stress in skeletal muscle (110, 294, 295). These proteins are particularly important to skeletal muscle function as mitochondrial generated reactive oxygen species production is a primary mechanism that underlies insulin resistance and metabolic inflexibility. AMPK is not only a master metabolic regulatory, controlling the shift of nutrient utilization in skeletal muscle, but senses cellular energy stats through AMP concentrations. Disruption of either of these proteins has a detrimental effect on skeletal muscle metabolism and should be investigated as being role players in the dicarbonyl proteome.

After characterizing the MG-GLO1 axis in the context of T2DM and exercise (see above) we utilized a human skeletal muscle cell culture model to investigate: molecular mechanisms of the diabetic milieu that may contribute to dicarbonyl stress, role players in the dicarbonyl proteome and potential therapeutic strategies. We developed our cell culture models so they would be most applicable to human physiology. Herein, we reported that both hyperglycemia and insulin stimulation have independent effects on the MG-GLO1 axis and that dicarbonyl stress effects skeletal muscle AMPK activity, as measured by

the surrogate marker of pACC. Additionally, metformin, a first line medication for T2DM displays not only glucose lowering effects, but reduces the progression of diabetic complications beyond what the glucose lowering effects would suggest. The biguanidine structure of metformin makes it a prime scavenger for MG and this has been verified by others in biological systems. We therefore sought to investigation the effect of metformin on the MG-GLO1 axis in skeletal muscle. Our results indicate that metformin protects skeletal muscle cells from dicarbonyl stress and increases GLO1 protein expression in human skeletal muscle cells. Important to our overall experimental design, we fully differentiated a human skeletal muscle cell line (Lonza) to myotubes, the mature, post-mitotic cell, prior to experimentation. This allows a better comparison to the skeletal muscle tissue present in human physiology.

Our initial hypothesis was that hyperglycemia would increase MG-modification of proteins in skeletal muscle cell culture, as others have shown this effect in insulin independent tissue. We performed a 24-hour hyperglycemic insult at 15 mM glucose (*ca.* 270 mg/dl) which is similar to the hyperglycemia observed in the postprandial state and 30 mM glucose (*ca.* 540 mg/dl) which would be observed in uncontrolled T2DM. However, MG-modifications were unchanged compared to control (5 mM glucose, *ca.* 90mg/dl). Others have shown that 5 days of hyperglycemia using 30 mM glucose is required to increase MG-modification of proteins, so we extended our model of hyperglycemia accordingly. The dramatic increase in MG-modified proteins (50% above control) occurred without changes in GLO1 protein expression. This suggests the increase in hyperglycemia-induced dicarbonyl stress stems from an overproduction of MG, likely through increased glycolytic flux, rather than through decreases in GLO1 protein expression. We cannot discount the possible loss of GLO1 enzymatic activity, as it was not measured in this experiment. Additionally, extended hyperglycemia may have alternate effects not analyzed by our methods like contributing to an increase in oxidative stress concomitant with mitochondrial dysfunction. Under these conditions, lipid peroxidation may occur and predominate as a means of MG generation. Future *in vitro* investigations should aim to quantify changes in GLO1 activity, mitochondrial function and intracellular ROS to better understand the cause of hyperglycemia-induced dicarbonyl stress. The addition of a GLO1 gain-of-function condition to this model (either through genetic overexpression or

via incubation with a GLO1 inducer formulation) would be useful to elucidate a potential protective mechanism of GLO1 enhancement.

To further characterize the effect of the diabetic milieu on MG and GLO1 biology, we stimulated HSKMCs with hyperinsulinemia (300 pM) for 30 and 180 minutes. We selected 300 pM because this concentration is similar to postprandial hyperinsulinemia in the plasma and it is also the final concentration used in our hyperinsulinemic-euglycemic clamp studies. The time course was selected as a physiologic mimic to the postprandial period *in vivo*. Hyperinsulinemia had no effect of MG-modification of proteins, but increased GLO1 protein expression at 30 minutes, returning near baseline at 180 minutes. This time course effect on insulin-stimulated GLO1 protein expression may be important in the physiologic hyperinsulinemia indicative of progressive skeletal muscle insulin resistance and progression to T2DM. These experiments, although simplistic, present novel data identifying that characteristics of the diabetic milieu (hyperglycemia and hyperinsulinemia) may have direct effects on the MG-GLO1 axis and warrants further investigation to determine the physiologic importance of these results.

Our second primary goal utilizing an *in vitro* model, was to identify potential role players in the dicarbonyl proteome. Recall, the dicarbonyl proteome consists of proteins that are not only modified by MG, but maintain an altered function after MG-modification with the potential to effect cellular signaling pathways and ultimately cell health. A hypothesis by Guggliucci et al. suggested that the AMPKγ subunit is particularly susceptible to MG attack with the potential to reduce kinase activity due to a high concentration of arginine residue within the AMP binding pocket (4). The AMPKγ subunit binds AMP and becomes active when the cell has low energy or is stressed. In this manner, AMPK controls cellular nutrient metabolism through phosphorylation cascades control anabolic/catabolic processes like nutrient utilization. Further, skeletal muscle metabolic inflexibility stems from an aberrant response to an increase in glucose availability. In healthy physiology, cells prefer to inhibit oxidation of fatty acids when glucose in available and optimize fatty acid oxidation when glucose (or available cell energy) is low. These processes are at least in part due to AMPK mediated regulation of enzymes like acetyl-CoA carboxylase. Whether these

signals dysregulated prior to glucose entry into cells (as is reduced with T2DM) is not fully understood, but it is known that exercise and metformin improve metabolic flexibility and have profound effects on AMPK.

Given its important role in skeletal muscle nutrient metabolism and adaptation to exercise, AMPK was a prime target for MG-modification and its potential as part of the dicarbonyl proteome was intriguing. We therefore performed immunoprecipitation the AMPKγ subunit and probed it via immunoblotting for MG-modifications in cells treated with increasing doses of MG (a model of dicarbonyl stress). However, we showed no difference in MG-modification of AMPKγ, even at the highest MG dose. Given these lack of findings, it stands to reason that our crude application of IP-AMPKγ:IB-MG to assess specific MG-modification may have been technically inappropriate. The experimental conditions involved high heat and an acidic pH which have been reported to affect our anti-MG antibody binding, potentially confounding the results of this particular experiment. Future studies should utilize the targeted mass spectrometry approach outlined by Thornalley et al. (135) to investigate specific MG-modifications on individual proteins.

In light of these results, we modified our approach to investigate AMPK enzymatic activity by a commonly used surrogate, pACC (a downstream phosphorylation target of AMPK). With this approach, we showed that experimental dicarbonyl stress inhibits the AMPK activity in response to AICAR, a potent AMPK agonist. We specifically chose AICAR as our AMPK stimuli, because AICAR is a functional AMP mimetic and activates AMPK by binding to the gamma subunit. Our original hypothesis, revolved around MG-modification of the gamma subunit, due to a high prevalence of arginine residues susceptible to MG-modification causing dysregulation of AMPK. Therefore, AICAR was the logical AMPK stimuli of choice. AICAR has also been shown to reduce IL6 and IL8 in skeletal muscle cells (296) whereas insulin stimulation has been shown to increase IL6 and TNFα (297). It is possible that these alternative effects of AICAR or insulin are playing confounding roles in skeletal muscle cell culture and future mechanistic studies should consider the independent effects of inflammation in the context of dicarbonyl stress and the use of AICAR to elucidate MG independent effects on AMPK stimulation. Despite our inability to show MG-specific modifications of AMPK, our results implicate that AMPK may be a part of the dicarbonyl proteome and requires further investigation.

Our final *in vitro* goal was identification of potential therapeutic strategies to combat dicarbonyl stress. Given our other interests, metformin was a front line therapeutic for type 2 diabetes, has a mechanism of action that involves stimulating AMPK, and has potent MG scavenging capabilities indicated by its biguanidine chemical structure and was a clear therapeutic of interest. To investigation this potential, we performed a titration curve of both MG (an experimental model of dicarbonyl stress) and metformin in human skeletal muscle cell culture. Our results are in agreement with others that metformin is protective to dicarbonyl stress, as we see a stepwise reduction in MG-modified proteins as metformin dose is increased in the cell culture media. Additionally, we provide novel insight into the potential role of metformin in GLO1 biology as GLO1 protein expression was increased with metformin. These implicates metformin's non-glucose lowering related benefits may occur through increasing GLO1 protein expression and reducing dicarbonyl stress. Future investigations should aim to identify GLO1 and MG characteristics in the tissue of individual with T2DM with and without the development of diabetic complications while under metformin prescription and utilize additional *in vitro* approaches to further characterize the effect of metformin on the MG-GLO1 axis.

When a redox imbalance occurs, NADH accumulates shifting the NAD+/NADH ratio. This inhibits enzymes whose reaction produces NADH (GAPDH) (298) and leads to ROS production (299). To compound this, NADH pressure on mitochondrial complex 1 can further exacerbate ROS generation (300). The combination of these perturbations have led others to suggest the redox imbalance of NADH/NAD+ is a major factor contributing to diabetic complications (301). The excess generation of MG from reduced GAPDH may be another route of redox imbalance contributing to diabetic complications, further evidencing this physiologic issue. Additionally, MG *per se* has been shown to modify GAPDH and alter its isoelectric point and mass. these modifications inhibit GAPDH enzyme function and may produce a viscous cycle of MG generation (99). However, we did not investigate redox imbalances in the skeletal muscle directly. Future investigations may consider measuring redox balance in the context of dicarbonyl stress and GLO1 biology due to the clear link with glycolytic regulation and GAPDH activity. In the context of redox balance, additional investigations into the sirtuins are particularly intriguing because of the role that

sirtuins play in connecting cell redox state with metabolic changes and adaptations to exercise. Ultimately, much is yet to be determined as to the role of MG-GLO1 in skeletal muscle in the context of insulin resistance and obesity. Clearly, there remains much to be learned about other potential contributing factors both in the regulation of the MG-GLO1 axis and in the potential effects of MG-modified proteins on skeletal muscle metabolic function. An integrated schematic that represents our current understanding of MG-GLO1 biology in skeletal muscle in the context of T2DM and exercise is presented in **Figure 39**. The data herein provide a framework for future investigations and represent an exciting development in MG, glyoxalase and diabetes research.

CHAPTER VI. CONCLUSION

This study provides an important link between dysregulation of the MG-GLO1 axis and skeletal muscle metabolic health. Using a clinical-translational approach we demonstrated aberrant regulation of the MG-GLO1 axis in the skeletal muscle of individuals with insulin resistance and T2DM compared to a lean, healthy reference group and implicated major role players in the diabetic milieu (glucose and insulin) as direct effectors of MG-GLO1 biology in tissue culture. We further elucidated skeletal muscle specific mechanisms effected by dicarbonyl stress and implicated the major metabolic regulatory protein, AMPK as a potential role player in the dicarbonyl proteome. Although, it is yet to be determined whether these phenotypic differences underlie the diabetic condition or are a repercussion of hyperglycemia and insulin resistance characteristic of T2DM, we sought to identify potential therapeutic strategies. We established a role for both aerobic exercise training and metformin therapy, two primary countermeasures to insulin resistance, in improving skeletal muscle GLO1 and protecting against dicarbonyl stress. This is an important step in understanding the role of the glyoxalase system and methylglyoxal in the context of T2DM and should be used as a platform to investigate therapeutic strategies and interventions to prevent the onset and progression of T2DM. Future investigations are warranted to determine the therapeutic potential of normalizing the MG-GLO1 axis in the prevention and treatment of insulin resistance and T2DM. The next logical progression of MG-GLO1 research in T2DM skeletal muscle should involve the use of exercise and therapeutics such as metformin or other GLO1 inducer therapies to investigate if the MG-GLO1 axis in T2DM skeletal muscle and whether it may play a protective role in mediating the diabetic condition.

CHAPTER VII. PRACTICAL APPLICATIONS

The elucidation of molecular mechanisms related to skeletal muscle insulin resistance is imperative to understanding both the etiology and pathology of T2DM. Given the damaging effects of hyperglycemia and the prime importance skeletal muscle plays on whole body glucose metabolism, therapeutic interventions targeting skeletal muscle insulin sensitivity have been a premier area of diabetic research. A recent clinical trial utilizing GLO1 inducer therapy improved whole body glucose metabolism, rivaling current pharmacologic therapy and implicating a regulatory role of GLO1 in highly metabolic tissue like skeletal muscle. The results of our investigations provide strong proof of concept that the MG-GLO1-NRF2-Keap1 axis is dysregulated in insulin resistant and diabetic skeletal muscle and that therapeutic interventions targeted at this system may prove to be an effective part of a comprehensive treatment plan for T2DM. Aerobic exercise training and metformin have long remained first-line therapies for insulin resistance and prevention of T2DM, and this study further evidences their therapeutic potential by extending their application to the MG-GLO1-NRF2-Keap1 axis in human skeletal muscle. Our clinical-translational approach enriches the current body of knowledge by identifying novel mechanisms that may contribute to the primary defect in the development of T2DM, skeletal muscle insulin resistance, and demonstrates value for the continued investigation of GLO1 inducer therapy for the prevention and treatment of T2DM.

CHAPTER VIII. PERSPECTIVE

We have presented novel insight into MG and GLO1 metabolism in the context of insulin resistance, T2DM and exercise. Skeletal muscle GLO1 protein expression in markedly reduced along the natural progression to T2DM confering susceptiblitiy to MG-directed protein modification when glucose flux is stimulated. We have identified novel dysregulation of GLO1 regulatory proteins with insulin stimulation that may underlie the observed GLO1 protein reductions, thereby identifying a novel skeletal muscle specific mechanism to combat dicarbonyl stress. Additionally, we have elucidated skeletal muscle specific mechanisms of dicarbonyl stress that may underlie T2DM disease phenotype in that MG can dramatically reduce AMPK activity *in vitro.* Furthermore, we identified that aerobic exercise training can rescue GLO1 protein expression in concert with improvements in skeletal msucle insulin sensitivity and glucose metabolism.

The bulk of current literature has been directed towards MG-GLO1 biology as it pertains to complications of T2DM, however little evidence exists in the highly metabolic skeletal muscle tissue. Given the development and complications of T2DM are secondary to skeletal muscle insulin resistance, understanding the role of the MG-GLO1 axis in this context will significantly advance the field. Herein, we provide a significant extension of the effects of dicarbonyl stress and GLO1 metabolism into the highly metabolic skeletal msucle tissue. This work provides an important transition from the role of dicarbonyl stress and GLO1 as merely an effect of skeletal muscle insulin resistance to a potential major role player in the intial decline in skeletal muscle health and the onset of insulin resistance. Certainly, this provides a strong potential for the role of dicarbonyl stress and GLO1 in not just the pathology, but the etiology of the natural history of T2DM.

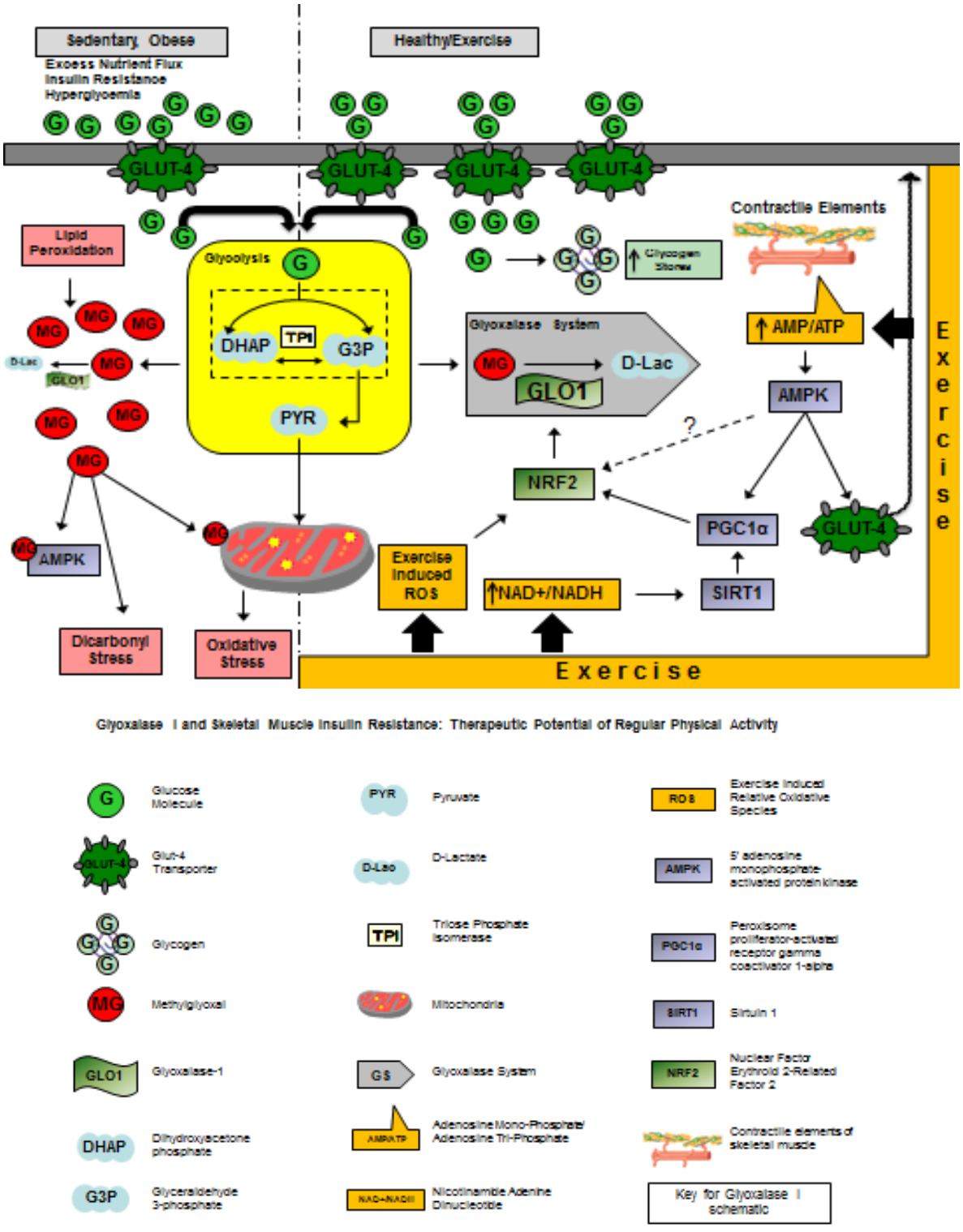

Figure 39. Integrated schematic of skeletal muscle MG-GLO1 physiology in the context of T2DM and exercise

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APPENDICES

Cell Culture Protocol.

Cell Culture Protocols for Human Skeletal Muscle Cells (HSKMC)

Maintaining Cell Culture Line – Thawing, Subculturing and Freezing

**Prior to all cell culture work, decontaminate all surfaces, equipment and containers with 70% EtOH solution. Use aseptic technique for all procedures.*

Thawing Human Skeletal Muscle Cells/Initiating Cell Culture Process

1. Prepare 5-6 mLs or 1ml/5cm^2 growth medium (SkBM) supplemented with growth factors (SkGM) in T25 flask and place in incubator (37°C, 5% CO_2) to warm ~30 minutes PRIOR to obtaining cells from LN_2.
2. Remove a cryovial of HSKMC from LN_2 and QUICKLY transfer to cell culture room.
    a. Relieve pressure of the vial – within cell culture hood, twist cap a quarter turn to release pressure and immediately retighten.
    b. Place cryovial into water bath (37°C) for 2 minutes. DO NOT submerge the cap in the water and DO NOT incubate for longer than 2 minutes.
3. Resuspend cells in cryovial (with ~1 mL growth medium)
4. Dispense cells into previously prepared culture flask. Rock flask gently to evenly distribute cells.
5. Incubate (37°C, 5% CO_2) for 24 hours to allow for cell attachment before replacing medium.
6. @80+% confluence, subculture all cells from T25 to T75

Subculturing Human Skeletal Muscle Cells

**Recommended seeding density for SkMC culture is 3,500 cells/cm^2 (Lonza)*

1. When cells are ~80% confluent, prepare all solutions and flasks necessary for subculture.
2. Remove medium from cell culture and wash cells in D-PBS to remove elements that may inhibit trypsin function (calcium, etc).
3. Detach cells from flask.

a. Cover cells with ~2mL trypsin (TrypLE). Be sure the entire surface of the flask is covered with trypsin.
b. Incubate in 37°C, 5% CO_2 for 2-6 minutes, viewing under the microscope intermittently, until ~90% of cells are rounded up.
c. Tap side of flask moderately to vigorously to release the cells that remain attached to the flask surface.
d. If cells do not detach, continue trypsin incubation for 30 more seconds, view, and tap again. Repeat as necessary until >95% of cells have detached into suspension.

4. Transfer the detached cells suspended in trypsin to sterile 15 mL falcon tube.
    a. Rinse flask with D-PBS to collect leftover cells and add this to 15mL falcon tube.
5. Deactivate trypsin.
    a. Add an equal volume of basal medium to 15 mL falcon tube. This prevents the trypsin from potentially harming cells.
6. View 'empty' flask under microscope, <5% of cells should be remaining.
7. Centrifuge harvested cells in 15 mL falcon tube at 220xg for 5 minutes to pellet the cells.
8. Decant the supernatant and resuspend the cells in 2-3 mL of growth medium (record this volume).
9. Determine cell count/density using a hemocytometer and Trypan Blue.
    a. Refer to 'Determining Cell Density and Viability' protocol
10. Aliquot desired volume of cells into respective vessels
    a. Transfer desired volume of cells into a new T-75 flask (or experimental vessel) with growth factor supplemented medium already warmed and present.
    b. Prepare a new T75 flask for continuous cell line propagation.
    c. If cell density permits, prepare an additional T-75 flask to be grown to confluence and returned to LN_2 for future cell culture experiments.
11. Change the growth medium the day after seeding and every other day thereafter. As the cells become more confluent, increase the volume of medium as follows:

➢ Under 25% confluence then feed cells 1 ml per 5 cm2
➢ 25-45% confluence then feed cells 1.5 ml per 5 cm2
➢ Over 45% confluence then feed cells 2 ml per 5 cm2

Freezing Human Skeletal Muscle Cells/Replenishing Cell Supply

1. When cells are ~80% confluent, prepare solutions and flasks necessary for freezing cells.
2. Wash cells in D-PBS to remove elements of the basal medium that may inhibit trypsin function (calcium, etc)
3. Detach cells from flask.
    a. Cover cells with ~2mL trypsin (TrypLE). Be sure the entire surface of the flask is covered with trypsin.
    b. Incubate in 37°C, 5% CO_2 for 2-6 minutes, viewing under the microscope intermittently, until ~90% of cells are rounded up.
    c. Tap side of flask moderately to vigorously to release the cells that remain attached to the flask surface.
    d. If cells do not detach, continue trypsin incubation for 30 more seconds, view, and tap again. Repeat as necessary until >95% of cells have detached into suspension.
4. Transfer the detached cells suspended in trypsin to sterile 15 mL falcon tube.
    a. Rinse flask with D-PBS to collect leftover cells and add this to 15mL falcon tube.
5. Deactivate trypsin.
    a. Add an equal volume of basal medium to 15 mL falcon tube. This prevents the trypsin from potentially harming cells.
6. View ‘empty’ flask under microscope, <5% of cells should be remaining.
7. Centrifuge harvested cells in 15 mL falcon tube at 220xg for 5 minutes to pellet the cells.
8. Decant the supernatant and resuspend the cells in 1 mL volumes of freezing medium depending on the density of cells. (~1/2 a confluent T75 per vial)

9. Place ~1mL aliquots of cell suspension into a labeled cryovial (passage x+1) and place into "Mr. Frosty" container in -80°C freezer for 24 hours.
10. Transfer cryovial from "Mr. Frosty" into LN_2 within the designated and labeled box.

Working with Myotubes – Differentiating and Harvesting

**Prior to all cell culture work, decontaminate all surfaces, equipment and containers with 70% EtOH solution. Use aseptic technique for all procedures.*

Differentiating Human Skeletal Muscle Cells/Formation of Myotubes

**Differentiation must occur in experimental vessel. Myotubes cannot be transferred to new vessels after differentiation.*

1. Culture myoblasts under standard culturing conditions in SkBM until culture has achieved ~95+% confluence.
2. Remove SkBM and replace with an equal volume of differentiation medium (DMEM-F12 supplemented with 2% horse serum).
3. Continue to culture the cells in the differentiation medium (replacing every other day) for ~3 to 5 days, or until myotubes are observed throughout the culture.
    a. Observe multinucleated (more than 3 nuclei) myotubes.
4. If the myotubes are to be used in assays that require an extended period in culture, following differentiation, replace differentiation medium with SkBM. For best performance, myotube cultures are best used by 2 weeks post differentiation.

Harvesting Human Skeletal Muscle Cells

1. Perform desired experimental procedures and incubations.
2. Remove medium (collect this if your target of interest may be released in the medium).
3. Rinse cells with D-PBS
4. Add 100uL of Lysis Buffer (supplemented with Protease/Phosphatase Inhibitors)
5. **Optional: If harvesting entire plate/flask for homogenization, place into -20°C for <5 minutes. This helps to prime the cells for removal from treatment vessel.*

6. Using a cell scraper, scrape cells from top to bottom. Be sure to scrape close to the edges of the well. It helps to tip the vessel at ~45 degrees, so that the cells are scraped into the homogenization buffer that collects at the bottom of the well.
7. Rotate plate 90° and repeat scraping process. Repeat until entire well surface has been scraped appropriately.
8. Collect cells suspended in the homogenization buffer into 1.5 mL Eppendorf tube.
9. Vortex cell suspension.
10. Rock on ice for 30 minutes to ensure cell lysis and homogenization.

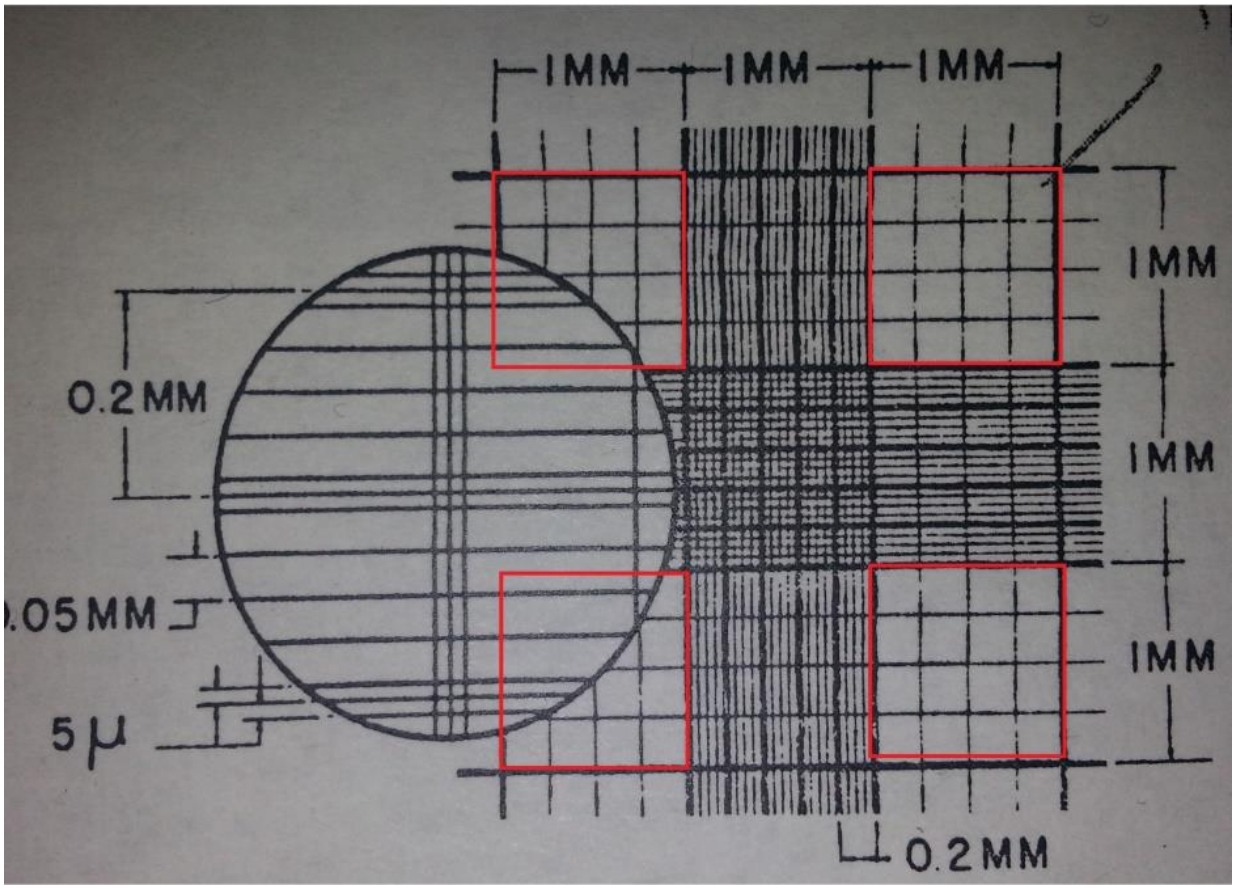
Experimental Procedures in Human Myotubes

Determining Cell Density and Viability

**Trypan Blue will not penetrate cells with an intact cell membrane. Dead cells will appear filled with the blue die. Viable cells will be outlined, but have a clear interior.*

1. Combine 15 uL of cell suspension with 15 uL Trypan blue in Eppendorf tube.
2. Inject 15 uL of the cell/Trypan mix into the hemocytometer and view under microscope.
3. Count viable cells to obtain an average # of cells in a 4x4 block. Ie. count total cells in all 4 corner boxes and divide by 4. This is your "cell count." See image.

Corner boxes are 4x4 squares - outlined in RED.



4. Determine percent viability:
    a. Count <u>dead</u> cells.
    b. Add viable and dead cell totals together.
    c. Viability = #Viable Cells/#Total Cells x100%
5. Determine cell density:
    a. Multiply <u>cell count</u> by 10,000 (volume factor converting to cells/mL)
    b. Multiply by dilution factor of 2 (1:1, cell suspension:Trypan Blue)
        i. Use this value to determine volume of cell suspension to load per vessel.
    c. Multiply by total volume of cell suspension stock to determine total cells
        i. Use this value to determine # of possible experiments.

<u>Acitretin Experiments</u>

**Use caution when handling Acitretin. Consult MSDS prior to use.*

1. Product Information
    a. Name: Acitretin-25MG
    b. Brand: Sigma
    c. Product Number: 44707
    d. CAS-Number: 55079-83-9
    e. Molecular Weight: 326.43g/mol
2. Background
    a. Acitretin is a synthetic retinoid that preferentially binds to cellular retinoic acid binding proteins (CRABPs). Acitretin, a retinoid that binds to nuclear receptors and regulates gene expression, is a potent activator of the α-secretase ADAM10 gene expression and apoptosis inducer via the CD95 signaling pathway. Acitretin is a systemic retinoid drug used in the treatment of severe psoriasis

Medium Preparations

Warming Aliquots of Growth/Differentiation Medium

**It is optimal to avoid warming and cooling the full supply of cell culture medium whenever possible. To achieve this, aliquot only the required amount of medium (into a 15 or 50 mL Falcon tube) under sterile conditions. Return stock medium to 4° storage and incubate aliquot as necessary.*

Supplementing Growth Factors (SkGM) to Growth Medium (SkBM)

**Growth factors (stored in -20°C) come in multi-vial kits contained in sealed plastic bags.*

1. Thaw all components from a single kit (bag) on ice.
2. In the hood, add all thawed components to a new 500 mL container of SkBM.
3. Fill out the Growth Factor Checklist included with the SkGM kit.
    a. Be sure to include date
    b. Place Growth Factor Checklist (peel off sticker) onto SkBM container.

4. Return the now GF-supplemented SkBM to 4° storage.

Preparing Differentiation Medium (DMEM-F12 supplemented with 2% horse serum)

1. Thaw horse serum (stored in -20°C) on ice.
2. In cell culture hood, aliquot ONLY the amount of DMEM F12 needed for your experiments into 50 mL falcon tubes. DO NOT add horse serum to DMEM stock – adding horse serum will reduce the shelf life to ~ 30 days.
3. In cell culture hood, add horse serum to DMEM aliquot to a final concentration of 2% (v/v) horse serum. Ie. 49 mL DMEM + 1 mL horse serum.
4. Return the now horse serum-supplemented DMEM to 4° storage.

Preparing Freezing Medium

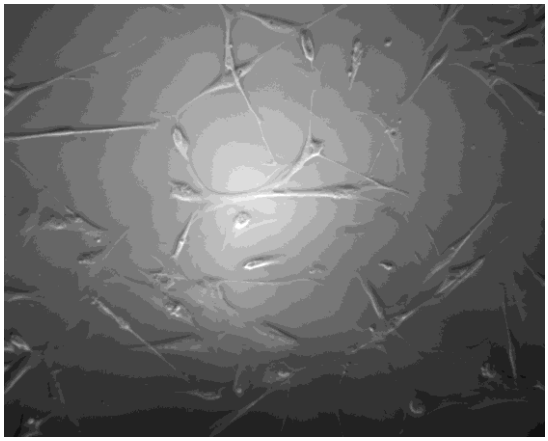
1. Combine the following ingredients into a 15 mL falcon tube.

✓ 10% (v/v) DMSO
✓ 10% (v/v) FBS (Fetal Bovine Serum)
✓ 80% (v/v) SkBM w/ Growth Factors

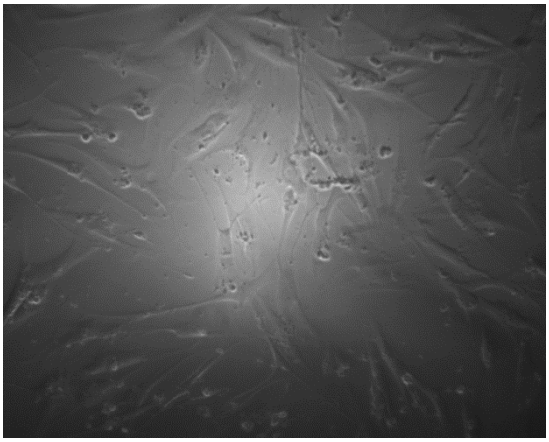
2. Store leftover freezing medium solution in -20°C

Cell Culture Images

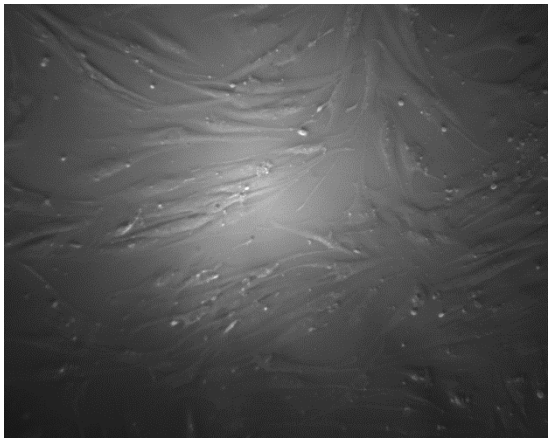
1) Myoblasts after 24-hour seeding in 6-well plate (seeded @3,500 cells/cm^2 or 40,000 cells per well) at ~30% confluence.



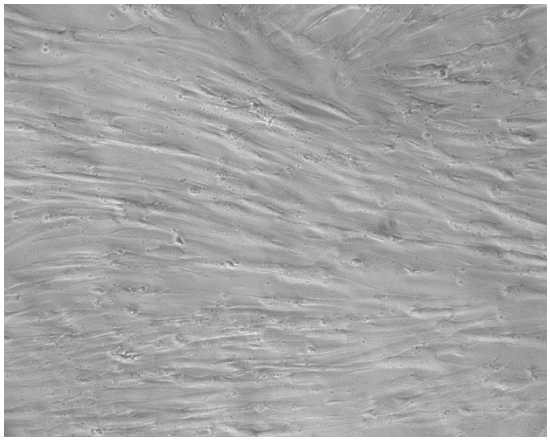
2) Myoblasts grown to ~65% confluence prior to differentiation.



3) Partially differentiated myotubes 2 days after differentiation medium.



3) Fully differentiated myotubes.



Product List

Product	Supplier	Cat. No.	Description
SkBM	Lonza	CC-3161	Basal Medium for Skeletal Muscle Cells
SkGM SingleQuots	Lonza	CC-4139	Growth Factors for SkBM
DMEM:F12	Lonza	BE04-687F/U1	Differentiation Medium
Horse Serum	Gibco	16050-130	Horse Serum
D-PBS	Gibco	14190-144	Wash buffer for cell culture
TrypLE	Gibco	12563-029	For cell transfer/ subculture
T-75 Flask	CytoOne	CC7682-4875	Polystyrene, Sterile
6-Well Plate	CytoOne	CC7682-7506	Flat Bottom, Tissue Culture Treated
Trypan Blue (0.4%)	Lonza	17-942E	Cell Staining
Cell Lysis Buffer (10x)	Cell Signaling	9803S	Homogenization
10 mL Sterile Pipettes	USA Scientific	1071-0810	Cell Culture Supply
1 mL Sterile Pipettes	USA Scientific	1070-1210	Cell Culture Supply

Metabolic Calculations.

Equation 1

Homeostatic model assessment for insulin resistance (HOMA-IR)

HOMA-IR = (fasting plasma glucose (mg/dl) x fasting insulin level (µU/ml))/405. (302)

Equation 2

Index of insulin sensitivity derived from an oral glucose tolerance test developed by Matsuda et al.

Matsuda index = 10,000/square root of [(fasting plasma glucose (mg/dl) x fasting insulin level (µU/ml)) x (average glucose during OGTT x average insulin during OGTT)]. (303)

Equation 3

Glycolysis under anaerobic conditions

$Glucose + 2\ Pi + 2\ ADP \rightarrow 2\ lactate + 2\ ATP + 2\ H_2O$

Equation 4

Glycolysis under aerobic conditions

$Glucose + 2\ Pi + 2\ NAD^+ + 2\ ADP \rightarrow 2\ pyruvate + 2\ ATP + 2\ NADH + 2\ H^+ + H_2O$

Equation 5

Respiratory exchange ratio (RER) calculation (304, 305)

$RER = VCO_2/VO_2$; $RER_{carbohydrate} = 1.0$; $RER_{palmitate} = 0.7$

Equation 6

Metabolic flexibility calculation (306, 307)

Metabolic flexibility = $RER_{insulin}$ - RER_{basal} (during the hyperinsulinemic-euglycemic clamp procedure)

VITA
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EDUCATION

University of Illinois at Chicago, Chicago, Illinois August 2012 - Present
Ph.D., Nutrition and Kinesiology – Expected Graduation Spring 2017
Mentor: Jacob M. Haus, Ph.D.
Coordinated Program in Dietetics – Expected Graduation Spring 2017
Advisor: Jennifer Bathgate, MS, RDN

Case Western Reserve University, Cleveland, Ohio August 2007–May 2012
B.A., Nutrition
Advisors: James Swain, Ph.D. and Hope Barkoukis, Ph.D.
Project: Nutritional Regulation of Skeletal Muscle Protein Synthesis
Completed Didactic Program in Dietetics

PROFESSIONAL POSITIONS

University of Illinois at Chicago, Chicago, Illinois August 2012 – Present

Teaching Assistant Department of Kinesiology and Nutrition, 2-4 courses equivalent to 20 course hours/semester
Advisor: Kirsten Straughan, MS, RDN

Lecturer Department of Kinesiology and Nutrition, ~5 lectures/semester at the graduate and undergraduate level

Graduate Research Assistant

- Experience using a clinical-translational research approach involving:
  - Human, cell culture, and enzymatic activity studies
- Clinical procedures:
  - Hyperinsulinemic-euglycemic clamp
  - Aerobic exercise training & maximal effort testing
  - Skeletal muscle biopsy & tissue processing
  - Nutrition counseling
  - IRB/Recruitment/Subject screening
  - Research design
- Cell culture:
  - Human skeletal muscle and HUVEC lines
  - Experimental design & method development
- Wetlab procedures:
  - Western blotting
  - Immunoprecipitation

- PCR
- ELISA
- Enzymatic activity assays
- Method design and development

Cleveland Clinic, Cleveland, Ohio June 2011 – August 2012
Undergraduate Research Assistant

- Undergraduate experience in clinical-translational research
- Manage individual research projects
- Peer-review papers for publication
- Nutrition and lifestyle counselor for STAMPEDE II study
- Undergraduate project:
  - Nuclear Orphan Receptors (NR4A) in Skeletal Muscle: Effects of a 12-Week Lifestyle Intervention

OTHER ACTIVITIES

Abstract Reviewer

- American Diabetes Association 74th Annual Sessions (2014)
- PLOS ONE (2014-present)

Academy of Nutrition and Dietetics, Healthy Aging Dietetic Practice Group

- Executive Committee Student Member (2016-2017)

Student Nutrition Association, University of Illinois at Chicago (2013-Present)

- Member (2013-Present)
- President (2014-2015)

AWARDS

Academic Awards

- President's Scholarship, Case Western Reserve University (4-year recipient, 2007-2010)
- Graduate Student Travel Stipend – Kinesiology and Nutrition Department, UIC (2012)
- Graduate Student Council Travel Stipend – Graduate College, UIC (2016)

Teaching Assistantship Awards

- Certificate of Excellence: Outstanding Graduate Teaching Assistant Award (Two-Time Recipient for the Academic Years 2012-2013, 2013-2014)

Graduate Research Awards

- Recognition for Achievement, Research and Excellence (RARE) – Kinesiology and Nutrition Department, UIC (2015)

Community Service Awards

- Kids Eat Right – Everyday Heroes (Academy of Nutrition and Dietetics, December 2015) http://bit.ly/1XGFgO0

PROFESSIONAL MEMBERSHIPS

Student Member of the AND, Academy of Nutrition and Dietetics (2010 – present)

- SCAN (Sports, Cardiovascular and Wellness Nutrition practice group) student member (2010-present)
- SCAN Symposium Attendee (2011, 2012, 2013)
- Healthy Aging DPG Executive Committee Student member (2015-present)

Student Member of ACSM, American College of Sports Medicine (2012-present)
- ACSM student member (2012-present)
- ACSM National Symposium attendee (2012)

Student Member of the ADA, American Diabetes Association (2013 – present)
- Abstract submission and poster presentation (2013)

Graduate Student Reviewer, University of Illinois, Chicago - Interdisciplinary Undergraduate Research Journal (2015)
- Review undergraduate research article submissions

RESEARCH SUPPORT
12/30/2015-12/30/2016: Total funds: $1000 *Sigma Xi, Grant in Aid of Research Award*. "Glyoxalase-1 in Skeletal Muscle with Aging and Exercise." The purpose of this work is to identify GLO1 abnormalities with aging and the benefits with exercise. ***Role: (Pre-doctoral) PI***

PUBLICATIONS
Trepanowski JF, **Mey J**, Varady KA. Fetuin-A: a novel link between obesity and related complications. Int J Obes (Lond) 2014 Dec 3. doi: 10.1038/ijo.2014.203. [Epub ahead of print] PMID: 25468829

Williamson DL, Dungan CM, Mahmoud AM, **Mey JT**, Blackburn BK, Haus JM. Aberrant REDD1-mTORC1 responses to insulin in skeletal muscle from type 2 diabetics. Am J Physiol Regul Integr Comp Physiol. 2015 Aug 12. doi: 10.1152/ajpregu.00285.2015. [Epub ahead of print] PMID: 26269521

Mahmoud AM, Szczurek MR, Blackburn BK, **Mey JT**, Chen Z, Robinson AT, Bian JT, Unterman TG, Minshall RD, Brown MD, Kirwan JP, Phillips SA, Haus JM. Hyperinsulinemia Augments Endothelin-1 Release and Impairs Vasodilation of Human Skeletal Muscle Arterioles. Physiol Rep. 2016 Aug;4(16). pii: e12895. Epub 2016 Aug 22.

OTHER SCHOLARLY WORKS
Mey, JT. Advanced Glycation Endproducts and Their Role in the Aging Process. The Spectrum. Healthy Aging: A Dietetic Practice Group of the Academy of Nutrition and Dietetics. (Winter) 2016.

ABSTRACTS
Haus JM**, Mey JT**, Solomon TP, Kasumov T, Zhang R, Kirwan JP. Methylglyoxal and glyoxal-derived hydroimidazolone-AGEs are reduced with aerobic exercise training plus a glycemic index diet in obese subjects. American College of Sports Medicine, San Franscisco, CA, 2012

Jacob T. Mey, Lee E. Nadolski, Thomas P. J. Solomon, Ciaran E. Fealy, Steven K. Malin, John P. Kirwan, Jacob M. Haus. Diminished Glyoxalase I Activity in Skeletal Muscle of Obese Individuals is Associated with Determinants of Metabolic Health – American Diabetes Association, Chicago 2013 - accepted as poster **(Presenting Author)**

Abeer M. Mahmoud, **Jacob T. Mey**, Thomas P.J. Solomon, Ciaran E. Fealy, Steven K. Malin, John P. Kirwan, Jacob M. Haus. Glyoxalase 1 protein expression in human skeletal muscle is normalized following aerobic exercise training in obese insulin resistant adults - Biochemical Society: Glyoxalase Centennial, Warwick, UK 2013.

Abeer M. Mahmoud, Brian K. Blackburn, Karia Coleman, **Jacob T. Mey**, Vikram S. Somal, Thomas P. J. Solomon, Ciaran E. Fealy, Steven K. Malin, John P. Kirwan, Jacob M. Haus Aerobic Exercise Induces

RAGE Shedding via ADAM10 in Human Skeletal Muscle - 13th Biennial Advances in Skeletal Muscle Biology in Health and Diseases Conference, Gainesville, FL 2014

Abeer M. Mahmoud, **Jacob T. Mey**, Thomas P.J. Solomon, John P. Kirwan, Jacob M. Haus. Aerobic Exercise Reduces Pro-inflammatory Cytokines in Skeletal Muscle Via Down Regulation of RAGE in Obese Insulin Resistant Adults – American Diabetes Association, Boston, MA 2015

Jacob M. Haus, Vikram S. Somal, Sarah S. Farabi, **Jacob T. Mey,** Brian K. Blackburn, Karia Coleman, Laurie Quinn. Acute Aerobic Exercise Increases Plasma Soluble Receptor of Advanced Glycation Endproducts (sRAGE) in Obese Insulin Resistant Adults – American Diabetes Association, Boston, MA 2015

Jacob T. Mey, Abeer M. Mahmoud, Jacob M. Haus. GLO1 Protein Expression In Reduced In Individuals With T2DM And May Be Related To Chronic Hyperinsulinemia – Experimental Biology, San Diego, CA 2016

Jacob T. Mey, Jacob M. Haus. Human Skeletal Muscle Proteins and Metabolic Pathways Are Susceptible To Dicarbonyl Stress – Experimental Biology, Chicago, IL 2017 (Submitted)

REFERENCES

Kirsten Straughan, RD, MS *Former Employer (University of Illinois at Chicago)*, 4 years (312-996-7890)

John Kirwan, PhD *Former Employer (Cleveland Clinic)*, 6 years (216-440-3900)

Jacob Haus, **PhD** *Current Mentor (University of Illinois at Chicago)*, 6 years (330-518-8225)