A PEDAGOGICAL AND PHYSIOLOGICAL APPROACH TO TREATING TYPE 2 DIABETES

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Abstract

Background: Lack of exercise and sedentary behavior are primary risk factors for the development of type 2 diabetes mellitus (T2DM). Furthermore, physical activity (PA) and exercise are effective therapeutic tools for reducing diabetic complications and may reduce mortality rates. Despite this evidence, only 65% of adults and 47% of college students in the United States meet the suggested physical activity guidelines, indicating that human behavior plays a fundamental role in the increasing prevalence of T2DM. Human behaviors are formed through a reasoning process. Therefore, a reasoning-based approach may prove efficacious in improving PA. Furthermore, the mechanisms behind the beneficial effect of exercise on T2DM remain elusive. Oxidative stress plays a causal role in diabetic complications. The transcription factor, NF-E2–related factor 2 (Nrf2) protects against oxidative stress and exercise increases Nrf2 in healthy humans. Therefore, research regarding the effect of exercise on Nrf2 in T2DM is warranted. **Purpose:** The purpose of this dissertation was to conduct two separate lines of research to explore behavioral and physiological aspects of exercise and diabetes. **Methods of study one:** College-aged individuals participated in an online higher-level reasoning based educational intervention designed to increase PA. Measures of PA and social-cognitive variables regarding PA were assessed pre and post intervention. **Methods of study two:** normal and diabetic (*db/db*) mice underwent an acute exercise bout and Nrf2 activity was examined. We further explored the role of O-GlcNAcylation on Nrf2 signaling using an O-linked N-acetylglucosamine transferase (OGT) knockdown mouse and

conducted *in vitro* studies in cardiomyocytes. **Results of study one:** The reasoning based intervention significantly increased the level of reasoning and leisure time PA amongst the experimental group. **Results of study two:** acute exercise increases Nrf2 protein content and mRNA expression Nrf2 transcription only in the normal mice. We also showed that protein O-GlcNAcylation is altered in the *db/db* mouse heart. Loss of the OGT enzyme resulted in drastic reduction in Catalase mRNA, indicating reduced Nrf2 transcription. Conversely, increasing O-GlcNAcylation in H9C2 cells augmented Nrf2 transcription. Lastly, we showed that Nrf2 is O-GlcNAcylated and *in silico* analysis identified Thr^{595} as a possible O-GlcNAcylation site near NLS and co-activation motif. **Conclusions:** The results from study one suggest that improving reasoning regarding PA improves leisure-time physical activity (LTPA) and cognitive domains associated with PA amongst college students. The results from study two suggest that and exercise-induced increase in the Nrf2 response is blunted in the *db/db* mouse heart, indicating that an acute-exercise elicits a lower Nrf2 response in diabetes. Furthermore our results demonstrate O-GlcNAc as a novel mechanism of regulation in the Nrf2 signaling cascade.

Acknowledgments

“If I have seen further, it is by standing upon the shoulders of giants” ~ Issac Newton

A doctoral thesis, while original in concept, is not the work of one mind. Nor is it the product of a sole individual’s effort; it is the consequence of the coalescing of minds and the synergistic result of all those involved. It is for these reasons, along with sincere humility, that I thank and acknowledge several people.

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Dedication

To my parents, for without you I would not be here. To my mentors, for without you I would be lost. To science, for without you my world would lack wonder.

Table of Contents

Authorization to Submit Dissertation ii

Abstract iii

Acknowledgments v

Dedication vii

Table of Contents viii

List of Figures xiv

List of Tables xvi

Chapter 1: Introduction 1

- Background of the Problem 1
- Setting of the Problem for Study 1 3
- Setting of the Problem for Study 2 5
- Statement of the Problem 8
- Specific Aims. 8
- Hypotheses 9
- Variables 9
- Delimitations 10
- Limitations 10
- Definitions 11

Chapter 2: Review of the Literature 15

- Epidemiology of Cardiovascular Disease and Diabetes 15
- Study 1: A Higher-Level Reasoning-based Approach to Improving Physical Activity Knowledge and Behaviors 15

Levels of cognition and orders of thinking. 18

Higher-level reasoning requires education, reflection, and practice. 20

Application of higher-level reasoning-based education interventions. 22

Framework to implement reasoning-based Intervention 26

Social Cognitive Theory and relevant constructs. 27

Social Cognitive Theory and exercise interventions. 30

Integrating reasoning constructs and SCT constructs. 34

Study 2: Physiological Mechanisms of Exercise and Diabetes 36

Properties of Nrf2. 37

Nrf2 and vascular complications. 38

Nrf2 and pathological cardiac hypertrophy. 41

Regulation of Nrf2. 43

Exercise increases Nrf2 activation and antioxidant gene expression. 49

Diabetes and exercise differentially alter protein O-GlcNAcylation 50

Summary 51

Chapter 4: A Reasoning-Based Educational Intervention Utilizing Social Cognitive Theory Improves Physical Activity Through Enhancing Reasoning, Goal Setting, and Self-Regulation. 54

Introduction 54

Methods 61

Human participants. 61

Educational intervention. 62

SCT variables of physical activity. 63

Physical activity data. 64
Levels of reasoning. 64
Data analysis. 65
Results 65
Effect of intervention reasoning levels. 65
Physical activity. 66
Social cognitive theory domains. 66
Discussion 67
Reasoning-based intervention improves reasoning about physical activity 67
Reasoning-based intervention improves physical activity and Social Cognitive Theory domains related to physical activity. 70
Strengths and limitations. 75
Conclusion. 76
References 79
Chapter 5: Exercise induced Nrf2 response is blunted in the Db/Db mouse and O-GlcNAc regulates Nrf2 activity 85
Abstract 87
Introduction 88
Methods 91
Animals 91
Exercise Protocol 92
Cell Culture/In Vitro 92

Western Blotting 93
RNA Isolation and qPCR 94
Immunoprecipitation 94
O-GlcNAcylation site prediction 95
Statistical analysis. 95
Results 96
Animal characteristics 96
Nrf2 response to acute exercise is blunted in the db/db mouse heart. 96
Protein O-GlcNAcylation and OGT protein content is altered in the db/db mouse heart. 97
Loss of OGT enzyme decreases Nrf2 gene products 99
O-GlcNAc increases Nrf2 transcription in response to cellular stress *in vitro* 100
Nrf2 is O-GlcNAcylated and prediction analysis of Nrf2 glyocsylation sites identifies Thr^{594} as a possible O-GlcNAcylation site. 102
Discussion 104
Exercise-induced Nrf2 response is attenuated in the *db/db* mouse heart 104
O-GlcNAc of high molecular weight proteins and levels of OGT are reduced in the *db/db mouse heart* 106
O-GlcNAc positively regulates Nrf2 106
Nrf2 is O-GlcNAcylation may occur near a localization and DNA-binding regulatory motif 109

Conclusions 112

Chapter 6: A Proposed Mechanism for Exercise Attenuated Methylglyoxal Accumulation: Activation of the ARE-Nrf Pathway and Increased Glutathione Biosynthesis 124

Background 124

Presentation of the hypothesis. 126

Evaluation of the hypothesis. 127

References 132

Chapter 7: Dysregulation of Nrf2 signaling in diabetes: An opportunity for a multi-target pharmaceutical approach 137

Introduction 137

Manuscript 137

Nrf2 in type 2 diabetes mellitus 139

Mechanisms of Nrf2 signaling and dysregulation in diabetes. 141

The Nrf2 transcription factor 142

Nrf2 is negatively regulated by Keap1 144

Nrf2 is negatively regulated by Glycogen Synthase Kinase 3 153

Transcriptional regulation of Nrf2 by acetylation/deacetylation 160

Future perspectives 164

References 167

References 177

Appendix A: Original Proposal 198

Appendix B: IRB Forms 240

Appendix C: IACUC Approval 242

Appendix D: Surveys and Questionnaires 244

Appendix D: Student Writing Samples 263

Early Writing Samples 263

Student 1. 263

Student 2. 266

Student 3. 269

End Writing Samples 272

Student 1. 272

Student 2. 275

Student 3. 277

List of Figures

Figure 2.1. The formal process of higher-level reasoning through education, reflection and practice. 21

Figure 2.2. Integration of the reasoning process into SCT variables of physical activity behavior change. 36

Figure 2.3. Nuclear translocation of Nrf2 and antioxidant gene expression. 38

Figure 2.4. The glyoxalase pathway and methylglyoxal detoxification. 41

Figure 2.5. Regulation of Nrf2 44

Figure 2.6. Effect of acetylation and deacetylation on Nrf2 transcription. 45

Figure 2.7. Role of HDAC2:Nrf2 association on Nrf2 activity. 46

Figure 2.8. O-GlcNAc modification of Nrf2 and role in localization and/or activity ... 48

Figure 3.1. Effect of intervention on reasoning levels.. 66

Figure 3.2. Integration of the reasoning process into SCT variables of physical activity behavior change 74

Figure 4.1.The effect of acute exercise on Nrf2 protein content and Nrf2-mediate gene products in C57/B6J and *db/db* mice. 97

Figure 4.2. The effect of acute exercise on protein O-GlcNAcylation and OGT protein content in C57/B6J and *db/db* mice. 99

Figure 4.3. Nrf2 function in OGT KD mice 100

Figure 4.4.The effect of angiotensin II, N-acetylglucosamine (GlcNAc), and Thiamet G on Nrf2 function in H9C2 cells..... 102

Figure 4.5. O-GlcNAcylation of Nrf2 and *in silico* prediction of protein glycosylation of Nrf2 by the YinOYang 1.2 server identifies Ser^{594} as a site possible site of O-GlcNAc modification 103

Figure 5.1. MGO formation from glucose and lipids and detoxification to pyruvate 125

Figure 5.2. Exercise and the antioxidant response element-nuclear respiratory factor (ARE-Nrf) pathway..... 129

Figure 6.1. Structures of Nrf2 and Keap1 and Keap1-mediated ubiquitination and GSK-3/β-TrCP mediated ubiquitination..... 143

Figure 6.2. Cytosolic regulation of Nrf2..... 159

Figure 6.3. Nuclear regulation of Nrf2. 162

Figure 6.4. Effect of diabetes on Nrf2 signaling and current mechanism of action of current therapeutics 165

List of Tables

Table 3.1. Effect of intervention on reasoning levels..... 78

Table 3.2. Effect of Intervention on Leisure Time Physical Activity..... 78

Table 3.3. Between group comparisons for social cognitive theory variables..... 78

Table 4.1. Animal Characteristics for exercised mice 123

Table 4.2. Primer Sequences 123

Chapter 1: Introduction

Background of the Problem

Lack of exercise and sedentary behavior are primary risk factors for the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Hu, Leitzmann, Stampfer, Colditz, Willet, & Rimm, 2001; LaMonte, Blair, & Church, 2005; Berlin & Colditz, 1990). Concurrently, increased levels of exercise reduce risk for development of T2DM and CVD (LaMonte, Blair, & Church, 2005; Kohl, Gordon, Villegas, & Blair, 1992; Berlin & Colditz, 1990). Furthermore, physical activity and exercise are effective therapeutic tools for reducing complications from these diseases and may reduce mortality rates (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000; Okada, et al., 2010; Boor, et al., 2009; Broderick, Poirier, & Gillis, 2005). Despite this evidence, only 65% of adults and 47% of college students in the United States meet the suggested physical activity guidelines, indicating that human behavior and lifestyle play a fundamental role in the increasing prevalence of T2DM. (State indicator report on physical activity, 2010; American College Health Association-National College Health Assessment II, 2009). Exercise and physical activity are a known necessity for optimal health, but that knowledge does not always generate behavior change. Education has always been argued as the means to alter behavior, but current educational models are not effective. Thus, there is a need to understand physical activity behavior, and to develop more effective methods to improve knowledge of the benefits of exercise and behaviors regarding exercise and physical activity in young adult populations of the United States.

(Haskell, et al., 2007; American College Health Association-National College Health Assessment II, 2009).

Physical activity and exercise are effective therapeutic tools for reducing complications from these diseases and may decrease mortality rates (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000; Okada, et al., 2010; Boor, et al., 2009; Broderick, Poirier, & Gillis, 2005); however, the physiological mechanisms behind exercise attenuated mortality and morbidity rates in T2DM are not completely understood. Currently, the majority of medical interventions utilize pharmacological agents to treat T2DM and subsequent complications; however, there is increasing evidence that exercise provides the same benefits without the potential side-effects of current medication (Sharoff, et al., 2010). Thus, there is a need to add knowledge to the literature surrounding the physiology of diabetic complications and to provide sound evidence of how exercise reduces complications and mortality in diabetes.

Evidence suggests diabetes increases the risk of developing CVD by three-fold (Saydah, Miret, Sung, Varas, Gause, & Brancati, 2001). Currently, 8.3% of the American population has been clinically diagnosed with diabetes and it is estimated that over 35% of individuals over the age of 20 years have hyperglycemic conditions (National diabetes fact sheet, 2011; Cowie, et al., 2009). Cardiovascular disease and complications from T2DM are among the leading causes of death in the United States (Hoyert & Xu, 2011). Given that the high prevalence of T2DM and the increased risk of comorbidities, it is abundantly clear that T2DM presents a major and escalating health concern for American society.

When taken together, both physiological and behavioral factors are central in the high prevalence of T2DM and related diseases. Therefore, the purpose of this dissertation is to conduct two separate studies to explore these two distinct, yet conceptually related facets of T2DM and to add to the literature on both aspects.

Setting of the Problem for Study 1

Lack of physical activity is a risk factor for the development of obesity, T2DM, and CVD (Hu, Leitzmann, Stampfer, Colditz, Willet, & Rimm, 2001; Powell, Thompson, Caspersen, & Kendrick, 1987; Myers, 2003). Furthermore, physical activity and exercise are effective therapeutic tools for reducing complications from these diseases (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000; Okada, et al., 2010; Boor, et al., 2009; Broderick, Poirier, & Gillis, 2005). Even with evidence highlighting the efficacy of physical activity in preventing and treating chronic disease, only 65% of adults and 47% of college students in the United States meet the suggested physical activity guidelines (State indicator report on physical activity, 2010; American College Health Association-National College Health Assessment II, 2009). The relationship between lack of physical activity and the staggering rates of CVD and T2DM in the adult population highlights a dire need to develop programs aimed at increasing physical activity amongst the adult populations of the United States.

Behaviors, such as physical activity behaviors, are a skill set. They are acquired over a long time, informed by consciously articulated principles and reasons that are otherwise ‘second-natured’ into the cognitive unconscious (Damasio, 2010, p. 287). This indicates that human behaviors are formed through a reasoning process.

Therefore, a reasoning-based approach may prove efficacious in improving knowledge, attitudes, and behaviors regarding exercise and physical activity.

Currently, there is a paucity literature investigating the use of high-ordered reasoning interventions to improve physical activity behaviors; however, there is evidence that reasoning-based interventions can improve knowledge, attitudes, and behaviors in other fields. Higher-ordered reasoning-based educational interventions altered behavior in college-aged students regarding ethics (Stoll & Dieter, 2013; Grant, 2012). While these data do not directly support the hypothesis that a reasoning-based intervention can significantly improve physical activity, it does present strong evidence that a reasoning-based curriculum can alter reasoning and behaviors regarding the area in which the curriculum is focused.

Effective educational interventions aimed at changing behavior require a theoretical framework to inform and guide the curriculum. Furthermore, the framework must address aspects of behavior specific to the target behavior, i.e., physical activity. Thus, a theoretical framework capable of explaining how individuals develop and sustain physical activity behavior is critical in the development of a reasoning-based program aimed at modifying behavior. Research has demonstrated the efficacy of using social cognitive theory (SCT) based education intervention programs to improve exercise behaviors in older adult population (Anderson, Winett, Wojcik, & Williams, 2010; Anderson-Bill, Suppini, & Apap, 2011; Hallam & Petosa, 20004; Doerksen, Umstattd, & McAuley, 2009). Despite the success of these interventions, there is little research exploring the efficacy of online SCT-based educational in improving exercise behaviors in college-aged populations.

Furthermore, online interventions provide a vector through which educational interventions can reach a wider audience and require fewer resources for implementation. Therefore, the purpose of study 1 is to develop a reasoning-based online educational intervention based in SCT theory and examines its efficacy in improving knowledge, attitudes, and behaviors towards exercise in a population of university students.

Setting of the Problem for Study 2

Diabetes affects 8.3% of the American population and increases the risk for associated complications including CVD and nephropathy (Cowie, et al., 2009). Exercise is an effective tool for reducing complications in diabetes and may reduce overall morbidity and mortality rates (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000). While benefits are well described in the literature, there is a lack of data describing physiological mechanisms for the therapeutic benefits of exercise in diabetes.

Oxidative and chemical stress is central to the development of diabetic complications and causes early cell death (Shen, Zheng, Metreveli, & Epstein, 2006; Kitada, Kume, Imaizumi, & Koya, 2011). A redox sensitive transcription factor, NF-E2–related factor 2 (Nrf2) and the Nrf2 antioxidant-response pathway are one of the major cellular defenses against the cytotoxic effects of oxidative stress (Zhong, Mishra, & Kowluru, 2013). The reduced activity of Nrf2 observed in humans and animals with diabetes has been suggested to have a causative effect in diabetic complications (Tan et al, 2011; Zhong et al., 2013), while evidence indicates that increasing Nrf2 activity in diabetic animals reduces complications (Zheng, et al.,

2011). Furthermore, human and animal studies have demonstrated increases in Nrf2 activity following exercise (Cartoni, et al., 2005; Baar, et al., 2002); however, a mechanism has not been fully elucidated.

Diabetes is associated with increased O-linked N-acetylglucosamine (O-GlcNAc) modification of proteins (Clark et al., 2003). O-GlcNAc is a sugar moiety that can post-translationally modify serine/threonine residues of proteins (Ande, Moulik, & Mishra, 2009; Chou, Hart, & Dang, 1995). Post-translational modifications of proteins, including O-GlcNAcylation can induce protein conformational changes. Conformational changes of proteins can alter cell signaling and metabolic processes in the cell.

In addition to O-GlcNAcylation, proteins can also be modified by phosphorylation. Phosphorylation is the addition of a phosphate molecule to a protein. Like O-GlcNAcylation, phosphorylation also induces conformational changes of proteins and alters cell signaling and metabolic processes. Phosphorylation and O-GlcNAcylation can modify the same amino acid residues (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011). Thus, phosphorylation and O-GlcNAcylation can compete for post-translational modifications; when an amino acid residue is O-GlcNAcylated it cannot be phosphorylated and vice versa. When a protein is phosphorylated it displays different functions and results in different cellular processes than when it is O-GlcNAcylated (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011).

Phosphorylation is controlled by kinases and phosphatases. Briefly, phosphate groups are enzymatically added to proteins by kinases and removed by

phosphatases. O-GlcNAcylation is also enzymatically regulated. The enzyme O-GlcNAc transferase (OGT) adds O-GlcNAc moiety to proteins while O- GlcNAcase (OGA) removes O-GlcNAc. There are hundreds of kinases and phosphatases; however attachment and removal of O-GlcNAc are catalyzed by only two enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase, respectively (Haltiwanger, Holt, & Hart, 1990; Braidman, et al., 1974). This suggests two things: first that the O-GlcNAc modification plays a critical role in cell signaling, and secondly, O-GlcNAc modification is more responsive to perturbations in the cellular environment and has a more ubiquitous role in cell signaling than a single phosphorylation action. Thus O-GlcNAcylation is likely to play an essential role in Nrf2 signaling.

Currently, there is a lack of research exploring the role of O-GlcNAc in the Nrf2 signaling cascade. Despite this substantial gap in the Nrf2 literature, cursory evidence suggests O-GlcNAc mediates some aspect of Nrf2 signaling. Ngoh and colleagues demonstrated that mRNA levels of Nrf2-mediated gene product catalase are increased when protein O-GlcNAcylation is upregulated by inhibiting OGA in the presence of oxidative stress in neonatal rat cardiomyocytes (Ngoh, Watson, Facundo, & Jones, 2011). Additionally when they overexpressed OGT using and adenovirus, reactive oxygen species (ROS) were greatly reduced in the neonatal rat cardiomyocytes treated with hydrogen peroxide (H_2O_2). Conversely overexpression of OGA increased ROS in neonatal rat cardiomyocytes. Despite this compelling evidence, Nrf2 was never examined in this paper and the role of O-GlcNAc on Nrf2 signaling has never been directly been explored.

The cursory evidence suggests that O-GlcNAc plays a role in Nrf2 signaling. As Nrf2 activity is regulated, in part, by posttranslational modifications, it is likely O-GlcNAc directly modifies Nrf2. The YinOYang1.2 prediction server utilized protein amino acid sequences to identify possible O-GlcNAc modification sites. Using the YinOYang1.2 prediction server to identify possible target amino acids, I identified a key amino acid residue, Thr^{594}, which is likely to be O-GlcNAcylated. Thr^{594} lies immediately adjacent to a motif, $P_{587}KSKKPD_{593}$, which harbors a nuclear localization signal and two key acetylation sites, which regulate Nrf2 transcription. This suggests that O-GlcNAc may indeed play a role in nuclear localization and/or binding affinity of Nrf2. Therefore, the purpose of study 2 is two examine the role of exercise on Nrf2 in an animal model of diabetes and to explore the role of O-GlcNAc on Nrf2 activity.

Statement of the Problem

The purpose of this dissertation is to examine two unique, but related facets of diabetes: 1) to increase participant knowledge of the interaction of exercise and health and improve exercise and physical activity behaviors and 2) to use an animal model to understand how exercise prevents and attenuates T2DM and its complications at a molecular level.

Specific Aims.

1. To create an online educational intervention to increase participants' higher-level reasoning and psychological mediators of behavior change in regards to exercise in college-aged individuals.

2. To utilize an online higher-level reasoning-based educational intervention to effectively increase participants' physical activity and exercise behavior in college-aged individuals.
3. To determine whether exercise increases Nrf2 and activation of the endogenous antioxidant system in diabetes.
4. To establish a role for O-GlcNAc modification and the OGT enzyme in the Nrf2 signaling cascade.

Hypotheses

1. **Study One**
    a. An online higher-level reasoning-based intervention utilizing Social Cognitive Theory will improve higher-level reasoning and psychological mediators of behavior change regarding physical activity and exercise in college-aged adults.
    b. An online higher-level reasoning program will improve exercise and physical activity behavior in college-aged adults.
2. **Study Two**
    a. Acute exercise will increase Nrf2 and alter O-GlcNAcylation in the diabetic mouse heart.
    b. O-GlcNAc modification will play a role in Nrf2 signaling.

Variables

1. **Pedagogy Study**
    a. **Independent Variables:** Higher-level reasoning-based Social Cognitive Theory intervention/ control, gender

   b. **Dependent Variables**: Measures of SCT related to physical activity (self-efficacy, self-regulation, goal setting), levels of physical activity, and reasoning related to physical activity.

2. **Physiology Studies**
   a. **Independent Variables:** Exercise/non-exercise, diabetic/non-diabetic, OGT KD/ WT, AngII and O-GlcNAc/Thiamet G.
   b. **Dependent Variables:** Nrf2 protein levels, OGT protein levels, O-GlcNAcylation of Nrf2 protein and global proteins, mRNA levels of GCLM, HMOX1, NQO1, and Catalase

Delimitations

1. Students for the intervention group will be those enrolled in an online physical activity and health-based course. There will be no randomization sampling procedure for this intervention.
2. Measuring biochemical aspects of the Nrf2 signaling pathway in exercised tissue is highly invasive; thus, human subjects will not be used to collect the physiological data. Instead, diabetic mouse models will be used.

Limitations

1. Students for the intervention group will not be randomly selected. Therefore, the sample may not be an accurate representation of the population.

2. All physiological data regarding hypotheses 1 and 2 will be derived from animal models. As a result this imposes some limitations in extrapolation to humans.

Definitions

- Acetylation – a post-translational modification in which an acetyl group is added onto a molecular compound.
- Advanced Glycation End Products (AGEs) – the end result of a chain of chemical reactions beginning with glycation. AGES are causal in multiple pathologies and are elevated in diabetes.
- Cardiac Hypertrophy – thickening of the heart muscle, specifically of the ventricular walls.
- Cardiovascular disease – disease of the heart or blood vessels.
- Glomeruli – a network of capillaries located at the beginning of the nephron in the kidney involved in filtering blood.
- Glutathione (GSH) – a tripeptide of glutamate, cysteine, and glycine. GSH is an endogenous antioxidant responsible for maintaining proper redox status *in vivo.*
- Glycation – a non-enzymatic reaction involving covalent bonding of a sugar with a protein or lipid.
- Goal setting – developing plans to accomplish chosen behaviors.
- HepG2 Cells – an *in vitro* cell model that allows for examination of hepatic (liver) cell function.

- Histone deacetylase (HDAC) – a class of enzymes that remove acetyl groups from lysine amino acids on histones and other proteins.
- Kelch-like ECH-associated protein 1 (Keap1) – cytosolic inhibitor of Nrf2.
- Myocardial fibrosis – excess fibrous connective tissue in the myocardium form in response to cardiac injury or insult.
- NIH 3T3 – standard fibroblast cell line that enables *in vitro* analysis of fibroblasts.
- Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) – Nrf2 is a basic leucine zipper (bZIP) transcription factor featuring a Cap ‘‘n’’ collar (CNC) structure and is the key transcription factor regulating the antioxidant response.
- O-linked attachment of β-N acetyl-glucosamine (O-GlcNAc) – a post-translational modification in which O-GlcNAc is added onto a molecular compound. O-GlcNAcylation has been implicated in the pathogenesis of diabetic conditions.
- Oxidative stress – an imbalance between the production of prooxidants (reactive oxygen/nitrogen species) and antioxidants.
- Phosphorylation – a post-translation modification in which a phosphate group is added to a molecule, altering its function. Phosphorylation plays a major role in determining cellular function and processes.
- Reactive oxygen/nitrogen species – chemically reactive molecules containing oxygen or nitrogen. Examples include: superoxide radical

($\bullet O_2^-$), hydroxyl radical (HO•), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($ONOO^-$).

- Self-efficacy – the confidence a person has in his or her ability to pursue a behavior, it is behavior-specific and a function of the present.
- Self-regulation – the sense that self-corrective adjustments are taking place as needed to stay on track toward achieving the purpose, and the sense that the corrective adjustments originate within the person.
- Small interfering RNA (siRNA) – interferes with the expression of specific genes through complementary nucleotide sequencing. SiRNA is used to effectively silence gene expression by destroying mRNA and preventing protein translation.
- Social Cognitive Theory (SCT) – Social cognitive theory posits that human behavior can be explained by behavior, environmental factors, and personal factors.
- Streptozotocin (STZ) – a toxic chemical that induces dysfunction of pancreatic beta cells. Large doses induce T1DM and STZ used to produce animal models of T1DM.
- Type 1 diabetes mellitus (T1DM) – a form of diabetes mellitus that results from autoimmune destruction of the insulin producing beta cells in the islet of Langerhans in the pancreas.
- Type 2 diabetes mellitus (T2DM) – a form of diabetes mellitus that arises from the development of peripheral insulin resistance and a subsequent

reduction in pancreatic function. Hyperglycemia, hyperlipidemia, insulin resistance, and/or insulin deficiency characterize T2DM.

- Ubiquitylation/ubiquitination – enzymatic post-translation modification process where an ubiquitin protein is attached to a substrate, effectively marking it for degradation in the proteasome.
- γ-glutamyl cysteine ligase-catalytic (GCLC) – The initial enzyme in the GSH biosynthesis pathway that catalyzes the condensation of cysteine and glutamate to form gamma-glutamylcysteine.
- γ-glutamyl cysteine ligase-modulatory (GCLM) – an enzyme involved in GSH biosynthesis that catalyzes the efficiency of GCLC.

Chapter 2: Review of the Literature

Epidemiology of Cardiovascular Disease and Diabetes

Cardiovascular disease (CVD) is the leading cause of death in the United States, accounting for 25% of all deaths in 2008 (Hoyert & Xu, 2011; Heron, 2008). Evidence suggests diabetes increases the risk of developing CVD three-fold and 25% of individuals with T2DM will develop other vascular complications, such as nephropathy, within 10 years of diagnosis (Saydah, Miret, Sung, Varas, Gause, & Brancati, 2001; Alder, Stevens, Manley, Bilous, Cull, & Holman, 2003). It is estimated the worldwide prevalence of T2DM amongst adults (aged 20-79 years) is 285 million and will reach 439 million by the year 2030 (Shaw, Sicree, & Zimmet, 2010). As of 2009, 8.3% of the American population has been clinically diagnosed with diabetes and it is estimated over 40% of individuals over the age of 20 years have hyperglycemic conditions (Cowie, et al., 2009; National diabetes fact sheet: National estimates and general information on diabetes and prediabetes, 2011). Furthermore, an estimated $113 billion was spend on diabetes-related health care in 2009 (Huang, Basu, O'Grady, & Capretta, 2009). Epidemiological evidence clearly indicates that CVD and T2DM present a major health and financial concern. (National diabetes fact sheet: National estimates and general information on diabetes and prediabetes, 2011).

Study 1: A Higher-Level Reasoning-based Approach to Improving Physical Activity Knowledge and Behaviors.

Lack of physical activity is a risk factor for the development of T2DM and CVD (Powell, Thompson, Caspersen, & Kendrick, 1987; Myers, 2003; Kriska, et al.,

2003). The reduced levels of physical activity and pervasiveness of sedentary behavior in our society is significantly contributing to the exponential increase in obesity and obesity-related diseases such as CVD and T2DM (Hu et al. 2001; Lamonte et al. 2005). Conversely, physical activity and exercise are effective therapeutic tools for reducing complications from these diseases (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht et al., 2000; Okada et al., 2010; Boor et al., 2009). Independent of specific physiological mechanisms, exercise clearly improves the development and progression of diabetes and diabetic complications (Okada et al., 2010; Boor et al., 2009; Hambrecht et al., 2000).

Despite evidence highlighting the efficacy of exercise in preventing and treating chronic disease, only 65% of adults and 47% of college students in the United States meet the suggested physical activity guidelines (State indicator report on physical activity, 2010; American College Health Association-National College Health Assessment II, 2009). The relationship between lack of physical activity and high prevalence of CVD and T2DM highlights a dire need to develop programs aimed at increasing physical activity amongst the adult populations of the United States.

Behaviors are a skill set, acquired over repeated practice sessions and over a long time, informed by consciously articulated principles and reasons but otherwise ‘second-natured’ into the cognitive unconscious (Damasio, 2010, p. 287). In essence, behaviors result from physical manifestations of non-conscious and conscious mental processes that are formed directly from knowledge and practiced application of that knowledge. Subsequently, through the training of the conscious

mind, one is able to affect the decisions of the non-conscious mind and behaviors at multiple levels of cognition. Therefore, I argue that in order to positively change physical activity behaviors, one must first begin with the conscious mind, through which one can influence the decision making of nonconscious mind.

Cognitive educational interventions and physical activity behavior. Damasio (2010) clearly indicates that cognition and human intelligence drive behavior. The notion that knowledge and intelligence drives behavior has been the primary focus of interventions aimed improving exercise and physical activity behavior. While most research regarding physical activity in college students is descriptive, there is some literature showing that cognitive education interventions exist. Some colleges and universities have educational curriculums directed toward increasing knowledge that physical activity and exercise are beneficial for health, with the ultimate goal of influencing physical activity/exercise behaviors. These programs focus on skill and knowledge development, and result in moderate, short-lived improvements in physical activity. Two large-scale studies have utilized cognitive educational interventions to improve physical activity and exercise behavior amongst college-aged student, project GRAD (Graduate Ready for Activity Daily) and the Active Recreation on Tertiary Education Campuses (ARTEC) program. Project GRAD examined the effect of a university course on 321 university students (Sallis, et al., 1999). The course was 21.5 months long and utilized labs and lectures to teach behavior change and physical activity skills. The intervention had small increases in leisure time physical activity in women but showed no effect on men and long-term changes in physical activity habits (Sallis, et al., 1999). ARTEC, which was shorter in

length (8 weeks), utilized similar methodology as project GRAD, but also offered students a free activity class of their choice, fitness assessments, swimming vouchers for a nearby facility, and on-campus media promotion. Similar to project GRAD, the ARTEC program did not indicate that the interventions were successful in establishing habitual physical activity patterns (Leslie, Fotheringham, Veitch, & Owen, 2000). These findings suggest that current physical activity interventions are not designed to facilitate long-term, habitual behavior change. Habits play a critical role in physical activity and exercise adherence, and as such the ultimate goal of physical activity and exercise interventions ought to be long-term and habitual behavior change (Kretchmar, 2001; Keating, Guan, Pinero, & Bridges, 2010).

The lack of long-term and habitual changes in physical activity and exercise behavior suggests such interventions are lacking the necessary components to stimulate such a change. A recent meta-analysis of college student physical activity behaviors found that there is a lack of multi-level approaches to college students' physical activity, and an absence of physical education pedagogy specialists' involvement in research on students' PA (Keating, Guan, Pinero, & Bridges, 2010). Additionally, Riebe et al. (2005) found that individuals who maintained long-term (24 months) regular exercise had higher use of experiential and behavioral processes. Together, this suggests current educational interventions lack a higher level of cognition or order of thinking

Levels of cognition and orders of thinking.

Researchers, such as Kohlberg and Piaget, have described a developmental conception of intelligence, with cognitive processes underlying the intelligence in a

chronological process (Kohlberg & Lickona, 1976; Kohlberg, 1981; Reimer, Paolitto, & Hersh, 1983, pg. 19). The cognitive education formats present in the aforementioned studies (Leslie et al., 2000; Sallis et al., 1999) utilize mechanistic explanations of how exercise and physical activity are beneficial for health. The pedagogical formats of these programs tend to focus on what is known as a first order level of reasoning. In his work, Kohlberg flushed out three levels of reasoning and cognitive development (Kohlberg & Lickona, 1976; Kohlberg, 1981). The first level of reasoning involves instrumental purpose and exchange. In this order, one makes decisions to serve one's own immediate needs and adopt a concrete individualistic perspective (Reimer, Paolitto, & Hersh, 1983). For example, at the first level of reasoning one might decide to engage in exercise to simply fit into a dress, or because they are told "it is the healthy thing to do". At this level, decision-making is fickle and behaviors are inconsistent. There is no greater purpose to one's chosen behavior.

The second level of reasoning is based upon the notion of mutual interpersonal expectations and the foundation of relationships (Reimer, Paolitto, & Hersh, 1983). In this order, one makes decisions based upon what others expect of them, and what the perspective of how those decisions influence social relationships. At the second level of reasoning one might decide to engage in exercise because their family or significant other believes it is the right thing to do, or that exercising helps retain their current relationships.

The third level of reasoning is the highest order of reasoning for at this level, the reasoned functions at a reflective level (Reimer, Paolitto, & Hersh, 1983). At this

highest order of reasoning an individual differentiates societal views from interpersonal motives and makes decisions following self-chosen principles. Their beliefs are rational and adopted a sense of personal commitment to themselves derived from a reflective process. Decisions in this highest order of reasoning are based upon personalized principles and behaviors are consistent, even in times of conflict. At this level of reasoning, one might engage in exercise or physical because it is their belief that exercise or physical activity is part of their nature and is good for their own well being.

Higher-level reasoning requires education, reflection, and practice.

As evidenced in Leslie et al. (2000) and Sallis, et al. (1999) current educational interventions have shown positive, but superficial changes in physical activity and exercise behavior. Long lasting change requires a change in higher order reasoning, and thus, is dependent upon reflection (Fishbein & Ajzen, 2010). The process of reflection begins with education, the development of knowledge. Education initiates the transformation of conscious reasoning to the nonconscious mind through reflection. Reflection through conscious deliberation occurs over extended periods, from days to weeks, not merely seconds or minutes. It is of vital importance that this reflection is reflection over knowledge, not over trivial matters, but the true knowledge of the matters at hand. In doing so, one is allowed to deliberate free from distractions, and to arrive at independently derived conclusions. Reflection is crucial; it is the keystone in the arch of changing reasoning. The theoretical notion of Damasio's ideas are supported in application by Piaget, Kohlberg, Reimer, Paolitto, Hersh, Stoll, and the entire field of moral reasoning which is predicated on the notion

that continual reflection and the practice that follows leads to higher order reasoning and behavior change (Kohlberg & Lickona, 1976; Kohlberg, 1981; Stoll & Dieter, 2013; Reimer, Paolitto, & Hersh, 1983; Inhelder & Piaget, 1999; Piaget, 1997; Beller & Stoll, 1992).

A well-reasoned mind is sharpened and reinforced through practice. The ultimate goal of practice and repetition is the indoctrination of skills into the unconscious mind; a goal that is no different for changing reasoning in regard to physical activity and health. Damasio is of the belief that,

> . . .The exercise of decisions can be honed into a skill with the help of nonconscious mind processing, the submerged operations of our mind in matters of general knowledge and reasoning often referred to as the cognitive unconscious" (Damasio, 2010, p. 288).

Our nonconscious processes are indeed capable of reasoning, which can be properly trained by past experiences, reflection, practice, and conscious decision making, to make in the heat of the moment decisions, where otherwise our reasoning might falter and undesired behaviors ensue.

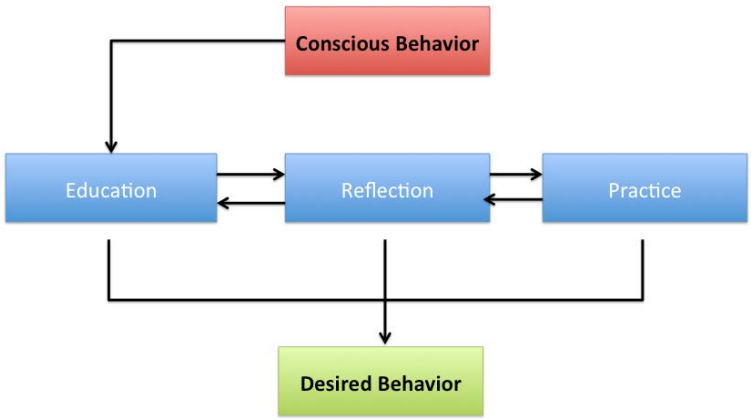

Figure 2.1. The formal process of higher-level reasoning through education, reflection and practice.

Behaviors are a skill set, acquired over repeated practice sessions and over a long time, informed by consciously articulated principles and reasons but otherwise 'second-natured' into the cognitive unconscious" (Damasio, 2010, p. 287). Therefore, an educational intervention program designed to positively change physical activity behaviors ought to utilize a curriculum that implements the three foundational steps in establishing reasoning: educational, reflection, and practice. Thus, the intervention implemented in this dissertation will improve self-efficacy, self-regulation, and goal setting through a reasoning-based approach in which the participants will learn foundation knowledge, continually reflect upon that knowledge, and practice implementing the knowledge and skills they develop along the way.

Application of higher-level reasoning-based education interventions.

Currently, there is no literature investigating the use of higher-level reasoning interventions to influence physical activity behaviors. However, different approaches to reasoning have been utilized to help explain physical activity and exercise behavior through the theory of reasoned action (TRA) and the theory of planned behavior (TPB). The theory of reasoned action was initially set forth by Ajzen and Fishben (1997), in which they suggested that human behavior is a function of one's intention to engage in a specific behavior. This intention is formed by the individual's attitude toward the behavior, the effect of others on that behavior, and the willingness to comply with the desires of others (Ajzen and Fishben, 1977). This model was later revised into the TPB (Ajzen, 1985,1991). In the revised TPB, Ajzen included perceived behavioral control, which explains a person's perceived self-efficacy in achieving the desired behavior.

The theory of reasoned action and the theory of planned behavior consistently show a positive correlational between intention to be active and physical activity amongst a wide demographic, including college students (Gordon, 2008; Biddle and Goudas, 1996; Blue, 1995; Ferguson et al., 1989; Godin and Shephard, 1986). Gordon (2008) found that TPB accounted for roughly 54% of the variance in intention to exercise and positively correlated (r=0.27) with self-reported physical activity in college-students. In a critical review of the literature, Blue (1995) found that both TRA and TPB have can be utilized to predict exercise behavior, but that the revised TPB was a more promising framework. In a follow-up meta-analysis, Hausenblas, Carron, & Mack (1997) also found that TPB more useful than TRA. Therefore, the following evidence on the efficacy intervention studies on improving physical activity will focus on TPB.

In addition to explanatory studies, intervention studies utilizing TBP have shown to be effective in increasing physical activity (Duangpunmat, Kalampakorn, & Pichayapinyo, 2013; Muzaffar, Chapman-Novakofski, Castelli, & Scherer, 2014). Duangpunmat and colleagues investigated the effective of a walking exercise program applying TPB in people at risk of hypertension in Thailand. The participants were 35-59 years old, with hypertension and were randomly selected into the intervention group (n = 34) and the comparison group (n = 34). The intervention including health information, benefits of walking exercise, group discussion in exercise barriers, modeling and experience exchange, walking exercise practice, and monitoring of their walking activity. In contrast, the comparison group received only health information at the beginning of the intervention. The authors found that

the intervention group made significant improvements in attitude towards walking exercise, perceived behavior control, subjective norm, whereas there was no improvement in the control group.

The Healthy Outcomes for Teens project was a randomized control trial that utilized the TPB in an online intervention program to improve behaviors for obesity and T2DM amongst adolescents (Muzaffar, Chapman-Novakofski, Castelli, & Scherer, 2014). 216 participants were recruited and randomized into the treatment group (n=127) the control group (n=89). The intervention website included interactive videos, narrated text, and knowledge/skill based games focused on diabetes, energy expenditure, physical activity guidelines, nutritional recommendations. The control website only included text and minimal images. In regards to physical activity, both groups saw an increase the predictability of TBP to explain intentions to exercise; however, the treatment group showed a greater increase than the control group. Unfortunately, the authors did not present actual physical activity data to compare the increase in intentions to actual physical activity.

Another randomized controlled trial evaluated the efficacy of a 4-week TPB intervention on physical activity in older adults diagnosed with T2DM or CVD (White, et al., 2012). 183 participants were recruited and randomized into an intervention group (n=130) or a control group (n=53). The 4-week intervention included sessions that explored the participants' attitudes and beliefs about healthy eating and physical activity, barriers that prevent them from making healthy eating choices and engaging in regular physical activity, and common triggers to unhealthy behaviors, and how unhealthy habits develop. The intervention also focused on planning for behavior

change, practicing the steps of effective planning, generating strategies to deal with barriers preventing them from meeting their healthy-eating and physical activity goals. The authors reported that intervention group showed short-term increases in physical activity as a result of planning. They ultimately concluded that TPB-based interventions might encourage physical activity among people with diabetes and cardiovascular disease (White, et al., 2012).

The results from the TPB studies indicate that reasoning is an integral part of physical activity behavior and that a reasoning-based intervention may prove to be a useful approach to improve physical activity. The TPB studies implement a distinct type of reasoning, one in which reasoning is a convergence of different aspects of human decision-making (i.e. attitude, beliefs, outside influences). Our approach to reasoning is distinct from the reasoning utilized in TPB, wherein we utilize a hierarchical approach to reasoning. As described above, we discuss reasoning in terms of levels of cognition with the end goal of reaching a higher-level of reasoning. Our approach is to bring participants to a new level of reasoning, not to improve the integration of different cognitive faculties as in TRA and TPB interventions. This specific approach, one of increasing the level of reasoning about physical activity through and education intervention has not been done before. However, there is evidence that reasoning-based interventions can improve cognitive faculties in the physically active realm, namely sport. Sharon Stoll, at the Center for ETHICS at University of Idaho, has utilized reasoning-based interventions to improve behaviors and moral reasoning in sport. Specifically, in a longitudinal study Stoll and colleagues showed that a reasoning-based intervention that involved education,

reflection, and practice increased in reasoning in the field of sport ethics and qualitative data demonstrated a change in moral-reasoning behavior in college-aged students (Stoll & Dieter, 2013).

While these data do not directly support the hypothesis that a reasoning-based intervention can significantly improve physical activity, it does present strong evidence that a higher-level reasoning-based curriculum can alter reasoning and behaviors regarding any area in which the curriculum is focused. To further support the efficacy of cognitive, reasoning-based approaches to changing reasoning and subsequent behavior, a similar educational intervention in journalism students resulted in increases in writing, reading, and reasoning of ethical thinking in regards to journalism (Grant, 2012).

Framework to implement reasoning-based Intervention.

Effective education behavioral interventions require a framework to guide program development and research in which one must address and carefully explain methods, methodology, and a theoretical perspective. Thus, a theoretical framework capable of explaining how individuals develop and sustain behavior is critical in the development of a reasoning-based program aimed at modifying behavior. There is substantial research demonstrating the efficacy of using social cognitive theory (SCT) based education intervention programs to improve exercise behaviors in adult population (Anderson, Winett, Wojcik, & Williams, 2010; Anderson-Bill, Suppini, & Apap, 2011; Hallam & Petosa, 20004; Doerksen, Umstattd, & McAuley, 2009). Despite the marked success of these interventions, there is little research exploring the efficacy of online SCT based educational in improving exercise behaviors in

college-aged populations. Therefore, a central aim of this dissertation is to develop a reasoning-based online educational intervention based in SCT theory and to examine its efficacy in improving knowledge, attitudes, and behaviors towards exercise in a population of university students.

Social Cognitive Theory and relevant constructs.

Social cognitive theory (SCT) posits that human behavior can be explained as a triadic reciprocal causation (Sharma & Romas, 2008). The angles of the tripod consist of behavior, environmental factors, and personal factors (i.e. cognitions, affect, and biological events). Ultimately, it is the interaction amongst these three aspects of SCT that results in behavior change. Furthermore, SCT rejects a dualism between personal agency and a social structure disembodied from human activity (Bandura, 2012). The unique ability of SCT to approach humans as sole individuals and members of society allows it to be applied in facilitating change in human behavior. It is this ability of SCT that spurred intervention programs built upon an SCT framework to improve leisure-time physical activity.

Presently, there are nine constructs within SCT, with each construct contributing to behavior change. While each construct contributes to behavior change, the amount each construct contributes to behavior change is contextual, being highly dependent on the chosen type of behavior. The literature regarding SCT based intervention programs suggests the three constructs which play the greatest role in changing behavior regarding exercise or leisure-time physical activity are self-efficacy, goal setting, and self-regulation.

Self-efficacy.

Self-efficacy is defined as the confidence a person has in his or her ability to pursue a behavior; it is behavior-specific and a function of the present (Sharma & Romas, 2008). Additionally, self-efficacy is a judgment of personal capability and is independent of, and orthogonal to self-esteem (Bandura, 2012). According to Bandura, unless an individual believes they are able to produce the desired changes through their own effort, there will be little motivation or incentive to put forth that effort.

The critical nature of self-efficacy in regards to facilitating behavior change has made it the focus of a number of intervention studies, including those aimed at increasing physical activity. Copious strategies aimed at improving self-efficacy have been established; however, four strategies are prominent in the literature: 1) Break down complex behaviors in practical and manageable steps, 2) use a demonstration from credible role model, 3) utilize persuasion and reassurance, and 4) attenuate stress (Sharma & Romas, 2008).

Self-efficacy is highly salient in regards to increasing physical activity as perceived inefficacy increases vulnerability to relapse into previous behaviors, which in this case would be sedentary behaviors (Bandura, 1982). Additionally, increased levels of self-efficacy increase intrinsic motivation (Bandura, 1982). It is important to note that increased intrinsic motivation as a result of improved self-efficacy occurs both dependent, and independent, of goal setting and goal attainment. This suggests that self-efficacy is a powerful construct of SCT in its own right, and as a factor influencing the goal-setting construct.

Goal setting.

Goal setting refers to developing plans to accomplish chosen behaviors (Sharma & Romas, 2008). Goal setting is critical in enhancing self-efficacy through promoting competence (Smith, Ntoumanis, & Duda, 2007), and is required for self-regulation to be effective. Therefore, proper goal setting is a critical component in successful behavior change within SCT. Effective goal setting requires individuals to set realistic and achievable, yet challenging goals (Frederick-Recascino & Schuster-Smith, 2003; Smith, Ntoumanis, & Duda, 2007; Standage & Ryan, 2012). This would be best achieved through providing support and structure to these goals such that the individual has clear expectations, comprehends how behavior influences outcome, and also receives feedback (Standage & Ryan, 2012). Additionally, the type of goals set can influence one's perception of competence; setting intrinsic, performance oriented goals are more likely to promote competence than extrinsic, outcome oriented goals (Anderman & Midgley, 1997).

Self-regulation.

Self-regulation is the sense that self-corrective adjustments are taking place as needed to stay on track toward achieving the purpose, and the sense that the corrective adjustments originate within the person (Carver & Scheier, 2011). Thus, the self-regulation construct is based on the assumption that behavior is goal directed and controlled through feedback. Additionally, this construct is based upon setting internal standards and engaging in self-evaluation of one's behavior (Sharma & Romas, 2008). Therefore, self-regulation is also an integral component to our second construct, goal-setting.

Self-regulation, while capable of being influence by external sources, is an internal construct. One must regulate both action and affect for effective behavior change. Regulation of action requires direct modification of physical behavior. Simply, to regulate action one must either reduce an unwanted behavior or increase a wanted behavior. In contrast, affect pertains to what an individual is feeling, their desires, and whether those desires are being met (Carver & Scheier, 2011). While a full explanation of feedback mechanisms influencing both action and affect is beyond the scope of this review, it is important to note that what occurs in the affect loop has direct influence on the action loop. There is substantial research exploring self-regulation of action in regards to physical activity, which will be discussed in the following sections. Presently, there is a lack of research exploring self-regulation of affect in regards to increasing exercise behaviors or leisure-time physical activity.

Social Cognitive Theory and exercise interventions.

Anderson-Bill, Suppini, and Apap (2011) examined the social cognitive determinates of nutrition and physical activity among web-health users enrolled in an online social cognitive theory based nutrition, physical activity and weight-gain prevention program. The authors utilized online surveys to measure nutritional and physical activity related variables of social cognitive theory including social support, self-efficacy, outcome expectations, and self-regulation. In regard to physical activity-related social cognitive characteristics, the authors found that inactive individuals generally did not perceive their friends and family members as taking steps to being physically active themselves. However, they did find the participants had moderate levels of confidence in their ability to increase physical activity in the

face of social, emotional, and logistical barriers, indicating moderate levels of self-efficacy. These scores varied depending on domain (e.g. social, emotion, physical), with self-efficacy scores being higher in regards to ability to exercise and lower in the social domain. Moreover, participants tended to believe physical activity was beneficial for their health. Additionally, using structural equation modeling (SEM), Anderson et al. (2011) found that social support and self-efficacy significantly influenced the levels of leisure-time physical activity ($P<0.01$). Taken together, these results indicate that social support and self-efficacy are critical in dictating leisure-time physical activity behavior.

Hallam & Petosa (2004) examined the efficacy of work-site exercise interventions on increasing selected SCT variables linked to adult exercise adherence over the span of six weeks, six months, and one year. The authors suggest the following three variables of SCT are highly associated with exercise behavior: self-efficacy, outcome-expectancy, and self-regulation. Briefly, the authors found a significant group-by-time increase in self-regulation in the treatment group and a significant decrease in the control group. The same trend followed with outcome-expectancy value with a significant group-by-time increase in the treatment group and a significant decrease in the control group. Again, the analysis revealed a significant group-by-time increase in self-efficacy in the treatment group and a significant decrease in the control group. Together, the above data suggest exercise intervention programs are efficacious in improving self-regulation, outcome-expectancy value, and self-efficacy.

Anderson et al. (2010) examined whether social cognitive (SCT) variables mediated treatment effects on physical activity and nutrition in the Guide-to-Health trial (GTH). Briefly, the GTH trial used an online intervention aimed at improving nutritional behaviors and increasing physical activity. The GTH was based on SCT theory and included 12 weekly modules. Using SEM, Anderson et al. (2010) found robust application of SCT domains in regards to mediation of the treatment effects on physical activity. Briefly, the intervention lead to increased physical activity, which was due to activity-related self-efficacy and self-regulation. GTH participants increased self-efficacy through frequent goal setting and planning and monitoring activity. Increased perceptions of social support mediated the effect of increased self-efficacy and self-regulation through enhancing the perceived social support of one's ability to self-regulate behaviors and succeed in those behaviors. This suggests social support plays a role in increasing physical activity by bolstering self-efficacy and self-regulation. Together, these data offer evidence that SCT-based online intervention programs increase physical activity through improving social support, self-efficacy, and self-regulation.

Doerksen, Umstattd, and McAuley (2009) examined the relationship amongst social cognitive factors and physical activity in first-year college students from a Midwestern university. Specifically, the authors measured exercise self-efficacy, outcome expectations, and physical activity goals and compared them with their levels of physical activity levels measured by an accelerometer. Doerksen et al. (2009) utilized bivariate analysis to examine associations among physical activity goals, self-efficacy, outcome expectations, and measures of physical activity. Briefly,

physical activity goals were the only measure positively associated with time spent engaging in moderate physical activity. High physical activity goals, internal feelings, and strong self-efficacy were positively associated with time spent engaging in vigorous physical activity. Additionally, both physical activity goals and self-efficacy were associated with overcoming interpersonal and stressful barriers to exercise and were significant predictors of vigorous physical activity. In agreement with previous studies such as Anderson et al. (2010), the overall regression equation using SCT variables accounted for 16% of the variance in moderate physical activity. Consistent with previous and subsequent research, these data suggest that constructs of SCT can partially explain physical activity behaviors in college students. Furthermore, in agreement with previous research, goal setting appears to play a significant role in determining amounts of physical activity, suggesting goal setting ought to be a critical component in curriculum/program development. The findings of Doerksen et al. (2009) indicate that SCT improving specific variables can positively change exercise behavior.

There have been online educational interventions aimed at improving physical activity levels amongst college students that have not employed SCT (Greene, et al., 2012). Greene et al. (2012) implemented a 10-lesson curriculum focusing on healthful eating and physical activity. Interestingly, Greene et al. (2012) found similar results as project GRAD (Sallis et al., 1999); the intervention did not improve levels of physical activity in college students. Additionally, Cardinal and Spaziani (2007) examined the effect of a 10-week, online educational intervention designed using techniques from the transtheoretical model (TTM) on exercise behavior amongst 109

college students. The authors found that the web-based participants experienced a 52% in physical activity. Web-Based participants experienced a 53% increase in their exercise behavior compared to control participants. Furthermore, the participants also saw increases in self-efficacy, and in the behavioral processes of change.

Comparing the results of these interventions with SCT based interventions suggests the utilization and incorporation of the self-efficacy, self-regulation, and goal setting components of SCT may be an integral part in the success of the observed behavior change in the studies by Anderson et al. (2010) and Doerksen et al. (2009). Therefore, we believe integrating reasoning constructs and SCT constructs will provide a novel and effective approach for an online educational intervention aimed at improving physical activity amongst college students.

Integrating reasoning constructs and SCT constructs.

A successful reasoning-based educational intervention based in SCT requires a conceptual model through which the reasoning constructs (i.e. education, reflection, and practice) are integrated with the selected SCT constructs (i.e. self-efficacy, self-regulation, and goal setting). A graphic representation of the integration of reasoning constructs and SCT constructs demonstrations how a reasoning-based intervention results in desired behavior change (2.1).

In this model we begin the intervention with education. This effectively establishes foundational knowledge that facilitates improvement in both self-efficacy and provides a basis for reflection. Reflection upon the foundational knowledge and skills developed through education the individual is able to engage in accurate self-

regulation of their behaviors and practice the desired behaviors. Reflection is essential in enhancing conscious decision making processes and is critical in translating into nonconscious decisions and the establishment of natural behaviors, actions, and responses (Damasio, 2010). The theoretical notion of Damasio's ideas are supported in application by Piaget, Kohlberg, Reimer, Paolitto, Hersh, Stoll, and the entire field of moral reasoning which is predicated on the notion that continual reflection and the practice that follows leads to higher order reasoning and behavior change (Kohlberg, 1981; Stoll & Dieter, 2013; Reimer, Paolitto, & Hersh, 1983; Inhelder & Piaget, 1999; Piaget, 1997).

Subsequently, goal setting in-turn provides continued opportunity for honest, measurable self-reflection and further promotes practice. Thus, through application of the reasoning process in an educational intervention one can improve self-efficacy, self-regulation, and goal setting, ultimately resulting in the desired behavior change.

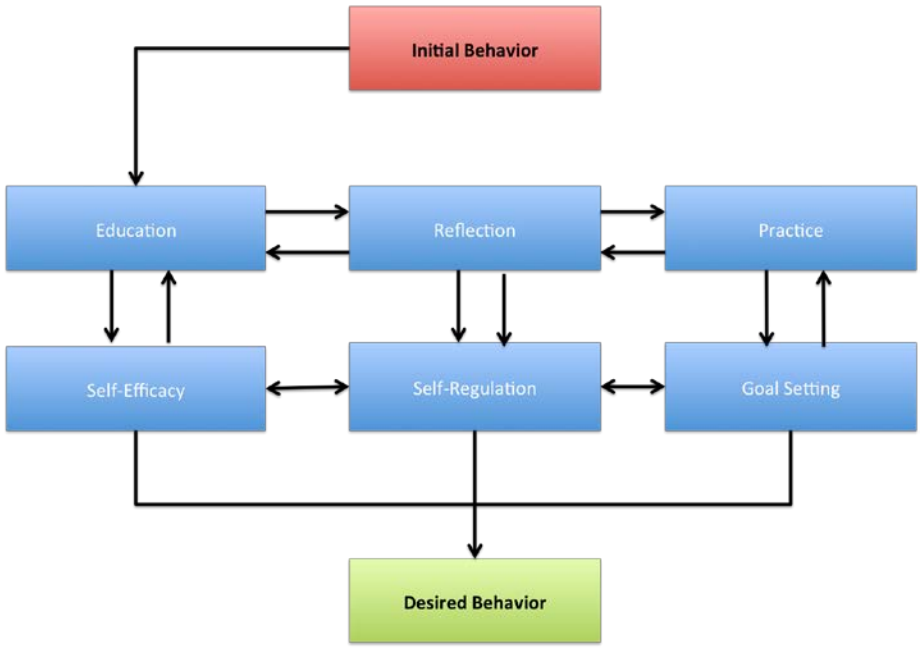

Figure 2.2. Integration of the reasoning process into SCT variables of physical activity behavior change.

Decreased levels of physical activity increase the risk of obesity, type 2 diabetes, heart disease, and many other chronic, lifestyle induced diseases (Powell, Thompson, Caspersen, & Kendrick, 1987; Myers, 2003; Kriska, et al., 2003). Implementing successful behavior change through a higher order reasoning-based intervention requires addressing the different aspects that influence that behavior. SCT utilizes behavioral, environmental, and personal factors in attempts to facilitate behavior change. While SCT based exercise interventions have been successfully utilized to influence positive human behavior and increase physical activity, there is little evidence regarding the efficacy of online reasoning-based SCT education interventions in improving exercise behaviors in college-aged individuals. Therefore, we propose that combining higher-order reasoning with SCT in an educational intervention will result in greater cognitive growth and physical activity and exercise behavior change.

Study 2: Physiological Mechanisms of Exercise and Diabetes

Chemical stress, including oxidative stress in skeletal muscle, has been implicated in the development of T2DM as well as the progression of diabetic complications, and causes premature cell death (Ogihara, et al., 2004; Matuzawa-Nagata, et al., 2008; Kowluru, Kowluru, Xiong, & Ho, 2006; DeRubertis, Craven, & Melhem, 2007). The redox sensitive transcription factor, NF-E2–related factor 2 (Nrf2) and the Nrf2 antioxidant response element pathway are one of the major cellular defenses against the cytotoxic effects of oxidative stress (Zhong, Mishra, & Kowluru, 2013). Reduced activity of Nrf2 observed in T2DM has been suggested to

have a causative effect in diabetic complications (Tan et al, 2011; Zhong et al., 2013), while evidence indicates that increasing Nrf2 activity in diabetic animals reduces complications (Zheng, et al., 2011).

In health individuals, Nrf2 is increased in response to the production of reactive oxygen species in acute hyperglycemia; however in T2DM we see decreased activity, suggesting the chronic hyperglycemia present in diabetes results in down regulation of Nrf2 (He, Kan, Cai, & Ma, 2009). It has been shown that diminished Nrf2 activity present in patients with T2DM contributes to increased oxidative stress, endothelial dysfunction, insulin resistance, and increased cardiac insult (Tan, et al., 2011; Cheng, Slow, & Mann, 2011). Overexpression of Nrf2 and pharmacologically enhanced activation of Nrf2 reduces oxidative stress and diabetic complications, including cardiovascular complications and diabetic nephropathy (Cheng, Cheng, Chiou, & Chang, 2012; Hsu, Lee, Li, Hsu, & Pan, 2013). Together, evidence highlights the importance of the Nrf2 pathway in T2DM and its subsequent complications.

Properties of Nrf2.

Nrf2 protects cells and tissues from a variety of endogenous and exogenous toxicants (Jaramillo & Zhang, 2013). The Nrf2 pathway is the major regulator of cytoprotective responses (Chen et al., 2006), is expressed in all tissues of the human body, and is essential in maintaining cellular homeostasis (Moi, Chan, Asunis, Cao, & Kan, 1994). Research regarding the role of Nrf2 in disease is burgeoning and evidence has indicated Nrf2 plays a central role in the development

of a variety of diseases including, T2DM, CVD, and cancer (Uruno, et al., 2013; Satoh, Moriguchi, Takai, Ebina, & Yamamoyo, 2013).

Nrf2 is a cap ‘n’ collar (CNC) basic-region leucine zipper transcription factor that provides cells the ability to adapt to oxidative stress and electrophiles by mediating the induction of the cytoprotective genes (Hayes & Ashford, 2012). These genes include the enzymes involved in glutathione (GSH) biosynthesis [i.e. γ-glutamyl cysteine ligase-catalytic (GCLC) and γ-glutamyl cysteine ligase-modulatory (GCLM), and phase II detoxifying enzymes such as heme oxygenase-1 (HMOX1), glutathione peroxidase (GPx), and superoxide dismutase (SOD)] (Osburn & Kensler, 2008). Induction of these genes requires interaction of the Nrf2 transcription factor with the antioxidant response element (ARE) (Nguyen, Sherratt, & Pickett, 2003) (Figure 2.3).

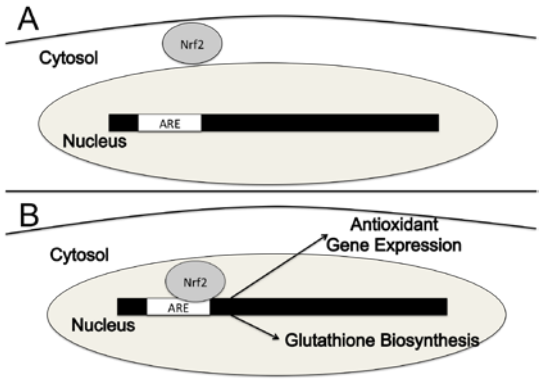

Figure 2.3. Nuclear translocation of Nrf2 and antioxidant gene expression.

Nrf2 and vascular complications.

Diabetes increases vascular complications, such as nephropathy, neuropathy, and retinopathy. Diabetic retinopathy leads to approximately 10,000 new cases of

blindness each year, and diabetic nephropathy is the leading cause of renal failure in the United States (Fong, Aiello, Ferris, & Klein, 2004; Association, 2004). There is increasing evidence that Nrf2 is critical in the development of these vascular complications in diabetes (Jiang, Huang, Lin, Zhang, Fang, & Zhang, 2010; Zheng, et al., 2011).

Nrf2 coordinates the endogenous antioxidant system (Uruno, et al., 2013). Given that oxidative stress is causal in diabetic nephropathy, and Nrf2 activity is decreased in T2DM it is likely that Nrf2 plays a central role in diabetic nephropathy. In support of this, examination of glomeruli of patients with diabetic nephropathy revealed that diabetic patients are under severe oxidative stress and the Nrf2-mediated antioxidant response is significantly different than non-diabetic controls. Jiang et al. (2010) also demonstrated that Nrf2 deficient ($Nrf2^{-/-}$) diabetic mice experience greater levels of oxidative stress and suffer greater levels of renal damage than diabetic mice with normal Nrf2 function.

In addition to reactive oxygen species, another cellular toxicant, methylglyoxal (MGO), is elevated in diabetes and is considered a major contributor to the development of insulin resistance and diabetic complications (Silva, Gomes, Ferreira, Freire, & Cordeiro, 2013; Rabbani, et al., 2011; Kilhovd, et al., 2003; Riboulet-Chavey, Pierron, Durand, Murdaca, Giudicelli, & Van Obberghen, 2006). MGO is involved in the pathogenesis of diabetic complications through a process known as glycation. Briefly, MGO reacts with intracellular and extracellular proteins and nucleic acids to form advanced glycation end-products which damage vascular tissue in a similar manner to oxidative stress (Berlanga, et al., 2005). There is

evidence that Nrf2 is involved in MGO-dependent vascular complications in diabetes through its role in MGO detoxification.

MGO detoxification is the process by which cells convert cytotoxic MGO to pyruvate, which can then be metabolized for energy. This process occurs via the GSH-dependent glyoxalase pathway (2.4) (Thornalley, 1998). Nrf2 activation results in transcription of GSH biosynthesis enzymes; thus, increased Nrf2 activation may reduce MGO accumulation by increasing availability of the rate limiting substrate, GSH. In support of this, pharmacological upregulation of Nrf2 by resveratrol in human liver cells reduced MGO accumulation and attenuated MGO-induced insulin resistance (Cheng, Cheng, Chiou, & Chang, 2012). Cheng et al. (2012) also demonstrated depletion of Nrf2, using silencing RNA (siRNA), resulted in reduced MGO detoxification (Cheng et al., 2012). Furthermore, MGO, MGO-derived advanced glycation end products, and tissue damage from intraperitoneal injections of MGO into mice pancreas was attenuated when the Nrf2 pathway was concomitantly activated pharmacologically (Hsu, Lee, Li, Hsu, & Pan, 2013). Individuals with uncontrolled T2DM have diminished GSH status; thus impaired GSH biosynthesis and status may play a major role in MGO-related diabetic complications (Sekhar, et al., 2011). When taken together, these data suggest increasing Nrf2 activity results in the attenuation of MGO accumulation, MGO-derived AGE formation, and tissue damage, ultimately reducing vascular complications associated with T2DM.

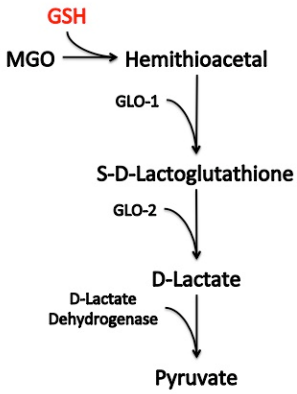

Figure 2.4. The glyoxalase pathway and methylglyoxal detoxification.

Nrf2 and pathological cardiac hypertrophy.

Hypertension is a hallmark consequence of diabetes and leads to pathological hypertrophy of the myocardium and cardiac dysfunction (Grossman, Shemesh, Shamiss, Thaler, Carroll, & Rosenthal, 1992). Furthermore, there is increased pathological hypertrophy in the diabetic heart independent of pressure overload; suggesting additional mechanisms are responsible for the increased hypertrophy (Grossman, Shemesh, Shamiss, Thaler, Carroll, & Rosenthal, 1992). Recent evidence suggests in the presence of oxidative stress, Nrf2 is involved in modulating hypertrophic signaling in the heart and may play a central role in regulating diabetic cardiomyopathy (Li, et al., 2009; Bai, et al., 2013).

Li et al. (2009) subjected wild type ($Nrf2^{+/+}$) and Nrf2 deficient ($Nrf2^{-/-}$) mice to two weeks of hypertension by transverse aortic constriction. Hypertension caused Nrf2 expression to transiently increase and then declined to basal levels (Li et al., 2009). Both wild type and Nrf2 deficient mice showed signs of pathological cardiac remodeling; however, $Nrf2^{-/-}$ mice experienced significantly greatly cardiac hypertrophy, myocardial fibrosis, overt heart failure and increased mortality than $Nrf2^{+/+}$ mice when exposed to hypertension (Li, et al., 2009). Furthermore, Li et al.

(2009) demonstrated that over expression of Nrf2 in rat neonatal cardiac myocytes and fibroblasts significantly inhibited hypertrophic factor–induced reactive oxygen species production and growth in both cardiomyocytes and cardiac fibroblasts, whereas knockdown of Nrf2 exerted opposite effects in both cells.

Increasing Nrf2 expression and transcription of Nrf2 regulated genes has been shown to prevent diabetic cardiomyopathy in mice (Bai, et al., 2013). Bai et al. (2013) treated diabetic mice with sulforaphane, a pharmacological Nrf2 activator, and measured blood and cardiac function. The authors found that mice treated with sulforaphane significantly increased Nrf2 activity and protein expression of Nrf2-regulated antioxidants. The increased Nrf2 activity prevented diabetes-induced cardiac hypertrophy and fibrosis, and almost eliminated diabetes-induced oxidative damage in the myocardium. Additionally, in rat heart cells, silencing Nrf2 with siRNA abolished the prevention of high glucose-induced fibrotic response by sulforaphane.

When considered together, the findings of Li et al. (2009) and Bai et al. (2013), suggest Nrf2 is a key mediator in pathological cardiac hypertrophy and heart failure in response to hypertension and oxidative stress. This novel interaction between diabetes-related cardiomyopathy and Nrf2 could provide insight into individual susceptibility to diabetic complications in the cardiovascular system and therefore should be investigated carefully (Howden, 2013). Furthermore, the exact mechanisms through which Nrf2-regulated signaling exert its cardio-protective effects are not well understood. Further exploration of Nrf-2 regulated signaling may provide a better understanding of the mechanisms regulating pathological cardiac hypertrophy and myocardial damage in the diabetic heart.

Regulation of Nrf2.

Nrf2 is negatively regulated by Keap1

Concentration and activity of Nrf2 are regulated at several levels, including degradation, translocation, post-translational modification, and translation. Nrf2 is a transcription factor, and as such, nuclear translocation from the cytosol is required in order for it to interact with DNA and encode cytoprotective genes. Nrf2 is located in the cytosol by a negative regulator Kelch-like ECH-associated protein 1 (Keap1). In normal, unstressed cells, Nrf2 protein is rapidly turned over in a Keap1-dependent manner through Cul3-Rbx1 ubiquitination and proteasomal degradation (Sun, Zhang, Chan, & Zhang, 2007; Eggier, Small, Hannink, & Mesecar, 2009) (Figure 2.5A). When cells are exposed to oxidative stress, electrophiles, or chemopreventive agents, Nrf2 in the cell escapes Keap1-mediated repression, translocates to the nucleus, and activates antioxidant responsive element-dependent gene expression to maintain cellular redox homeostasis (Zhang, 2006) (Figure 2.5B). Given that diabetes results in increased oxidative stress, it is unlikely the reduced Nrf2 activity in diabetes is due to increased inhibition by Keap1. Therefore, we suggest that T2DM reduces Nrf2 activity at the level of transcription, in the nucleus.

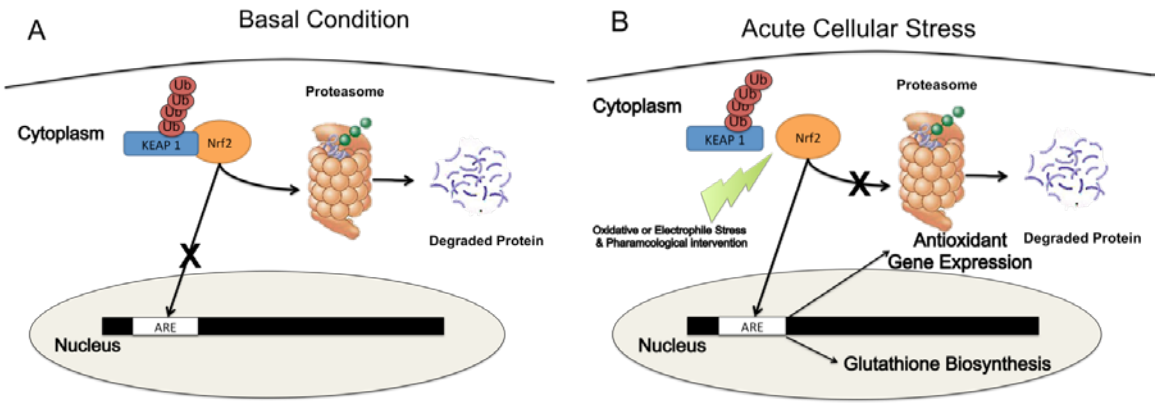

Figure 2.5. Regulation of Nrf2

Acetylation of Nrf2 and Gene Expression.

Regulation of Nrf2 gene transcription is controlled by other post-translational modifications, specifically acetylation. After Nrf2 disassociates from Keap1 under stressed conditions, it translocates to the nucleus. Once Nrf2 arrives in the nucleus it is acetylated, which results in increased gene transcription (Bannister & Miska, 2000). Evidence indicates that acetylation of Nrf2 in human colon cells increases binding of Nrf2 to DNA and increases transcription of Nrf2 induced genes, and that acetylation of Nrf2 occurs within the nucleus and has no effect on basal Nrf2 protein stability in human mammary cells (Sun, Chin, & Zhang, 2009). Sun et al. (2009) demonstrated that acetylation induces promoter-specific DNA binding of Nrf2, with acetylation directly influencing the Nrf2 regulated enzyme glutamate-cysteine ligase, the first rate-limiting enzyme of glutathione synthesis. In support of the findings of Sun et al. (2009), acetylation conditions in human hepatocytes cells resulted in increased nuclear localization of Nrf2 (Kawai, Garduno, Theodore, Yang, & Arinze, 2011). Kawai et al. (2011) also demonstrated that deacetylation conditions increased cytoplasmic rather than nuclear localization of Nrf2 (Kawai, Garduno, Theodore,

Yang, & Arinze, 2011). Together, evidence indicates nuclear acetylation of Nrf2 results in binding to the antioxidant response element and gene transcription, and that deacetylation disengages it from the antioxidant response element, thereby resulting in transcriptional termination and subsequently in its nuclear export and degradation (see Figure 2.6). Therefore, increasing acetylation of Nrf2 increases expression while deacetylation decreases expression of Nrf2 regulated genes.

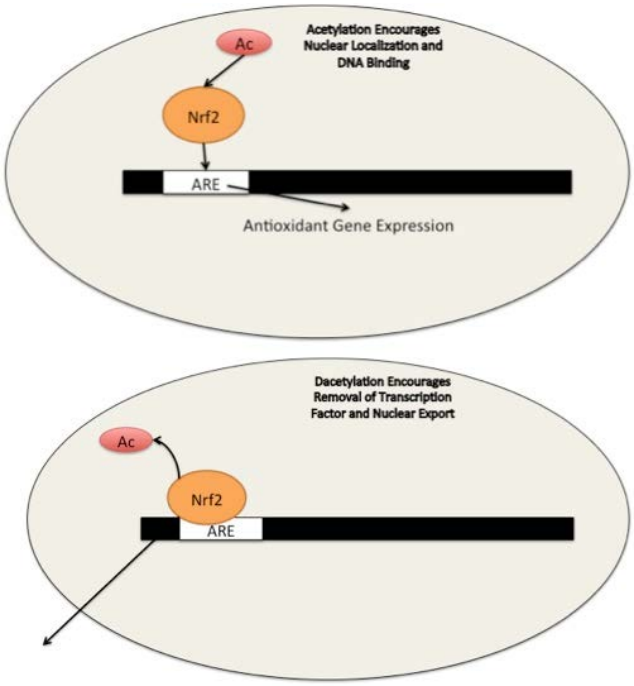

Figure 2.6. Effect of acetylation and deacetylation on Nrf2 transcription.

Acetylation and deacetylation of transcription factors is regulated through enzymatic reactions. For example, histone deacetylases (HDAC) are a class of enzymes that deacetylate transcription factors and other proteins involved in DNA transcription. HDAC 2 is a class I histone deacetylase that is phosphorylated and has been shown to form a complex with Nrf2. When Nrf2 complexes with phosphorylated HDAC2, Nrf2 is deacetylated, preventing *in vivo* binding to the

antioxidant response element in DNA (Lee, et al., 2012) (see Figure 2.7). Preventing Nrf2 deacetylation by inhibiting HDAC2 activity with trichostatin A increased Nrf2 binding to antioxidant response elements (Wang, et al., 2012), suggesting HDAC activity plays an important role in regulating Nrf2 activity.

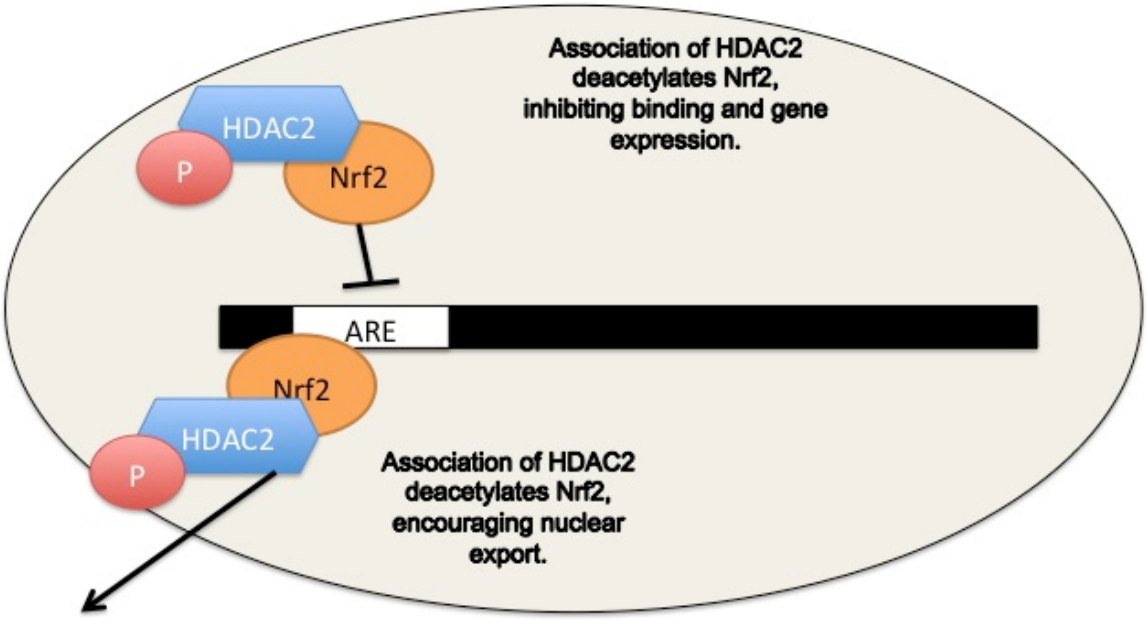

Figure 2.7. Role of HDAC2:Nrf2 association on Nrf2 activity.

O-GlcNAc modification and Nrf2

The post-translational O-linked attachment of the beta-N-acetylglucosamine (O-GlcNAc) sugar moiety modifies serine/threonine residues of thousands of structural proteins and transcription factors, whereby it can regulate transcriptional activity. O-GlcNAc cycling is controlled by the O-GlcNAc transferase (OGT) enzyme, which adds O-GlcNAc serine and threonine residues, and O-GlcNAcase (OGA), which removes it. O-GlcNAc modification is linked to cellular metabolism. Approximately 2–4% of glucose entering the cell is shuttled through the hexosamine biosynthetic pathway (HBP), whose end product is UDP-GlcNAc, the substrate for O-GlcNAc

modification. As T2DM is a metabolic disease, it is no surprise that aberrant O-GlcNAcylation is involved in disturbed cell signaling in the myocardium, including the regulation of transcription factors (Kim, Woo, Joo, & Moon).

To date, no studies have been conducted on the role of O-GlcNAc in the Nrf2 signaling cascade, although substantial cursory evidence suggests O-GlcNAc mediates some aspect of Nrf2 signaling. For example, increasing O-GlcNAc levels in the presence of oxidative stress in neonatal rat cardiomyocytes (NRCMs) increases the Nrf2-mediated gene product catalase (Ngoh, Watson, Facundo, & Jones, 2011). Furthermore, adenoviral overexpression of OGT reduced ROS while overexpression of OGA increased ROS in NRCMs treated with H_2O_2. Additionally, they demonstrated that overexpression OGA in NRCMs reduced mRNA expression of Catalase and that inhibiting OGA with PUGNAc increased Catalase expression. Despite this compelling evidence, Nrf2 was never examined in this paper and the role of O-GlcNAc on Nrf2 signaling has never been directly been explored.

Advances in bioinformatics have allowed for the development of *in silico* analysis capable of predicting possible sites of O-GlcNAc modification on proteins. Using the YinOYang1.2 prediction server, we identified a key amino acid residue, Thr^{594}, which is likely to be O-GlcNAcylated. This residue is a likely candidate to regulate Nrf2 function as it lies immediately adjacent to a nuclear localization signal, $P_{587}KSKKPD_{593}$, in the NEH3 domain of the Nrf2 protein (Theodore, et al., 2008). In addition to harboring a NLS, the $P_{587}KSKKPD_{593}$ motif is involved in transcriptional regulation of Nrf2 via acetylation (Figure 2.8). As previously mentioned, acetylation of Nrf2 by CREB-binding protein (CBP) induces transcription and promotes nuclear

localization and mutation studies have identified Lys^{588} and Lys^{591} as acetylation sites essential for Nrf2 transcription (Kawai, Garduno, Theodore, Yang, & Arinze, 2011). This suggests that O-GlcNAc may indeed play a role in nuclear localization of Nrf2. Thus, one possibility is that O-GlcNAc plays a role in altering the localization and/or activity of Nrf2.

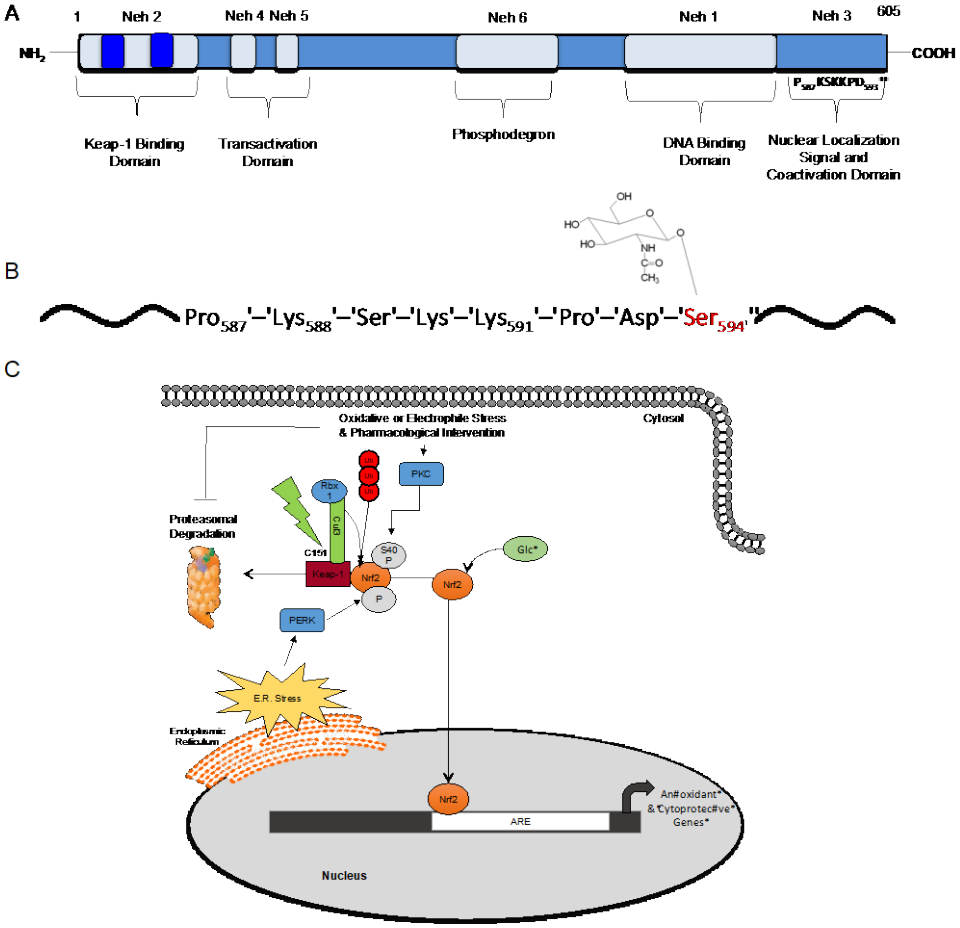

Figure 2.8. O-GlcNAc modification of Nrf2 and role in localization and/or activity. A) Structure of Nrf2 protein with NLS motif in the Neh domain. B) O-GlcNAc modification of Ser^{594}. C) Potential role of O-GlcNAcylation of Nrf2. O-GlcNAcylation of Ser^{594} may cause localization of Nrf2 via the nuclear localization signal or increase Nrf2 DNA-binding by increasing affinity for acetylation by CBP

Exercise increases Nrf2 activation and antioxidant gene expression.

Nrf2 activation occurs when it is release from its cytosolic inhibitor, Keap1, and expression of Nrf2 regulated antioxidant genes occurs when Nrf2 binds to the antioxidant response element of DNA. Recent evidence from animal model research indicates that exercise results in increased Nrf2 activation and expression of Nrf2 regulated genes (Baar, et al., 2002; Gounder, et al., 2012; Muthusamy, et al., 2012). Baar et al. (2002) reported a 56% increase in Nrf-2 expression in skeletal muscle of mice after an acute bout of swimming. Similarly, two consecutive days of 90 minutes/day of treadmill exercise increased Nrf2 protein expression roughly 25% and 100% in young and old rat heart muscle respectively (Gounder, et al., 2012). Muthusamy et al. (2012) found that acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in mouse heart muscle. Utilizing $Nrf2^{-/-}$ Muthusamy et al. (2012) also demonstrated that disruption of Nrf2 increases the heart to oxidative stress and that the beneficial effects of exercise on cardiac antioxidant mechanisms are dependent on Nrf2 function.

In addition to the benefits of acute exercise on Nrf2 function, chronic, moderate, intensity exercise proves to be effective in increasing Nrf2 activation in rats (Ashgar, George, & Lokhandwala, 2007). Ashgar et al. (2007) demonstrated that exercising sixty minutes a day, five days a week for six weeks on a treadmill increased nuclear levels of Nrf2 50% in rat models. Gounder et al. (2012) also found that impaired Nrf2 signaling was associated with GSH depletion and oxidative stress and that moderate intensity exercise improved Nrf2 signaling, GSH levels and reduced oxidative stress. There is also evidence that acute and chronic exercise in humans leads to

upregulation of the Nrf2 pathway (Cartoni, et al., 2005). Cartoni et al. (19) found Nrf2 levels increased 5-fold 24 hours post exercise in skeletal muscle cells of trained male cyclists after an acute bout of cycling exercise.

There is clear evidence that decreased Nrf2 activity is causal in diabetic complications and that exercise increases Nrf2 activity; however, no studies to date have examined the effect of exercise on Nrf2 activity in diabetes. Furthermore, there is no research to elucidate a mechanism by which exercise improves Nrf2 activity and expression of Nrf2 associated genes in T2DM. Therefore, the primary purpose of these studies is to examine the effect of exercise on signaling mechanisms of Nrf2 activity in the myocardium in an animal model of diabetes.

Diabetes and exercise differentially alter protein O-GlcNAcylation

A unique feature of the heart is its ability to differentially adapt to stress. Exercise and diabetes expose the heart to distinct, opposing stresses and the heart adapts physiologically in response to exercise while it adapts pathologically in diabetes. Interestingly, exposing the diabetic heart to exercise causes physiological cardiac hypertrophy and benefits the diabetic heart. The Marsh lab has previously shown that exercise and diabetes have opposite effects on the O-GlcNAc modification of transcription complexes that regulate cardiac remodeling in response to stress. Briefly, they demonstrated that association of OGT and mSin3A (a co-repressor in the REST complex) with HDAC1/2 was reduced in the db hearts, and that exercise returned this to normal levels by increasing O-GlcNAc modification. As the mSin3A/REST complex is involved in regulated diabetic cardiomyopathy, the authors concluded that diabetes and exercise oppositely affect interactions between

pro-hypertrophic transcription factors and suggested that an increasing total O-GlcNAc is a mechanism by which exercise benefits in T2DM hearts (Cox and Marsh, 2013). As our *in silico* analysis indicated that O-GlcNAcylation might increase Nrf2 nuclear localization, we hypothesized that acute exercise would increase Nrf2 and alter O-GlcNAcylation in the diabetic mouse heart and that O-GlcNAc modification plays a role in Nrf2 signaling.

Summary

Cardiovascular disease (CVD) and complications from T2DM are among the leading causes of death in the United States (Hoyert & Xu, 2011). Currently, 8.3% of the American population has been clinically diagnosed with diabetes and it is estimated that over 40% of individuals over the age of 20 have hyperglycemic conditions (Cowie, et al., 2009; National diabetes fact sheet, 2011). Evidence suggests diabetes increases the risk of CVD by three-fold (Saydah, Miret, Sung, Varas, Gause, & Brancati, 2001). The high prevalence and mortality rates of CVD and T2DM present a major challenge to our health-care system.

Lack of physical activity is a risk factor for the development of obesity, T2DM, and CVD (Hu et al., 2001; LaMonte et al., 2005; Berlin & Colditz, 1990). Furthermore, physical activity and exercise are effective therapeutic tools in reducing complications from these disease, including attenuating advanced glycation end-product formation and attenuating pathological cardiac hypertrophy in the diabetic heart (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000; Okada, et al., 2010; Boor, et al., 2009; Broderick, Poirier, & Gillis, 2005). Currently, only 65% of adults and 47% of college students in the United States meet the suggested physical

activity guidelines, highlighting the need to develop programs aimed at increasing physical activity amongst the adult and young adult populations of the United States (State indicator report on physical activity, 2010; American College Health Association-National College Health Assessment II, 2009). Behaviors such as physical activity are a skill set. They are acquired over repeated practice sessions and over a long time, informed by consciously articulated principles and reasons but otherwise ‘second-natured’ into the cognitive unconscious” (Damasio, 2010, p. 287). This suggests the formal process of reasoning is required to establish behavioral patterns. Additionally, human behavior can be explained as a triadic reciprocal causation: behavior, environmental factors, and personal factors (i.e. cognitions, affect, and biological events) (Sharma & Romas, 2008). Therefore, the implementation of a reasoning-based educational intervention designed to improve the determinants of human behavior may indeed result in improved physical activity amongst young adult populations.

Exercise has been shown to be effective tool for reducing complications in T2DM. Despite the well-documented benefits of exercise, there is a lack of data describing physiological mechanisms for the therapeutic benefits of exercise in T2DM. Oxidative and chemical stress is central to the development of diabetic complications and causes early cell death (Shen, Zheng, Metreveli, & Epstein, 2006; Kitada, Kume, Imaizumi, & Koya, 2011). A redox sensitive transcription factor, NF-E2–related factor 2 (Nrf2) and the Nrf2 antioxidant response pathway is one of the major cellular defenses against the cytotoxic effects of oxidative stress (Zhong, Mishra, & Kowluru, 2013). The Nrf2 pathway is downregulated in T2DM (Tan, et al.,

Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo, 2011) and plays a role in development diabetic complications including nephropathy and pathological cardiac hypertrophy (Li, et al., 2009). Furthermore, exercise increases Nrf2 content and activity in normal animals and humans (Baar et al., 2005; Cartoni et al., 2002).

The exact mechanisms regarding reduced basal activity of Nrf2 in diabetes how exercise affects Nrf2 in diabetes remain elusive. Nrf2 is regulated, in part, by posttranslational modifications. O-GlcNAc is a sugar moiety that can post-translationally modify serine/threonine residues of proteins (Ande, Moulik, & Mishra, 2009; Chou, Hart, & Dang, 1995). Prediction analysis indicates that Nrf2 is O-GlcNAcylated and that this may regulate both localization and activity of Nrf2. As O-GlcNAc cycling is aberrant in diabetes, we propose that O-GlcNAc modification regulates Nrf2 activity and may be responsible for reduced basal activity of Nrf2 in diabetes and may also affect how exercise affects Nrf2 in diabetes.

Chapter 3: A Reasoning-Based Educational Intervention Utilizing Social Cognitive Theory Improves Physical Activity Through Enhancing Reasoning, Goal Setting, and Self-Regulation.

Introduction

Lack of exercise and physical activity, along with sedentary behavior are primary risk factors for the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Hu, Leitzmann, Stampfer, Colditz, Willet, & Rimm, 2001; LaMonte, Blair, & Church, 2005; Berlin & Colditz, 1990). Conversely, increased levels of exercise reduce risk for development of T2DM and CVD (LaMonte, Blair, & Church, 2005; Kohl, Gordon, Villegas, & Blair, 1992; Berlin & Colditz, 1990). Furthermore, physical activity and exercise are effective therapeutic tools for reducing complications from these diseases and may reduce mortality rates (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000; Okada, et al., 2010; Boor, et al., 2009; Broderick, Poirier, & Gillis, 2005). Despite this evidence, only 65% of adults and 47% of college students in the United States meet the suggested physical activity guidelines, indicating that lifestyle plays a fundamental role in the increasing prevalence of T2DM. (State indicator report on physical activity, 2010; American College Health Association-National College Health Assessment II, 2009).

Exercise and physical activity are a known necessity for optimal health, but that knowledge does not always generate behavior change. Education has always been argued as the means to alter behavior, but current educational models appear not to be effective (Leslie, Fotheringham, Veitch, & Owen, 2000; Sallis J. F., et al.,

1999). Thus, there is a need to develop more effective methods to improve behaviors regarding exercise and physical activity in young adult populations of the United States. (Haskell, et al., 2007; American College Health Association-National College Health Assessment II, 2009).

Some colleges and universities have educational curriculums directed toward increasing knowledge that physical activity and exercise are beneficial for health, with the ultimate goal of influencing physical activity/exercise behaviors. These programs focus on skill and knowledge development, and result in moderate, short-lived improvements in physical activity (Leslie et al., 2002; Sallis et al., 1999). Two large-scale studies have utilized cognitive educational interventions to improve physical activity and exercise behavior amongst college-aged student, project GRAD (Graduate Ready for Activity Daily) and the Active Recreation on Tertiary Education Campuses (ARTEC) program. Project GRAD examined the effect of a university course on 321 university students (Sallis et al., 1999). The course was 21.5 months long and utilized labs and lectures to teach behavior change and physical activity skills. The intervention resulted in small increases in leisure-time physical activity in women but no effect on men and no long-term changes in physical activity habits were observed (Sallis et al., 1999). ARTEC, which was shorter in length (8 weeks), utilized similar methodology as project GRAD, but also offered students a free activity class of their choice, fitness assessments, swimming vouchers for a nearby facility, and on-campus media promotion. Similar to project GRAD, the ARTEC program did not indicate that the interventions were successful in establishing habitual physical activity patterns (Leslie, Fotheringham, Veitch, & Owen, 2000).

These findings suggest that current physical activity interventions are not designed to facilitate long-term, habitual behavior change. Habits play a critical role in physical activity and exercise adherence, and as such the ultimate goal of physical activity and exercise interventions ought to be long-term and habitual behavior change (Kretchmar, 2001; Keating, Guan, Pinero, & Bridges, 2010).

The lack of long-term and habitual changes in physical activity and exercise behavior with skill and knowledge focused interventions suggests that present interventions are lacking the necessary components to stimulate such a change. A recent meta-analysis of college student physical activity behaviors found that there is a lack of multi-level approaches to college students' physical activity, and an absence of physical education pedagogy specialists' involvement in research on students' physical activity (Keating, Guan, Pinero, & Bridges, 2010). Behaviors, such as physical activity behaviors, are a skill set. They are acquired over a long time, informed by consciously articulated principles and reasons that are otherwise 'second-natured' into the cognitive unconscious (Damasio, 2010, p. 287). This indicates that human behaviors are formed through a reasoning process. Therefore, a reasoning-based approach may prove efficacious in improving knowledge, attitudes, and behaviors regarding exercise and physical activity.

Effective educational interventions aimed at changing behavior require a theoretical framework to inform and guide the curriculum. Furthermore, the framework must address aspects of behavior specific to the target behavior, i.e., physical activity. Thus, a theoretical framework capable of explaining how individuals develop and sustain physical activity behavior is critical in the development of a

reasoning-based program aimed at modifying behavior. Research has demonstrated the efficacy of using social cognitive theory (SCT) based education intervention programs to improve exercise behaviors in older adult population (Anderson, Winett, Wojcik, & Williams, 2010; Anderson-Bill, Suppini, & Apap, 2011; Hallam & Petosa, 20004; Doerksen, Umstattd, & McAuley, 2009). Recently, Dennis et al (2012) demonstrated that an SCT based intervention is able modify behavior in college-aged students. The authors demonstrated that a 14-week SCT based intervention was effective in preventing increases in body fat in college freshman, indicating that SCT based interventions can influence health behaviors in college-aged individuals.

Currently, there is no literature investigating the use of higher-level reasoning interventions to influence physical activity behaviors. However, different approaches to reasoning have been utilized to help explain physical activity and exercise behavior through the theory of planned behavior. The theory of planned behavior (TPB) consistently shows a positive correlational between intention to be active and physical activity amongst a wide demographic, including college students (Gordon, 2008; Biddle and Goudas, 1996; Blue, 1995; Ferguson et al., 1989; Godin and Shephard, 1986). Gordon (2008) found that TPB accounted for roughly 54% of the variance in intention to exercise and positively correlated (r=0.27) with self-reported physical activity in college-students. Blue (1995) found that TPB can be utilized to predict exercise behavior, but that the revised TPB was a more promising framework. In a meta-analysis, Hausenblas, Carron, & Mack (1997) also found that TPB was useful in prediction of physical activity.

In addition to prediction studies, intervention studies utilizing TBP have shown to be effective in increasing physical activity (Duangpunmat, Kalampakorn, & Pichayapinyo, 2013; Muzaffar, Chapman-Novakofski, Castelli, & Scherer, 2014). Duangpunmat and colleagues investigated the effective of a walking exercise program applying TPB in adults with hypertension in Thailand. The authors found that the intervention group made significant improvements in attitude towards walking exercise, perceived behavior control, subjective norm, whereas there was no improvement in the control group. The Healthy Outcomes for Teens project was a randomized control trial that utilized the TPB in an online intervention program to improve behaviors for obesity and T2DM amongst adolescents (Muzaffar, Chapman-Novakofski, Castelli, & Scherer, 2014). Two hundred sixteen participants were recruited and randomized into the treatment group (n=127) the control group (n=89). In regards to physical activity, both groups saw an increase in the predictability of TBP to explain intentions to exercise; however, the treatment group showed a greater increase than the control group. Unfortunately, the authors did not present actual physical activity data to compare the increase in intentions to actual physical activity. Another randomized controlled trial evaluated the efficacy of a 4-week TPB intervention on intentions for physical activity in older adults diagnosed with T2DM or CVD (White, et al., 2012). 183 participants were recruited and randomized into an intervention group (n=130) or a control group (n=53). The authors reported that intervention group showed short-term increases in physical activity as a result of planning. They ultimately concluded that TPB-based interventions might encourage

physical activity among people with diabetes and cardiovascular disease (White, et al., 2012).

The results from the TPB studies indicate that reasoning is an integral part of physical activity behavior and that a reasoning-based intervention may prove to be a useful approach to improve physical activity. The TPB studies implement a distinct type of reasoning, one in which reasoning is a convergence of different aspects of human decision-making (i.e. attitude, beliefs, outside influences). Our approach to reasoning is distinct from the reasoning utilized in TPB, wherein we utilize a hierarchical approach to reasoning. We discuss reasoning in terms of levels of cognition with the end goal of reaching a higher-level of reasoning. Our approach is to bring participants to a new level of reasoning, not to improve the integration of different cognitive faculties as in TRA and TPB interventions.

Researchers have described a developmental conception of intelligence, with cognitive processes underlying the intelligence in a chronological process (Reimer, Paolitto, & Hersh, 1983, pg. 19). The cognitive education formats present in most studies utilize mechanistic explanations of how exercise and physical activity are beneficial for health and the pedagogical formats of current educational physical activity intervention programs for college students tend to focus on what is known as a first order level of reasoning (Leslie, Fotheringham, Veitch, & Owen, 2000; Sallis, et al., 1999). The first level of reasoning involves instrumental purpose and exchange. In this order, one makes decisions to serve one's own immediate needs and adopt a concrete individualistic perspective (Reimer, Paolitto, & Hersh, 1983). For example, at the first level of reasoning one might decide to engage in exercise to

simply fit into a dress or suit, or because he/she is told “it is the healthy thing to do”. At this level, decision-making is vacillating and behaviors are inconsistent. There is no greater purpose to one’s chosen behavior. Philosophically, there are three levels of reasoning and cognitive development (Kohlberg, 1981). The second level of reasoning is based upon the notion of mutual interpersonal expectations and the foundation of relationships (Reimer, Paolitto, & Hersh, 1983). In this order, one makes decisions based upon what others expect, and the perspective of how these decisions influence social relationships. At the second level of reasoning, one might decide to engage in exercise because family or significant others believe it is the right thing to do, or one may exercise to retain current relationships. The third level of reasoning is the highest order of reasoning. Third order reasoning is a reflective level of reasoning (Reimer, Paolitto, & Hersh, 1983). At this highest order, an individual differentiates societal views from interpersonal motives and makes decisions following self-chosen principles. Beliefs are rational and adopted from a sense of personal commitment derived from a reflective process. Decisions in this highest order of reasoning are based upon personalized principles and behaviors are consistent, even in times of conflict. At this level of reasoning, one might engage in exercise or physical because of a belief that exercise or physical activity is part of their nature and is essential for personal self and well-being. Currently, there are no interventions that utilize this specific reasoning-based approach to influence physical activity behaviors and we believe improving reasoning through this approach can lead to positive changes in physical activity amongst college-aged students.

Despite some success of TPB and SCIT interventions, there is little research exploring the efficacy of online ordered reasoning-based educational interventions in improving exercise behaviors in college-aged populations. Furthermore, online interventions provide a vector through which educational interventions can reach a wider audience and require fewer resources for implementation, Therefore, the purpose of the present study is to examine the efficacy of an ordered reasoning-based online educational intervention based in SCT theory on improving knowledge, attitudes, and behaviors towards exercise in a population of university students.

Methods

Study participants.

Participants were be recruited from a northwest university. The participants were be between the ages of 18 and 22 years and were physically capable of engaging in physical activity. The experimental group consisted of students enrolled in an undergraduate health and nutrition class. Sixteen participants enrolled in the course and 4 students dropped out, leaving a total of 12 participants in the experimental group. The control group consisted of undergraduate students enrolled in a sport in society course in which the students engaged in lectures and writing about sports and physical activity. The control group was informed that they were involved as a control group in a research study. Thirty-two students enrolled in the course and 7 students dropped out, leaving a total of 25 participants. The study was approved by the University of Idaho Institutional Review Board (exempt status number 13-215).

Educational intervention.

The educational intervention took place during a 16-week online course and consisted of 6 distinct modules (experimental group only). Module 1 established foundational knowledge about the relationship between physical activity and health. Module 1 required students to complete the HBS, write a reflective response about the importance of physical activity in health and to reflect upon how this information might inform their future lifestyle habits.

Module 2 through 3 was aimed at improving self-efficacy in physical activity and nutritional habits. The content helped students to develop knowledge and skills to increase physical activity by providing information regarding different exercise modalities, and structuring exercise programs. Modules 2 through 3 required participants to submit questions they have regarding how to improve their self-efficacy in regard to physical activity. These questions were directly answered using a podcast format in which the students listened to the following week. The participants were also required to write a reflective paper in which they discuss the tools and skills they acquired in modules 2 and 3.The reflective paper is based upon a higher level of reasoning as noted by Reimer, Paolitto, and Hersh (1983). This is designed to increase information retention and allow the participants to realize the skills garnered from the modules.

Module 4 was aimed at increasing self-regulation through reading case-studies highlighting the importance of self-regulation and the articles provided information on improving self-regulation. Participants were required to write a

reflective paper discussing what they learned about the importance and application of self-regulation in regards to physical activity.

Module 5 improved goal-setting skills in the participants. The module included a lecture on the key points of goal setting and how to apply them to physical activity. The participants were then required to develop specific physical activity goals (e.g. how will you choose to be physically active, what form of exercise will you partake in, how will you structure your physical activity, how will you maintain your health through exercise?).

Module 6 required the participants to reflect upon the knowledge and skills they gain from the course and apply them to physical activity in the context of health. The participants then engaged in an online discussion in which they discussed what they learned from the intervention, how their self-efficacy, goal-setting, and self-regulation improved in regards to physical activity.

SCT variables of physical activity.

Participants came in and completed demographic information, physical activity, and the Health Beliefs Survey (HBS) questionnaires to assess SCT variables of physical activity, requiring about 35 minutes. The SCT variables we assessed included self-efficacy, self-regulation, and goal setting. The HBS was completed during the first week of the intervention and then again upon completion of the intervention. The survey has been used in previous SCT based exercise intervention research and has shown to be valid and reliable in young adults aged 18-25 years (Anderson, Winett, Wojcik, & Williams, 2010; Anderson-Bill, Suppini, & Apap, 2011; Rovniak, Anderson, Winett, & Stephens, 2002).

Physical activity data.

The official long form English version of the International Physical Activity Questionnaire (IPAQ) was used. The participants came in, the IPAQ was explained and completed during the first week of the intervention and then again upon completion of the intervention. We report total leisure-time physical activity (LTPA) captured by scoring the IPAQ as per the IPAQ scoring guide. Briefly, total LTPA in MET-minutes/week was calculated as the sum of (3.3 * walking minutes * walking days) + (4.0 * moderate-intensity activity minutes * moderate-intensity days) + (8.0 * vigorous-intensity activity minutes * vigorous-intensity days). To maintain consistency with the instrument, all data are reported in MET-minutes per week. Dinger, Behrens, and Han (2006) examined the validity and reliability of the long form English IPAQ using pedometers and accelerometers. Dinger et al. (2006) found that the IPAQ was significantly correlated (r=0.30-0.47, p<0.01) with steps/day from both the accelerometer and pedometer and had an intraclass correlation coefficients ranging from 0.71-0.89, indicating moderate to high reliability in college students.

Levels of reasoning.

In order to capture the reasoning process the responses from the writing assignment of the participants were recorded and analyzed for themes to explore development of the participants' reasoning. Initial analysis was conducted through immersion into the data as described in Pope, Zieband, and Mays (2000). The responses blindly reviewed by one person to reduce interrater variability and were categorized into one of the three levels of reasoning as established by Kohlberg

(Kohlberg, 1981). Levels of reasoning were examined at the beginning, middle (week 8), and end of the 16-week program.

Data analysis.

Independent sample *t*-tests were used to examine difference between groups at baseline and paired *t*-tests were used to examine differences in physical activity and SCT variables at the beginning and end of the intervention. Bonferroni corrections were applied for the *t*-tests to control for experimentwise error. Repeated measures analysis of variance (ANOVA) was conducted to examine changes of reasoning at three different stages of the intervention. Statistical analysis was conducted using SPSS (Version 22.0, IBM Corp, Armonk, NY) and R (version 3.1.0). Significance for all measures of this study as set at $\alpha=0.05$.

Results

Effect of intervention reasoning levels.

We assessed the levels of reasoning through the participants writing about physical activity. As we wanted to maintain a true control group in which they did not engage in reflective thinking about physical activity, only the intervention group engaged in the reflective writing. We found that the intervention significantly increased the level of reasoning amongst the experimental group ($p<0.05$). We found incremental increases in the mean reasoning throughout the intervention at the pre-test, mid-point, and post-test respectively (3.1). This improvement in reasoning occurred alongside significant increases in the goal-setting and self-regulation domains of SCT as well as increased levels of LTPA, suggesting that

improving reasoning regarding physical activity improves LTPA and cognitive domains associated with physical activity amongst college students.

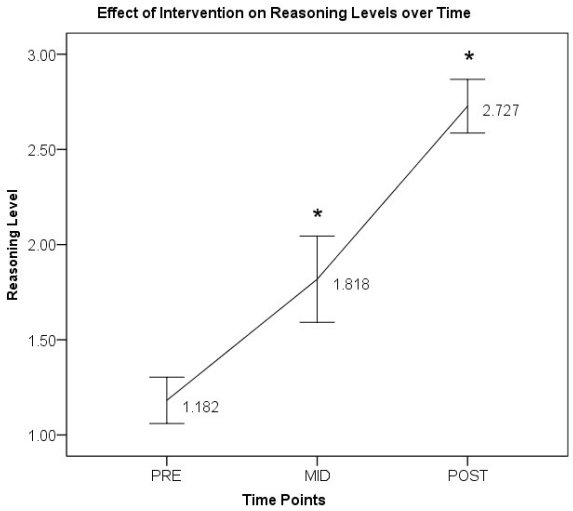

Figure 3.1. Effect of intervention on reasoning levels. *Indicates significant difference (p<0.05). Error bars represent standard deviation.

Physical activity.

We determined the effect of the reasoning-based intervention on leisure time physical activity (LTPA. There was no difference between group differences in LTPA at the start of the intervention (*p=0.246*) (Table 2). There was a non-significant decrease in LTPA in the control group (∆-832.00 MET-minutes/week, p=0.281) (Table 3 and 3.1). Conversely, the experimental group saw a significant increase in LTPA (∆2928.33 MET-minutes/week, p=0.018) (Table 2). These results suggest that a reasoning based intervention can effectively increase LTPA amongst college students.

Social cognitive theory domains.

We determined the efficacy of the reasoning-based intervention on domains of SCT by comparing the following variables from the Health Beliefs Survey within and between the control and experiment groups: 1) physical activity goal setting (PA

goal), 2) physical activity self-regulation (PA self-regulation), and 3) physical activity self-efficacy (PA self-efficacy). The control group saw no change in PA goal (Δ -0.15, *p*=.553), PA self-regulation (Δ 0.23, *p*=.39), or PA self-efficacy (Δ 17.88, *p*=.181) (Table 3). Conversely, the experimental group saw an increase in PA goal (Δ 0.58, *p*=0.03) and PA self-regulation (Δ 0.45, *p*=.037) but not in PA self-efficacy (Δ 6.54, *p*=.127 (Table 2). We also found that at baseline, the two groups were different in PA Goal (3.14 control group vs. 4.0 experimental group, *p*<.05), PA self-regulation (2.19 control group vs. 3.2 experimental group, *p*<.05), and PA self-efficacy PA self-regulation (2.19 control group vs. 3.2 experimental group, *p*<.05) (Table 3). The differences between groups at baseline presents a limitation to the results and is likely due to the characteristics of the samples or self selection. These shall be discussed at length in the discussion.

Discussion

Reasoning-based intervention improves reasoning about physical activity

Our study demonstrated for that an online reasoning-based intervention can improve reasoning about physical activity amongst college students and that this improvement in reasoning coincided with increased psychological domains related to physical activity behavior and levels of physical activity. At the outset of the intervention, the average reasoning level of the participants was 1.2, indicating the participants were operating near the first order level of reasoning. This indicates their reason for engaging in exercise or physical activity was that of instrumental purpose. At this level of reasoning one might decide to engage in exercise because one is told “it is the healthy thing to do” and there is no greater purpose to one’s chosen

behavior. We also observed that lower levels of physical activity were associated with this lower level of reasoning. At the midpoint of the intervention, the average reasoning level of the participants was 2.2, indicating they were reasoning near the second level of reasoning. Reasoning at the second level is based upon the notion of mutual interpersonal expectations and the foundation of relationships (Reimer, Paolitto, & Hersh, 1983). In this order, one makes decisions based upon what others expect, and the perspective affect how decisions influence social relationships. At the second level of reasoning one might decide to engage in exercise because family or significant others believe it is the right thing to do, or one may exercise to retain current relationships. At the end of the intervention the average reasoning level of the participants was 2.8, indicating they were operating near the third level of reasoning. At this level, reasoning functions at a reflective level, and an individual differentiates societal views from interpersonal motives and makes decisions following self-chosen principles (Reimer et al., 1983). Their beliefs are rational and adopt a sense of personal commitment to self, derived from a reflective process. At the third level of reasoning, one might engage in exercise or physical because of a belief that exercise or physical activity is essential for personal self and well-being. Decisions in this highest order of reasoning are based upon personalized principles and behaviors are consistent, even in times of conflict.

In this study, we observed higher levels of physical activity associated with higher levels of reasoning. Most importantly, the significant improvement of reasoning regarding physical activity as it relates to health occurred alongside an increase in physical activity. Additionally, we found that improving reasoning also

coincided with improvements in psychological domains that predict physical activity. Therefore, we propose that improving the reasoning of college age students in regards to physical activity and health may improve physical activity levels.

While there is no existing literature investigating the use of higher-level reasoning interventions to influence physical activity behaviors, different approaches to reasoning have been utilized to help explain physical activity and exercise behavior, namely the theory of planned behavior (TPB). TBP-based intervention studies have also shown a reasoning based approach is effective in increasing physical activity (Duangpunmat, Kalampakorn, & Pichayapinyo, 2013; Muzaffar, Chapman-Novakofski, Castelli, & Scherer, 2014). Duangpunmat and colleagues investigated the effective of a walking exercise program applying TPB in people at risk of hypertension in Thailand and found that the intervention group made significant improvements in attitude towards walking exercise, perceived behavior control, subjective norm, whereas there was no improvement in the control group. Additionally, a randomized controlled trial evaluated the efficacy of a 4-week TPB intervention on physical activity in older adults diagnosed with T2DM or CVD (White, et al., 2012). The authors reported that the intervention group showed short-term increases in physical activity and ultimately concluded that TPB-based interventions might encourage physical activity among people with diabetes and cardiovascular disease (White, et al., 2012).

The results from the current study, along with the work of Duangpumat et al. (2014) and White et al. (2012) indicate that reasoning is an integral part of physical activity behavior and that a reasoning-based intervention may prove to be a useful

approach to increase physical activity levels. The TPB studies implement a distinct type of reasoning, one in which reasoning is a convergence of different aspects of human decision-making (i.e. attitude, beliefs, outside influences). Our approach to reasoning is distinct from the reasoning utilized in TPB, wherein we utilize a hierarchical approach to reasoning. Therefore, further investigation on the efficacy of these different approaches to reasoning and physical activity is warranted.

Reasoning-based intervention improves physical activity and Social Cognitive Theory domains related to physical activity.

We found that a reasoning-based educational intervention program improved leisure time physical activity approximately 1960 MET-minutes/week amongst our participants while the control group saw a non-significant decrease of approximately 832 MET-minutes/week.

Our study demonstrated for the first time that a hierarchical, online reasoning-based intervention can successfully improve self-regulation (Δ 0.45, p=0.037) and goal setting (Δ 0.58, p=0.03) in college students as it relates to physical activity as measured by the Health Beliefs Survey. Previous research has shown the positive correlation between these variables and physical activity in varying populations and that improving goal-setting and self-regulation leads to improved physical activity (Anderson et al., 2010; Anderson et al., 2011; Doerksen, Umstattd, & McAuley, 2009; Hallam & Petosa, 2004). Therefore, we believe the increase in physical activity was a result of increasing the participants reasoning about physical activity and health while concurrently improving their goal-setting and self-regulation through a reasoning-based SCT educational intervention.

Doerksen, Umstattd, and McAuley (2009) measured exercise self-efficacy and physical activity goals amongst college students and compared those with their levels of physical activity as measured by an accelerometer. Doerksen et al. (2009) found that physical activity goals were positively associated with time spent engaging in moderate physical activity and that high physical activity goals along with strong self-efficacy were positively associated with time spent engaging in vigorous physical activity. Additionally, both physical activity goals and self-efficacy were associated with overcoming interpersonal and stressful barriers to exercise and were significant predictors of vigorous physical activity. Anderson et al. (2011) and Anderson et al. (2010) found that self-regulatory behaviors was a strong predictor of physical activity amongst elderly populations in an online intervention aimed at improving physical activity. Furthermore, they found improving goal-setting behaviors and establishing goals added considerable strength to these self-regulatory behaviors. Hallam and Petosa (2004) also demonstrated that improving self-regulation and self-efficacy in an adult population improved physical activity levels.

Our findings are in concordance with Doerksen et al. (2009), Anderson et al. (2009), and Hallam and Petosa (2004) in that improving these variables coincided with an increase in physical activity levels. In contrast with Doerksen et al. (2009), Anderson et al. (2009), and Hallam and Petosa (2004), we did not observe an increase in self-efficacy in the experimental group (p=.127*)*. We believe this result can be partly explained by the initially high levels of self-efficacy in the experimental group and the small sample size of the study. When considered together, these data

suggest that a reasoning-based educational intervention increases goal setting and self-regulation in college students and that improving these domains improves physical activity.

A model of reasoning, SCT, and human behavior

Damasio (2010) articulates that behaviors are a skill set, acquired over repeated practice sessions and over a long time, informed by consciously articulated principles and reasons but otherwise 'second-natured' into the cognitive unconscious. In essence, behaviors result from physical manifestations of non-conscious and conscious mental processes that are formed directly from knowledge and practiced application of that knowledge through the reasoning process.

In his work, Damasio (2010) describes the components and formal process of reasoning and the importance of reflection on reasoning. The process of reflection begins the development of knowledge through education. Education initiates the transformation of conscious reasoning to the nonconscious mind through reflection. Conscious decisions begin with reflection, simulation, and testing in the conscious mind (Damasio, 2010). Reflection through conscious deliberation occurs over extended periods, from days to weeks, not merely seconds or minutes. Therefore, it is important that educational interventions be intensive and require reflective thinking to improve reasoning. Through intensive reflection and the development of higher-level reasoning, we can establish a form of reasoning within the non-conscious mind. This reasoning, once it has been properly trained may lead to beneficial decisions (Damasio, 2010). In essence, our intervention increased the level of

conscious reasoning, which then improved nonconscious reasoning and behaviors regarding physical activity.

This process of establishing “second-natured” behaviors through consciously articulated principles has been shown to occur physiologically in the brain. Higher order thinking, reasoning, and conscious decision making occur in the prefrontal cortex. It is within the prefrontal cortex that we find the seat of intellection, of cognitive functioning, of personality and identity, and of the integration of emotions and thoughts (Tancredi, 2005). Additionally, the prefrontal cortex is associated with complex behaviors and the establishment of principles (Frith, Friston, Liddle, & Frackowiak, 1991). Conversely, the nonconscious mind is located within the limbic system. It is within the limbic system where our immediate actions and decisions are made; no reflection or deep thought is required to make our decision, the behavior has become “who we are” (Mega, Cummings, Salloway, & Malloy, 1997). Engaging in reflection and reasoning has been shown to transition decision-making regarding behaviors from the prefrontal cortex to the limbic system (Pally, 2007). Thus, the process of reflection and reasoning results in neurobiological changes in decision making. Ultimately, this provides a physiological basis for reflection and reasoning altering our thought processes and decisions.

The theoretical notions of Damasio’s ideas are supported in application by the findings of our study, wherein we find that reflection and reasoning about physical activity increases physical activity behavior. Reasoning and behaviors are reinforced through practice, with the ultimate goal of practice and repetition as the indoctrination of skills into the unconscious mind; a goal that is critical for changing

physical activity behavior. Thus, it is critical that educational interventions aimed at improving physical activity, utilize a reflective, reasoning-based approach.

A successful reasoning-based educational intervention based in SCT requires a conceptual model through which the reasoning constructs (i.e. education, reflection, and practice) are integrated with the selected SCT constructs (i.e. self-efficacy, self-regulation, and goal-setting). A graphic representation of the integration of reasoning constructs and SCT constructs demonstrations how a reasoning-based intervention results in desired behavior change (3.2).

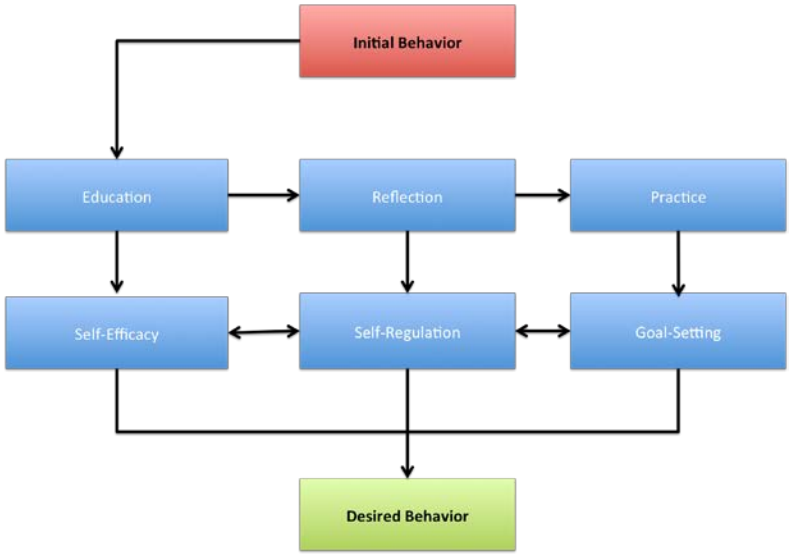

Figure 3.2. Integration of the reasoning process into SCT variables of physical activity behavior change.

This models begins with education to effectively establish foundational knowledge that facilitates improvement in both self-efficacy and provides a basis for reflection. Reflection upon the foundational knowledge and skills developed through education the individual is able to engage in accurate self-regulation of their behaviors and practice the desired behaviors. Reflection is essential in enhancing conscious decision making processes and is critical in translating into nonconscious

decisions and the establishment of natural behaviors, actions, and responses (Damasio, 2010). Subsequently, goal-setting in-turn provides continued opportunity for honest, measurable self-reflection and further promotes practice. Thus, through application of the reasoning process in an educational intervention one can improve self-efficacy, self-regulation, and goal-setting, ultimately resulting in the desired behavior change.

Strengths and limitations.

This was the first study to adopt a reasoning-based educational model to improve physical activity. The intervention changed the level of reasoning about physical activity amongst college-aged students. Furthermore, as indicated by the changes in reasoning, the participants developed a value base about activity. Lifestyle habits are a system of individual differences in the habitual use of declarative and procedural knowledge structures that intervene between personal values and situation-specific product perceptions and behaviors (Brunso, Scholdere, & Grunert, 2004). Thus, the values developed in this intervention are likely to establish a foundation of behavior, and as such, establish a value base for physical activity which should promote a life-time of physical activity.

One limitation present in the study was our small sample size. Despite the small sample size we were able to find significant changes in reasoning, goal setting, self-regulation and physical activity amongst the experimental group. This suggests that the intervention can successfully improve reasoning and physical activity behaviors amongst college students. There were also differences present between the groups at baseline, indicating the two samples were not identical and

self-selection bias may indeed be present. Additionally, due to the nature of the reasoning-based intervention and the need for a true control group, we did not collect data on the reasoning levels of the control group. As this study was an initial inquiry into the efficacy of a reasoning-based intervention on physical activity levels among college-aged individuals, it is possible that being in a health and nutrition class stimulated them to increase PA and self-efficacy. Future studies to compare the efficacy of this method with other methodologies will elucidate this caveat in our study. Despite these limitation we feel that the data presented in this paper highlights the potential for a reasoning-based program to improve psychological domains related to physical activity and levels of leisure time physical activity and provides rationale for further inquiry.

Conclusion.

Decreased levels of physical activity increase the risk of obesity, type 2 diabetes, heart disease, and many other chronic, lifestyle induced diseases (Powell, Thompson, Caspersen, & Kendrick, 1987; Myers, 2003; Kriska, et al., 2003). Implementing successful behavior change through a higher order reasoning-based intervention requires addressing the different aspects that influence that behavior. SCT utilizes behavioral, environmental, and personal factors in attempts to facilitate behavior change. While SCT-based exercise interventions have been successfully utilized to influence positive human behavior and increase physical activity, there is little evidence regarding the efficacy of online reasoning-based SCT education interventions in improving exercise behaviors in college-aged individuals. Therefore, we propose that combining higher-order reasoning with SCT in an educational

intervention results in improved reasoning about physical activity as well as exercise behavior change.

Table 3.1. Effect of intervention on reasoning levels

	Pre (n=11)		Mid (n=11)		Post (n=11)	
	Mean	SD	Mean	SD	Mean	SD
Reasoning Level	1.18	0.42	1.82	0.75	2.73*	0.4

* Indicates significant difference in reasoning level from Pre-intervention

Table 3.2. Effect of Intervention on Leisure Time Physical Activity

	CTL Pre (n=23)		*CTL Post (n=23)*		*EXP Pre (n=11)*		*EXP Post (n=11)*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total MET-min/week LTPA	2876.28	2067.7	2043.83	2186.8	2341.29.5	1197.93	4278.83*	2775.01

* Indicates significant difference from pre-test (p<0.05)

Table 3.3. Between group comparisons for social cognitive theory variables

	CTL Pre (n=23)		*CTL Post (n=23)*		*EXP Pre (n=11)*		*EXP Post (n=11)*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PA Goal	3.14	0.88	3.08	1	4.0*	0.73	4.31*†	0.68
PA Self-Regulation	2.19	0.85	2.5	0.99	3.2*	1.2	3.95*†	0.85
PA Self-Efficacy	61.21	18.1	70.04	19.04	85.81*	11.788	92.35*	7.86

* Indicates significant difference from CTL group (P<.05)

† Indicates significant different from Pre-test (P<.05)

References

Alder, A., Stevens, R., Manley, S., Bilous, R., Cull, C., & Holman, R. (2003). Development and progression of nephropathy in type 2 diabetes: The United Kingdom Prospective Diabetes Study. *Kidney International, 63*(1), 225-232.

(2009). *American College Health Association-National College Health Assessment II.* American College Health Association.

Anderman, E. M., & Midgley, C. (1997). Changes in acheivement goal orientations, perceived academic competence, and grades across transition to middle-level school. *Contemporary Educational Psychology, 22*, 269-298.

Anderson, E. S., Winett, R. A., Wojcik, J. R., & Williams, D. M. (2010). Social cognitive mediators of change in a group randomized nutrition and physical activity intervention: Social support, self-efficacy, outcome expectations and self-regulation in the guide-to-health trial . *Journal of Health Psychology, 15*(1), 21-32.

Anderson-Bill, E. S., Suppini, A., & Apap, D. (2011). Social cognitive determinants of nutrition and physical activity among web-based health users enrolling in and online intervention: The influence of social support, self-efficacy, outcome expectations, and self-regulation. *Journal of Medical Internet Research, 13*(1), e28.

Bandura, A. (2012). Social Cognitive theory. In P. Van Lange, A. Kruglanski, & E. Higgins (Eds.), *Handbook of Theories of Social Psychology.* London: SAGE Publications Ltd.

Berlin, J. A., & Colditz, G. A. (1990). A meta-analysis of physical activity in in the prevention of coronary heart disease. *American Journal of Epidemiology, 132*(4), 612-628.

Boor, P., Celec, P., Behuliak, M., Grancic, P., Kebis, A., KukanqM., . . . Sebekova, K. (2009). Regular moderate exercise reduces advanced glycation and ameliorates early diabetic nephropathy in obese Zucker rats. *Metabolism, 58*, 1669-1677.

Broderick, T., Poirier, P., & Gillis, M. (2005). Exercise training restores abnormal myocardial glucose utilization and cardiac function in diabetes. *Diabetes/Metabolism Research and Reviews, 21*(1), 44-50.

Brunso, K., Scholdere, J., & Grunert, K. (2004). Closing the gap between values and behavior -- a means-end theory of lifestyle. *Marketing Communications and Consumer Behavior, 56*(6), 665-670.

Damasio, A. (2010). *Self comes to mind: Constructing the conscious brain.* New York, NY: Random House.

Dennis, E., Potter, K., Estabrooks, P., & Davy, B. (2012). Weight gain prevention for college freshman: Comparing two social cognitive theory-based interventions with and without explicit self-regulation training. *Journal of Obesity*, 803769.

Dinger, M., Behrens, T., & Han, J. (2006). Validity and reliability of the international physical activity questionnaire in college students. *American Journal of Health Education*, *37*(6), 337-343.

Doerksen, S. E., Umstattd, M., & McAuley, E. (2009). Social cognitive determinants of moderate and vigorous physical activity in college freshman. *Journal of Applied Sports Psychology, 39*(5), 1201-1213.

Frith, C., Friston, K., Liddle, P., & Frackowiak, R. (1991). Willed action and the prefrontal cortex in man: A study in PET. *Proceedings of the Royal Society of London*, 241-246.

Hallam, J. S., & Petosa, R. (20004). The long-term impact of a four-session work-site intervention on selected social variables linked to adults exercise adherence. *Health Education Behavior, 31*(88), 88-100.

Hamblin, M., Friedman, D., Hill, S., Caprioli, R., Smith, H., & Hill, M. (2007). Alterations in the diabetic myocardial proteome coupled with increased myocardial oxidative stress underlies diabetic cardiomyopathy. *Journal of Molecular and Cellular Cardiology, 42*(4), 884-895.

Hambrecht, R., Wolf, A., Gielen, S., Linke, A., Hofer, J., Erbs, S., . . . Schuler, G. (2000). Effect of exercise on coronary endothelial function in patients with coronary artery disease. *The New England Journal of Medicine, 342*, 454-460.

Haskell, W. L., Lee, I., Pate, R., Powell, K., Blair, S., Franklin, B., . . . Bauman, A. (2006). Physical activity and public health: Updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. *Medicine and Science in Sports and Exercise*, *39*(8), 1423-34

Hu, F., Leitzmann, M., Stampfer, M., Colditz, G., Willet, W., & Rimm, E. (2001). Physical activity and television watching in relation to risk for type 2 diabetes mellitus in men. *Archives of nternal Medicine, 161*(12), 1542-1548.

Keating, X. D., Guan, J., Pinero, J., & Bridges, D. (2010). A meta-analysis of college students' physical activity behaviors. *Journal of American College Health, 54*(2), 116-126.

Kohl, H. W., Gordon, N., Villegas, J., & Blair, S. (1992). Cardiorespiratory fitness, glycemic status, and mortality in men. *Diabetes Care, 15*, 185-192.

Kohlberg, L. (1981). *The philosophy of moral development: Moral stages and the idea of justice.* New York: Harper & Row.

Kohlberg, L., & Lickona, T. (1976). Moral stages and moralization: The cognitive-developmental approach. *Moral Development and Behavior: Theory, Research, and Social Issues*. New York: Rinehart and Winston.

Kretchmar, R. S. (2001, 53 318-325). Duty, habit, and meaning: Different faces of adherence. *Quest*.

LaMonte, M. J., Blair, S., & Church, T. (2005). Physical activity and diabetes prevention. *Journal of Applied Physiology, 9*, 1205-1213.

Leslie, E., Fotheringham, M., Veitch, J., & Owen, N. (2000). A university campus physical activity promotion program. *Health Promotion Journal of Australia, 10*, 51-54.

Mega, M., Cummings, J., Salloway, S., & Malloy, P. (1997). The limbic system: an anatomic, phyolgenetic, and clinical perspective. *Journal of Neuropsychiatry,* 315-350.

Myers, J. (2003). Exercise and cardiovascular health. *Circulation, 107*, e2-e5.

Okada, S., Hiuge, A., Makino, H., Nagumo, A., Takaki, H., Konishi, H., . . . Miyamoto, Y. (2010). Effect of exercise intervention on endothelial function and

incidence of cardiovascular disease in patients with type 2 diabetes. . *Journal of Atheroscolerosis and Thrombosis, 17*(8), 828-833.

Pally, R. (2007). The predicting brain: Unconscious repetition, conscious reflection and therapeutic change. *The International Journal of Psychoanalysis, 88*, 861-881.

Pope, C., Ziebland, S., & Mays, N. (2000). Qualitative research in health care. Analyzing qualitative data. *British Medical Journal*, *320*, 114-116.

Powell, K. E., Thompson, P., Caspersen, C., & Kendrick, J. (1987). Physical activity and the incidence of coronary heart disease. *Annual Reviews of Public Health, 8*, 253-287.

Reimer, J., Paolitto, D., & Hersh, R. (1983). *Promoting moral growth: From Piaget to Kohlberg.* Prospect Heights: Waveland Press.

Rovniak, L. S., Anderson, E., Winett, R., & Stephens, R. (2002). Social cognitive determinants of physical activity in young adults: A prospective structural equation analysis. *Annals of Behavioral Medicine, 24*(2), 149-156.

Sallis, J. F., Nichols, J., Sarkin, J., Johnson, M., Caparosa, S., Thompson, S., & Alcarez, J. (1999). Evaluation of a university course to promote physical activity: Project GRAD. *Research Quarterly for Exercise and Sport, 70*(1), 1-19.

Soorappan, R. N., Devdoss, D., Kannan, S., Olsen, C., Gounder, S., Davidson, C., . . . Abel, E. (2012). Endurance exercise induces cardiac hypertrophy in aged Nrf2-/- mice. *Circulation Research, 111*, A30.

Standage, M., & Ryan, R. (2012). Self-determination theory and exercise motivation: Facilitating self-regulatory processes to support and maintain health and well-

being. In G. C. Roberts, & D. Treasure, *Advances in Motivation in Sport and Exercise.* Champaign, IL: Human Kinetics.

(2010). *State indicator report on physical activity.* Center for Disease Control and Prevention, U.S. Department of Health and Human Services, Atlanta.

Tanasescu, M., Leitzmann, M., & Rimm, E. (2003). Physical activity in relation to cardiovascular disease and total mortality among men with type 2 diabetes. *Circulation, 107*, 2435-2439.

Tancredi, L. R. (2005). *Hardwired Behavior.* New York, NY: Cambridge University Press.

Chapter 4: Exercise induced Nrf2 response is blunted in the Db/Db mouse and O-GlcNAc regulates Nrf2 activity

"The beautiful thing about setbacks is they introduce us to our strengths". ~ Robin Sharma

Research in science is unpredictable, and often we have to overcome unforeseen obstacles and pursue interesting lines of inquiry. Such was the case in this dissertation; the research presented in this chapter has deviated slightly from the original research proposal. The original proposal included a chronic exercise study to examine the effect of chronic exercise training on Nrf2 in diabetes. Due to unforeseen and unpredictable circumstances, the research project deviated slightly from the original proposal. Below is a summary of events that preclude the data from the chronic exercise study being incorporated into this dissertation.

During the exercise intervention two of the diabetic mice died during training sessions in uncontrollable accidents, effectively reducing our sample size. During our drug trial we administered doses of fk228 that were identical to published literature, however we found that this dose with lethal in 100% of our mice. As both animals and the drug were extremely expensive, this project had to be sideline until full analysis of the pharmacokinetics of the drug could be investigated. Lastly, we completed the chronic exercise training in the control and diabetic mice; however, due to an outbreak of Parvo virus in the vivarium, we were unable to have sedentary tissue to compare the tissue with. As the diabetic mice are expensive and the

outbreak is still no contained, we are unable to finish the tissue analysis of the chronically exercised mice.

The aforementioned events were unforeseen and unavoidable. Despite these setbacks, we decided to follow up interesting findings in the acute exercise studies with several more research experiments to elucidate mechanisms of regulation of Nrf2 that would be pertinent to our findings. These experiments required a substantial amount of additional work. The additional experiments were fruitful and elucidated a novel mechanism of regulation in Nrf2 signaling that may have vast implications for diabetes and cancer research. In the end, the research remained focus on the effect of exercise on Nrf2 function in diabetes and the role of O-GlcNAc modification in response to exercise and its role in Nrf2 signaling.

The Ph.D. degree is a process of learning how to apply knowledge and making novel contributions to your field with intellectual rigor. The experience of dealing with adversity and learning to adjust in the face of setbacks greatly enriched the Ph.D. experience. During this process I have become adept at applying newly acquired knowledge and have made novel discoveries and contributions to our field. As a Ph.D. is a process of learning, the original proposal for this study is presented in Appendix A, so that others may see how and why research changes, and to inspire future students to use setbacks as inspiration to push forward and pursue interesting questions.

Exercise induced Nrf2 response is blunted in the Db/Db mouse and O-GlcNAc regulates Nrf2 activity

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Abstract

Background: Oxidative stress is a central feature of diabetes and plays a causal role in the pathogenesis of diabetic complications. The redox sensitive transcription factor, NF-E2–related factor 2 (Nrf2) protects against oxidative stress and exercise increases Nrf2 in healthy humans. Therefore, research regarding the effect of exercise on Nrf2 in T2DM is warranted. **Methods:** Normal and diabetic (*db/db*) mice were subjected to an acute exercise test and western blotting and qPCR were utilized to assess how exercise affects the Nrf2 transcription. We further explored the role of OGT and O-GlcNAcylation on Nrf2 signaling using a tamoxifen-inducible cardiomyocytes-specific OGT knockdown (OGTKD) mouse and *in vitro* studies in cardiomyocytes (H9C2 cell line). **Results:** Acute exercise increases Nrf2 protein content in normal mice (p=0.048) and mRNA expression of the Nrf2-mediated gene products Catalase and NQO1 (*$p<0.05$ for all comparisons)*, yet no changes were observed in the diabetic mice. We also showed that protein O-GlcNAcylation (p<0.01 for all comparisons) and levels of the 110kDa isoform of the OGT enzyme is altered

in the *db/db* mouse heart (*p*=0.002). Loss of the OGT enzyme resulted in drastic reduction in Catalase mRNA (*p*=0.033). Conversely, increasing O-GlcNAcylation in H9C2 cells augmented Nrf2 transcription of Catalase, GCLM, and HMOX1 (*p<.05 for all comparisons).* Lastly, *in silico* analysis identified Thr^{595} as a possible O-GlcNAcylation site near NLS and co-activation motif. **Conclusion:** An exercise-induced increase in the Nrf2 response is blunted in the *db/db* mouse, which may be due to aberrant O-GlcNAc modification. Furthermore, O-GlcNAcylation may play a regulatory role in the localization and/or transcription of the Nrf2 transcription factor.

Introduction

Type 2 diabetes mellitus (T2DM) is reaching pandemic levels, affecting approximately 350 million people worldwide. In 2012, the estimated cost of diagnosed diabetes in the United States was $245 billion (Danaei, et al., 2011). T2DM is characterized by metabolic dysfunction and increases debilitating complications such as cardiomyopathy, nephropathy, neuropathy, and overall mortality (Alder, Stevens, Manley, Bilous, Cull, & Holman, 2003; Kilhovd, Berg, Birkeland, Thorsby, & Hanssen, 1999). The high prevalence and large financial burden associated with the disease emphasizes the need to develop effective therapeutic strategies that simultaneously and comprehensively target the many aberrant aspects of the diabetic milieu. While the positive effects of exercise on multiple aspects of the diabetic milieu are well described in literature (Short, et al., 2003; Lumini et al., 2008), the molecular mechanisms are not well understood. Developing a better understanding of molecular mechanisms behind the beneficial effect of exercise in diabetes may lead to more effective therapeutic agents.

Substantial evidence has demonstrated that oxidative stress plays a causal role in insulin resistance, diabetic cardiomyopathy, neuropathy, and nephropathy (Jing, et al., 2011; Bonnard, et al., 2008). Patients with T2DM have a diminished capacity of the endogenous antioxidant system and oxidative stress is a central feature underlying both the pathogenesis of T2DM as well as associated comorbidities. Exercise has been shown to improve the endogenous antioxidant system via upregulation of antioxidant gene expression (Gomez-Cabrera, Domenech, & Vina, 2008). Thus, it possible that exercise exerts its beneficial effects in diabetes via improving the endogenous antioxidant system.

The redox sensitive transcription factor, NF-E2–related factor 2 (Nrf2) is one of the major cellular defenses against the cytotoxic effects of oxidative stress (Zhong, Mishra, & Kowluru, 2013; Bonnard, et al., 2008). In response to acute stress, the Nrf2 signaling pathway induces the expression of cytoprotective genes such as SOD, HMOX1, NQO1, GCLC, and GCLM (He, Kan, Cai, & Ma, 2009). Interestingly, the chronic cytotoxic insult of the diabetic condition results in decreased Nrf2 content and transcriptional activity in mouse models of type 2 diabetes mellitus (T2DM) and in cross-sectional human studies (Tan, et al., 2011; Miao, Gonzalo, Lantin, & Natarajan, 2004; Siewart, Gonzalez, Santillan, Lucero, Ojeda, & Gimenez, 2013). Furthermore, defective Nrf2 signaling has been shown to contribute to diabetic complications (Velmurugan, Sundaresan, Gupta, & White, 2013). Transgenic overexpression of Nrf2 by knockout of its cytosolic inhibitor, Keap1, suppresses the onset of diabetes in the *Db/Db* mouse and in high-calorie-diet induced obesity (Uruno, et al., 2013). Small molecule activators of Nrf2 attenuate the onset of

diabetes and diabetic nephropathy and cardiomyopathy in animal models (Zheng, et al., 2011; Uruno, et al., 2013; Palsamy & Subramanian, 2011; Bai, et al., 2013). . This evidence highlights Nrf2 as a central mediator of diabetic complications.

Recent evidence from both human and animal studies demonstrates that exercise increases both the content and activity of Nrf2 in skeletal muscle and myocardium in healthy subjects (Cartoni, et al., 2005; Baar, et al., 2002). Currently, there is a lack of information regarding the cause for reduced Nrf2 function and the effect of exercise on Nrf2 in diabetes. Therefore, a major purpose of this paper is to explore the effect of exercise on Nrf2 and a novel mechanism of Nrf2 regulation, O-GlcNAc modification.

The Nrf2 signaling cascade is multi-staged and is regulated by post-translational modifications. The post-translational O-linked attachment of the beta-N-acetylglucosamine (O-GlcNAc) sugar moiety modifies serine/threonine residues of thousands of structural proteins and transcription factors, whereby it can regulate transcriptional activity. O-GlcNAc cycling is controlled by the O-GlcNAc transferase (OGT) enzyme, which adds O-GlcNAC serine and threonine residues, and O-GlcNAcase (OGA), which removes it. O-GlcNAc modification is linked to cellular metabolism. Approximately 2–4% of glucose entering the cell is shuttled through the hexosamine biosynthetic pathway (HBP), whose end product is UDP-GlcNAc, the substrate for O-GlcNAc modification. As T2DM is a metabolic disease, it is no surprise that aberrant O-GlcNAcylation is involved in disturbed cell signaling in the myocardium, including the regulation of transcription factors (Kim, Woo, Joo, & Moon).

Increasing O-GlcNAc levels in the presence of oxidative stress increases Nrf2-mediated gene products, suggesting O-GlcNAcylation may play a role in the activity of Nrf2 (Ngoh, Watson, Facundo, & Jones, 2011). However, the role of O-GlcNAc on Nrf2 signaling has never been directly been explored. We have previously demonstrated that exercise alters global O-GlcNAc levels, O-GlcNAcylation of transcription factors, and the association of OGT with transcription factors and transcription complexes in animal models of T2DM (Cox & Marsh, 2013). Therefore, we hypothesized that acute exercise would increase Nrf2 and alter O-GlcNAcylation in the diabetic mouse heart and that O-GlcNAc modification plays a role in Nrf2 signaling.

Methods

Animals

The procedures in this study followed the guidelines of the Washington State University Institutional Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85– 23, revised 1996). 8-week-old type 2 diabetic mice (B6.BKS (D)-Leprdb /J, db+/db+ (*db*) and age-matched C57BL/6J non-diabetic lean background strain controls (C57) were purchased from Jackson Laboratories (Bar Harbor, ME). To control for activity, mice were singly housed without environmental enrichment in a climate controlled vivarium on a 12:12 light:dark cycle. Mice consumed water and standard chow ad libitum.

The tamoxifen-inducible, cardiomyocyte-specific OGT knockdown (OGTKD) mouse model which was generated by crossing loxP-flanked OGT knockdown mice

(OGTtm1Gwh/J) with B6;129-Tg(Myh6-creEsr1)1Jmk/J mice carrying a mutant estrogen receptor ligand binding domain fusion protein-Cre (MerCreMer) under transcriptional control of the α-myosin heavy chain gene promoter as previously described (Watson, et al., 2010). Inducible cardiomyocyte specific OGT knockdown (KD) and WT mice were treated tamoxifen or vehicle (corn oil) treated at 5 weeks to generate the OGT KD. Mice lacking MerCreMer were used as 'wild type' controls (WT).

Exercise Protocol

For the acute exercise, *db/db* and C57/B6J mice underwent a VO_2 max test. The VO_2 max test was completed as previously described (Rocco, LeValley, Eldridge, Marsh, & Rodgers, 2014) with one change for the *db/db* mice. The grade was reduced to 5% for the *db/db* mice to ensure the mice reached maximal oxygen consumption prior to volitional exhaustion. Similar workload mechanical and metabolic workloads have been shown to elicit a Nrf2 response in mice (Muthusamy, et al., 2012).The mice were sacrificed 15 minutes after cessation of the test. Ventricular tissue was harvested from the mice and frozen in liquid nitrogen and in 4% paraformaldehyde for later analysis.

Cell Culture/In Vitro

H9C2 neonatal rat myoblasts from ATCC were used for these experiments. One day prior to treatment, the medium was replaced with normal glucose (5.6mM), low serum (1% FBS), and 1% L-glutamine. Cells were then treated with combinations of Angiotensin II (AngII), Glc-NAc, and ThiametG, a selective inhibitor of OGA.Treatment concentrations were as follows: AngII (1μM), Glc-NAc (10mM),

Thiamet G (10nM). Cells were harvested 24 hours after treatment. Samples for RNA analysis were treated with RNAprotect Cell Reagent (Qiagen,Valencia, CA) and stored at -80°C. Samples for western blotting and immunoprecipitation were treated with homogenization buffer (RIPA Buffer 50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% v/v protease inhibitor, 1%v/v phosphatase inhibitor, and 0.1% PUGNAc), homogenized, centrifuged at 13,000 rpm for 10 min; the supernatant was then removed at stored at -80°C.

Western Blotting

Heart tissue was homogenized in Tissue Protein Extraction Reagent (Sigma-Aldrich, St. Louis, MO); 20 mM sodium fluoride; 1 mM sodium orthovanadate; 3% protease inhibitor cocktail (Sigma); 0.02% PUGNAc, an OGA inhibitor (Sigma), was also added in order to inhibit removal of O-GlcNAc from proteins. Total protein was quantified using a modified Lowry assay (BioRad, Hercules, CA) and on a NanoDrop 2000™ spectrophotometer (Thermo Fisher Scientific, Rockford,IL) and equal protein concentrations were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were probed overnight at 4°C with primary antibodies (anti-Nrf2 (D1Z9C), Cell Signaling; anti-OGT (50271), -Nrf2 (181455) and –calsequestrin (3516), Abcam; anti-OGlcNAc (CTD 110.6), gift from Mary-Ann Accavitti, University of Alabama Birmingham), then probed with appropriate secondary antibody for 1 hour at room temperature. Chemiluminescent substrates (Thermo Fisher Scientific, Rockford,IL) were used to detect horseradish peroxidase activity on a ChemiDoc (BioRad). ImageJ software (National Institutes of Health, Bethesda, MD) was used

to conduct densitometry for protein levels on duplicate blots. All protein levels were normalized to the loading control calsequestrin.

RNA Isolation and qPCR

RNA was isolated from whole tissue with a fibrous tissue RNA isolation kit (Qiagen,Valencia, CA); RNA from cells was isolated using a an RNA isolation kit (Qiagen,Valencia, CA). All isolated was RNA quantified on a NanoDrop 2000™ spectrophotometer (Thermo Fisher Scientific, Rockford,IL). Complementary DNA (cDNA) was created from isolated RNA using a cDNA synthesis kit (Thermo Fisher Scientific) and amplified using a GeneAmp PCR System 9700 (Applied Biosystems, Grand Island, NY). Quantitative polymerase chain reaction (qPCR) was performed in triplicate with SYBR green fluorescence chemistry (Qiagen). The negative control utilized water instead of cDNA for the reaction. Thermal cycling was performed on an iCycler iQTM Real Time PCR Detection System (Biorad) using the following cycle: 95°C for 10 min, and 40 cycles of 95°C for 30 sec and Tm for 10 sec. Melt curve analysis was utilized to confirm primer specificity. The qPCR data were analyzed utilizing the ΔΔCT method with normalization to a housekeeping gene (GAPDH and RPLP0) as previously described (Livak & Schmittgen, 2001). Primer were custom designed and details can be found in Table 2.

Immunoprecipitation

Ventricular tissue was homogenized in RIPA, 1% phosphatase inhibitor, 2% protease inhibitor (Sigma), and 0.02% PUGNAc. Lysates were assayed for total protein in the same manner as for Western blotting. Equal protein concentrations of each sample were precleared over protein A/G agarose beads (Thermo Fisher

Scientific) at 4°C for 4 hours. 5 µl of Nrf2 antibody was added to precleared supernatants and incubated overnight at 4°C. The samples were then added to washed beads and incubated for 2 hours at 4°C. After incubation the samples were spun at 13,000 x G for 2 minutes, and supernatant was removed. The beads were washed 6 times and then eluted at 100°C for 5 min. The eluents were then loaded and ran on SDS page as described above. The negative control was ventricular lysate that was immunoprecipitated without antibody.

O-GlcNAcylation site prediction

The YinOYang 1.2 prediction server (http://www.cbs.dtu.dk/services/YinOYang/) was used to identify possible sites of O-GlcNAc modification in the murine Nrf2 protein. Analysis of the murine Nrf2 protein was conducted by entering the proteins FASTA sequence into the server. This server identifies consensus sequences that have potential for both O-GlcNAc modification and phosphorylation.

Statistical analysis.

Data were tested for normality using Shapiro-Wilk test and appropriate transformations were made. Two-way ANOVA was utilized to examine effects of exercise and genotype on protein content and mRNA levels; Student *t*-tests were used in cases where single comparisons were made (i.e. OGT KD data). One-way ANOVA was utilized for cell culture data to compare treatments on Nrf2 protein and mRNA of Nrf2-mediated gene products. Tukey's HSD was used for post-hoc analysis for all multiple comparisons. All statistical analysis was done using the R statistical computing software. Statistical significance for all measures of this study were set at $\alpha=.05$.

Results

Animal characteristics

Physical characteristics of the exercised mice are presented in Table 1. Briefly, the *db/db* mice weighed more ($p<0.00001$), had higher glucose levels ($p=0.0006$), and had a lower heart weight to body weight ratio (HW:BW) ($p=0.0005$) but no difference in heart weight($p=0.21$).

Nrf2 response to acute exercise is blunted in the db/db mouse heart.

Here we show for the first time that the *db/db* mouse has a blunted Nrf2 response to exercise. We measured Nrf2 protein content and mRNA of Nrf2-mediated genes in heart tissue from 7-week-old diabetic (*db/db)* and non-diabetic (C57/B6J) mice that were subjected to an acute maximal exercise test. In 7 week-old mice, there was no difference in Nrf2 content in the sedentary mice ($p=.43$) (4.1 A and B). Acute maximal exercise induced an increase in Nrf2 content in the non-diabetic mice ($p=.048$), but not in the diabetic mice (Figure 4.1 A and B). Acute exercise also increased mRNA levels of Nr2-mediated genes in the control mice but not in the diabetic mice. Catalase and NQO1 expression were increased in the acutely exercised control mice ($p=0.0005$ and $p<0.00001$, respectively) while the diabetic mice showed no increase. Catalase and NQO1 expression in the acutely exercised control mice was also higher than both the sedentary and exercised diabetic mice ($p<0.05$ for all comparisons). GLCM expression showed a similar response to exercise as Catalase and NQO1; however, the changes in GCLM were not significant at a level of $\alpha=0.05$. Together, these data suggests that the response of Nrf2 to acute maximal exercise is blunted in the *db/db* mouse heart.

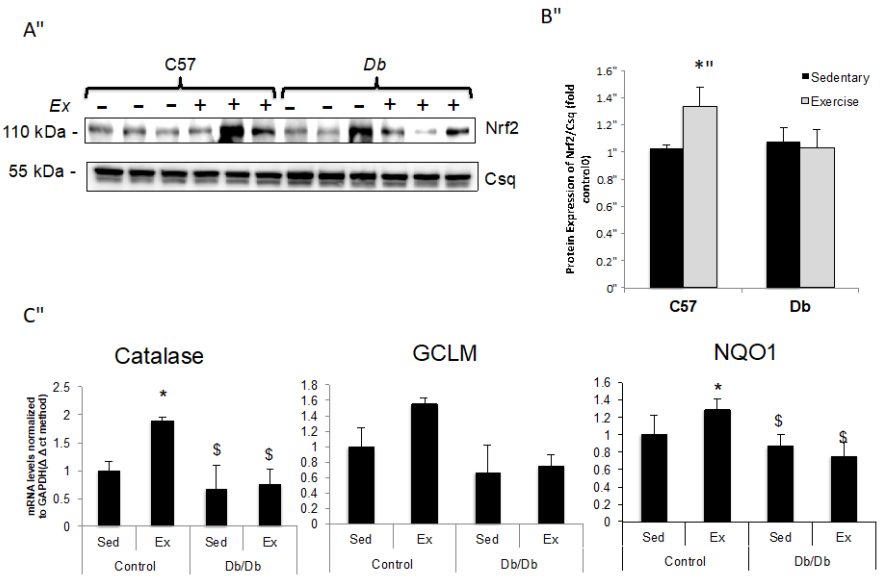

Figure 4.1.The effect of acute exercise on Nrf2 protein content and Nrf2-mediate gene products in C57/B6J and *db/db* mice. A) Representative Western blot of Nrf2 protein content and loading control calsequestrin. B) Quantification of Western blot by densitometry. C) mRNA levels of Catalase, GCLM, NQO1. n=5 for each genotype in all experiments. * indicates significant effect of exercise within genotype (p<0.05). $ indicates significant difference from exercised control mice (p<0.05).

Protein O-GlcNAcylation and OGT protein content is altered in the db/db mouse heart.

O-GlcNAc cycling is aberrant in the diabetic heart; therefore, we examined the hearts of the exercised mice for global levels of protein O-GlcNAcylation to determine how maximal exercise effects protein O-GlcNAcylation in the *db/db* mouse heart. Total O-GlcNAc levels after exercise were not different between genotype and acute maximal exercise did not significantly alter levels of total O-GlcNAc in either the *Db/Db* or control mouse hearts. Densitometric analysis of O-GlcNAc levels was also performed over the high, medium, and low molecular weight ranges as previously described (Cox & Marsh, 2013). When analyzed by molecular weight, we found that both the sedentary and exercised *db* mouse hearts had lower

levels of high molecular weight O-GlcNAc when compared to both sedentary and exercise controls (all comparisons $p<0.01$) (Figure 4.2A and 4.2B). No other differences were observed in O-GlcNAc levels. Protein levels of the nucleocytoplasmic 110kDa isoform form of OGT were modestly increased by exercise in the control mice ($p=0.103$), although this was not significant at our *a priori* determined level of significance. Protein levels of OGT in the exercised control mice were higher than both the sedentary and exercised diabetic mice ($p=0.002$ and $p=0.01$, respectively) and there was change in OGT levels by exercise in the diabetic mice (Figure 4.2C and 4.2D).

The Nrf2 transcription factor lies in the high-molecular weight region and we observed a positive relationship between high-molecular weight O-GlcNAc levels, indicating O-GlcNAcylation may influence Nrf2. We analyzed the levels of high-molecular weight O-GlcNAcylation and mRNA levels of the Nrf2 gene products and found that high-molecular weight O-GlcNAcylated protein levels were positively correlated with mRNA levels of the Nrf2 mediated gene products ($p=.021$, $R^2=0.43$) (Figure 4.2E). Together, these results suggest that O-GlcNAc modifications of high molecular weight proteins may be altered in the *db* mouse heart and acute exercise does not alter the genotype effect on global protein O-GlcNAc modification and that diabetic mice have altered levels of 110kDa OGT. Furthermore, there may be a positive relationship between OGT and O-GlcNAc modification and Nrf2.

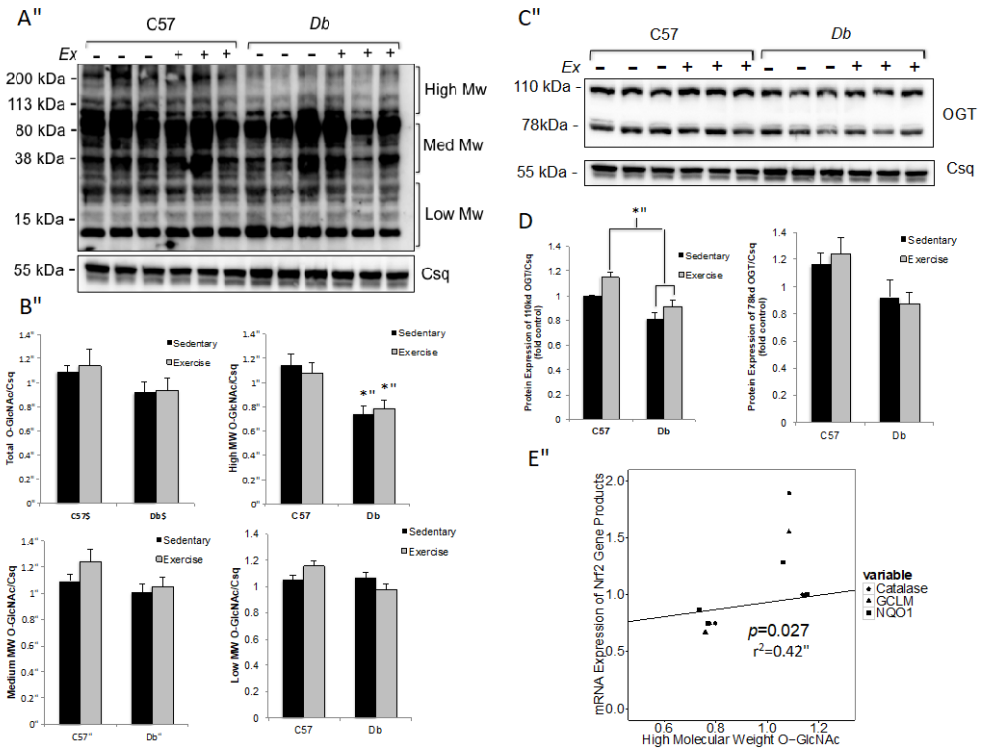

Figure 4.2. The effect of acute exercise on protein O-GlcNAcylation and OGT protein content in C57/B6J and *db/db* mice. A) Representative Western blot of protein O-GlcNAcylation and loading control calsequestrin. B) Quantification of O-GlcNAc Western blot by densitometry. C) Representative Western blot of OGT protein content and loading control calsequestrin. D) Quantification of O-OGT Western blot by densitometry. E) Correlation analysis of high molecular weight O-GlcNAc and mRNA expression of Nrf2 Gene products. * indicates significant difference from C57/B6J mice (p<0.05). n=5 for all experiments

Loss of OGT enzyme decreases Nrf2 gene products

We then examined the role of O-GlcNAc on Nrf2 *in vivo* by observing the effect of the loss of the OGT enzyme on Nrf2 in the mouse myocardium. We compared whole heart tissue from inducible cardiomyocyte specific OGT knockdown (KD) mice with WT mice for Nrf2 protein content and mRNA of the Nrf2-mediated gene product Catalase. We found that Nrf2 protein content is increased 2-fold in the KD heart (p=0.033) (Figure 4.3A and 4.3B). Despite the increase in Nrf2 protein content, the KD mouse had a 5-fold reduction of mRNA content of the Nrf2 gene product

Catalase when compared with WT (p=.027) (4. 3C). The marked reduction of the Nrf2 transcript Catalase in the OGT KD mouse suggest that OGT and O-GlcNAcylation play a vital role in the Nrf2 signaling cascade.

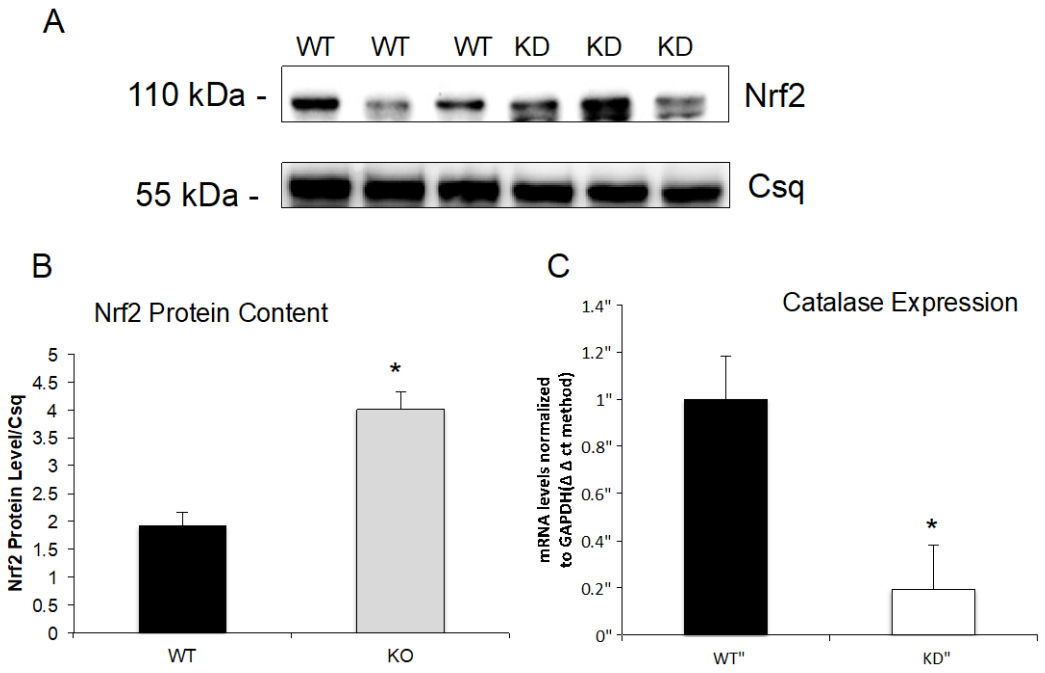

Figure 4.3. Nrf2 function in OGT KD mice. A) Representative Western blot of Nrf2 protein content and loading control calsequestrin in WT and OGT KD mice. B) Quantification of Western blot by densitometry. C) mRNA levels of Catalase in the OGT KD mouse compared to control. n=5 for all experiments. * indicates significant difference between WT and OGT KD (p<0.05)

O-GlcNAc increases Nrf2 transcription in response to cellular stress *in vitro*

Based upon the results of the *in vivo* data, we hypothesized that O-GlcNAc might serve as a positive regulator of Nrf2. To examine this hypothesis we interrogated cardiomyoblasts (H9C2 cells) to determine the role of O-GlcNAc in Nrf2 signaling in response to a cellular stress. Angiotensin II (Ang II) has been shown to induce both a hypertrophic stimulus and oxidative stress in the myocardium (Dai, et al., 2011; Watkins, Borthwick, & Arthur, 2011). Therefore, we treated H9C2 cells with

Ang II and increased O-GlcNAc with N-acetylglucosamine or by inhibiting O-GlcNAcase with Thiamet G (TG).

We found that AngII increased Nrf2 protein content (p=0.04) and increasing O-GlcNAc by administering GlcNAc or TG attenuated the AngII induced increase in Nrf2 protein content (p=0.49 and p=0.20, respectively) (Figure 4.4A and 4.4B). Although AngII increased protein content, it did not significantly increase mRNA levels of the Nrf2-mediated genes, Catalase, GCLM, and HMOX1 at a significance level of α=.05 (Figure 4.4A and 4.4B). Interestingly, we found that increasing O-GlcNAc levels with either N-acteylglucosamine (GlcNAc) or the PUGNAc inhibitor Thiamet G, increased mRNA levels of Nrf2 mediated genes in AngII treated cardiomyoblasts despite no increase in Nrf2 protein content. Catalase expression was increased only in the AngII + GlcNAc treated cells (p=0.04) (Figure 4.4C). GCLM was increased in both the AngII + GlcNAc (p=0.017) and the AngII + Thiamet G (p=0.027) treated cardiomyoblasts (Figure 4.4C). HMOX1 was also increased in the AngII + GlcNAc (p=0.027) and the AngII + Thiamet G (p=0.0005) treated cardiomyoblasts (Figure 4.4C). Together, this evidence suggests that in the presence of cellular stress O-GlcNAc augments Nrf2 signaling in cardiomyoblasts independent of increased Nrf2 protein content.

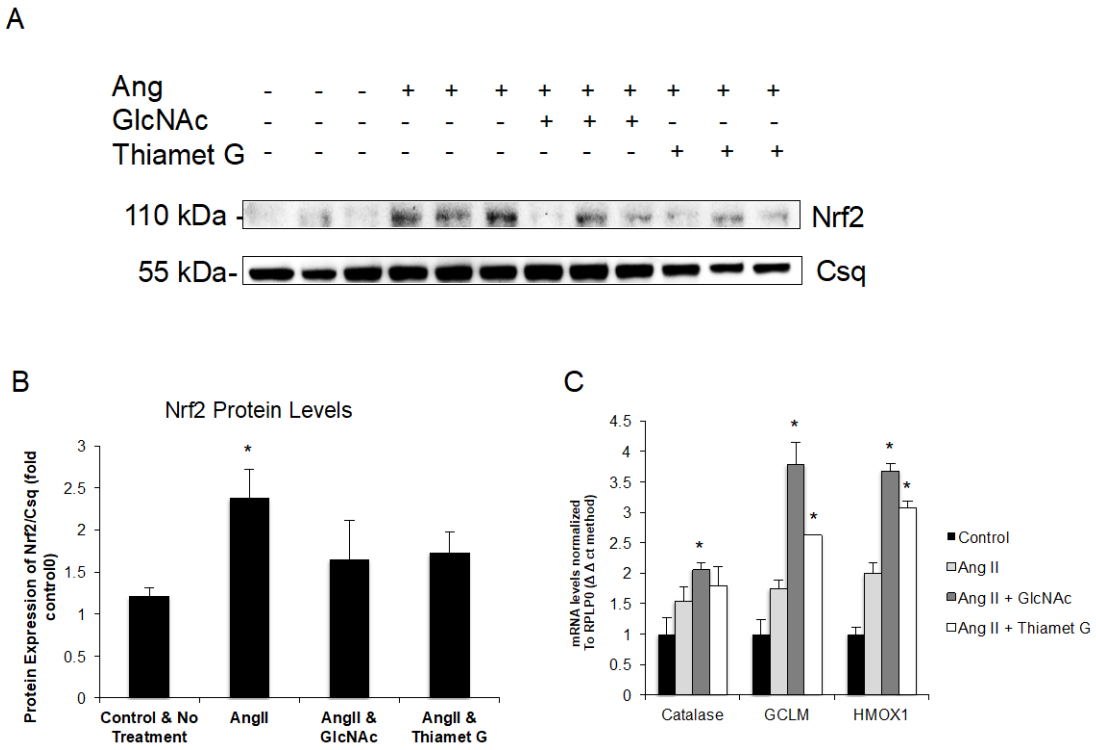

Figure 4.4.The effect of angiotensin II, N-acetylglucosamine (GlcNAc), and Thiamet G on Nrf2 function in H9C2 cells. A) Representative Western blot of Nrf2 protein content and loading control calsequestrin. B) Quantification of Western blot by densitometry. C) mRNA levels of Catalase, GCLM, and HMOX1. n=4 for each condition experiments. * indicates significant effect of treatment from control (p<0.05).

Nrf2 is O-GlcNAcylated and prediction analysis of Nrf2 glyocsylation sites identifies Thr^{594} as a possible O-GlcNAcylation site.

Accumulating data presented provide strong evidence that the O-GlcNAc PTM is involved in the Nrf2 signaling cascade. A thorough review of the literature revealed no data on O-GlcNAc modification of Nrf2. Therefore, we performed immunoprecipitation of Nrf2 from heart tissue of the C57/B6J and performed Western blotting for O-GlcNAcylation of the Nrf2 protein. We were able to demonstrate for the first time that Nrf2 is indeed O-GlcNAcylated *in* vivo (Figure 4.5A). We then utilized computational modeling and performed an *in silico* analysis

of the murine Nrf2 protein using YinOYang 1.2 prediction server. The analysis identified several possible O-GlcNacylation sites (Figure 4.5B). Based upon current knowledge of the Nrf2 peptide and known roles of specific residues in the Nrf2 signaling cascade, we identified Thr^{594} as a key residue. The murine Nrf2 protein harbors a nuclear localization signal, the $P_{587}KSKKPD_{593}$ motif, in the NEH3 domain (Theodore, et al., 2008) (Figure 4.5C and 4.5D). Additionally, acetylation of two lysine residues within this motif, Lys^{588} and Lys^{591}, by CREB-binding protein (CBP) are involved in DNA binding and upregulation of Nrf2 transcription (Kawai, Garduno, Theodore, Yang, & Arinze, 2011). As Thr^{594} lies immediately adjacent to this motif, this finding suggests that O-GlcNAcylation of this reside may regulate the localization and/or activity of Nrf2. This finding coincides with our results that O-GlcNAc positively regulates Nrf2 transcriptional activity and provides a possible explanation for the blunted exercise-induced Nrf2 response observed in our *db/db* mice.

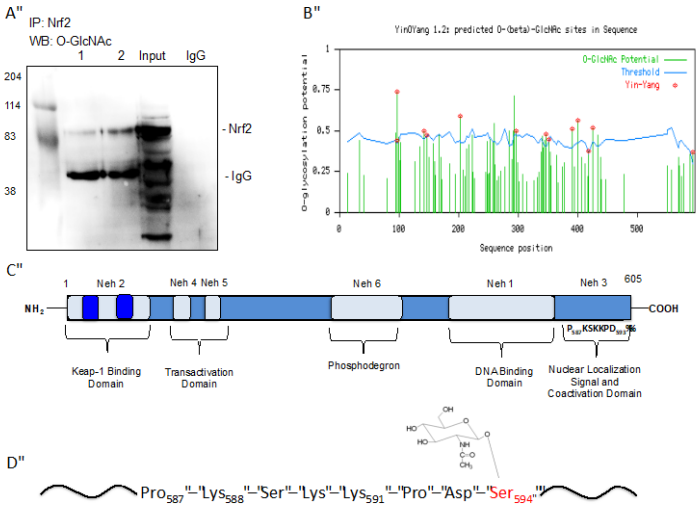

Figure 4.5. O-GlcNAcylation of Nrf2 and *in silico* prediction of protein glycosylation of Nrf2 by the YinOYang 1.2 server identifies Ser^{594} as a site possible site of O-GlcNAc modification. A) Immunoprecipitation of Nrf2 and Western blot with O-GlcNAc. B) Potential sites of O-GlcNAc modification. C)

Structure of the Nrf2 protein and the NLS motif ($P_{587}KSKKPD_{593}$) within the Neh3 domain. D) Possible O-GlcNAcylation of Ser^{594}

Discussion

Oxidative stress is a central feature in the pathogenesis of diabetes and its subsequent complications. Function of Nrf2 transcription factor, which coordinates the endogenous antioxidant system, is downregulated in the diabetic heart and is being heavily investigated as a therapeutic target for T2DM. Exercise has been shown increase Nrf2 function in normal rodents and humans, however the effect of exercise on Nrf2 in diabetes had not previously been studied. Additionally, Nrf2 signaling is regulated, in part, by posttranslational modifications. The O-GlcNAc modification has been shown to reduce oxidative stress in cardiomyocytes, suggesting a role of O-GlcNAc in Nrf2 function. In this paper we show that 1) the exercise-induced Nrf2 response is attenuated in the *db/db* mouse heart, 2) O-GlcNAc of high molecular weight proteins is reduced in the *db/db* mouse heart and this is unaffected by exercise, 3) loss of cardiac OGT *in vivo* results in a marked decrease in Nrf2 gene products, 4) O-GlcNAc augments Nrf2 transcription in the presence of cellular stress in cardiomyocytes, 5) identified a potential O-GlcNAcylation site of Nrf2 immediately adjacent to a motif that regulates nuclear localization and DNA binding, and 6) established an association between O-GlcNAc, OGT, and Nrf2.

Exercise-induced Nrf2 response is attenuated in the *db/db* mouse heart

We hypothesized that acute exercise would increase Nrf2 in the diabetic heart; however, the findings of this study were in contrast to our hypothesis. The data from our exercised mice provide evidence that the exercise-induced increase in Nrf2

signaling is blunted in the *db/db* mouse. The control mice exhibited an increase in Nrf2 protein content and transcription as assessed by mRNA levels of Nrf2-mediated genes, while the *db/*db mouse showed no change in either Nrf2 protein level or mRNA levels of Nrf2-mediated genes (Figure 4.1). Our findings from the control mice are in concordance with several other papers (Muthusamy, et al., 2012; Cartoni, et al., 2005; Asghar, George, & Lokhandwala, 2007). Muthusamy et al. demonstrated that an acute bout of exercise increased Nrf2 binding to the ARE in mice and showed similar levels of increased of mRNA as shown in our study (Muthusamy, et al., 2012). We found that acute exercise increased Catalase, GCLM, and NQO1 2-fold, 1.6-fold, and 1.3-fold respectively; Muthusamy et al. found that exercise increased mRNA levels 2-fold, 2-fold, and 1.5 fold respectively. Furthermore, human studies have demonstrated that Nrf2 mRNA is increased in skeletal muscle tissue approximately 1.5 fold immediately following a maximal exercise test on a cycle ergometer (Cartoni, et al., 2005).

The increase Nrf2 and mRNA levels of Nrf2-mediated genes was not observed in the diabetic mice, suggesting diabetic mice have an attenuated exercise-induced Nrf2 response (Figure 4.1). While no other studies have directly examined the effect of exercise on Nrf2 in diabetes, Laher *et al.* showed that acute exercise induces a roughly 2-fold increase in the expression of Catalase and GGLC in normal mice but not in *db/db* mice (Laher, et al., 2013), effectively demonstrating that the endogenous antioxidant system response to acute exercise is reduced in the *db/db* mouse. As Nrf2 regulates both Catalase and GCLC, the findings of Laher *et al.*

support our conclusion of an attenuated exercise-induced Nrf2 response in the *db/db* mouse and provides further explanation for the results of their paper.

O-GlcNAc of high molecular weight proteins and levels of OGT are reduced in the *db/db mouse heart*

In this paper we also showed that exercised control mice show higher expression of the nucleocytoplasmic isform of OGT (110kDa) when compared to diabetic mice, and that O-GlcNAcylation of higher molecular weight proteins was reduced in the diabetic mouse myocardium (Figure 4.2). These findings corroborate earlier work demonstrating that O-GlcNAc modification is aberrant in the diabetic heart (Fülöp, et al., 2007) and that 15 and 30 minutes of exercise alters O-GlcNAc levels (Medford, Porter, & Marsh, 2013). Our finding of increased 110kDa OGT in the C57/B6J mice is intriguing as the 110kDa isoform is the only nuclear form of OGT, suggesting exercise may result in increased O-GlcNAcylation of nuclear proteins, such as transcription factors. This finding is consistent with previous work from our lab where we showed that nuclear protein O-GlcNAcylation is elevated following acute exercise in C57/B6J (Medford, Porter, & Marsh, 2013).

O-GlcNAc positively regulates Nrf2

O-GlcNAc can function as a nutrient and cell stress sensor and acute increases in O-GlcNAc are cardioprotective in ischemia reperfusion, via augmenting the antioxidant response system (Laczy, Marsh, Brocks, Wittman, & Chatham, 2010; Ngoh, Watson, Facundo, & Jones, 2011). As Nrf2 coordinates the antioxidant response and O-GlcNAc increases this response in oxidative environments, we hypothesized that increased levels of O-GlcNAcylation would correspond with

increased mRNA levels of Nrf2-mediated genes. Therefore, we examined the relationship between the exercised-induced changes in Nrf2 transcription and changes in O-GlcNAcylation of high molecular weight proteins and levels of 110kDa OGT. Our analysis revealed a modest positive association with the Nrf2 mediated genes, Catalase, GCLM, and HMOX1. When taken together, this data suggest a positive association between the exercise-induced Nrf2 response and levels of high molecular weight O-GlcNAc and levels of OGT enzyme.

The findings from our exercise mice and the association between Nrf2 and O-GlcNAc led us to the second major aim of this paper; to examine the role of O-GlcNAC on Nrf2 activity. To explore how changes in O-GlcNAc affect Nrf2 we examined how the loss of OGT affects Nrf2 *in vivo* and how increasing O-GlcNAc affects Nrf2 *in vitro*. In this paper we demonstrated that loss of the OGT enzyme effects Nrf2 function *in vivo.* In the OGT knockdown animals there was a marked increase in Nrf2 protein content, yet marked reduction in mRNA levels of the Nrf2-gene product Catalase. This finding was intriguing as increases in Nrf2 protein content typically increase Nrf2 activity (Chen, et al., 2006; Li, Johnson, Calkins, Wright, Svendsen, & Johnson, 2005). The drastic reduction in mRNA levels of Catalase suggests that OGT and O-GlcNAc modification regulates Nrf2 transcription. The decreased Nrf2 transcription in the OGT KD was consistent with the positive association we saw between O-GlcNAc and Nrf2 gene products in the exercised mice. Research in *C. elegans* has demonstrated a similar finding on the role of O-GlcNAc in the oxidative stress response. Null mutation of the homologous OGT enzyme in *C. elegans* greatly reduces oxidative stress resistance, while null

mutation of OGA is resistant to oxidative stress, further suggesting that O-GlcNAc is an integral and perhaps essential part of the endogenous antioxidant system (Rahman, Stuchlick, El-Karim, Stuart, Kipreos, & Wells, 2010).

The *in vivo* data from our exercised and OGT knockdown animals revealed a distinct connection between the OGT enzyme, O-GlcNAcylation, and Nrf2 signaling. After showing that knockdown of OGT reduced Nrf2 transcription, we hypothesized that increasing O-GlcNAc in the presence of cellular stress would augment Nrf2 transcription. To explore this hypothesis we augmented O-GlcNAc in stressed cardiomyocytes *in vitro*. The Nrf2-mediated gene products Catalase, GLCM, and HMOX1 were increased only in cells where O-GlcNAc was augmented and this occurred without increasing protein levels of Nrf2; further supporting the notion that O-GlcNAc increases Nrf2 at a transcriptional level and not by increasing protein stability. This data may help explain how acute increases in O-GlcNAc are cardioprotective via reducing oxidative stress in ischemia reperfusion studies and in cells treated with H_2O_2 (Laczy, Marsh, Brocks, Wittman, & Chatham, 2010; Ngoh, Watson, Facundo, & Jones, 2011). The Jones lab has demonstrated that increasing O-GlcNAc attenuates oxidative stress and augments Nrf2 transcriptional activity in the presence of cellular stress (Ngoh, Watson, Facundo, & Jones, 2011). They showed that adenoviral overexpression of OGT reduced ROS while overexpression of OGA increased ROS in H_2O_2 neonatal rat cardiomyocytes (NRCMs). Furthermore they demonstrated that overexpression OGA in NRCMs reduced mRNA expression of Catalase and that inhibiting OGA with PUGNAc increased Catalase expression. These data support our *in vivo* data from the OGT KD mice and further suggest that

acute increases in O-GlcNAc can serve as a positive regulator of Nrf2 transcription. Although we cannot entirely rule out that the increased O-GlcNAc in our cells presented an additional stress which caused the augmented Nrf2 response to Ang II, our *in vivo* data from the OGT KD mice, coupled with the findings from the Jones lab, indicate that O-GlcNAc regulates Nrf2 transcription.

When considered in its entirety, these data indicates the blunted Nrf2 response seen in the *db/db* mouse may be due to altered O-GlcNAc signaling. Increased levels of O-GlcNAc are often considered maladaptive and pathological in diabetes. As such, our findings may seem paradoxical. However, there appears to be an important and distinct difference between acute and chronic increases in O-GlcNAc, whereby chronic elevations of O-GlcNAc are indeed maladaptive yet acute increases are beneficial. Furthermore, chronic elevation of the O-GlcNAc moiety in diabetes does not result pan O-GlcNAc modification of proteins. In fact, diabetes can result in decreased O-GlcNAcylation of transcription factors. For example, *in vivo* evidence has demonstrated that diabetes inhibits O-GlcNAcylation of the Sp1 transcription factor (Majumdar, Wright, Markowitz, Martinez-Hernandez, Raghow, & Solomon, 2004), indicating that despite elevated O-GlcNAc levels, diabetes can reduce O-GlcNAc modification of certain transcription factors.

Nrf2 is O-GlcNAcylation may occur near a localization and DNA-binding regulatory motif

Currently, precise mechanism by which the Nrf2 exercise-induced response is blunted in diabetic mice or the exact role O-GlcNAc and OGT plays in this signaling cascade remains elusive. We successfully demonstrated that Nrf2 is O-

GlcNAcylated in the mouse heart (Figure 4.5A), suggesting O-GlcNAc directly affects Nrf2 function. One possibility is that O-GlcNAc plays a role in altering the localization and/or activity of Nrf2. Murine Nrf2 harbors two nuclear localization signal (NLS) motifs in murine Nrf2, one located in the NEH2 domain (R_{42}QKDYELEKQKK$_{53}$) and one in the NEH3 domain (P_{587}KSKKPD$_{593}$) (Theodore, et al., 2008). In addition to harboring a NLS, the P_{587}KSKKPD$_{593}$ motif is involved in transcriptional regulation of Nrf2 via acetylation. Acetylation of Nrf2 by CREB-binding protein (CBP) induces transcription and promotes nuclear localization and mutation studies have identified Lys^{588} and Lys^{591} as acetylation sites essential for Nrf2 transcription (Kawai, Garduno, Theodore, Yang, & Arinze, 2011). *In silico* analysis of the murine Nrf2 peptide by the YinOYang glycosylation prediction server revealed that Thr^{594}, which lies immediately adjacent to the NLS and acetylated Lys^{588} and Lys^{591} in the NEH3 domain, is likely to be O-GlcNAcylated (Gupta & Brunak, 2002). This suggests that O-GlcNAc may indeed play a role in nuclear localization of Nrf2. Indeed, O-GlcNAcylation of a serine residue (Ser^{484}) in the NLS of the sp1 transcription factor enables nuclear localization and DNA binding, while RNAi of OGT reduces Sp1 nuclear localization and transcription. Thus, this mechanism of regulation is entirely plausible. Furthermore, O-GlcNAc modification can induce conformational changes in proteins and it has been shown that protein glycosylation can increase the binding affinity for CBP; therefore, O-GlcNAcylation of Thr^{594} may also increase Nrf2 transcription via acetylation increased CBP binding (Gewinner, Hart, Zachara, Cole, Beisenherz-Huss, & Groner, 2003). Such a mechanism is consistent with our data suggesting that O-GlcNAc positively regulates Nrf2

transcriptional activity and provides a testable hypothesis to explain the blunted exercise-induced Nrf2 response observed in our *db/db* mice.

It is also entirely plausible that O-GlcNAcylation of other potential sites as identified by our prediction analysis regulate Nrf2. The YinOYang1.2 server also predicted that Ser^{347} and Ser^{351} may be modified by O-GlcNAc. These residues lie within a GSK-3/ β-TrCP mediated phosphodegron in the Neh6 domain of Nrf2 where phosphorylation of a serine residue cluster (335, 338, 342, 347, 351, and 355) promotes ubiquitination by β-TrCP E3 ligases (Rada et al., 2012). As O-GlcNAc can compete for phosphorylation sites, it is possible that O-GlcNAc of Ser^{347} and Ser^{351} would increase Nrf2 activity by preventing GSK-3/ β-TrCP mediated degradation. Based upon the data available, we believe this is not the most likely mechanism. Firstly, Hayes lab identified Ser^{335} and Ser^{338} as the binding sites for GSK-3 phosphorylation site through which β-TrCP serves as a receptor for the Skp1-Cul1-RBX/Roc1 ubiquitin ligase complex (Chowdry et al., 2013). Secondly, our *in vitro* data demonstrate a marked increase in Nrf2 transcription independent of increases in Nrf2 protein content. Thus, our proposed model of O-GlcNAc increasing nuclear localization and/or augmenting acetylation is more biologically parsimonious. Future studies should involve investigation of site-specific O-GlcNAcylation of Nrf2 using mass spectrometry and the role O-GlcNAcylation in the localization, transcriptional activity, and protein stability of Nrf2.

A limitation of this study was the use of the leptin receptor deficient *db/db* mouse model. Leptin plays a critical role in regulating metabolic pathways in the heart and has been shown to alter Nrf2 signaling in the *db/db* mouse (More, Wen,

Thomas, Aleksunes, & Slitt, 2012; Atkinson, Fischer, & Lopaschuk, 2002). However, our use additional *in vivo* and *in vitro* models to examine Nrf2 attenuated the effect of this limitation on the conclusions we drew from our data.

Conclusions

The findings of this study demonstrate that an exercise-induced increase in Nrf2 signaling is blunted in diabetes. Despite the evidence that diabetics have a blunted acute response to exercise, exercise should not be ruled out as a therapeutic tool. Where acute exercise fails to elicit substantial beneficial responses in biochemical signaling, chronic exercise has proven to be beneficial in the same pathways (Cao, et al., 2012; Sriwijitkamol, et al., 2007). Future research is warranted to determine how chronic exercise alters Nrf2 functioning in the diabetic heart.

We have also shown for the first time that O-GlcNAc modification and the OGT enzyme are involved in the regulation of Nrf2. We demonstrated that loss of the OGT enzyme significantly reduces Nrf2 transcription and that acutely increasing O-GlcNAc can increase Nrf2 transcription in stressed cells. This finding is significant in the field of chronic disease as O-GlcNAc and Nrf2 are implicated in a wide range of pathological conditions, including diabetes, Alzheimers, and cancer. As such, the findings from this paper give rise to a new area of investigation for Nrf2 in chronic disease.

References

Alder, A., Stevens, R., Manley, S., Bilous, R., Cull, C., & Holman, R. (2003). Development and progression of nephropathy in type 2 diabetes: The United Kingdom Prospective Diabetes Study. *Kidney International*, *63*(1), 225-232.

Ande, S. R., Moulik, S., & Mishra, S. (2009). Interaction between O-GlcNAc modification and tyrosine phosphorylation of prohibitin: Implication for a novel binary switch. *PLOS ONE*, *4*(2), p. e4586.

Asghar, M., George, L., & Lokhandwala, M. (2007). Exercise decreases oxidative stress and inflammation and restores renal dopamine D1 receptor function in old rats. *American Journal of Physiology Renal Physiology*, *293*, F914-F919.

Atkinson, L., Fischer, M., & Lopaschuk, G. (2002). Leptin activates cardiac fatty acid oxidation independent of changes in the AMP-activated protein kinase-acetyl-CoA carboxylase-malonyl-CoA axis. *Journal of Biological Chemistry, 277*, 29424-29430.

Baar, K., Wende, A., Jones, T., Marison, M., Nolte, L., Chen, M., et al. (2002). Adaptations of skeletal muscle to exercise: Rapid increase in the transcriptional coactivator PGC-1. *FASEB J, 16*(14), 1879-1886.

Bai, Y., Cui, W., Xin, Y., Miao, X., Barati, M., Zhang, C., et al. (2013). Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. *Journal of Molecular and Cellular Cardiology, 57*, 82-95.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M., Morio, B., et al. (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal

muscle of diet-induced insulin-resistant mice. *The Journal of Clinical Investigation, 118*(2), 789-800.

Broderick, T., Jankowski, M., Wang, D., Danalache, B., Parrott, C., & Gutkowska, J. (2012). Downregulation in GATA4 and downstream structural and contractile genes in the db/db mouse heart. *ISRN Endocrinology*, 1-12.

Bullen, J., Balsbaugh, J., Chanda, D., Shabanowitz, J., Hunt, D., Neumann, D., et al. (2014). Cross-talk between two essential nutrient senstive enzymes: O-GlcNAc transferase (OGT) and AMP-activated protein kinase (AMPK). *Journal of Biological Chemistry*, *15*, 10592-10606.

Cao, S., Li, B., Yi, X., Chang, B., Lian, Z., Zhang, Z., et al. (2012). Effects of exercise on AMPK signaling and downstream components to PI3K in rat with type 2 diabetes. *PLoSOne*, *7*(12).

Cartoni, R., Leger, B., Hock, M., Praz, M., Crettenand, A., Pich, S., et al. (2005). Mitofusins 1/2 and ERRα expression are increased in human skeletal muscle after physical exercise. *Journal of Physiology*, *567* (1), 349-358.

Chen, X., Dodd, G., Thomas, S., Zhang, X., Wasserman, M., Rovin, B., et al. (2006). Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. *American Journal of Physiology Heart and Circulatory Physiology*, *290*, H1862-H1870.

Chou, T. Y., Hart, G., & Dang, C. (1995). c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas. *The Journal of Biological Chemistry*, *270*(32), 18961-18965.

Cowie, C. C., Rust, K., Ford, E., Eberhardy, M., Byrd-Holt, D., Li, C., et al. (2009). Full accounting of diabetes and pre-diabetes in the U.S. population in 1988-1994 and 2005-2006. *Diabetes Care*, *32*(2), 287-294.

Cox, E. J., & Marsh, S. (2013). Exercise and diabetes have opposite effects on the assembly and O-GlcNAc modification of the mSin3A/HDAC1/2 complex in the heart. *Cardiovascular Diabetology*, *12*(101).

Dai, D. F., Johnson, S. C., Villarin, J. J., Chin, M. T., Nieves-Cintron, M., Chen, T., et al. (2011). Mitochondrial oxidative stress mediates angiotensin II–induced cardiac hypertrophy and Gαq overexpression–induced heart failure. *Circulation Research*, *108*, 837-846.

Danaei, G., Finucane, M., Lu, Y., Singh, G., Cowan, M., Paciorek, C., et al. (2011). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: Systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet*, *378*(9785), 31-40.

Fülöp, N., Mason, M., Dutta, K., Wang, P., Davidoff, A., Marchase, R., et al. (2007). Impact of Type 2 diabetes and aging on cardiomyocyte function and O-linked N-acetylglucosamine levels in the heart. *American Journal of Physiology Cell Physiology*, *292*, C1370-C1378.

Galmozzi, A., Mitro, N., Ferrari, A., Gers, E., Gilardi, F., Godio, C., et al. (2013). Inhibition of class I histone deacetylases unveils a mitochondrial signature and enhances oxidative metabolism in skeletal muscle and adipose tissue. *Diabetes*, *62*(3), 732-742.

Gewinner, C., Hart, G., Zachara, N., Cole, R., Beisenherz-Huss, C., & Groner, B. (2003). The coactivator of transcription CREB-binding protein interacts preferentially with the glycosylated form of STAT5. *The Journal of Biological Chemistry*, *279*, 563-3572.

Gomez-Cabrera, M.-C., Domenech, E., & Vina, J. (2008). Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. *Free Radical Biology and Medicine*, *44*(2), 126-131.

Gupta, R., & Brunak, S. (2002). Prediction of glycosylation across the human proteome and the correlation to protein function. *Pacific Symposium on Biocomputing*, *7*, 310-322.

Hart, G. W., Slawson, C., Ramirez-Correa, G., & Lagerlof, O. (2011). Cross talk between O-GlcNAcylation and phosphorylation: Roles in signaling, transcription, and chronic disease. *Annual Review of Biochemistry*, *80*, 825-858.

He, X., Kan, H., Cai, L., & Ma, Q. (2009). Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes. *Journal of Molecular and Cellular Cardiology*, *46*(1), 47-58.

Jing, E., Emanuelli, B., Hirschey, M., Boucher, J., Lee, K., Lombard, D., et al. (2011). Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(35), 14608-14613.

Kawai, Y., Garduno, L., Theodore, M., Yang, J., & Arinze, I. (2011). Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. *Journal of Biological Chemistry*, *286*(9), 7629-7640.

Kilhovd, B., Berg, T., Birkeland, K., Thorsby, P., & Hanssen, K. (1999). Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. *Diabetes Care*, *22*, 1543-1548.

Kim, H., Woo, J., Joo, H., & Moon, W. Cardiac transcription factor Nkx2.5 is downregulated under excessive O-GlcNAcylation condition. *PLOS ONE*, *7* (6), e38053.

Laczy, B., Marsh, S., Brocks, C., Wittman, I., & Chatham, J. (2010). Inhibition of O-GlcNAcase in perfused rat hearts by NAG-thiazolines at the time of reperfusion is cardioprotective in an O-GlcNAc-dependent manner. *American Journal of Physiology Heart and Circulatory Physiology*, *299* (5), H1715-1727.

Laher, I., Beam, J., Botta, A., Barendret, R., Sulistyoningrum, D., Devlin, A., et al. (2013). Short-term exercise worsens cardiac oxidative stress and fibrosis in 8-month-old db/db mice by depleting cardiac glutathione. *Free Radical Research*, *47*(1), 44-54.

Lee, D. Y., Lee, C., Lin, T., Lim, S., Zhou, J., Tseng, Y., et al. (2012). Role of histone deacetylases in transcription factor regulation and cell cycle modulation in endothelial cells in response to disturbed flow. *Proceedings of the National Academy of Science*, 1-6.

Li, J., Johnson, D., Calkins, M., Wright, L., Svendsen, C., & Johnson, J. (2005). Stabilization of Nrf2 by tBHQ confers protection against oxidative stress-induced cell death in human neural stem cells. *Toxicological Sciences*, *83*, 313-328.

Liu, X., Peyton, K., Shebib, A., Wang, H., Korthuis, R., & Durante, W. (2011). Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival. *American Journal of Physiology Heart and Circulatory Physiology*, *300*, H84-H93.

Livak, K. J., & Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods*, *25*, 402-408.

Majumdar, G., Wright, J., Markowitz, P., Martinez-Hernandez, A., Raghow, R., & Solomon, S. (2004). Insulin stimulates and diabetes inhibits O-linked N-acetylglucosamine transferase and O-glycosylation of Sp1. *Diabetes*, *53*(12), 3184-3192.

Medford, H. M., Porter, K., & Marsh, S. (2013). Immediate effects of a single exercise bout on protein O-GlcNAcylation and. *American Journal of Physiology Heart and Circulatory Physiology*, *305*, H114-H123.

Miao, F., Gonzalo, I., Lantin, L., & Natarajan, R. (2004). In vivo chromatin remodeling events leading to inflammatory gene transcription under diabetic conditions. *The Journal of Biological Chemistry*, *279*, 18091-18097.

Mo, C., Wang, L., Zhang, J., Numazawa, S., Tang, H., Han, X., et al. (2014). The crosstalk between Nrf2 and AMPK signal pathways is important for the anti-

inflammatory effect of berberine in LPS-stimulated macrophages and endotoxin-shocked mice. *Antioxidants and Redox Signaling*, *20*(4), 574-588.

More, V., Wen, X., Thomas, P., Aleksunes, L., & Slitt, A. (2012). Severe diabetes and leptin resistance cause differential hepatic and renal transporter expression in mice. *Comparative Hepatology*, *11*(1), 1-14.

Muthusamy, V. R., Kannan, S., Sadhaasivam, K., Gounder, S., Davidson, C., Boeheme, C., et al. (2012). Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium. *Free Radical Biology & Medicine*, *52*, 366-376.

Naveira, L., Mercado, N., & Ito, K. (2011). AMPK signalling regulates Nrf2 localization and activity via sirtuins in a monocytic cell line. *European Respiratory Journal*, *38*, 424.

Ngoh, G., Watson, L., Facundo, H., & Jones, S. (2011). Augmented O-GlcNAc signaling attenuates oxidative stress and calcium overload in cardiomyocytes. *Amino Acids*, *40*(3), 895-911.

Palsamy, P., & Subramanian, S. (2011). Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2–Keap1 signaling. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1812*(7), 719-731.

Rahman, M., Stuchlick, O., El-Karim, E., Stuart, R., Kipreos, E., & Wells, L. (2010). Intracellular protein glycosylation modulates insulin mediated lifespan in C.elegans. *Aging*, *2*(10), 678-690.

Rocco, A. B., LeValley, J., Eldridge, J., Marsh, S., & Rodgers, B. (2014). A Novel protocol for assessing exercise performance and dystropathophysiology in the mdx mouse. *Muscle & Nerve*

Salazar, M., Rojo, A., Velasco, D., de Sagarra, R., & Cuadrado, A. (2006). Glyocgen synthase kinase-3b inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. *The Journal of Biological Chemistry*, *281*, 1484-14851.

Shao, D., Oka, S., Liu, T., Zhai, P., Ago, T., Sciaretta, S., et al. (2014). A redox-dependent mechanism for regulation of AMPK activation by Thioredoxin1 during energy starvation. *Cell Metabolism*, *19*(2), 232-245.

Siewart, S., Gonzalez, I., Santillan, L., Lucero, R., Ojeda, M., & Gimenez, M. (2013). Downregulation of Nrf2 and HO-1 expression contributes to oxidative stress in type 2 diabetes mellitus: A study in Juana Koslay City, San Luis, Argentina. *Journal of Diabetes Mellitus*, *3*(2), 71-78.

Sriwijitkamol, A., Coletta, D., Wajcberg, E., Balbontin, G., Reyna, S., Barrientes, J., et al. (2007). Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: A time-course dose-response study. *Diabetes*, *56* (3), 836-848.

Sun, Z., Chin, Y., & Zhang, D. (2009). Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Molecular and Cellular Biology*, *29*(10), 2658-2672.

Szendroedi, J., Schmid, A., Meyerspeer, M., Cervin, C., Kacerovsky, M., Smekal, G., et al. (2009). Impaired mitochondrial function and insulin resistance of skeletal muscle in mitochondrial diabetes. *Diabetes Care*, *32*(4), 677-679.

Tan, Y., Ichikawa, T., Li, J., Yang, H., Chen, X., Goldblatt, C., et al. (2011). Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo. *Diabetes*, *60*, 625-633.

Theodore, M., Kawai, Y., Yang, J., Kleschenko, Y., Reddy, S., Villalta, F., et al. (2008). Multiple nuclear localization signals function in the nuclear import of the transcription factor Nrf2. *The Journal of Biological Chemistry*, *283*, 8984-8994.

Trivedi, C., Zhu, W., Wang, Q., Jia, C., Kee, H., Li, L., et al. (2010). Hopx and Hdac2 interact to modulate Gata4 acetylation and embryonic cardiac myocyte proliferation. *Developmental Cell*, *19*(3), 450-459.

Uruno, A., Furusawa, Y., Yagishita, Y., Fukutomi, T., Muramatsu, H., Negishi, T., et al. (2013). The Keap1-Nrf2 system prevents onset of diabetes mellitus. *Molecular and Cellulary Biology*, *33*(15), 2996-3010.

Velmurugan, G., Sundaresan, N., Gupta, M., & White, C. (2013). Defective Nrf2-dependent redox signalling contributes to microvascular dysfunction in type 2 diabetes. *Cardiovascular Research*, *100*(1), 143-150.

Wang, B., Zhu, X., Kim, Y., Li, J., Huang, S., Saleem, S., et al. (2012). Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. *Free Radical Biology & Medicine*, *52*, 928-936.

Watkins, S., Borthwick, G., & Arthur, H. (2011). The H9C2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro. *In Vitro Cellular & Developmental Biology*, *47*(2), 125-131.

Watson, L., Facundo, H., Ngoh, G., Ameen, M., Brainard, R., Lemma, K., et al. (2010). O-linked β-N-acetylglucosamine transferase is indispensable in the failing heart . *Proceedings of the National Academy of Sciences*, *107*(41), 17797-17802.

Yu, S., Khor, T. O., Cheung, K., Li, W., Wu, T., Huang, Y., et al. (2010). Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. *PLOS ONE*, *5*(1), e8579.

Zheng, H., Whitman, S., Wu, W., Wondrak, G., Wong, P., Fang, D., et al. (2011). Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy. *Diabetes*, *60*(11), 3055-3066.

Zhong, Q., Mishra, M., & Kowluru, R. (2013). Transcription factor Nrf2-mediated antioxidant defense system in the development of diabetic retinopathy. *Investigational Opthamology and Visual Science*, *54*(6), 3941-3948.

Table 4.1. Animal Characteristics for exercised mice

	Db/Db (n=10)	Control (n=10)
Body weight (g)	32.49 ± 1.25*	20.41 ± .45
Heart wt (mg)	0.11 ± 0.006	0.102 ± 0.005
Glucose (ng/dl)	318.6 ± 91.3*	93.8 ± 15.02
HW:BW (mg/g)	3.4 ±0.03	4.9 ± 0.01

All values are presented as mean ± SE

Table 4.2. Primer Sequences

Gene	Primer	Sequence
Mouse GAPDH	Forward	TGTGATGGGTGTGAACGAGAA
	Reverse	CATGAGCCCTTCCACAATGCCAAA
Mouse *GCLM*	Forward	GAGTTCCCAAATCAGCCCCG
	Reverse	CCACTGCATGGGACATGGTG
Mouse *CAT*	Forward	CTTCTGGAGTCTTCGTCCCG
	Reverse	GTGACCATCGGGAATCCCTC
Mouse *NQO1*	Forward	CATTGGCCACACTCCACCAG
	Reverse	CTCCCAGACGGTTTCCAGAC
Rat RPLP	Forward	ATCGTCTTTAAACCCCGCGT
	Reverse	TAGTTGGACTTCCAGGTCGC
Rat *GCLM*	Forward	GAGCAGCTGTACCAGTGGG
	Reverse	GTGGCATCACACAGCAGGAG
Rat *CAT*	Forward	CAGGAGGCCATCCCTTACAC
	Reverse	TGAGTACCTCCCACCTCGTG
Rat *HMOX1*	Forward	CAGGAGGCCATCCCTTACAC
	Reverse	TGAGTACCTCCCACCTCGTG

Chapter 5: A Proposed Mechanism for Exercise Attenuated Methylglyoxal Accumulation: Activation of the ARE-Nrf Pathway and Increased Glutathione Biosynthesis

In addition to the studies conducted and presented above. We have written and published a formal hypothesis regarding how using exercise to improve the Nrf2 system translates into improved cellular detoxification of the cytotoxic chemical methylgyloxal. This article, which is published in *Medical Hypotheses*, is presented in this chapter.

Citation

Dieter, B. P., & Vella, C. A. (2013). A proposed mechanism for exercise attenuated methylglyoxal accumulation: Activation of the ARE-Nrf pathway and increased glutathione biosynthesis. *Medical Hypotheses*. doi:10.1016/j.mehy.2013.08.034

Background

Recent evidence suggests that individuals with type 1 and type 2 diabetes (T2DM) experience increased accumulation of methylglyoxal (MGO) in whole blood (1). Methylglyoxal is an endogenous metabolite and a known intracellular precursor of advanced glycation endproducts (AGEs) (2). High serum levels of MGO have been correlated with MGO-derived AGEs in individuals with type 2 diabetes (T2DM) (1). Furthermore, there is human and animal evidence to suggest that MGO is causal in copious pathologies related to AGE accumulation including heart disease, hypertension and nephropathy (1,3-5).

In addition to its causal role in AGE-related pathologies, MGO modification of proteins exacerbates insulin resistance by inhibiting insulin-dependent insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation and phosphoinositide 3-kinase (PI3K) activity, and reducing protein kinase B (PKB) phosphorylation in cultured rat skeletal muscle cells (6). The findings of Riboulet-Chavey et al. (6) indicate that MGO is not only involved in diabetic complications, but in furthering the progression of insulin resistance.

MGO is a by-product of metabolism and may serve important roles in cell signaling, such as inhibiting glycolysis (7). However, excess MGO results in toxicity through formation of AGEs and has been suggested to play a causal role in the pathology of diabetic complications (8-9). To maintain normal physiological levels and prevent accumulation, MGO is detoxified through the glutathione (GSH) dependent glyoxalase system (Figure 5.1). Therefore, the detoxification of MGO is heavily reliant upon GSH concentrations. Masterjohn (10) demonstrated that depletion of hepatic GSH biosynthesis in rats increased hepatic MGO accumulation, suggesting that depletion of GSH impairs MGO detoxification.

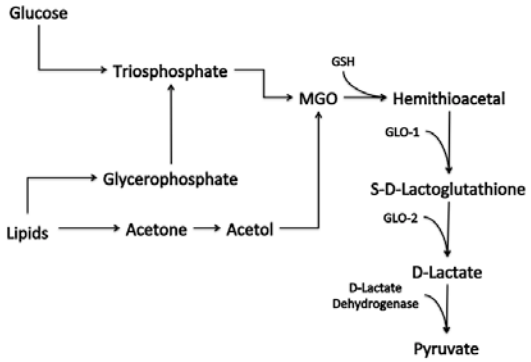

Figure 5.1. MGO formation from glucose and lipids and detoxification to pyruvate

GSH biosynthesis and recycling is heavily dependent upon the antioxidant response element-nuclear respiratory factor pathway (ARE-Nrf) (11-13). Pharmacological and dietary intervention studies have demonstrated that activation of this pathway increases intracellular GSH and glyoxalase enzymes and reduces MGO levels (11,12). Recent *in vivo* evidence suggests that pharmacological agents that increase expression of ARE-Nrf2 results in increased GSH and attenuated MGO levels in serum and organ tissue (14). Together, this evidence suggests activation of the ARE-Nrf improves MGO detoxification thereby attenuating MGO-derived AGE related complications.

There is evidence suggesting the ARE-Nrf pathway is also upregulated during exercise (15-17). Specific to T2DM, acute bouts of exercise have been shown to restore reduced Nrf-1 mRNA expression in streptozotocin induced diabetic rats on a high-fat diet (18). There is also evidence demonstrating increased Nrf1/2 expression with exercise in humans across wide age ranges and exercise modalities (15, 19). Individuals with uncontrolled T2DM have diminished GSH status (20), which may contribute to the MGO accumulation observed in these individuals (1). Currently, indirect evidence suggests that exercise training may improve GSH status through the ARE-Nrf pathway in individuals with T2DM, thereby improving increasing MGO detoxification and attenuating MGO-derived AGE related complications.

Presentation of the hypothesis.

We propose that exercise will increase GSH status through the upregulation of the ARE-Nrf pathway, thereby improving MGO detoxification and attenuating MGO accumulation. This hypothesis describes a potential mechanism through

which exercise can improve diabetic complications by reducing MGO accumulation. Additionally, this hypothesis presents a mechanism through which exercise can improve MGO-induced insulin resistance.

Evaluation of the hypothesis.

Glutathione status and MGO detoxification

MGO detoxification occurs through the GSH-dependent glyoxalase system (21). Therefore, GSH status will dictate the ability of a cell to detoxify MGO. Masterjohn demonstrated that suppression of hepatic GSH biosynthesis in rats increased hepatic MGO accumulation (10). Additionally, Masterjohn et al. (2012) found that increasing GSH concentrations by administering γ-tocopherol eliminated postprandial increases in plasma MGO in humans. These findings suggest increased GSH concentrations will result in improved MGO detoxification.

ARE-Nrf1/2 pathway activation increases glutathione status.

The ARE-Nrf pathway is critical in maintaining GSH status. Under normal physiological conditions, Nrf2 is located in the cytoplasm and kept dormant by a cytoplasmic repressor named Kelch-like ECH-associated protein 1 (Keap1) (2). In response to oxidative stress, Nrf2 dissociates from its cytosolic inhibitor Keap1, translocates to the nucleus, and binds to antioxidant-response elements (AREs) in the promoters of target genes (23). This leads to transcriptional induction of several cellular defense genes, including GSH biosynthetic enzymes glutathione cysteine ligase modifier subunit (GCLM) and GSH cysteine ligase catalytic subunit (GCLC), GSH-dependent antioxidant enzymes (glutathione peroxidase 2 (GPX2) and glutathione *S*-transferases (GST) (24).

Nrf2 knockout mice have decreased expression of GSH S-transferases and the catalytic subunit of glutamate cysteine ligase, a key enzyme in GSH metabolism (25). These mice displayed decreased GSH status, reduced GSH dependent detoxification, and failure to adapt to oxidative stress (25). Additional evidence from animal models indicates age-related decreases in GSH biosynthesis are a result of reduced nuclear Nrf2 (26). *In vitro* evidence suggests that the ARE-Nrf pathway is critical to maintaining GSH status in human cells (27). Taken together, these findings provide evidence suggesting that Nrf2 plays a major role in GSH biosynthesis and maintenance of adequate GSH levels.

Exercise increases ARE-Nrf1/2 activation and expression

Low to moderate amounts of reactive oxygen species (ROS) produced during skeletal muscle work are a part of “hormesis”, which describes the generally favorable biological responses to low exposure to toxins and other stressors (2). Exercise training results in increased levels of oxidative stress, thereby upregulating antioxidant defense mechanisms in various tissues, including GSH (28). In response to exercise-induced oxidative stress, Nrf2 dissociates from its cytosolic inhibitor Keap1, translocates to the nucleus, and binds to antioxidant-response elements (AREs) in the promoters of target genes, including GSH biosynthesis (Figure 5.2). This suggests that exercise will result in increased ARE-Nrf activation and GSH biosynthesis.

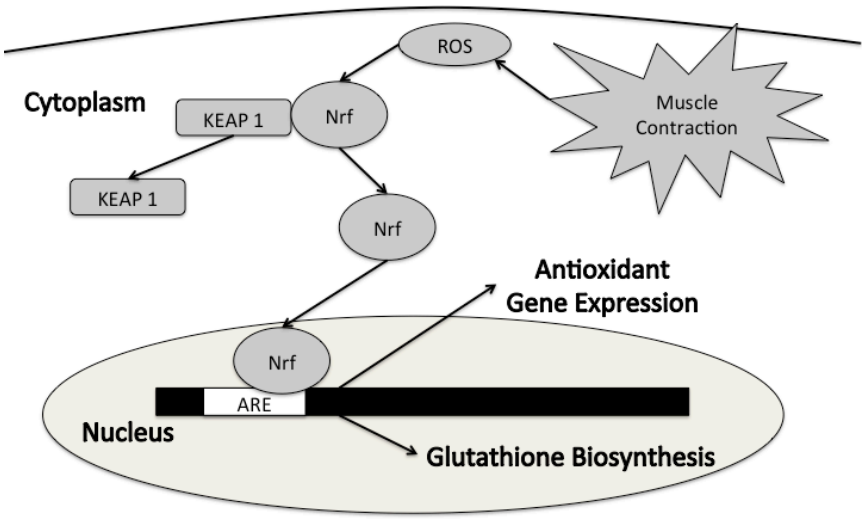

Figure 5.2. Exercise and the antioxidant response element-nuclear respiratory factor (ARE-Nrf) pathway. Muscle contraction results in reactive oxygen species and changes in the cells redox status. Oxidation of Kelch-like ECH-associated protein 1 (Keap-1) causes Nrf to detach and translocate to the nucleus. Nrf then binds to the ARE, activating antioxidant genes, resulting in glutathione (GSH) biosynthesis.

Research from rodent models has found a 50% increase of Nrf-1 expression after an acute bout of swimming or running (16,17). Similarly, Baar et al. (16) reported a 56% increase in Nrf-2 expression in mice after an acute bout of swimming. Baar et al. hypothesized the increase observed in Nrf1/2 was due to increased peroxisome proliferator-activated receptor-gamma coactivator 1 PGC-1 activation. Furthermore, acute bouts of exercise have been shown to restore reduced Nrf-1 mRNA expression in streptozotocin-induced diabetic rats on a high-fat diet (18).

Acute and chronic exercise in humans leads to upregulation of the ARE-Nrf

pathway (19,29). Cartoni et al. (19) found Nrf-2 levels increased 5-fold 24 hours post-exercise in skeletal muscle cells of trained male cyclists after an acute bout of cycling exercise. Short et al. (29) demonstrated that 16 weeks of moderate-intensity aerobic exercise increased Nrf-1 activity in men and women over a wide age range (21-87 years). Furthermore, this change was not affected by age.

Exercise and MGO detoxification

MGO-derived AGEs are increased in individuals with T2DM (1). There is substantial evidence to suggest that MGO is causal in several pathologies related to AGE accumulation including heart disease, hypertension and nephropathy (5,30, 31). Additionally, MGO furthers the progression of insulin resistance (6).

MGO detoxification occurs through the GSH-dependent glyoxalase system and impaired GSH status results in increased MGO concentrations (10,32), whereas improving GSH status significantly and substantially decreases MGO concentrations (22). Individuals with uncontrolled T2DM have diminished GSH status (20); thus impaired GSH biosynthesis and status may be one mechanism responsible for increased MGO accumulation and MGO-derived AGE's in T2DM.

Activation of the ARE-Nrf pathway leads to the transduction of GSH biosynthetic enzymes (24). Exercise-induced oxidative stress has been shown to increase activation of the ARE-Nrf pathway and GSH biosynthesis, and improve GSH status. Recent animal data from Boor et al. (33) demonstrated that exercise effectively reduces advanced glycation and ameliorates diabetic nephropathy and

reduces levels of oxidized proteins. These findings occurred independent of change in glycemic conditions, supporting the hypothesis presented here.

Consequences of the hypothesis

Based upon the circumstantial evidence, we hypothesize that exercise improves MGO detoxification and attenuates MGO accumulation by increasing GSH biosynthesis and improving GSH status through activation of the ARE-Nrf pathway. This testable hypothesis describes a potential mechanism through which exercise can improve MGO-induced insulin resistance and diabetic complications by reducing MGO accumulation. If proven to be accurate, it would provide mechanistic evidence for exercise as an effective nonpharmacological approach to reduce glycation (33). Additionally, it would provide evidence for the benefit of exercise in the prevention of complications and overall metabolic function in patients with T2DM.

References

1) Kilhovd B, Giardino I, Torjesen K, et al. Increased serum levels of the specific AGE-compound methylglyoxal-derived hydroimidazolone in patients with type 2 diabetes. Metabolism, 2003, 52, 163-167.
2) Golbidi S, Badran M, Laher I. Antioxidant and anti-inflammatory effects of exercise in diabetic patients. Exp Diabetes Res, 2012, 941868.
3) Wang X, Desai K, Chang T, Wu L, Vascular methylglyoxal metabolism and the development of hypertension. J Hypertens 2005, 23, 1565-1573.
4) Rabbani N, Godfrey L, Xue M, et al. Glycation of LDL by methylgloxal increases arterial atherogenicity. Diabetes, 2011, 60, 1973-1980.
5) Mostafa AA, Randell E, Vasdev S, et al. Plasma protein advanced glycation end products, carboxymethyl cysteine, and carboxyethyl cysteine, are elevated and related to nephropathy in patients with diabetes. Mol Cell Biochem, 2007, 302, 35-42.
6) Riboulet-Chavey A, Pierron A, Durand I, Murdaca J, Giudicelli J, Van Obberghen E. Methylglyoxal impairs the insulin signaling pathways independently of the formation of intracellular reactive oxygen species. Diabetes, 2006, 55, 1289-1299.
7) Leoncini G, Maresca M, Buzzi E. Inhibition of the glycolytic pathway by methylglyoxal in human platelets. Cell Biochem Funct, 1989, 7, 65-70.
8) Berlanga J, Cibrian D, Guillen I, et al. Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds. Clin Sci, 2005 109, 83-95.

9) Ratliff DM, Vander Jagt D, Eaton R, Vander Jagt D. Increased levels of methylglyoxal-metabolizing enzymes in mononuclear and polymorphonuclear cells from insulin-dependent diabetic patients with diabetic complications: Aldose reductase, glyoxalase I, and glyoxalase II. J Clin Endocrinol Metab, 1996, 81, 488-492.

10) Masterjohn C. Regulation of methylglyoxal accumulationby glutathione and dietary antioxidants. Doctoral dissertation, 2012, Retrieved from ProQuest Dissertations and Theses. (Accession Order No. AAT 3533954)

11) Ha K, Chen Y, Sternberg P. Increased glutathione synthesis through an ARE-Nrf1-dependent pathway by zinc in the RPE: Implication for protection against oxidative stress. Invest Ophthalmol Vis Sci, 2006, 47, 2709-2715.

12) Kim AD, Zhang R, Kang K, You H, Hyun J. Increased glutathione synthesis following Nrf2 activation by vanadyl sulfate in human chang liver cells . Int J Mol Sci, 2011, 12, 8878-8894.

13) Shih A, Johnson D, Wong G, et al. Coordinate regulation of glutathione biosynthesis and release by Nrf-2 expression glia potently protects neurons from oxidative stress. J Neurosci, 2003, 23, 3394-3406.

14) Lee BH, Hsu W, Chang Y, Kuo H, Hsu Y, Pan T. Ankaflavin: A natural novel PPARγ agonist upregulates Nrf2 to attenuate methylglyoxal-induced diabetes in vivo. Free Radic Biol Med, 2012, 53, 2008-2016.

15) Sriwijitkamol A, Coletta D, Wajcberg E, et al. Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes. Diabetes, 2007, 56, 836-848.

16) Murakami T, Shimomura Y, Yoshimura A, Sokabe M, Fujitsuka N. Induction of nuclear respiratory factor-1 expression by an acute bout of exercise in rat muscle. Biochim Biophys Acta, 1998, 1281, 113-122.

17) Baar K, Wende A, Jones T. Adaptations of skeletal muscle to exercise: Rapid increase in the transcriptional coactivator PGC-1. FASEB J, 2002, 16, 1879-1886.

18) Cao S, Li B, Yi X, et al. Effects of exercise on AMPK signaling and downstream components to PI3K in rat with type 2 diabetes. 2012, PLOSone, 7

19) Cartoni R, Leger B, Hock M, et al. Mitofusins 1/2 and ERRα expression are increased in human skeletal muscle after physical exercise. J Physiol, 2005, 567, 349-358.

20) Sekhar RV, McKay SV, Patel SG, et al. Glutathione synthesis is diminshed in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine. Diabetes Care, 2011, 34, 162-167.

21) Thornalley PJ. Pharmacology of methylglyoxal: Formation, modification of proteins and nucleic acids, and enzymatic detoxification-a role in pathogenesis and antiproliferative chemotherapy. Gen Pharmacol, 1996, 27, 565-573.

22) Masterjohn C, Mah E, Guo Y, Koo S, Bruno R. γ-Tocopherol abolishes postprandial increases in plasma methylglyoxal following an oral dose of glucose in healthy, college-aged men. J Nutr Biochem, 2012, 23, 292-298.

23) Harvey C, Thimmulappa R, Singh A, et al. Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. Free Radic Biol Med, 2009, 46, 443-453.

24) Kensler T, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol, 2007, 47, 89-116.

25) Chanas S, Jiang Q, McMahon M, et al. Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. Biochem J, 2002, 365, 405-416.

26) Suh JH, Shenvi SV, Dixon BM, et al. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci USA, 2004, 101, 3381-3386.

27) Hansen J, Watson W, Jones D. Compartmentation of Nrf-2 redox control: Regulation of cytoplasmic activation by glutathione and DNA binding by thioredoxin-1. Toxicol Sci, 2004, 82, 308-317.

28) Elokda A, Nielsen D. Effects of exercise training on the glutathione antioxidant system. Eur J Cardiovasc Prev Rehabil, 2007, 14, 630-637.

29) Short K, Vittone J, Bigelow M, et al. Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. Diabetes, 2003, 52, 1888-1896.

30) Brouwers O, Niessen P, Haenen G, et al. Hyperglycaemia-induced impairment of endothelium-dependent vasorelaxation in rat mesenteric

arteries is mediated by intracellular methylglyoxal levels in a pathway dependent on oxidative stress. Diabetologia, 2010, 53, 989-1000.

31) Wang X, Desai K, Chang T, Wu L. Vascular methylglyoxal metabolism and the development of hypertension. J Hypertens, 2005, 23, 1565-1573.

32) Ferguson G, Booth I. Importance of glutathione for growth and survival of escherichia coli cells: Detoxification of methylglyoxal and maintenance of intracellular K+. J Bacteriol, 1998, 180, 4314-4318.

33) Boor P, Celec P, Behuliak M, et al. Regular moderate exercise reduces advanced glycation and ameliorates early diabetic nephropathy in obese Zucker rats. Metabolism, 2009, 58, 1669-1677.

Chapter 6: Dysregulation of Nrf2 signaling in diabetes: An opportunity for a multi-target pharmaceutical approach

The findings of the study presented in chapter 5, *Exercise Improves Nrf2 Content and Activity in db/db Mice Via O-GlcNAc and HDAC2 Signaling Mechanisms,* highlighted a novel approach to improving Nrf2 signaling in diabetes. The mechanism proposed from this study may be used in conjunction with current Nrf2 activators to optimize Nrf2 in diabetic populations. This finding motivated a through review of the literature regarding current therapeutic Nrf2 agonists. Based upon this review of the literature, I have also written and submitted a formal review to the Nature Reviews publication: *Clinical Pharmacology & Therapeutics,* describing the dysregulation in Nrf2 signaling in diabetes and propose a novel, multistage approach to pharmacologically targeting Nrf2 in T2DM. This paper is currently under review after minor revisions were requested.

Introduction

Oxidative stress is a central feature of diabetes and plays a causal role in the pathogenesis of diabetic complications. The nuclear factor-like 2 (Nrf2) transcription factor mediates the induction of antioxidant and cytoprotective genes and is a major regulator of the endogenous antioxidant and detoxification systems. Multiple aspects of the Nrf2 signaling pathway are aberrant in diabetes and simultaneously targeting the dysregulated aspects of the Nrf2 signaling pathway ought to be considered.

Manuscript

Diabetes affects over 360 million people worldwide and increases the risk for cardiovascular disease (CVD), nephropathy, and neuropathy.[1, 2] Compelling

evidence exists to suggest that oxidative stress plays a causal role in the pathogenesis of diabetic complications such as nephropathy, neuropathy, and cardiac hypertrophy, suggesting oxidative stress is a central feature of the disease.[3-5] Improving the endogenous cellular antioxidant and detoxification system is under investigation as a therapeutic approach to reducing oxidative stress and attenuating complications in diabetes. Nrf2 is a cap ‘n’ collar (CNC) basic-region leucine zipper transcription factor that functions as a major regulator of the endogenous antioxidant and detoxification system and provides cells the ability to adapt to oxidative stress and electrophiles by mediating the induction of the cytoprotective genes. It is expressed in all tissues of the human body, and is essential in maintaining cellular redox status.[6]

Nrf2 is decreased in diabetic mice and patients with type 2 diabetes mellitus (T2DM), which contributes to increased oxidative stress, endothelial dysfunction, insulin resistance, nephropathy, and increased cardiac insult. [7-9] Genetic overexpression of Nrf2 prevents the onset of T2DM in mice and small molecule activation of Nrf2 reduces oxidative stress, and a myriad of diabetic complications, including cardiovascular complications, nephropathy, and neuropathy. [9-11] The potential of the Nrf2 pathway as a panacea for the features of the diabetic milieu has given rise to research aimed at developing pharmacological therapies that target the Nrf2 pathway. Nrf2 is regulated through a multi-stage signaling process involving cytosolic regulation, nuclear translocation, DNA binding, and nuclear export. Recent evidence has demonstrated that the diabetic milieu results in dysregulation in multiple aspects of the Nrf2 signaling pathway. [7, 11-13] Current therapeutic

approaches focus on targeting a single aspect of the Nrf2 pathway. Developing an optimal therapeutic strategy for improving Nrf2 function would involve simultaneous targeting of the dysregulated aspects of the signaling pathway. Therefore, the purpose of this review is to discuss the effect of diabetes on Nrf2, the dysregulation of nrf2 signaling in diabetes, and discuss current therapeutics that differentially target the Nrf2 pathway.

Nrf2 in type 2 diabetes mellitus

Hyperglycemia, the hallmark feature of T2DM, causes increased production of reactive oxygen species (ROS) and other cytotoxic molecules such as methylglyoxal and advanced glycation end products. Initial cell culture studies demonstrated that Nrf2 was critical in reducing hyperglycemia induced production of ROS, and methylglyoxal. [14] Xue and colleagues found that incubating human microvascular HMEC-1 endothelial cells with 25 mmol/l glucose resulted in a three-fold increase in ROS and methylglyoxal and administering the Nrf2 activator sulforaphane (SFN) attenuated the hyperglycemia induced increased in ROS and methylglyoxal. [14] Additionally, when Nrf2 was knocked down with siRNA, the protective effect of SN was removed; indicating Nrf2 was indeed responsible for reducing the hyperglycemia induced oxidative stress *in vitro.* [14]

He and colleagues also demonstrated that Nrf2 is activated in response to the oxidative and chemical stress caused by hyperglycemia. [15] They showed that Nrf2 mRNA was significantly increased after 24 hours when treated with glucose at concentrations of 20 mM for 6 hours or 40 mM for 18 hours in neonatal cardiomyocytes obtained from Nrf2 WT and that this effect was not present in Nrf2

KO mice.[15] In WT cells, hyperglycemia increased mRNA levels of the downstream Nrf2 gene products tO1 and HO-1 almost three fold and two-fold, respectively. Additionally, ROS levels and apoptosis were significantly increased in the Nrf2 KO cells compared to WT. He and colleagues also demonstrated that 2 weeks after inducing type I diabetes in C57BL/6 mice with a single dose of streptozotocin (STZ) there is a significant upregulation of Nrf2 downstream genes NQO1 and HO1 mRNA expression.

While acute hyperglycemia causes an increase in Nrf2 function, the chronic hyperglycemic milieu of the diabetic condition results in decreased Nrf2 function. [7, 16-18] Tan and colleagues demonstrated that at the onset of diabetes in mice Nrf2 expression was increased at 2 months but decreased at 5 months and that expression of Nrf2 was decreased in humans with late-stage T2DM. Furthermore, immunochemical staining in normal and T2DM human hearts showed that Nrf2 was significantly decreased in late stage, failing, diabetic hearts compared with the non-diabetic hearts. [7] Two further studies have validated these findings when they demonstrated that Nrf2 protein content and mRNA levels of NQO1 and HO-1 were increased at 3 months but decreased at 6 months in STZ-induced diabetic mice. [11,16] Diet-induced models of T2DM in C57BL/6J mice significantly reduced Nrf2 in skeletal muscle and the downstream product of Nrf2, HO-1, when compared with controls. [17] Additionally, Nrf2 along with several downstream products, NQO1, GSR, GSTA2, TXNDR1, GCLC, and GCLM, is reduced in the arterioles of adult *db/db* mice when compared to controls. [19]

Research in humans has also shown that Nrf2 function is decreased in subjects with T2DM. [18] Siewart and colleagues compared pro-oxidant status and mRNA expression of Nrf2 and HO-1 in 40 patients with T2DM and 30 age-matched controls. Using the thiobarbituric acid-reactive substances (TBAR'S) method, the authors found that blood from patients with T2DM exhibit a roughly 100% increase in oxidative stress compared to healthy controls. Furthermore, Nrf2 and HO-1 gene expression was significantly lower in leukocytes from patients with T2DM when compared to healthy controls.

The initial increase in Nrf2 occurs concomitantly with increases in ROS suggesting Nrf2 increases in response to the cytotoxic insult of hyperglycemia, while long-term studies demonstrate that after the initial increase in Nrf2 function there is a marked decrease in both content and activity of Nrf2 and its downstream antioxidant products. These *in vivo* and *in vitro* experiments provide substantial evidence for the following hypothesis: expression of Nrf2 is initially increased in response to the onset of the diabetic milieu, which is then decreased in late stage diabetes. This suggests that therapies targeting the Nrf2 pathway in late-stage diabetes should be aimed at increasing Nrf2 activity.

Mechanisms of Nrf2 signaling and dysregulation in diabetes.

Decreased Nrf2 content and activity is associated with the diabetic state and plays a critical role in diabetic complications. For over a decade, Nrf2 has been investigated in cancer research; it is only more recently that Nrf2 has been the focus of diabetic research. Given the highly coordinated nature of Nrf2 signaling, it is reasonable to assume that signaling dysregulation is involved the Nrf2 dysfunction

observed in T2DM. As such, several reviews have been published describing the mechanisms of Nrf2 signaling in the context of cancer cells, however, no reviews have been published describing the current research on Nrf2 signaling in T2DM. Therefore, one aim of this review is to discuss the relevant mechanisms of Nrf2 signaling in non-diabetic and diabetic states. Furthermore, Nrf2 is currently being intensely researched as a therapeutic target for pharmaceuticals, thus it is highly salient that we develop an understanding of Nrf2 signaling dysregulation in T2DM.

The Nrf2 transcription factor

Nrf2 is a cap 'n' collar (CNC) basic-region leucine zipper transcription factor that is highly conserved amongst vertebrates, is constitutively expressed, and homozygous KO mice develop normally, indicating it is dispensable for mouse development. [20, 21] Nrf2 contains six highly conserved Nrf2-ECH homology (Neh) domains (Figure 6.1). The Neh 1 domain is the DNA-binding domain that interacts with small Maf proteins and incurs post-translational modifications, such as acetylation, that influence DNA-binding affinity.[17, 22, 23] The Neh2 domain serves as the binding domain for the Kelch domain of Keap-1 and contains seven lysine residues for ubiquitin conjugation, thus allowing negative regulation of Nrf2 via proteasome-mediated degradation.[24, 25] The Neh 3 domain lies at the C-terminus and has been shown to be requisite for transcription through the recruitment of CHD6, a coactivator that contains a helicase and chromodomain. [21] Neh 4 and 5 are transcription transactivation domains which bind to p300-CBP and act synergistically to optimize activation of reporter gene expression. [26] The Neh 6 domain functions to regulate protein stability through the formation of a phosphodegron via GSK-3

mediated phosphorylation of serine residues and β-TrCP mediated ubiquitination. [27-30]

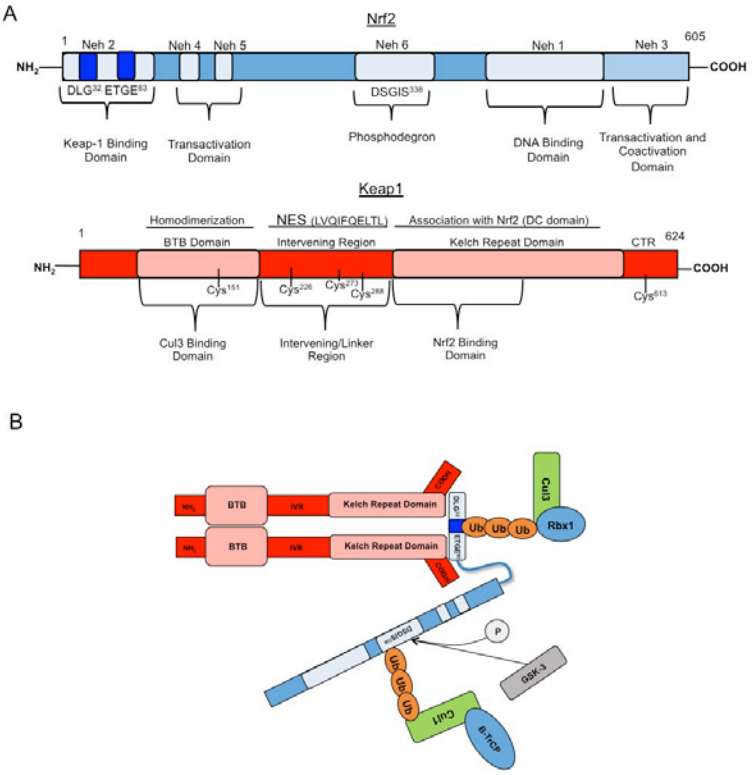

Figure 6.1. Structures of Nrf2 and Keap1 and Keap1-mediated ubiquitination and GSK-3/β-TrCP mediated ubiquitination. A) Human Nrf2 contains 589 amino acids and six highly conserved Nrf2-ECH homology (Neh) domains. Neh 1 domain is the DNA-binding domain that interacts with small Maf proteins, HATs, and HDACs. The Neh2 domain contains seven lysine residues for ubiquitin and serves as the binding domain for the Kelch domain of Keap-1. The Neh 3 domain is requisite for transcription through the recruitment of CHD6, a coactivator that contains a helicase and chromodomain. Neh 4 and 5 are transcription transactivation domains that bind to p300/CBP and act synergistically to optimize activation of reporter gene expression. The Neh 6 domain is a serine rich domain that is believed be involved in regulation of degradation and regulation through phosphorylation. Human Keap1 contains 624 amino acids and five domains. The BTB domain functions with the N-terminus to mediate homodimerization and binding of Keap1 to Cul3. The Kelch domain functions with the C-terminus to mediate the binding of Nrf2 to Keap1. B) Degradation of Nrf2 is regulated through the Keap1-Cul3-Rbx E3 ubiqutin ligase complex in the Neh2 domain and by the Glycogen Synthase Kinase 3/β-TrCP axis via a phosphodegron in the Neh6 domain.

Nrf2 is negatively regulated by Keap1

Keap1-dependent Cul3-Rbx1 degradation

Nrf2 is located in the cytosol where it is associated with a negative regulator, Kelch-like ECH-associated protein 1 (Keap1). The Kelch domain of Keap1 binds to an ETGE motif in the NEH2 amino-terminal regulatory domain of Nrf2, and in unstressed conditions, the Nrf2 protein is rapidly turned over in through Keap-1 dependent manner Cul3-Rbx1 ubiquitination and proteasomal degradation. [31] Thus, under basal conditions, the Neh2 domain of Nrf2 functions as a redox-sensitive degron via Keap1, which associates with Cul3 and Rbx, forming an E3 ubiquitin ligase complex that actively targets the lysine residues of Nrf2 for ubiquitination (Figure 6.2).[25]

When cells are exposed to oxidative stress, electrophiles, or chemopreventive agents, Nrf2 escapes Keap1-mediated repression, allowing it to translocate from the cytosol to the nucleus, and induce the ARE response. Based upon our current understanding, it appears that cellular stress or pharmacological therapy mainly disrupts the Keap1-Nrf2 complex, thereby increasing Nrf2 protein stability through direct modification of Keap1 and Nrf2 residues. Keap1 is rich in cysteine residues, which often act as a molecular switch triggered by changes in intracellular redox status. *In vitro* studies demonstrated that formation of the Keap1-Nrf2 complex is dependent upon the presence of strong reducing agents and that thiol-reactive reactive compounds can alter the Keap1-Nrf2 interaction, suggesting that cysteine residues in Keap1 may play a role in Keap1 mediated repression of Nrf2. [32] Keap1 mutation studies demonstrated that C273 and C288 are essential for Keap1-

dependent repression of Nrf2 under basal conditions. Additionally, these studies demonstrated C151 is required for Keap1-dependent inhibition of Nrf2 by pharmacological agent, and electrophilic and oxidative stress. [33]

The Toledano lab elucidated a molecular mechanism through which Keap1-Nrf2 interaction is inhibited under oxidative conditions. They demonstrated that under basal conditions, Keap1 carries a disulfide bridge between Cys^{226} and Cys^{613}, and exposure to H_2O_2, increases this disulfide bridge and initiates formation of an intermolecular disulfide linking two KEAP1 molecules via Cys^{151}, which then allows for increased Nrf2 Stabilization.[34] Independently, the Hayes lab demonstrated that Cys^{226} and Cys^{613} are required for the $Zn2^{+}$ sensor, which allows Keap1 to sense Cd^{2+}, As^{3+}, Se^{4+}, and Zn^{2+}. This findings indicates that the Keap1-Nrf2 complex is involved not only in sensing oxidative stress but also as a metal(oid) sensor. [35] When considered together, these studies support the hypothesis that the cysteine residues of Keap1 act as a molecular switch, thereby enabling Keap1 to regulate Nrf2 and respond appropriately to changes in the intracellular redox status and other cellular stresses.

Most evidence suggests the majority of the disruption occurs through direct modification of the Keap1-Nrf2 complex; however, several studies have shown that the protein kinase C (PKC) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) pathways can also cause disruption of the Keap1-Nrf2 complex (Figure 6.2). Two independent groups have demonstrated that antioxidant therapy modifies the complex through the PKC pathway. [36, 37] Huang and colleagues and Bloom and Jaiswal demonstrated that PKC phosphorylates Nrf2 at Ser40, which lies

in the Neh2 domain that interacts with the Kelch-domain of Keap1, and releases Nrf2 from Keap1. Bloom and Jaiswal also demonstrated that Ser40 phosphorylation was not required for protein stability or nuclear accumulation.[36] The unfolded protein response (UPR) and endoplasmic reticulum (ER) stress has also been shown to disrupt the Keap-Nrf2 complex and activate Nrf2 and its target genes. [38] Immunoprecipitation of PERK from 3T3 cells transduced with a retrovirus-encoding PERK demonstrated that Nrf2 was a direct PERK substrate. In the same paper, The Diehl Lab demonstrated that the UPR and ER stress activated PERK rapidly phosphorylates Nrf2 and is necessary and sufficient for PERK-dependent dissociation of Nrf2 from Keap1, nuclear import, and prevents reassociation with Keap1. [38]

This data suggests that stability of Nrf2 is primarily controlled by the Nrf2-Keap1 complex, which is regulated through 2 distinct mechanisms: 1) Nrf2 can be released from Keap1 through modification of cysteine residues in response to thiol-reactive compounds, 2) through signaling pathways that sense cell stress through oxidative stress-independent mechanisms (i.e. ER stress via the PERK pathway or chemopreventative therapy via the PKC pathway) (Figure 6.2).

Keap1 dependent nuclear export

Recent studies have demonstrated that Keap1 also plays a role in regulating post-transcriptional nuclear export of Nrf2. [39-41] Immunofluorescence studies have shown that Keap1 detaches from the cytoskeleton and translocates to the nucleus after exposure to oxidative stress. [40] Velichkova and Hasson demonstrated that Keap1 possessed a nuclear export signal (NES), a LVQIFQELTL motif within the

intervening region, and that mutation in the NES of Keap1 resulted in nuclear accumulation of both Keap1 and Nrf2, suggesting that the NES of Keap1 plays a primary role in nuclear export of Nrf2. The Zhang lab confirmed these findings and demonstrated that Keap1 nuclear translocation is independent of Nrf2 and the Nrf2-Keap1 complex does not bind to the ARE. [41] These findings suggest that in the presence of oxidative stress Nrf2 escapes Keap1, translocates to the nucleus wherein it transcribes antioxidant genes and once cellular redox homeostasis is recovered, Keap1 translocates into the nucleus and complexes with the disengaged Nrf2. The Keap1-Nrf2 complex is then exported from the nucleus where it associates with the E3 ubiquitin ligase, thereby resulting in degradation of Nrf2. [41] The Jaiswal lab identified a specific residue for this mechanism of nuclear export when they demonstrated that phosphorylation of Tyr^{85} was required for Keap1-mediated nuclear export of Nrf2.[42]

Keap1 regulates Nrf2 protein stability via Cul3-Rbx1 ubiquitination and proteasomal degradation by binding an ETGE motif in Neh2 domain. Additionally, Keap1 contains a NES, a motif LVQIFQELT within the intervening region, which regulates nuclear export of Nrf2 through interaction with a phosphorylated Tyr85, which is then marked for degradation by the E3 ubiquitin ligase. Thus, Keap1 plays a critical role in governing Nrf2 function.

Keap1 is increased in diabetes

When bound to Keap1, Nrf2 is ubiquitinated and degraded by the proteasome in a rapid manner, effectively preventing Nrf2 from transcribing Nrf2-dependent phase 2 gene products. Furthermore, Keap1 regulates post-transcriptional

repression of Nrf2-via nuclear export and subsequent proteasomal degradation. Together, these mechanisms reduce Nrf2 protein stability and transcription of Nrf2-mediated genes.

Keap1 levels are elevated in fibroblasts taken from diabetic rats and co-immunoprecipitation analysis has demonstrated that association of Nrf2 with Keap1 is significantly increased in the diabetic fibroblasts when compared to non-diabetic controls. Furthermore, Nrf2 is degraded by the 26S proteasome in diabetic fibroblasts at a greater rate than in control fibroblasts.[12] Additionally, Keap1 mRNA is elevated in high-fat diet induced models of diabetes in mice.[43]

The Shinohara lab has recently demonstrated that increased Keap1 in diabetes may due to epigenetic mechanisms. They found that CpG islands in Keap1 promoter were demethylated in cataractous lenses from diabetic patients and that treating human lens epithelial cells with 5-aza-2′deoxycytidine (5-Aza), a demethylation agent, induces a 10-fold increase in *Keap1*mRNA, a 3-fold increase levels of *Keap1* protein, and reduced Nrf2 function. Thus, demethylation of the CpG Our results indicated that demethylation of the CpG islands in the *Keap1* promoter will activate the expression of *Keap1* protein, which then increases the targeting of Nrf2 for proteosomal degradation. Decreased Nrf2 activity represses the transcription of many antioxidant enzyme genes and alters the redox-balance towards lens oxidation. Thus, demethylation of the CpG islands in the Keap1 promoter may be a contributor factor to increased Keap1 levels observed in diabetes and that Nrf2 protein stability is reduced in T2DM, at least in part, through increased levels of Keap1.

Targeting Keap1-Nrf2 dysregulation

The antioxidants sulforaphane (SFN) and tetrahydroxyquinone (tBHQ) disrupt the Keap1-Nrf2 complex and have been shown to be effective in reducing or preventing diabetic complications by increasing Nrf2 content and activity in animal models.[8, 11, 44] It was originally believed that SFN and tBHQ increased Nrf2 activity by modifying cysteine residues of the Nrf2-Keap1 complex and caused dissociation in a manner similar to ROS.[32] This theory would present a paradox as the evidence has shown that increased ROS causes Nrf2 to dissociate and translocate to the nucleus and that late T2DM is associated with increased ROS but decreased Nrf2. The evidence that treating oxidative stressed diabetic cells and animals with tBHQ or SFN increases Nrf2 signaling suggests these compounds have a different mechanism of action. Several research groups have been attempting to elucidate exactly how SFN and tBHQ increase Nrf2 content and activity. Based upon current evidence, SFN and tBHQ disrupt the Nrf2-Keap1 complex by inhibiting the activity of the Keap1-Cul3 ubiquitin ligase. Kobayashi and colleagues demonstrated that tBHQ blocked ubiquitination of Nrf2. [45] Zhang confirmed this and demonstrated that SFN functions in the same manner. [46] This presents an interesting question, how does preventing ubiquitination of Nrf2 increase its activity? Zhang presented the following saturation model to answer that question: during unstressed, basal conditions, large amounts of Keap1 molecules exist in relation to Nrf2, and as such the majority of newly synthesized Nrf2 binds to Keap1 and is degrading through proteasomal degradation.[46] Upon administration of tBHQ or SFN, Nrf2 degradation is suppressed by blocking ubiquitination and its subsequent degradation. The amount of Nrf2 in the

cell eventually saturates the binding capacity of Keap1, resulting in free Nrf2, which is capable of transcription. This makes SFN and tBHQ an attractive target for improving Nrf2 function in diabetes by reducing the ubiquitination caused by increased levels of Keap1 present in the diabetic state.[12]

The triterpenoid derivative of dihydro-CDDO-trifluorethyl amid (Dh404) has also been shown to directly disrupt Keap1 association and reduce Keap1-dependent suppression of Nrf2.[47] Ichikawa and colleagues used a balanced Keap1-Nrf2 expression system and residue mutations of C151S, C273S, and C288S to examine whether Dh404 increases Nrf2 by directly disrupting the Keap1-Nrf2 complex. They found that Dh404 increased Nrf2 activity in all cells except those with Nrf2 and C151S Keap1 mutant. Furthermore, Dh404 increased Nrf2 activity and inhibited the formation of ROS in in H9C2 cardiomyocytes. Together, this evidences indicates that Dh404 directly disrupts the Keap1-Nrf2 complex by interacting with the C151 residue in Keap1, thereby increasing Nrf2 stability. Importantly, the mechanism of Dh404 is independent of SFN and tBHQ, suggesting they may have synergistic effects when administered in combination.

Targeting the Keap1-Nrf2 complex with SFN, tBHQ, and Dh404 *in vivo* has shown promise in attenuating diabetic complications in animal models. Bai and colleagues demonstrated that administration of SFN to diabetic mice upregulated Nrf2 expression and its downstream genes and concurrently preserved ejection fraction, reduced protein expression and mRNA of the hypertrophic marker ANP, attenuated cardiac hypertrophy, reduced fibrosis and TGF-β expression, decreased inflammation and TNFα, and reduced oxidative stress in the myocardium.[11] SFN has

been shown to have similar effects in diabetic nephropathy. 3 months of SFN treatment attenuated loss of kidney function, fibrosis and TGF-β expression, reduced inflammation and TNFα, and oxidative stress in the kidneys of diabetic mice [48]. The improved renal function the diabetic mice by SFN treatment occurred concomitantly with increased Nrf2 expression in the kidneys of the diabetic mice. Bai et al. demonstrated that SFN upregulates renal expression of Nrf2 and its downstream gene products at both mRNA and protein level.[11] Importantly, *in vitro* studies from the Cui et al. paper demonstrated that silencing Nrf2 abolished SFN's prevention of hyperglycemia induced fibrosis, indicating the therapeutic benefits of SFN treatment occur predominately via Nrf2. Additionally, a recent randomized double-blind placebo controlled trial found that a supplemental source of SFN, broccoli sprout powder, reduced markers of oxidative stress in patients with T2DM.[49]

tBHQ disrupts the Keap1-Nrf2 complex via the same mechanism as SFN and has shown similar results as SFN in animal models of diabetic nephropathy. Feeding diabetic mice a diet containing 1% tBHQ significantly reduced the levels of serum and glomerular malondialdehyde, kidney weight and proteinuria, as well as decreased levels of fibronectin while concomitantly increasing Nrf2 protein expression and nuclear accumulation and expression of HO-1 and γ-GCS. [8]

Dh404 has been efficacious in attenuating diabetes-associated nephropathy via increasing Nrf2 function. Tan and colleagues demonstrated that dh404 improves diabetic kidney disease and atherosclerosis in an inverse dose-dependent manner. [50] A low dose but not a high dose of dh404 lessened diabetes-associated atherosclerosis while concomitantly decreasing oxidative stress (markers) and TNF-

α, ICAM-1, VCAM-1 and MCP-1. They also demonstrated that dh404 attenuates loss of renal function and glomerular and renal tubular injury in diabetic mice. Corresponding in vitro studies in NRK cells showed that low doses dh404 increased the Nrf2 gene products HO-1, NQO1 and GSH-S transferase, and inhibited TGFβ-mediated pro-fibrotic fibronectin and IL-6.[50] It is pertinent to note that analogues of Dh404, namely bardoxolone methyl, have shown to have adverse effects. However, it was believed that impure compounds were responsible for the adverse effects in those studies.[51] Follow up studies demonstrated that using structural analogs lacking the impurities and at appropriate doses were unable to reproduce the adverse effects and that dh404 is in fact well tolerated and exhibits efficacy in rodent models of T2DM.[52] It is believed these negative side effects were due to impure compounds and that dh404 is both efficacious and well tolerated in several animal models and in humans. As dh404 has shown promise in animal models and human trials have already been conducted, both the dose and source of dh404 need to be given thorough consideration in future research and human trials.

SFN and tBHQ increase Nrf2 stability by preventing Keap1-mediated ubiquitination and degradation of Nrf2. Dh404 increases Nrf2 protein stability through a dissimilar mechanism by causing dissociation of the Nrf2 complex. In combination therapy, dh404 may increase Keap1-Nrf2 disassociation while SFN or tBHQ may reduce ubiquitination and improve Nrf2 protein stability. Therefore, a therapy utilizing both approaches may prove synergistic in improving Nrf2 function in diabetes and research exploring this is needed.

Nrf2 is negatively regulated by Glycogen Synthase Kinase 3

Glycogen Synthase Kinase 3/β-TrCP regulates Nrf2 protein stability

Degradation of the Nrf2 transcription factor also occurs in a Keap1-independent manner by the Glycogen Synthase Kinase 3/β-TrCP axis via a phosphodegron in the Neh6 domain.[28-30] Rada and colleagues initially demonstrated that Glycogen Synthase Kinase 3 (GSK-3) regulates Nrf2 protein levels independently of Keap1. [31] Administration of SB216763, a GSK-3 inhibitor, to $Keap1^{-/-}$ mice and $Keap1^{+/+}$ mice SB216763 increased Nrf2 protein levels by increasing its half-life in both the $Keap1^{-/-}$ and WT mice, suggesting GSK-3 alters protein levels through regulation of Nrf2 stability independently of Keap1.[29] In the same paper, utilizing bioinformatics and a series of elegant experiments, the authors were able to demonstrate that GSK3-3 phosphorylates a serine residue cluster (335, 338, 342, 347, 351, and 355) in a β-TrCP destruction motif within the Neh6 domain of Nrf2, and that serine to alanine mutation of these residues prevented GSK-3/β-TrCP dependent degradation. These initial findings indicate that the Neh6 domain in the Nrf2 transcription factor functions as a phosphodegron in which GSK-3 phosphorylates serine residues within a β-TrCP destruction motif that is then ubiquitinated by the β-TrCP E3 ligase.

Recently the Hayes lab identified the binding site for the GSK-3 phosphorylation site through which β-TrCP serves as a receptor for the Skp1-Cul1-RBX/Roc1 ubiquitin ligase complex. [30] Utilizing biotinylated-peptide pull-down assays, they identified $DSGIS^{338}$ as the binding site for β-TrCP, and that phosphorylation of Ser^{335} and Ser^{338} increased β-TrCP binding. Together, these findings indicate that GSK-3 represses Nrf2 by phosphorylating Ser^{335} and Ser^{338} within a phosphodegron in the

Neh6 domain, which increases binding of β-TrCP and subsequent ubiquitination and degradation.

GSK-3 activity is, at least in part, negatively regulated via the PI3K and PKB/Akt pathway. Several studies have demonstrated that inhibition of PI3K and PKB/Akt pathway increases GSK-3 activity and downregulates Nrf2.[29, 30] As there is aberrant PI3K and PKB/Akt signaling in diabetes, the GSK-3/ β-TrCP axis is likely to be involved in Nrf2 dysregulation in Diabetes.

GSK-3β-Fyn axis regulates nuclear export of Nrf2

Deacetylation of Nrf2 causes transcriptional termination and disengages it from the ARE.[53] Upon disengagement from the ARE Nrf2 must then be exported from the nucleus where it is degraded by the proteasome. Currently, there are two major mechanisms for nuclear export of Nrf2; the GSK3B-Fyn pathway and Keap1-mediated nuclear export.

Upon completion of Nrf2 activation, the Src kinase, Fyn, phosphorylates Nrf2 at Tyr^{568}, and causes nuclear export by Crm1 and ultimately degradation of Nrf2.[39] When treated with xenobiotics or hydrogen peroxide, Fyn is exported out of the nucleus, thereby allowing Nrf2 to bind to the ARE and induce transcription. Allowing accumulation of Fyn by removing its NES motif renders Nrf2 ineffective and increases susceptibility to cellular death. Nuclear accumulation of Fyn is regulated by Glycogen synthase kinase -3β (GSK-3β). GSK-3β phosphorylates Fyn, causing it to localize to the nucleus where it phosphorylates Nrf2 at Tyr^{568}, resulting in nuclear export and degradation.

Exposing granule neurons to oxidative stress by administrating H_2O_2 inhibits GSK-3β activation and promotes nuclear accumulation of Nrf2.[54] Furthermore, inhibiting GSK-3β with the compound TDZD-8 resulted in large increases of nuclear Nrf2. These studies, along with several others suggest that in the presence of oxidative stress GSK-3β-regulated nuclear translocation of Fyn is inhibited, thereby reducing nuclear export of Nrf2.[55] It has been hypothesized that there may be two prominent phases of the Nrf2 stress response: 1) an early phase (0-4 hrs.) in which Nrf2 translocates to the nucleus and the oxidative stress suppresses the GSK-3β pathways, thereby reducing nuclear export, 2) a delayed response (5-8 hours) in which unknown kinases activate the GSK-3β pathways.[56] This hypothesis poses several novel questions and potentially rewarding areas of investigation. I propose there may be an alternative hypothesis that explains GSK-3β-regulated control of Fyn. I propose the GSK-3β-Fyn axis works on a feedback mechanism whereby GSK-3β constitutively activates Fyn and causes nuclear translocation under basal conditions and when exposed to oxidative and/or cellular stress, GSK-3β-regulated activation of Fyn is inhibited, and Nrf2 is allowed to accumulate in the nucleus and transcribe antioxidant genes. When the antioxidant products of Nrf2 transcription have attenuated the cellular stress, the GSK-3β-inhibitory signal is removed and Fyn translocation is restored, exporting Nrf2, thereby attenuating the activity of the endogenous antioxidant system. Such a mechanism is parsimonious and would be effective in maintaining the redox status of the cell.

GSK-3 functions to downregulate Nrf2 through two distinct mechanisms, 1) through the formation of a phosphodegron via the GSK-3/ β-TrCP axis, and 2) by

enhancing nuclear export of Nrf2 via the GSK-3β-Fyn axis. The dual role of GSK-3 in regulating Nrf2 makes it an attractive potential target for improving Nrf2 function.

Diabetes increases GSK-3 activity

GSK-3 decreases protein stability through the formation of a phosphodegron and ubiquitination via β-TrCP. GSK-3 is constitutively active at basal conditions and is inhibited via the IRS-1/PI3K and Akt pathways, and inhibition of the PI3K/Akt pathway increases Nrf2 degradation.[29, 30] Hayes and Dinkova-Kostova noted that this mode of regulation has been overlooked because GSK-3 is inhibited under conventional cell-culture conditions.[57] As such, no studies have explicitly demonstrated that GSK-3/TrCP mediated degradation of Nrf2 is increased in diabetes. However, GSK-3 is elevated in rodent models and humans of diabetes.[58, 59] Furthermore, PI3K/Akt signaling is diminished in insulin-resistant rodents models and in the skeletal muscle of obese humans with T2DM.[60, 61] This suggests increased degradation of Nrf2 via the GSK-3/β-TrCP axis is a plausible, and highly probable mechanism for decreased Nrf2 function in diabetes. Research is needed to establish the state of this mechanism in the function of Nrf2 in diabetes.

Gsk-3 is also involved in reducing nuclear accumulation of Nrf2 via the GSK-3β/Fyn axis and evidence from animal models of diabetes suggest this mechanism is increased in the diabetic state. Bitar and Al-Mulla demonstrated that GSK-3β/Fyn mediated nuclear export of Nrf2 was increased in diabetes when they showed increased GSK-3β activity and expression of Fyn alongisde diminished accumulation of nuclear Nrf2 and Nrf2 gene products in primary fibroblasts from diabetic mice.[12] Furthermore, they demonstrated that siRNA-mediated downregulation of GSK-3

restored nuclear accumulation of Nrf2 signaling in the diabetic fibroblasts; suggesting inhibition of GSK-3β can improve Nrf2 function in diabetes. Independently of Bitar and Al-Mulla, the Calvert lab has also demonstrated that nuclear expression of Fyn is increased in hearts of *db/db* mice when compared to controls.[13] Together, these findings indicate that augmentation in GSK-3B-Fyn signaling in T2DM reduces nuclear accumulation of Nrf2 and transcription of its antioxidant gene products.

Increased GSK-3 in diabetes can reduce Nrf2 protein stability through the phosphodegron in the Neh6 domain and decrease nuclear accumulation through nuclear export via the GSK-3β-Fyn mechanism. As such, GSK-3 inhibition is an attractive target to improve Nrf2 function in diabetes.

GSK-3 inhibition in diabetes

GSK-3 inhibitors have shown efficacy in treating diabetes, primarily for their role in restoring defects in glycogen synthesis and glucose uptake.[62, 63] While the majority of GSK-3 inhibition in diabetes has focused on improving insulin sensitivity, inhibition of GSK-3 inhibition has also been shown to improve Nrf2 function in cell culture and in non-diabetic and diabetic animal models.

In initial *in vitro* studies*,* Rojo and colleagues treated N2A neuroblasts with the GSK-3 inibitors10 mM lithium or 30 μM TDZD8 and then submitted them to SFN or tBHQ. Lithium or TDZD8 increased luciferase activity of an ARE-LUC reporter 2-fold.[54] Rada and colleagues found demonstrated that the GSK-3 inhibitor SB216763 increased Nrf2 protein levels in the liver and hippocampus of C57/BL6 mice.[28] *In vitro* work by Bitar and Al-Mulla showed that GSK-3 inhibition with lithium normalizes

basal and inducible levels of Nrf2 and transcriptional activity in diabetic rat fibroblasts.[12]

When TDZD8 and lithium are combined with SFN or tBHQ, there is a synergistic effect in increasing Nrf2 transcription.[12, 28, 54] Rojo and colleagues demonstrated that combination of either lithium or TDZD8 with SFN increased HO-1 levels to a greater extent than any treatment alone, suggesting that combination therapies targeting different mechanisms of Nrf2 signaling have additive effects.[28] Furthermore, Rojo et al. demonstrated that combining inhibition of GSK-3 with the Keap1-Nrf2 disrupter tBHQ induced a roughly 4-fold increase in Nrf2 protein levels and transcription in the diabetic fibroblasts, which was greater than either treatment in isolation.[54]

GSK-3 regulates Nrf2 stability and nuclear export in a Keap1-independent manner. Recent evidence has demonstrated that combination therapy of the GSK-3 inhibitor TDZD-8 with the Keap1-Nrf2 modifying tBHQ have a synergistic effect on Nrf2 transcriptional activity in diabetic rat fibroblasts.[13] This provides a strong case for combining therapies that target different aspects of the Nrf2 signaling pathway in diabetes.

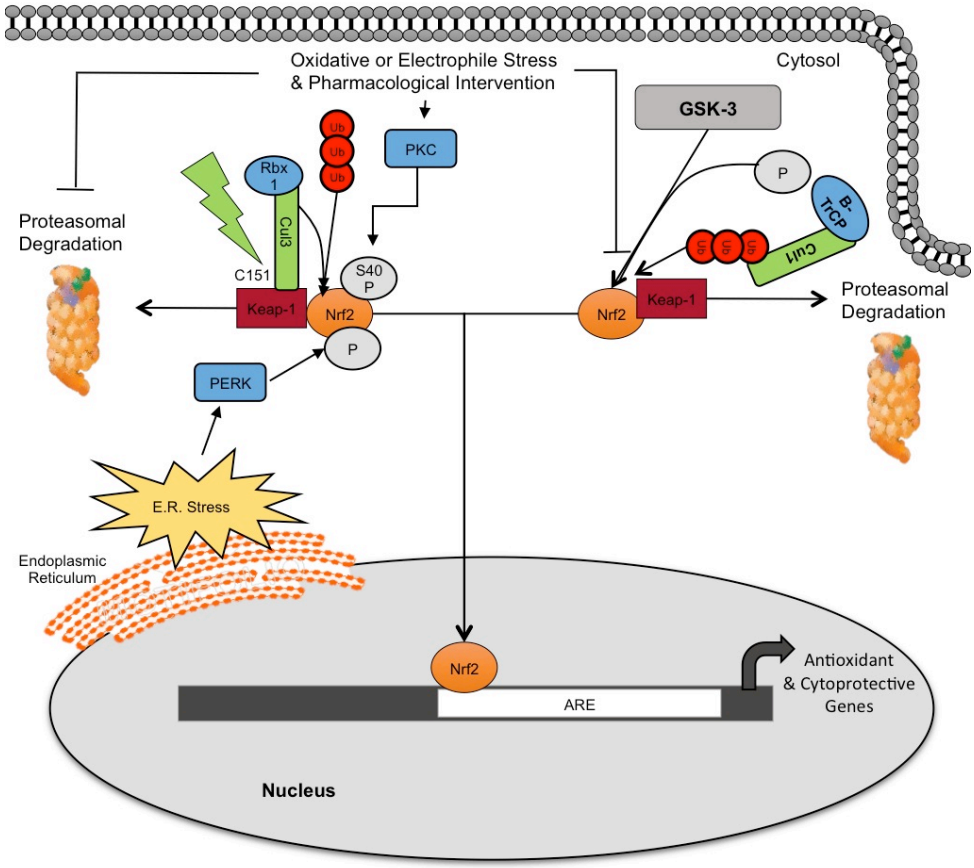

Figure 6.2. Cytosolic regulation of Nrf2. Under unstressed conditions, Nrf2 is located in the cytosol where it is associated with a negative regulator Kelch-like ECH-associated protein 1 (Keap1), which is associated with Cul3 and Rbx, forming an E3 ubiquitin ligase complex that actively targets the lysine residues of Nrf2 for ubiquitination. Nrf2 protein is rapidly turned over in a Keap1-dependent manner through Cul3-Rbx1 ubiquitination and proteasomal degradation. When cells are exposed to oxidative stress, electrophiles, or pharmacological agents, Nrf2 escapes Keap1-mediated repression, allowing it to translocate from the cytosol to the nucleus. Cellular stress can disrupt the Nrf2-Keap1 complex directly through directly modifying cysteine residues, the PKC pathway, or via the PERK pathway as a result of ER stress. Degradation of the Nrf2 transcription factors also occurs in a Keap1-indepdent manner by the Glycogen Synthase Kinase 3/β-TrCP axis via a phosphodegron in the Neh6 domain [28-30]. Briefly, GSK3 phosphorylates Ser^{335} and Ser^{338} in the $DSGIS^{338}$ of Nrf2 which increases β-TrCP binding and ubiquitination.

Transcriptional regulation of Nrf2 by acetylation/deacetylation

Nrf2 forms heterodimers with small v-maf musculoaponeurotic fibrosarcoma oncogene family (Maf) proteins and activates gene expression directly through the ARE.[64-66] Transcriptional activity of Nrf2 and its nucleo-cytoplasmic localization are regulated via acetylation and deacetylation of lysine residues by the histone acetyltransferase P300/CREB-binding protein (CBP) and histone deacetylase (HDAC) proteins

Katoh and colleagues found that two transactivation domains, Neh4 and Neh5, bind to CREB-binding protein (CBP) and are critical in attaining maximal activation of Nrf2-dependent gene expression as measured by reporter assays. [26] CBP induces acetylation of Nrf2, which increases binding of Nrf2 to the gene promoter in the ARE, and increases dependent Nrf2-dependent transcription.[53] Mutation of Lys^{588} and Lys^{591} to alanine or arginine of Nrf2 reduces Nrf2-dependent gene transcription and abolishes the acetylation and transcription activating effect of CREB-binding protein, identifying Lys^{588} and Lys^{591} as critical sites of acetylation and transcription of Nrf2-dependent genes.[53]

Kawai et al. demonstrated that the deacteylase sirtuin 1 (SIRT1) decreases acetylation of Nrf2 and Nrf2-dependent transcription.[53] Furthermore, acetylation of Nrf2 is recapitulated when transfected the cells with negative SIRT1 or administered the SIRT1 inhibitors nicotinamide or EX-527.[53] In addition to SIRT1, HDAC proteins have been shown to regulate nuclear Nrf2 function. Chromatin immunoprecipitation (ChIP) studies in vascular endothelial cells have shown that association of class I HDACs with Nrf2 results in deacetylation and inhibits its *in vivo* binding to the ARE

and NQO1 expression.[67] Conversely, Nrf2 was able to bind to the ARE and maintain NQO1 expression after transfecting the cells with HDAC-1/2/3-specific siRNA. This indicates that HDAC proteins, specifically class I HDACs, associate with Nrf2 and modulate its transcriptional activity.

Kawai and colleagues showed that acetylation/deacetylation also regulates nucleo-cytoplasmic localization. Acetylation of Nrf2 increases nuclear localization while deacetylation conditions promote cytoplasmic localization.[53] The authors hypothesized that nuclear acetylation of Nrf2 results in binding of to the ARE and augments transcription, while deacetylation disengages Nrf2 from the ARE, terminating transcription, and encourages nuclear export.[53] When considered together, these findings indicate that hyperacetylation results in increased expression of Nrf2 regulated genes and hypoacetylation results in decreased expression of Nrf2 regulated genes (Figure 6.3). This evidence suggests that Nrf2 activity is modulated by class I HDACs in a variety of cells and types of cell stress and that Nrf2 is primarily regulated by HDACs by the following mechanisms: 1) HDACs associate with Nrf2 and deacetylate it, thereby reducing binding of Nrf2 to DNA for transcription, 2) HDACs modify histone proteins and modulate chromatin to condense it, thereby reducing the availability of the DNA for Nrf2 to bind and engage in transcription.

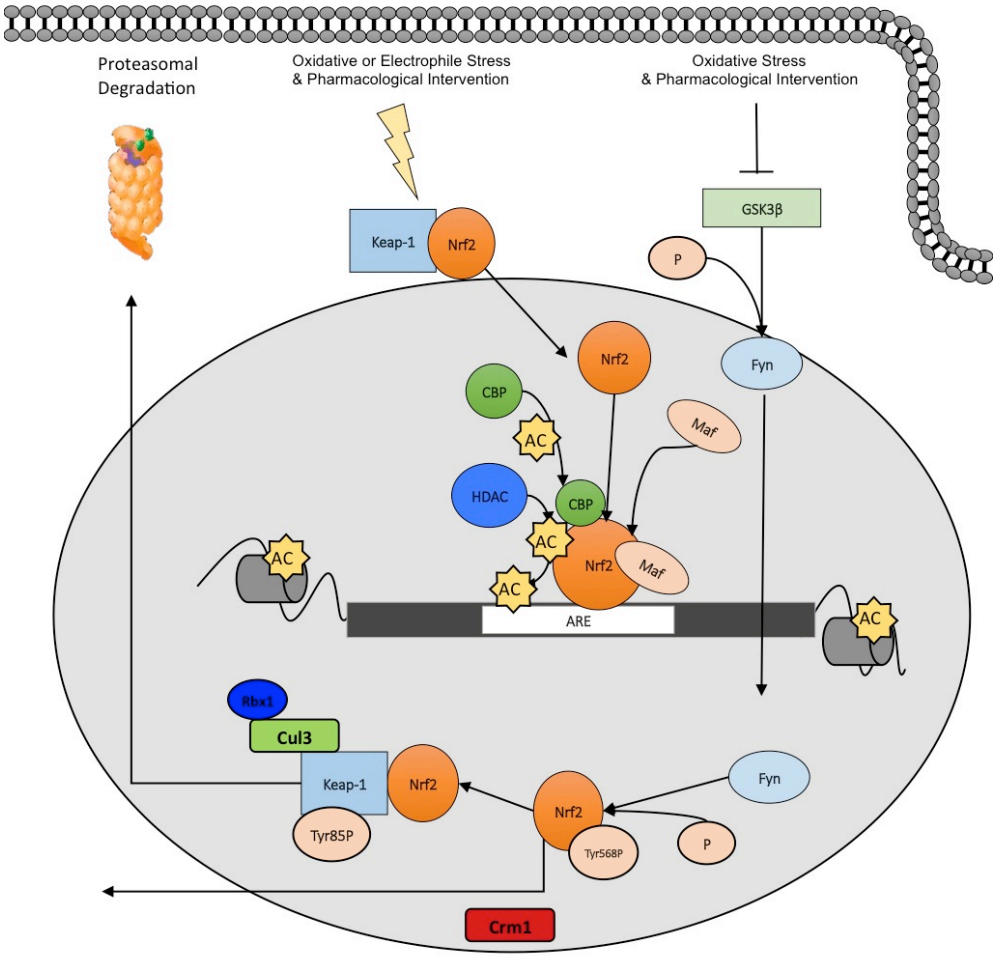

Figure 6.3. Nuclear regulation of Nrf2. Nrf2 forms heterodimers with Maf proteins to control differential gene regulation through the antioxidant response element (ARE). Acetylation of Nrf2 by the co-activator p300/CBP increased binding of Nrf2 to promoters in the ARE. Deacetylation by SIRT1 and other class I HDACs disengages Nrf2 from the ARE, thereby terminating transcription. Upon disengagement from the ARE, Fyn phosphorylates Tyr568 of Nrf2, resulting in Keap1 independent nuclear export via Crm1. Nrf2 can also complex with Keap1-Cul3-Rbx1, which is then ubiquitinated, exported, and degraded by the proteasome.

HDAC inhibitors to improve Nrf2 in diabetes

Abnormalities in epigenetic regulation by class I HDACs have been associated with T2DM.[68] Class I HDAC activity is elevated in diabetic mice and is associated with metabolic dysfunction, hypertrophic cardiomyopathy, renal impairment and are

considered to be regulators of diabetic complications.[68-70] Small molecule inhibitors of class I HDACs have therapeutic potential in improving metabolic dysfunction, and attenuating diabetic cardiomyopathy, nephropathy, and neuropathy in animal models of diabetes.[68, 71]

Currently the effect of class I HDAC inhibitors on Nrf2 function have not been explored in diabetic models; however, evidence from non-diabetic studies indicate that HDAC inhibitors are efficacious in increasing Nrf2 function.[79-81] Suberoylanilide hydroxamic acid (SAHA), a class I and II HDAC inhibitor, upregulates transcription of the Nrf2 gene products GCLC and GLCM, without increasing expression of Nrf2 in U937 cells.[72] The pan HDAC inhibitor trichostatin A (TSA) restores the expression of Nrf2 as well as transcription of NQO1 in a TRAMP C1 cell line.[73] TSA has also been shown to increase Nrf2 binding to the ARE and transcription of HO1. Wang and colleagues demonstrated that TSA increases Nrf2 nuclear translocation, enhances Nrf2-ARE binding, and upregulates expression of HO1 in cortical neuronal cells. [74] Additionally, Correa and colleagues demonstrated that increased HDAC activity decreased levels of Nrf2 and the downstream target γGCL-M in astroglial cells and valproic acid (VPA) restored the Nrf2 function and protected against oxidative-stress induced cell death in astroglial cells.[75] *In* vivo experiments have show that TSA protects against cerebral ischemia in mice, at least in part, through increased Nrf2 activity and that the protective effect of TSA was abolished in Nrf2-deficient mice.[74]

As previously mentioned, SFN has shown to be efficacious in attenuating diabetic cardiomyopathy and nephropathy in animal models of diabetes via improving Nrf2 function. In addition to the ability of SFN to repress Keap1-mediated

degradation of Nrf2, it is also an HDAC inhibitor. Thus, it is likely that SFN improves Nrf2 function in diabetes, at least it part, through its ability to act as an HDAC inhibitor. Future experiments exploring SFN as a potential therapeutic for diabetes and diabetic complications ought to explore this mechanism of action.

The role of HDAC proteins on Nrf2 function has been overlooked in the diabetic condition. Based upon the evidence that class I HDACs reduce Nrf2 function and inhibition of these HDACs restore Nrf2 function, HDAC inhibition may provide a powerful therapeutic target in the Nrf2 signaling pathway.

Future perspectives

The redox sensitive transcription factor Nrf2 is one of the major cellular defenses against the cytotoxic effects of oxidative stress and is currently being investigated as a therapeutic target for diabetic complications.[6] Evidence indicates that multiple aspects of the Nrf2 signaling pathway are dysregulated in T2DM, including: 1) increased expression of the Keap1, 2) increased GSK-3 activity, 3) upregulated nuclear Fyn expression, and 4) possibly decreased acetylation via increased HDAC activity (Figure 6.4). Currently most therapeutic strategies for increasing Nrf2 activity are aimed at targeting one aspect of the Nrf2 signaling pathway. While this approach has shown to be modestly effective in reducing diabetic complications such as nephropathy, hypertrophic cardiomyopathy, and neuropathy in animals and to reduce oxidative stress in humans, it is likely that combination therapy designed to target multiple aspects of Nrf2 signaling simultaneously may lead to more powerful outcomes. Data from *in vitro* data studies have shown that combination of a Keap1-Nrf2 disruptor with a GSK-3 inhibitor, were

more effective in improving Nrf2 content and its downstream gene products in diabetic fibroblasts than either treatment in isolation.[12] Therefore, research aimed at developing a better understanding of Nrf2 signaling in diabetes and the development combination therapies to optimize Nrf2 activity in diabetes is warranted.

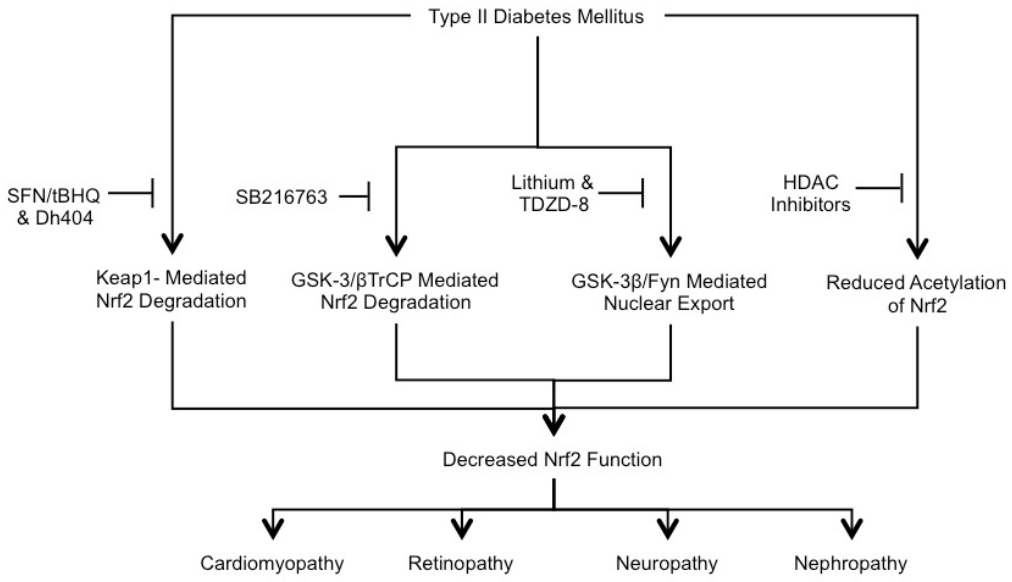

Figure 6.4. Effect of diabetes on Nrf2 signaling and current mechanism of action of current therapeutics. Sulforaphane and tert-Butylhydroquinone (tBHQ) prevents ubiquitination of Nrf2, thereby reducing proteasomal degradation. The triterpenoid derivative of dihydro-CDDO-trifluorethyl amid (Dh404) has been shown to directly disrupt Keap1 association and reduce Keap1-dependent suppression of Nrf2, thereby increasing Nrf2 stability. Inhibition of GSK-3 by SB216763 increases Nrf2 protein stability by preventing phosphorylation of Ser^{335} and Ser^{338} DSGIS338 and β-TrCP mediated ubiquitination. TDZD-8 and Lithium are GSK-3B inhibitors that prevents GSK-3B-Fyn mediated nuclear export of Nrf2 and normalizes basal and inducible levels of Nrf2 in diabetic rat fibroblasts [13]. Class I HDACs deacetylate Nrf2, which reduces DNA binding and increases nuclear export and cytosolic accumulation. Class I HDAC inhibitors reduce deacetylation of Nrf2, thereby promoting DNA binding and Nrf2 activity.

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Conflict of Interest

The author has no conflict of interest to disclose

Author Contribution

BD reviewed the literature and wrote manuscript

References

1. Cowie, C.C. et al. Full accounting of diabetes and pre-diabetes in the U.S. population in 1988-1994 and 2005-2006. Diabetes Care 32, 287-94 (2009).
2. Whiting, D.R., Guariguata, L., Weil, C. & Shaw, J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract 94, 311-21 (2011).
3. Tuttle, K.R., Anderberg, R.J., Cooney, S.K. & Meek, R.L. Oxidative stress mediates protein kinase C activation and advanced glycation end product formation in a mesangial cell model of diabetes and high protein diet. Am J Nephrol 29, 171-80 (2009).
4. Aksakal, E. et al. The role of oxidative stress in diabetic cardiomyopathy: an experimental study. Eur Rev Med Pharmacol Sci 15, 1241-6 (2011).
5. Vincent, A.M., Brownlee, M. & Russell, J.W. Oxidative stress and programmed cell death in diabetic neuropathy. Ann N Y Acad Sci 959, 368-83 (2002).
6. Zhong, Q., Mishra, M. & Kowluru, R.A. Transcription factor Nrf2-mediated antioxidant defense system in the development of diabetic retinopathy. Invest Ophthalmol Vis Sci 54, 3941-8 (2013).
7. Tan, Y. et al. Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo. Diabetes 60, 625-33 (2011).
8. Li, H. et al. Attenuation of glomerular injury in diabetic mice with tert-butylhydroquinone through nuclear factor erythroid 2-related factor 2-dependent antioxidant gene activation. Am J Nephrol 33, 289-97 (2011).

9. Cheng, A.S., Cheng, Y.H., Chiou, C.H. & Chang, T.L. Resveratrol upregulates Nrf2 expression to attenuate methylglyoxal-induced insulin resistance in Hep G2 cells. J Agric Food Chem 60, 9180-7 (2012).
10. Uruno, A. et al. The Keap1-Nrf2 system prevents onset of diabetes mellitus. Mol Cell Biol 33, 2996-3010 (2013).
11. Bai, Y. et al. Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. J Mol Cell Cardiol 57, 82-95 (2013).
12. Bitar, M.S. & Al-Mulla, F. A defect in Nrf2 signaling constitutes a mechanism for cellular stress hypersensitivity in a genetic rat model of type 2 diabetes. Am J Physiol Endocrinol Metab 301, E1119-29 (2011).
13. Peake, B.F. et al. Hydrogen sulfide preconditions the db/db diabetic mouse heart against ischemia-reperfusion injury by activating Nrf2 signaling in an Erk-dependent manner. Am J Physiol Heart Circ Physiol 304, H1215-24 (2013).
14. Xue, M. et al. Activation of NF-E2-related factor-2 reverses biochemical dysfunction of endothelial cells induced by hyperglycemia linked to vascular disease. Diabetes 57, 2809-17 (2008).
15. He, X., Kan, H., Cai, L. & Ma, Q. Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes. J Mol Cell Cardiol 46, 47-58 (2009).
16. Miao, X. et al. Sulforaphane prevention of diabetes-induced aortic damage was associated with the up-regulation of Nrf2 and its down-stream antioxidants. Nutr Metab (Lond) 9, 84 (2012).

17. He, H.J. et al. Curcumin attenuates Nrf2 signaling defect, oxidative stress in muscle and glucose intolerance in high fat diet-fed mice. World J Diabetes 3, 94-104 (2012).
18. Siewart, S.G., I; Santillan, L; Lucero, R; Ojeda, MS; Gimenez, MS. Downregulation of Nrf2 and HO-1 expression contributes to oxidative stress in type 2 diabetes mellitus: A study in Juana Koslay City, San Luis, Argentina. journal of Diabetes Mellitus 3, 71-78 (2013).
19. Velmurugan, G.V., Sundaresan, N.R., Gupta, M.P. & White, C. Defective Nrf2-dependent redox signalling contributes to microvascular dysfunction in type 2 diabetes. Cardiovasc Res 100, 143-50 (2013).
20. Chan, K., Lu, R., Chang, J.C. & Kan, Y.W. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci U S A 93, 13943-8 (1996).
21. Nioi, P., Nguyen, T., Sherratt, P.J. & Pickett, C.B. The carboxy-terminal Neh3 domain of Nrf2 is required for transcriptional activation. Mol Cell Biol 25, 10895-906 (2005).
22. Motohashi, H., Katsuoka, F., Engel, J.D. & Yamamoto, M. Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. Proc Natl Acad Sci U S A 101, 6379-84 (2004).
23. Sun, Z., Chin, Y.E. & Zhang, D.D. Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. Mol Cell Biol 29, 2658-72 (2009).

24. Tong, K.I. et al. Keap1 recruits Neh2 through binding to ETGE and DLG motifs: Characterization of the two-site molecular recognition model. Mol Cell Biol 26, 2887-900 (2006).
25. Zhang, D.D., Lo, S.C., Cross, J.V., Templeton, D.J. & Hannink, M. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. Mol Cell Biol 24, 10941-53 (2004).
26. Katoh, Y. et al. Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. Genes Cells 6, 857-68 (2001).
27. McMahon, M., Thomas, N., Itoh, K., Yamamoto, M. & Hayes, J.D. Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. J Biol Chem 279, 31556-67 (2004).
28. Rada, P. et al. Structural and functional characterization of Nrf2 degradation by the glycogen synthase kinase 3/beta-TrCP axis. Mol Cell Biol 32, 3486-99 (2012).
29. Rada, P. et al. SCF/{beta}-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. Mol Cell Biol 31, 1121-33 (2011).
30. Chowdhry, S. et al. Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. Oncogene 32, 3765-81 (2013).
31. Eggler, A.L., Small, E., Hannink, M. & Mesecar, A.D. Cul3-mediated Nrf2 ubiquitination and antioxidant response element (ARE) activation are

dependent on the partial molar volume at position 151 of Keap1. Biochem J 422, 171-80 (2009).

32. Dinkova-Kostova, A.T. et al. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. Proc Natl Acad Sci U S A 99, 11908-13 (2002).
33. Zhang, D.D. & Hannink, M. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 23, 8137-51 (2003).
34. Fourquet, S., Guerois, R., Biard, D. & Toledano, M.B. Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation. J Biol Chem 285, 8463-71 (2010).
35. McMahon, M., Lamont, D.J., Beattie, K.A. & Hayes, J.D. Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. Proc Natl Acad Sci U S A 107, 18838-43 (2010).
36. Bloom, D.A. & Jaiswal, A.K. Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. J Biol Chem 278, 44675-82 (2003).
37. Huang, H.C., Nguyen, T. & Pickett, C.B. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem 277, 42769-74 (2002).

38. Cullinan, S.B. et al. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol 23, 7198-209 (2003).
39. Kaspar, J.W. & Jaiswal, A.K. Tyrosine phosphorylation controls nuclear export of Fyn, allowing Nrf2 activation of cytoprotective gene expression. FASEB J 25, 1076-87 (2011).
40. Velichkova, M. & Hasson, T. Keap1 regulates the oxidation-sensitive shuttling of Nrf2 into and out of the nucleus via a Crm1-dependent nuclear export mechanism. Mol Cell Biol 25, 4501-13 (2005).
41. Sun, Z., Zhang, S., Chan, J.Y. & Zhang, D.D. Keap1 controls postinduction repression of the Nrf2-mediated antioxidant response by escorting nuclear export of Nrf2. Mol Cell Biol 27, 6334-49 (2007).
42. Kaspar, J.W., Niture, S.K. & Jaiswal, A.K. Antioxidant-induced INrf2 (Keap1) tyrosine 85 phosphorylation controls the nuclear export and degradation of the INrf2-Cul3-Rbx1 complex to allow normal Nrf2 activation and repression. J Cell Sci 125, 1027-38 (2012).
43. Yu, Z. et al. Oltipraz upregulates the nuclear factor (erythroid-derived 2)-like 2 [corrected](NRF2) antioxidant system and prevents insulin resistance and obesity induced by a high-fat diet in C57BL/6J mice. Diabetologia 54, 922-34 (2011).
44. Zheng, H. et al. Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy. Diabetes 60, 3055-66 (2011).
45. Kobayashi, A. et al. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol Cell Biol 26, 221-9 (2006).

46. Zhang, D.D. Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab Rev 38, 769-89 (2006).
47. Ichikawa, T. et al. Dihydro-CDDO-trifluoroethyl amide (dh404), a novel Nrf2 activator, suppresses oxidative stress in cardiomyocytes. PLOS ONE, 4, e8391 (2009).
48. Cui, W. et al. Prevention of diabetic nephropathy by sulforaphane: Possible role of Nrf2 upregulation and activation. Oxid Med Cell Longev 2012, 821936 (2012).
49. Bahadoran, Z. et al. Broccoli sprouts reduce oxidative stress in type 2 diabetes: a randomized double-blind clinical trial. Eur J Clin Nutr 65, 972-7 (2011).
50. Tan, S.M. et al. A derivative of Bardoxolone methyl, dh404, in an inverse dose-dependent manner, lessens diabetes-associated atherosclerosis and improves diabetic kidney disease. Diabetes (2014).
51. Zoja, C. et al. Analogs of bardoxolone methyl worsen diabetic nephropathy in rats with additional adverse effects. Am J Physiol Renal Physiol 304, F808-19 (2013).
52. Chin, M. et al. Bardoxolone methyl analogs RTA 405 and dh404 are well tolerated and exhibit efficacy in rodent models of Type 2 diabetes and obesity. Am J Physiol Renal Physiol 304, F1438-46 (2013).
53. Kawai, Y., Garduno, L., Theodore, M., Yang, J. & Arinze, I.J. Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. J Biol Chem 286, 7629-40 (2011).

54. Rojo, A.I., Sagarra, M.R. & Cuadrado, A. GSK-3beta down-regulates the transcription factor Nrf2 after oxidant damage: relevance to exposure of neuronal cells to oxidative stress. J Neurochem 105, 192-202 (2008).
55. Shin, S.M., Cho, I.J. & Kim, S.G. Resveratrol protects mitochondria against oxidative stress through AMP-activated protein kinase-mediated glycogen synthase kinase-3beta inhibition downstream of poly(ADP-ribose)polymerase-LKB1 pathway. Mol Pharmacol 76, 884-95 (2009).
56. Niture, S.K., Khatri, R. & Jaiswal, A.K. Regulation of Nrf2-an update. Free Radic Biol Med 66, 36-44 (2014).
57. Hayes, J.D. & Dinkova-Kostova, A.T. The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. Trends Biochem Sci 39, 199-218 (2014).
58. Eldar-Finkelman, H., Schreyer, S.A., Shinohara, M.M., LeBoeuf, R.C. & Krebs, E.G. Increased glycogen synthase kinase-3 activity in diabetes- and obesity-prone C57BL/6J mice. Diabetes 48, 1662-6 (1999).
59. Nikoulina, S.E. et al. Potential role of glycogen synthase kinase-3 in skeletal muscle insulin resistance of type 2 diabetes. Diabetes 49, 263-71 (2000).
60. Goodyear, L.J. et al. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. J Clin Invest 95, 2195-204 (1995).
61. Bjornholm, M., Kawano, Y., Lehtihet, M. & Zierath, J.R. Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in

skeletal muscle from NIDDM subjects after in vivo insulin stimulation. Diabetes 46, 524-7 (1997).

62. Ring, D.B. et al. Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. Diabetes 52, 588-95 (2003).
63. Cline, G.W. et al. Effects of a novel glycogen synthase kinase-3 inhibitor on insulin-stimulated glucose metabolism in Zucker diabetic fatty (fa/fa) rats. Diabetes 51, 2903-10 (2002).
64. Toki, T. et al. Human small Maf proteins form heterodimers with CNC family transcription factors and recognize the NF-E2 motif. Oncogene 14, 1901-10 (1997).
65. Itoh, K. et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236, 313-22 (1997).
66. Hirotsu, Y. et al. Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks. Nucleic Acids Res 40, 10228-39 (2012).
67. Lee, D.Y. et al. Role of histone deacetylases in transcription factor regulation and cell cycle modulation in endothelial cells in response to disturbed flow. Proc Natl Acad Sci U S A 109, 1967-72 (2012).
68. Galmozzi, A. et al. Inhibition of class I histone deacetylases unveils a mitochondrial signature and enhances oxidative metabolism in skeletal muscle and adipose tissue. Diabetes 62, 732-42 (2013).

69. Yoshikawa, M., Hishikawa, K., Marumo, T. & Fujita, T. Inhibition of histone deacetylase activity suppresses epithelial-to-mesenchymal transition induced by TGF-beta1 in human renal epithelial cells. J Am Soc Nephrol 18, 58-65 (2007).
70. Noh, H. et al. Histone deacetylase-2 is a key regulator of diabetes- and transforming growth factor-beta1-induced renal injury. Am J Physiol Renal Physiol 297, F729-39 (2009).
71. Christensen, D.P. et al. Histone deacetylase (HDAC) inhibition as a novel treatment for diabetes mellitus. Mol Med 17, 378-90 (2011).
72. Hu, Y. et al. Overcoming resistance to histone deacetylase inhibitors in human leukemia with the redox modulating compound beta-phenylethyl isothiocyanate. Blood 116, 2732-41 (2010).
73. Yu, S. et al. Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. PLOS ONE 5, e8579 (2010).
74. Wang, B. et al. Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. Free Radic Biol Med 52, 928-36 (2012).
75. Correa, F., Mallard, C., Nilsson, M. & Sandberg, M. Activated microglia decrease histone acetylation and Nrf2-inducible anti-oxidant defence in astrocytes: Restoring effects of inhibitors of HDACs, p38 MAPK and GSK3beta. Neurobiol Dis 44, 142-51 (2011).

References

Ajzen, I. (1985). From Intentions to Actions: A Theory of Planned Behavior. In J. Kuhl & J. Beckmann (Eds.), *Action Control* (pp. 11–39). Springer Berlin Heidelberg.

Ajzen, I. (1991). The theory of planned behavior. *Organizational behavior and human decision processes , 50* (2), 179-211.

Ajzen, I., & Fishbein, M. (1977). Attitude-behavior relations: a theoretical analysis and review of empirical research. *Psychological Bulletin, 84*, 888-918.

Ajzen, I., & Fishbein, M. (1980). *Understanding attitudes and predicting social behavior.* Englewood Cliffs, NJ: Prentice-Hall.

Alder, A., Stevens, R., Manley, S., Bilous, R., Cull, C., & Holman, R. (2003). Development and progression of nephropathy in type 2 diabetes: The United Kingdom Prospective Diabetes Study. *Kidney International*, *63*(1), 225-232.

(2009). American College Health Association-National College Health Assessment II. *American College Health Association*.

Ande, S. R., Moulik, S., & Mishra, S. (2009). Interaction between O-GlcNAc modification and tyrosine phosphorylation of prohibitin: Implication for a novel binary switch. *PLOS ONE, 4*(2), p. e4586.

Anderman, E. M., & Midgley, C. (1997). Changes in achievement goal orientations, perceived academic competence, and grades across transition to middle-level school. *Contemporary Educational Psychology*, 22, 269-298.

Anderson, E. S., Winett, R. A., Wojcik, J. R., & Williams, D. M. (2010). Social cognitive mediators of change in a group randomized nutrition and physical

activity intervention: Social support, self-efficacy, outcome expectations and self-regulation in the guide-to-health trial. *Journal of Health Psychology, 15*(1), 21-32.

Anderson-Bill, E. S., Suppini, A., & Apap, D. (2011). Social cognitive determinants of nutrition and physical activity among web-based health users enrolling in and online intervention: The influence of social support, self-efficacy, outcome expectations, and self-regulation. *Journal of Medical Internet Research, 13*(1), e28.

Asghar, M., George, L., & Lokhandwala, M. (2007). Exercise decreases oxidative stress and inflammation and restores renal dopamine D1 receptor function in old rats. *American Journal of Physiology Renal Physiology*, 293, F914-F919.

Association, A. D. (2004). Nephropathy in Diabetes. *Diabetes Care, 27*(suppl 1), s79-s83.

Atkinson, L., Fischer, M., & Lopaschuk, G. (2002). Leptin activates cardiac fatty acid oxidation independent of changes in the AMP-activated protein kinase-acetyl-CoA carboxylase-malonyl-CoA axis. *Journal of Biological Chemistry*, 277, 29424-29430.

Baar, K., Wende, A., Jones, T., Marison, M., Nolte, L., Chen, M., et al. (2002). Adaptations of skeletal muscle to exercise: Rapid increase in the transcriptional coactivator PGC-1. *FASEB J, 16(*14), 1879-1886.

Bai, Y., Cui, W., Xin, Y., Miao, X., Barati, M., Zhang, C., et al. (2013). Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of

Nrf2 expression and transcription activation. *Journal of Molecular and Cellular Cardiology*, 57, 82-95.

Bandura, A. (1982). Self-efficacy mechanism in human agency. *American Psychology, 37*(2), 122-147.

Bandura, A. (2012). Social cognitive theory. In P. Van Lange, A. Kruglanski, & E. Higgins (Eds.), *Handbook of Theories of Social Psychology*. London: SAGE Publiciations Ltd.

Bannister, A. J., & Miska, E. (2000). Regulation of gene expression by transcription factor acetylation. *Cellular and Molecular Life Sciences*, 57, 1184-1192.

Berlanga, J., Cibrian, D., Guillen, I., Freyre, F., Alba, J., Lopez-Saura, P., et al. (2005). Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds. *Clinical Science*, 109, 83-95.

Berlin, J. A., & Colditz, G. A. (1990). A meta-analysis of physical activity in in the prevention of coronary heart disease. *American Journal of Epidemiology, 132*(4), 612-628.

Biddle, S., & Goudas, M. (1996). Analysis of children's physical activity and its association with adult encouragement and social cognitive variables. *Journal of School Health, 66*, 75-78.

Blue, C. (1995). The predictive capacity of the theory of reasoned action and the theory of planned behavior in exercise research: an integrated literature review. *Research in Nursing & Health, 18*(2), 105-21.

Bonnard, C., Durand, A., Peyrol, S., Chanseaume, E., Chauvin, M., Morio, B., et al. (2008). Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *The Journal of Clinical Investigation, 118*(2), 789-800.

Boor, P., Celec, P., Behuliak, M., Grancic, P., Kebis, A., KukanqM., et al. (2009). Regular moderate exercise reduces advanced glycation and ameliorates early diabetic nephropathy in obese Zucker rats. Metabolism, 58, 1669-1677.

Braidman, I., Carroll, M., Dance, N., Robinson, D., Poenaru, L., Weber, A., et al. (1974). Characterisation of human N-acetyl-beta-hexosaminidase C. *FEBS Letters, 41*(2), 181-184.

Broderick, T., Poirier, P., & Gillis, M. (2005). Exercise training restores abnormal myocardial glucose utilization and cardiac function in diabetes. *Diabetes/Metabolism Research and Reviews*, 21, 44-50.

Brunso, K., Scholdere, J., & Grunert, K. (2004). Closing the gap between values and behavior -- a means-end theory of lifestyle. *Marketing Communications and Consumer Behavior, 56*(6), 665-670.

Cao, S., Li, B., Yi, X., Chang, B., Lian, Z., Zhang, Z., et al. (2012). Effects of exercise on AMPK signaling and downstream components to PI3K in rat with type 2 diabetes. *PLOS ONE, 7*(12), e51709.

Cardinal, B. J., & Spaziani, M. D. (2007). Effects of Classroom and Virtual" Lifetime Fitness for Heath" Instruction on College Students' Exercise Behavior. *The Physical Educator, 64*(4).

Cartoni, R., Leger, B., Hock, M., Praz, M., Crettenand, A., Pich, S., et al. (2005). Mitofusins 1/2 and ERRα expression are increased in human skeletal muscle after physical exercise. *Journal of Physiology, 567*(1), 349-358.

Carver, C. S., & Scheier, M. (2011). Self-regulation of action and affect. *In Handbook of Self-Regulation* (pp. 3-22). New York: Guilford Press.

Chen, X., Dodd, G., Thomas, S., Zhang, X., Wasserman, M., Rovin, B., et al. (2006). Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. *American Journal of Physiology Heart and Circulatory Physiology*, 290, H1862-H1870.

Cheng, A., Cheng, Y., Chiou, H., & Chang, T. (2012). Resveratrol upregulates Nrf2 expression to attenuate methylglyoxal induced insulin resistance in Hep G2 cells. *Journal of Agriculture and Food Chemistry, 60*(36), 9180-9187.

Chou, T. Y., Hart, G., & Dang, C. (1995). c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas. *The Journal of Biological Chemistry, 270*(32), 18961-18965.

Cowie, C. C., Rust, K., Ford, E., Eberhardy, M., Byrd-Holt, D., Li, C., et al. (2009). Full accounting of diabetes and pre-diabetes in the U.S. population in 1988-1994 and 2005-2006. *Diabetes Care, 32*(2), 287-294.

Cox, E. J., & Marsh, S. (2013). Exercise and diabetes have opposite effects on the assembly and O-GlcNAc modification of the mSin3A/HDAC1/2 complex in the heart. *Cardiovascular Diabetology, 12*(101), 1-15.

Dai, D. F., Johnson, S. C., Villarin, J. J., Chin, M. T., Nieves-Cintron, M., Chen, T., et al. (2011). Mitochondrial oxidative stress mediates angiotensin II–induced

cardiac hypertrophy and Gαq overexpression–induced heart failure. *Circulation Research*, 108, 837-846.

Damasio, A. (2010). Self comes to mind: Constructing the conscious brain. New York, NY: Random House.

Danaei, G., Finucane, M., Lu, Y., Singh, G., Cowan, M., Paciorek, C., et al. (2011). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: Systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet, 378*(9785), 31-40.

DeRubertis, F. R., Craven, P., & Melhem, M. (2007). Acceleration of diabetic renal injury in the superoxide dismutase knockout mouse. *Metabolism*, 56, 1256-1264.

Doerksen, S. E., Umstattd, M., & McAuley, E. (2009). Social cognitive determinants of moderate and vigorous physical activity in college freshman. *Journal of Applied Sports Psychology, 39*(5), 1201-1213.

Eggier, A. L., Small, E., Hannink, M., & Mesecar, A. (2009). Cul3-mediated Nrf2 ubiquitination and antioxidant response element (ARE) activation are dependent on the partial molar volume at position 151 of Keap1. *The Biochemical Journal, 422*(1), 171-180.

Ferguson, K., Yesalis, C., Pomrehn, P., & Kirkpatrick, M. (1989). Attitudes, knowledge, and beliefs as predictors of exercise intent and behavior in school children. *Journal of School Health, 59*, 112-115.

Fishbein, M., & Ajzen, I. (2010). Predicting and changing behavior: The reasoned action approach. New York: Psyschology Press.

Fong, D. S., Aiello, L., Ferris, F., & Klein, R. (2004). Diabetic retinopathy. *Diabetes Care, 27*(10), 2540-2553.

Frederick-Recascino, C. M., & Schuster-Smith, H. (2003). Competition and intrinsic motivation in physical activity: A comparison of two groups. *Journal of Sport Behavior, 26*(3), 240-254.

Frith, C., Friston, K., Liddle, P., & Frackowiak, R. (1991). Willed action and the prefrontal cortex in man: A study in PET. *Proceedings of the Royal Society of London,* 241-246.

Fülöp, N., Mason, M., Dutta, K., Wang, P., Davidoff, A., Marchase, R., et al. (2007). Impact of Type 2 diabetes and aging on cardiomyocyte function and O-linked N-acetylglucosamine levels in the heart*. American Journal of Physiology Cell Physiology*, 292, C1370-C1378.

Gewinner, C., Hart, G., Zachara, N., Cole, R., Beisenherz-Huss, C., & Groner, B. (2003). The coactivator of transcription CREB-binding protein interacts preferentially with the glycosylated form of STAT5. *The Journal of Biological Chemistry*, 279, 563-3572.

Godin, G., & Shephard, R. (1986). Psychosocial factors influencing intentions to exercise of young students from grades 7 to 9. *Research Quarterly for Exercise and Sport, 57*, 41-52.

Gomez-Cabrera, M.-C., Domenech, E., & Vina, J. (2008). Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. *Free Radical Biology and Medicine, 44*(2), 126-131.

Gordon, M. (2008). Extending the theory of planned behavior in the exercise domain: A study of community college students in an urban multicultural setting. *FIU Electronic Theses and Dissertation*, 1-158.

Gounder, S., Kannan, S., Devadoss, D., Miller, C., Whitehead, K., Odelberg, S., et al. (2012). Impaired transcriptional activity of Nrf2 in age-related myocardial oxidative stress is reversible by moderate exercise training. *PLOS ONE, 7*(9), e45697.

Grant, T. (2012). Comparing the principle-based SBH Maieutic method to traditional case study methods of teaching media ethics. Unpublished Dissertation.

Greene, G. W., White, A., Hoerr, S., Lohse, B., Schembre, S., Riebe, D., et al. (2012). Impact of an online healthful eating and physical activity program for college students. *American Journal of Health Promotion, 27*(2), e47-e56.

Grossman, E., Shemesh, J., Shamiss, A., Thaler, M., Carroll, J., & Rosenthal, T. (1992). Left ventricular mass in diabetes-hypertension. *The Journal of the American Medical Association Archives of Internal Medicine, 152*(5), 1001-1004.

Gupta, R., & Brunak, S. (2002). Prediction of glycosylation across the human proteome and the correlation to protein function. *Pacific Symposium on Biocomputing*, 7, 310-322.

Hallam, J. S., & Petosa, R. (20004). The long-term impact of a four-session work-site intervention on selected social variables linked to adults exercise adherence. *Health Education Behavior, 31*(88), 88-100.

Haltiwanger, R. S., Holt, G., & Hart, G. (1990). Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins. Identification of a uridine diphospho-N-acetylglucosamine:peptide beta-N-acetylglucosaminyltransferase. *Journal of Biological Chemistry, 265*(5), 2563-2568.

Hambrecht, R., Wolf, A., Gielen, S., Linke, A., Hofer, J., Erbs, S., et al. (2000). Effect of exercise on coronary endothelial function in patients with coronary artery disease. *The New England Journal of Medicine*, 342, 454-460.

Hart, G. W., Slawson, C., Ramirez-Correa, G., & Lagerlof, O. (2011). Cross talk between O-GlcNAcylation and phosphorylation: Roles in signaling, transcription, and chronic disease. *Annual Review of Biochemistry*, 80, 825-858.

Haskell, W. L., Lee, I., Pate, R., Powell, K., Blair, S., Franklin, B., . . . Bauman, A. (2006). Physical activity and public health: Updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. *Medicine and Science in Sports and Exercise*, 39(8), 1423-34.

Hausenblas, H. A., Carron, A. V., & Mack, D. E. (1997). Application of the theories of reasoned action and planned behavior to exercise behavior: A meta-analysis. *Journal of Sport and Exercise Psychology*, 19, 36–51

Hayes, J. D., & Ashford, M. (2012). Nrf2 orchestrates fuel partitioning for cell proliferation. *Cell Metabolism*, 16, 139-141.

He, X., Kan, H., Cai, L., & Ma, Q. (2009). Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes. *Journal of Molecular and Cellular Cardiology, 46*(1), 47-58.

Heron, M. (2008). *Deaths: Leading causes for 2008*. Center for Disease Control and Prevention, U.S. Department of Health and Human Services.

Howden, R. (2013). Nrf2 and cardiovascular defense. *Oxidative Medicine and Cellular Longevity*, 104308, 1-10.

Hoyert, D. L., & Xu, J. (2011). Deaths: Preliminary Data for 2011. Center for Disease Control and Prevention, U.S. Department of Health and Human Services.

Hsu, W., Lee, B., Li, C., Hsu, Y., & Pan, T. (2013). Monascin and AITC attenuate methylglyoxal-induced PPAR phosphorylation and degradation through inhibition of the oxidative stress/pkc pathway depending on Nrf2 activation. *Journal of Agricultural and Food Chemistry*, 6, 5996-6006.

Hu, F., Leitzmann, M., Stampfer, M., Colditz, G., Willet, W., & Rimm, E. (2001). Physical activity and television watching in relation to risk for type 2 diabetes mellitus in men. *Archives of Internal Medicine*, 161 (12), 1542-1548.

Huang, E. S., Basu, A., O'Grady, M., & Capretta, J. (2009). Projecting the future diabetes population size and related costs for the U.S. *Diabetes Care*, 32, 225-2229.

Jaramillo, M. C., & Zhang, D. (2013). The emerging role of the Nrf2–Keap1 signaling pathway in cancer. *Genes & Development*, 27, 2179-2191.

Jiang, T., Huang, Z., Lin, Y., Zhang, Z., Fang, D., & Zhang, D. (2010). The protective role of Nrf2 in Streptozotocin-induced diabetic nephropathy. *Diabetes*, 59, 850-860.

Jing, E., Emanuelli, B., Hirschey, M., Boucher, J., Lee, K., Lombard, D., et al. (2011). Sirtuin-3 (Sirt3) regulates skeletal muscle metabolism and insulin signaling via altered mitochondrial oxidation and reactive oxygen species production. *Proceedings of the National Academy of Sciences of the United States of America, 108*(35), 14608-14613.

Kawai, Y., Garduno, L., Theodore, M., Yang, J., & Arinze, I. (2011). Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. . *Journal of Biological Chemistry, 286*(9), 7629-7640.

Keating, X. D., Guan, J., Pinero, J., & Bridges, D. (2010). A meta-analysis of college students' physical activity behaviors. *Journal of American College Health*, *54*(2), 116-126.

Kilhovd, B., Berg, T., Birkeland, K., Thorsby, P., & Hanssen, K. (1999). Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. *Diabetes Care*, 22, 1543-1548.

Kilhovd, B., Giardino, I., Torjesen, K., Berg, T., Thomalley, P., Brownlee, M., et al. (2003). Increased serum levels of the specific AGE-compound methylglyoxal-derived hydroimidazolone in patients with type 2 diabetes. *Metabolism, 52*(2), 163-167.

Kim, H., Woo, J., Joo, H., & Moon, W. Cardiac transcription factor Nkx2.5 is downregulated under excessive O-GlcNAcylation condition. *PLOS ONE, 7*(6), e38053.

Kitada, M., Kume, S., Imaizumi, N., & Koya, D. (2011). Resveratrol improves oxidative stress and protects against diabetic nephropathy through normalization of MnSOD dysfunction in AMPK/SIRT1-independent pathway. *Diabetes, 60*(2), 634-643.

Kohl, H. W., Gordon, N., Villegas, J., & Blair, S. (1992). Cardiorespiratory fitness, glycemic status, and mortality in men. *Diabetes Care*, 15, 185-192.

Kohlberg, L. (1981). *The philosophy of moral development: Moral stages and the idea of justice.* New York: Harper & Row.

Kohlberg, L. (1981). Essays on moral development. In *The philosophy of moral development.*

Kohlberg, L., & Lickona, T. (1976). Moral stages and moralization: The cognitive-developmental approach. *Moral Development and Behavior: Theory, Research, and Social Issues*. New York: Rinehart and Winston.

Kowluru, R. A., Kowluru, V., Xiong, Y., & Ho, Y. (2006). Overexpression of mitochondrial superoxide dismutase in mice protects the retina from diabetes-induced oxidative stress. *Free Radical Biology and Medicine*, 41, 1191-1196.

Kretchmar, R. S. (2001, 53 318-325). Duty, habit, and meaning: Different faces of adherence. Quest .

Kriska, A. M., Saremi, A., Hanson, R., Bennett, P., Kobes, S., Williams, D., et al. (2003). Physical activity, obesity, and the incidence of type 2 diabetes in a high-risk population. *American Journal of Epidemiology, 158*(7), 669-675.

Laczy, B., Marsh, S., Brocks, C., Wittman, I., & Chatham, J. (2010). Inhibition of O-GlcNAcase in perfused rat hearts by NAG-thiazolines at the time of reperfusion is cardioprotective in an O-GlcNAc-dependent manner. *American Journal of Physiology Heart and Circulatory Physiology, 299*(5), H1715-1727.

Laher, I., Beam, J., Botta, A., Barendret, R., Sulistyoningrum, D., Devlin, A., et al. (2013). Short-term exercise worsens cardiac oxidative stress and fibrosis in 8-month-old db/db mice by depleting cardiac glutathione. *Free Radical Research, 47*(1), 44-54.

LaMonte, M. J., Blair, S., & Church, T. (2005). Physical activity and diabetes prevention. *Journal of Applied Physiology*, 9, 1205-1213.

Lee, D. Y., Lee, C., Lin, T., Lim, S., Zhou, J., Tseng, Y., et al. (2012). Role of histone deacetylases in transcription factor regulation and cell cycle modulation in endothelial cells in response to disturbed flow. *Proceedings of the National Academy of Science*, 1-6.

Leslie, E., Fotheringham, M., Veitch, J., & Owen, N. (2000). A university campus physical activity promotion program. *Health Promotion Journal of Australia*, 10, 51-54.

Li, J., Ichikawa, T., Villacorta, L., Janicki, J., Brower, G., Yamamoto, M., et al. (2009). Nrf2 protects against maladaptive cardiac response to hemodynamic stress. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 29, 1843-1850.

Li, J., Johnson, D., Calkins, M., Wright, L., Svendsen, C., & Johnson, J. (2005). Stabilization of Nrf2 by tBHQ confers protection against oxidative stress-induced cell death in human neural stem cells. *Toxicological Sciences*, 83, 313-328.

Livak, K. J., & Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods*, 25, 402-408.

Majumdar, G., Wright, J., Markowitz, P., Martinez-Hernandez, A., Raghow, R., & Solomon, S. (2004). Insulin stimulates and diabetes inhibits O-linked N-acetylglucosamine transferase and O-glycosylation of Sp1. *Diabetes, 53*(12), 3184-3192.

Matuzawa-Nagata, N., Takamura, T., Ando, H., Nakamura, S., Seiichiro, K., Misu, H., et al. (2008). Increased oxidative stress precedes the onset of high-fat diet–induced insulin resistance and obesity. *Metabolism, 57*(8), 1071-1077.

Medford, H. M., Porter, K., & Marsh, S. (2013). Immediate effects of a single exercise bout on protein O-GlcNAcylation and. *American Journal of Physiology Heart and Circulatory Physiology, 305*, H114-H123.

Mega, M., Cummings, J., Salloway, S., & Malloy, P. (1997). The limbic system: An anatomic, phylogenetic, and clinical perspective. *Journal of Neuropsychiatry*, 315-350.

Miao, F., Gonzalo, I., Lantin, L., & Natarajan, R. (2004). In vivo chromatin remodeling events leading to inflammatory gene transcription under diabetic conditions. *The Journal of Biological Chemistry*, 279, 18091-18097.

Moi, P., Chan, K., Asunis, I., Cao, A., & Kan, Y. (1995). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Science*, *91*(21), 9926-9930.

More, V., Wen, X., Thomas, P., Aleksunes, L., & Slitt, A. (2012). Severe diabetes and leptin resistance cause differential hepatic and renal transporter expression in mice. *Comparative Hepatology, 11*(1), 1-14.

Muthusamy, V. R., Kannan, S., Sadhaasivam, K., Gounder, S., Davidson, C., Boeheme, C., et al. (2012). Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium. *Free Radical Biology & Medicine*, 52, 366-376.

Myers, J. (2003). Exercise and cardiovascular health. *Circulation*, 107, e2-e5.

(2011). *National diabetes fact sheet: National estimates and general information on diabetes and prediabetes*. Centers for Disease Control and Prevention, U.S. Department of Health and Human Services, Atlanta.

Ngoh, G., Watson, L., Facundo, H., & Jones, S. (2011). Augmented O-GlcNAc signaling attenuates oxidative stress and calcium overload in cardiomyocytes. *Amino Acids, 40*(3), 895-911.

Nguyen, T., Sherratt, P., & Pickett, C. (2003). Regulatory mechanisms controlling gene expression mediated by the antioxidant reponse element. *Annual Review of Pharmacology and Toxicology,* 43, 233-260.

Ogihara, T., Asano, T., Katagiri, H., Sakoda, H., Anai, M., Shojima, N., et al. (2004). Oxidative stress induces insulin resistance by activating the nuclear factor-κB

pathway and disrupting normal subcellular distribution of phosphatidylinositol 3-kinase. *Diabetologia, 47*(5), 794-805.

Okada, S., Hiuge, A., Makino, H., Nagumo, A., Takaki, H., Konishi, H., et al. (2010). Effect of exercise intervention on endothelial function and incidence of cardiovascular disease in patients with type 2 diabetes. Journal of *Atheroscolerosis and Thrombosis, 17*(8), 828-833.

Osburn, W. O., & Kensler, T. (2008). Nrf2 signaling: An adaptive response pathway for protection against environmental toxic insults. *Mutation Research*, 659, 31-39.

Pally, R. (2007). The predicting brain: Unconscious repitition, conscious reflection and therapeutic change. *The International Journal of Psychoanalysis*, 88, 861-881.

Palsamy, P., & Subramanian, S. (2011). Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2–Keap1 signaling. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1812*(7), 719-731.

Piaget, J. (1997). *The moral judgment of the child.* New York: Free Press Paperbacks.

Powell, K. E., Thompson, P., Caspersen, C., & Kendrick, J. (1987). Physical activity and the incidence of coronary heart disease. *Annual Reviews of Public Health,* 8, 253-287.

Rabbani, N., Godfrey, L., Xue, M., Shaheen, F., Geoffrion, M., Milne, R., et al. (2011). Glycation of LDL by methylgloxal increases arterial atherogenicity. *Diabetes, 60*(7), 1973-1980.

Rahman, M., Stuchlick, O., El-Karim, E., Stuart, R., Kipreos, E., & Wells, L. (2010). Intracellular protein glycosylation modulates insulin mediated lifespan in C.elegans. *Aging, 2(*10), 678-690.

Reimer, J., Paolitto, D., & Hersh, R. (1983). Promoting moral growth: From Piaget to Kohlberg. Prospect Heights: Waveland Press.

Riboulet-Chavey, A., Pierron, A., Durand, I., Murdaca, J., Giudicelli, J., & Van Obberghen, E. (2006). Methylglyoxal impairs the insulin signaling pathways independently of the formation of intracellular reactive oxygen species. *Diabetes, 55*(5), 1289-1299.

Rocco, A. B., LeValley, J., Eldridge, J., Marsh, S., & Rodgers, B. (2014). A Novel protocol for assessing exercise performance and dystropathophysiology in the mdx mouse. *Muscle & Nerve*.

Rovniak, L. S., Anderson, E., Winett, R., & Stephens, R. (2002). Social cognitive determinants of physical activity in young adults: A prospective structural equation analysis. *Annals of Behavioral Medicine, 24*(2), 149-156.

Sallis, J. F., Nichols, J., Sarkin, J., Johnson, M., Caparosa, S., Thompson, S., et al. (1999). Evaluation of a university course to promote physical activity: Project GRAD*. Research Quarterly for Exercise and Sport, 70*(1), 1-10.

Satoh, H., Moriguchi, T., Takai, J., Ebina, M., & Yamamoyo, M. (2013). Nrf2 prevents initiation but accelerates progression through the Kras signaling pathway during lung carcinogenesis. *Cancer Research*, 73, 4158.

Saydah, S. H., Miret, M., Sung, J., Varas, C., Gause, D., & Brancati, F. (2001). Postchallenge hyperglycemia and mortality in a national sample of U.S. adults. *Diabetes Care*, 24, 1397-1402.

Sekhar, R. V., McKay, S. V., Patel, S. G., Guthikonda, A. P., Reddy, V. T., Balasubramanyam, A., et al. (2011). Glutathione synthesis is diminshed in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine. *Diabetes Care*, 34, 162-167.

Sharma, M., & Romas, J. (2008). Theoretical foundations of health education and health promotion. Sudbury, MA: Jones and Bartlett Publishers.

Sharoff, C. G., Hagobian, T., Malin, S., Chipkin, S., Yu, H., Hirshman, M., et al. (2010). Combining short-term metformin treatment and one bout of exercise does not increase insulin action in insulin-resistant individuals. *American Journal of Physiology, Endocrinology, and Metabolism*, 298, E815-E823.

Shaw, J., Sicree, R., & Zimmet, P. (2010). Global estimates of the prevlaence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice, 87*(1), 4-14.

Shen, X., Zheng, S., Metreveli, N., & Epstein, P. (2006). Protection of cardiac mitochondria by overexpression of MnSOD reduces diabetic cardiomyopathy. *Diabetes*, 55, 798-805.

Siewart, S., Gonzalez, I., Santillan, L., Lucero, R., Ojeda, M., & Gimenez, M. (2013). Downregulation of Nrf2 and HO-1 expression contributes to oxidative stress in type 2 diabetes mellitus: A study in Juana Koslay City, San Luis, Argentina. *Journal of Diabetes Mellitus, 3*(2).

Silva, M. S., Gomes, R., Ferreira, A., Freire, A., & Cordeiro, C. (2013). The glyoxalase pathway: The first hundred years . . . and beyond. *Biochemistry Journal*, 453, 1-15.

Smith, A., Ntoumanis, N., & Duda, J. (2007). Goal striving, goal attainment, and well-being: Adapting and testing the self-concordance model in sport. *Journal of Sport & Exercise Psychology, 29*(6), 763-782.

Sriwijitkamol, A., Coletta, D., Wajcberg, E., Balbontin, G., Reyna, S., Barrientes, J., et al. (2007). Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: A time-course dose-response study. *Diabetes, 56*(3), 836-848.

Standage, M., & Ryan, R. (2012). Self-determination theory and exercise motivation: Facilitating self-regulatory processes to support and maintain health and well-being. In G. C. Roberts, & D. Treasure, *Advances in Motivation in Sport and Exercise*. Champaign, IL: Human Kinetics.

Stoll, S. K., & Dieter, B. (2013). *Competing with honor: To dope or not to dope*. World Anti-Doping Agency.

Sun, Z., Zhang, S., Chan, J., & Zhang, D. (2007). Keap1 controls postinduction repression of the Nrf2-mediated antioxidant response by exporting nuclear export of Nrf2. *Molecular and Cellular Biology, 27*(18), 6334-6349.

Tan, Y., Ichikawa, T., Li, J., Si, Q., Yang, H., Chen, X., et al. (2011). Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo. *Diabetes*, 60, 625-633.

Tan, Y., Ichikawa, T., Li, J., Yang, H., Chen, X., Goldblatt, C., et al. (2011). Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo. *Diabetes*, 60, 625-633.

Tanasescu, M., Leitzmann, M., & Rimm, E. (2003). Physical activty in relation to cardiovascular disease and total mortality among men with type 2 diabetes. *Circulation*, 107, 2435-2439.

Tancredi, L. R. (2005). Hardwired Behavior. New York, NY: Cambridge University Press.

Theodore, M., Kawai, Y., Yang, J., Kleschenko, Y., Reddy, S., Villalta, F., et al. (2008). Multiple nuclear localization signals function in the nuclear import of the transcription factor Nrf2. *The Journal of Biological Chemistry*, 283, 8984-8994.

Uruno, A., Furusawa, Y., Yagishita, Y., Fukutomi, T., Muramatsu, H., Negishi, T., et al. (2013). The Keap1-Nrf2 system prevents onset of diabetes mellitus. Molecular and Cellulary Biology, *33*(15), 2996-3010.

Velmurugan, G., Sundaresan, N., Gupta, M., & White, C. (2013). Defective Nrf2-dependent redox signalling contributes to microvascular dysfunction in type 2 diabetes. Cardiovascular Research, *100*(1), 143-150.

Wang, B., Zhu, X., Kim, Y., Li, J., Huang, S., Saleem, S., et al. (2012). Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. Free Radical Biology & Medicine, 52, 928-936.

Watkins, S., Borthwick, G., & Arthur, H. (2011). The H9C2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro. In Vitro Cellular & Developmental Biology, *47*(2), 125-131.

Watson, L., Facundo, H., Ngoh, G., Ameen, M., Brainard, R., Lemma, K., et al. (2010). O-linked β-N-acetylglucosamine transferase is indispensable in the failing heart. Proceedings of the National Academy of Sciences, *107*(41), 17797-17802.

Yu, S., Khor, T. O., Cheung, K., Li, W., Wu, T., Huang, Y., et al. (2010). Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. *PLOS ONE, 5*(1), e8579.

Zhang, D. D. (2006). Mechanistic studies of the Nrf2-KEAP1 signaling pathway. Drug Metabolism Reviews, 38, 769-789.

Zheng, H., Whitman, S., Wu, W., Wondrak, G., Wong, P., Fang, D., et al. (2011). Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy. *Diabetes, 60*(11), 3055-3066.

Zhong, Q., Mishra, M., & Kowluru, R. (2013). Transcription factor Nrf2-mediated antioxidant defense system in the development of diabetic retinopathy. *Investigational Opthamology and Visual Science, 54*(6), 3941-3948.

Appendix A: Original Proposal

Chapter 1: Introduction

Background of the Problem

Lack of exercise and sedentary behavior are primary risk factors for the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Hu, Leitzmann, Stampfer, Colditz, Willet, & Rimm, 2001; LaMonte, Blair, & Church, 2005; Berlin & Colditz, 1990). Concurrently, increased levels of exercise reduce risk for development of T2DM and CVD (LaMonte, Blair, & Church, 2005; Kohl, Gordon, Villegas, & Blair, 1992; Berlin & Colditz, 1990). Furthermore, physical activity and exercise are effective therapeutic tools for reducing complications from these diseases and may reduce mortality rates (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000; Okada, et al., 2010; Boor, et al., 2009; Broderick, Poirier, & Gillis, 2005). Despite this evidence, only 65% of adults and 47% of college students in the United States meet the suggested physical activity guidelines, indicating that human behavior and lifestyle play a fundamental role in the increasing prevalence of T2DM. (State indicator report on physical activity, 2010; American College Health Association-National College Health Assessment II, 2009). Exercise and physical activity are a known necessity for optimal health, but that knowledge does not always generate behavior change. Education has always been argued as the means to alter behavior, but current educational models are not effective. Thus, there is a need to understand this aspect of physical activity behavior, and to develop more effective methods to improve knowledge of the benefits of exercise and behaviors regarding exercise and physical activity in young adult populations of the United

States. (Haskell, et al., 2006; American College Health Association-National College Health Assessment II, 2009).

Physical activity and exercise are effective therapeutic tools for reducing complications from these diseases and may decrease mortality rates (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000; Okada, et al., 2010; Boor, et al., 2009; Broderick, Poirier, & Gillis, 2005); however, the physiological mechanisms behind exercise attenuated mortality and morbidity rates in T2DM are not completely understood. Currently, the majority of medical interventions utilize pharmacological agents to treat T2DM and subsequent complications; however, there is increasing evidence that exercise provides the same benefits without the potential side-effects of current medication (Sharoff, et al., 2010). Thus, there is a need to add knowledge to the literature surrounding the physiology of diabetic complications and to provide sound evidence of how exercise reduces complications and mortality in diabetes.

Evidence suggests diabetes increases the risk of developing CVD by three-fold (Saydah, Miret, Sung, Varas, Gause, & Brancati, 2001). Currently, 8.3% of the American population has been clinically diagnosed with diabetes and it is estimated that over 35% of individuals over the age of 20 years have hyperglycemic conditions (National diabetes fact sheet, 2011; Cowie, et al., 2009). Cardiovascular disease (CVD) and complications from T2DM are among the leading causes of death in the United States (Hoyert & Xu, 2011). Given that the high prevalence of T2DM and the increased risk of comorbidities, it is abundantly clear that T2DM presents a major and escalating health concern for American society.

When taken together, both physiological and behavioral factors are central in the high prevalence of T2DM and related diseases. Therefore, the purpose of this dissertation is to conduct two separate studies to explore these two distinct, yet conceptually related facets of T2DM and to add to the literature on both aspects.

Setting of the Problem for Study 2

Diabetes affects 8.3% of the American population and increases the risk for associated complications including CVD and nephropathy (Cowie, et al., 2009). Exercise is an effective tool for reducing complications in diabetes and may reduce overall morbidity and mortality rates (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000). While benefits are well described in the literature, there is a lack of data describing physiological mechanisms for the therapeutic benefits of exercise in diabetes.

Oxidative and chemical stress is central to the development of diabetic complications and causes early cell death (Shen, Zheng, Metreveli, & Epstein, 2006; Kitada, Kume, Imaizumi, & Koya, 2011). A redox sensitive transcription factor, NF-E2–related factor 2 (Nrf2) and the Nrf2 antioxidant-response pathway is one of the major cellular defenses against the cytotoxic effects of oxidative stress (Zhong, Mishra, & Kowluru, 2013). The reduced activity of Nrf2 observed in humans and animals with diabetes has been suggested to have a causative effect in diabetic complications (Tan et al, 2011; Zhong et al., 2013), while evidence indicates that increasing Nrf2 activity in diabetic animals reduces complications (Zheng, et al., 2011). Furthermore, human and animal studies have demonstrated increases in Nrf2

activity following exercise (Cartoni, et al., 2005; Baar, et al., 2002); however, a mechanism has not been fully elucidated.

Diabetes is associated with increased O-linked N-acetylglucosamine (O-GlcNAc) modification of proteins (Clark et al., 2003). O-GlcNAc is a sugar moiety that can post-translationally modify serine/threonine residues of proteins (Ande, Moulik, & Mishra, 2009; Chou, Hart, & Dang, 1995). Post-translational modifications of proteins, including O-GlcNAcylation can induce protein conformational changes. Conformational changes of proteins can alter cell signaling and metabolic processes in the cell.

In addition to O-GlcNAcylation, proteins can also be modified by phosphorylation. Phosphorylation is the addition of a phosphate molecule to a protein. Like O-GlcNAcylation, phosphorylation also induces conformational changes of proteins and alters cell signaling and metabolic processes. Phosphorylation and O-GlcNAclyation can modify the same amino acid residues (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011). Thus, phosphorylation and O-GlcNAcylation can compete for post-translational modifications; when an amino acid residue is O-GlcNAcylated it cannot be phosphorylated and vice versa. When a protein is phosphorylated it displays different functions and results in different cellular processes than when it is O-GlcNAcylated (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011).

Phosphorylation is controlled by kinases and phosphatases. Briefly, phosphate groups are enzymatically added to proteins by kinases and removed by phosphatases. O-GlcNAclyation is also enzymatically regulated. The enzyme O-

GlcNAc transferase (OGT) adds O-GlcNAc moiety to proteins while O- GlcNAcase (OGA) removes O-GlcNAc. There are hundreds of kinases and phosphatases; however attachment and removal of O-GlcNAc are catalyzed by only two enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase, respectively (Haltiwanger, Holt, & Hart, 1990; Braidman, et al., 1974). This suggests two things: first that the O-GlcNAc modification plays a critical role in cell signaling, and secondly, O-GlcNAc modification is more responsive to perturbations in the cellular environment and has a more ubiquitous role in cell signaling than a single phosphorylation action. Thus O-GlcNAcylation is likely to play a essential role in Nrf2 signaling.

Acetylation is another post-translational modification in which of an acetyl group to a protein. Like phosphorylation and O-GlcNAcylation, acetylation can change the structure and function of a protein. Acetylation of Nrf2 within the nucleus increases Nrf2 binding to the DNA and transcription of Nrf2 regulated genes, whereas deacetylation of Nrf2 reduces Nrf2 binding to the DNA and decreases transcription of Nrf2 regulated genes. A class of enzymes known as histone deacetylases (HDAC) play an integral role of reducing Nrf2 transcription in pathological conditions by deacetylating Nrf2 (Yu, et al., 2010). Specifically, histone deacteylase 2 (HDAC 2) is a class I histone deacetylase complexes with Nrf2. When HDAC2 is phosphorylated, it complexes with Nrf2, and deacetylates it, preventing *in vivo* binding to the antioxidant response element in DNA, thereby reducing the ability of Nrf2 to function (Lee, et al., 2012). There is evidence that HDAC2 is O-GlcNAcylated (Medford, Porter, & Marsh, , 2013). Given that O-GlcNAcylation competes and often inhibits phosphorylation due to possible competition for binding

sites, or protein conformational changes caused by post translational modification, we hypothesize that O-GlcNAc opposes phosphorylation of HDAC2. Therefore, we propose that O-GlcNAc modification of HDAC2 will prevent phosphorylation of HDAC2 and reduce Nrf2:HDAC2 complexing. O-GlcNAc modification of HDAC2 would then improve Nrf2 signaling be preventing deacetylation of Nrf2 by HDAC2.

Research has previously shown that HDAC2 activity and phosphorylation is increased in diabetic mice (Cox & Marsh, 2013). Furthermore, there is evidence that OGT:HDAC2 complexing, is reduced in T2DM mice, and that exercise removes the difference. This evidence indicates that O-GlcNAcylation of HDAC2 is reduced in diabetic mice and that exercise increases GlcNAcylation of HDAC2. Therefore, we hypothesize that T2DM increases Nrf2:HDAC2 complexing and that exercise reduces Nrf2:HDAC2 complexing as a result of increased O-GlcNAcylation of HDAC2, thereby increasing Nrf2 activity.

Statement of the Problem

The purpose of this dissertation is to examine two unique, but related facets of diabetes: 1) to increase participant knowledge of the interaction of exercise and health and improve exercise and physical activity behaviors and 2) to use an animal model to understand how exercise prevents and attenuates T2DM and its complications at a molecular level.

Specific Aims.

1. To create an online educational intervention to increase participants' higher-level reasoning and psychological mediators of behavior change in regards to exercise in college aged individuals.

2. To utilize an online higher-level reasoning-based educational intervention to effectively increase participants' physical activity and exercise behavior in college aged individuals.
3. To determine whether diabetes increases Nrf2:HDAC2 complexing and whether 8 weeks of aerobic exercise reduces Nrf2:HDAC2 complexing as a result of O-GlcNAcylation of HDAC2 in the skeletal muscle of a type 2 diabetic mouse model.

Hypotheses

1. **Pedagogy Study**
    a. An online higher-level reasoning-based intervention utilizing social cognitive theory will improve higher-level reasoning and psychological mediators of behavior change regarding physical activity and exercise in college-aged adults.
    b. An online higher-level reasoning program will improve exercise and physical activity behavior in college-aged adults.
2. **Physiology Study**
    a. Mice with diabetes will show decreased Nrf-2 content and 8 weeks of aerobic exercise will increase Nrf-2 content in the skeletal muscle of diabetic mice.
    b. Mice with diabetes will show increased skeletal muscle Nrf2:HDAC2 complexing and 8 weeks of aerobic exercise will reduce Nrf2:HDAC2 complexing in the skeletal muscle of diabetic mice.

c. 8 weeks of aerobic exercise will increase O-GlcNAcylation of HDAC2 in skeletal muscle of mice with diabetes, thereby reducing Nrf2:HDAC2 complexing.

Variables

3. **Pedagogy Study**
    a. **Independent Variables:** Higher-level reasoning-based Social Cognitive Theory intervention/ control, gender
    b. **Dependent Variables**: Measures of SCT related to physical activity (self-efficacy, self-regulation, goal-setting), levels of physical activity, and reasoning related to physical activity.
4. **Physiology Studies**
    a. **Independent Variables:** Exercise/non-exercise, diabetic/non-diabetic.
    b. **Dependent Variables:** HDAC2 levels, Nrf2 protein levels, O-GlcNAc of Nrf2 protein and HDAC2 proteins, and Nrf2:HDAC2 complexing.

Delimitations

3. Students for the intervention group will be those enrolled in an online physical activity and health-based course. There will be no statistically accurate sampling procedure for this intervention.
4. Measurement of HDAC2, Nrf2 and O-GlcNAc in exercised tissue is highly invasive; thus, human subjects will not be used to collect the physiological data. Instead, diabetic mouse models will be used.

Limitations

3. Students for the intervention group will not be randomly selected. Therefore, the population may not be an accurate representation of the population.
4. All physiological data regarding hypotheses 1 and 2 will be derived from animal models. As a result this imposes some limitations in extrapolation to humans.

Definitions

- Cardiovascular disease – disease of the heart or blood vessels.
- Histone deacetylase – a class of enzymes that remove acetyl groups from lysine amino acids on histones and other proteins.
- Type 1 diabetes mellitus (T1DM) – a form of diabetes mellitus that results from autoimmune destruction of the insulin producing beta cells in the islet of Langerhans in the pancreas.
- Type 2 diabetes mellitus (T2DM) – a form of diabetes mellitus that arises from the development of peripheral insulin resistance and the subsequent reduction in pancreatic function. T2DM is characterized by hyperglycemia, hyperlipidemia, insulin resistance, and/or insulin deficiency.
- Oxidative stress – an imbalance between the production of prooxidants (reactive oxygen/nitrogen species) and antioxidants.
- Reactive oxygen/nitrogen species – chemically reactive molecules containing oxygen or nitrogen. Examples include: superoxide radical

($\bullet O_2^-$), hydroxyl radical (HO•), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^-$).

- Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) – Nrf2 is a basic leucine zipper (bZIP) transcription factor featuring a Cap ‘‘n’’ collar (CNC) structure and is the key transcription factor regulating the antioxidant response.
- Glycation – a non-enzymatic reaction involving covalent bonding of a sugar with a protein or lipid.
- Advanced Glycation End Products (AGEs) – the end result of a chain of chemical reactions beginning with glycation. AGES are causal in multiple pathologies and are elevated in diabetes.
- Glutathione (GSH) – a tripeptide of glutamate, cysteine, and glycine. GSH is an endogenous antioxidant responsible for maintaining proper redox status *in vivo.*
- γ-glutamyl cysteine ligase-catalytic (GCLC) – The initial enzyme in the GSH biosynthesis pathway that catalyses the condensation of cysteine and glutamate to form gamma-glutamylcysteine.
- γ-glutamyl cysteine ligase-modulatory (GCLM) – an enzyme involved in GSH biosynthesis that catalyzes the efficiency of GCLC.
- Glomeruli – a network of capillaries located at the beginning of the nephron in the kidney involved in filtering blood.
- HepG2 Cells – an *in vitro* cell model that allows for examination of hepatic (liver) cell function.

- Small interfering RNA (siRNA) – interferes with the expression of specific genes through complementary nucleotide sequencing. siRNA is used to effectively silence gene expression by destroying mRNA and preventing protein translation.
- Cardiac Hypertrophy – thickening of the heart muscle, specifically of the ventricular walls.
- Myocardial fibrosis – excess fibrous connective tissue in the myocardium form in response to cardiac injury or insult.
- Kelch-like ECH-associated protein 1 (Keap1) – cytosolic inhibitor of Nrf2.
- Ubiquitylation/ubiquitination – enzymatic post-translation modification process where a ubiqutuin protein is attached to a substrate, effectively marking it for degradation in the proteasome.
- NIH 3T3 – standard fibroblast cell line that enables *in vitro* analysis of fibroblasts.
- Phosphorylation – a post-translation modification in which a phosphate group is added to a molecule, altering its function. Phosphorylation plays a major role in determining cellular function and processes.
- Acetylation – a post-translational modification in which an acetyl group is added onto a molecular compound.
- O-linked attachment of β-N acetyl-glucosamine (O-GlcNAc) – a post-translational modification in which O-GlcNAc is added onto a molecular compound. O-GlcNAclyation has been implicated in the pathogenesis of diabetic conditions.

- Streptozotocin (STZ) – a toxic chemical that induces dysfunction of pancreatic beta cells. Large doses induce T1DM and is used to produce animal models of T1DM.
- Social Cognitive Theory (SCT) – Social cognitive theory (SCT) posits that human behavior can be explained by behavior, environmental factors, and personal factors.
- Self-efficacy –defined as the confidence a person has in his or her ability to pursue a behavior, it is behavior-specific and a function of the present.
- Goal setting –developing plans to accomplish chosen behaviors.
- Self-regulation – the sense that self-corrective adjustments are taking place as needed to stay on track toward achieving the purpose, and the sense that the corrective adjustments originate within the person.

Chapter 2: Review of the Literature

Epidemiology of Cardiovascular Disease and Diabetes

Cardiovascular disease (CVD) is the leading cause of death in the United States, accounting for 25% of all deaths in 2008 (Hoyert & Xu, 2011; Heron, 2008). Evidence suggests diabetes increases the risk of developing CVD three-fold and 25% of individuals with T2DM will develop other vascular complications, such as nephropathy, within 10 years of diagnosis (Saydah, Miret, Sung, Varas, Gause, & Brancati, 2001; Alder, Stevens, Manley, Bilous, Cull, & Holman, 2003). It is estimated the worldwide prevalence of T2DM amongst adults (aged 20-79 years) is 285 million and will reach 439 million by the year 2030 (Shaw, Sicree, & Zimmet, 2010). As of 2009, 8.3% of the American population has been clinically diagnosed with diabetes and it is estimated over 40% of individuals over the age of 20 years have hyperglycemic conditions (Cowie, et al., 2009; National diabetes fact sheet: National estimates and general information on diabetes and prediabetes, 2011). Furthermore, an estimated $113 billion was spend on diabetes-related health care in 2009 (Huang, Basu, O'Grady, & Capretta, 2009). Epidemiological evidence clearly indicates that CVD and T2DM present a major health and financial concern. (National diabetes fact sheet: National estimates and general information on diabetes and prediabetes, 2011).

Study 2: Physiological Mechanisms of Exercise and Diabetes

Chemical stress, including oxidative stress in skeletal muscle, has been implicated in the development of T2DM and progression of diabetic complications, and causes premature cell death (Ogihara T. , et al., 2004; Matuzawa-Nagata, et al.,

2008; Kowluru, Kowluru, Xiong, & Ho, 2006; DeRubertis, Craven, & Melhem, 2007). The redox sensitive transcription factor, NF-E2–related factor 2 (Nrf2) and the Nrf2 antioxidant response element pathway is one of the major cellular defenses against the cytotoxic effects of oxidative stress (Zhong, Mishra, & Kowluru, 2013). Reduced activity of Nrf2 observed in T2DM has been suggested to have a causative effect in diabetic complications (Tan et al, 2011; Zhong et al., 2013), while evidence indicates that increasing Nrf2 activity in diabetic animals reduces complications (Zheng, et al., 2011).

Nrf2 is increased in response to the production of reactive oxygen species in acute hyperglycemia; however in T2DM we see decreased activity, suggesting the chronic hyperglycemia present in diabetes results in down regulation of Nrf2 (He, Kan, Cai, & Ma, 2009). It has been shown that diminished Nrf2 activity present in patients and subjects with T2DM contributes to increased oxidative stress, endothelial dysfunction, insulin resistance, and increased cardiac insult (Tan, et al., 2011; Cheng, Slow, & Mann, 2011). Overexpression of Nrf2 and pharmacologically enhanced activation of Nrf2 reduces oxidative stress and diabetic complications, including cardiovascular complications and diabetic nephropathy (Cheng, Cheng, Chiou, & Chang, 2012; Hsu, Lee, Li, Hsu, & Pan, 2013). Together, evidence highlights the importance of the Nrf2 pathway in T2DM and its subsequent complications.

Properties of Nrf2.

Nrf2 protects cells and tissues from a variety of endogenous and exogenous toxicants (Jaramillo & Zhang, 2013). The Nrf2 pathway is the major regulator of

cytoprotective responses, is expressed in all tissues of the human body, and is essential in maintaining cellular homeostasis. Research regarding the role of Nrf2 in disease is burgeoning and evidence has indicated Nrf2 plays a central role in the development of a variety of diseases including, T2DM, CVD, and cancer (Uruno, et al., 2013; Satoh, Moriguchi, Takai, Ebina, & Yamamoyo, 2013).

Nrf2 is a cap ‘n’ collar (CNC) basic-region leucine zipper transcription factor that provides cells the ability to adapt to oxidative stress and electrophiles by mediating the induction of the cytoprotective genes (Hayes & Ashford, 2012). These genes include the enzymes involved in glutathione (GSH) biosynthesis [i.e. γ-glutamyl cysteine ligase-catalytic (GCLC) and γ-glutamyl cysteine ligase-modulatory (GCLM), and phase II detoxifying enzymes such as heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), and superoxide dismutase (SOD)] (Osburn & Kensler, 2008). Induction of these genes requires interaction of the Nrf2 transcription factor with the antioxidant response element (ARE) (Nguyen, Sherratt, & Pickett, 2003) (Figure 3).

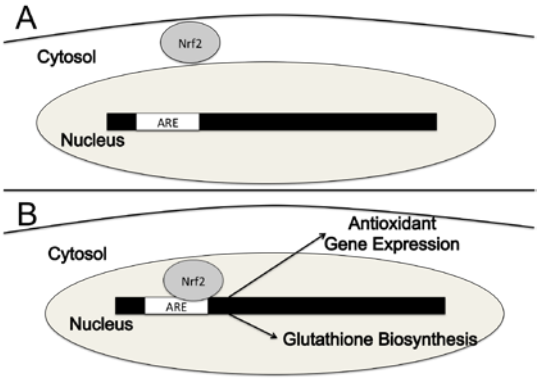

Figure 3. Nuclear translocation of Nrf2 and antioxidant gene expression.

Nrf2 and Vascular Complications.

Diabetes increases vascular complications, such as nephropathy, neuropathy, and retinopathy. Diabetic retinopathy leads to approximately 10,000 new cases of blindness each year, and diabetic nephropathy is the leading cause of renal failure in the United States (Fong, Aiello, Ferris, & Klein, 2004; Association, 2004). There is increasing evidence that Nrf2 is critical in the development of these vascular complications in diabetes (Jiang, Huang, Lin, Zhang, Fang, & Zhang, 2010; Zheng, et al., 2011).

Nrf2 coordinates the endogenous antioxidant system (Uruno, et al., 2013). Given that oxidative stress is causal in diabetic nephropathy, and Nrf2 activity is decreased in T2DM it is likely that Nrf2 plays a central role in diabetic nephropathy. In support of this, examination of glomeruli of patients with diabetic nephropathy revealed that diabetic patients are under severe oxidative stress and the Nrf2-mediated antioxidant response is significantly different than non-diabetic controls. Jiang et al. (2010) also demonstrated that Nrf2 deficient ($Nrf2^{-/-}$) diabetic mice experience greater levels of oxidative stress and suffer greater levels of renal damage than diabetic mice with normal Nrf2 function.

In addition to reactive oxygen species, another cellular toxicant, methylglyoxal (MGO), is elevated in diabetes and is considered a major contributor to the development of insulin resistance and diabetic complications (Silva, Gomes, Ferreira, Freire, & Cordeiro, 2013; Rabbani, et al., 2011; Kilhovd, et al., 2003; Riboulet-Chavey, Pierron, Durand, Murdaca, Giudicelli, & Van Obberghen, 2006). MGO is involved in the pathogenesis of diabetic complications through a process

known as glycation. Briefly, MGO reacts with intracellular and extracellular proteins and nucleic acids to form advanced glycation end-products which damage vascular tissue in a similar manner to oxidative stress (Berlanga, et al., 2005). There is evidence that Nrf2 is involved in MGO-dependent vascular complications in diabetes through its role in MGO detoxification.

MGO detoxification is the process by which cells convert cytotoxic MGO to pyruvate, which can then be metabolized for energy. This process occurs via the GSH-dependent glyoxalase pathway (Figure 5) (Thornalley, 1998). Nrf2 activation results in transcription of GSH biosynthesis enzymes; thus, increased Nrf2 activation may reduce MGO accumulation by increasing availability of the rate limiting substrate, GSH. In support of this, pharmacological upregulation of Nrf2 by reseveratrol in human liver cells reduced MGO accumulation and attenuated MGO-induced insulin resistance (Cheng, Cheng, Chiou, & Chang, 2012). Cheng et al. (2012) also demonstrated depletion of Nrf2, using silencing RNA (si-RNA), resulted in reduced MGO detoxification (Cheng et al., 2012). Furthermore, MGO, MGO-derived advanced glycation end products, and tissue damage from intraperitoneal injections of MGO into mice pancreas was attenuated when the Nrf2 pathway was concomitantly activated pharmacologically (Hsu, Lee, Li, Hsu, & Pan, 2013). Individuals with uncontrolled T2DM have diminished GSH status; thus impaired GSH biosynthesis and status may play a major role in MGO-related diabetic complications (Sekhar, et al., 2011). When taken together, these data suggest increasing Nrf2 activity results in the attenuation of MGO accumulation, MGO-derived AGE

formation, and tissue damage, ultimately reducing vascular complications associated with T2DM.

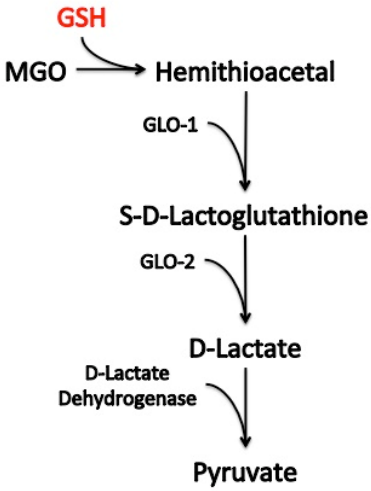

Figure 4. The glyoxalase pathway and methylglyoxal detoxification.

Nrf2 and Pathological Cardiac Hypertrophy.

Hypertension is a hallmark symptom of diabetes and leads to pathological hypertrophy of the myocardium and cardiac dysfunction (Grossman, Shemesh, Shamiss, Thaler, Carroll, & Rosenthal, 1992). Furthermore, there is increased pathological hypertrophy in the diabetic heart independent of pressure overload; suggesting additional mechanisms are responsible for the increased hypertrophy (Grossman, Shemesh, Shamiss, Thaler, Carroll, & Rosenthal, 1992). Recent evidence suggests in the presence of oxidative stress, Nrf2 is involved in modulating hypertrophic signaling in the heart and may be a central role in regulating diabetic cardiomyopathy (Li, et al., 2009; Bai, et al., 2013).

Li et al. (2009) subjected wild type ($Nrf2^{+/+}$) and Nrf2 deficient ($Nrf2^{-/-}$) mice to two weeks of hypertension by transverse aortic constriction. Hypertension caused

Nrf2 expression to transiently increase and then declined to basal levels (Li et al., 2009). Both wild type and Nrf2 deficient mice showed signs of pathological cardiac remodeling; however, $Nrf2^{-/-}$ mice experienced significantly greatly cardiac hypertrophy, myocardial fibrosis, overt heart failure and increased mortality than $Nrf2^{+/+}$ mice when exposed to hypertension (Li, et al., 2009). Furthermore, Li et al. (2009) demonstrated that over expression of Nrf2 in rat neonatal cardiac myocytes and fibroblasts significantly inhibited hypertrophic factor–induced reactive oxygen species production and growth in both cardiomyocytes and cardiac fibroblasts, whereas knockdown of Nrf2 exerted opposite effects in both cells.

Increasing Nrf2 expression and transcription of Nrf2 regulated genes has been shown to prevent diabetic cardiomyopathy in mice (Bai, et al., 2013). Bai et al. (2013) treated diabetic mice with sulforaphane, a pharmacological Nrf2 activator, and measured blood and cardiac function. The authors found that mice treated with sulforaphane significantly increased Nrf2 activity and protein expression of Nrf2-regulated antioxidants. The increased Nrf2 activity prevented diabetes-induced cardiac hypertrophy and fibrosis, and almost eliminated diabetes induced oxidative damage in the myocardium. Additionally, in rat heart cells, silencing Nrf2 with siRNA abolished the sulforaphane's prevention of high glucose-induced fibrotic response.

When considered together, the findings of Li et al. (2009) and Bai et al. (2013), suggest Nrf2 is a key mediator in pathological cardiac hypertrophy and heart failure in response to hypertension and oxidative stress. This novel interaction between diabetes-related cardiomyopathy and Nrf2 could provide insight into individual susceptibility to diabetic complications in the cardiovascular system and therefore

should be investigated carefully (Howden, 2013). Furthermore, the exact mechanisms through which Nrf2-regulated signaling exerts its cardio-protective effects are not well understood and further exploration of Nrf-2 regulated signaling may provide a better understanding of the mechanisms regulating pathological cardiac hypertrophy and myocardial damage in the diabetic heart.

Exercise increases Nrf2 activation and antioxidant gene expression.

Nrf2 activation occurs when it is release from its cytosolic inhibitor, Keap1, and expression of Nrf2 regulated antioxidant genes occurs when Nrf2 binds to the antioxidant response element of DNA. Recent evidence from animal model research indicates that exercise results in increased Nrf2 activation and expression of Nrf2 regulated genes (Baar, et al., 2002; Gounder, et al., 2012; Muthusamy, et al., 2012). Baar et al. (2002) reported a 56% increase in Nrf-2 expression in skeletal muscle of mice after an acute bout of swimming. Similarly, two consecutive days of 90 minutes/day of treadmill exercise increased Nrf2 protein expression roughly 25% and 100% in young and old rat heart muscle respectively (Gounder, et al., 2012). Muthusamy et al. (2012) found that acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in mouse heart muscle. Utilizing $Nrf2^{-/-}$ Muthusamy et al. (2012) also demonstrated that disruption of Nrf2 increases the heart to oxidative stress and that the beneficial effects of exercise on cardiac antioxidant mechanisms are dependent on Nrf2 Function.

In addition to the benefits of acute exercise on Nrf2 function, chronic, moderate, intensity exercise proves to be effective in increasing Nrf2 activation in rats (Ashgar, George, & Lokhandwala, 2007). Ashgar et al. (2007) demonstrated that exercising

sixty minutes a day, five days a week for six weeks on a treadmill has been shown to increase nuclear levels of Nrf2 50% in rat models. Gounder et al. (2012) also found that impaired Nrf2 signaling was associated with GSH depletion and oxidative stress and that moderate exercise improved Nrf2 signaling, GSH levels and reduced oxidative stress. There is also evidence that acute and chronic exercise in humans leads to upregulation of the Nrf2 pathway (Cartoni, et al., 2005). Cartoni et al. (19) found Nrf2 levels increased 5-fold 24 hours post exercise in skeletal muscle cells of trained male cyclists after an acute bout of cycling exercise.

There is clear evidence that decreased Nrf2 activity is causal in diabetic complications and that exercise increases Nrf2 activity; however, no studies to date have examined the effect of exercise on Nrf2 activity in diabetes. Furthermore, there is no research to elucidate a mechanism by which exercise improves Nrf2 activity and expression of Nrf2 associated genes in T2DM. Therefore, the primary purpose of these studies is to examine the effect of exercise on signaling mechanisms of Nrf2 activity in cardiac and skeletal muscle in animal model of diabetes.

Regulation of Nrf2.

Concentration and activity of Nrf2 are regulated at several levels, including degradation, translocation, post-translational modification, and translation. Nrf2 is a transcription factor, and as such, nuclear translocation from the cytosol is required in order for it to interact with DNA and encode cytoprotective genes. Nrf2 is located in the cytosol by a negative regulator Kelch-like ECH-associated protein 1 (Keap1). In normal, unstressed cells, Nrf2 protein is rapidly turned over in a Keap1-dependent manner through Cul3-Rbx1 ubuitylation and proteasomal degradation (Sun, Zhang,

Chan, & Zhang, 2007; Eggier, Small, Hannink, & Mesecar, 2009) (Figure 6A). When cells are exposed to oxidative stress, electrophiles, or chemopreventive agents, Nrf2 in the cell escapes Keap1-mediated repression, translocates to the nucleus, and activates antioxidant responsive element-dependent gene expression to maintain cellular redox homeostasis (Zhang D. D., 2006) (Figure 6B). Given that diabetes results in increased oxidative stress, it is unlikely the reduced Nrf2 activity in diabetes is due to increased inhibition by Keap1. Therefore, we suggest that T2DM reduces Nrf2 activity at the level of transcription, in the nucleus.

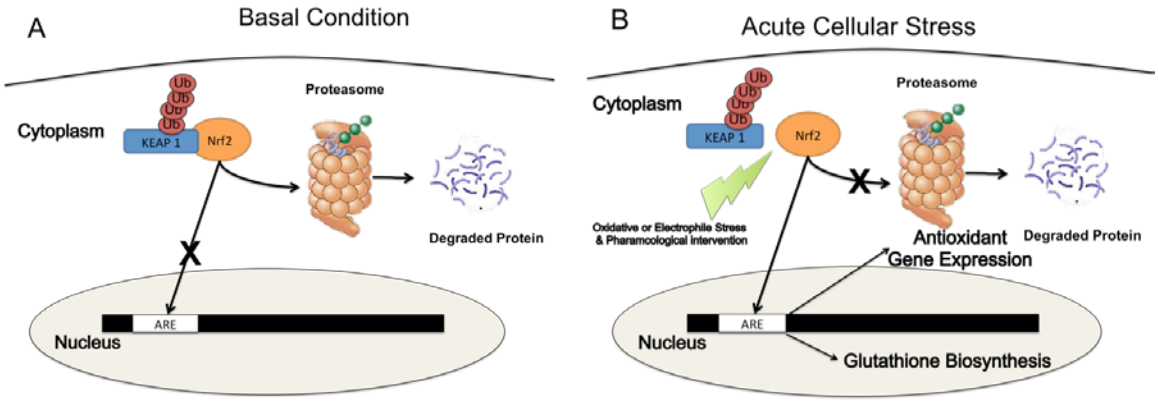

Figure 5. Regulation of Nrf2

Acetylation of Nrf2 and Gene Expression.

Regulation of Nrf2 gene transcription is controlled by other post-translational modifications, specifically acetylation. After Nrf2 disassociates from Keap1 under stressed conditions, it translocates to the nucleus. Once Nrf2 arrives in the nucleus it is acetylated, which results in increased gene transcription (Bannister & Miska, 2000). Evidence indicates that acetylation of Nrf2 in human colon cells increases binding of Nrf2 to DNA and increases transcription of Nrf2 induced genes, and that

acetylation of Nrf2 occurs within the nucleus and has no effect on basal Nrf2 protein stability in human mammary cells (Sun, Chin, & Zhang, 2009). Sun et al. (2009) demonstrated that acetylation induces promoter-specific DNA binding of Nrf2, with acetylation directly influencing the Nrf2 regulated enzyme glutamate-cysteine ligase, the first rate-limiting enzyme of glutathione synthesis. In support of the findings of Sun et al. (2009), acetylation conditions in human hepatocytes cells resulted in increased nuclear localization of Nrf2 (Kawai, Garduno, Theodore, Yang, & Arinze, 2011). Kawai et al. (2011) also demonstrated that deacetylation conditions increased cytoplasmic rather than nuclear localization of Nrf2 (Kawai, Garduno, Theodore, Yang, & Arinze, 2011). Together, evidence indicates nuclear acetylation of Nrf2 results in binding to the antioxidant response element and gene transcription, and that deacetylation disengages it from the antioxidant response element, thereby resulting in transcriptional termination and subsequently in its nuclear export and degradation (see Figure 7). Therefore, increasing acetylation of Nrf2 increases expression while deacetylation decreases expression of Nrf2 regulated genes.

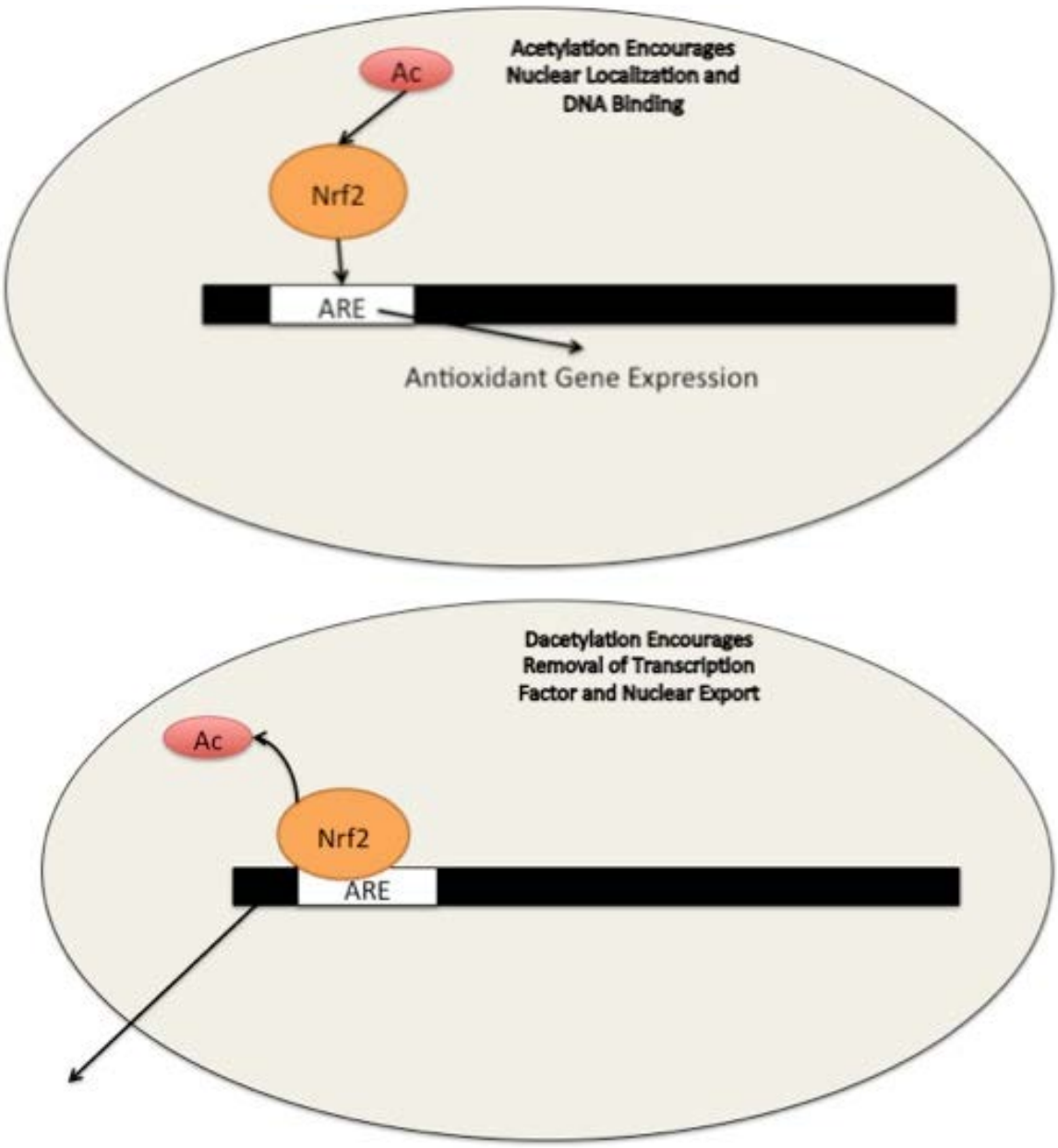

Figure 6. Effect of acetylation and deacetylation on Nrf2 transcription.

Acetylation and deacetylation of transcription factors is regulated through enzymatic reactions. For example, histone deacteylases (HDAC) are a class of enzymes that deacetylate transcription factors and other proteins involved in DNA transcription. HDAC 2 is a class I histone deacetylase that is phosphorylated and

has been shown to form a complex with Nrf2. When Nrf2 complexes with phosphorylated HDAC2, Nrf2 is deacetylated, preventing *in vivo* binding to the antioxidant response element in DNA (Lee, et al., 2012) (see Figure 8). Preventing Nrf2 deacetylation by inhibiting HDAC2 activity with trichostatin A increased Nrf2 binding to antioxidant response elements (Wang, et al., 2012), suggesting HDAC activity plays an important role in regulating Nrf2 activity.

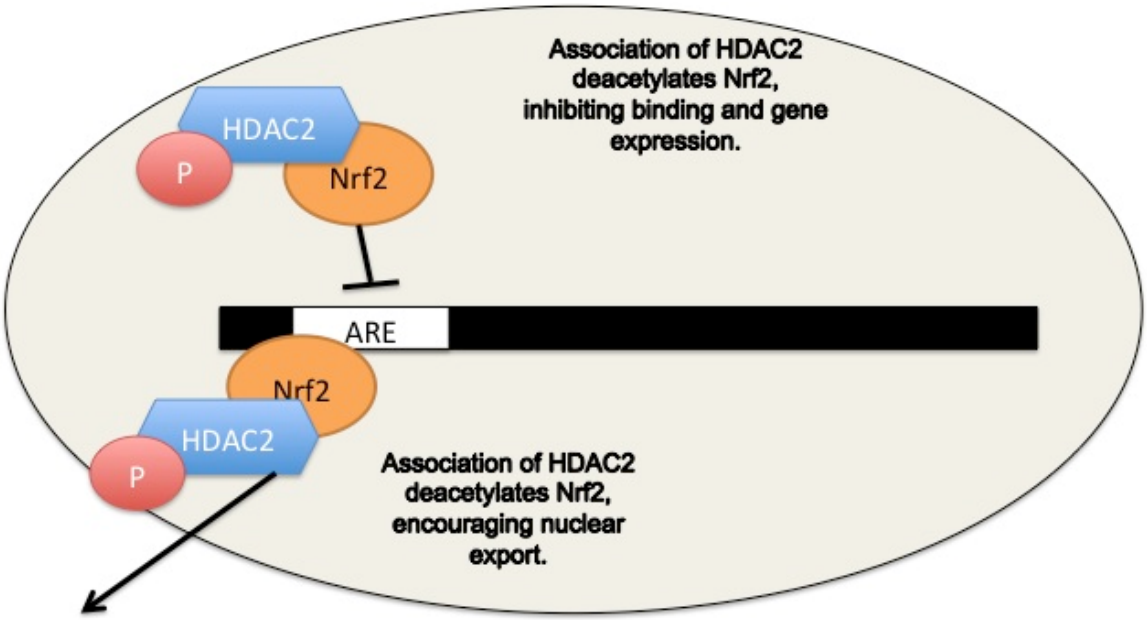

Figure 7. Role of HDAC2:Nrf2 association on Nrf2 activity.

Nrf2 and HDAC signaling in Diabetes

Nrf2 activity is down regulated in diabetes and the Keap1-Nrf2 system has been shown to prevent T2DM (Uruno, et al., 2013). This evidence indicates that Nrf2 signaling is dysregulated in diabetes; however the underlying mechanisms are not well understood. One possible mechanism is increased association of HDAC2:Nrf2. As previously mentioned, HDAC2 is phosphorylated and complexes with Nrf2 to inhibit binding and reduce Nrf2 activity (Lee et al., 2012). There is evidence that

HDAC2 activity and phosphorylation of HDAC2 is increased in diabetic mice (Cox & Marsh, 2013). Therefore, we propose that diabetes increases HDAC2:Nrf2 complexing, thereby reducing Nrf2 activity.

Exercise reduces Nrf2:HDAC2 complexing

Currently there is no literature examining the effect of exercise on Nrf2:HDAC2 complexing in diabetes; however, there is evidence that mimicking the hemodynamic environment produced by exercise in cell culture decreases HDAC2 content and phosphorylation, increases Nrf2 acetylation, and transcription of Nrf2 regulated genes (Lee, et al., 2012). This suggests that exercise may reduce Nrf2:HDAC2 complexing; however, there is no published research to support this, nor has a possible mechanism been described. Therefore, a central aim of this study is to establish a potential mechanism for the effect of exercise on Nrf2:HDAC2 complexing in skeletal muscle.

O-GlcNAc modification of proteins and diabetes.

O-GlcNAc is a sugar moiety that can post-translationally modify serine/threonine residues of proteins (Ande, Moulik, & Mishra, 2009; Chou, Hart, & Dang, 1995). Post-translational modifications of proteins, including O-GlcNAcylation can induce protein conformational changes. These changes can alter cell signaling and metabolic processes in the cell. Changes in O-GlcNacylation of proteins has been shown to be involved in the pathogenesis of insulin-resistance and hypertrophic signaling in the diabetic heart (Vosseller, Wells, Lane, & Hart, 2002; Marsh, Dell'Italia, & Chatham, 2011). Furthermore, exercise has been shown to return the O-GlcNAc modifications of proteins in diabetic mice to the same levels as

healthy controls (Cox & Marsh, 2013). Therefore, exercise may improve Nrf2 signaling by changing O-GlcNAc modification of Nrf2 or proteins involved in the Nrf2 signaling pathway.

Exercise reduces Nrf2:HDAC2 complexing through O-GlcNAcylation.

As previously mentioned, Nrf2 activity is regulated by post-translational modifications. O-GlcNAcylation is a post-translational modification that can induce protein conformational changes and is analogous to phosphorylation. O-GlcNAclyation and phosphorylation can modify serine and threonine residues and that cross talk between O-GlcNAcylation and phosphorylation is extensive (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011). Thus, there is competition between O-GlcNAcylation and phosphorylation for serine and threonine residues (Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011). Furthermore, O-GlcNAcylation of a protein may induce conformation changes of the proteins structure such that phosphorylation of distant amino acid residues is no longer possible. There is evidence that HDAC2 is O-GlcNAcylated (Medford, Porter, & Marsh, 2013). Due to possible competition for binding sites, or protein conformational changes caused by post-translational modification, it is likely that that O-GlcNAc opposes phosphorylation of HDAC2. Thus, it is likely that O-GlcNAcylation may play a role in the nuclear regulation of Nrf2 and the rate and level of transcription.

The enzyme O-GlcNAc transferase (OGT) adds O-GlcNAc moiety to proteins while O- GlcNAcase (OGA) removes O-GlcNAc. Cox and Marsh (2013) have demonstrated that OGT:HDAC2 association is reduced in diabetes, suggesting decreased O-GlcNAcylation of HDAC2. Cox and Marsh (2013) have also

demonstrated that phosphorylation of HDAC2 is increased in diabetes. As previously mentioned, phosphorylated HDAC2 associates with Nrf2, deacetylates it, and reduces transcription. Conisdering that O-GlcNAcylation and phosphorylation can have opposite effects on signaling mechanisms, and they display a reciprocal relationship in diabetes, we propose that Nrf2:HDAC2 complexing is increased in T2DM. Furthermore, we propose that O-GlcNAc modification of HDAC2 will prevent Nrf2:HDAC2 association, thereby allowing Nrf2 to bind to the ARE and induce gene expression (see Figure 9).

Exercise has been shown to increase O-GlcNAcylation of HDAC2 in the diabetic heart (Cox & Marsh, 2013). In their study, Cox and Marsh (2013) showed that OGT:HDAC2 association was reduced in diabetic mice, indicating reduced O-GlcNAcylation of HDAC2 and that four weeks of exercise increased levels of OGT:HDAC2 in the such that they were no longer significantly different than the health control mice. This evidence suggests that exercise increases O-GlcNAcylation of HDAC2. Based upon this body of evidence, we hypothesize that exercise increases Nrf2 activity in diabetes by increasing O'GlcNAcylation of HDAC2, thereby reducing Nrf2:HDAC2 complexing in skeletal muscle.

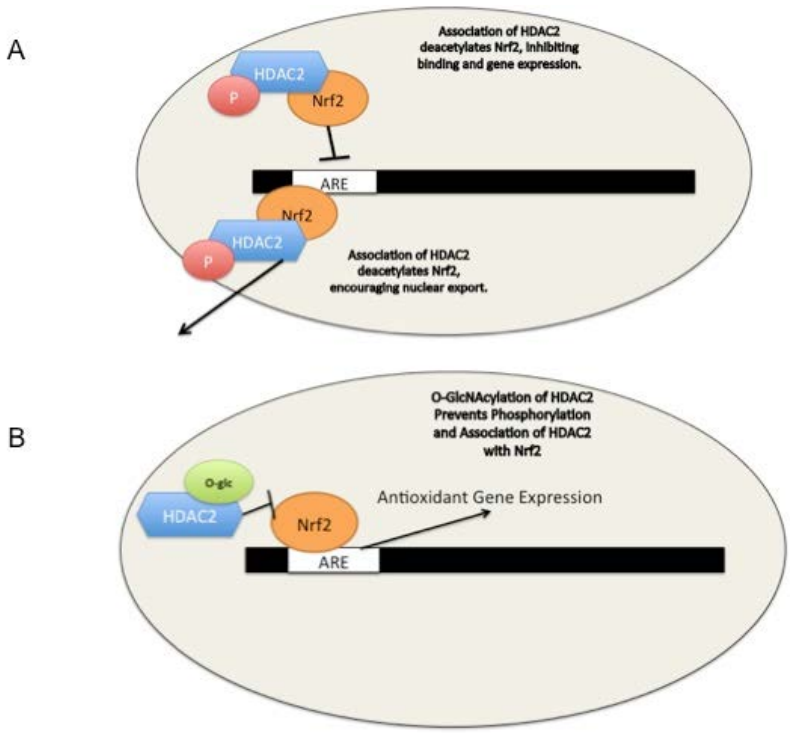

Figure 8. O-GlcNacylation of HDAC2 prevents phosphorylation and association of HDAC2 with Nrf2.

Summary.

Cardiovascular disease (CVD) and complications from T2DM are among the leading causes of death in the United States (Hoyert & Xu, 2011). Currently, 8.3of the American population has been clinically diagnosed with diabetes and it is estimated that over 40% of individuals over the age of 20 have hyperglycemic conditions (Cowie, et al., 2009; National diabetes fact sheet, 2011). Evidence suggests diabetes increases the risk of CVD by three-fold (Saydah, Miret, Sung, Varas, Gause, & Brancati, 2001). The high prevalence and mortality rates of CVD and T2DM present a major challenge to our health-care system.

Lack of physical activity is a risk factor for the development of obesity, T2DM, and CVD (Hu et al., 2001; LaMonte et al., 2005; Berlin & Colditz, 1990). Furthermore, physical activity and exercise are effective therapeutic tools in reducing complications from these disease, including attenuating advanced glycation end-product formation and attenuating pathological cardiac hypertrophy in the diabetic heart (Tanasescu, Leitzmann, & Rimm, 2003; Hambrecht, et al., 2000; Okada, et al., 2010; Boor, et al., 2009; Broderick, Poirier, & Gillis, 2005). Currently, only 65% of adults and 47% of college students in the United States meet the suggested physical activity guidelines, highlighting the need to develop programs aimed at increasing physical activity amongst the adult and young adult populations of the United States (State indicator report on physical activity, 2010; American College Health Association-National College Health Assessment II, 2009). Behaviors such as physical activity are a skill set. They are acquired over repeated practice sessions and over a long time, informed by consciously articulated principles and reasons but otherwise ‘second-natured’ into the cognitive unconscious” (Damasio, 2010, p. 287). This suggests the formal process of reasoning is required to establish behavioral patterns. Additionally, human behavior can be explained as a triadic reciprocal causation: behavior, environmental factors, and personal factors (i.e. cognitions, affect, and biological events) (Sharma & Romas, 2008). Therefore, the implementation of a reasoning-based educational intervention designed to improve the determinants of human behavior may indeed result in improved physical activity amongst young adult populations.

Exercise has been shown to be effective tool for reducing complications in T2DM. Despite the well documented benefits of exercise, there is a lack of data describing physiological mechanisms for the therapeutic benefits of exercise in T2DM. Oxidative and chemical stress is central to the development of diabetic complications and causes early cell death (Shen, Zheng, Metreveli, & Epstein, 2006; Kitada, Kume, Imaizumi, & Koya, 2011). A redox sensitive transcription factor, NF-E2–related factor 2 (Nrf2) and the Nrf2 antioxidant response pathway is one of the major cellular defenses against the cytotoxic effects of oxidative stress (Zhong, Mishra, & Kowluru, 2013). The Nrf2 pathway is downregulated in T2DM (Tan, et al., Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo, 2011) and plays a role in development diabetic complications including nephropathy and pathological cardiac hypertrophy (Li, et al., 2009). The exact mechanisms regarding reduced basal activity of Nrf2 in diabetes are not well understood. Recent research has shown that the acetylation posttranslation modification of Nrf2 significantly impacts its activity (Sun et al., 2009; Wang et al., 2012). HDAC2 is phosphorylated and associates with Nrf2 to deacetylate it and inhibit transcription (Wang, et al., 2012). In addition to phoshorylation, O-GlcNAcylation can induce protein conformational changes and O-GlcNAclyating and phosphorylation regulate each other. Work from the Marsh lab has shown that HDAC2 is O-GlcNAcylated (Medford, Porter, & Marsh, 2013). Due to possible competition for binding sites, or protein conformational changes caused by post translational modification, it is likely that that O-GlcNAc opposes phosphorylation of HDAC2. The Marsh lab has also previously shown that

HDAC2 activity and phosphorylation is increased in diabetic mice (Cox & Marsh, 2013). This suggests that HDAC2:Nrf2 association may be increased in T2DM, resulting in the decreased activity demonstrated by Tan et al. (2011). Furthermore, OGT:HDAC2 complexing is reduced in diabetic (db/db) mice, but exercise exercise removed the difference (Cox & Marsh, 2013). Therefore, we hypothesize that diabetes will inrease Nrf2:HDAC2 complexing and that exercise will reduce Nrf2:HDAC2 complexing as a result of increased O-GlcNAcylation of HDAC2 in skeletal muscle.

Chapter 3: Methods

The purpose of this dissertation is two examine two unique, but related facets of diabetes: 1) to increase participant knowledge of the interaction of exercise and health and improve exercise and physical activity behaviors and 2) to use an animal model to understand how exercise prevents and attenuates diabetes and its complications at a molecular level.

Methods for Study 1

Human participants.

Participants will be recruited from a northwest university. The participants will be between the ages of 18 and 22 years and will be physically capable of engaging in physical activity. The study was approved by the University of Idaho Institutional Review Board (exempt status number 13-215) and participants will be informed of any possible risk and discomfort associated with the experimental procedure prior to signing an informed consent form. See Appendix for IRB approval and consent form.

SCT variables of physical activity.

Participants will complete demographic information, physical activity, and the Health Beliefs Survey (HBS) questionnaires to assess SCT variables of physical activity, requiring about 35 minutes. The HBS will be completed during the first week of the intervention and then again upon completion of the intervention. The survey has been used in previous SCT based exercise intervention research and has shown to be valid and reliable in young adults aged 18-25 years (Anderson, Winett, Wojcik, & Williams, 2010; Anderson-Bill, Suppini, & Apap, 2011; Rovniak, Anderson,

Winett, & Stephens, 2002). The survey can be found in its entirety in appendix A. Briefly, the Cronbach Alpha values of the self-efficacy, self-regulation, and goal-setting subscales are 0.95, 0.85, and 0.91, respectively.

Physical Activity Data.

The official long form English version of the International Physical Activity Questionnaire (IPAQ) will be used. Dinger, Behrens, and Han (2006) examined the validity and reliability of the long form English IPAQ using pedometers and accelerometers. Dinger et al. (2006) found that the IPAQ was significantly correlated ($r=0.30-0.47$, $p<0.01$) with steps/day from both the accelerometer and pedometer and had an intraclass correlation coefficients ranging from 0.71-0.89, indicating moderate to high reliability in college students.

The questionnaire consists of 27 questions that cover 4 domains of physical activity: work, transport, domestic and garden, and leisure-time. The questions in IPAQ are designed to provide domain-specific scores for walking, moderate-intensity, and vigorous-intensity activity. The multiple domains of the IPAQ permit analysis of physical activity levels from different aspects of physical activity. All questions refer to the previous 7 days, thus the results are reflective of a limited window of time. Results are presented as an estimation of energy expenditure in metabolic equivalent-minutes per week (MET hours/week). The IPAQ will be scored based as instructed in the IPAQ instrument.

Qualitative data.

In order to capture the reasoning process the responses of the participants will be recorded and analyzed for themes and to explore development of the

participants' reasoning. Initial analysis will be conducted through immersion into the data as described in Pope, Zieband, and Mays (2000). The data will then be examined for themes and coded and categories into major themes. Coding and categorizing of the data will be conducted as described in Glaser and Strauss (2012) and Bradley, Curry, and Devers (2007). Hierarchical analysis of the data will then be conducted in order to find relationships between the participant's responses and the constructs of SCT. This will be achieved by segregate responses under SCT constructs and examining which experiences and/or perspectives influence the specific constructs. Upon completion of theme establishment, development of the coding structure, and hierarchical analysis, diagrams will be constructed to explain relationships between perspectives, experiences, emotions, and SCT constructs. Assessing reasoning process will be conducted by an expert in the reasoning field.

Educational intervention.

The educational intervention will take place during a 16-week online course and consisted of 6 distinct modules. Module 1 establishes foundational knowledge about the relationship between physical activity and health. It will utilize voiced-over video lectures discussing the implications of sedentary behavior and disease and the role physical activity plays in health. Module 1 requires students to complete the HBS and engage in online discussion about the importance of physical activity in health and to reflect upon how this information might inform their future lifestyle habits.

Module 2 through 3 will be aimed at improving self-efficacy in physical activity and nutritional habits. The content includes voiced-over video lectures aimed at developing the knowledge and skills necessary to improve physical activity by

providing information regarding different exercise modalities, structuring exercise programs, and resources on how and where to learn more. Modules 2 through 3 will require participants to submit questions they have regarding how to improve their self-efficacy in regard to physical activity as well as their nutritional habits. These questions will be answered using a podcast format in which the student will be able to listen to. The participants will also be required to right a reflective paper in which they discuss the tools and skills they acquired in modules 2 and 3.The reflective paper is based upon a higher level of reasoning as noted by Reimer, Paolitto, and Hersh (1983). This is designed to increase information retention and allow the participants to realize the skills they have garnered from the modules.

Module 4 will be aimed at increasing self-regulation. The participants will read case-studies highlighting the importance of self-regulation and articles that provide information on improving self-regulation. Participants will be required to write a reflective paper discussing what they learned about the importance and application of self-regulation in regards to physical activity.

Module 5 will improve goal-setting skills in the participants. The module will include a lecture on the key points of goal setting and how to apply them to physical activity. The participants will be required to develop specific physical activity goals (e.g. how will you choose to be physically active, what form of exercise will you partake in, how will you structure your physical activity, how will you maintain your health through exercise?).

Module 6 will provide the participants to reflect upon the knowledge and skills they gain from the course and apply them to physical activity in the context of health.

The participants will engage in an online discussion in which they will be required to discuss what they learned from the intervention, how their self-efficacy, goal-setting, and self-regulation improved in regards to physical activity.

Data analysis.

One-way analysis of variance (ANOVA) will be used to examine differences in physical activity and SCT variables over time and to test for differences between gender. Appropriate post-hoc tests will be conducted in the presence of significance. Significance for all measures of this study will be set at $\alpha=.05$.

Methods for Study 2

Animal care and facilities.

The procedures in this study will follow the guidelines of the Washington State University Institutional Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85–23, revised 1996). 8-week-old type 2 diabetic mice (B6.BKS(D)-*Leprdb*/J, *db+/db+* (*db*) and age-matched C57BL/6J non-diabetic lean background strain controls (C57) will be purchased from Jackson Laboratories (Bar Harbor, ME). To control for activity, mice will be singly housed without environmental enrichment in a climate-controlled vivarium on a 12:12 light:dark cycle. Mice will consume water and standard chow ad libitum, except for one overnight fast per week prior to blood glucose measurement.

Experimental groups.

Both type 2 diabetic mice *db/db* and age-matched C57BL/6J non-diabetic mice will be randomized to either drug treatment of vehicle control groups. These groups

will then be randomized into either exercise protocol or sedentary control. The experimental groups can be seen visually in Figure 10.

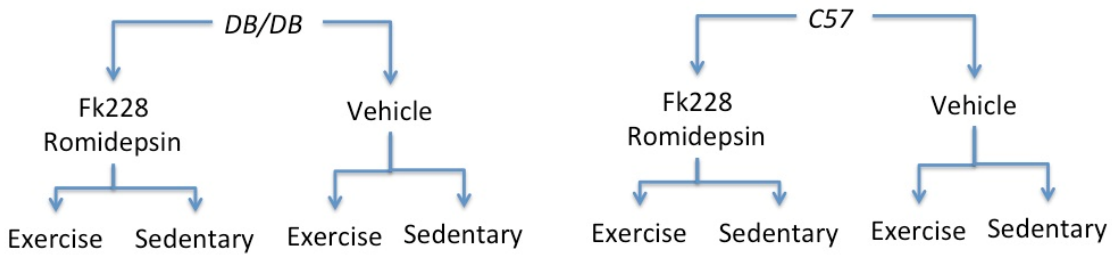

Figure 9. Experimental Groups

VO_2 Max Testing

In order to determine accurate relative workload for each group *db/db* and C57 mice will undergo a VO_2 max test. The VO_2 test was completed as previously described (Rocco, LeValley, Eldridge, Marsh, & Rodgers, 2014) with one change for the *db/db* mice. The grade was reduced to 5% for the *db/db* mice to ensure the mice reached maximal oxygen consumption prior to volitional exhaustion. The mice were sacrificed immediately following the test, tissue was harvested from the soleus muscle, and frozen in liquid nitrogen and in 4% paraformaldehyde for later analysis.

Exercise protocol.

Mice from each group, C57 and *db/db*, will be randomized to either exercise or sedentary groups and housed in groups of no more than 5 mice per cage. All mice will undergo an initial accommodation period of one week which will comprise 5 days of sitting on the treadmill with the belt turned off for 10 minutes, followed by slow walking at 0.5 m/min for 20 minutes. Exercise intensity will be determined by

maximal oxygen consumption test (VO_2 max) and matched for each subject. Based on the results of this VO2 protocol, mice in the exercise groups will be trained 5 days a per week at a workload corresponding to 60-70% of VO2 max for a duration of 20, 30, 40, 50, and 60 minutes during weeks 1, 2, 3, 4, and 5-8, respectively. This exercise intensity corresponds to the clinical guidelines for humans with diabetes and will therefore provide the most appropriate data for human disease (Colberg et al., 2010)

Drug treatment.

C57 and *db/db* mice will also be randomized into groups receiving either Fk228 Romidepsin or a saline vehicle. Fk228 Romidepsin is a selective and potent HDAC1/2 inhibitor (Furumai, et al., 2002). Both vehicle and drug will be delivered using intraperitoneal injections. A pilot study will be conducted to determine the minimal effective dosage and confirm drug efficacy on HDAC2 activity. 0.5 μg/g and 2.5μ/g have been shown to be effective dosages in in recent literature (Kosugi, et al., 2001; Nishida, et al., 2004).

Tissue harvesting.

Following an overnight fast, mice will decapitated and blood glucose will be measured in neck blood. Skeletal muscle from the soleus will be excised and stored in 4% paraformaldehyde and snap-frozen in liquid nitrogen, and stored at −80°C.

Nrf2 and HDAC2 protein analysis.

Western blotting.

Tissue will be homogenized in Tissue Protein Extraction Reagent (Sigma-Aldrich, St. Louis, MO); 20 mM sodium fluoride; 1 mM sodium orthovanadate; 3%

protease inhibitor cocktail (Sigma); and 0.02% PUGNAc, an OGA inhibitor (Sigma), to inhibit O-GlcNAc removal from proteins. Total protein will be quantified with a modified Lowry assay (BioRad, Hercules, CA). Proteins will be separated by SDS-PAGE and transferred onto PVDF membranes, which will be probed overnight at 4°C with primary NRF2 antibody ((D1Z9C) XP® Rabbit mAb #12721, Cell Signaling, MA) and HDAC2 antibody (Rabbit mAB #2545, Cell Signaling, MA), then probed with the appropriate secondary antibodies for 1 hour at room temperature (see Table X for antibody details). Chemiluminescent substrates (Thermo Fisher Scientific, Rockford, IL) will be used to detect horseradish peroxidase activity on a ChemiDoc (BioRad). Protein levels will be quantified on duplicate blots with standard densitometry using ImageJ software (National Institutes of Health, Bethesda, MD), and normalized to the loading control calsequestrin.

O-GlcNac of HDAC2 and Nrf2 and HDAC2:Nrf2 association.

Immunoprecipitation.

Skeletal muscle tissue will be homogenized in Tissue Protein Extraction Reagent, 1% phosphatase inhibitor, 2% protease inhibitor (Sigma), and 0.02% PUGNAc, an inhibitor of O-GlcNAc removal. Lysates will be assayed for total protein as described for Western blotting. Samples will be diluted to equal protein concentrations and precleared over protein A/G agarose beads (Thermo Fisher Scientific) at 4°C for 4 hours. Precleared supernatants will then be added to 25 ul of beads that will be been incubated with primary HDAC2 and Nrf2 antibody for 4 hours at 4°C. IP will be performed overnight at 4°C; beads will then be washed and eluted for 5 min at at 100°C. Eluents will then be assayed for co-immunoprecipitated

proteins using immunoblotting. The positive control will be skeletal muscle lysate and negative control will skeletal muscle lysate that will be immunoprecipitated without antibody. Co-immunoprecipitated proteins with then be probed with either O-GlcNAc, HDAC2, or Nrf2 antibody. will then normalized to the level of captured target protein for analysis.

Immunohistochemistry.

Immunohistochemistry (IHC) will be used to examine the effect of O-GlcNAclyation of HDAC2 on Nrf2 nuclear translocation. IHC will be performed as previously described (Tan, et al., Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo, 2011). Briefly, skeletal muscle tissue will be harvested and perfusion fixed with 4% paraformaldehyde, stored in 70% ethanol and then paraffin embedded and section at 5 μm and mouted on slides. Slides will be deparaffinized in xylene, rehydrated in ethanol, and blocked with 5% goat serum in 1% bovine serum overnight at 4°C; appropriate secondary antibodies will be conjugated to either Alexa Fluor 488 (green) or 594 (red) (Invitrogen, Carlsbad CA) to visualize specific proteins; nuclei will be identified using 4’,6-diamidino-2-phenylindole (DAPI). Image acquisition will be performed using an epiflourescence microscope.

Data analysis.

Based upon the experimental design of this study, a randomized factorial design will be used for statistical analysis. All physiological data will be tested for normality using a Shapiro-Wilks test and appropriate transformations will be made. Kruskal-Wallis tests will be used in cases where data is resistant to transformation.

One-way ANOVAs will be used to tests difference between groups for the following dependent variables: Nrf2 protein expression, O-GlcNAc of Nrf2, O-GlcNAc of HDAC2, Nrf2:HDAC2 complexing. Necessary post-hoc analysis will be conducted on all significant results. Significance for all measures of this study will be set at $\alpha=.05$.

Appendix B: IRB Forms

University of Idaho
Office of Research Assurances (ORA)
Institutional Review Board (IRB)
875 Perimeter Drive, MS 3010
Moscow ID 83844-3010

Phone: 208-885-6162
Fax: 208-885-5752
irb@uidaho.edu

September 5, 2013

To: Sharon Stoll
Cc: Brad Dieter

From: IRB, University of Idaho Institutional Review Board

Subject: Exempt Certification for IRB project number 13-215

Determination: August 30, 2013
Certified as Exempt under category 2 at 45 CFR 46.101(b)(2)
IRB project number 13-215: Assessment of Nutritional Knowledge and Attitudes of College Students and Athletes

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This study may be conducted according to the protocol described in the Application without further review by the IRB. As specific instruments are developed, each should be forwarded to the ORA, in order to allow the IRB to maintain current records. Every effort should be made to ensure that the project is conducted in a manner consistent with the three fundamental principles identified in the Belmont Report: respect for persons; beneficence; and justice.

It is important to note that certification of exemption is NOT approval by the IRB. Do not include the statement that the UI IRB has reviewed and approved the study for human subject participation. Remove all statements of IRB Approval and IRB contact information from study materials that will be disseminated to participants. Instead please indicate, "The University of Idaho Institutional Review Board has Certified this project as Exempt."

Certification of exemption is not to be construed as authorization to recruit participants or conduct research in schools or other institutions, including on Native Reserved lands or within Native Institutions, which have their own policies that require approvals before Human Subjects Research Projects can begin. This authorization must be obtained from the appropriate Tribal Government (or equivalent) and/or Institutional Administration. This may include independent review by a tribal or institutional IRB or equivalent. It is the investigator's responsibility to obtain all such necessary approvals and provide copies of these approvals to ORA, in order to allow the IRB to maintain current records.

This certification is valid only for the study protocol as it was submitted to the ORA. Studies certified as Exempt are not subject to continuing review (this Certification does not expire). If any changes are made to the study protocol, you must submit the changes to the ORA for determination that the study remains Exempt before implementing the changes. The IRB Modification Request Form is available online at: http://www.uidaho.edu/ora/committees/irb/irbforms

Certificate of Completion

The National Institutes of Health (NIH) Office of Extramural Research certifies that **Brad Dieter** successfully completed the NIH Web-based training course "Protecting Human Research Participants".

Date of completion: 09/28/2011

Certification Number: 774429

Appendix C: IACUC Approval

Subject: IACUC Approval for Amendment to add personnel to ASAF# 04142-009

Body:

TO: Susan Marsh

FROM: Rani Muthukrishnan, for Stephen A. Greene, Chair, The Institutional Animal Care and Use Committee, IACUC

DATE: 11/14/2013

TITLE: Exercise, O-GlcNAc and the diabetic heart

ASAF: 04142-009

The Animal Subjects Protocol Amendment to, #04142-009, to add the following personnel--

Dieter, Brad

has been approved on 11/14/2013.

If you have any other changes before the protocol's annual review is due, please inform the IACUC by submitting an amendment to the IACUC Program Coordinator at the Office of Research Assurances (campus zip 3005).

All IACUC approved protocols and amendments are subject to Post Approval Review. Contact Gary Turner (335-8043) to schedule one.

If you have any questions please contact the Program Coordinator listed below.

Thank you,

Rani Muthukrishnan, PhD.
IACUC Program Coordinator
Institutional Animal Care and Use Committee (IACUC)
Office of Research Assurances
Washington State University
Pullman, WA 99164
Phone: 509-335-7951
Fax: 509-335-6410
Email: rani_m@wsu.edu

Notification Details

Subject: IACUC Approval for Amendment to add personnel to ASAF# 03900-012

Body:

TO: Susan Marsh

FROM: Rani Muthukrishnan, for Stephen A. Greene, Chair, The Institutional Animal Care and Use Committee, IACUC

DATE: 11/14/2013

TITLE: O-GlcNAc and exercise training

ASAF: 03900-012

The Animal Subjects Protocol Amendment to, #03900-012, to add the following personnel--

Dieter, Brad

has been approved on 11/8/2013.

If you have any other changes before the protocol's annual review is due, please inform the IACUC by submitting an amendment to the IACUC Program Coordinator at the Office of Research Assurances (campus zip 3005).

All IACUC approved protocols and amendments are subject to Post Approval Review. Contact Gary Turner (335-8043) to schedule one.

If you have any questions please contact the Program Coordinator listed below.

Thank you,

Rani Muthukrishnan, PhD.
IACUC Program Coordinator
Institutional Animal Care and Use Committee (IACUC)
Office of Research Assurances
Washington State University
Pullman, WA 99164
Phone: 509-335-7951
Fax: 509-335-6410
Email: rani_m@wsu.edu

Source: IACUC - Sub Activity

Priority: Med

Appendix D: Surveys and Questionnaires

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the

prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

Yes ☐

No ☐ → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ **days per week**

No vigorous job-related physical activity ☐ → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

_____ **days per week**

No moderate job-related physical activity ☐ → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

No job-related walking ☐ → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

No traveling in a motor vehicle ☐ → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

No bicycling from place to place ☐ → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

No walking from place to place ☐ → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

No vigorous activity in garden or yard ☐ → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

No moderate activity in garden or yard ☐ → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

No moderate activity inside home ☐ → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

No walking in leisure time ☐ → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

No vigorous activity in leisure time ☐ → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

No moderate activity in leisure time ☐ → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Physical Activity Beliefs
Strategies

Please, tell us what strategies you have you used in the past 3 months to successfully walk or do other exercise.

Use this scale to tell us how often in the past month you did the following:

1	2	3	4	5
Never	Seldom	Occasionally	Often	Repeatedly

In the past month how often did you:	How Often (1-5)
1. Set aside time each day to walk or do other exercise?	
2. Make a plan to walk or do other exercise?	
3. Keep or make a new plan based on how well you were doing with your walking or other exercise?	
4. Set a goal for the number of days you walked or exercised each week?	
5. Keep track of how many steps you take each day?	
6. Keep track of the number of days you walked or exercised each week?	
7. Keep track of how long your walks or exercise sessions were?	
8. Plan to walk or exercise 5 days a week?	
9. Plan to make your walking or exercise sessions a little longer?	
10. Set goals for how long your walking or exercise sessions will be?	
11. Plan your walking or other exercise sessions so they are enjoyable?	
12. Get together with someone else to walk or do other exercise?	
13. Keep track of how much you enjoy your walking or other exercise?	
14. Keep track of how fast you walked or how hard you did other exercise?	

Physical Activity Beliefs
Social Support

What do the members of your family do and think about walking or other exercise? We just want your opinion even if you are not sure.

Use this scale to tell us if you agree with the following statements:

1 Strongly Disagree — **2** — **3** — **4** — **5** Strongly Agree

The members of my family …	Agree or Disagree 1-5
1. make time to walk or do other exercise.	
2. set goals to walk or exercise.	
3. plan to walk or do other exercise.	
4. exercise or walke most days of the week.	
5. make their walks or other exercise as enjoyable as possible.	
6. keep track of their walking or other exercise.	
7. keep or make new plans based on how well they are doing with their walking or other exercise.	
8. set goals to walk faster or exercise longer.	

Physical Activity Beliefs
Social Support

What do your closest friends do and think about walking or other exercise? We just want your opinion even if you are not sure.

Use this scale to tell us if you agree with the following statements:

1	2	3	4	5
Strongly Disagree				Strongly Agree

My closest friends …	Agree or Disagree 1-5
1. make time to walk or do other exercise.	
2. set goals to walk or exercise.	
3. plan to walk or do other exercise.	
4. exercise or walke most days of the week.	
5. make their walks or other exercise as enjoyable as possible.	
6. keep track of their walking or other exercise.	
7. keep or make new plans based on how well they are doing with their walking or other exercise.	
8. set goals to walk faster or exercise longer.	

Physical Activity Beliefs
Self Efficacy

Use any number from 0 to 100 on the following scale to tell how certain you are that you can – all or most of the time:

0	----------	50	----------	100
Certain I CAN NOT		Somewhat certain I can		Certain I CAN

How certain are you that you can …	How certain? (0-100)
1. get up early during the week to walk or do other exercise?	
2. get together with someone else to walk or do other exercise?	
3. walk most days of the week?	
4. keep track of when and how long you walk or do other exercise?	
5. go to social events or fun activities only after reaching your waking goal?	
6. take small breaks during the day to take a walk or do other exercise?	
7. begin walking again if you miss a day or two?	
8. increase the enjoyment of your walks or other exercise?	
9. make a plan to walk or do other exercise?	
10. plan your walks or other exercise so you will enjoy them?	
11. each week, increase how long you walk or do other exercise?	
12. find a place to walk during bad weather?	
13. change your normal routine to get in a walk or do other exercise?	
14. stay up later to make time for taking a walk or do other exercise?	

Physical Activity Beliefs
Self Efficacy

Use any number from 0 to 100 on the following scale to tell how certain you are that you can – all or most of the time:

0	----------	50	----------	100
Certain I CAN NOT		Somewhat certain I can		Certain I CAN

How certain are you that you can walk or do other exercise when …	
15. you are feeling stressed?	
16. you are tired?	
17. your family wants more time?	
18. your muscles might be a little sore?	
19. you get busy at work?	
20. you have social activities?	
21. you have chores or errands to do?	
22. you need a babysitter to do so?	
23. you are feeling depressed?	

Physical Activity Beliefs
Self Efficacy

These questions ask about what you expect will happen *if you were take a walk or do other exercise most days of the week.* They also ask about how much it would matter to you for these things to happen.

Use this scale to tell us if you agree the following will happen:

1	2	3	4	5
Strongly Disagree				Strongly Agree

Use this scale to tell us how much it will matter:

1	2	3	4	5
It will not matter at all				It will matter very much

If I slowly and steadily build up to walking or doing other exercise most days of the week, I expect I will ...	Do you agree? (1-5)	Will it matter? (1-5)
1. decrease my chance of becoming ill or disabled.		
2. have to give up some of my normal activities.		
3. have to take more time than usual to plan my day.		
4. have one more thing to worry about getting done.		
6. not have enough time for other things I want to do.		
6. have to change my normal routine.		
7. sleep better.		
8. have less time to spend with my family.		
9. have less time to spend with my friends.		
10. fit into my clothes better.		
11. manage my weight better.		
12. feel less stress.		

Physical Activity Beliefs
Self Efficacy

Use this scale to tell us if you agree the following will happen:

1	2	3	4	5
Strongly Disagree				Strongly Agree

Use this scale to tell us how much it will matter:

1	2	3	4	5
It will not matter at all				It will matter very much

If I slowly and steadily build up to walking or doing other exercise most days of the week, I expect I will ...	Do you agree? (1-5)	Will it matter? (1-5)
13. be less irritable.		
14. enjoy it		
15. feel bored.		
16. dislike it.		
17. find it pleasurable		
18. be no fun at all		
19. be very energizing		
20. make me feel depressed.		
21. be happier.		
22. feel good physically		
23. feel very invigorated		
24. be frustrated.		
25. be gratified.		
26. feel exhilarated.		

Physical Activity Beliefs
Self Efficacy

Use this scale to tell us if you agree the following will happen:

1	2	3	4	5
Strongly Disagree				Strongly Agree

Use this scale to tell us how much it will matter:

1	2	3	4	5
It will not matter at all				It will matter very much

If I slowly and steadily build up to walking or doing other exercise most days of the week, I expect I will ...	Do you agree? (1-5)	Will it matter? (1-5)
27. feel a strong sense of accomplishment.		
28. not want to do anything else.		
29. be very absorbed by it.		
30. feel refreshed.		

Appendix D: Student Writing Samples

Early Writing Samples

Student 1.

Physical Activity

Introduction- In this paper we will discuss physical activity as a whole. The different types such as aerobic or anaerobic. Amounts in a scale of minutes in a session or day. Intensity as in how strenuous physical activity needs to be. Finally benefit in a sense of how physical activity can improve your daily life and long term health as a whole. We also will explore the health costs and benefits of an active life versus a sedentary lifestyle.

Before we get into amount or intensity of physical activity, lets first look at the different types of activity. The four types are endurance or aerobic, strength or anaerobic, balance, and flexibility. One of the two main types of physical activity is aerobic. Aerobic exercise is doing things like running long distance, jogging, fast walking, biking, hiking or swimming. This type of exercise improves the health of the heart, lungs, and circulatory system. Keeping these parts of the body strong will help fight off diseases such as heart disease and diabetes that usually develop as an individual gets older.

The second major type of activity is anaerobic. This type of activity is doing things like weight lifting, resistance running, or resistance bands. This type of exercise is meant to strengthen muscles. Anaerobic activates are meant to work muscles instead of the respiratory system because they use energy systems that do not use much oxygen due to the capacity of the respiratory system. It is important to

know the difference in the two different kinds of physical activity because both are needed to maintain the body's many systems and functions.

Taking what we now know in the difference between aerobic and anaerobic activity, we can look at the intensity of each kind of physical activity and how that affects the quality and amount of the particular activity. When looking at an overview of physical activity most people know that 150 to 300 minutes of moderate to vigorous activity is recommended per week. What most people do not know is if there is a smaller amount of time that they can put in so they can get the same or close results from a health standpoint. That being said one cannot make up for lifting extremely light weights for longer instead of lifting a challenging weight that will break down and build muscles stronger.

Intensity is an important part of every physical activity, because if you do anything at a slow enough pace then it will not do anything beneficial for your body. This means that depending on every individual's age, weight, history, current physical shape, etc. That they know what is a light activity compared to a very hard activity. As I said before, it varies from person to person on what is "difficult" or "easy". However this is a very important part of physical activity in gaging how long the exercise should be for.

Knowing the amount of each activity is also very important in knowing how many times a day or more popular a week. In our reading of "Physical Activity for Health: What Kind? How Much? How Intense? On Top of What?" it states that the desired amount for the majority of Americans that do not reach the required 150-300 minutes is simply a "some is better than nothing" approach. Although this sounds

like a defeatist attitude two thirds of Americans do not even reach the recommended amount of 150-300 minutes of moderate to vigorous activity. With these facts to be evident the approach of “some is better than none” is as good as it gets. In knowing these facts we can postulate that if the two thirds of Americans did do just a little bit more than they would see a massive improvement of health from a preventative standpoint as far as heart and circulatory system diseases go.

Now how does this help us as athletes when we stop being physically active for the sake of competition? The two most important things to know is first the connection between physical activity as a whole, keeping in mind the intensity, frequency, type, and amount and overall physical health. The second is the rate of which sedentary individuals try to become active too fast and sustain injuries as the result.

The most important I believe to focus on is how crucial getting 300+ minutes of moderate to vigorous physical activity is in disease prevention and overall physical health. Getting the recommended amount of activity is not just for maintaining a healthy weight. The benefits to this amount of physical activity is that it will help prevent heart disease, stroke, high blood pressure, diabetes, breast and colon cancer, and some other injuries. Not only can it help prevent getting major diseases such as this but getting the recommended amount will prevent depression, sleep quality, and overall cognitive function as well. In the elderly populations this amount of exercise will help prevent things like osteoporosis and arthritis. To think that millions of Americans can prevent early death by one of the aforementioned diseases is an amazing thought.

The second reason why we as athletes should continue to be physically active is because once an individual becomes completely sedentary for a certain period of time they increase their risk for injury when they begin to be physically active again. These injuries mainly come in the form of musculoskeletal injuries from doing exercises too often, having too much of the physical activity in one day or week, or doing the wrong type of exercise.

The message in the readings and from this class as a whole when it comes to the connection between physical activity and health is that it is much easier and healthier to stay physically active and reap the many rewards throughout life than it is to stop once competition is over and try to not be sedentary after a long period. In conclusion, every individual should keep in mind the type, intensity, amount, and duration of each physical activity and should reach the recommended amount of physical activity of 150-300 minutes of moderate to vigorous activity a week. In reaching this physical activity goal each week after competition is over we can prevent many diseases and be healthy for years to come.

Student 2.

Physical Activity

Physical activity is an important aspect of life for all ages. Without exercise the human body would not be healthy at all. Exercising results in many physical benefits such as maintaining a healthy weight, conserving lean muscle, and how our bodies look. Additionally, it has great physiological benefits. In this essay I will

exemplify the different modes of physical activity, the physiological benefits that come along with exercise, the appropriate amount of exercise and the types of exercise that people should partake in.

There are multiple types of physical activities that people can partake in to create an overall healthier and happier lifestyle. Different activities range from aerobic exercises, which put more of an emphasis on cardiovascular gains such as, walking, basketball, swimming and soccer these type of activities improve the efficiency and capacity of the cardiorespritory system. Anaerobic activities is intense enough to trigger lactic and acid formation, the type of activities include power lifting and sprinting. The anaerobic activities require energy production systems that do not use oxygen because they exceed the capacity of the cardiorespritory system to distribute oxygen and other metabolites. Both groups of physical activity provide a wide spectrum of health benefits. Those health benefits include reductions in risk of gaining diseases, in fact the first the coronary heart disease was the first condition whose incidence was shown to be reduced by regular physical activity, these same physical activities can improve a person's functional ability as well. Recent studies on the relations between physical activity and health show that physical activity reduces the risk of early death, coronary heart disease, strokes, high blood pressure, type 2 diabetes, breast and colon cancer, excessive weight gain (which is a big problem in the US), injurious falls, depression and loss of cognitive functions. Those facts will take me into my next topic of discussion in this paper which is the physiological benefits of physical activity.

Exercising benefits that because it stimulated brain chemicals that may leave a person feeling happier and more relaxed. Studies show that the physiological changes caused by various physical activities overlap on some cases. But some of the activities are associated with physiological changes more than others, such as aerobic activities, they cause changes to the cardiovascular system that improve the capacity and efficiency of the delivery of oxygen and glucose to tissues that need them. They also improve cellular systems that release energy from substrates that can be used for movement. But different activities cause different effects for example; ambulatory activities strengthen your muscles and bones along the axial skeleton and lower extremities, while swimming has a greater impact on the upper body musculature and less impact on the skeletal systems. The physiological response to exercise is dependent on the intensity, duration and frequency of the exercise as well as the environmental conditions, and age. Exercise for older people is crucial. They need to know the correct amount of time, energy, and amount of exercise they need so that it will affect their bodies in the most positive way. For older adults who don't usually exercise they need to take it a step by step process when speaking about the intensity of the activities. For example if they are working out for the first time they will find that they gained a good amount of benefits from a low intensity and small amount of time activity. If they were to go too long and too hard they could possibly end up negatively affecting their health and body. The same goes for younger adults, a small amount of exercise is better than no exercise at all.

Our bodies are engineered to meet the needs of our ancestor's time which mean we still need physical activity to be healthy. This means that an inactive lifestyle is not safe to live, for inactive people even small increases in the volume of exercises will provide important health gains. Basically people need to make sure that they are not sedentary, once again some activity is better than none. Studies show that 100-300 minutes a week of moderate intensity physical activities provide substantial health benefits for the general adult population. Benefits that are just as good as the moderate intensity activities can be reached with about 75 minutes per week of vigorous intensity activity or by a combination of moderate and vigorous activity.

Physical activity is essential for a healthy lifestyle; one must not be sedentary. With a lack of exercise, one increases the likelihood of getting a sickness or disease. Studies show that physical activity benefits a person's life in numerous amounts of ways ranging from preventing or managing a wide range of health problems all the way to boosting your energy and mood throughout your days.

Student 3.

Physical Activity

Nutrition and exercise are very important aspects in physical health and the overall length of life one lives. The thing that many people do not realize is that without both at the same time, you are only guaranteed to obtain half of the potential that your body's peak performance could be. Student athletes have it the best and the worst when it comes to nutrition and health. Many have access to top-of-the-line

resources and exercise programs where knowledge is easily accessible. The problem lies with how each individual chooses to use the information presented before them. There are many student athletes that can literally eat anything they want without having any major or noticeable setbacks in their performance. While it seems like a blessing, what usually occurs with this section of student athletes is that once their time of performance is up they continue to live with the same habits they have built up for years while not putting in the same amount or intensity level of exercise they have been. This results overall in the person becoming unhealthy after they have moved on to having a regular life.

Being a student athlete since I was a freshman in High School, I have kept up the same basic routine of exercise and diet throughout the years. The only changes I have had are dietary changes and the intensity of the exercise increasing. I was one of those student athletes in high school where I could eat anything I wanted without having problems. It did not catch up to me until I had to grey shirt a semester before coming to the University of Idaho. It was extremely noticeable and held me back for a long period of time. Maintaining a healthy diet and exercise level is important to ensuring you live a long healthy life. It is very hard to keep motivated when you are done with athletics because once you are done putting your body through the grinder for years, the last thing that is on your mind is the continuance of that. I can attest that I have definitely thought before, “Once I am done with collegiate athletics, I am not going to work out for a month.” While this seems like a nice treat, it actually can end up hurting your body a lot more. Reading these articles and my personal experiences in the past have led me to decide that rather than take a break from

working out altogether, I am going to merely shift the focus on my workouts. Being a football player, workouts focus on leg strength, upper body strength, and fast-twitch muscle movement. Once I am done with the sport, I doubt I am going to have to bash into 300 pound lineman every single day. This got me thinking that I would better be served doing anaerobic activities that I enjoy, as well as lifting light weights for high repetitions to tone muscle groups. My focus would be on losing and then eventually maintaining a healthy body weight that I could operate on a daily basis with.

I thought the articles were very informative. They shared really good information that dealt with intensity level of exercises, and basically stated that little low intensity exercise is better than doing absolutely nothing at all. I think I will be in a great position when I graduate in December because I have so much knowledge on what needs to be done; the thing that remains is whether or not I act on it.

End Writing Samples

Student 1.

The fact that all of these top athletes gained a substantial amount of weight soon after retirement isn't that surprising. It makes sense that they would have the same eating habits, not thinking they needed to change, and they just stop working out like they use to. It would also seem like if an athlete was kept to a strict diet that they would want to branch out to many types of junk food to compensate for times they have been missing out on, adding more calories that they aren't burning off. The same goes with beer consumption like the article mentioned, not being able to burn off extra calories that unnecessary food was adding. What I liked to see was that it wasn't just college athletes adjusting to life afterward; it was also hall of fame athletes from all sports. I agree with Jeff Ruland's statement "You can work out all you want, but no matter what you do, it's just not the same as running up and down the court," and Golic's statement "I wasn't working out for football anymore, so I didn't have the same drive." I've experienced this even in just the three weeks on Christmas break, working out on my own at my own pace but coming back to work out in January with the team again and not having the stamina like I did four weeks prior. It's really not that easy to get in the swing of working out when you don't have motivation like doing well in a sport. Even when you do go and work out of a consistent basis it isn't the same as a sports workout that you have done in the past. You're not striving to get better every day because there's nothing you need to be better for; your team isn't there, there is no reason to compete and no one to

compete with. I also thought the second articles point about a “gym strike” was interesting. It makes sense that athletes would want a long break from treating their bodies the way that they do, I know I have felt like that a lot in the past. Putting your body through long hours of heavy training multiple times a week for months on end makes you feel like you deserve a break. I like how the article also mentioned the likelihood of returning to training because of personal appearance and the desire to find a relationship. It was kind of humorous that married athletes are less likely to go back to hard training because they aren’t worried about their appearance anymore. It was nice to get an inside look into what could happen after I end my sport. Now I have a few years to get my head wrapped around the idea that I’m not going to be playing for forever and I can plan for the future.

Lately I’ve been thinking a lot about how I will act and train after my sports career is over. Right now it is still hard to believe that I only have three years left and I don’t really want to dwell on that. While I’m still in my sport I want to gradually increase the amount of nutrient dense, healthy foods I consume and decrease the greasy, fat filled, fast food I consume. I want to create a diverse diet of all the essential nutrients I need. That being said, I don’t want to decrease the amount of food I eat, for now I actually want to increase the food intake to increase my weight. When it comes time to switch up training and nutrition I plan on slowly decreasing calorie intake by a certain goal a week until I reach a reasonable amount based on my training regimen. As for training, first thing I will do is take one to two weeks off right after my last competition to get that break in. To avoid a prolonged gym strike I will begin a new type of training regimen. I feel like if I don’t start something new

right away I will become like so many of those athletes, out of shape and out of motivation. I understand I won't be lifting for any specific use but for myself. Even though that is the case, I will keep on with the same exercise regimen, only at my own pace and with a few alterations to add to it and make longer work outs. I want to focus more of an effort on cardio. The way I workout for my sport there is little cardio work expect sprint work emphasized at different times of the year. I would like to focus on more long distant runs, starting with one mile after my short gym strike. I will start off with doing one mile every few days, improving time as I go, eventually adding more distance to my runs. I may switch up cardio workouts in the sense that one day I will go for a certain distance and the next I will go for a certain time. Along with running I plan on biking and other things to improve cardiovascular health. My number one priority after sports isn't to look good or be skinny; it's to make sure I have the best heart health possible. My thought process is that if I focus on the steps to maintain a healthy heart in diet and exercise my physical appearance will follow.

The articles mentioned that most athletes become unmotivated after athletics because there's no goal to work for, but I believe my case will be different because I have that important goal of heart health to work for. As I have said in an earlier essay, there is a large history of heart disease on both sides of my family that I want to make a hard effort to avoid as much as possible if not completely. I'm not just completely focused on the physical side of health either. It's no secret that those who are or were involved with football have been at higher risk for mental issues down the road. I have been doing a small amount of research on the risks, issues, and long term effects of small and large, frequent blows to the head. I want to do a

little more research to find ways to keep my brain at full functioning longer and reduce risks of issues such as dementia. I fear that I'm likely to end up with Alzheimer's in my old age because of repeated blows to the head while playing sports and heredity; I also have a history of Alzheimer's on my father's side of the family. If I focus on my main goals, heart health and mental function, I feel like transitioning from the hard training lifestyle of an athlete to an everyday pedestrian won't be that big of a change; I just need to keep my motivation strong.

Student 2.

Being physically something is something that I plan on doing for the rest of my life. It is something that has not only changed my life for the better, but also my family, and my friends. I might not be able to play football or any physically demanding sport like that for the rest of my life, however there are many different alternatives to staying physically fit and active without having to try and knock someone out.

If I were to make goals for when my time is done with football, I would say that my overall complete goal would just be to stay active and fit for as long as I live. That's a pretty broad and bold goal, but I feel like trying to live that type of life style will not only be beneficial to me, but also to those around me. Short term goal could be things like stretching at least twice a day for a week, educating myself on new ways of exercising and also educating others on the importance of being physically active.

Since the profession I am choosing to go into requires me to deal with physically active individuals, I feel that the goals I have set will be easy to reach.

Like I have stated before being physically active is something that has been huge in my life. It has helped me become a better athlete, and most importantly a better person. The reason I say this is because working out provided almost like an escape to other things that were going on around me, in there I got the solitude that I wanted, nothing else mattered. This is one of the main reasons why I want to be a strength and conditioning coach, to provide athletes the guidance to get the best out of their bodies, but also to develop them as a person. I feel like a weight room can really help shape a person, because at that point where a workout gets rough or you've had a long day you have to make a choice, either to attack it, or give in. It's a great tool to help develop mental strength and self-esteem, it's you against you in there, only you know what your best is, now you just have to demand it out of yourself.

My plan for staying fit after football season will consists of many different things. One of them being cross fit, the person who trains me when I'm home lets me workout at his gym for free and has even offered me an opportunity to work for him when I graduate. The other motivating factor for doing cross fit is what I mentioned in paper 4, my brother, I will finally get to train with him and compete with him day in and day out. Another way I plan on staying physically active is hiking, or hill runs. Hiking is probably one of my most favorite things to do when I am home, during winter break I went on at least 5 hikes a week, even if they were not all extremely long, they were still a nice little change.

The last way I plan on staying healthy is through football itself. Not playing necessarily, but coaching. I plan on going back to my old High school and helping

coach the linebackers and also the Strength and Conditioning aspect. Coaching high school kids is something that will definitely keep me in shape, my brother is actually the JV offensive coordinator and whatever he makes his players do as conditioning, he does it as well. I feel like this is a great strategy because we all know what it's like to be dying during sprints, while we watched our out of shape just demand us to go faster. I also want to be able to effectively demonstrate proper footwork and technique for the linebackers that I am coaching.

Overall seeing the slides on goal settings didn't really make me change my mind on my goals or change how I originally planned them, however the slides did provide me with a refresher from my sport psychology that I took back in Jr college. Whether it's on the football field, in the gym, or out on a mountain every day, being physically active is something that will forever be part of me.

Student 3.

I plan to transition from being a D1 athlete to a runner involved in all the local fun road races and events. I will be able to accomplish all the things I have always wanted to do: run a half-marathon, full-marathon, and some sort of triathlon. After college, I will finally be able to relax and choose what activity I can do each day. For example, run a trail run one day, bike to Pullman another, and play tennis on Sundays without the worry of twisting an ankle. During my season, all I do is run. The training is intense but it is solely running. Once I graduate, I'll be able to broaden my activities and not just be so focused on running and school. Instead, I can be focused on exercising, healthy living, and balancing activities. A lot of people get bored with the same activities, so having a variety of activities will keep me

interested and intrigued. Also running every day is a lot of wear and tear on my body, mixing it up will keep my body injury free and more balanced. I'm excited to broaden my activities and explore different sports.

Reading this article, shocked me because I did not realize so many ex-athletes struggled with weight gain. Once I finish, I will definitely take a break and relax for a while. However, I won't be able to stay down for long. Even when we had breaks between Cross Country and Track season, I have trouble staying sedentary; I prefer to be active and busy. I love working out and feeling like I can eat anything. I run so that I can eat. I love food. I love health. I love life. Therefore, I doubt I will just be a couch potato. I will definitely not run every day with the same intensity but I'll take hikes and bike rides etc… If I can't run one day, it won't kill me, I'll do something else. That will be my retirement. My retirement won't be a retirement from working out, it will be a retirement of strict running routine intensity.

In regards to calories, I actually eat dramatically less when I'm not running. I usually gain weight when my season starts, which is muscle, but also I think fat because I'm constantly hungry and eating whatever I want. When I am not running, I watch what I eat more carefully. Surprisingly, I am way more self-conscious about what I eat when I'm not running. Apparently, this isn't normal according to the article. My appetite just isn't very big without burning all those calories during my intense workouts. Therefore, I think that once I graduate, I will just naturally go back to consuming the amount of calories I am currently burning. During the summer, when I was running alone, I was able to really concentrate on my eating habits and get to a good balance. Once school starts though, my eating goes to the back burner

because I am just doing all that I can to keep my energy and intensity up along with balancing school.

The article also talked about alcohol being a problem because it has so many calories. I doubt this will be an issue for me because the only time I drink is right after season with my college friends. Thus, once I am out of college I will hardly drink because I don't like beer and I don't enjoy sitting around drinking them. Also, I do not enjoy golfing so it will not be my form of exercise.

Going to the gym will be hard. I have never been a gym type of person, I love the outdoors and running so going to the gym to lift will be difficult to get used to. But I want to be active and healthy, so if I need to go to the gym in addition to running I will. During my season, I have to go to the gym to cross train so I will probably try to keep that routine up. Especially if I can't run one day, I would want to cross train. Some seasons we have weight days where we have a certain routine we have to accomplish. Therefore, I have learned some valuable techniques to carry with me into my retirement.

The way for me to succeed in all of this is to have a set goal. I want to sign up for events, running or triathlon, and make a day-by-day plan months in advance. This will help me to stick to my plan, have a goal to look forward to, and see the benefits of my hard work just like in college. My sister runs marathons, teaches spin class, and does cross fit. She signs up for races frequently and just ran the Boston Marathon. I really respect her and look up to her. I want to be like her because she is very dedicated to sign up for events and then plan her training for that event. Once I set a goal for myself, I accomplish it. I am very driven and love a challenge. The

Spartan Race looks like so fun, I've always wanted to run it! Also, triathlons have been a life time goal for me so I'm excited to finally be able to complete that. Besides, I am not the type of person to become sedentary.

Apparently, the first year is the hardest for athletes, which is understandable. I will definitely take a break to recover and let my body and mind have a rest. However, I'll still be active by hiking, biking, tennis, swimming, etc… I think I'll give myself a month and then I'll get back to running and start looking for races to enter in. Really, exercising is a lifestyle. I'm an adventurous and curious person, I love being busy and active. If I haven't done something active one day, I hate it, I have to go outside every day. I love the outdoors and adventures.

The key is to be aware of my nutrition and the change in my calories burned. Also, I need to have a plan and a goal. I will do this by signing up for events and then setting a pre-race planner. Also, watching my calorie intake is important, making sure I'm burning the right amount of calories for the amount of exercise I am completing.