ABSTRACT

The Acute Effects of *Pterocarpus Marsupium* Supplementation on Insulin-Dependent and Insulin-Independent Signaling Pathways at Rest, After an Oral Glucose Tolerance Test and After Intense Exercise in Overweight Pre-Diabetic Females.

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Increasing physical activity and modifying diet to incorporate more phytonutrients has been suggested as a way to prevent the progression of insulin resistance (IR) into cardiometabolic disease. *Pterocarpus Marsupium* (PM), a phytonutrient compound shown to improve hyperglycemia, could potentially improve IR. The primary purpose of this study was to examine the effects of PM on insulin sensitivity and blood lipids in overweight and obese, sedentary women classified as prediabetic. Further, to examine the effects of acute ingestion of PM on insulin- and exercise-mediated glucose disposal following an oral glucose tolerance test (OGTT) and aerobic exercise bout, and to understand the mechanisms by which PM supplementation may affect specific muscle gene expression.

A double-blind, randomized, placebo controlled study was conducted. Participants consumed 250 mg of either PM (n=8) or placebo (PL, n=8) twice daily for 6 days. Blood and muscle samples were obtained prior to supplementation, prior to and following OGTT, and prior to and following an exercise bout. Following OGTT, plasma

glucose levels were higher at 30 minutes ($p<0.001$) and 1 hour post-OGTT ($p=0.001$) in both PM and PL groups. Similarly, plasma insulin levels were higher 30 minutes ($p=0.023$), 1 hour ($p=0.023$), and 2 hours post-OGTT ($p=0.003$) in both PM and PL groups. Additionally, serum glucose levels decreased from 30 minutes to 1 hour postexercise ($p = 0.011$), and from 30 minutes to 2 hours postexercise ($p = 0.013$) in both PM and PL groups. A significant increase in mRNA expression of Akt2 ($p = 0.001$), AMPK ($p = 0.001$), AS160 ($p = 0.02$), and PPARα ($p = 0.025$) was observed one hour after OGTT. The findings suggest that 250 mg of PM twice daily does not improve insulin sensitivity or fasting lipid levels in this population. However, this is the first study to report the novel finding of an increase in skeletal muscle mRNA expression of Akt2, AMPK, AS160, and PPARα in response to an OGTT. The observed increase, and subsequent decrease, in serum glucose levels after brief, maximal exertion exercise was also novel.

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By

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A Dissertation

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LIST OF ABBREVIATIONS

4E-BP1 - eukaryotic initiation factor 4E binding protein 1

AKT – protein kinase B

BCAA – branched-chain amino acids

dL - deciliter

eIF – eukaryotic initiation factor

g – gram

GDP – guanine diphosphate

GLUT4 – glucose transporter 4

GTP – guanine triphosphate

IGF-1 – insulin-like growth factor-1

IRS-1 – insulin receptor substrate-1

kg – kilogram

mg – milligram

mL – milliliter

mM – millimolar

mRNA – messenger ribonucleic acid

mTOR – mammalian target of rapamycin

P70-S6K - P70 Ribosomal Protein S6 Kinase

PI-3 kinase – phosphotidylinositol 3-kinase

RDA – recommended dietary allowance

RP S6 – ribosomal protein S6

RPE – rating of perceived exertion

Ser – serine

Thr – threonine

Tyr - tyrosine

tRNA – transfer ribonucleic acid

U – units or international units

VO_2 max – maximal oxygen uptake

µg - microgram

µL – microliter

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CHAPTER ONE

Introduction

Insulin resistance (IR) is a defect in the transportation of glucose into a cell where the tissue becomes resistant to the effects of normal circulating levels of insulin. Insulin resistance is viewed as part of a cluster of cardiovascular and diabetic (cardiometabolic) risk factors termed Metabolic Syndrome (MetS). Traditionally, MetS also includes hypertension, visceral adiposity, atherosclerotic dyslipidemia, and a prothrombotic and proinflammatory state (Grundy et al., 2005). However, MetS is recently being perceived as a cluster of risk factors linked to IR instead of a cluster of risk factors including IR.

Preliminary reports for 2007 show that cardiometabolic disease accounts for three of the top seven leading causes of death in the United States (Xu, Kochanek, & Tejada-Vera, 2009). The most common cardiovascular diseases, heart disease and stroke, account for greater than 35% of all deaths in the United states, while diabetes mellitus, a chronic disease resulting in increased concentration of glucose in the blood, is the seventh leading cause of death (Xu et al., 2009). The prevalence of diabetes mellitus has tripled over the past 25 years (Xu et al., 2009). It is currently estimated that, 23.6 million Americans suffered from diabetes mellitus (U.S. Department of Health and Human Services [USDHHS], 2009). The majority of diabetics are Type II diabetics (non-insulin dependent) that cannot effectively utilize circulating levels of glucose mainly due to peripheral IR. The Center for Disease Control expects the incidence of diabetes mellitus to double by 2050, with the largest increase occurring in Type II diabetics (USDHHS, 2009).

Given the link between MetS and IR, it is imperative that people with MetS find ways to reduce risk factors associated with cardiometabolic diseases. Lifestyle change is the first-line of defense against the further progression of such cardiometabolic risk factors progressing into chronic diseases such as Type II diabetes or possible complications such as myocardial infarction or stroke. Of particular importance is decreasing fat mass, increasing physical activity, and modifying diet to an anti-atherogenic type diet (National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III [NCEP], 2002).

The NCEP (2002) recommends increasing physical activity for persons with MetS, but fails to provide recommendations for exercise intensities and/or modalities. Research demonstrates that exercise at higher aerobic intensities (70 – 85% VO_{2max}) are effective at improving cholesterol and lipid levels (King, Haskell, Young, Oka, & Stefanick, 1995). Further, a recent review showed that high intensity interval training (90-95% peak heart rate) not only improves maximum oxygen uptake, but increases cardiomyocyte calcium handling (Wilsoloff, Ellingsen, & Kemi, 2009).

Notably, aerobic exercise at similar high intensities (Okura et al., 2007; Short et al., 2003; Winnick et al., 2007) has also been shown to improve insulin sensitivity in subjects with insulin resistance. Though previous research has established that muscle contraction itself can stimulate glucose uptake within the cell; specific intracellular mechanisms involved in the translocation of the glucose transporter-4 (GLUT4) have only been recently been identified (Abbott, Edelman, & Turcotte, 2009; Holloszy, 2005; Sakamoto & Holman, 2008; Winder, Taylor, & Thomson, 2006). Adenosine

monophospate protein activated kinase (AMPK) is nutrient sensitive and is activated with a change in the ratios of AMP:ATP and Cr:PCr during exercise. Activation of AMPK (Fisher, Gao, Han, Holloszy, & Nolte, 2002; Jessen et al., 2003) has been shown to increase glucose uptake via insulin-independent mechanisms. Continued long-term exercise increases the expression of various proteins important in the insulin signaling cascade, such as Akt (Christ-Roberts et al., 2004) and a 160 kd substrate of Akt (AS160) (Sriwijitkamol et al., 2007), which, when phosphorylated, leads to GLUT4 translocation to the plasma membrane, thus ensuring glucose uptake. In addition to exercise-induced glucose uptake, insulin can itself cause glucose uptake within the cell via insulin dependent mechanisms. Insulin has to bind to an insulin receptor, activating the phosphorylation of phosphatidylinositol 3 kinase (PI3K), which phosphorylates the membrane bound phospholipid phosphatidylinositol 4,5-bisphospate (PIP2) to make phosphatidylinositol 3,4,5-triphospate (PIP3). Phosphorylation of PIP2 to yield PIP3 results in a lipid-binding site for serine/threonine kinase Akt via protein kinase D (PKD) activation (Sesti, 2006). The protein Akt then phosphorylates AS160, which then allows for GLUT4 translocation (Ramm, Larance, Guihaus, & James, 2006). It is clear that glucose uptake can be mediated by 2 pathways independent (exercise-mediated) or dependent (insulin-mediated) of insulin.

In addition to the aforementioned pathways, exercise can also induce changes in expression of metabolic genes such as the peroxisome proliferator-activated receptors (PPARs) and peroxisome proliferator-activated receptor co-coactivators (PGCs) (Short et al., 2003; Spangenburg, Brown, Johnson, & Moore, 2009) These genes are intimately, but also redundantly involved in the regulation of substrate utilization and mitochondrial

biogenesis. Peroxisomes are membrane bound organelles, found in all Eukaryotes. One major function of the peroxisome is to break down fatty acids (FA) in a process called β-oxidation (Tabak, Braakman, & Distel, 1999). In mammals the peroxisome is not the only place where β-oxidation occurs, as it also occurs in the mitochondria. Peroxisomes break down fatty acids that are otherwise too long for the mitochondria (Tabak et al., 1999). By way of degradation, once a FA is short enough it can be transported to the mitochondria where it is further degraded and used as a fuel source (van den Bosch, Schutgens, Wanders, & Tager, 1992). The three PPAR isoforms, -α, -β/δ, and -γ are members of the steroid/thyroid superfamily of nuclear receptors (Mojotima, 1993) that overlap in function and are distributed differently according to tissue type. PPARα and PPARβ/δ are predominantly found in skeletal muscle and have important roles in regulating FA catabolism (Gilde, 2003), while PPARγ is found primarily in adipose tissue and is partly responsible for controlling adipo- and lipogenesis (Muoio & Koves, 2007). The PPAR co-activator PGC-1α regulates many genes involved in FA catabolism via co-activation of the PPARs (Vega, Huss, & Kelley, 2000). Continued aerobic exercise and some PPAR agonists induce skeletal muscle fiber type switching to that of a slow a twitch fiber better suited to catabolize FA (De Souza, Cornwell, Dai, Caguyong, & Ulrich, 2006; Russell et al., 2003).

People that are IR exhibit metabolic inflexibility where they do not correctly utilize glucose or FA (Novo et al., 2008). Under normal postprandrial conditions, triglyceride (TG) lipolysis is suppressed, which decreases the amount of free fatty acids (FFA) in circulation (Novo et al., 2008). Consequently, there is an excess of FFA that can decrease insulin sensitivity in skeletal muscle (Ginsberg, 2003). Increased levels of

circulating TG can also accelerate hepatic gluconeogenesis (Vitarius, 2005). Thus, any means, such as exercise, that can increase activation of the PPARs and PGCs, increase mitochondrial and peroxisome biogenesis, and increase utilization of FFA may improve IR and associated risk of cardiometabolic disease.

To help prevent further advancement of IR into chronic cardiometabolic disease, people are also encouraged to eat a diet that incorporates a lower percentage of total calories from fat, reduces dietary trans fat and cholesterol, and incorporates a greater percentage of plant stanols, sterols and soluble fiber (NCEP, 2002). Stanols and sterols are structurally similar to cholesterol in animals, function in a similar fashion in plant cell membranes (Katan MB, 2003), and are considered phytonutrients (Bland, 1996). Recently, several phytonutrients have been purported to help improve glucose disposal and/or hyperlipidemia (Bhathena & Velasquez, 2002; Bland, 1996; de Kleijn, van der Schouw, Wilson, Grobbee, & Jacques, 2002; Manickam, Ramanathan, Farboodniay Jahromi, Chansoria, & Ray, 1997). Unfortunately, a large portion of the population does not meet the recommended daily consumption of fruits and vegetables (Casagrande, Wang, Anderson, & Gary, 2007). Furthermore, the amounts of plant sources needed to meet the recommended levels of phytonutrients are difficult to achieve. Thus, any means to improve dietary consumption of phytonutrients that may help improve one or more of the cardiovascular risk factors of MetS is clearly warranted.

One commercially available product containing several potential beneficial phytonutrient compounds is *Pterocarpus marsupium* (PM) extract. *Pterocarpus marsupium* and isolated compounds from PM, such as pterostilbene, have been demonstrated to be hypolipidemic (Rimando, Nagmani, Feller, & Yokoyama, 2005), anti-

oxidant (Remsberg et al., 2008), anti-inflammatory (Remsberg et al., 2008), and can up-regulate glucose transport (Anandharajan, Pathmanathan, Shankernarayanan, Vishwakarma, & Balakrishnan, 2005; Manickam et al., 1997). Several compounds characterize extracts of PM, but the most potent effects have been attributed to a compound called pterostilbene. Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) is a polyphenolic phytoestrogen found in high concentrations in red wine and a variety of plant sources such as grape skin, berries, and pomegranates. Pterostilbene is also found in many plants, and used to treat diabetes and inflammatory diseases in many Eastern medical practices.

Pterocarpus marsupium is commonly used in Ayurvedic medicine and has been compared to drugs often prescribed to treat diabetes, such as Metformin, which is mainly an antihyperglymemic drug. The exact mechanism for how metformin works is unkown. However, it does suppress hepatic gluconeogenesis (Kirpichnikov, McFarlane, & Sowers, 2002; Radziuk, Zhang, Wiernsperger, & Pye, 1997), and may increase insulin sensitivity via AMPK stimulation in myocytes and adipocytes (Suwa, Egashira, Nakano, Sasaki, & Kumagai, 2006). The benefit to increasing insulin sensitivity is obvious, as the peripheral tissue, such as skeletal muscle, in people with IR are resistant to the effects of insulin. Less obvious, perhaps, is the benefit to a decrease in hepatic glucose production. Increased hepatic gluconeogenesis is mainly responsible for fasting hyperglycemia in a diabetic population (De Fronzo, Bonadonna, & Ferannini, 1992). Therefore, it would be beneficial to reduce hepatic gluconeogenesis in this population.

Studies have also compared PM to Rosiglitizone, which belongs to a family of drugs called thiazolidinediones (TZD) (Anandharajan et al., 2005). It is believed TZDs

increase insulin sensitivity in skeletal muscle, suppress hepatic glucose production, and activate AMPK (Ye et al., 2006). Thiazolidinediones also target PPARs in order to improve insulin sensitivity, with PPARγ being the primary target (Moller & Greene, 2001). The effects of TZDs on PPARγ are thought to occur primarily in adipocytes by increasing lipogenesis, decreasing circulating levels of free fatty acids (FFA), and decreasing inflammatory cytokines (Gervois, Fruchart, & Staels, 2007), but TZDs may also may exert their insulin-sensitizing effects on PPARγ in muscle (Norris et al., 2008). Not surprisingly, PM, as well as the chief active ingredient pterostilbene, has been shown to effectively activate PPARs in a similar manner to that of TZDs and fibrates (Anandharajan et al., 2005; Mizuno et al., 2008; Rimando et al., 2005). The fibrates are a class of drugs used to effectively lower lipid levels via activation of PPARα. A study of a fibrate, known to be a PPARα agonist, in hampsters resulted in improved TG levels, and also increased peroxisomal and mitochondrial increased β-oxidation (Minnich, Tian, Byan, & Bilder, 2001).

Purposes of the Study

People with IR are at an elevated risk for cardiometabolic disease. Therefore, any method that could improve insulin sensitivity and the metabolic inflexibility of IR warrants further study. Diet and exercise are typically the first line of defense for attenuating the effects of IR, but many people fail to meet current diet and exercise guidelines (Casagrande et al., 2007). As such, phytonutrient dietary supplementation may have a positive impact on characteristics of the IR and MetS. *Pterocarpus marsupium* has been used for centuries in Ayurvedic medicine to treat hypoglycemia and dyslipidemia, and the extract of PM is also used in products available over the counter at

health food stores in the United States. Since PM is commonly used in Ayurvedic medicine, is commercially available and its mechanisms of action are thought to be similar to drugs taken by people with diagnosed IR; this extract was used in the current study.

The purpose of this study was four-fold. First, this study sought to examine the effects of a short-term (6 day) PM supplementation on insulin sensitivity and blood lipid profiles of overweight and obese, pre-diabetic, sedentary women. Secondly, this study sought to determine if an acute (5 day) ingestion of PM would enhance insulin-mediated glucose disposal up to 2 hours following an OGTT. Third, the study sought to determine if an acute ingestion (6 day) of PM would enhance exercise-mediated glucose disposal up to 2 hours following an aerobic exercise bout. Fourth, to understand the mechanisms by which PM supplementation elicits its effects within the muscle genes involved in insulin signaling and glucose metabolism were examined.

Hypotheses

Based on the purposes of the study, the following hypotheses have been formulated. To facilitate better understanding of which hypotheses relate to which purpose, the hypotheses have been separated into the categories: fasting glucose and insulin, OGTT glucose and insulin, GXT glucose, fasting lipid, fasting skeletal muscle mRNA expression, OGTT skeletal muscle mRNA expression, and GXT skeletal muscle mRNA expression.

Fasting Glucose and Insulin

H_1: Serum levels of fasting glucose will decrease further in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_2: Serum levels of fasting insulin will decrease further in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_3: Measures of insulin resistance (HOMA-IR) will be lower in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

OGTT Glucose and Insulin

H_4: *Pterocarpus marsupium* group will display lower increases in overall serum glucose levels compared to placebo group during the oral glucose tolerance test (OGTT).

H_5: *Pterocarpus marsupium* group will display lower increases in overall serum insulin levels compared to placebo group during the oral glucose tolerance test (OGTT).

H_6: The measure of insulin sensitivity ($OGTT_{ISI}$) will be higher for the *Pterocarpus marsupium* group compared to placebo group during the oral glucose tolerance test (OGTT).

GXT Glucose

H_7: *Pterocarpus marsupium* group will display smaller increases in serum glucose levels compared to the placebo group following the graded exercise test (GXT).

Fasting Lipid

H_8: Serum levels of fasting HDL levels will increase to a greater extent in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_9: Serum levels of fasting LDL levels will decrease to a greater extent in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_{10}: Serum levels of fasting total cholesterol levels will decrease to a greater extent in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_{11}: Serum levels of fasting triglyceride levels will decrease to a greater extent in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

Fasting Skeletal Muscle mRNA Expression

H_{12}: Skeletal muscle gene expression of AMPK mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_{13}: Skeletal muscle gene expression of Akt2 mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_{14}: Skeletal muscle gene expression of AS160 mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_{15}: Skeletal muscle gene expression of PPARα mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_{16}: Skeletal muscle gene expression of PPARγ mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

H_{17}: Skeletal muscle gene expression of PGC-1α mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group over the course of the study.

OGTT Skeletal Muscle mRNA Expression

H_{18}: Skeletal muscle gene expression of AMPK mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the oral glucose tolerance test (OGTT).

H_{19}: Skeletal muscle gene expression of Akt2 mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the oral glucose tolerance test (OGTT).

H_{20}: Skeletal muscle gene expression of AS160 mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the oral glucose tolerance test (OGTT).

H_{21}: Skeletal muscle gene expression of PPARα mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the oral glucose tolerance test (OGTT).

H_{22}: Skeletal muscle gene expression of PPARγ mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the oral glucose tolerance test (OGTT).

H_{23}: Skeletal muscle gene expression of PGC1α mRNAα will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the oral glucose tolerance test (OGTT).

GXT Skeletal Muscle mRNA Expression

H_{24}: Skeletal muscle gene expression of AMPK mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the grade exercise test (GXT).

H_{25}: Skeletal muscle gene expression of Akt2 mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the graded exercise test (GXT).

H_{26}: Skeletal muscle gene expression of AS160 mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the graded exercise test (GXT).

H_{27}: Skeletal muscle gene expression of PPARα mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the graded exercise test (GXT).

H_{28}: Skeletal muscle gene expression of PPARγ mRNA will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the graded exercise test (GXT).

H_{29}: Skeletal muscle gene expression of PGC1 mRNAα will be greater in the *Pterocarpus marsupium* group compared to the placebo group following the graded exercise test (GXT).

Delimitations

This study was completed with the following delimitations, limitations, and assumptions:

1) Sixteen, overweight or obese, sedentary pre-diabetic women (18-35 y) participated in this study.

2) Participants were recruited from the general population of Waco, TX, and student population at Baylor University by flyers posted throughout campus and through newspaper advertisements.
3) Muscle biopsies from the lateral thigh were collected during the pre-supplementation testing session; collected prior to the OGTT, 30 minutes post-OGTT, 1 hour post-OGTT, and 2 hours post-OGTT during the second testing session; and collected prior to the GXT, 30 minutes post-GXT, 1 hour post-GXT, and 2 hours post-GXT during the third testing session.
4) Venous blood were collected during the pre-supplementation testing session; prior to the OGTT, 30 minutes post-OGTT, 1 hour post-OGTT, and 2 hours post-OGTT during the second testing session; and collected prior to the GXT, 30 minutes post-GXT, 1 hour post-GXT, and 2 hours post-GXT during the third testing session.
5) Participants completed an OGTT during the second testing session and a GXT during the third testing session.
6) Participants were randomly assigned to one of two supplement groups: *Pterocarpus marsupium* or placebo.
7) All participants in the study did not participate in any other forms of vigorous exercise during the duration of the study and did not modify their nutritional intake in any manner.
8) All testing was done in the Exercise and Biochemical Nutrition Lab and Exercise and Sport Nutrition Lab at Baylor University in the Marrs-McLean Gym according to all policies and procedures within each respective laboratory.

Limitations

1) The sample size was limited to those who came forward to participate in the study, which limited the scope of conclusions that could be inferred to a larger population.
2) The motivation and willingness of each participant to maximally exert themselves during the third testing session during the GXT.
3) The sensitivity of the technologies and protocols utilized to identify quantifiable changes in the criterion variables.
4) The daily schedules of each participant and the inherent circadian rhythms that exist for all humans as a result of slightly different testing times, stresses, etc.
5) The ability of the homeostasis model assessment (HOMA-IR) index of insulin resistance and Matsuda insulin resistance index to accurately estimate insulin function.

Assumptions

1) Participants fasted for 8 - 12 hours prior to reporting for each testing session.
2) Participants presented as pre-diabetic yet did not have contraindications to any of the prescribed treatments involved with this protocol.
3) Participants were sedentary.
4) All participants followed all instructions throughout the familiarization session, the OGTT testing session, the GXT, and maximally exerted themselves during the GXT.
5) All assay reagents and equipment used in the sample analysis was accurate and reliable in quantification of the criterion variables.

6) All methods were previously established and were accurate and reliable methods for determination of the criterion variables.

Definition of Key Terms

1) AMPK –AMP activated protein kinase is an energy sensitive kinase activated by in low-energy states.
2) Akt – This protein is also known as protein kinase B (PKB) and is a downstream target of IRS-1. Akt is a key protein in cell signaling and specifically has the ability to phosphorylate serine 2448 on mTOR.
3) AS160 – AS160 is a substrate of Akt necessary for the translocation of GLUT4.
4) Eukaryotic initiation factors (eIF) – A special set of proteins that help regulate translation initiation.
5) Eukaryotic initiation factor 4E (eIF4E) – A key protein in the Akt-mTOR signal transduction pathway. When eIF4E is activated, it can bind to eIF4G and help facilitate translation initiation. Eukaryotic initiation factor 4E binding protein (4E-BP1) regulates the activity of this protein.
6) Eukaryotic initiation factor 4E binding protein 1 (4E-BP1) – This protein is a key factor in the Akt-mTOR signal transduction pathway. When hypophosphorylated, it binds eIF4E and prevents it from combing with eIF4G. This ultimately inhibits translation initiation. However, when it becomes hyperphosphorylated, most likely via mTOR, it does not bind eIF4E and allows translation initiation to ensue.
7) Eukaryotic initiation factor 4F complex (eIF4F) – A combination of three eukaryotic initiation factors that include eIF4A, eIF4G, and eIF4E. The complete eIF4F complex helps regulate the binding of mRNA to the 40S ribosomal subunit.

8) Eukaryotic initiation factor 4G (eIF4G) – One of the three eukaryotic initiation factors that comprise the eIF4F complex. Eukaryotic initiation factor 4G serves as a docking protein for eIF4A and eIF4E. When the complete eIF4F complex comes together it promotes the binding of mRNA to the 40S ribosomal subunit and ultimately translation initiation.
9) Insulin – An anabolic hormone that is released from the pancreas and when it binds to its receptor, it instigates various metabolic processes and can activate the proteins in the Akt-mTOR cell signaling pathway.
10) Insulin-like growth factor-1 (IGF-1) – A growth factor that has the ability to bind to its receptor, and to a lesser affinity, the insulin receptor, and activate the proteins in the Akt-mTOR cell-signaling pathway.
11) Insulin receptor substrate-1 (IRS-1) – A cytoplasmic protein that is stimulated by insulin, insulin-like growth factor-1, and possibly growth hormone. IRS-1 has the ability to bind to and activate (phosphorylate) Akt.
12) Mammalian Target of Rapamycin (mTOR) – Is a protein kinase that is involved with cell signaling and plays a role in regulating translation initiation. It is thought to be an activator of the downstream targets 4E-BP1 and P70-S6K.
13) Messenger RNA (mRNA) – The DNA of a gene is transcribed into mRNA. The mRNA is carried to the ribosome and serves as a template for the synthesis of polypeptides (proteins).
14) Phosphorylation – The process of adding a phosphate to a compound or protein through an enzyme. Through the procedure of adding a phosphate, a protein/enzyme can be activated or deactivated.

15) Protein degradation - Also known as protein breakdown and proteolysis. This is the process of breaking down proteins into their individual amino acids. During states of muscle atrophy, protein breakdown exceeds muscle protein synthesis.

16) Protein synthesis – The process of translating mRNA in protein as directed by the genetic code. During states of muscle hypertrophy, protein synthesis exceeds muscle protein breakdown.

17) Pterostilbene – This is a structural analog of resveratrol (*trans*-3,5,4′-trihyrdoxystilbene) which, naturally occurring in plants such as grapes, blueberries, and peanuts.

18) P70 Ribosomal Protein S6 Kinase (also denoted as S6K1, P70-S6K, and $p70^{S6K}$) – This 70 kilodalton protein kinase is a key enzyme in the Akt-mTOR signal transduction pathway. It is thought to be activated by mTOR and appears to activate ribosomal protein S6.

19) Resveratrol – This is a naturally occurring polyphenol found in grapes, berries, and Ayurvedic medicines.

20) Serine/threonine kinase – These kinases phosphorylate serines or threonine kinases at the OH group of their side chains.

CHAPTER TWO

Literature Review

Overview of Glycemic Control

Insulin is an anabolic peptide hormone produced in the pancreatic β-cell in response to increased glucose levels in plasma and is responsible for the uptake of glucose, amino acids (AA), and fatty acids (FA) in adipose, skeletal, and liver tissues (Young, 1963). Insulin is partially responsible for regulation of glucose homeostasis in several tissues. It reduces hepatic glucose production by decreasing gluconeogenesis and glycogenolysis and by increasing glucose uptake into peripheral tissues such as skeletal muscle and adipose tissue (DeFronzo, 1988; Shulman, 2000).

Glucagon, on the other hand, is produced by the pancreatic alpha cell and functions to raise plasma glucose levels when they get too low by increasing glycogen and gluconeogenesis. Insulin and glucagon vary inversely with each other. A rise in insulin is accompanied by a fall in glucagon. However, they both are released in order to maintain glucose homeostasis. When there is an increase in plasma glucose following a meal, there is a resultant increase in insulin and concomitant decrease in glucagon. When plasma glucose levels fall below 50 mg/dl, glucagon is released (Benson, Johnson, Palmer, Werner, & Ensinck, 1977). Insulin secretion is minimal or ceases when plasma glucose levels reach 80-85 mg/dl. Insulin can be thought of as being dominant during times of anabolism, where glucagon plays a role during times of catabolism. The

coordination of insulin and glucagon production is closely mediated as a protective mechanism to ensure the body has adequate glucose to maintain activity levels and vital functions.

Mechanisms of Insulin-dependent Glucose Uptake

Plasma insulin binds to its receptor triggering activation of phosphatidylinositol 3-kinase (PI3K). PI3K phosphorylates the membrane bound phospholipid phosphatidylinositol 4,5-bisphospate (PIP2) to make phosphatidylinositol 3,4,5-triphospate (PIP3) Phosphorylation of PIP2 to get PIP3 results in a lipid-binding site for serine/threonine kinase Akt. Akt is phosphorylated by protein kinase PKD-1 (Sesti, 2006). To regulate muscle hypertrophy, this cascade would result in the phosphorylation of mTOR, 4EBP-1 and p70S6K, leading to increased translation in the same manner as insulin-like growth factor-1 (IGF-1) activates the PI3K/Akt/mTOR pathway (Han, Tong, Zhu, Ma, & Du, 2008; Latres et al., 2005). However, plasma insulin controls glucose homeostasis via a slight variation in the aforementioned pathway. The insulin receptor is comprised of four subunits. There are two α-peptide subunits where insulin binds, and two β-peptide subunits that span the membrane (Karlsson & Zierath, 2007). Once insulin binds to the insulin receptor, autophosphorylation of tyrosine kinase domains (TKD) on the β-peptide subunits occurs. The TKD are docking sites for insulin receptor substrates (IRS) that are downstream of the insulin receptor (Karlsson & Zierath, 2007). Phosphorylation of IRS causes it to bind to and activate Src homology domains (SH2), including the regulatory subunit of PI3K (p85) (Sesti, 2006).

Via insulin signaling, transport of glucose into the cell requires phosphorylation of the regulatory subunit p85 of PI3K, which results in phosphorylation of PIP2 to make

PIP3 (Latres et al., 2005). An increase in PIP3 leads to activation of PDK, which will activate the serine/threonine kinases Akt and the atypical protein kinase C (PKC) (Sesti, 2006). PKC is represented by two important isoforms known as PKCζ/λ. PKCζ/λ, which has been shown to translocate from low-density microsomes to the plasma membrane in conjunction with an increase in GLUT4 translocation and glucose uptake in L6 muscle cells (Liu et al., 2006). The authors suggest that PKCζ mediates the effect of insulin on glucose uptake via actin remodeling. PKCλ knockout mice were engineered and these mice had a reduction of GLUT4 translocation, diminished glucose transport, and developed insulin resistance (Farese et al., 2007). These studies suggest that atypical PKCζ/λ plays a role in the translocation of GLUT4.

However, there appeared to be a missing link in the insulin-dependent translocation of GLUT4. The above pathway steps were known, but it was not fully understood how these steps lead to GLUT4 translocation. Recent literature has pointed to a novel substrate of Akt (AS160) as a potential key to the missing link in the cascade (Kane et al., 2002). In 3T3LI adipocytes, the phosphorylation of AS160 is required for insulin-induced translocation of GLUT4 (Sano et al., 2003). The mRNA of Akt substrate AS160 is highly prevalent in human skeletal and cardiac muscle in comparison to other tissue (Matsumoto et al., 2004), and in rat skeletal muscle, has been shown to respond to insulin in a dose-dependent manner (Bruss, Arias, Lienhard, & Cartee, 2005).

The activation of Akt by insulin results in phosphorylation of multiple phosphomotifs on AS160, which is believed to either inhibit the activation of Rab-GTPase proteins linked with GLUT4 vesicles or cause AS160 to dissociate from GLUT4 vesicles, or both (Cartee & Katsuhiko, 2009). Rab GTPase is a member of a superfamily

of small GTPases called Ras. Rab GTPases functions to regulate specific steps of signaling pathways near plasma membranes and recruit specific effecter proteins to membranes (Stenmark & Olkkonen, 2001). AS160 contains a Rab GTPase-activating protein domain that plays an important role in insulin triggered GLUT4 translocation (Kane et al., 2002; Sano et al., 2003). GLUT4 is sequestered in the cytosol and has to translocate to plasma membrane to ferry glucose into the cell. It is believed that AS160 inhibits GLUT4 under basal conditions and insulin-dependent phosphorylation of AS160 by Akt reverses inhibition (Ramm et al., 2006). It has also been shown that AS160 partially binds directly to the GLUT4 vesicle and that insulin signaling releases AS160 into the cytosol (Larance et al., 2005).

Overview of Insulin Resistance

Glucose is transported into skeletal muscle and adipose cells via GLUT4 after insulin has bound to its receptor (Birnbaum, 1989; Guma, Zierath, Wallberg-Henriksson, & Klip, 1995). The binding of insulin to the insulin receptor sets off a cascade of events culminating in the translocation of GLUT4 to the membrane surface to ferry glucose into the cell for utilization. Liver, fat, and muscle tissue can develop a defect in the transportation of glucose into the cell where tissue becomes resistant to the effects of normal circulating levels of insulin, hence the term insulin resistance. Insulin resistance plays a role in Type II diabetes, and is associated with other diseases such as polycystic ovarian syndrome, obesity, and dyslipidemia.

Proposed Deficiencies in Insulin Signal Transduction

In order for tissue to be insulin resistant, there must be a defect in signal transduction (as previously mentioned) resulting in translocation of GLUT4. In such a complex pathway, there can be numerous points for the defect to take place, or the defect could be a result of multiple sites of dysfunction (and to explore all of these possible points is beyond the scope of this dissertation). One potential site is the insulin receptor itself. Two different isoforms in the receptor are known with the difference in the two resulting from alternative splicing at exon II (Sesti, 2006). Therefore, the isoforms are known as Exon II^+ and Exon II^-. Exon II^+ is associated with increased expression in people with hyperglycemia and hyperinsulinemia (Norgren, Zierath, Wedell, Wallberg-Henriksson, & Luthman, 1994; Sesti, 2006; Shier & Watt, 1989). Exon II^- is known to have a higher affinity for insulin than II^+ and is a better activator of PI3K (Sesti, 2006). Impaired phosphorylation of IRS and reduced phosphorylation of PI3K has been observed in the skeletal muscle of obese individuals and non-obese subjects with Type II (Brozinik Jr., Roberts, & Dohm, 2003; Goodyear et al., 1995; Kim, Nikoulina, Ciaraldi, Henry, & Kahn, 1999). Defects in GLUT4 expression and function have also been observed, contributing to impaired whole-body glucose uptake. GLUT4 content is decreased in obese subjects with and without Type II, and in insulin resistant offspring in patients with NIDDM (Sheperd & Khan, 1999).

Mechanisms of Insulin-independent Glucose Uptake

In the case of an exercising animal, the rise in glucagon and decrease in insulin is partially due to the increase in insulin sensitivity resulting from contracting skeletal muscle. In regards to exercise-induced insulin sensitivity, it was reported that hypoxia,

electrically stimulated muscle contractions, and acute bouts of exercise have been shown to stimulate transport of glucose in the absence of insulin (Holloszy, 2005). Insulin and muscle contraction both stimulate glucose transport into the cell, but do so via differing mechanisms. Contraction stimulated uptake of glucose can be initiated by the release of calcium from the sarcoplasmic reticulum (Finck et al., 2005). The release of calcium activates calcium/calmodulin-dependent protein kinase found in skeletal muscle. Glucose can also be transported into skeletal muscle cells by GLUT4 through activation of the AMPK pathway. The AMPK pathway is nutrient sensitive and is activated with a change in the AMP:ATP and Cr:PCr ratio during exercise. AMPK turns on catabolic pathways that will generate ATP, while turning off those anabolic pathways that use ATP (Towler & Hardie, 2007).

Activities such as exercise that increase metabolic stress and increase ATP consumption will increase the AMP:ATP ratio (Towler & Hardie, 2007). This in turn will activate AMPK. Consequently, exercise also causes Ca2+ to be released from the sarcoplasmic reticulum. In recent years, it has been demonstrated that the release of calcium and the stimulation of the calcium/calmodulin pathway can increase the activity of AMPK (Towler & Hardie, 2007). AMPK is phosphorylated at Threonine^{172} (Thr^{172}) of the α-subunit by upstream kinase complexes of LKB1 tumor suppressor, STRAD, and MO25 (Boudeau et al., 2004; Hawley et al., 2003; Marignani et al., 2007). Addition of Ca2+ ionophores increases Thr^{172} phosphorylation and AMPK activity (Towler & Hardie, 2007). In some circumstances Thr^{172} is phosphorylated by upstream kinases Ca2+/calmodulin-dependent protein kinase kinase (CAMKK) (Raney & Turcotte, 2008; Shen, Zhu, Tong, Ren, & Du, 2007). Thus, any stressor that causes an increase in

cytoplasmic Ca2+ will cause an increase in ATP demand and a shift in the AMP:ATP ratio. Towler and Hardie (2007) propose that AMPK activity may be increased simply by an increase in ATP demand, owing to the fact that Ca2+ is immediately pumped out of the cytoplasm via ATP-driven pumps.

Effects of Exercise on Insulin Sensitivity

In instances of chronic exercise, insulin sensitivity (defined as a shift in the insulin dose-response curve to the left with decreasing insulin concentration needed to cause 50% of the maximal response (Holloszy, 2005)) has been shown to increase in younger people, improve citrate synthase and cytochrome c oxidase, and mRNA and protein levels of GLUT4 (Short et al., 2003). Short et al. used a 16-week aerobic exercise program to study the effect of age on exercise-induced changes on PGC-1α, GLUT4, and nuclear transcription factors that regulate mitochondrial genes for cytochrome c (Short et al., 2003). Short et al. trained subjects 3 times a week for 20 minutes at 70% max heart rate, which was gradually increased until subjects were cycling four times a week for 40 minutes at 80% max heart rate during the last month of the training program. Insulin sensitivity was increased by an average of 26%, but improvement was inversely related to age. Both cytochrome c oxidase and citrate synthase increased. The authors concluded that aerobic exercise training increased glucose transport capacity, but the changes were not related to changes in insulin sensitivity (Short et al., 2003). Although, insulin sensitivity was not improved, the change in glucose transport in response to exercise, points to an increased insulin responsiveness, which is defined as an increase in glucose transport induced by a maximally effective insulin concentration with a proportional upward shift of the dose response curve (Holloszy, 2005). It has been shown that genes

encoding for cytochrome c and GLUT4 are regulated by cytosolic calcium (Freyssenet, Di Carlo, & Hood, 1999; Ojuka et al., 2002) and PGC-1α (Michael et al., 2001). Thus increases in cytosolic Ca2+, which also activates AMPK, from chronic exercise can improve mitochondrial function and GLUT4 translocation.

Christ-Roberts et al. (Christ-Roberts et al., 2004) sought to determine how improved insulin signaling (GLUT4 expression and glycogen synthase activity) contributed to improved insulin sensitivity using an eight-week exercise program. Training increased insulin-stimulated glucose disposal in non-diabetic and diabetic subjects. Glycogen synthase activity, GLUT4, and Akt protein expression increased in both groups (Christ-Roberts et al., 2004). However, exercise training did not enhance IRS-1/PI3K activity. Thus, eight weeks of exercise training increased insulin-stimulated glucose disposal primarily by increasing GLUT4 protein expression without enhancing insulin-stimulated PI3K signaling. The training-induced improvement in glucose disposal was primarily accounted for by increased insulin-stimulated glucose storage.

The previous two studies used aerobic exercise to study the effect of exercise on insulin sensitivity and GLUT4 content of skeletal muscle in response to prolonged exercise bouts of 8 (Christ-Roberts et al., 2004) and 16 weeks (Short et al., 2003). While aerobic exercise causes an increase in GLUT4 content in skeletal muscle, it does not always equate to increases in insulin sensitivity or an increase in the activation of the PI3K/Akt pathway. However, neither study examined the effect of aerobic exercise on AMPK and its involvement in GLUT4 activation.

AMPK is thought to be an important mediator of substrate metabolism in skeletal muscle (Koshinaka et al., 2004). Six male subjects cycled for 60 minutes at about 72%

$_{VO2peak}$ (McGee et al., 2003), this resulted in an increase in Nuclear AMPK-α2. The authors suggested that nuclear translocation of AMPK may mediate the effects of exercise on skeletal muscle gene and protein expression (McGee et al., 2003). Another study using rats subjected to six hours of swimming per day for 10 days (Kubota, Koshinaka, Kawata, Loike, & Oshida, 2008). GLUT4 protein was increased in exercising rats well above that of non-exercising rats. AMPK was also elevated above that of non-exercising rats, but not significantly (Kubota et al., 2008). However, the authors conceded that their protocol might not have been of significant intensity to illicit a significant response, as AMPK activation is intensity dependent (Sriwijitkamol et al., 2007). It has been shown by two different sets of researchers that increased activation of AMPK after aerobic exercise, with both acute (Fisher et al., 2002) and repeated exercise bouts (Jessen et al., 2003) is involved in the increase in glucose disposal after exercise. The involvement of AMPK in increasing glucose disposal may be due to an association with AS160.

Downstream of AMPK is AS160 (Treebak et al., 2006). Treebak et al. reported that mice deficient in AMPK had decreased levels of phosphorylated AS160 (Treebak et al., 2006). Additionally, adenosine anolog 5-aminoimidazole-4-carboxaminde ribonucleoside (AICAR) affected AMPK activation via the α-catalytic subunit and subsequent AS160 phosphorylation was inhibited in AMPK-α2-inactive mice (Kramer et al., 2006). However, AMPK-α2-inactive mice only demonstrated partial inhibition of contraction stimulated AS160 phosphorylation (Kramer et al., 2006), demonstrating that while AMPK activation increases AS160 phosphorylation and the resultant GLUT4 translocation it is not the sole regulatory pathway in insulin-independent glucose uptake.

In isolated rat muscle, 5 minutes of contraction resulted in an increase in phosphorylated AS160 (Bruss et al., 2005). Additionally when rats performed four 30-minute bouts of swimming with 5-minute rest periods between swimming bouts, immediately after exercise phosphorylated AS160 increased significantly compared to controls (Arias, Kim, Funai, & Cartee, 2007). In humans, cycling for 60 and 90 minutes at 70% VO_{2peak} increased AS160 phosphorylation in the vastus lateralis muscle (Deshmukh et al., 2006; Howlett, Mathews, Garnham, & Sakamoto, 2008; Treebak et al., 2007). However, phosphorylated AS160 was not increased in vastus lateralis after shorter exercise bouts of similar intensity (Treebak et al., 2007), or resistance training did not increase phosphorylated AS160 immediately after exercise (Howlett, Sakamoto, Garnham, Cameron-Smith, & Hargreaves, 2007). AS160 phosphorylation was shown to increase up to 1h and 2h after resistance training (Dreyer et al., 2008). The two studies employing resistance training as an exercise modality used different protocols, but neither protocol elicited increased in phosphorylation of AS160 immediately post exercise (Dreyer et al., 2008; Howlett et al., 2007). Howlett, Sakamoto, Garnham, Cameron-Smith, & Hargreaves (2007) even performed analysis of phosphorylated AS160 in the presence of insulin. The explanation for the failure of AS160 to be phosphorylated immediately post-resistance exercise was accompanied by a decreased capacity for AS160 to bind to 14-3-3 proteins (Howlett et al., 2007), with which AS160 binds to in the cytosol (Ramm et al., 2006), thus inhibiting Rab GTPase activity. Interestingly, endurance exercise demonstrated an increased capacity for binding with 14-3-3 (Howlett et al., 2008).

It is apparent that exercise stimulates improvement in glucose uptake and that AMPK plays a pivotal role via AS160. However, AMPK activation from chronic exercise also leads to better use of FA as a fuel source via the phosphorylation of acetyl CoA carboxylase, inhibiting FA formation, and inhibiting the formation of malonyl-CoA (Saha & Ruderman, 2003). This also inhibits malonyl-CoA decarboxylase, which is responsible for degrading malonyl-CoA. Malonyl-CoA is an allosteric regulator of carnitine palmitoyltransferase-1 (CPT-1), which controls the entrance of long-chain fatty acyl CoA into the mitochondria. Thus, increased AMPK activation also leads to increased FA oxidation after exercise. It is thought that AMPK is an upstream regulator of hormone sensitive lipase (HSL) (Witczak, Sharoff, & Goodyear, 2008). Hormone sensitive lipase hydrolyzes the conversion of triglycerides into FFA and glycerols, which is thought to be the rate-limiting step in the skeletal muscle lipolysis (Kiens, 2006). The phosphorylation of HSL at Ser^{565} by AMPK after muscle contraction (Donsmark, Langfort, Holm, Ploug, & Galbo, 2004) suggests the potential for AMPK to mediate intramuscular triglyceride breakdown.

Nonetheless, phosphorylation states of proteins, are relatively transient interactions and do not explain long-term beneficial effects of exercise on substrate utilization. It has been suggested that AMPK activation increases FA oxidation, in skeletal muscle in particular, via activation of PPAR-α and PGC-1α (Lee et al., 2006). The co-activator PGC-1α is a member of a transcriptional co-activator family interacting with transcriptional factors to coordinate mitochondrial biogenesis and promote the remodeling of muscle fiber type to a tissue more compatible with fatty acid oxidation like that of Type 1 muscle fiber (Liang & Ward, 2006). This has been observed in cell culture

and murine models with use of AICAR (Lee et al., 2006), and after chronic AMPK activation from endurance training (Baar, 2004). However, AMPK appears to be attenuated in the skeletal muscle of both obese and lean Type II diabetic persons after an acute bout of exercise when compared to lean subjects (Sriwijitkamol et al., 2007). This is not surprising considering that in this study lean Type II diabetic participants had reduced PGC-1α levels (Sriwijitkamol et al., 2007). Similarly, obese people have been shown to have reduced PGC-1α levels as a result of over-feeding (Muoio & Koves, 2007). Ultimately this also leads to attenuated AS160 activation in these populations.

Pharmacological Intervention of IR

Insulin resistance and Type II are typically treated using various pharmacological therapies including metformin and TZDs. Metformin is a biguanide that was originally obtained from *Galega officianalis*, a botanical also known as 'goat's rue' or 'French lilac' which can be found in the northern hemisphere and was used for centuries to treat symptoms of diabetes mellitus (Straughan, 2007). A biguanide is a drug, based on the molecular formula $C_2H_7N_5$, which act as antihyperglycemic agent. Originally, three biguanides were extracted from *Galega officianalis*, but of the three, metformin is the only one currently being prescribed for diabetes. Phenformin and buformin, the other two biguanides, have a propensity to induce lactacidosis in patients (Salpeter, Greyber, Pasternak, & Salpeter, 2006). Metformin can induce lactacidosis, but this typically only occurs with high dosages (Salpeter et al., 2006). Figure 1 depicts the biguanide structure and the structure of metformin.

Metformin is absorbed mainly in the small intestine and is believed to have 50-60% bioavailability (Schafer, 1983). This biguanide is stable, is excreted in urine in an

unchanged form, and is non-plasma protein binding (Mehnert, 2001). The literature typically states that metformin should be thought of as an anti-hyperglycemic not a hypoglycemic drug. Metformin works to lower blood glucose up to 3.5 mmol/l in a dose dependent manner by delaying the enteric absorption of glucose (De Fronzo, Barzilai, & Simonson, 1991), stimulating glucose uptake by myocytes and adipocytes (Galuska, Zierath, Thorne, Sonnenfeld, & Wallberg-Henriksson, 1991), and inhibiting hepatic

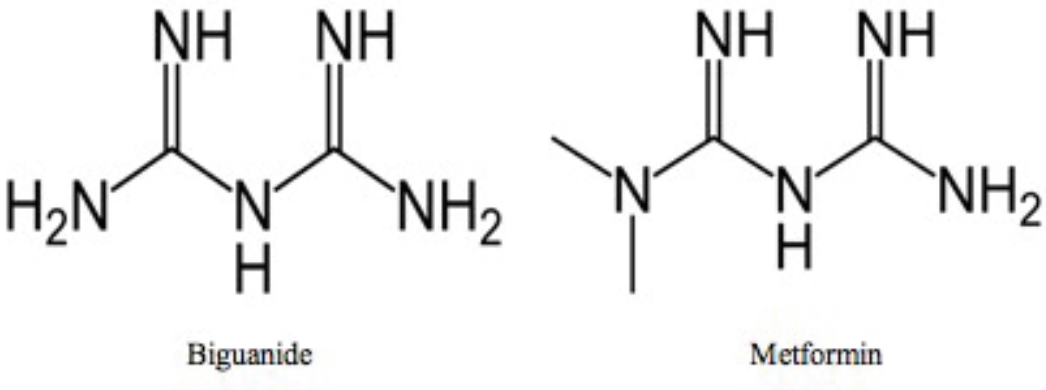

Figure 1. The above figure depicts the general structure of a biguanide, on the left, and the structure of the anhtihyperglycemic biguanide drug metformin, on the right.

gluconeogenesis and glycogenolysis (Bailey, 1992). Additionally, metformin does not increase insulin secretion. Metformin has also been reported to reduce body weight (Salpeter et al., 2006), provide modest improvements in lipid profiles (Lund et al., 2007; Mourao, Sa, Guedes, & Dib, 2006; Stumvoll, Nurjahan, Perriello, Dailey, &Gerich, 1995), and possibly affect LDL and HDL particle size (Lawrence, Reid, & Taylor, 2004).

Mechanistically, metformin is thought to inhibit hepatic gluconeogenisis by inhibiting hepatic lactate uptake (Radziuk et al., 1997), as well as increasing ATP. Furthermore, ATP is an allosteric regulator of pyruvate kinase (PK), which results in an

increased PK flux (Large & Beylot, 1999). In the liver, metformin may also inhibit pyruvate carboxylase-phosphoenolpyruvate carboxy kinase activity as well as oppose the gluconeogenic action of glucagon (Kirpichnikov et al., 2002). Relative to skeletal muscle, metformin enhances exocytosis of GLUT4 and GLUT1 to the plasma membrane and may increase the capacity of said transporters to carry transfer glucose across the membrane (Bailey & Turner, 1996; Wiernsperger & Bailey, 1999).

The TZDs increase insulin sensitivity in skeletal muscle and suppress hepatic glucose production (Day, 1999). It is believed that TZDs also activate AMPK (Ye et al., 2006). The TZD, Rosiglitazone is believed to enhance insulin sensitivity be stimulating PPARγ in insulin sensitive tissue as well as mimicking insulin, which causes a slow anti-hyperglycemic affect (Day, 1999). However, the exact mechanism is somewhat unclear. Improvement in insulin sensitivity by TZDs occurs mostly in skeletal muscle via activation of the PI3K/Akt pathway (Inzucchi et al., 1998; Petersen KF, 2000). Troglitazone, another TZD, was found to restore insulin-stimulated PI3K activity and increase p110β protein levels in skeletal muscle of type 2 diabetic subjects (Kim et al., 2002). Rosiglitiazone enhanced peripheral glucose uptake in newly diagnosed Type II patients via insulin and exercise mediated pathways while metformin did not (Hällsten et al., 2002).

Pterocarpus Marsupium

Pterocarpus marsupium, also known as the Indian kino tree, is a member of the Legumaniaceae family, and is a large deciduous tree native to the west peninusula of India (Saxena & Vikram, 2004). PM has been used in Ayurvedic medicine for several centuries to the treat symptoms of diabetes and to lower lipid levels (Sharma, 1996). In

rats and hamsters, and human cell cultures, PM has been used to study several of the individual components of the metabolic syndrome (Grover, Vats, & Yadav, 2005; Remsberg et al., 2008; Rimando et al., 2005).

Kar, Choudhary, and Bandyopadhyay (Kar, Choudhary, & Bandyopadhyay, 2003) used crude ethanolic extracts of 30 full grown matured plants to assess hypoglycemic activity of these extracts in male albino rats. Diabetes was induced via interperitoneal injection of alloxan monohydrate in order to partially destroy pancreatic β-cells. Groups of five animals were selected for each botanical extract at 250 mg/kg of body weight. Baseline blood glucose was measured and re-sampled at one-week intervals. If adequate lowering of blood glucose levels were not achieved within two weeks with a single dosage of 250 mg/kg the dosage was doubled and given twice daily. Of the thirty, only eight extracts were effective within one week with the single 250 mg/kg dosage. PM was the fourth most affective at lower blood glucose (Kar et al., 2003). In a similar study, the heartwood of PM alone was given to alloxan-induced diabetic male and female Wistar rats (Mukhtar, Ansari, Ali, Bhat, & Naved, 2005). Five days after hypergycemia was induced, the rats were separated into three groups of five. One group was not given alloxan and used as controls. Of the other three groups, one served as a diabetic control and received chow and distilled water, another group received the anti-diabetic drug glicazide (a drug usually given along with Metformin or TZDs) and chow, while the last group was given 250 mg/kg of aqueous extract of PM. Treatment lasted for ten days. Blood samples were draw prior to initiating treatment, 1 hour, 3 hours, after initial treatment, and on days 1, 3, 7, and 10. PM significantly lowered blood

glucose levels compared to diabetic controls and glicazide treated rats (Mukhtar et al., 2005).

Aqueous extract of PM*, Ocimum sanctum, and Trigonella foenumgraecum* were prepared due to their ability to exert hypoglycemic/anti-hyperglycemic effects (Grover Vats, & Yadav, 2005). The three extracts were given to groups of rats fed a 66% fructose diet in order to induce metabolic syndrome. There were five groups of rats total. One group was a fed a normal chow diet, one was fed the high-fructose diet alone, one groups was fed the high-fructose diet plus PM (1 g • kg^{-1} • day^{-1}), another was fed the high-fructose diet plus *Ocimum sanctum* (200 mg • kg^{-1} • day^{-1}), while the final group was fed the high-fructose diet plus *Trigonella foenumgraecum* (2 g • kg^{-1} • day^{-1}) for thirty days. Compared to controls, fructose fed rats had increased levels of serum glucose, insulin, and triglyceride levels. All three plant extracts significantly lowered the serum glucose, but only PM prevented hypertriglyceridaemia and hyperinsulinemia (Grover et al., 2005).

In another study, aqueous extract of PM was studied to assess the mechanisms of how it might exert its anti-diabetic effects (Grover, Vats, & Yadav, 2002). Male albino rats were fed a standard chow diet then randomized into three groups of six rats. One group was kept as controls, while two groups were administered streptozotocin to induce hyperglycemia for a period of ten days. One of the hyperglycemic groups was used as diabetic control, and the other group was given PM for 30 days. After thirty days of treatment, all animals were sacrificed and organs and tissues were sampled. Again, PM effectively lowered blood glucose levels by up to 60%. In the diabetic controls, liver and kidney weight increased significantly ($p < 0.0005$) versus controls. *Pterocarpus marsupium* extract prevented this gain in kidney, but not liver weight. *Pterocarpus*

marsupium extract also partially prevented increased glycogen content in the diabetic kidney and the decrease in glycogen content in diabetic liver and skeletal muscle. Diabetic controls had a decrease in hexokinase, phosphofructokinase, and glucokinase compared to controls. In rats treated with PM the change in phosphofructokinase was corrected and the change in hexokinase and glucokinase was partially corrected (Grover et al., 2002).

Methanolic extract of PM and 7-O-α-L-rhamnopyranosyloxy-4'-methoxy-5-hydroxy isoflavone isolated from PM were used to examine activation of cellular targets of *PM* (Anandharajan et al., 2005), in yet another study. L6 myotubes were cultured and subjected to glucose uptake after being starved for 5 hours. The culture were then incubated with PM and 7-O-α-L-rhamnopyranosyloxy-4'-methoxy-5-hydroxy isoflavone. After incubation the cells were rinsed and incubated again. Next, glucose uptake was terminated by aspiration of the media. Cells were then washed three times. RT-PCR was carried out. *Pterocarpus marsupium* incubated cells demonstrated a two-fold increase of 2-deoxy glucose compared to controls. Cells were also analyzed by PT-PCR for transcriptional activity of GLUT4, PPARγ, and PI3K. Both PM and 7-O-α-L-rhamnopyranosyloxy-4'-methoxy-5-hydroxy isoflavone showed elevated expression of GLUT4 that was comparable to the results of cells cultured with insulin and rosiglitizone (Anandharajan et al., 2005). Cells cultured in PM and 7-O-α-L-rhamnopyranosyloxy-4'-methoxy-5-hydroxy isoflavone showed increased expression of PPARγ comparable to rosiglitizone, while only PM cultured cells showed increased expression which was comparable to insulin (Anandharajan et al., 2005).

In human clinical studies, PM has also been shown to be affective. In one study, ten patients were given water that was stored in a container made of the hardwood of PM for one month (Kedar & Chakrabarti, 1981). Blood glucose levels decreased after a minimum of two weeks, and remained at normal levels until the patients were taken off of the medication. In another study, the efficacy of PM for treating newly diagnosed or untreated Type II patient given the extract for 12 weeks was studied (Indian Council of Medical Research [ICMR], 1998). Two grams were initially given for four weeks. If no significant change was observed in that time, the dosage was doubled. Ninety-three patients finished the study. Blood glucose levels, fasting and postprandial, decreased significantly by 32mg% and 45mg%, respectively (ICMR, 1998).

Specific Extracts of PM

The previously mentioned research articles mostly concern PM extracts as a whole; however, numerous compounds have been isolated from PM in an attempt to identify the most potent compounds. Some of those compounds have been purported to have antihyperlipidemic effects (Jahromi & Ray, 1993), antioxidant effects (Satheesh & Pari, 2006), and insulin mimetic effects (Ahmad, Khalid, Khan, Rastogi, & Kidwai, 1989). (Jahromi & Ray, 1993) examine the antihyperlipidemic effects of the flavonoids marsupsin, pterosupin, and liquiritgenin from decocted PM extract and PM extract. Male albino Charles Foster rats were given high fat diets and Triton to induce hyperlipidemia. Rats were kept on high fat diets and given either water as a control, Cholestyramine as a reference, marsupsin, pterosupin, and liquiritgenin at doses of 40 mg/kg body weight (Jahromi & Ray, 1993). *Pterocarpus marsupium* extract, given at 120 mg/kg body weight, significantly reduced serum cholesterol, triglycerides, and LDL- and VLDL-

cholesterol without any effect on HDL-cholesterol. Pterosupin and liquiritgenin effectively lowered serum cholesterol, LDL-cholesterol, and raised HDL-cholesterol (Jahromi & Ray, 1993).

One potent compound isolated from PM is (-) epicatechin (Ep). Epicatechin was purported to have insulin mimetic effects and to be insulinogenic (Ahmad et al., 1989). It stimulated uptake of oxygen in fat cells and increased glycogen content of rat diaphragms. Epicatechin also inhibited theophylline lipolysis in isolate fat pads. However, Ep has not been shown to share binding sites with insulin and does not affect the release of glucagon (Ahmad et al., 1989). In vitro, Ep was also shown to increase cAMP, a second messenger in signal transducion, content in pancreastic islets associated with increased insulin secretion (Ahmad, Khan, Rastogi, Chaubey, & Kidwai, 1991). It was also associated with the conversion of proinsulin to insulin.

The most researched compound isolated from PM extract is pterostilbene. Pterostilbene, a structural analog of resveratrol (*trans*-3,5,4′-trihyrdoxystilbene), which occurs naturally in plants such as grapes and peanuts. Resveratrol has been shown to regulate inflammation (Csiszar et al., 2006; Martin, Villegas, Sanchez-Hildalgo, & Alarcon, 2006; Vignaud, Cebrian, Martelly, Caruelle, & Ferry, 2005), be an antioxidant and regulate lipid peroxidation, regulate cell-proliferation and apoptosis (Manna, Mukhopadhyay, & Aggarwal, 2000; Surh, 2003), and regulate GLUT transport via activation of AMPK (Breen, Sanli, Giacca, & Tsiani, 2008; Park et al., 2007). Structurally, pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystibene) is identical to resveratrol, but is methylated at carbons 3 and 5 of the secondary aromatic ring.

Resveratrol and pterostilbene are also found in plants used to treat diabetes and inflammatory diseases in the Ayurvedic system of medicine.

Comparison of Resveratrol and Pterostilbene

Resveratrol is highly studied for its chemopreventative effects in cancer cells, cardiovascular disease, and its antioxidant capabilities. However, it has been reported that resveratrol has low oral bioavailability (Goldberg, Yan, & Soleas, 2003; Walle, Hsieh, DeLegge, Oatis, & Walle, 2004). The low bioavailability of resveratrol, or most polyphenols, is believed to be due to the free hydroxyl groups on both aromatic rings, allowing the compound to be freely glucoronidated and sulfated (Otake, Hsieh, & Walle, 2002). With this thought in mind, Wen and Walle (Wen & Walle, 2006) compared methylated and unmethylated polyphenols in pooled human liver S9 fraction and human colon adenocarcinoma cells. Among the unmethylated polyphenols studied were resveratrol and quercetin, another highly studied polyphenol. Four methylated flavones were also studied and were found to result in increased hepatic metabolic stability and improved intestinal absorption (Wen & Walle, 2006). The authors found that blocked hydroxyl groups via methylation removed the influence of the conjugation pathways. Thus reduced clearance in intestinal epithelial cells and in the liver may provide higher oral absorption.

As pterostilbene is a natural dimethylated analog of resveratrol, this may lead to higher oral bioavailablity than resveratrol. However, the research on this topic is unclear whether this is actually the case. For example, in a study designed to examine the pharmacometrics and pharmacokinetics of trans-pterosilbene, male Sprague-Dawley rats were intravenously dosed with 20 mg/kg of the dimethyl resveratrol analog trans-

pterostilbene (Remsberg et al., 2008). Remsberg et al. found that glucoronidatied pterostilbene levels agreed with previous studies reporting that resveratrol existed predominately in its glucoronidated form (Remsberg et al., 2008; Wenzel & Somoza, 2005). Additionally, similar to trans-resveratrol, trans-pterostilbene appeared to excrete mainly via non-renal routes with a half-life of less than one hour (Remsberg et al., 2008).

Applied Studies Involving Pterostilbene Administration

Based on the structural similarities to resveratrol, pterostilbene has been studied in many of the same models to test its effectiveness in preventing cancer and cardiovascular disease, as well as treating hyperlipediemia and hyperglycemica. However, most studies have been performed using murine models or *in vitro* human cell lines. As such, there is a paucity of data related to the use of pterostilbene in human models.Based on the structural similarities and the reported similarities in metabolism, it is no surprise that pterostilbene has been shown to be as potent as resveratrol or other polyphenols, such as quercetin. Recently, pterostilbene and resveratrol were used to explore their effects on the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse epidermis (Cichocki et al., 2008). Both resveratrol and pterostilbene were effective at reducing pro-carcinogenic processes such as activator protein-1 and NF-κB. There was also reduced activation of cyclooxygenase-2 (COX-2) and iNOS with both polyphenols. In most assays run in the study, pterostilbene was as effective as resveratrol or significantly more potent than resveratrol (Cichocki et al., 2008). Although, as stated previously, pterostilbene was metabolized in a similar manner as resveratrol the authors suggested the greater potency of pterostilbene might be due to its higher bioavailability via methylation.

The Effects of Pterostilbene on IR and Factors of Metabolic Syndrome

Three phenolic constituents of the heartwood of *Pterocarpus marsupium,* marsupsin, pterosupin, and pterostilbene, were given to hyperglycemic rats. Marsupsin and pterostilbene significantly lowered the blood glucose level, with effects comparable to metformin (Manickam et al., 1997). Pterostilbene was also found to lower plasma lipoprotein and cholesterol in hypercholesterolemic hamsters and to be an agonist for peroxisome proliferator-activated receptor alpha (PPAR-α) (Rimando et al., 2005). It was investigated whether resveratrol and three analogues, one of which is pterostilbene, would activate the PPAR-α, which may mediate the effects of fibrates. Resveratrol and its analogues were compared to ciprofibrate in H4IIEC3 cells. Of the analogues, pterostilbene resulted in the highest amount of PPAR-α induction. In fact, maximal induction with pterostilbene was higher than with ciprofibrate. Hypercholesterolemic hamsters were given pterostilbene, which resulted in lowered LDL and plasma glucose as well as increased HDL when compared to controls (Rimando et al., 2005).

Pterostilbene was used in streptozotocin-nicotinamide-induced diabetic rats to assess anti-oxidant status (Satheesh & Pari, 2006). Superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and reduced glutathione were significantly decreased in liver and kidney of diabetic animals when compared with normal control. Pterostilbene (40 mg/kg) treatment for six weeks significantly improved the activities of these, and lipid peroxidation was in the liver and kidneys were normalized, showing the anti-oxidant activity of pterostilbene (Satheesh & Pari, 2006).

Another study using diabetic rats was done to examine the effects of pterostilbene on key enzymes in hepatic glucose metabolism, and the action of pterostilbene was

compared to that of metformin (Pari & Satheesh, 2006). Rats were given streptozotocin-nicotinamide to induce diabetes then were administered pterostilbene orally. Six weeks of pterostilbene (40 mg/kg) significantly decreased plasma glucose and increased plasma insulin levels diabetic rats. Pterostilbene also significantly reduced glycosylated hemoglobin and increased total hemoglobin levels. Hepatic hexokinase was significantly increased. Furthermore, glucose-6-phosphatase and fructose-1,6-bisphosphatase were significantly decreased (Pari & Satheesh, 2006).

Summary

It appears that exercise and insulin exert their effects on glucose transport via integration of different pathways that converge at a common point in the cascade at AS160, and that AS160 is regulated by the action of 14-3-3 (Howlett., Sakamoto, Garnham, Cameron-Smith & Hargreaves, 2007; Kramer et al., 2006; Ramm et al., 2006). Thus, the glucose uptake in response to exercise does not actually improve insulin sensitivity, but causes translocation of GLUT4 via a non-insulin-dependent pathway. Therefore, in regards to glucose uptake via muscle contraction, the term insulin sensitivity is a misnomer. Regardless, exercise improves the ability of GLUT4 to facilitate glucose entry into skeletal muscle.

As stated previously, AMPK is one mechanism by which glucose disposal is increased during exercise. However, the role of AMPK appears to be dependent on work intensity. Activation of AMPK, at rest and during low intensities (>40% VO_2), results in the phosphorylation of ACC, activation of PGC-1 and different PPARs, as well as increased levels of CPT-1. Together this sequence of events leads to increased utilization of FA as a fuel source in the short term and increased mitochondrial biogenesis in the

long term. Thus, AMPK plays an integral role in the metabolic milieu and the switching of substrates as a fuel source. Although, data in humans is scarce, studies in murine models using PM have demonstrated a propensity to lower serum glucose, which could possibly extend the body's improved ability to dispose of glucose during and after exercise. Furthermore, PM and isolated compounds from PM have been purported to not only decrease inflammation, improve lipid profiles, and decrease glycosylated hemoglobin, but more importantly to increase activation of AMPK, independent of exercise. This information, coupled with the fact that IR individuals have abnormalities in lipid and glucose metabolism, have an attenuated AMPK response to exercise, and have reduced basal activation of PGC-1; it is clear that any increase in AMPK activity could be beneficial. Moreover, since PM has been purported to decrease inflammation, improve lipid profiles, and decrease glycosylated hemoglobin, to name a few, if PM functions in *in vivo* human models via increasing AMPK as research suggests, administering PM may also provide an alternative means of preventing or delaying the progression of IR to diabetes and to improve postexercise substrate utilization.

CHAPTER THREE

Methods

Participants

Sixteen obese or overweight sedentary, prediabetic as determined by handheld glucometer, [fasting plasma glucose: 100-125 mg/dL] premenopausal females between 18 and 40 years of age were recruited and finished this study. Initially, nineteen obese or overweight, sedentary women between the ages of 18 and 40 years of age started the study. However, three participants dropped out of the study. Two participants were in the PL group, and one participant was in the PM group. Of those that dropped out, two did not finish the study due to time constraints, while the third did not provide reason for dropping out of the study. Participants were declared sedentary if they did not participate in any form of exercise for 30 minutes at least 5 days a week for a minimum of one year. Additionally, all participants were not on any form of oral contraceptives for at least two months prior to study commencement; oral contraceptives appear to reduce glucose tolerance by reducing peripheral tissue insulin sensitivity (Kim et al., 2002). Prior to participation in the study, all participants provided written physician approval. Participants who qualified for the study were cleared for participation by successfully completing a series of health screening examinations (i.e., health screening questionnaire, blood pressure assessment, fasting glucose assessment) by study personnel.

Participants were not allowed to participate if they met any of the following criteria: 1) had any metabolic disorders including known electrolyte abnormalities; heart disease, arrhythmias, diabetes, thyroid disease or hypogonadism; a history of

uncontrolled hypertension, hepatorenal, musculoskeletal, autoimmune, or neurological disease; if they were taking thyroid, hyperlipidmeic, hypoglycemic, or antihypertensive; 2) had ingested any thermogenic, anti-oxidant, or phytonutrient nutritional supplements for a 2-month time period prior to beginning the study and did not take any additional nutritional supplement (except a vitamin/mineral supplement) or contraindicated prescription medication during the protocol. All participants meeting entrance criteria signed informed consent statements in compliance with the Human Participants Guidelines of Baylor University and the American College of Sports Medicine.

Study Site

All testing sessions were conducted in the Exercise & Sport Nutrition Laboratory (ESNL) and the Exercise & Biochemical Nutrition Laboratory (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University.

Independent and Dependent Variables

The independent variables were nutritional supplementation (either placebo or *Pterocarpus marsupium*), graded exercise test, and oral glucose tolerance test. Dependent variables included body weight, measurements of glucose and insulin, muscle gene expression of Akt2, AMPK, AS160, PPAR -α and -γ, and PGC-1α, serum clinical safety markers, uric acid, triglycerides, total cholesterol, low-density lipoprotein, and high-density lipoprotein.

General Study Overview

Table 1 presents the general research design and time course of assessments administered in this study. Participants expressing interest in participating in this study

were interviewed on the phone to determine whether they appeared to qualify for participation in this study. Participants believed to meet eligibility criteria were then invited to attend an entry and familiarization and presupplementation testing session. The entry and familiarization session included a thorough medical history, signing an informed consent, a fasting glucose test, as well as an exercise history form. The presupplementation testing session included a second fasting glucose test, measurement of body mass, total body water, body composition, and resting HR and BP. Additionally, baseline blood and muscle samples were sampled. Before leaving the laboratory, the participants were assigned to a randomized, double-blinded group. The participants either received a seven-day supply of *Pterocarpus marsupium* or placebo. Participants supplemented for seven days. However, on the sixth day the participants returned to the lab for an OGTT, and on the seventh day returned for a GXT.

Familiarization and Initial Glucose Screening

Participants expressing interest in participating in this study were invited to attend a familiarization and initial glucose screening session. Participants were instructed to refrain from exercise for 48 hours and fast for 8 - 12 hours prior to pretesting and for all scheduled assessments. On reporting to the lab, participants completed a medical history questionnaire and personal information forms regarding exercise habits and health status (Appendix A and B) and underwent a general physical examination to determine whether they met eligibility criteria. Participants were required to obtain medical clearance from their personal physician prior to participating in baseline assessments. Once medical clearance was obtained, participants were familiarized to the study protocol via a verbal and written explanation outlining the study

design. Body mass was measured to ensure participants met the entry criteria for BMI (≥ 25).

Table 1

Treatment Timeline

Familiarization Session Testing (T0)	Pre-Supplementation Testing (T1)	Days 1-5 Pre-Testing Supplementation	Day 6 Oral Glucose Tolerance Testing (T2)	Day 7 Exercise Testing (T3)
Familiarization session & Informed Consent	Blood glucose screening	Begin supplementation with placebo, or pterostilbene	Body Mass/ HR/BP/ Reported Side Effects Questionnaire	Body Mass/ HR/BP/ Reported Side Effects Questionnaire
Review medical history Blood glucose screening	Body Mass/ BIA/DEXA HR/BP/ Reported Side Effects Questionnaire	Monitor dietary intake	Blood & muscle biopsy sample	Blood & muscle biopsy sample
Complete paperwork. Health Status	Pre-supplementation blood & biopsy sampling Randomized, double-blind, placebo-controlled assignment		Glucose (75g) administration	Graded exercise test (GXT)
			30min post-glucose blood sample	30min post-exercise blood sample
			1hr post-glucose blood & biopsy sample	1hr post-exercise blood & biopsy sample
			2hr post-glucose blood sample	2hr post-exercise blood sample
			Continue supplement intake & dietary monitoring	

Fasting blood glucose was assessed using a ReliOn® Ultima glucometer and testing strips (ReliOn®; Bedford, MA;) to determine if participants were pre-diabetic

(glucose: 100-125 mg/dL). The instruction manual for the ReliOn® Ultima glucometer and testing strips states that results typically vary by no more than 2.9% to 5.1%. The means, standard deviations (SD), and percent coefficient of variation (CV) (mean ± SD (CV)) normative values, for mid-low to mid-high range glucose samples, as reported by the ReliOn® Ultima manufacurer, are 76 ± 3.0 (3.5%) and 218 ± 7.0 (3.4%). Participants meeting all entry criteria were familiarized to the study protocol by way of a verbal and written explanation outlining the study design. After meeting all inclusion criteria and familiarization of the study, participants signed university-approved informed consents granted by the Institutional Review Board for Human Subjects of Baylor University (Appendix C). Participants were then asked to return to the lab in two days for a second fasting blood glucose measurement to confirm pre-diabetic status based on fasting blood glucose as measured by a handheld glucometer. Baseline testing was done if the second fasting glucose measure was at or near 100 mg/dL.

Baseline Testing/Pre-Supplementation (Day 1- T1)

On reporting to the laboratory, fasting blood glucose was assessed again in an attempt to avoid a false classification of pre-diabetes. Once a fasting glucose level between 100-125 mg/dL had been confirmed, the baseline testing session proceeded. Height, total body mass (kg) (Cardinal Detecto Scale; Webb City, MO), waist circumference (a tension-regulated retractable cloth tape measure; GF Health Products Inc.; Atlanta, GA), and intracellular, extracellular, and total body water (TBW) (BIA; Xitron Technologies Inc.; San Diego, CA) were measured. The BIA has been shown to be acceptable for assessing short-term changes in TBW within individuals over time with a precision of ±0.5% TBW (Wells et al., 1999). Percent body fat, fat mass, and fat-free

masses were determined using Dual Energy X-ray Absorptiometer (DEXA; Hologic® Discovery™ DEXA - QDR Series; Bedford, MA). The accuracy of the DEXA is approximately ±2.9% in men and ±2.6% in women for fat mass, lean mass, and total mass as assessed by direct comparison with hydrodensitometry and scale weight (Prior et al., 1997).

Following anthropometeric measurements, participants had resting heart rate and blood pressure measured using standard procedures. Participants then donated approximately 10-15 ml of fasting blood using venipuncture techniques of an antecubital vein in the forearm according to standard procedures. Blood samples were allowed to stand at room temperature for 10 min and then centrifuged. For each sample, the plasma and/or serum was removed and frozen at -80°C for later analysis. Percutaneous muscle biopsies (10-15 mg), using a fine needle aspiration technique, were obtained from the vastus lateralis muscle. After removal, adipose tissue was trimmed from the muscle specimens and immediately frozen in liquid nitrogen and then stored at -80°C for later analysis.

Supplementation

Following baseline measurements, subjects were matched based on body composition and randomly assigned in a double-blind fashion to ingest capsules containing 250 mg cellulose placebo twice daily or 250 mg of *Pterocarpus Marsupium* twice daily, at breakfast and at noon, for the duration of the study. *Pterocarpus Marsupium* extract sold under the name Silbinol® (Sabinsa Corporation; Piscataway, NJ) and Nutricology® cellulose (Nutricology Inc.; Alameda, CA) placebo supplements were prepared in Capsuline® vegetarian gel capsules using a Capsuline® 60 capsule filling

machine (Capsuline Inc.; Pompano Beach, FL). Sabinsa Corporation provided documentation that *Pterocarpus marsupium* was 5.4% pterostilbene and 0.27% epicatechin as confirmed by HPLC. Supplements were prepared in gel capsule form and packaged generically for double blind administration. Supplementation compliance was monitored by having the participants fill out supplement logs recording the amount of supplement ingested during each day of the supplementation period as well as bringing remaining capsules to the second testing session (T2). Participants were instructed to consume the supplement for 5 days prior to the second testing session. Participants also filled out a reported side effects questionnaire at baseline, T2, and T3.

Oral Glucose Tolerance Test (Second Testing Session – T2)

Participants refrained from exercise for 48 hours and fasted for 8 - 12 hours. Upon arrival, participants ingested their respective supplement then had total body mass, total body water (total, intracellular, and extracellular), waist circumference, heart rate, and blood pressure assessed. Next, a baseline blood sample (15 ml) was drawn using standard phlebotomy techniques and muscle tissue was sampled from the vastus lateralis muscle (15-20 mg) using fine needle aspiration techniques. The participant then ingested an oral dosage of 75 g of glucose solution (Casco-Nerl TRUTOL Glucose Tolerance Beverages; East Rutherford, RI) within a five-minute time frame. Participants were instructed to remain in a rested state during the two hours following the glucose administration. Additional blood samples (5 ml) were drawn at 30, 60, and 120 minutes post-ingestion while muscle tissue (15-20 mg) was sampled as 60 minutes post-ingestion. Blood samples were analyzed for glucose and insulin levels to measure glucose clearance levels.

The OGTT was to determine the amount of time it took a bolus of glucose to be cleared from the blood; individuals who are insulin resistant require a longer period of time and a greater amount of insulin to clear the same amount of glucose from their blood. Whole-body insulin sensitivity is best calculated from an equation that utilizes the reciprocal of the product of insulin and glucose concentrations (Matsuda & De Fronzo, 1999). In normal, non-insulin resistant individuals fasting plasma glucose levels should be below 6.1 mmol/l (109.8 mg/dL). Impaired fasting glycemia is between 6.1 and 7.0 mmol/l (109.8 and 126 mg/dL). Levels above 7.0 mmol/l (126 mg/dL) are indicative of diabetes mellitus. Two-hour glucose levels should be below 7.8 mmol/l (140.4 mg/dL). Levels between 7.8 and 11.1 mmol/l (140.4 and 199.8 mg/dL) are indicative of impaired glucose tolerance, while glucose levels above 11.1 mmol/l (199.8 mg/dL) are diagnostic of diabetes mellitus.

Quantification of Insulin Resistance

The homeostasis model assessment of IR (HOMA IR) was used to quantify IR from fasting serum. The following formula was used for HOMA-IR: (fasting plasma glucose x fasting plasma insulin)/405 (Matthews et al., 1985). After the OGTT, insulin resistance was determined with the Matsuda OGTT insulin sensitivity index using OGTT insulin values (Matsuda & DeFronzo, 1999). The Matsuda OGTT insulin sensitivity index has been validated against the hyperinsulinemic, euglycemic clamp in research ($r = 0.73$, $P < 0.0001$) (Matsuda & DeFronzo, 1999). For this whole body model (hepatic and peripheral) of insulin resistance, the composite insulin sensitivity index is:

$$\frac{10000}{\sqrt{[(FSG \times FSI) \times (\mu OGTT_{[glucose]} \times (\mu OGTT_{[insulin]})]}}$$

where FSG is the fasting serum glucose concentration, FSI is the fasting serum insulin concentration, and μOGTT[glucose] and μOGTT[insulin] are the respective mean serum glucose and insulin concentrations during the OGTT (Matsuda & DeFronzo, 1999).

Acute Exercise Bout (Testing Session 3 – T3)

Twenty-four hours following T2 testing, participants returned to the laboratory in a fasted state of (8-12 hours.) Upon arrival, participants ingested their respective supplement then had total body mass, total body water (total, intracellular, and extracellular), waist circumference, heart rate, and blood pressure assessed. Next, a baseline blood sample (15 ml) was drawn using standard phlebotomy techniques and muscle tissue was sampled from the vastus lateralis muscle (15-20 mg) using the fine needle aspiration technique. Each participant completed a graded exercise test (GXT), and the test was performed until volitional exhaustion. Additional blood samples (5 ml) were drawn at 30, 60, and 120 minutes post-ingestion while muscle tissue (15-20 mg) was sampled as 60 minutes post-ingestion.

For the GXT, participants were first seated next to the treadmill and resting heart rate and blood pressure were measured. A sterile mouthpiece attached to a head harness was secured on the participant. The participant then had a nose clip placed on their nose. Once the participant was ready to begin the test protocol, she straddled the treadmill with both legs while the treadmill was turned on at a speed of 2.0 mph and at a 0% grade. The participant then used one foot to repeatedly swipe the belt in order to gauge the speed of

the motion. Once the participant was familiar with this speed, she stepped onto the belt while still gripping the handrail with both hands. Once the participant became comfortable walking on the treadmill, she let go of the handrail and began walking freely. The participant then performed a standard symptom-limited Modified Balke treadmill maximal exercise test using the protocol in Table 2. Only participants who were low-to-moderate risk with no contraindications to exercise, based on the American College of Sports Medicine (ASCM) guidelines, were eligible to exercise.

Table 2

Modified Balke Protocol

Stage	Speed	Grade (%)	Duration (min.)
1	3.4	0	1
2	3.4	1	1
3	3.4	2	1
4	3.4	3	1
5	3.4	4	1
6	3.4	5	1
7	3.4	6	1
8	3.4	7	1
9	3.4	8	1
10	3.4	9	1
11	3.4	10	1
12	3.4	11	1
13	3.4	12	1

Resting expired gases were collected using the Parvo Medics 2400 TrueMax Metabolic Measurement System (Sandy, UT). From these gases the participant's oxygen uptake (VO_2) and respiratory exchange ratio (RER) was measured throughout the duration of the test to assess their aerobic capacity and substrate utilization. Based on the ACSM guidelines, HR, BP, and a 12-lead electrocardiogram (ECG) were used to ensure

no contraindications existed before the GXT. Heart rate, BP, and ECG were also monitored during the GXT.

Muscle Biopsy Sampling

Percutaneous muscle biopsies (10-15 mg) using a fine needle aspiration technique were obtained from the middle portion of the vastus lateralis muscle of the leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. The leg in which the initial biopsy was performed was randomly assigned. Biopsies for T2 and T3 were performed alternating from the randomly chosen leg to the opposite leg and then back to the initial leg. For subsequent biopsies, muscle tissue was extracted from the same location by using the previous location and depth markings on the needle. Pre-biopsy preparations included removing hair from the procedure area and wiped with 70% isopropyl alcohol prep pads. The biopsy site was sterilized with a 10% povidone-iodine solution and anesthetized with 1 ml of 1% Lidocaine. After local anesthesia, a pilot puncture was made with an 18-gauge needle, after which a 16-gauge biopsy needle was inserted. Once the muscle sample had been obtained, pressure was immediately applied to the biopsy site and an adhesive bandage immediately applied. Bleeding was minimal due to the small puncture-type opening; therefore, only a standard band-aid type bandage was needed to cover the puncture. The biopsy needles were discarded as hazardous waste in an appropriately labeled plastic sharps container.

Post-biopsy, participants were instructed to leave the band-aid in place for 12 hours (unless unexpected bleeding or pain occurred) and asked to contact the lab immediately if they felt there was a problem. The time course of the study allowed for daily follow-up to occur, in order to ensure all biopsy locations healed correctly and were

free of infection. After the testing sessions, the participants were advised to refrain from vigorous physical activity during the first 24 hours post-biopsy to minimize pain and possible bleeding of the area. If needed, the participant was instructed that non-prescription analgesic medication such as Acetaminophen could be ingested to relieve pain if needed. However, medications such as aspirin, Nuprin, Bufferin, or Advil were discouraged as these medications may lead to ecchymosis at the biopsy site. Soreness of the area typically occurred for approximately 24 hours post-biopsy.

Assessment of Hemodynamic Safety Markers

Resting hemodynamic safety markers (HR and BP) were assessed at the beginning of each testing session. Heart rate was determined by palpation of the radial artery using standard procedures. Blood pressure will be assessed in the seated position using standard procedures with an aneroid sphygmomanometer

Venous Blood Sampling

Participants donated approximately 3-4 teaspoons of fasting venous blood (approximately 10-15 milliliters) during each baseline blood draw, approximately 5 mL during the post-OGTT blood draws, and approximately 10-15 mL during the post-GXT blood draws. All blood samples were obtained from the antecubital vein using standard phlebotomy procedures by study personnel trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. The phlebotomists and lab technicians wore personal protective clothing (gloves, lab coats, etc.) when handling blood samples. Subjects were seated in a phlebotomy chair. Their arm was cleaned with a sterile alcohol wipe and sterile gauze. A standard rubber

tourniquet was then placed on the brachium (upper arm) and was tight enough to visibly indent the skin, but not cause the participant discomfort. A 22-gauge sterile needle attached to a plastic Vacutainer™ holder was inserted into the vein using standard procedures. Two plasma tubes and one serum separation tube were used in succession for blood collection. Once samples were obtained, the needle was removed and discarded as hazardous waste in a plastic sharps container.

The site of the blood draw was cleaned with a sterile alcohol wipe and gauze and a sterile Band-Aid will be placed on the site. The blood collection tubes were labeled and placed in a test tube rack. Laboratory technicians (who had received blood borne pathogen training and were wearing personal protective clothing) allowed the serum to clot for 10-mintues, centrifuged the serum and/or plasma samples at 2,400 rpm for 15 minutes, and then stored the serum at -80 degrees C.

Serum Analysis

Insulin (intra-assay CV 5.3%; inter-assay CV 5.6%) was analyzed using a commercially available enzyme-linked immunoabsorbent assay (ELISA) kit (catalog 0030, Alpha Diagnostic International, San Antonio, TX,). The serum samples were diluted 1:10 with diluent buffer and added to the pre-coated plate. One hundred µl of antibody-enzyme conjugate was dispensed into each well and then left to incubate for 30 minutes using a Jitterbug orbital microplate shaker (Boekel Scientific, Philadelphia, PA). The plate was then washed 5 times with 300 µl in each well with a MultiWash Advantage automated microplate washer (Tri Continent Scientific, Grass Valley, CA). Next, 200 µl of HRP-substrate mix was dispensed into each well and allowed to incubate at room temperature for 15 minutes. Finally, 50 µl of stop solution was added to all wells and the

plate was immediately read using a Wallac Victor^{2} ^{TM}1420 multilabel microplate reader (Turku, Finland) at wavelength of 450 nm. All standards, controls, and unknowns were assayed in duplicate, and Mikrowin 2000 (Mikrotek Laborsysteme, Germany) data-reduction software was used to quantify insulin concentration.

Serum and plasma samples were sent to Quest Diagnostics (Irving, TX) for analysis of glucose, total protein, blood urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, AST, ALT, GGT, albumin, globulin, sodium chloride, calcium, carbon dioxide, total bilirubin, alkaline phosphatase, LDL, HDL, total cholesterol, triglycerides, and HDL/cholesterol ratio.

Assessment of Metabolic Gene Expression

Based on previous guidelines (D. S. Willoughby & Wilborn, 2006), approximately 10 mg of muscle tissue was placed in a microfuge tube and homogenized with a plastic pestle in 500 µl of monophasic solution of phenol and guanidine isothiocyanate contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). The muscle homogenate was allowed to stand for five minutes at room temperature to allow for the complete dissociation of nucleoprotein complexes at which point 100 µl of chloroform was added. The solution was mixed and allowed to sit at room temperature for ten minutes and then centrifuged at 12,000 x g for 15 minutes. The supernatant was then removed and transferred to a new microfuge tube, and then 250 µl of isoproponal was added. The solution was mixed and allowed to sit at room temperature for ten minutes and centrifuged at 12,000 x g for ten minutes. The supernatant was then discarded and the RNA pellet was washed with 500 µl 75% ethanol and centrifuged for five minutes at 7,500 x g. The ethanol wash was repeated a second time at which point

the supernatant was removed and the RNA pellet allowed to air dry for ten minutes prior to being dissolved in 50 µl of nuclease-free water. The RNA samples were stored at -80°C until later analysis.

The RNA extracted from each muscle sample was reverse transcribed to generate cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). A reverse transcription reaction mixture [2 µg of cellular RNA, 5x reverse transcription buffer (20 mM Tris-HCL, pH 8.3; 50 mM KCl; 2.5 mM MgCl2; 100 µg of bovine serum albumin/ml), a dNTP mixture containing 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.8 µM MgCl2, 0.5 µg/µl of oligo(dT) 15 primer, and 25 u/µg of MMLV RNAase H+ reverse transcriptase enzyme (Bio-Rad, Hercules, CA, USA)] was added to 2 µg of skeletal muscle RNA and incubated at 25°C for five minutes, 42°C for 30 minutes, and then heated to 85°C for ten minutes. The cDNA concentration was determined spectrophotometerically using (Helio γ, Thermo Electron, Milford, MA) by optical density (OD) at 260 nm using an OD_{260} equivalent to 50 µg/µl (D. S. Willoughby, Stout, J. R., & Wilborn, C. D., 2007) and the starting cDNA template concentration was standardized by adjusting all samples to 200 ng prior to amplification. The cDNA was then stored at -80°C until later analysis.

Oligonucleotide Primers for PCR

The mRNA sequences of human skeletal muscle genes (GLUT4, AMPK, PPAR -α and -γ, and PGC-1-α) published in the NCBI Entrez Nucleotide database (www.ncdi.nlm.hih.gov) were used to construct oligonucleotide PCR primers using Beacon Designer software (Bio-Rad, Hercules, CA, USA). Table 3 shows the sense

sequence, anti-sequence, amplicon size, and NCBI accession number for each gene assessed in the study. The sense and anti-sense primers were synthesized commercially (Integrated DNA Technologies, Coralville, IA). β-actin was used as an external control standard for each reaction due to its consideration as a constitutively expressed "housekeeping gene," and the fact that it has been shown to be an appropriate external reference standard in real-time PCR in human skeletal muscle following acute exercise (Willoughby, Stout, & Wilborn, 2007).

Real-Time PCR Amplification

Based on previous guidelines (Willoughby & Wilborn, 2006), 200 ng of cDNA were added to each of the 25 μl PCR reactions using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

Table 3

Oligonucleotide Primers used for RT-PCR

Primer Name	NCBI Accession Number	Sense Sequence (5' → 3')	Anti-Sense Sequence (5' → 3')	Amplicon Size (bp)
β-Actin	NM_001101	ATC GTG GAC ATT AAG	GTC ATC ACC ATT GGC AAT	102
AMPK alpha2	EF056019	TGA TGA TGA AGT GGA GCA GAG	GCC AGT GAG AGA GCC AGA AAG	139
GLUT4	M20747	CCA TTG TTA TCG GCA TTC	GGA TGA TGT AGA GGT AGC	165
PGC-1α	NM_013261	GGA GAG GCA GAG GCA GAA GG	AAG CAT CAC AGG TAT AAC GGT AGG	191
PPARγ	L40904	ATG ACA GAC CTC AGA CAG ATT G	ATT GTT GGC AGT GGC TCA G	148
PPARα	EU395809	GGC GAG GAT AGT TCT GGA AGC	CAC AGG ATA AGT CAC CGA GGA G	132
AS160	AB449885	AGC AAT GAG TCC CTA AGT G	CTC GTT CCT GTC CAA TCC	144
AKT2	M95936	GTC GCC AAC AGC CTC AAG	ACC GCC ACT TCC ATC TCC	104
IRS-1	NM_005544	GAG AGC AGC GGT GGT AAG	GGC AAT GAG TAG TAG GAG AGG	149

Each reaction contained the following mixtures: [10x PCR buffer, 0.2 μM dNTP mixture, 2.0 μM of a cocktail containing both the sense and antisense RNA oligonucleotide primers, 2 mM $MgCL_2$, 1.0 u/μl of hot-start iTaq DNA polymerase, SYBR Green I dye, and nuclease-free dH_2O], and was amplified using real-time quantitative polymerase chaine reaction (PCR) (iCycler IQ Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA). The amplification profile was run for 40-cycles employing a denaturation step at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. Fluorescence was measured after each cycle, resulting from the incorporation of SYBR green dye into the amplified PCR product. To help control for differences in amplification efficiency during thermocycling, all PCR reactions were each gene were prepared from the same stock solution. The specificity of the PCR was demonstrated with absolute negative controls using separate PCR reactions containing no cDNA template or primers. A single gene product was confirmed using DNA melt curve analysis. The relative expression of mRNA was assessed by determining the ratio between the C_T values of each target mRNA and the C_T values for β-actin for each muscle sample obtained at each of the three testing sessions. Test-retest reliability of performing this procedure of mRNA expression on samples in this laboratory has demonstrated low mean coefficients of variation and high reliability (1.6%, intraclass $r = 0.95$).

Qualitative Gene Expression: Gel Electrophoresis Imaging

To verify positive amplification, aliquots (20 μl) of the PCR reaction mixtures were electrophoresed in 1.5% agarose gels in 1X Tris-Acetate-EDTA (TAE) buffer. As shown in Figure 2, the gel was stained with ethidium bromide (present in the TAE buffer

at 1 μg/ml) and illuminated with UV transillumination (Chemi-Doc XRS, Bio-Rad, Hercules, CA, USA). From the left, the molecular weight marker, β-actin, AMPK, PPARα, PGC-1α, PPARγ, GLUT4, Akt2, AS160 were loaded into the 1.5% agarose gel.

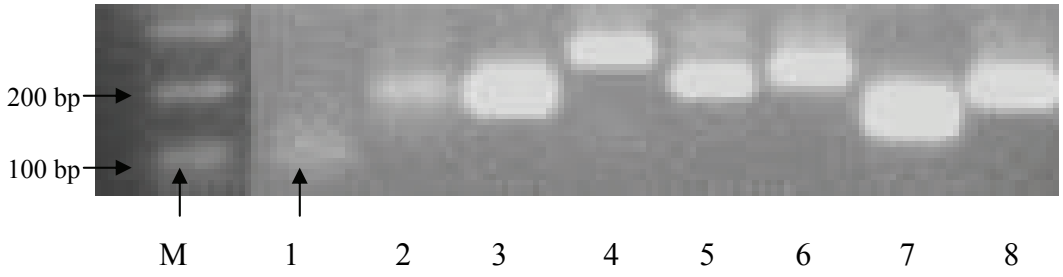

Figure 2. Illustration of PCR amplicons for the mRNA targets for one study participant at all four time points run on a 1% agarose gel. M = base pair marker, 1 = β-Actin, 2 = AMPK, 3 = PPARα, 4 = PGC-1α, 5 = PPARγ, 6 = GLUT4, 7 = Akt2, 8 = AS160.

Dietary Records

In order to assess the average daily macronutrient consumption of fat, carbohydrate, and protein, the participants were required to keep four-day dietary records during the supplementation period. The four-day food log consisted of three-week days and one-weekend day. The dietary records were analyzed with the Food Processor Dietary Assessment Software program (ESHA Research Inc., Salem, OR).

Reported Side Effects from Supplementation

To determine whether the participants suffered any negative side effects from the control or experimental supplements, participants reported by questionnaires administered in a confidential manner whether they tolerated the supplement, supplementation protocol, as well as reported any medical problems/symptoms they may have encountered throughout the supplementation period.

Statistical Analysis

For blood and muscle samples taken at baseline, T1, T2, and T3, a 2 x 3 (Group x Time) factor multivariate analysis of variance (MANOVA) with repeated measures on the time factor was used to determine differences between groups. Differences between groups in muscle gene expression at T2 and T3 were determined via a 2 x 2 factorial MANOVA for repeated measures. Differences between groups in blood variables at T2 were determined via a 2 x 4 factorial MANOVA for repeated measures. Differences between groups in glucose variable at T3 were determined using a 2 x 4 factoral Analysis of Variance (ANOVA) for repeated measures. For all MANOVAS and the ANOVA, the two factors were group (PM or PL) and time. An independent sample t-test was used to analyze difference in Matsuda Index. Significant differences in mean values for main effects or interactions were determined by performing independent sample t-tests. The statistical software Predictive Analytics Software (PASW, formerly SPSS statistics) version 18.0 was used with an alpha (type I error rate) <0.05 used to determine statistical significance.

CHAPTER FOUR

Results

The purpose of the present study was to determine whether acute supplementation with PM extract would improve fasting glucose levels via the insulin dependent or non-insulin-dependent signaling pathway at rest, after an oral glucose tolerance test (OGTT), and after a single bout of exercise at maximal exertion. It also investigated whether acute administration of PM improved lipid profiles. Since limited studies exist using this extract in humans and even fewer exist that study the mechanism with which this extract functions, muscle specific expression of genes involved in glucose disposal, fuel utilization, and mitochondrial biogenesis were examined.

Participants

Sixteen, sedentary, overweight or obese females, qualified as prediabetic via handheld glucometer, signed informed consent statements, and were assigned to either a placebo (PL) or a supplement group (PM), and started experimental testing. Baseline demographics for the sixteen participants are presented in Table 4. At baseline, there were no significant differences between groups with regards to age ($p = 0.355$), height ($p = 0.817$), body weight ($p = 0.622$), percent body fat ($p = 0.587$), and BMI ($p = 0.973$).

Supplement Compliance

Participants were provided with a coded opaque envelope containing fourteen encapsulated cellulose placebo or fourteen encapsulated PM sealed in an airtight plastic bag. Participants recorded the time of ingestion for each capsule using four-day food

logs. All participants returned with the correct amount of capsules (four) following the five-day supplementation period. During the second and third testing sessions, participants were observed ingesting the remaining pills. Therefore, the supplement compliance rate was considered to be 100%.

Table 4

Study Participant Demographics

Variable	PL (n=8)	PM (n=8)
Age (yrs)	30 ± 5.31	28 ± 5.66
Height (cm)	161.29 ± 7.07	162.05 ± 5.77
Weight (kg)	105.19 ± 33.3	112.94 ± 27.88
BMI (kg/m^2)	40.12 ± 10.5	46.81 ± 4.84
Body Fat (%)	46.72 ± 5.26	46.81 ± 4.84

Note: Data are presented as means ± standard deviations

Dietary Analysis

All participants were instructed to consume their usual diet, but were required to keep a four-day food log during the week of the study. A one-way ANOVA was performed to assess differences in the groups for daily intake of total calories, fat, carbohydrate, and protein. There were no significant differences between groups in all dietary intake variables (see Table 5).

Reported Side Effects and Clinical Chemistry Markers

Reported side effects questionnaires administered at baseline, the second testing session, and final testing sessions revealed no side effects due to the supplementation. A

2 x 2 (group x time) factorial MANOVA with repeated measures was performed on clinical chemistry markers of kidney and liver function. The within-subjects factor was time with two levels (baseline = T1 and immediately before a GXT = T3). The between-subjects factor was group with two levels (PL or PM). Multivariate analysis did not reveal significant differences for group ($p = 0.915$), time ($p = 0.921$), or for a group by time interaction ($p = 0.616$). Data are presented in Table 6 below.

Table 5

Comparison of Nutritional Intake Variables between PL and PM Groups

Variable	PL (n=8)	PM (n=8)
Total Calories	2034 ± 647.68	1948 ± 691.93
Carbohydrates (g)	259 ± 89.25	249 ± 90.00
Protein (g)	79.90 ± 35.37	127 ± 156.60
Fat (g)	73.86 ± 21.85	72.70 ± 27.81

Note: Data are presented as means ± standard deviations

Blood Variable Analysis

A 2 x 4 (group x time) factorial MANOVA for repeated measures was used to investigate the effect of the PM supplementation on glucose regulation following OGTT. A 2 x 4 (group x time) factorial ANOVA was used to investigate the effects of PM supplementation on glucose regulation following GXT. Follow-up univariate ANOVA or t-tests were used to investigate differences between individual groups or time points—a Bonferroni correction was used in these cases. A t-test for independent samples was used to assess differences in $OGTT_{ISI}$ using the Matsuda index.

Table 6

Baseline Comparison of Serum Clinical Chemistry Variables between PL and PM Groups over the Course of the Study

Variable	PL		PM	
	T1	T3	T1	T3
Uric Acid (mg/dL)	5.22 ± 1.55	5.05 ± 1.69	6.22 ± 1.16	5.76 ± 0.69
BUN (mg/dL)	11.00 ± 3.46	12.88 ± 4.36	11.63 ± 3.89	9.33 ± 2.49
Creatinine (mg/dL)	0.73 ± 0.11	0.74 ± 0.14	0.78 ± 0.12	0.74 ± 0.14
Total Protein (g/dl)	7.08 ± 0.65	6.95 ± 0.56	7.34 ± 0.21	7.00 ± 0.36
Total Bilirubin (mg/dl)	0.35 ± 0.12	0.34 ± 0.18	0.33 ± 0.07	0.30 ± 0.08
ALP (U/L)	65.63 ± 21.50	67.13 ± 23.10	63.50 ± 21.69	58.43 ± 14.15
AST (U/L)	15.13 ± 5.11	16.25 ± 3.69	24.13 ± 12.68	22.57 ± 7.54
ALT (U/L)	17.88 ± 12.04	17.50 ± 10.11	32.50 ± 26.29	28.86 ± 14.54

Note: Data are presented as means ± standard deviations.

Baseline Glucose and Insulin Levels

Within-subjects factor was time with three levels (baseline = T1, prior to OGTT = T2, and prior to GXT = T3), and between-subjects factor was group with two levels (PL or PM). Machly's test for sphericity was not significant ($p > 0.10$); therefore, the null hypothesis and assumed sphericity. No significant differences were found between-subjects or within-subjects for serum glucose, serum insulin, or HOMA-IR, and no group by time interactions were present ($p > 0.05$). Results for glucose, insulin, and HOMA-IR are displayed in Table 7. Figure 3 shows changes in glucose and insulin over time.

Table 7

Baseline Comparisons of Serum Glucose, Insulin, and HOMA-IR between PL and PM Groups over the Course of the Study

Variable	*Group*	*T1*	*T2 Baseline*	*T3 Baseline*
Glucose	*PL*	95.63 ± 14.11	99.75 ± 18.67	98.88 ± 18.23
	PM	100.00 ± 14.60	96.62 ± 9.35	94.50 ±9.56
Insulin	*PL*	6.87 ± 4.76	9.57 ± 7.16	7.14 ± 4.86
	PM	6.89 ± 6.53	8.36 ± 7.51	8.87 ± 6.00
HOMA-IR	*PL*	1.71 ± 1.30	2.047 ± 1.43	1.83 ± 1.36
	PM	1.23 ± 0.62	1.92 ± 1.60	2.03 ± 1.28

Note. Data are presented as means ± standard deviations. All glucose values are expressed as mg/dL. All insulin values are expressed as ulU/mL. HOMA-IR is a ratio number.

Hypothesis one states that serum levels of fasting glucose will decrease further in the PM group compared to the PL group over the course of the study. While lower levels of fasting glucose were evident over time, the amount of improvement in PM was similar to the PL group, and thus no significance between groups was evident. As such, the null hypothesis was accepted. However, it is worth noting that while the average blood glucose levels did not change between groups, the variability appeared to change a between the groups. The variability improved in the PM group and became larger in the PL group over time. The SD for the PM and PL groups at T1 were 14.60 and 14.11, respectively, but by T3 the SD for the PM group was half of the SD for the PL group. Hypothesis two states that serum levels of fasting insulin will decrease further in the PM group than for PL group over the course of the study. Conversely, similar increases in serum insulin levels over time were evident in both PL and PM groups, and thus no significance between groups was found. As such, the null hypothesis is accepted since

there was no difference between groups for fasting serum insulin. The third hypothesis predicted that the PM group would have lower HOMA-IR ratios than the PL group. The PM group showed an increase in HOMA-IR from T1 to T3, but the there was not a significant change, nor a significant difference from PL. Therefore, the null hypothesis is accepted.

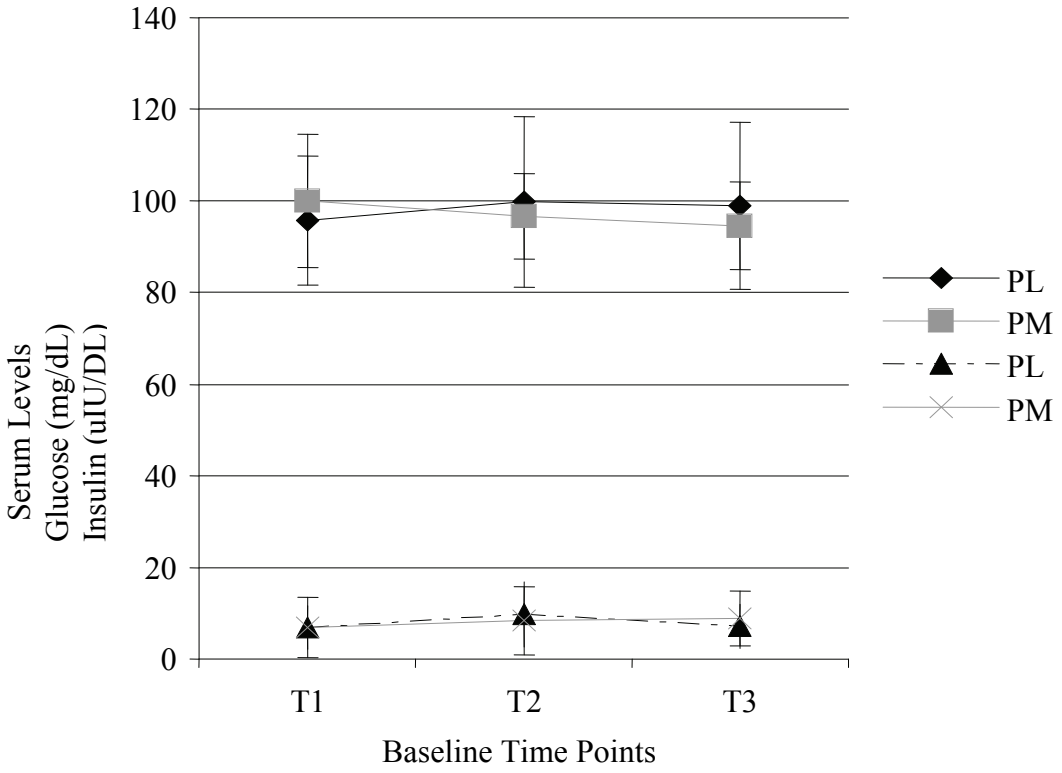

Figure 3. Changes in serum glucose are displayed in the top two lines and changes serum insulin are displayed in the bottom two lines. Data are presented as means ± standard deviations.

Baseline Serum Lipid Levels

Serum lipid variables are displayed in Table 8. The within-subjects factor was time with two levels (baseline = T1 and immediately before a GXT = T3). The between-subjects factor was group with two levels (PL or PM). Machly's test for sphericity was significant ($p < 0.10$), so the alternative hypothesis was accepted and sphericity could not

be assumed. Multivariate analysis revealed no main effect for time, group, or group by time interaction ($p > 0.05$) using Huynh-Feldt adjustment.

Table 8

Comparisons of Serum Lipid Levels between PL and PM Groups over the Course of the Study

Variable	*Group*	*T1*	*T3 Baseline*
Total Cholesterol	*PL*	168.63 ± 21.58	163.00 ± 18.45
	PM	176.63 ± 38.83	173.00 ± 33.34
HDL	*PL*	44.88 ± 15.03	42.63 ± 11.70
	PM	37.50 ± 9.47	38.29 ± 10.99
Total Cholesterol:HDL	*PL*	4.33 ± 2.31	4.15 ± 1.57
	PM	4.81 ± 0.94	4.71 ± 0.98
LDL	*PL*	95.75 ± 16.32	95.25 ± 19.39
	PM	111.88 ± 32.87	110.00 ± 28.32
Triglycerides	*PL*	132.88 ± 140.17	125.75 ± 98.18
	PM	135.75 ± 36.39	123.14 ± 20.21

Note. Data are presented as means ± standard deviations. All lipid variables are in mg/dL except total cholesterol:HDL.

Hypothesis eight states that fasting HDL levels will increase to a greater extent in the PM group compared to the placebo group over the course of the study. Though conflicting results were observed with fasting serum HDL levels increasing in the PM group and decreasing in the PL groups over time, no significant differences were detected between groups. Thus, the null hypothesis was accepted. The ninth hypothesis states that fasting serum LDL levels will decrease to a greater extent in the PM group compared to the placebo group over the course of the study. No significant changes in serum LDL

levels were detected in either group over time. As such, the null hypothesis was accepted.

Assessment of Blood Glucose and Insulin at T2 (OGTT)

Serum glucose and insulin variables are displayed in Figure 4. The within-subjects factor was time with four levels: prior to OGTT, 30 minutes post-glucose ingestion, 1 hour post-glucose ingestion, and 2 hr post-glucose ingestion. The between-subjects factor was group with two levels (PL or PM). Machly's test for sphericity was significant ($p < 0.10$) so the alternative hypothesis was accepted and sphericity was not assumed.

Multivariate analysis revealed a significant main effect for time ($p = 0.000$), with subsequent univariate analysis indicating a significant increase in plasma glucose ($p<0.001$) and insulin ($p=0.007$) levels over time. Pairwise comparison analysis showed a significant increase in plasma glucose levels from baseline to 30 minutes post-OGTT ($p<0.001$) and from baseline to 1 hour post-OGTT ($p=0.001$) Further, pairwise comparison analysis also showed a significant increase in plasma insulin levels from baseline to 30 minutes post-OGTT ($p=0.023$), baseline to 1 hour post-OGTT ($p=0.023$), and baseline to 2 hours post-OGTT ($p=0.003$). No significant group or group by time interaction was observed ($p > 0.05$).

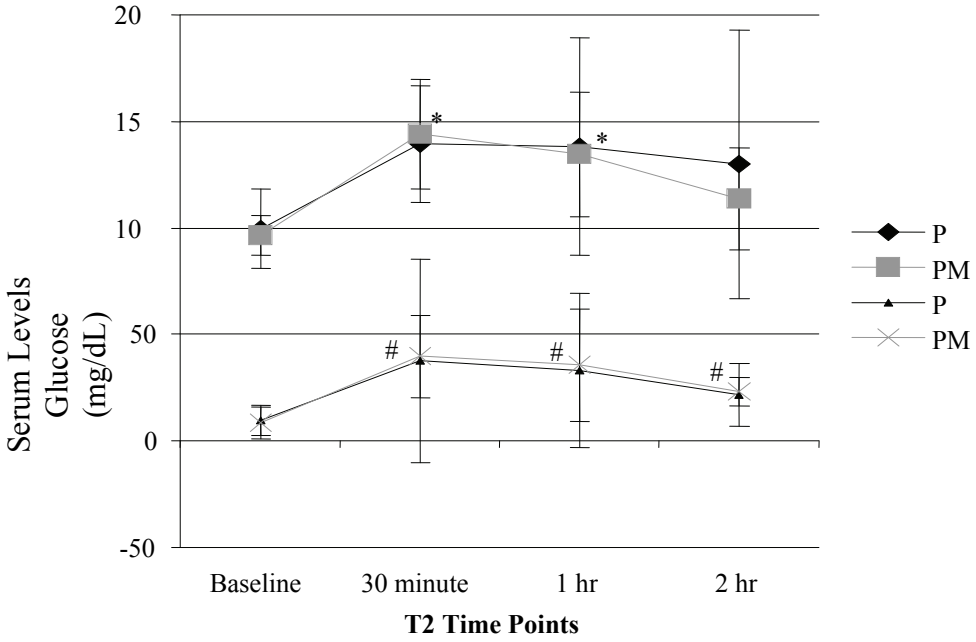

Figure 4. Changes in serum glucose are displayed in the top two lines and changes serum insulin are displayed in the bottom two lines. Data are presented as means ± standard deviations. * indicates a significant difference from baseline for glucose. # indicates a significant difference from baseline for insulin.

The fourth hypothesis stated that the PM group would display lower increases in overall serum glucose levels compared to placebo group during the OGTT. However, since a significant difference between the groups was not found. The null hypothesis was accepted. The null hypothesis was also accepted for the fifth hypothesis, which, states that the PM group would display lower increases in overall serum insulin levels compared to placebo group during the OGTT. A significant difference was not observed between the groups

Analysis of Matsuda's Insulin Sensitivity Index ($OGTT_{ISI}$)

Students t-test revealed no significant differences *(*$p = .986$*)* between the PL (9.14 ± 4.83) and PM (9.19 ± 5.63) groups. Hypothesis six states that the measure of insulin sensitivity ($OGTT_{ISI}$) will be higher in the PM group compared to placebo group during

the OGTT. Since no significant difference was found between groups, the null hypothesis was accepted.

Assessment of Serum Glucose Levels at T3 (GXT)

Figure 5 displays changes in glucose levels prior to, and following, the GXT. The between-subjects factor was group with two levels (PL or PM). The within-subjects factor was time with four time points: immediately prior to the GXT, 30-minutes post, one-hour post, and two hours post-GXT. Mauchly's test of sphericity was not significant ($p = 0.145$). Thus, the test was retained and sphericity was assumed. Univariate analysis showed a significant main effect for time ($p = 0.007$), with no group ($p = 0.549$) or group by time interactions ($p = 0.923$). Subsequent pairwise comparisons using a Bonferroni correction factor revealed a significant decrease in serum glucose levels from 30-minute to one-hour post exercise ($p = 0.011$), and from 30-minute to 2 hours post exercise ($p = 0.013$)

Hypothesis seven states that the PM group will display lower increases in serum glucose levels compared to the PL group following the GXT. Since no significant difference was found between groups at any time point, the null hypothesis was accepted.

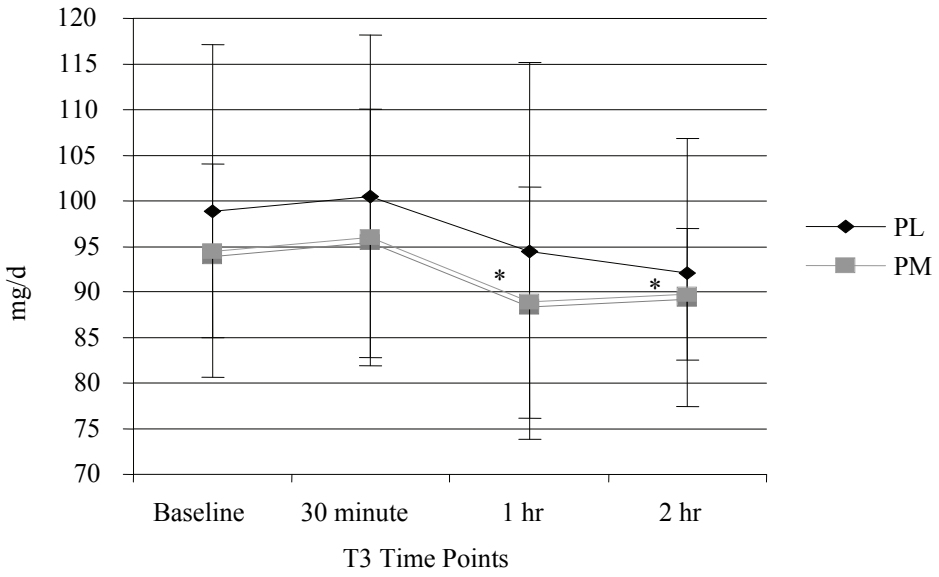

Figure 5. Changes in serum glucose for PL and PM groups prior to, and following the GXT are displayed. Data are presented as means ± standard deviations. * indicates significantly different from 30 minute glucose levels.

Analysis of Muscle-Specific Genes

Skeletal muscle genes involved in glucose, fatty acid metabolism, and mitochondrial biogenesis were analyzed in this study. More specifically, the mRNA expression of Akt2, AMPK, AS160, PPARα, PPARγ, and PGC-1α were determined. A 2 x 3 factorial MANOVA with repeated measures was conducted to evaluate the acute effects of supplementation on mRNA expression of the above genes at baseline, prior to OGTT, and prior to GXT. To further elucidate the acute effects of supplementation on the aforementioned muscle genes, two separate 2 x 2 factorial MANOVA with repeated measures was conducted prior to, and following an OGTT, and prior to, and following a GXT.

mRNA Expression of Muscle-Specific Genes

The within-subjects factor was time with three levels (baseline = T1, immediately before OGTT = T2, and immediately before a GXT = T3). The between-subjects factor was group with two levels (PL or PM). Table 9 displays baseline means (SD) at each baseline time point. Multivariate analysis did not reveal significance for group ($p = 0.499$), for time ($p = 0.904$), nor for a group by time interaction ($p = 0.537$).

Hypotheses 12 through 17 states that skeletal muscle gene expression of Akt2, AMPK, AS160, PPARα, PPARγ, and PGC-1α would be greater in the PM group compared to the PL group over the course of the study. However, no significant differences were found between groups. As such, the null hypothesis was accepted for hypotheses 12 through 17.

Muscle-Specific Gene Expression at T2 (OGTT)

The within-subjects factor was time with two levels (immediately before glucose ingestion = T1 and one-hour post-glucose ingestion = T2). The between-subjects factor was group with two levels (PL or PM). Multivariate analysis revealed a significant difference for time ($p = 0.006$), but did not reveal significance for a group effect ($p = 0.693$) or for a group by time interaction ($p = 0.444$). Univariate results were then further analyzed to determine which variables were significant. Mauchly's test ($p = 0.000$) for sphericity was retained, and sphericity was not assumed. A Huynh-Feldt adjustment was used. Univariare analysis revealed a significant increase in Akt2 ($p = 0.001$), AMPK ($p = 0.001$), AS160 ($p = 0.02$), and PPARα ($p = 0.025$) at one-hour post-OGTT.

Table 9

Baseline Comparisons of Muscle mRNA Expression of Genes between PL and PM Groups over the Course of the Study

Gene	Group Assignment	T1	T2	T3
Akt2	*PL*	0.94 ± 0.84	0.95 ± 0.06	0.96 ± 0.07
	PM	0.92 ± 0.10	0.92 ± 0.08	0.93 ± 0.09
AMPK	*PL*	1.11 ± 0.11	1.12 ± 0.11	1.12 ± 0.06
	PM	1.06 ± 0.09	1.04 ± 0.08	1.05 ± 0.09
AS160	*PL*	0.91 ± 0.07	0.93 ± 0.05	0.97 ± 0.09
	PM	0.90 ± 0.09	0.91 ± 0.05	0.89 ± 0.06
PPARα	*PL*	0.97 ± 0.07	1.00 ± 0.05	1.00 ± 0.05
	PM	0.95 ± 0.08	0.95 ± 0.05	0.94 ± 0.05
PPARγ	*PL*	1.06 ± 0.07	1.87 ± 2.26	1.07 ± 0.07
	PM	1.04 ± 0.10	1.09 ± 0.06	1.02 ± 0.07
PGC-1α	*PL*	1.00 ± 0.09	1.02 ± 0.09	1.06 ± 0.07
	PM	1.00 ± 0.15	0.97 ± 0.07	0.94 ± 0.08

Note. Data are presented as means ± standard deviations. All data is expressed as the ratio between the variable of interest and the internal standard (β-Actin).

Hypotheses 18 through 23 states that expression for muscle genes Akt2, AMPK, AS160, PPARα, PPARγ, and PGC-1α will be greater in the PM group compared to the PL group following the OGTT. However, no significant differences were found between

groups. As such, the null hypotheses were accepted for hypotheses 18 through 23. Table 10 displays changes for muscle gene expression prior to and one-hour post-glucose ingestion for both PL and PM groups.

Muscle-Specific Gene Expression at T3 (GXT)

Data for muscle gene expression across GXT is presented in Table 11. The within-subjects factor was time with two levels (immediately before GXT = T1 and one-hour post-GXT = T2). The between-subjects factor was group with two levels (PL or PM). Multivariate analysis revealed a strong trend for group ($p=0.061$). No main effect for time ($p = 0.143$) or group by time ($p = 0.969$) interaction was detected.

Hypotheses 24 through 29 states that expression for will be greater for PM group compared to the PL group following the graded exercise test GXT. The null hypotheses for 24 through 29 were accepted, as significance was not observed between the groups.

Table 10

Comparisons of Muscle mRNA Expression of Genes between PL and PM Groups prior to, and following, OGTT

Gene	Group Assignment	T1	T2
Akt2*	*PL*	0.96 ± 0.06	1.02 ± 0.12
	PM	0.92 ± 0.08	0.97 ± 0.08
AMPK*	*PL*	1.11 ± 0.11	1.16 ± 0.09
	PM	1.05 ± 0.08	1.14 ± 0.08
AS160*	*PL*	0.93 ± 0.05	0.97 ± 0.07
	PM	0.91 ± 0.05	0.96 ± 0.05
PPARα*	*PL*	0.99 ± 0.05	1.03 ± 0.07
	PM	0.95 ± 0.05	1.01 ± 0.07
PPARγ	*PL*	1.86 ± 2.27	1.03 ± 0.07
	PM	1.09 ± 0.06	1.05 ± 0.08
PGC-1α	*PL*	1.02 ± 0.09	0.99 ± 0.03
	PM	0.97 ± 0.07	1.04 ± 0.09

Note. Data are presented as means ± standard deviations. All data is expressed as the ratio between the variable of interest and the internal standard (β-Actin).
* indicates significant time effect.

Table 11

Comparisons of Muscle mRNA Expression of Genes between PL and PM Groups prior to, and one-hour following, GXT

Gene	Group Assignment	T1	T2
Akt2	*PL*	0.96 ± 0.07	1.00 ± 0.09
	PM	0.93 ± 0.08	0.95 ± 0.13
AMPK	*PL*	1.12 ± 0.06	1.17 ± 0.10
	PM	1.05 ± 0.09	1.10 ± 0.12
AS160	*PL*	0.97 ± 0.09	0.98 ± 0.05
	PM	0.89 ± 0.07	0.92 ± 0.07
PPARα	*PL*	1.01 ± 0.05	1.04 ± 0.07
	PM	0.94 ± 0.05	.98 ± 0.09
PPARγ	*PL*	1.07 ± 0.07	1.10 ± 0.10
	PM	1.02 ± 0.07	1.04 ± 0.10
PGC-1α	*PL*	1.06 ± 0.07	1.07 ± 0.07
	PM	0.94 ± 0.08	0.95 ± 0.06

Note. Data are presented as means ± standard deviations. All data is expressed as the ratio between the variable of interest and the internal standard (β-Actin).

CHAPTER FIVE

Discussion

The primary purpose of the current study was to examine the acute effects of *Pterocarpus marsupium* plant extract on blood lipid profiles and insulin resistance in overweight and obese, pre-diabetic, sedentary women. There were three additional aims of the study. First, to determine if an acute five-day ingestion of PM would enhance insulin-mediated glucose disposal up to 2 hours following an OGTT. Second, to determine if an acute six-day ingestion of PM would enhance exercise-mediated glucose disposal up to 2 hours following an aerobic exercise bout. Finally, to understand the mechanisms by which PM supplementation may elicit effects within the muscle, the expression of genes involved in insulin and non-insulin dependent glucose disposal and substrate metabolism was determined.

The major finding of the current study was that acute PM supplementation of 250 mg twice daily was unable to improve blood lipid levels, insulin sensitivity, and both insulin- and exercise-mediated glucose disposal in overweight, prediabetic sedentary women. Since statistical analysis failed to support any of the alternative hypotheses set forth prior to study commencement, all null hypotheses were accepted. Despite no significant effects from PM supplementation, several conclusions can be drawn from the results of the study and will be discussed under the following headings: assessment of glucose pathology, acute effects of PM supplementation on blood lipid profiles and insulin resistance, acute effects of PM supplementation on insulin-mediated glucose disposal and acute effects of PM supplementation on exercise-mediated glucose disposal.

Assessment of Glucose Pathology

Fasting glucose values greater than 100 mg/dL were an inclusion criterion for the study. Every participant met this criterion when capillary blood was tested with a glucometer on two separate occasions. Some participants had values lower than 100 mg/dL on one occasion but given the reported variation of precision testing of the glucometer (2.9% to 5.1%) they were allowed into the study. Research also indicated that capillary glucose could be 10–15 mg/dL lower than venous glucose samples. The mean glucometer reading for PL was 105.63 ± 9.44 mg/dL for the first reading and 103.60 ± 13.83 mg/dL for the second reading. The mean glucometer reading for PM was 107.43 ± 9.47 mg/dL for the first reading and 109.50 ± 15.87 mg/dL for the second reading. Using the glucometer measurements, all participants qualified as having impaired fasting glucose. However, later, baseline serum glucose levels were later independently analyzed by Quest Diagnostics (Dallas, TX.), only seven of the sixteen participants actually had impaired fasting glucose. Of those seven participants, three were in the PL group and four were in the PM group, with one participant from each group having a fasting glucose level exceeding 126 mg/dL (i.e. diabetic), even though the diagnosis of diabetes was an exclusionary criterion for this study. Four participants, two from each group, had two-hour post-OGTT values that exceeded 140 mg/dL, which is the diagnostic criterion for impaired glucose tolerance.

Acute Effects of PM Supplementation of Blood Lipid Profiles and IR

Pterocarpus marsupium has been used in Ayurvedic medicine for several centuries to treat hyperglycemia and to lower lipid levels. *Pterocarpus marsupium* and extracts of the compound have been shown to lower lipid levels in a manner similar to

that of TZDs and fibrates (Anandharajan et al., 2005; Jahromi & Ray, 1993; Mizuno et al., 2008; Rimando et al., 2005). Similarly, studies in murine models and humans have been shown to reduce levels of elevated blood glucose (Grover et al., 2002; Grover et al., 2005; Kar et al., 2003; Kedar & Chakrabarti, 1981; Mukhtar et al., 2005; ICMR, 1998). However, this study contradicts previous findings that PM supplementation improves lipid and/or glucose levels. Failure to improve lipid levels and/or lower levels of blood glucose with PM supplementation could be due to factors such as dosage and/or supplement duration.

Studies showing improvement in fasting lipid and/or glucose levels in murine models implemented PM dosages ranging from 250 mg/kg (Kar et al., 2003) to 1 $g \bullet kg^{-1} \bullet day^{-1}$ (Grover et al., 2002), whereas in diabetic human studies, dosages ranged from 2 g/day to 4 g/day (ICMR, 1998). The Sabinsa Corporation suggests dosages of 450 to 500 mg should be effective at improving hyperglycemia and lipid levels, based on limited previous research examining COX-2 (an enzyme involved in inflammation and pain) inhibition following 450 mg of PM supplementation in healthy human volunteers (Hougee et al., 2005). Since, the current study was novel in using this particular supplement in unhealthy humans, it was prudent to continue to stay within manufacturer guidelines. Duration of the supplementation period could also explain the non-significant effects of PM supplementation on lipid and/or glucose levels in the present study. Previous studies reporting improvement in lipid levels and/or fasting glucose following PM supplementation were longer in duration than the present study, in which, seven days was implemented. Studies using hamsters and rats showed improved blood glucose levels in as little as one week (Kar et al., 2003). One study in newly diagnosed Type II diabetic

humans reported improvements in glucose levels during a 12-week trial of PM supplementation using 2 – 4 g/day (ICMR, 1998). Fasting and postprandial glucose levels were assessed every four weeks. Both fasting and postprandial glucose levels were reduced at four weeks in most patients (ICMR, 1998). Improvement in blood glucose levels was observed in as little as 2 weeks in a study that recruited 10 patients with hyperglycemia, but not diabetes (Kedar & Chakrabarti, 1981). Patients were given water stored that had been stored in a PM heartwood container for one month, however, concentrations of PM could not be determined (Kedar & Chakrabarti, 1981). Differences between findings using PM in these two studies may have been due to the population being studied and/or the concentration of PM administered in the study. Whether dosage and/or duration of supplementation utilized in the current study accounts for why blood lipids and glucose levels were unaffected requires further investigation.

Acute Effects of PM Supplementation on Insulin-mediated Glucose Disposal

The responses in glucose and insulin after the OGTT were not surprising as the purpose of the OGTT (consuming 75 g of glucose) was to observe an increase in circulating plasma glucose and the subsequent increase in insulin levels to assess the ability of insulin to clear glucose from circulation. Serum glucose levels significantly increased from baseline to 30 minutes post-glucose ingestion and remained elevated above baseline at the two-hour time point. A similar response was also observed for insulin, which increased above baseline measurements and remained elevated up to two hours post-glucose ingestion. The response for glucose in the current study fell between the responses observed in participants that demonstrated normal glycemic handling and those that displayed impaired glucose tolerance in (Matsuda and DeFronzo, 1999) and

(Morino et al. 2005). Our findings are more than likely a representation of the population used since heterogeneous fasting glucose levels were present in the current study compared to being distinctly IR or normal as reported by Matsuda and DeFronzo (1999) and Morin et al. (2005). The response in insulin over time was similar to that observed by others (Matsuda & De Fronzo, 1999; Morino et al., 2005). Although the maximum insulin levels were not quite as high in this study compared to those reported for individuals with normal or impaired glucose tolerance (Matsuda & DeFronzo, 1999), the insulin levels were similar to those observed in controls in a study examining IR in children of type II diabetics (Morino et al., 2005). The discrepancies observed between Matsuda et al. (Matsuda & DeFronzo, 1999) and (Morino et al., 2005) are unclear as both studies used a 75 g glucose dose for the OGTT.

HOMA-IR and OGTT was used in the current study to determine whether PM improved insulin sensitivity compared to the cellulose PL group. The purpose of the HOMA-IR calculation was to assess insulin and glucose in a fasted state, which is essentially governed by communication between insulin, glucagon, and hepatic glucose production. An OGTT was employed to assess whole body (hepatic and peripheral) differences in insulin sensitivity between the groups. It was believed that five days of supplementation with PM would provide enough improvement in glucose regulation to observe a difference in groups following an OGTT. Since acute PM supplementation did not have a significant impact on serum insulin and/or glucose levels over time, it was not surprising that whole body differences in insulin sensitivity were similar between groups. Failure to observe an improvement in whole body insulin sensitivity, as assessed by the OGTT, may be due to a limitation in study design. An OGTT testing session at baseline

may have provided benefit to the study, allowing for better comparison in improvement in whole body insulin sensitivity over time.

Skeletal muscle mRNA expression of Akt2, AMPK, AS160, and PPARα significantly increased from baseline to one-hour post OGTT. It is well established that a key action of insulin is to stimulate glucose uptake into cells by inducing translocation of the glucose transporter, GLUT4, to the plasma membrane. Binding on insulin to its receptor leads to phosphorylation of its downstream targets PI3K and AKT, which are known to play a role in GLUT4 translocation (Lizcano & Alessi, 2002; Saltiel & Kahn, 2001). Furthermore, the specific activation of Akt and AS160 has been observed in IR and first-degree relatives of Type II diabetics (Karlsson, Ahlsen, Zierath, Wallberg-Henriksson, & Koistinen, 2006). The function of AS160 in insulin-mediated GLUT4 translocation is linked to its interaction with a novel binding partner, 14-3-3 (Ramm et al., 2006). It has been demonstrated that 14-3-3 interacts with AS160 in an insulin- and Akt-dependent manner via the Akt Thr^{642} phosphorylation site (Ramm et al., 2006), and potentially this interaction may lead to dissociation of AS160 from the Glut4 storage vesicles. Recently, Howlett et al. (2007) supported the physiological role of 14-3-3 by demonstrating an increase in its binding capacity of AS160 following an increase in insulin in human skeletal muscle.

To the author's knowledge, no studies have examined the effects on insulin directly on skeletal muscle mRNA gene expression of Akt2, AMPK, AS160, and PPARα following acute insulin response. Since Akt2 and AS160 mRNA levels increased in response to insulin, it can be speculated that both Akt2 and AS160 proteins would also increase as both are directly involved in the insulin-mediated glucose transport. Indeed, in

the present study, insulin increased in response to glucose ingestion. But more importantly, serum glucose levels gradually declined following the OGTT, indicating that the rise in insulin-stimulated glucose transport via insulin binding to the insulin receptor. Given the lack of research in this area, further research is clearly warranted to understand changes at the molecular level following an acute insulin response.

As mentioned previously, a significant increase in AMPK and PPARα gene expression was also observed. Although it may seem counter intuitive, since AMPK and PPARα are involved in non-insulin dependent signaling and fatty acid metabolism, the observed increased expression of mRNA in these genes could be due to decreased levels of circulating FFA in circulation. The AMPK and PPARα genes are regulated by various stimuli such as diet (Muoio & Koves, 2007; Yeo et al., 2008), exercise (Lee-Young, Canny, Myers, & McConell, 2009; Watt, Southgate, Holmes, & Febbraio, 2004), and sleep (Chikahisa, Fujiki, Kitaoka, Shimizu, & Sei, 2009; Shirai, Oishi, Kudo, Shibata, & Ishida, 2007). A stimulus pertinent to post-OGTT activation of these genes may have to do with FFA uptake. In a recent study to understand FFA kinetics, normal weight participants, obese participants, and obese diabetic participants were subjected to an OGTT with glucose, insulin, and FFA measured before and after glucose ingestion (Boston & Moate, 2008). The study determined the effects of gender, activity, and body mass index on FFA clearance. In every group, FFA increased immediately following glucose ingestion, but by one-hour post ingestion, FFA levels had declined to well below fasting levels (Boston & Moate, 2008). In order for FFA to be cleared from circulation, the FAs would have to be taken up, which in skeletal muscle would mean an increase in activity of proteins such as CD36, Acyl-CoA, and CPT. In skeletal muscle, CD36 is

responsible for the uptake of FFA, while Acyl-CoA and CPT are for their transport into the mitochondria (Long et al., 2005). It was recently shown that in the presence of glucose there is an increase in FFA oxidation and storage and activation of AMPK in glycolytic skeletal muscle (Fosgerau et al., 2006). In addition, AMPK is thought to regulate FA oxidation via PPARα (Lee et al., 2006). Initially, FFA levels were to be assessed in this study, but due to funding constraints their assessment was not prioritized. As such, while the above points are plausible the effect of FFA clearance is speculative, and more research needs to be done.

Acute Effects of PM Supplementation on Exercise-Mediated Glucose Disposal

In the present study, increases in blood glucose levels after the GXT were expected in both the PL and PM groups as the liver is releasing glucose to meet the demand of the exercise bout (i.e. uptake of glucose elsewhere). Findings in the current study contradict those reported for the glucose response to a GXT in active, non-obese and sedentary, moderately-obese men (White, Ismail, & Bradley, 1978). During exercise in the active group, mean glucose concentration increased, non-significantly, with a steady increase as exercise intensity increased, and then increased significantly during recovery (White et al., 1978). In the sedentary group glucose declined during the GXT and increased during recovery (White et al., 1978). Differences in results between the current study and that using the cycle ergometer were more than likely due to type of GXT that was performed. The GXT on the cycle ergometer involved a 10-minute low intensity warm-up prior to the incremental increase in intensity. Additionally, the GXT on the cycle ergometer was stopped once HR reached 160 bpm. This corresponded to 92% and 88% predicted VO_{2Max} for the active, non-obese and sedentary, moderately

obese men, respectively (White et al., 1978). The GXT on the cycle ergometer was not a true maximal GXT, thus maximal exertion was not achieved for either the active, non-obese or sedentary, moderately obese men. Additionally, the average length of the GXT in the current study (7.5 minutes and 6.7 minutes for the PL group and PM group, respectively) was shorter than the warm-up time of the GXT on the cylce ergometer used in active, non-obese and sedentary, moderately obese men (White et al., 1978). Therefore, the women in the current study performed at higher intensities, but for much shorter duration. It is worth noting that an increase in circulating glucose post-exercise, as observed this study, is not entirely surprising. At high intensities such as those that occur during maximal exercise tests, hepatic glucose production is greatest (Wahren, Felig, Ahlborg, & Jorfeldt, 1971) and often exceeds muscle uptake (Kjaer, 1998). This phenomenon was observed in the current study with a subsequent decline in circulating glucose levels.

The significant decline in glucose levels observed after exercise was likely a result of continued glucose uptake by peripheral tissues while hepatic glucose production declined after exercise ceased. This was also an expected observation as increased post-exercise glucose uptake is expected after acute bouts of high intensity (> 85% VO_{2max}) exercise (Koshinaka, Kawasaki, Hokari, & Kawanaka, 2009). It has been previously thought that this is due to increased insulin sensitivity, but it may actually be due, in part, to increased insulin responsiveness (Holloszy, 2005) as well as the continued increase in non-insulin dependent mechanism such as increased AMPK (Fisher et al., 2002; Jessen et al., 2003) and AS160 (Dreyer et al., 2008) after exercise. While increased insulin sensitivity is a decrease in the insulin concentration needed to cause half of the maximal

response, increased insulin responsiveness is an increase in glucose disposal due to a maximally effective concentration (Holloszy, 2005). In other words, increased insulin responsiveness is when a greater amount of glucose is taken up into the cell at the same relative amount of insulin stimulus as before exercise. Regardless of whether the post-exercise glucose response observed in the current study can be explained, further research needs to be carried out to investigate the glucose response to in overweight and obese sedentary women.

Study Implications and Future Research Needs

Results in this study suggest that acute supplementation with 250 mg twice daily of PM, sold under the name Silbinol®, does not improve insulin sensitivity or fasting lipid levels. Serum glucose and insulin increased in response to ingesting 75 g of glucose, as did skeletal muscle mRNA expression of Akt2, AMPK, AS160, and PPARα. Additionally, it was the observed that serum glucose levels briefly increased then significantly decreased after a brief, maximal exertion bout of exercise.

A number of limitations existed in the current study. These included: small sample size, varying degrees of participant insulin sensitivity, dosage and purity of supplement, and length of the study. All limitations may have potentially contributed to the limited significant findings in this study. Before future studies are performed, these limitations need to be addressed. Power calculations performed prior to beginning the study predicted a power of .80 could be obtained with only six participants per group. Therefore, it was assumed that eight participants would be sufficient. However, the small sample size probably increased type II error, hence, failing to reject a null hypothesis when it is in fact not true. Additionally, the range of fasting glucose levels makes it

difficult to infer any observations to the pre-diabetic population, as was initially intended, and likely caused undue variability in the findings. The decision to use some participants that had values lower than 100 mg/dL on one occasion, as measured by a handheld glucometer, was based on the error of the glucometer, which was < 5.1%. Thus, a sample that would read 96 mg/dL on the glucometer could be 92 mg/dL, or it could very well be 100 mg/dL. According to glucometer readings all participants would have qualified as having impaired fasting glucose on at least one occasion, but research shows that glucometers may be less sensitive to fasting blood glucose values (Marley et al., 2007). Future studies seeking to use a classification of pre-diabetic as an inclusion criterion should attempt to utilize serum or plasma glucose levels instead of capillary glucose measures via glucometer readings. However, funding was also a limitation as more blood draws increases study cost. The use of a glucometer in this study was a means to reduce study costs.

Additionally, the dosage of PM may have been problematic. Future studies should seek to use larger dosages as the 500 mg/day dosage in this study did not result in significant changes in the chemical safety panel. Much larger doses may be feasible as a recent study comparing cardiotonic effects of aqueous extracts of PM to digoxin showed that while digoxin was fatal at 0.5 mg/ml, PM was not fatal until 4 mg/ml (Mohire, Salunkhe, Bhise, & Yadav, 2007). Additionally, the original intension was to use pterostilbene isolated from the plant *Pterocarpus marsupium*, but the product sent to use by the Sabinsa Corporation contained only 5.4% pterostilbene. Attempts to find other sources of pterostilbene resulted in high-grade pterostilbene that was either too expensive or not for human consumption. As such, the study was carried out using *Pterocarpus*

marsupium extract that is sold under the name Silbinol®. Other studies using *Pterocarpus marsupium* need to be carried out using a larger sample size, a longer time frame, and a standardized diet between groups. Though the changes in fasting glucose, HDL, LDL, and triglycerides were not significant, the change in each variable in the PM group displayed an improved lipid profile that may have been significant given a larger sample size or a longer study.

Finally, given recent interest into PGC-1α, future studies should explore the paradoxical role of muscle mitochondrial function and insulin-stimulated muscle glucose metabolism in human pre-diabetic and diabetic populations. Recently it has been suggested that under normal physiologic conditions of exercise, PGC-1α induces a coordinated program of increased energy delivery and increased mitochondrial biogenesis and fatty acid oxidation to meet the increased energy demands of working skeletal muscle. In contrast, in a non-exercising muscle (as seen in the current study) this coordinated program may result in a mismatch between increased fatty acid uptake and that of mitochondrial fat oxidation, resulting in a net increase of intramyocellular fat and diacylglycerol content and insulin resistance (Choi et al., 2008). Choi and colleagues (2008) fed a high fat diet to transgenic mice over expressing muscle specific PGC-1α for 3 weeks (Choi et al., 2008). The PGC-1α transgenic mice displayed increases in mitochondrial density and oxidative phosphorylation-related genes (Choi et al., 2008), but when fed a high fat diet, these mice became insulin resistant compared to wild-type mice fed the same high-fat diet. Neither group was allowed to exercise; therefore, the increase in PGC-1α was not due to normal physiological function. Choi et al. (2008) also observed that, per unit of mitochondrial mass, there was a partial down-regulation of

mitochondrial activity. Furthermore, a recent study of lipid induced insulin resistance, similar to what occurs with inactive individuals on a high-fat diet, suggested that the presence of larger quantities of intramuscular triglycerides may actually lead to increased fatty acid usage as a fuel (Koves et al., 2008), which would mean increased PGC-1α expression in the absence of improved metabolic flexibility. Indeed in the current study, an increase in PGC-1α gene expression was observed in the placebo group over time, though not statistically significant. Taken together, whether increase in markers of oxidative phosphorylation and/or mitochondrial biogenesis in the absence of increased glucose disposal and insulin responsiveness leads to mitochondrial distress clearly warrants further investigation.

Conclusions

Findings from the current study suggest that acute supplementation with 250mg twice daily of PM, sold under the name Silbinol®, does not improve insulin sensitivity or lipid levels. Supplementation with PM may still reduce hyperglycemia and hyperlipidemia, but a larger dose may be necessary to provide such benefit. A few findings in this study were novel. To the authors' knowledge this is the first study to report an increase in skeletal muscle mRNA expression of Akt2, AMPK, AS160, and PPARα in response to ingesting 75 g of glucose. While increased expression of Akt2 and AS160 skeletal muscle mRNA should be expected after an OGTT, the findings of increased expression AMPK and PPARα skeletal muscle mRNA after ingestion of glucose in a situation where plasma FFA are increased (i.e. a fasting state or after exercise), could hint at why the increased presence of intramuscular is observed in people with people lipotoxicity and endurance trained athletes (van Loon & Goodpaster, 2006).

An additional finding that was novel in this study was the observed increase and subsequent significant decrease in serum glucose levels after a brief, maximal exertion bout of exercise. While novel, this finding is not particularly concerning as circulating levels of glucose remained within in healthy fasted ranged (60 – 100 mg/dL). If anything this provides further evidence for the use of exercise to help maintain healthy circulating levels of glucose. However, caution must be exercised when inferring findings of this study, as not all participants were actually pre-diabetics. Since this study presents several novel findings, more research needs to be conducted in a population of obese or overweight females with elevated fasting glucose levels below the pre-diabetic level.

APPENDICES

APPENDIX A

Personal Demographic Form

Baylor University
Exercise & Sport Nutrition Laboratory

Personal Information

Name:

Address:

City: _____ State: _____ Zip Code: _____ SS# _____

Home Phone: ( ) _____ Work Phone: ( ) _____

Beeper: ( ) _____ Cellular ( ) _____

Fax:: ( ) _____ email address: _____

Birth date: _____ / _____ / _____ Age: _____ Height: _____ Weight: _____

Exercise History/Activity Questionnaire

1. Describe your typical occupational activities.

2. Describe your typical recreational activities.

3. Describe any exercise training that you routinely participate.

4. How many days per week do you exercise/participate in these activities?

5. How many hours per week do you train?

6. How long (years/months) have you been consistently training?

APPENDIX B

Medical History Inventory

BAYLOR UNIVERSITY
EXERCISE & SPORT NUTRITION LABORATORY

Directions. The purpose of this questionnaire is to enable the staff of the Exercise and Sport Sciences Laboratory to evaluate your health and fitness status. Please answer the following questions to the best of your knowledge. All information given is CONFIDENTIAL as described in the Informed Consent Statement.

Name:_____ Age _____Date of Birth_____

Name and Address of Your Physician:_____

MEDICAL HISTORY

Do you have or have you ever had any of the following conditions? (Please write the date when you had the condition in blank).

____ Heart murmur, clicks, or other cardiac findings?
____ Frequent extra, skipped, or rapid heartbeats?
____ Chest Pain of Angina (with or without exertion)?
____ High cholesterol?
____ Diagnosed high blood pressure?
____ Heart attack or any cardiac surgery?
____ Leg cramps (during exercise)?
____ Chronic swollen ankles?
____ Varicose veins?
____ Frequent dizziness/fainting?
____ Muscle or joint problems?
____ High blood sugar/diabetes?
____ Thyroid Disease?
____ Low testosterone/hypogonadism?
____ Gluacoma?
____ Asthma/breathing difficulty?
____ Bronchitis/Chest Cold?
____ Cancer, Melanoma,
____ Stroke or Blood Clots?
____ Emphysema/lung disease?
____ Epilepsy/seizures?
____ Rheumatic fever?
____ Scarlet fever?
____ Ulcers?
____ Pneumonia?
____ Anemias?
____ Liver or kidney disease?
____ Autoimmune disease?
____ Nerve disease?
____ Psychological Disorders?

Do you have or have you been diagnosed with any other medical condition not listed?

_____
_____
_____
_____

Please provide any additional comments/explanations of your current or past medical history.

_____
_____
_____
_____

Please list any recent surgery (i.e., type, dates etc.).

_____
_____

_____
_____

List all prescribed/non-prescription medications and nutritional supplements you have taken in the last 3 months.

_____
_____

What was the date of your last complete medical exam?

_____
_____

Do you know of any medical problem that might make it dangerous or unwise for you to participate in this study (including strength and maximal exercise tests) ____ If yes, please explain: _____

_____
_____

Recommendation for Participation

____ No exclusion criteria presented. Subject is cleared to participate in the study.
____ Exclusion criteria is/are present. Subject is not cleared to participate in the study.

Signed: _____ Date: _____

APPENDIX C

Application to the Baylor IRB
For Review of Research/Activity Proposal

Part 1: Signature Page

1. Name Matthew Cooke, PhD
2. Email Address (optional) Matt_Cooke@baylor.edu
3. Complete Mailing Address P.O. Box 97313
4. Position Assistant Professor
5. Faculty Advisor (if researcher is Graduate Student) _____
6. Department/School HHPR/SOE
7. Telephone # x4025 FAX # _____
8. Are you using participants in research (***Y*** or N) or in teaching exercises (Y or ***N***)?
9. Title of the research project/teaching exercise:

   Effects of Resveratrol and Pterostilbene Supplementation on Insulin and Exercise-Mediated Signaling Pathways for Glucose Uptake in Overweight Insulin-Resistant Females: A Double-Blind, Clinically-Controlled Study

10. Please return this signed form along with all the other parts of the application and other documentation to the University Committee for Protection of Human Subjects in Research; Dr. Matt Stanford, Chairman, Department of Psychology and Neuroscience, Baylor University, P.O. Box 97334, Waco, Texas 76798-7334. If you have questions, or if you would like to see a copy of the OHRP Report on protection of human subjects in research, contact Dr. Stanford at extension 2961.

_____

11/26/08

Signature of Principal Investigator Date

_____

Signature of Faculty Advisor (required if researcher is a Graduate Student)

Departmental Review: _____

Department Chair or the Chair's Designate

Part 2: Introduction & Rationale

Type II diabetes mellitus is a critical health concern that has tripled in prevalence over the past 25 years, with 24.1 million Americans (8% of the population) suffering from this condition in 2007 (3). This condition is typically preceded by an initial insulin resistant state where the insulin-responsive cells in the body do not appropriately react to circulating insulin, which results in an increase in blood glucose levels and disturbances in fatty acid metabolism. Insulin serves to lower blood glucose levels via an insulin-signaling pathway within adipocytes and muscle cells that result in translocation of the GLUT4 glucose transporter to the cell membrane. The first line of defense to prevent the development or further progression of Type II diabetes mellitus is lifestyle change. Of particular importance is losing weight, increasing physical activity, and modifying diet to an anti-atherogenic type diet (2, 4, 13).

Insulin binding is the primary stimulus for glucose uptake in muscle cells and adipocytes at rest or after a meal. Glucose, however, is taken up into muscle cells during moderate to intense exercise via insulin-independent signaling pathways. Insulin-mediated glucose uptake proceeds via the phosphatidylinositol 3-kinase (PI3-K) pathway and phosphorylation of protein kinase B (PKB; Akt) and Akt Substrate of 160 kD (AS160). Glucose uptake into muscle cells during moderate to intense exercise, however, is mediated by insulin-independent signaling pathways, likely via activation of AMP-activated protein kinase (AMPK). AMPK, which responds to increases in adenosine monophosphate (AMP):adenosine triphosphate (ATP) ratio during exercise (particularly at higher intensities), serves to phosphorylate AS160 and relieve the GLUT4 sequestration to its intracellular storage site and facilitate its translocation to the plasma membrane. Additionally, AMPK has also been shown to increase insulin sensitivity by phosphorylating specific serine residues on the insulin receptor. Recent evidence points to a convergence between the insulin and contraction-mediated signaling pathways at AS160; however this concept requires further *in vivo* research. (11).

In addition to weight reduction and physical exercise, dietary adjustments are also essential to lifestyle modifications recommended for obese or diabetic individuals. Included in these recommended dietary changes is increased intake of fruits and vegetables. Fruits and vegetables are important dietary components that can provide beneficial antioxidants and phytonutrients. Resveratrol and pterostilbene are two such phytonutrients that are found in high concentrations in red wine and a variety of plant sources such as grape skin, berries, pomegranates, and peanuts (10, 12). Over the past 5 years, research into these 2 botanical compounds has expanded, with benefits from supplementation including: improved insulin sensitivity, weight loss, cardioprotection, cancer growth inhibition, and decreased mortality. Although interest in such compounds has increased, limited research has examined their purported effects in humans. In particular, the mechanism by which both supplements are said to improve insulin-sensitivity, by enhancing GLUT4 translocation, requires further investigation. A few studies have suggested that improved insulin-sensitivity from resveratrol and pterostilbene supplementation is similar to that of metformin (a commonly used drug to treat Type II diabetes); enhancing GLUT4 translocation via AMPK stimulation (12). However, more research is needed to confirm such observations. Additionally, exercise also stimulates GLUT4 translocation, albeit via insulin-independent mechanisms. Administration of resveratrol and pterostilbene could provide a synergistic enhancement of glucose uptake during exercise, but more importantly, continue to stimulate glucose uptake after exercise.

The primary purpose of this study is to determine the effects of two polyphenolic (resveratrol or pterostilbene) supplements on glucose/insulin kinetics and markers of inflammation at rest and following an exercise bout. Further, the study will also determine the effects of prolonged polyphenolic supplementation on markers of the metabolic syndrome. In so doing, this study will involve three specific aims:

Specific Aim 1: To determine the acute (4 days) and chronic (8 weeks) effects of pterostilbene and resveratrol supplementation on A) glucose/insulin kinetics (serum glucose, insulin) as determined by an oral glucose tolerance test (OGTT), B) skeletal muscle expression of genes involved in GLUT4 translocation (GLUT4, AMPK, AS160, Akt), C) serum levels of other relevant hormones (estrogen, leptin, adiponectin, cortisol), and D) serum and whole blood analyses for general clinical safety markers will be assessed.

Specific Aim 2: To determine the acute (5 days) and chronic (8 weeks) effects of pterostilbene and resveratrol supplementation on A) glucose/insulin kinetics (serum glucose, insulin), B) plasma inflammatory markers (TNF-α, CNTF, IL-6, IL-16), C) lipid peroxidation, and D) skeletal muscle expression of genes involved in GLUT4 translocation (GLUT4, AMPK, AS160, Akt, aPKC, CAMK, PPARα,γ,δ) prior to and following an exercise bout.

Specific Aim 3: To determine the effects of 4 weeks and 8 weeks of polyphenolic supplementation in conjuction with an exercise training program on A) serum lipid profiles, B) muscle markers of lipid metabolism (ACC, CPT1) clinical safety markers (HR, BP, complete serum metabolic panel), C) gene expression of NF-kB, SIRT1, and PPARα,γ,δ D)body composition, E) waist circumference, F) resting energy expenditure, and G) cardiopulmonary fitness.

Part 3: Methodology

Participants

Approximately 45 sedentary, overweight, pre-diabetic [fasting plasma glucose: 100-125mg/dL (6)] premenopausal females between 18 and 35 years of age, will be recruited for this study. Participants will be declared sedentary if they have not exercised for 30 minutes a day at least 5 days a week for a year or more. Additionally, oral contraceptives appear to reduce glucose tolerance by reducing peripheral tissue insulin sensitivity. Since oral contraceptives may increase glucose intolerance, all participants will be women who have not taken oral contraceptives for at least two months. Prior to participation in the study, all potential participants must provide written physician approval. Participants who qualify for the study will be cleared for participation by successfully completing a series of health screening examinations (i.e., health screening questionnaire, blood pressure assessment, fasting glucose assessment) by qualified research staff members.

All participants will not be allowed to participate if they meet any of the following criteria: 1) have current or past history of anabolic steroid use; 2) have any metabolic disorders including known electrolyte abnormalities; heart disease, arrhythmias, diabetes, thyroid disease or hypogonadism; a history of medically controlled hypertension, hepatorenal, musculoskeletal, autoimmune, or neurologic disease; if they are taking thyroid, hyperlipidmeic, hypoglycemic, anti-hypertensive, or androgenic medications; 3) have ingested any ergogenic levels of creatine, HMB, thermogenics, ribose, pro-hormones (i.e., DHEA, androstendione, etc.) or other purported anabolic or ergogenic nutritional supplements for a 2-month time period prior to beginning the study; 4) do not take any additional nutritional supplement or contraindicated prescription medication during the protocol. All participants meeting entrance criteria will sign informed

consent statements in compliance with the Human Participants Guidelines of Baylor University and the American College of Sports Medicine.

Study Site

All exercise sessions will be supervised by qualified individuals and will take place at the Student Life Center or at Russell Gymnasium at Baylor University or at the WRS Athletic Club. The majority of the training sessions will take place at Russell Gymnasium and will be supervised by National Strength and Conditioning Association (NSCA) – Certified Strength and Conditioning Specialists (CSCS). Testing sessions will be conducted in the Exercise & Sport Nutrition Laboratory (ESNL) and the Exercise & Biochemical Nutrition Laboratory (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University.

Independent and Dependent Variables
Table 1 shows the general research design and time course for assessments. The independent variables will be physical activity, diet, nutritional supplementation, subject's age, and the number of testing/evaluation times during the study. Dependent variables will include body composition measurements (body weight, whole body DEXA scans and total body water), resting energy expenditure and respiratory quotient, maximal oxygen consumption, fasting whole blood and plasma analysis, inflammatory and anti-inflammatory cytokine measures, lipid metabolism status, muscle protein and gene expression measures, serum clinical safety markers, uric acid, triglycerides, total cholesterol, low-density lipoprotein, high-density lipoprotein, resting blood pressure and heart rate, and estimated energy intake.

Entry
Participants expressing interest in participating in this study will be interviewed on the phone to determine whether they appear to qualify to participate in this study (Please refer to Selection Criteria listed below). Participants believed to meet eligibility criteria will then be invited to attend an entry/familiarization and baseline session.

Familiarization Session/Baseline Testing/Pre-Supplementation (Day 1- T1)
Participants will be instructed to refrain from exercise for 24 hours and fast for 12 hours prior to pre-testing and for all scheduled assessments. Upon reporting to the lab, participants will complete questionnaires regarding exercise habits and health status (Symptoms Inventory). All participants will report to the lab in the morning for all scheduled assessments. Fasting blood glucose will initially be assessed using a OneTouch® glucometer (Lifescan, Inc.) to ensure that they are prediabetic (glucose: 100-125mg/dL [6]). Participants meeting entry criteria will be familiarized to the study protocol by way of a verbal and written explanation outlining the study design. After meeting all inclusion criteria, familiarization of the study, and signing of the informed consent, participants will then begin baseline testing.

Resting energy expenditure (REE) will be determined by analysis of the gases expired by the participant while resting in a supine position (Parvo Medics, Provo, UT). Total body mass (kg), total body water (total, intracellular, and extracellular), percent body fat, fat mass, and fat-free mass, will be assessed. Following these assessments, participants will have resting heart rate and blood pressure determined using standard procedures. Participants will then donate approximately 10-15 ml of fasting blood using venipuncture techniques of an antecubital vein in the forearm according to standard procedures. Blood samples will be allowed to stand at room temperature for 10 min and then centrifuged. For each sample, the plasma and/or serum will be removed and frozen at -80°C for later analysis. Percutaneous muscle biopsies (10-15 mg), using a fine needle aspiration technique, will be obtained from the middle portion of the vastus lateralis muscle of the

dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm under local ansesthesia (2% Xylocaine with epinephrine). After removal, adipose tissue will be trimmed from the muscle specimens and immediately frozen in liquid nitrogen and then stored at -80°C for later analysis.

Supplementation Protocol
Following baseline measurements, subjects will be matched based on age, body composition, and fasting glucose to randomly and blindly ingest (meaning neither the investigators nor the participants will know which groups until the end of the study) capsules containing a cellulose placebo, 250mg of resveratrol, or 250mg of pterolstilbene twice daily for the duration of the study. Supplements will be prepared in gel capsule form and packaged in generic bottles for double blind administration by Sabinsa Corporation. Supplementation compliance will be monitored by having the participants fill out supplement logs recording the amount of supplement ingested during each day of the supplement period. Participants will be instructed to consume the supplement for 4 days prior to the second testing session (T2).

Testing Sessions T2
Upon arriving, participants will have their total body mass (kg), total body water (total, intracellular, and extracellular), heart rate and blood pressure assessed. An indwelling catheter will be inserted into the participant's antecubital vein of the forearm according to standard procedures, and approximately 10-15ml of blood will be sampled. Next a percutaneous muscle biopsy sample (10-15 mg), using a fine needle aspiration technique, will be obtained from the middle portion of the vastus lateralis muscle. An OGTT will then be performed, where the participant will ingest 1.75g of glucose per kilogram of body weight (max = 75 g glucose). Additional venous blood samples (5 ml) will be obtained by an indwelling venous catheter at 30 minutes, 1hr, and 2hrs after glucose ingestion. A 2hr post-glucose ingestion muscle biopsy will also be obtained. Participants will be instructed to remain in a rested state during the 2hrs following the glucose administration.

Testing Sessions T3
Participants will have total body mass (kg), total body water (total, intracellular, and extracellular), heart rate, and blood pressure measured. An indwelling catheter will again be inserted and approximately 10-15 ml of blood will be sampled. Similarly, a percutaneous muscle biopsy sample (10-15 mg), using a fine needle aspiration technique will also be taken. It should be noted that both blood and muscle sampling will be taken on the contralateral arm and leg that was tested during T2. A 12-lead electrocardiogram (ECG) will be used to ensure that there are no contraindications during exercise testing based on the ACSM guidelines. Oxygen uptake (VO_2) will be measured every 15 sec via an open-circuit sampling system. Throughout the treadmill test, blood pressure will be accessed via standard procedures, while heart rate and rhythm will be assessed using an ECG. Blood samples (10-15mL) via an indwelling venous catheter will be taken at 30min, 1hr, and 2hrs following the cardiopulmonary exercise test to assess glucoregulation, insulin sensitivity, inflammatory markers, and lipid peroxidation markers post-exercise. Muscle biopsy samples will be obtained prior to and 2hrs post-exercise for assessment of protein and gene expression of glucoregulatory intermediates and certain inflammatory markers. Again, subjects will remain in the laboratory in a rested state for the 2hrs following the exercise bout. Participants will be instructed to continue consuming the supplement and begin an 8 week structured, energy restricted, low glycemic index diet and supervised, structured, circuit-training exercise program that includes both aerobic and resistance-training elements. Participants will return to return to the laboratory in 4 weeks for blood donation, hemodynamic, body composition and cardiopulmonary assessment.

Testing Sessions T4

Upon arriving, participants will have their total body mass (kg), total body water (total, intracellular, and extracellular), heart rate and blood pressure assessed. Resting energy expenditure (REE) will be determined by analysis of the gases expired by the participant while resting in a supine position (Parvo Medics, Provo, UT). Participants will then donate approximately 10-15 ml of fasting blood using venipuncture techniques of an antecubital vein in the forearm according to standard procedures. Subjects will then perform a graded-exercise test on a treadmill following the same guidelines as mentioned above.

Testing Sessions T5 & T6

Following the 8 week training program, subjects will return to the laboratory for testing sessions 5 and 6. Testing sessions 5 and 6 will mirror testing sessions 2 and 3, respectively to determine the chronic effects of supplementation in conjunction with exercise training on insulin-mediated glucose uptake and proteins and genes involved in exercise-mediated glucose uptake. The only addition is that REE will also be assessed at the beginning of testing session 5. Further, clinical safety panels will be used to determine the safety of the supplement. Training volume, dietary intake and supplementation will be monitored during the 8 week study intervention by training and supplementation logs and 4-day dietary recall analysis sheets.

Nutritional Intervention

Each participant will follow a structured low glycemic index diet during the eight-week study. The diet will involve the following protocol in which each participant will be requested to: 1) consume two servings of low-fat dairy each day, 2) consume two pieces of fruit per day, or one cup of canned or frozen fruit, 3) at lunch and dinner, consume low-starch vegetables, eating at least two cups of different kinds at each meal, 4) consume four ounces of meat protein (beef, pork, veal, poultry, fish, shellfish) at lunch and dinner, and 5) limit starchy vegetables (potatoes, corn, rice), grain-based foods (breads, pasta, salty snacks), flour-based foods (pastries, cakes, cookies, donuts), candy, sugar-containing beverages including juices. A typical dietary meal plan is presented in Table 2.

Exercise Protocol

The exercise intervention will be 8 weeks in duration. All groups will participate in a resistance training circuit three times a week. Circuit training will consist of 12 stations with 8-12 repetitions, following intensity guidelines of the American College of Sports Medicine (ACSM). Aerobic activity will be three days a week and intensities will follow ACSM guidelines. An overview of the exercise training program is presented in Tables 3 and 4.

Assessment of Body Composition (T1 – T6)

Total body mass (kg) will be determined on a standard dual beam balance scale (Detecto). Total body water (total, intracellular, and extracellular) will be determined with bioelectrical impedance (BIA; Xitron) while percent body fat, fat mass, and fat-free mass, will be determined using a Dual Energy X-ray Absorptiometer (DXA; Hologic QDR-4500W).

Assessment of Hemodynamic Safety Markers (T2, T4, T5)

In order to assess any hemodynamic safety concerns regarding the effects of the diet, exercise program, and polyphenol supplement, after 1 week, 4 weeks, and 8 weeks participants will undergo the assessment of resting hemodynamic safety markers (heart rate and blood pressure). Heart rate will be determined by palpation of the radial artery using standard procedures.

Assessment of Dietary Analysis (T2, T4, T5)
In an attempt to determine compliance with the low glycemic diet, and to also assess the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet, each participant will be required to keep four-day dietary records during weeks 1, 4, and 8 of the study. The dietary records will be analyzed with the Food Processor dietary assessment software program (ESHA Research Inc., Salem, OR).

Assessment of Serum Lipids (T1, T3, T4, T6)
Using a Dade Dimension clinical chemistry analyzer (Dade-Behring, Inc., Newark, DE) blood samples will be assayed for serum lipid levels (total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides). This assay will help evaluate the effects of the exercise and the supplementation regimen on serum lipids.

Reported Side Effects from Supplementation (T3, T4, T6)
To determine whether the participants suffered any side effects from the placebo or supplement, after weeks 1, 4, and 8 participants will report by questionnaire administered in a confidential manner whether they tolerated the supplement, supplementation protocol, as well as report any medical problems/symptoms they may have encountered throughout the study.

In Vivo Biochemical Analyses
A standard clinical chemistry panel will be run by Quest Diagnostics, Inc. (Madison, NJ), whereas all other analytical procedures will be conducted in line with established and published protocols in the Exercise and Biochemical Nutrition Laboratory at Baylor University. Clinical blood counts will be determined via Cell Dyne 3500. Serum insulin, cortisol, and estrogen will be determined by way of enzyme-linked immunoabsorbent assay (ELISA) at a wavelength of 450 nm. Insulin sensitivity will be determined by way of the Matsuda OGTT insulin sensitivity index using OGTT insulin values (9). In addition, insulin resistance will be determined Homeostatic model assessment (HOMA) of insulin resistance (8). Bioplex bead analysis will be used to determine plasma leptin, adiponectin, TNF-α, CNTF, IL-6, IL-10, and IL-16 levels. The skeletal muscle gene expression of GLUT4, AMPK, AS160, Akt, aPKC, CAMK, NF-kB, SIRT1, and PPARα,γ,δ will be performed using RT-PCR. Oligonucleotide primers will be designed using Primer Express from known human mRNA sequences available online through the NCBI database. The quantity of mRNA will be determined relative to the expression of β-actin, and ΔC_T values will be used to compare gene expression. The specificity of the PCR will be demonstrated with an absolute negative control reaction containing no cDNA template, and single gene products confirmed using DNA melt curve analysis. Protein levels of GLUT4, AMPK, AS160, Akt, PPARα,γ,δ, CAMK, and NF-kB will be measured with an ELISA kit.

Data Analysis, Presentation, & Publication
Data will be analyzed using with SPSS for Windows Version 16 software (SPSS Inc., Chicago, IL). Body composition, aerobic capacity, glucose/insulin kinetics, protein and mRNA expression data will be analyzed via separate (time x supplement) factorial multivariate, repeated measures analyses of variance (MANOVAs). Data will be considered significantly different when the probability of error is 0.05 or less. Tukey's honestly significant differences post-hoc procedures will be performed when a significant interaction is observed. Data will be presented at an appropriate scientific conference (e.g., American College of Sports Medicine, Experimental Biology, etc) and published in a peer reviewed scientific journal (e.g., Diabetes, Medicine & Science in Sport and Exercise, Nutrition, International Journal of Sport Nutrition and Exercise Metabolism, etc).

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Research Team

Matt Cooke, PhD. Dr. Cooke is an Associate Professor in the Department of Health, Health, Human Performance, & Recreation at Baylor University. Dr. Cooke has previous research experience in the sport nutrition industry, specifically in the areas of protein supplementation and training adaptations. He will serve as one of the principal investigators of the study assisting with all aspects of data collection and analysis.

Geoffrey Hudson, MA, CSCS. Mr. Hudson is currently a Ph.D. student in Exercise, Nutrition and Preventative Health at Baylor University and a research assistant in the Exercise and Sport Nutrition Laboratory at Baylor University. Mr. Hudson has previous research experience in the areas of sport nutrition supplementation, anaerobic performance enhancement, thermoregulation,

and the metabolic syndrome. He will serve as one of the principal investigators of the study assisting with all aspects of data collection and analysis and manuscript preparation.

Brian Shelmadine, MA, CSCS. Mr. Shelmadine is currently a Ph.D. student in Exercise, Nutrition and Preventative Health at Baylor University and a research assistant in the Exercise and Sport Nutrition Laboratory at Baylor University. Mr. Shelmadine has previous research experience in the areas of sport nutrition supplementation, anaerobic performance enhancement, strength and conditioning, and the metabolic syndrome. He will serve as one of the principal investigators of the study assisting with all aspects of data collection and analysis and manuscript preparation.

Thomas Buford, MS, CSCS. Mr. Buford is currently a Ph.D. student in Exercise, Nutrition and Preventative Health at Baylor University and a research assistant in the Exercise and Sport Nutrition Laboratory at Baylor University. Mr. Buford has previous research experience in the areas of sport nutrition supplementation, anaerobic performance enhancement, strength and conditioning, and muscle physiology. He will assist with data collection.

Mike Greenwood, PhD, FNSCA, CSCS*D, FACSM, FISSN. Dr. Greenwood is Professor and Graduate/Research Coordinator in the Department of HHPR at Baylor University. Dr. Greenwood's research expertise is in exercise nutrition predominately creatine supplementation and strength related exercise programming. He will advise on experimental design and procedures.

Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS, CISSN. Dr. Willoughby is an Associate Professor of Exercise and Muscle Physiology and Biochemistry in the Department of Health, Human Performance, & Recreation at Baylor University. He is also an Associate Professor of Baylor's Biomedical Science Institute. Dr. Willoughby is an internationally recognized exercise biochemist and molecular physiologist. He has conducted a vast amount of research focusing on the biochemical and molecular regulatory mechanisms regarding exercise performance and nutrition. Dr. Willoughby will be the principal supervisor of the project. He will oversee all aspects of the study and advise on the biochemical and clinical chemistry assays involved in the project.

Procedures

Medical Monitoring. Interested participants will be invited to familiarization sessions. During this time, participants will sign consent forms and complete medical history information. Participants will then undergo a general exam by trained laboratory personnel to determine whether the subject meets entry criteria to participate in the study. This exam will include evaluating the medical and training history questionnaires and performing a general physical examination according to ACSM exercise testing guidelines. Based on this examination, participants will be assessed for their risk of cardiovascular disease and contraindications to exercise and then a recommendation will be made on whether the participant meets entry criteria and may therefore participate in the study. Trained, non-physician exercise specialists certified in CPR will supervise participants undergoing testing and assessments. A telephone is in the laboratory in case of any emergencies, and there will be no less than two researchers working with each subject during testing sessions. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance. Participants will be informed to report any unexpected problems or adverse events

they may encounter during the course of the study to Matthew Cooke, Ph.D. If clinically significant side effects are reported, the participants will be referred to discuss the problem with Ronald Wilson, MD for medical follow-up. Dr. Wilson is one of the Sports Medicine physicians for Baylor University and is associated with the Department of HHPR. He has agreed to provide medical support and consultation for this study and to the ESNL. Dr. Wilson will evaluate the complaint and make a recommendation whether any medical treatment is needed and/or whether the participant can continue in the study. If Dr. Wilson feels medical follow-up is necessary, the participant will be referred to obtain medical treatment from their personal physician. This is a similar referral/medical follow-up system that Baylor athletes are provided with the exception that participants in this study will not be provided medical care. New findings and/or medical referrals of unexpected problems and/or adverse events will be documented, placed in the participants research file, and reported to the Baylor IRB committee.

Screening for Cardiopulmonary Disease Risk and Exercise Contraindications. All participants will have their risk of cardiopulmonary disease and possible contraindications to exercise assessed by Exercise Physiologists in accordance to standard procedures described by the American College of Sports Medicine's (ACSM) (*ACSM's Guidelines for Exercise Testing and Prescription, 6th ed. Williams & Wilkins Publishers, 2000)*. These guidelines are outlined and presented below:

ACSM Risk Stratification Criteria for Cardiovascular Disease

Low Risk

Younger individuals (men < 45 years of age; women < 55 years of age) who are asymptomatic for
cardiovascular disease and possess no more than one positive cardiovascular disease risk factor.

Moderate Risk

Older individuals and/or those who are asymptomatic for cardiovascular disease and possess two or more cardiovascular disease risk factors.

High Risk

Individuals with one or more signs/symptoms suggestive of cardiovascular disease.

ACSM Criteria for Signs and Symptoms Suggestive of Cardiovascular Disease

1. Pain, discomfort (or other anginal equivalents) in the chest, neck, jaw, arms, or other areas that may be
   due to myocardial ischemia.
2. Shortness of breath at rest or with mild exertion.
3. Dizziness or syncope.
4. Orthopnea or paroxysmal nocturnal dyspnea.
5. Ankle edema.
6. Palpitations or tachycardia.
7. Intermittent claudication.
8. Known heart murmur.
9. Unusual fatigue or shortness of breath with usual activities.

ACSM Absolute and Relative Contraindications to Exercise

Absolute Contraindications
1. Unstable angina.
2. Uncontrolled dysrhythmias.
3. Recent EKG changes and cardiac events.
4. Acute myocarditis or pericarditis.
5. Acute pulmonary embolism or acute myocardial infarction.
6. Severe aortic stenosis.
7. Dissecting aneurysm.
8. Acute infections.

Relative Contraindications
1. Left main coronary stenosis.
2. Severe hypertension (> 200/110).
3. Tachycardia or bradycardia.
4. Uncontrolled metabolic disease.
5. High-degree AV block.
6. Chronic infectious disease.
7. Cardiomyopahty and outflow obstructions.
8. Stenotic valve disease.
9. Ventricular aneurysm.

Assessment of Hemodynamic Safety Markers (Heart Rate, Heart Rhythm, & Blood Pressure). Heart rate will be determined by an ECG. For the ECG, the subject's skin will be prepared for placement of 10 ECG electrodes. Electrode sites will be cleansed with sterile alcohol gauze using a circular motion. The site will be allowed to air dry or will be dried with a gauze pad. Electrodes will then be placed on the right subclavicular fossa (RA), left subclavicular fossa (LA), right abdomen (RL), left abdomen (LL), 4th intercostals space at the right sternal border (V1), 4th intercostals space at the left sternal border (V2), equidistant between V2 and V4 (V3), 5th intercostal space at the midclavicular line (V4), 5th intercostal space at the anterior axillary line (V5), and 5th intercostals space at the axillarly line (V6) of the chest. The subject will then be attached to a Quinton 710 ECG. Blood pressure will be assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Reported Side Effects from Supplements. Participants will report, by questionnaire administered in a blinded manner, whether they tolerated the supplement, supplementation protocol, as well as report any medical problems/symptoms they may have encountered throughout the protocol of the study.

Estimated Energy Intake/Dietary Inventories. The participants will be required to keep four-day dietary records at three time points (T2, T4, T5) that will be evaluated with the Food Processor dietary assessment software program (ESHA Research Inc., Salem, OR) to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet.

Resting Energy Expenditure Test. Participants will remove shoes and lie supine on a plinth. A sterile hood will be placed over the participant's head. Once the participant is ready to begin the test protocol, the subject will begin to have their expired gases monitored continuously throughout the exercise test. Resting expired gases will be collected using the Parvo Medics

2400 TrueMax Metabolic Measurement System (Sandy, UT). Participants will be asked to remain motionless, yet awake for twenty minutes.

Body Composition Assessments. Participants will undergo body composition tests in the ESNL. Prior to each assessment, height will be measured using standard anthropometry and total body weight will be measured using a calibrated electronic scale with a precision of +/- 0.02 kg. Body composition will then be determined using a calibrated Hologic 4500W dual-energy x-ray absorptiometry (DEXA) by licensed personnel with limited x-ray technology. The DEXA body composition test will involve having the participant lie down on their back in a standardized position in a pair of shorts/t-shirt or a gown. A low dose of radiation will then scan their entire body for approximately six minutes. The DEXA segments regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments for determination of fat, soft tissue (muscle), and bone mass. Radiation exposure from DEXA for the whole body scan is approximately 1.5 mR per scan. This is similar to the amount of natural background radiation a person would receive in one month while living in Waco, TX. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose will be less than 5 mR for the entire study.

Total body water will then be estimated using a Xitron 4200 Bioelectrical Impedance Analyzer (San Diego, CA) which measures bio-resistance of water and body tissues based on a minute low energy, high frequency current (500 micro-amps at a frequency of 50 kHz) transmitted through the body. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of this device has been approved by the Food and Drug Administration (FDA) to assess total body water and the current to be used has been deemed safe. This is measured through four electrodes placed on the body: one electrode will be placed on the posterior surface of the right wrist, in between the radial and ulna styloid processes (wrist bones), another electrode will be placed on the posterior surface of the right hand at the distal base of the second metacarpal; the third electrode will be placed on the anterior surface of the right foot at the distal end of the first metatarsal. Participants will lie on a table in the supine position and electrodes will be connected to the analyzer. After the subject is connected, age, gender, weight, height, and activity level are entered into the unit by the technician. After the unit has measured the resistance, which takes approximately 30 seconds, the unit then calculates total body water and body water percent.

Aerobic Exercise Capacity Test. Participants will first be seated next to the treadmill and resting heart rate and blood pressure will be measured. A sterile mouthpiece attached to a head harness will be secured on the participant. The participant will then have a noseclip placed on their nose. Resting expired gases will be collected using the Parvo Medics 2400 TrueMax Metabolic Measurement System (Sandy, UT). Once the participant is ready to begin the test protocol, he/she will straddle the treadmill with both legs while the treadmill is turned on at a speed of 2.0 mph and at a 0% grade. The participant will then use one foot to repeatedly swipe the belt in order to gauge the speed of the motion. Once the participant is familiar with this speed, he/she will step onto the belt while still gripping the handrail with both hands. Once the participant becomes comfortable walking on the treadmill, he/she will let go of the handrail and begin walking freely. The participant will then perform a standard symptom-limited Modified Balke treadmill maximal exercise test using the following speeds and grades:

Table 1.

Stage	Speed	Grade (%)	Duration (min.)
1	3.4	0	1
2	3.4	1	1
3	3.4	2	1
4	3.4	3	1
5	3.4	4	1
6	3.4	5	1
7	3.4	6	1
8	3.4	7	1
9	3.4	8	1
10	3.4	9	1
11	3.4	10	1
12	3.4	11	1
13	3.4	12	1

Blood Samples. Participants will donate approximately 3-4 teaspoons of fasting venous blood (approx 10-15 milliliters) during each baseline blood draw, ~5mL during the post-OGTT blood draws, and approx 10-15mL during the post-GXT blood draws. Blood samples obtained during testing sessions 2, 3, 5 and 6 will be via standard indwelling venous catheter, while blood samples obtained during testing session 1 and 4 will be via standard sterile venipuncture technique. All blood samples will be obtained from the antecubital vein using standard phlebotomy procedures by Matthew Cooke, Ph.D. or laboratory technician's trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. The phlebotomists and lab technicians will wear personal protective clothing (gloves, lab coats, etc.) when handling blood samples. Subjects will be seated in a phlebotomy chair. Their arm will be cleaned with a sterile alcohol wipe and sterile gauze. A standard rubber tourniquet will then be placed on the brachium (upper arm) and will be tight enough to visibly indent the skin, but not cause the patient discomfort. For venipunctures, an antecubital vein will be palpated and then a 22 gauge sterile needle attached to a plastic vacutainer holder will be inserted into the vein using standard procedures. One plasma vacutainer tube (Green top), one serum separation vacutainer tube (Red tops), and one whole blood vacutainer (Purple top) will be inserted into the vacutainer holder for blood collection in succession using multiple sample phlebotomy techniques. Once samples are obtained, the vacutainer holder and needle will be removed. The needle will then be discarded as hazardous waste in a plastic sharps container. For the indwelling catheter sampling, the entry site will be thoroughly cleaned with an alcohol prep pad and allowed to dry and also sterilized with 10% povidone-iodine solution and allowed to dry. The participant will be instructed to lower their arm and make a fist several times in order to maximize venous engorgement. The appropriate vein will be selected. If a suitable vein is difficult to identify, the arm may be covered with a warm, moist compress to help with peripheral vasodilatation. If after a meticulous search no suitable veins are found, then the tourniquet will be released from above the elbow and placed around the forearm to search in the distal forearm, wrist and hand. To puncture the vein, the 20 gauge catheter will be held in the dominant hand. With the bevel up, enter the skin at about a 30-degree angle and in the direction of the vein. After entering the skin, the angle of the catheter will be reduced until it is nearly parallel to the skin. If the vein appears to "roll" (move around freely under the skin), the venipuncture will begin by applying counter tension against the skin just below the entry site using the thumb of the non-dominant hand. The skin

will be pulled distally toward the wrist in the opposite direction the needle will be advancing, being careful not to press too hard which will compress blood flow in the vein and cause the vein to collapse. The catheter will be advanced into the vein until blood is seen in the "flash chamber" of the catheter. After entering the vein, the plastic catheter (which is over the needle) will be advanced into the vein while leaving the needle stationary. The hub of the catheter will be all the way to the skin puncture site. The tourniquet will be released. Gentle pressure will be applied over the vein just proximal to the entry site to prevent blood flow. The needle will be removed from within the plastic catheter and disposed in an appropriate sharps container. The catheter will be taped in place using strips of tape and a sterile dressing. Once sampling is complete, the site of the blood draw will then be cleaned with a sterile alcohol wipe and gauze and a sterile Band-Aid will be placed on the site. The blood collection tubes will be labeled and placed in a test tube rack. Laboratory technicians (who have received blood borne pathogen training and will be wearing personal protective clothing) will centrifuge the serum and/or plasma samples, transfer serum and/or plasma into labeled storage containers, and store at -80°C for later analysis.

Muscle Biopsies. Percutaneous muscle biopsies (10-15 mg), using a fine needle aspiration technique, will be obtained from the middle portion of the vastus lateralis muscle of the dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. Muscle biopsies will be performed by Dr. Matt Cooke or an appropriately trained graduate student. Muscle biopsy training requires that graduate students successfully perform a minimum of 3 biopsy procedures on non-research participants under Dr. Willoughby or Dr. Cooke's direct supervision before they are allowed to perform them on research participants. Graduate students must then successfully perform 15 biopsies on research participants under direct supervision before they are allowed to perform muscle biopsies without supervision. The procedures used are previously-established procedures by Darryn Willoughby, Ph.D., who has extensive experience in performing muscle biopsies as a part of his research. For subsequent biopsies, muscle tissue will be extracted from the same location by using the previous location and depth markings on the needle. Pre-biopsy preparations will include removing hair from the procedure area and wiped with 70% isopropyl alcohol prep pads. The biopsy site will be numbed with 1cc of 2% Xylocaine with epinephrine. After local anesthesia, the biopsy site will be sterilized with 10% povidone-iodine solution and a pilot hole will be made with an 18-gauge needle. The biopsy procedure will then begin and take 15-20s to perform. Once the muscle sample has been obtained, pressure will be immediately applied to the biopsy site and an adhesive bandage immediately applied. Bleeding is minimal due to the small puncture-type opening; therefore, only a standard band-aid type bandage is needed to cover the puncture. The biopsy needles will be discarded as hazardous waste in an appropriately-labeled plastic sharps container. The site of the biopsy will be cleaned with a sterile alcohol wipe and gauze. The alcohol wipe and gauze then will be discarded in an appropriately labeled biohazard waste receptacle. The tissue sample will be stored at -80 degrees C for future analyses. Written instructions for post-biopsy care will be given to the subjects. The participant will be instructed to leave the band-aid in place for 12 hours (unless unexpected bleeding or pain occurs) and asked to contact the lab immediately if they feel there is a problem. The time course nature of the study will allow for daily follow-up to occur in order to ensure all biopsy locations are healing correctly and free of infection. Aside from the testing sessions, the participant will be further advised to refrain from vigorous physical activity during the first 24 hours post-biopsy. These suggestions will minimize pain and possible bleeding of the area. If needed, the subject may take non-prescription analgesic medication such as Acetaminophen to relieve pain if needed. However, medications such as aspirin, Nuprin, Bufferin, or Advil will be discouraged as these medications may lead to ecchymosis at the biopsy site. Soreness of the area may occur for about 24 hours post-biopsy.

Equipment

Digital Scale. Total body weight will be determined using a digital scale accurate to ±0.02 kg. The scale is calibrated by placing certified 25-kg weights and balancing the scale. Other than general instructions, special skills are not required to measure body weight.

Mercurial Sphygmomanometer. Blood pressure will be assessed by auscultation of the brachial artery using a mercurial sphygmomanometer using standard clinical procedures.

Bioelectrical Impedance Analyzer (BIA). The Xitron 4200 Bioelectrical Impedance Analyzer (San Diego, CA) which measures bio-resistance and body composition based on a minute low energy, high frequency current transmitted through the body from surface electrodes attached to standardized anatomical locations on the dorsal surface of the right hand and foot while the subject lies motionless in a supine position. The analyzer is calibrated internally to a standard electrical current by pressing the calibration key located on the unit. A trained research assistant will perform this procedure.

Dual-Energy X-Ray Absorptiometer (DEXA). Body composition measurements will be determined by qualified personnel (in compliance with State Regulations) using a Hologic 4500W dual energy x-ray absorptiometer (Waltman, MA). This system segments regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments (i.e., bone mass, fat mass, and fat-free/soft tissue mass). Quality control (QC) calibration procedures will be performed on a spine phantom (Hologic X-CALIBER Model DPA/QDR-1 anthropometric spine phantom) prior to each testing session. In addition, weekly calibration procedures will be performed on a density step calibration phantom.

Resting Energy Expenditure. Resting energy expenditure measurements will be obtained using Parvo Medics 2400 TrueMax metabolic measurement system (Sandy, UT).

Aerobic Exercise Capacity Testing. Maximal cardiopulmonary measurements will be obtained using Parvo Medics 2400 TrueMax metabolic measurement system (Sandy, UT). Participants will be attached to the Quinton 710 ECG (Bothell, WA) and walk on a Quinton QStressTm65 treadmill (Bothell, WA).

Muscle Biopsy Needle. The muscle biopsy technique (as mentioned above) will be performed with a TRU-CORE® 1 Automatic Reusable Biopsy Instrument (Angiotech, Medical Device Technologies, INC., Gainsville, Florida, USA).

Clinical Chemistry Analysis. Serum samples will be shipped to Quest Diagnostics, Inc. (Madison, NJ) and a standard clinical chemistry panel will be run. Cell-Dyne 3500 (Abbott Diagnostics, Dallas, TX) hematology analyzer will be used to measure whole-blood markers [leukocytes, lymphocyte differentials (neutrophils, lymphocytes, monocytes, eosinophils, basophils), red blood cells, hemoglobin, hematocrit, platelets].

Serum & Plasma Analysis. Blood samples will also be used to assess hormone profiles insulin, estrogen, and cortisol, and the levels of the inflammation-related, interleukin-1β (IL-1β), interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF), and tumor necrosis factor-α (TNF-α) spectrophotometrically using either enzyme-linked immunoabsorbent assays (ELISA) or enzyme immunoassys (EIA) with a Wallac Victor-1420 micoplate reader (Perkin-Elmer Life Sciences, Boston, MA) or the Bio-Plex bead-based multiplex assays (Luminex xMAP technology) by Bio-

Rad Laboratories, Inc. (Hercules, CA; #7000005KYMR). The assays will be performed at either 405 or 450 nm wavlength against a known standard curve.

Muscle Protein Gene Expression. Muscle protein samples will be used to isolate total RNA, reverse transcribed, and then used to perform real-time polymerase chain reaction using specific oligonucleotide primers to amplify target genes using an iQ real-time PCR system (Bio-Rad, Hercules, CA).

Participants

Recruitment

Approximately 45 sedentary, overweight, pre-diabetic [fasting plasma glucose: 100-125mg/dL (4)] premenopausal females between 18 and 35 years of age, will be recruited for this study. Participants will be declared sedentary if they have not exercised for 30 minutes a day at least 5 days a week for a year or more. Additionally, oral contraceptives appear to reduce glucose tolerance by reducing peripheral tissue insulin sensitivity. Since oral contraceptives may increase glucose intolerance, all participants will be women who have not taken oral contraceptives for at least two months. Prior to participation in the study, all potential participants must provide written physician approval. Participants who qualify for the study will be cleared for participation by successfully completing a series of health screening examinations (i.e., questionnaire, blood pressure assessment) by qualified research staff members. A recruitment flyer that will be posted on campus, at area fitness centers, and on the Internet (http://www3.baylor.edu/HHPR/research/subjects/) and sent via campus mail is attached.

Selection Criteria

Participants will not be allowed to participate in the study if they:

1. have a body mass index less than 25.
2. have been involved in a habitual exercise training program (minimum of 2.5 hours/week for 1 year);
3. have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism;
4. have a history of pulmonary disease, medically controlled hypertension, hepatorenal disease, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, cancer, peptic ulcers, anemia, or bleeding disorders;
5. have liver, kidney disease, or heart disease;
6. are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g, thyroid, insulin, etc), psychotropic, or neuromuscular/neurological medications;
7. have taken nutritional supplements or prescription medications that may affect body composition and/or muscle mass (e.g., creatine, HMB) within three months prior to the start of the study.
8. have any absolute or relative contraindication for exercise testing or prescription as outlined by the American College of Sports Medicine;
9. report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study.

Compensation or Incentives

Participants completing all familiarization and testing sessions as well as turning in all required materials (i.e., dietary and training logs) in the study will be paid $150. Participants may receive information regarding results of these tests if they desire. If participants are Baylor students, they will not receive any academic credit for participating in this study.

Potential Risks

The resveratrol and pterostilbene supplements to be investigated in this study have been studied for various medical uses in rats and in vitro, but limited research has been performed in humans. However, recent research has demonstrated that oral administration of these compounds is not associated with any significant medical side effects. Pterocarpus marsupium Roxb. extract (450 mg), one source of pterostilbene, was recently used to study Cox-2 inhibition in humans (5). No adverse side effects were reported in healthy human volunteers. Resveratrol is currently under study in Phase II clinical trials for use in prevention of colon cancer (www.cancer.org). Thus, resveratrol has passed Phase-I trials for safe dose ranges, any side effects, and how the body copes with the drug (1). Additionally, both compounds are found in ayurvedic medicines, which have been commonly used for hundreds of years, to treat hyperlipidemia and hyperglycemia. Moreover, these supplements are currently available in over the counter nutritional supplements sold in the United States. As with the vast majority of nutritional supplements, however, the FDA may not have evaluated the safety or marketing claims of resveratrol and pterostilbene.

Participants who meet eligibility criteria will be exposed to a low level of radiation during the DEXA body composition tests, which is similar to the amount of natural background radiation a person would receive in one month while living in Waco. In addition, a very low level of electrical current will be passed through each participant's body using a bioelectrical impedance analyzer. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of the BIA and DEXA analyzers has been shown to be safe methods of assessing body composition and total body water and is approved by the FDA.

Participants will donate samples of venous blood during the study using standard phlebotomy procedures. This procedure may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising. The subject may also experience some dizziness, nausea, and/or faint if they are unaccustomed to having blood drawn. Participants will likely experience short-term muscle soreness and moderate fatigue, and may experience muscle strains/pulls during their routine resistance-training program.

Subjects donating muscle biopsies may experience some anxiety before this procedure regarding a perception of pain or discomfort. The biopsy procedure may cause a small amount of bleeding and/or pain as the pilot hole is made and the sample is extracted from the muscle. However, once the anesthesia takes affect, there is usually only mild pressure and a small amount of bleeding as the needle is inserted and extracted. During the biopsy procedure, subjects may experience a slight localized cramping followed by brief and minor aching but these symptoms usually go away when the needle is withdrawn. Frequently, subjects feel little or no sensation at all. Although the muscle selected for biopsy (vastus lateralis) has no major blood vessels or nerves in the areas where the biopsy needle will be inserted, there is the rare occurrence of compressing or cutting small nerve branches, which can sometimes cause temporary tingling and numbness in the skin. These responses, when they have occurred, have dissipated in a few days or weeks. After the needle is withdrawn, pressure is applied to the site of the incision to prevent any unwarranted

bleeding (there is usually very little bleeding). After the biopsy, the muscle is likely to be moderately sore for about 24 hours. This soreness is similar to muscle soreness following unusually vigorous exercise or a muscle injury especially if muscle is compressed against a bone (e.g., "charley horse"). Complications accompanying this procedure are rare and no complications have been observed in subjects who have donated biopsies in the EBNL in previous studies. The primary risks, include bleeding, hematoma (bruising), infection, and slight scarring of the skin have occurred, however. Some individuals may develop keloid scarring at the site of incision. Also, some individuals may have an allergic reaction to the anesthetic, such as a local rash, or difficulty breathing. To minimize these risks, the amount of anesthetic used will be approximately 2-4% of the maximal dose for a normal sized individual. Every precaution will be made to keep these risks to a minimum. Additionally, these potential risks can be prevented and/or treated with rest, ice, compression, elevation, and adhering to post-biopsy care instructions. Taking a mild non-prescription pain medication such as Tylenol, providing the subject can tolerate these medications, is also recommended for pain. In all these procedures, care is taken to employ precautions to avoid infection, including the "universal precautions" for the handling of blood and infectious materials. Muscle biopsies do not cause any permanent damage with the exception of a small scar which should become undetectable over time, although some individuals may develop keloid scarring at the site of the incision.

Laboratory personnel represent trained, non-physician, certified exercise specialists (American Society of Exercise Physiologies Certified Exercise Physiologist, Certified Strength & Conditioning Specialists, and/or American College of Sports Medicine Health Fitness Instructor$_{SM}$, Exercise Technologist$_{SM}$, or Exercise Specialists$_{SM}$). All personnel involved in collecting data will be certified in CPR, which is also a condition to holding these professional certifications. A telephone and automated electronic defibrillator (AED) is located in the laboratory in case of any emergencies and there will be no less than two researchers working with each subject during testing. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance.

Potential Benefits

The main benefit that participants may obtain from this study is that if these two thermogenic nutritional supplements are effective there is a possibility that they may experience decrements in percent body fat during training as well as improved health profiles. Participants may also gain insight about their health and fitness status from the assessments to be performed. However, even if no individual benefit is obtained, participating in this study will help to determine whether ingesting this nutritional supplement affects training adaptations and body composition. This information will be helpful to athletes and non-athletes alike who use thermogenic supplements during training to know whether they are effective or not.

Assessment of Risk

Because they are relatively new to the market and little research conducted in humans, the potential medical benefits of resveratrol and pterostilbene are not yet fully known; although, these compounds are available in a number of over the counter nutritional supplements. Initial results in humans and rats suggest that these supplements may provide benefit by reducing lipid levels, improving insulin sensitivity, weight loss, cardioprotection, cancer growth inhibition, and increased mortality (10-12). However, additional well-controlled research is necessary before conclusions can be drawn. This study will help determine whether ingesting either resveratrol or

pterostilbene may prove to be effective at improving insulin sensitivity in overweight prediabetic females. The only known side effects associated with these polyphenols are found with extremely high and unrealistic doses and include: mild anaemia, an increased liver weight, and increased serum cholesterol.

The risk of supplementation of these compounds at the levels to be evaluated in this study is low. The greatest risk associated with participating in this study will be the participants initiating an exercise-training program. Since the participants to be used in this study are prediabetic, are required to have physician clearance, and would be told to begin exercising and eating a healthier diet, these risks would be no different than if they participated in their own training program or exercised at a local gym. Additionally, each circuit training session will supervised by a Certified Strength and Conditioning Specialist. Therefore, the potential benefits of subjects participating in this study outweigh the potential risks.

Compensation for Illness or Injury

Each participant will agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss suffered as a result of participation in this study regardless of the cause of injury, damage, or loss.

Confidentiality

Information obtained from this research (including questionnaires, medical history, laboratory findings, or physical examination) will be kept confidential to the extent permitted by law. However, according to FDA regulations, records will be open to FDA representatives to review if necessary. This may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken throughout this study. Records of the research may also be subpoenaed by court order or may be inspected by federal regulatory authorities. Data derived from this study may be used in reports, presentations and publications. However, participants will not be individually identified unless they give their written consent.

Data Presentation & Publication

Data will be presented at an appropriate scientific conference (e.g., American College of Sports Medicine, International Society of Sports Nutrition, Experimental Biology, etc.) and published in a peer reviewed scientific journal (e.g., Journal of Nutrition, International Journal of Sport Nutrition and Exercise Metabolism, etc.).

Statement on Conflict of Interest

Funding for the study has been obtained from Sabinsa, Inc. (Piscataway, NJ) through research grants awarded to Baylor University. Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Table 2. Overview of a Typical Daily Meal Plan

Meal	Example Meals
Breakfast	Meal replacement shake 1 cup low-fat milk 1 piece of fruit Coffee or tea
Mid-morning snack	½ snack bar Tea or coffee
Lunch	Large (> 2 cups) salad (a variety of raw vegetables) Low-fat salad dressing 4 ounces lean cold cuts 6 small crackers No-calorie carbonated beverage
Mid-afternoon snack	½ snack bar No-calorie beverage
Dinner	4 ounces meat ½ baked potato Small pat margarine 2 cups low-starch vegetables (two different kinds) Water
Evening snack	1 cup low-fat milk Cut up raw vegetables

Table 3. Overview of the Resistance Training Program

Weeks	Modalities (type of exercise)	Intensity (% 1-Rep Max)	Sets/Reps	Frequency (Days/Week)
1-2	upper-body exercises (chest, shoulders, back, arms, abs); lower-body exercises (gluteals, quadriceps, hamstrings)	50%-55%	2/15-20	3
3-4	upper-body exercises (chest, shoulders, back, arms, abs); lower-body exercises (gluteals, quadriceps, hamstrings)	50%-55%	2/15-20	3
5-6	upper-body exercises (chest, shoulders, back, arms, abs); lower-body exercises (gluteals, quadriceps, hamstrings)	55%-60%	3/12-15	3
7-8	upper-body exercises (chest, shoulders, back, arms, abs); lower-body exercises (gluteals, quadriceps, hamstrings)	55%-60%	3/12-15	3

Table 4. Overview of the Aerobic Training Program

Weeks	Modalities (type of exercise)	Intensity (% HRR)	Minutes	Frequency (Days/Week)
1-2	Walking, Jogging	45%-55%	35-40	3
3-4	Walking, Jogging	55%-60%	35-40	3
5-6	Walking, Jogging	55%-60%	40-45	3
7-8	Walking, Jogging	60%-65%	40-45	3

APPENDIX D

Informed Consent

BAYLOR UNIVERSITY

Department of Health, Human Performance, & Recreation
Informed Consent Form

Title of Investigation:	Effects of Resveratrol and Pterostilbene Supplementation on Insulin and Exercise-Mediated Signaling Pathways for Glucose Uptake in Overweight Insulin-Resistant Females: A Double-Blind, Clinically-Controlled Study
Principal Investigator:	Mathew Cooke, PhD Assistant Professor, Department of HHPR, Baylor University
Co-investigators:	Geoffrey Hudson, MA, CSCS Exercise and Biochemical Nutrition Lab, Baylor University Brian Shelmadine, MA, CSCS Exercise and Biochemical Nutrition Lab, Baylor University Thomas Buford, MS, CSCS Exercise and Biochemical Nutrition Lab, Baylor University Mike Greenwood, PhD, FNSCA, CSCS*D, FACSM, FISSN Professor, Department of HHPR, Baylor University Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS, CISSN Associate Professor, Department of HHPR, Baylor University Rodney Bowden, PhD Associate Dean, Graduate Studies and Research Associate Professor, Health Education Ronald Wilson, MD Exercise and Sport Nutrition Lab, Baylor University
Sponsors:	Sabinsa, Inc. (Piscataway, NJ)

Rationale:
Diabetes mellitus is a critical health concern that has tripled in prevalence over the past 25 years. The CDC estimated 17.9 million Americans suffered from this condition in 2007 and that another 5.7 million Americans were estimated that to have diabetes but were undiagnosed. Of the two most common kinds of diabetes, Type 2 diabetes is the most prevalent, making up 90-95 percent all diagnosed cases in adults. Type 2 diabetes is preceded by an initial insulin resistant state

where the insulin-responsive cells in the body do not appropriately react to circulating insulin, which results in an increase in blood glucose levels and disturbances in fatty acid metabolism. Insulin binding is the primary stimulus for glucose uptake in muscle cells and fat cells at rest or

after a meal. Insulin serves to lower blood glucose levels via an insulin-signaling pathway within fat cells and muscle cells that results in movement of the glucose transporter 4 (GLUT4) glucose transporter to the cell membrane. This initial insulin-resistant state leading to Type 2 diabetes is preceded by Pre-diabetes where people can have impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or both. If a person has IFG, the fasting glucose level will be between 100-125 mg/dL. If a person has IGT, after given oral glucose, their blood glucose level is between 140-200 mg/dL after two hours.

It is not a given that those with pre-diabetes will progress to Type 2 diabetes. Progression can be prevented. The first line of defense to prevent the development or further progression of Type 2 diabetes mellitus is lifestyle change. Of particular importance is losing weight, increasing physical activity, and eating better. The goal is to improve insulin's ability to get glucose into muscle cells. One way to improve this ability is with exercise. However, during moderate-intense exercise glucose uptake is accomplished through insulin-independent mechanisms. During exercise, insulin levels in the blood decrease. Thus, glucose is taken into the cell via different means. Ultimately, either the insulin-mediated pathway or the non-insulin mediated pathway, induced by exercise, causes GLUT4 to move to the plasma membrane. During exercise the liver produces more glucose for the muscles to use as a fuel source. However, after exercise has stopped, muscles take in less glucose via the insulin-independent pathway, but the liver continues to produce glucose. At this time insulin levels in the blood begin to increase. However, insulin-resistant people are resistant to the effects of insulin and often suffer from post-exercise hyperglycemia.

Often drugs such as Metformin are prescribed to improve insulin sensitivity. Metformin, one of the most frequently prescribed diabetes medications, also enhances GLUT4 transport and insulin sensitivity in muscle and fat cells via insulin-independent mechanisms. Resveratrol and pterostilbene are two compounds found in high concentrations in red wine and a variety of plant sources such as grape skin, berries, pomegranates, and peanuts that are believed to improve insulin sensitivity in a manner similar to Metformin. These two compounds are found in a variety of nutritional supplements available at most health food stores. Over the past 5 years, research into these 2 botanical compounds has expanded, with benefits from supplementation including: improved insulin sensitivity, weight loss, cardioprotection, cancer growth inhibition, and increased mortality. Although interest in such compounds has increased, limited research has examined their purported effects in humans. Administration of resveratrol and pterostilbene could not only improve glucose uptake at rest, they could provide a synergistic enhancement of glucose uptake during exercise, but more importantly, continue to stimulate glucose uptake after exercise and hopefully attenuate a post-exercise hyperglycemic response.

Thus, the primary overall purpose of this study is to determine the effects of resveratrol or pterostilbene supplementation on glucose/insulin kinetics and markers of inflammation at rest and following an exercise bout. Further, this study will also determine the effects of prolonged supplementation and exercise training on additional cardiovascular risk factors often found with insulin resistance. These include elevated LDL, triglycerides, total cholesterol, decreased HDL levels, and an overall state of increased inflammation.

Description of the Study:

I will be one of 45 eumenorrheic, physically inactive (not presently engaged in a regular, structured exercise program) females between the ages of 18-35 with a body mass index (BMI) ≥ 25 who will participate in this study. During an initial familiarization session, I will be informed of the requirements of the study and sign an informed consent statement in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. A medically trained individual will examine me to determine if I am qualified to participate in this study. If I am cleared to participate in the study, I will be familiarized to the testing procedures. Familiarization will take approximately 30 minutes to complete. Once I complete the familiarization session, I will complete pre-supplementation testing.

I will not exercise for 48 hours nor eat for 12 hours prior to all testing sessions (including the initial familiarization session). On reporting to the lab, I will be weighed and seated in a comfortable chair where my resting blood pressure will be measured using a standard sphygmomanometer and heart rate determined by assessing pulse rate from my wrist. I understand that I will then have my resting energy expenditure measured. This will involve lying down on my back on an exam table for 20 minutes while a clear plastic bubble is placed over my head. I will then have my body composition determined by using a Hologic 4500W dual energy x-ray absorptiometer (DEXA). This will involve lying down on my back on the DEXA exam table in a pair of shorts or a gown for about 6 minutes. I understand that a low dose of radiation will scan my entire body to determine the amount of fat weight, muscle weight, and bone weight. I understand that I will be exposed to an X-ray dose that is similar to the amount of natural background radiation a person would receive in one month while living in Waco. I understand that I will then donate about 15 milliliters (3-4 teaspoons) of venous blood from a vein in my arm. Blood samples will be obtained using standard/sterile procedures using a needle inserted into a vein in my arm. I understand that personnel who will be taking my blood are experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. This will take about 5-minutes. .

I understand that I will also donate a muscle biopsy sample prior to beginning supplementation. I understand that Matthew Cooke, PhD and trained research assistants will be responsible for collecting muscle biopsy samples. The muscle biopsy procedure basically involves sterilizing and anesthetizing or numbing (2% Xylocaine with epinephrine) the biopsy site on the outside middle of my thigh. Then, a pilot hole (about the diameter of hypodermic needle) is made in my skin and fascia in order to provide access to underlying muscle. Approximately 10 to 15 milligrams of muscle tissue is then extracted from the thigh muscle using a sterile muscle biopsy needle according to standard clinical procedures. Once the muscle sample has been obtained, pressure will be immediately applied to minimize bleeding. Since the site is small with minimal bleeding; I understand only a small bandage will be required to cover the biopsy site. I will then be given a list of post-biopsy instructions on how to clean and care for the incision in order to promote healing. I understand that I will have to return to the lab within 24 to 72 hours so that the biopsy sites can be checked for infection and improper healing. I understand that I will begin ingesting the appropriate supplement the following day.

I understand that after baseline testing, I will be matched based on age, body composition, and fasting glucose to orally ingest capsules containing a placebo, 250mg of resveratrol, or 250mg of pterolstilbene twice daily for the duration of the study. Resveratrol and pterostilbene are two polyphenolic botanical supplements that are found in high concentrations in red wine and a

variety of plant sources such as grape skin, berries, pomegranates, and peanuts. Both resveratrol and pterostilbene are purported to improve insulin sensitivity, weight loss, cardioprotection, cancer growth inhibition, and mortality. I understand that the supplements will be prepared in capsule form and presented to me packaged in a blinded format (where I don't know which one I am taking). I understand that my compliance in taking the supplements will be monitored by returning empty bottles to the EBNL when I report back for testing after 4 days, 4 weeks, and 8 weeks of supplementation. I understand that if I do not take my supplements I will be removed from the study.

I understand that during the supplementation period I will be required to participate in a structured exercise training program 3 times weekly, along with a daily energy-controlled diet. Study personnel will provide me with the guidelines for the exercise and diet program and I will be required to document my exercise training and dietary intake for review by one of the study's investigators. After baseline testing, I understand that following 4 days, 4 weeks, and 8 weeks of supplementation, I will be scheduled to return to the lab for subsequent testing.

I understand that after taking the supplement for four days I will return to lab for a second testing session. I understand that I will be required to have my heart rate, blood pressure, and total body water determined. I understand that an indwelling catheter will be inserted into my antecubital vein using standard techniques and a blood sample will be taken. I understand that I will also donate a muscle biopsy sample. I will then undergo an oral glucose tolerance test, The oral glucose tolerance test will require me to ingest 75g of glucose and remain in the laboratory for 2 hours. Additional blood samples will be drawn at 30 minutes and one hour post glucose ingestion. At 2 hours post glucose ingestion, a blood and muscle sample will be taken. In addition, I will turn in a 4-day dietary record and complete a report of side effects from supplementation questionnaire to determine if I have experienced any unexpected problems or adverse events from participating in this study.

I will return to the lab the following day for testing session number three. I understand that I will again have my heart rate, blood pressure, and body composition assessed. I understand that I will then donate another blood and muscle biopsy using the same techniques as described in testing session two. I understand that the blood and muscle biopsy sample will be taken on the contralateral arm and leg, respectively. I understand that I will then undergo a maximal cardiopulmonary treadmill test. For the maximal cardiopulmonary exercise treadmill test, I understand that I will then be positioned on the treadmill and a sterile mouthpiece will be placed in my mouth and a mouthpiece holder will be placed on my head. I understand that a nose clip will be placed on my nose and that the air I breathe will be measured for oxygen and carbon dioxide content. Once the equipment is attached, I will be given instructions to begin walking on the treadmill. I will then perform an exercise test that involves increasing the speed and grade I am walking on the treadmill until I reach my maximal effort. I understand that heart rate and my ratings of exertion will be monitored throughout the test. I understand that my heart rate and rhythm will be monitored by an electrocardiogram, where electrodes will be placed at standardized locations on my shoulders, hips, and chest. Once I reach my maximum, I understand that I will undergo a slow walking and seated recovery period. This test will take about 20 minutes to complete. Following the exercise test, additional blood samples will be drawn at 30 minutes and one hour post glucose ingestion. At 2 hours post glucose ingestion, a blood and muscle sample will be taken.

After testing session three I will then begin my exercise and nutrition program, and continue taking the supplement. I understand that after four weeks I will return to the laboratory for a fourth testing session. I understand I will be required to have my heart rate, blood pressure, and

body composition determined, and undergo a maximal cardiopulmonary treadmill test. In addition, I will turn in a 4-day dietary record, four weeks of exercise training logs, and complete a report of side effects from supplementation questionnaire to determine if I have experienced any unexpected problems or adverse events from participating in this study.

After testing session four, I will continue taking my supplement and resume my exercise and nutrition program for an additional four weeks. I understand that after a total of 8 weeks (four weeks following testing session four), I will return to the laboratory for a fifth and six testing session. I understand that I will be required to undergo the exact same battery of tests performed during the second and third testing sessions. In addition, I will turn in a 4-day dietary record, four weeks of exercise training logs, and complete a report of side effects from supplementation questionnaire to determine if I have experienced any unexpected problems or adverse events from participating in this study. I understand that if clinically significant side effects are reported, I will report those to Matthew Cooke, Ph.D, Geoffrey Hudson, MA, or Brian Shelmadine, MA. I may then be referred to discuss the problem with the ESNL physician, Dr. Ronald Wilson to determine whether any medical treatment is needed and/or whether I can continue in the study. I understand that if I fail to report my progress and health status to the research assistant I may be removed from the study.

I agree to do my best to: 1) follow the instructions outlined by the investigators; 2) show up to all scheduled testing times; 3) take the supplements as instructed, and 4) comply with the exercise training and diet protocol. I agree not to take any other nutritional supplements or performance enhancing aids during this study (i.e., vitamins/minerals, creatine, HMB, androstenedione, DHEA, etc). In addition, I agree not to take any non-medically prescribed medications and to report any medication that is prescribed for me to take during this study. I understand that if I take any other nutritional supplements or medications during the course of the study that may affect body composition, or blood hormone levels that I may be removed from the study.

Exclusionary Criteria

I understand that in order to participate in the study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 1) my body mass index (BMI) is not at least 25; 2) my resting blood glucose is not between 100mg/dL and 120mg/dL; 3) I have taken oral contraceptives in the past 2 months; 4) I have menstrual irregularity (e.g., oligomenorrhea or amenorrhea); 5) I have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; 6) I have a history of pulmonary disease, medically controlled hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 7) I am taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g, thyroid, insulin, etc), psychotropic, neuromuscular/neurological; 8) I have a chronic infection (e.g., hepatitis, HIV, etc.); or 9) a known bleeding disorder.

I have reported all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that I am presently taking. I have completed medical history questionnaires and am not aware of any additional medical problems that would prevent me from participating in this study. I agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that I take during the course of the investigation to Matthew Cooke, Ph.D. (254-710-4025), Geoffrey Hudson, MA (254-710-4012), or Brian Shelmadine, MA (254-710-4012).

Risks and Benefits

I understand that the supplements to be investigated in this study have been studied for use as an anti-diabetic supplement in rats, have been used in few humans studies, but based on clinical trials it has been demonstrated that oral administration of these compounds is not associated with any significant medical side effects. These nutrients are currently available in over the counter nutritional supplements sold in the United States. As with any food or nutritional supplement, possible side effects may include stomach upset, gastrointestinal distress, allergic reactions, changes in mood and vigor, and/or changes in training adaptations. However, as with the vast majority of nutritional supplements, I understand that the FDA may not have evaluated the safety or marketing claims of resveratrol or pterostilbene.

I understand that I will be exposed to a low level of radiation during the DEXA body composition tests, which is similar to the amount of natural background radiation a person would receive in one month while living in Waco, TX. This analyzer is commercially available and has been used in the health care industry as a means to assess body composition for over 10 years. The use of the DEXA analyzer has been shown to be a safe method of assessing body composition and is approved by the FDA.

I also understand that I will have about 3-4 teaspoons (~7-15milliliters) of blood drawn from a vein in my forearm using a sterile needle and blood tubes by an experienced phlebotomist 18 times over the nine weeks of this study. This procedure may cause a small amount of pain when the needle is inserted into my vein as well as some bleeding and bruising. I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn.

I understand that I will have nine muscle biopsies performed by trained muscle physiologists over the nine weeks of this study. I understand that there may be some pain, minimal bleeding, and some residual bruising and soreness involved in this procedure. Specifically, I understand that there is a potential risk of contracting a community MRSA infection by participating in a muscle biopsy. I understand that if I feel it necessary I may take a non-prescription analgesic medication such as Tylenol to relieve pain if needed and that some soreness of the area may occur for about 24 hours after the biopsy. I will also be advised to avoid such medications such as aspirin, Advil, Bufferin, or Nuprin, as they may lead to bruising at the biopsy site.

I understand that my exercise program may cause symptoms of fatigue, shortness of breath, and/or muscular fatigue/discomfort and soreness. I understand that I may also experience muscle strains/pulls during my exercise program. However, these risks will be similar to the risk of participating in any exercise program. I also understand that trained, non-physician exercise specialists certified in CPR will supervise exercise assessments. I understand that a telephone and an automated electronic defibrillator are in the laboratory in case of any emergencies and that there will be no less than two researchers working with me during each testing session. I understand that emergency procedures are posted in the lab in the unlikely event that any emergency may arise.

I understand that the main benefit that may be obtained from this study is to determine whether providing 250mg of resveratrol or 250mg of pterostilbene, coupled with an exercise training and diet program, is effective at decreasing fat mass, and improving body composition, metabolism, cardiopulmonary performance, serum lipids, inflammatory markers, and hormones.

Alternative Treatments

This is not a medical treatment. Therefore, if medical treatment is needed, I must continue to obtain treatment for any medical problem I might have from my personal physician.

Costs and Payments

If I am a Baylor University student, I will not receive any academic credit for participating in this study. I understand that if I am an intercollegiate scholarship athlete I may not be eligible to receive payment to participate in this study. If eligible I will be paid $150 for completing the familiarization and all experimental testing sessions over the course of the nine weeks. I also understand that I will be given free blood assessments during the course of the study as described above and may receive information regarding results of these tests if I desire.

New Information

Any new information obtained during the course of this research that may affect my willingness to continue participation in this study will be provided to me. In addition, I will be informed of any unusual/abnormal clinical findings in which medical referral to my personal physician may be warranted. If I desire, I may request that this information be provided to my physician.

Confidentiality

I understand that any information obtained about me in this research, including questionnaires, medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, I understand in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review my records from this study which may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken about my participation in this study. In addition, I understand that my records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities. I understand that data derived may be used in reports, presentations, and publications. However, I will not be individually identified unless my consent is granted in writing. Additionally, that confidentiality will be maintained by assigning code numbers to my files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. I understand that once blood samples are analyzed that they will be discarded.

Right to Withdrawal

I understand that I am not required to participate in this study and I am free to refuse to participate or to withdraw from the study at any time. Further, that my decision to withdraw from the study will not affect my care at this institution or cause a loss of benefits to which I might be otherwise entitled. If there is concern about my medical safety, I may be referred to seek medical attention.

Compensation for Illness or Injury

I understand that if I am injured as a direct result of taking part in this study, I should consult my personal physician to obtain treatment. I understand that the cost associated with the care and treatment of such injury will be the responsibility of me or my insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study. I understand that Baylor University, the investigator's institutions, and the grant sponsor have not budgeted funds to compensate me for injury or illness that may result from my participation in this study and thus will not be accountable for illness or injury acquired during the course of this study. However, I may be referred to my personal physician if any clinically significant medical/psychological findings are observed during the course of this study.

I agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss I suffer as a result of my participation in this study regardless of the cause of my injury, damage or loss.

Statement on Conflict of Interest

Funding for the study has been obtained from Sabinsa, Inc. (Piscataway, NJ) through research grants awarded to Baylor University. Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Voluntary Consent

I certify that I have read this consent form or it has been read to me and that I understand the contents and that any questions that I have pertaining to the research have been, or will be answered by Matthew Cooke, PhD (Assistant Professor, Department of Health, Human Performance & Recreation, 118 Marrs McLean Gymnasium, Baylor University, phone: 254-710-4025), Geoffrey Hudson, MA (Doctoral Research Assistant Department of Health, Human Performance & Recreation, 117 Marrs McLean Gymnasium, Baylor University, phone: 254-710-4012), or Brian Shelmadine, MA (Doctoral Research Assistant Department of Health, Human Performance & Recreation, 117 Marrs McLean Gymnasium, Baylor University, phone: 254-710-4012). My signature below means that I am at least 18 years of age and that I freely agree to participate in this investigation. I understand that I will be given a copy of this consent form for my records. If I have any questions regarding my rights as a research subject in this study, I may contact Baylor's University Committee for Protection of Human Subjects in Research. The chairman is Dr. Matt Stanford, Professor, Department of Psychology and Neuroscience, P.O. Box 97334, Waco, TX 76798-7334, phone number 254-710-2961.

Date _____ Participant's Signature _____

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

Date _____ Investigator's Signature _____

APPENDIX E

Wound Care for the Muscle Biopsy Procedure Handout

Complications resulting from the muscle biopsy procedure are rare. Furthermore, after the procedure, you can reduce your risk of chance of infection by adhering to the following course of action for wound care:

For approximately 24 hours post biopsy

- leave the bandage on for 24 hours (unless unexpected bleeding or pain occurs, which should be immediately reported to the lab)
- lightly clean around the bandage if necessary
- reapply a fresh adhesive bandage if necessary
- refrain from vigorous physical activity during the first 24 hours post biopsy

After approximately 24-48 hours post biopsy

- reapply a fresh adhesive bandage after 24-36 hours
- return to normal hygiene practices after 48 hours unless complications arise

Possible Pain Side Effects

Soreness of the area may occur for about 24 hours post biopsy. Following the procedures outlined above should significantly minimize pain and possible bleeding of the area. However, some subjects experience no significant pain post biopsy.

☺ If needed, the subject may take non-prescription analgesic medication such as Tylenol to relieve pain if needed.

☹ Medications such as aspirin, Advil, Bufferin, or Nuprin, are discouraged as they may lead to excess bruising at the biopsy site.

If any questions or complications arise please contact:

Matthew Cooke, Ph.D.
Room # 118 Marrs McLean Gym
(254) 710-4025

or

Geoffrey Hudson, MA.
Room # 117 Marrs McLean Gym
(254) 710-4012

APPENDIX F

Dietary Log

Baylor University
Exercise & Sport Nutrition Laboratory

NAME _____Date_____

INSTRUCTIONS

1. Record everything you eat for 4 days (including one weekend day). If you eat pretzels, record how many. If you eat a bag of chips, record the number of ounces. For drinks, record the number of cups or ounces. Record everything you drink except water.
2. Record the Food, Amount, Brand Name, and Preparation Methods. For example: baked vs. fried chicken; 1 cup of rice; 2 teaspoons of margarine; 1 cup of 2% milk; McDonald's, Healthy Choice, or Frosted Flakes.
3. Record immediately after eating. Waiting until that night may make it difficult to remember all foods and quantities.

Food (include brand) no.)	**Method of Preparation**	**Quantity (cups, oz.,**
BREAKFAST:		
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
LUNCH:		
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
DINNER:		
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
SNACKS:		
_____	_____	_____
_____	_____	_____
_____	_____	_____

APPENDIX G

Activity Log

Baylor University
Exercise & Sport Nutrition Laboratory

NAME _____ Date _____

INSTRUCTIONS

1. Record all daily physical activity including type, duration and distance covered

Day covered	**Type (i.e. cycling, running)**	**Duration**	**Distance**

APPENDIX H

Reported Side Effects Form

Subject #: _____ Date: _____

Testing Session	1	2	3
Rate the ***frequency*** of the following symptoms according to the scale where: 0 = none 1 = minimal (1-2 per/wk) 2 = slight (3-4 per/wk) 3 = occasional (5-6 per/wk) 4 = frequent (7-8 per/wk) 5 = severe (9 or more per/wk)			
Dizziness?			
Headache?			
Fast or racing heart rate?			
Heart skipping or palpitations?			
Shortness of breath?			
Nervousness?			
Blurred Vision?			
Any other unusual or adverse effects?			
Rate the ***severity*** of the following symptoms according to the scale where: 0 = none 1 = minimal 2 = slight 3 = moderate 4 = severe 5 = very severe			
Dizziness?			
Headache?			
Fast or racing heart rate?			
Heart skipping or palpitations?			
Shortness of breath?			
Nervousness?			
Blurred Vision?			
Any other unusual or adverse effects?			

PLEASE REMEMBER TO REPORT ANY SIDE EFFECTS IMMEDIATELY.

Directions: If necessary, please contact either Darryn Willoughby, Ph.D. at 254-710
3504 or Matt Cooke, Ph.D. at 254-710-4025. You may also e-mail either at Darryn_Willoughby@baylor.edu or Matt_Cooke@baylor.edu.

Thanks for your participation!

APPENDIX I

Medical Waiver

BAYLOR
UNIVERSITY

Dear Medical Provider:

One of your patient's would like to participate in a study entitled "Effects of Resveratrol and Pterostilbene Supplementation on Insulin and Exercise-Mediated Signaling Pathways for Glucose Uptake in Overweight Insulin-Resistant Females: A Double-Blind, Clinically-Controlled Study" that is being conducted by Matthew Cooke, Ph.D. (254-710-4025; Matthew_Cooke@Baylor.edu) through the Exercise & Biochemical Nutrition Laboratory at Baylor University. In order to do so, she must meet the entrance criteria described below and have approval from her personal physician to participate in the study. The study will involve overweight, pre-diabetic, sedentary pre-menopausal females who have not taken oral contraceptives for the two months prior to the study. They also will be subjected to an oral glucose tolerance test and a maximal graded exercise test on a treadmill on two occasions. Assessments will include obtaining venous blood and muscle samples from the thigh, body composition measurements, and changes in maximal oxygen consumption. In addition, each participant will participate in a dietary intervention and circuit training exercise program for two months. Details about the study are described in the attached study outline and consent form. If you feel she meets the entrance criteria and/or any existing medical condition that she may have is under control and would not be a limitation for her to participate in the study, please sign the medical clearance below. If you feel the need to contact me, please feel free to do so.

Subjects
Approximately 45 overweight, sedentary, pre-diabetic, pre-menopausal females will participate in this study. I understand that in order to participate in this study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 1) I do not have physician's approval; 2) I have been on hormone replacement therapy three months prior; 3) I do not have medically-managed hypertension, glucose-intolerance, type II diabetes, thyroid conditions, hyperlipidemia and/or other controlled medical conditions that my physician feels would benefit me from participation in this study; 4.) I have taken nutritional supplements that may affect my antioxidant status (e.g., green tea, fish oil, Vitamins C and E) for three months prior to the study 5.) I report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study.

Medical Clearance

I medically clear _____ to participate in this study.

Physician's Name _____ Date _____

Physician's Signature _____

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