ATTENUATION OF HEPATIC ER STRESS BY
BITTER MELON IN HIGH-FAT DIET-FED MICE

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE
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BY

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DEDICATION

To my family.

I could never have gotten this far without all of your love and support.
ACKNOWLEDGEMENTS

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I thank Dr. Jinzeng Yang and Dr. Harry C. Bittenbender for their counsel and guidance while serving on my committee.

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I would also like to thank Dr. John Paul Bingham for all of his advice in helping me to make the transition from a student to a professional.

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ABSTRACT

Nearly 70 percent of the adult population in the United States is obese. Obesity is a growing concern as it is a risk factor for developing metabolic disorders, type II diabetes (T2D) and cardiovascular disease. More than 23 million people in the United States are diagnosed T2D. T2D develops when an individual suffers from chronic insulin resistance, which is due to impaired insulin signaling and reduced insulin sensitivity.

During obesity, insulin signaling can be impaired via endoplasmic reticulum (ER) stress. Obesity causes cellular stress, which results in an accumulation of unfolded or misfolded proteins. This accumulation of proteins in the ER leads to ER stress. The cell attempts to decrease ER stress and return to homeostasis through the unfolded protein response (UPR). The UPR increases expression of molecular chaperone Grp78, increased phosphorylation of eukaryotic initiation factor 2α (eIF2α) and up-regulation of the activating transcription factor ATF4. These three proteins are central to the UPR and are seen in increasing levels during cellular stress. This ER stress response also activates genes such as c-Jun N-terminal kinase (JNK), which cause the phosphorylation of insulin receptors and decrease levels of insulin signaling.

There are various treatments available for T2D, including dietary management, oral medications and insulin injections. Over time, most diabetics become less sensitive to these medications and are required to combine different treatments. As a result,
diabetic individuals are 60 percent more likely to use a type of complementary or alternative medicine.

Momordica charantia, commonly known as bitter melon (BM), is an alternative medicine that has been traditionally used in Ayurvedic and Chinese medicine to treat diabetes and its complications. Our lab has previously demonstrated that BM decreases weight gain and improves hepatic insulin signaling and resistance. The aim of this study was to investigate the effect of BM on the UPR in mice fed a high-fat-diet (HFD). C57BL/6 mice were randomized into three groups: 1) control, 2) HFD, 3) HFD + BM. After 16 weeks, livers were excised and analyzed for ER stress proteins, Grp78, ATF4 and phosphorylated eIF2α via Western blotting.

Data analyses indicate that HFD significantly elevated ER stress associated proteins Grp78, ATF4 and eIF2α-P, while a HFD supplemented with BM significantly decreased eIF2α-P expression and normalized ER stress proteins Grp78 and ATF4. These data indicate that improvement in BM-associated hepatic insulin signaling is in part due to decreased ER stress. Therefore, BM has potential to be a dietary therapy for reducing HFD-associated diabetes.
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<th>Description</th>
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<tbody>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate-Activated Protein Kinase</td>
</tr>
<tr>
<td>ApoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating Transcription Factor 4</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating Transcription Factor 6</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BM</td>
<td>Bitter Melon</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BMJ</td>
<td>Bitter Melon Juice</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Complementary and alternative medicine</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Diseases</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>eIF2α-P</td>
<td>Phosphorylated Eukaryotic Initiation Factor 2α</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose Transporter 4</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose Tolerance Test</td>
</tr>
<tr>
<td>Grp78</td>
<td>78 kDa Glucose-Regulated Protein</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
</tr>
<tr>
<td>HFD + BM</td>
<td>High-Fat + Bitter Melon Diet Group</td>
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<tr>
<td>HFD</td>
<td>High-Fat Diet</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol Requiring Kinase</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin Receptor Substrate</td>
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<td>ITT</td>
<td>Insulin Tolerance Test</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-Terminal Kinase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>NaF</td>
<td>Sodium Fluoride</td>
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</table>
NHOPI  Native Hawaiian and Other Pacific Islander

NMR  Nuclear Magnetic Resonance

PERK  Pancreatic ER Kinase (PKR)-like ER Kinase

PI3K  Phosphatidylinositol 3-Kinase

PMSF  Phenylmethylsulfonyl Fluoride

PPARγ  Peroxisome Proliferator-Activated Receptor γ

ROS  Reactive Oxygen Species

RPM  Revolutions per Minute

SDS-PAGE  Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SEM  Standard Error of the Mean

T2D  Type II Diabetes

TAG  Triacylglycerol

TF  Transcription Factor

USA  United States of America

UPR  Unfolded Protein Response
CHAPTER 1

1 Introduction

1.1 Obesity and Metabolic Syndrome

Obesity is a risk factor for developing metabolic syndrome and associated diseases. Given the prevalence of obesity throughout the world it is considered a global problem. In the United States, approximately 68 percent of adult Americans are overweight, 34 percent of which are obese [1]. Similarly, approximately 20 percent of children ages 2-11 and 10 percent of adolescents ages 12-19 are obese [2].

Obesity demographics differ among ethnicity, gender and age. A higher percent of the male population is overweight, while there is an equal gender distribution in the obese population and a greater proportion of females categorized as extremely obese [3]. In addition to gender, specific ethnicities have been found to be more likely to become overweight or obese. The African-American female population is 82 percent overweight or obese, which the Caucasian female population is 60 percent overweight or obese. The Hispanic female population has an overweight or obese prevalence of 75 percent, which is also significantly higher than the Caucasian population. This trend is not seen in African-American men, but can be seen, to a lesser extent, in Hispanic men [3]. Conversely, the Asian-American population distribution of overweight individuals is slightly less than the Caucasian population. However, the Asian-American population shows a 60 percent decrease in their obese statistics [4]. Within this Asian-American population, all Asian ethnicities are less likely to be
overweight or obese than whites, with some ethnicities, such as Filipinos, being more likely to be obese than others (Table 1).

In Hawaii, ethnic populations display differing levels of obesity. The Chinese and Korean populations have the lowest average BMI, followed by an increasing average BMI in Japanese, Caucasians, Hispanics, African-Americans and finally Native Hawaiians (Figure 1) [5, 6]. Native Hawaiians and Pacific Islanders are 60 percent more likely to become obese than the Caucasian population and three times more likely to become obese than other Asian American populations [4]. This prevalence of obesity in different populations throughout the United States and Hawaii is important as metabolic complications such as insulin resistance, type II diabetes (T2D), cardiovascular diseases (CVD), high blood pressure and neurodegenerative diseases can manifest as complications.

<table>
<thead>
<tr>
<th>Ethnic population</th>
<th>Overweight Population (%)</th>
<th>Obese Population (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Whites</td>
<td>34.6</td>
<td>23.6</td>
</tr>
<tr>
<td>All Asians</td>
<td>33.4</td>
<td>8.1</td>
</tr>
<tr>
<td>Chinese</td>
<td>21.8</td>
<td>4.2</td>
</tr>
<tr>
<td>Filipino</td>
<td>33.0</td>
<td>14.1</td>
</tr>
<tr>
<td>Asian Indian</td>
<td>34.4</td>
<td>6.0</td>
</tr>
<tr>
<td>Japanese</td>
<td>25.9</td>
<td>8.7</td>
</tr>
<tr>
<td>Vietnamese</td>
<td>19.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Korean</td>
<td>27.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Other Asian &amp; NHOLI</td>
<td>29.2</td>
<td>12.5</td>
</tr>
</tbody>
</table>


**Figure 1.** Obesity prevalence among ethnic populations in the State of Hawaii. The obesity prevalence in the Native Hawaiian and Pacific Islander populations is more than double that of other ethnic populations in the state. [Adapted from: Shim MJ, Chung C, Katz A, by Gender E: Self-reported Diabetes in Hawaii: 1988-1993. 1995.]

### 1.2 Insulin Resistance

Insulin is a hormone that is involved in the storage and synthesis of macromolecules in cells (Figure 2). These macromolecules include glucose, amino acids and free fatty acids. Insulin is necessary for both peripheral glucose uptake in muscle and adipose tissue and the inhibition of hepatic glucose production in the liver. Insulin signaling deficits can manifest as hepatic or peripheral insulin resistance and may lead to
serious complications [7]. Individuals suffering from chronic insulin resistance and impaired insulin secretion in their tissues are at risk for developing T2D.

**Figure 2.** Metabolic pathways dependent on insulin signalling. Insulin is responsible for the uptake of glucose, amino acids and fatty acids as well as important for the degradation and synthesis and storage of carbohydrates, lipids and proteins.  

### 1.3 Type II Diabetes

More than 23 million people throughout the United States suffer from T2D. In Hawaii, diabetes is one of the top five causes of death among Native Hawaiians [8]. Individuals in Hawaii that were of Japanese and Filipino decent were also found to have a greater risk of becoming insulin resistant diabetic than Caucasians, despite a lower average BMI [5]. These statistics are mirrored in the diagnosis of both lean and
overweight patients with metabolic syndrome. However, all of these patients show an increase in liver fat, despite their differences in overall BMI [9].

Patients with T2D suffer from chronic insulin resistance. As a result, these individuals are unable to metabolize glucose for energy. According to the American Diabetes Association (ADA) there are multiple and varied complications associated with T2D. In general, all patients suffer from a decreased quality of life and increased health care costs. Patients suffering from T2D are more likely to be diagnosed with glaucoma or cataracts in their eyes, hearing loss, nerve damage, high blood pressure or skin disorders. More serious complications involve an increased risk of a stroke, CVD, kidney disease, decreased circulation, amputation and ketoacidosis. In severe cases, ketoacidosis can lead to coma or death.

1.4 Treatment for Type II Diabetes

There are various treatments available for diabetes. The T2D therapies that are most commonly prescribed are oral medications and insulin injections. According to the American Diabetes Association, current oral medications fall into three main categories: alpha-glucosidase inhibitors, insulin sensitizers or insulin secretagogues. Alpha-glucosidase inhibitors, such as acarbose and miglitol, slow or block the digestion of starch so that glucose enters the blood stream slowly, effectively preventing a spike in blood glucose levels after eating. Some side effects of alpha-glucosidase inhibitors are gas and diarrhea.

There are two types of insulin sensitizers: biguanides and thiazolidinediones. Biguanides, such as metformin, reduce the amount of glucose synthesized in the liver
by suppressing gluconeogenesis and glycogenolysis. Diarrhea and lactic acidosis can be side effects of this medication [10]. Thiazolidinediones, such as rosiglitazone and pioglitazone, activate peroxisome proliferators-activated receptors (PPARs) and decrease insulin resistance through a decrease in gluconeogenesis. Thiazolidinediones may increase your risk of liver problems and heart failure.

The final class of oral medication is insulin secretagogues, which trigger insulin secretion. There are two types of insulin secretagogues: sulfonylureas and meglitinides. Sulfonylureas, such as glipizide and glyburide, stimulate the pancreas to release more insulin by blocking potassium channels. If potassium channels are blocked in pancreatic β-cells, calcium will build up within the cells and cause an increase in insulin secretion [11]. These medications can have various side effects, including hypoglycemia, jaundice and cholestasis. Meglitinides also stimulate insulin secretion from β-cells by binding to potassium channels. However, meglitinides bind to a different site and induce the release of potassium from the cell. This release depolarizes the cell and opens calcium channels. Increases in calcium levels within the cell again promote insulin secretion. Given this increase in insulin, meglitinides also increase the risk of hypoglycemia and may cause weight gain.

Oral drugs have been found to be initially successful, although patients tend to see a decrease in effectiveness over time as the pancreas begins to fail and not produce insulin. Overall, these oral medications also have various side effects, including weight gain, fatigue, nausea, and kidney toxicity [12]. Over time, individuals may no longer respond sufficiently to these oral medications and insulin injections may be prescribed to help counteract hypoinsulinemia. Insulin side effects include weight
gain, hypoglycemia and, in high doses, arterial plaques. These therapies target glucose or insulin levels directly, while other obesity or insulin related complications may result from protein interactions upstream of insulin signaling [12]. Additionally, the association between increases in liver fat and metabolic syndrome makes insulin resistant studies in hepatic tissue important in developing anti-diabetic therapies. A medication that can improve insulin sensitivity along with regulating glucose and/or insulin levels in hepatic tissue would have the potential to decrease insulin resistance, T2D and its complications.

In order to determine the efficacy of any new therapies, the molecular mechanisms underlying obesity and hepatic insulin resistance must be examined, specifically in liver tissue. Further study of methods to attenuate insulin resistance can be examined through induction of obesity, insulin resistance and diabetes in an animal model that can mimic the human condition. Furthermore, a preventative model, in which obesity and insulin resistance were prevented, would be beneficial for prevention of obesity associated complications.

1.5 Molecular Mechanisms of Obesity, Insulin Signaling and Diabetes

Free fatty acids, reactive oxygen species, hyperglycemia and hypoinsulinemia have been found to increase in obese individuals. When an individual is obese they have an energy imbalance due to chronic overloading of calories when they ingest more than they use. This causes an accumulation of fat through an increase in size or number of adipocytes, as well as an increase in intracellular lipids [13]. This increase in adipose tissue can lead to cellular dysfunction, including abnormalities in
adipokines, increased levels of free fatty acids and inflammation (Figure 3). When adipose tissue is inflamed, macrophages are recruited to the cells and release free fatty acids, adipokines and inflammatory cytokines. This response leads to increased lipid storage, lipotoxicity and insulin resistance (Figure 4) [14].

Insulin resistance can manifest in both peripheral and hepatic tissues. Insulin decreases hepatic glucose production via suppression of gluconeogenesis and glycogenolysis, promotes storage of glucose in skeletal muscle and inhibits lipolysis [15]. Chronic inflammation in adipose tissue inhibits insulin action and results in insulin resistance (Figure 5). Of specific interest to this study, obesity has been shown to decrease insulin sensitivity in liver tissue [16].

Mice with increased triglyceride levels and hepatic insulin resistance were found to have deficits in insulin signaling [17]. Insulin signaling involves many proteins, the most important of which are insulin receptors and insulin receptor substrates (Figure 6). Insulin receptors are transmembrane cell surface receptors that are activated by extracellular insulin. Once bound by insulin, these receptors initiate a cellular response by activating insulin receptor substrate (IRS) proteins via phosphorylation on tyrosine residues. Once activated, IRS proteins activate other proteins involved in various signaling cascades and cellular functions, including glucose metabolism [18]. Mouse studies have demonstrated that insulin receptors are involved in glucose metabolism using insulin receptor knockout mice. Knockout mice were diagnosed with early postnatal diabetes and soon died of ketoacidosis [19]. Additionally, insulin insensitivity has been shown in mice where IRS-1 proteins have been phosphorylated on serine or threonine residues, rather than on tyrosine residues. This serine or
Threonine phosphorylation of IRS-1 result in a conformation change that prevents IRS-1 from interacting with insulin receptor kinases and other proteins, resulting in insulin insensitivity [18].

**Figure 3.** The mechanisms through which obesity causes cellular stress. [Adapted from: de Ferranti S, Mozaffarian D: The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clin Chem* 2008, 54:945-955.]
Figure 4. Obesity can result in insulin resistance in various tissues. Chronic overloading of adipocytes cause by a high-fat-diet induces cellular stress that contributes to the chronic inflammatory state in the adipose tissue. Macrophages and other inflammatory cells are recruited into the adipose tissue to promote the release of free fatty acids, adipokines and inflammatory cytokines. Increase in circulating concentrations of free fatty acids, lipid mediators, inflammatory cytokines, insulin and leptin, together with reductions in circulating adiponectin concentrations, leads to ectopic lipid stores, lipotoxicity and insulin resistance in non-adipose tissues. [Adapted from: Iyer A, Brown L: Lipid mediators and inflammation in glucose intolerance and insulin resistance. Drug Discovery Today: Disease Mechanisms 2011.]
**Figure 5.** Peripheral and hepatic insulin resistance. Insulin suppresses hepatic glucose production, promotes skeletal muscle glucose disposal and inhibits lipolysis.

Inflammation in adipose tissue caused by obesity gives rise to excessive production of pro-inflammatory cytokines. These cytokines attenuate insulin action in these tissues.

[Source: Kalupahana NS, Moustaid-Moussa N, Claycombe KJ: Immunity as a link between obesity and insulin resistance. *Molecular Aspects of Medicine* 2011.]
**Figure 6.** Insulin receptor proteins. The insulin receptor is a tyrosine kinase which catalyses the phosphorylation of cellular proteins which initiate signaling pathways. These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation, and gene expression, glucose, lipid and protein metabolism. [Source: Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 2001, 414:799-806.]
1.6 Endoplasmic Reticulum Stress and Diabetes

Obesity can induce hepatic and peripheral insulin resistance by increasing tissue specific endoplasmic reticulum (ER) stress. However, given the association of the liver with protein synthesis and the previous link between metabolic syndrome and the liver, this study focused on hepatic insulin resistance in liver tissue.

Overall, increased levels of metabolic and inflammatory stress, caused by a high-fat diet, are inversely related to levels of insulin signaling [20]. During obesity, excess lipid storage causes cellular stress and has been seen to cause ER stress [13]. Additionally, increased plasma levels of glucose, insulin and free fatty acids can lead to metabolic stress via reactive oxygen species (ROS) which in turn cause an accumulation of improperly or unfolded proteins and further aggravate ER stress [16].

An accumulation of unfolded or misfolded proteins in the ER lumen result in ER stress. ER transmembrane stress sensors are activated at this accumulation of unfolded and misfolded proteins and initiate the unfolded protein response (UPR). The UPR has two mechanisms to reduce ER stress. The early response is to decrease protein synthesis, increase protein folding and degradation of misfolded proteins. If the cells cannot be restored to homeostasis the late response to reduce ER stress is to destroy the cells via apoptosis [21].

When ER stress levels are high, ER stress sensor inositol requiring kinase (IRE1) over-activates the serine kinase c-Jun N-terminal kinase (JNK) via phosphorylation (Figure 7). JNK then phosphorylates the serine residue on IRS-1, preventing
phosphorylation on the tyrosine residue. This inability to phosphorylate the tyrosine residue leads to a decrease in insulin receptor signaling. Thus the mechanism linking obesity and T2D via insulin resistance has been shown to be ER stress [22]. This knowledge of the mechanism between obesity and initial insulin resistance has the potential to be applied as a target for new and improved diabetes therapies. However, additional information on the molecular effect of these therapies within the ER stress response must be investigated to determine their mechanism and effectiveness.

Within the ER, the pancreatic ER kinase (PKR)-like ER kinase (PERK) pathway is one method of stress response (Figure 8) [23]. When unfolded proteins accumulate in the ER, the ER chaperone 78 kDa glucose-regulated protein (Grp78) dissociates from PERK receptors. PERK receptors are then activated via dimerization and autophosphorylation. Once active, PERK phosphorylates eukaryotic initiation factor 2α (eIF2-α), which inhibits the initiation of general translation. However, phosphorylated eIF2-α (eIF2-α-P) activates a select number of proteins, including activating transcription factor 4 (ATF4). ATF4 then translocates to the nucleus and induces a stress response. As this pathway is instrumental in initiation of an ER stress response, monitoring the levels of these proteins provides a correlation to ER stress levels.

In vivo models of ER stress have shown that mice on a high-fat diet (HFD) have increased levels of eIF2-α signaling, lipid accumulation and insulin resistance in hepatic tissue [24]. Transgenic inhibition of the hepatic eIF2-α signaling pathway reduced hepatic insulin synthesis. This inhibition also affected insulin sensitivity in
peripheral tissues, which may indicate cross-talk between the liver and peripheral organs. Cross-talk between peripheral and hepatic tissues may have implications for a therapy which can attenuate insulin resistance throughout the body, rather than specific organs. The link between obesity, ER stress and insulin resistance has been verified in a clinical study in which patients undergoing gastric bypass were followed one year pre- and post-operation. As they lost weight post-operation, these diabetic subjects demonstrated improved insulin sensitivity and significant decreases in ER stress associated proteins in liver, muscle and adipose tissues [20]. The ER stress proteins that were down regulated included JNK, Grp78, and eIF2-α-P. Given the relationship between obesity, ER stress and diabetes, a therapy targeting ER stress could be developed to treat T2D or to prevent insulin resistance in high-risk individuals. As there is no current therapy that targets ER stress natural products may provide a resource for developing new therapies.
**Figure 7.** ER stress links obesity and insulin resistance. Metabolic and inflammatory stresses caused by protein misfolding in the ER. The ER attempts to cope with stress by activating the UPR. If the UPR fails to restore homeostasis molecular pathways leading to insulin resistance are initiated. Impaired insulin signaling can alleviate intracellular stress, but compromises systemic glucose regulation.

Figure 8. The unfolded protein response. When unfolded or misfolded proteins accumulate in the ER Grp78 activates ER stress receptors PERK, ATF6 and IRE1 via dissociation. The first stress receptor, PERK, blocks general protein synthesis and initiates translation of ATF4 through phosphorylation of eIF2-α. ATF4, a transcription factor (TF), translocates to the nucleus and induces genes required for the restoration of ER homeostasis. The second stress receptor, ATF6, is activated by limited proteolysis after translocation to the golgi apparatus. ATF6, which is a TF and a stress receptor, then activates transcription of the TF XBP1. The final stress receptor, IRE1, activates XBP1 by splicing its mRNA. Spliced XBP1 translocates to the nucleus and controls the transcription of chaperones and proteins involved in protein degradation. These receptors work in a sequential fashion to reduce ER stress and restore homeostasis by preventing protein synthesis and promoting protein degradation. [Source: Szegezdi E, Logue SE, Gorman AM, Samali A: Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 2006, 7:880-885.]

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**Figure 8.** The unfolded protein response. When unfolded or misfolded proteins accumulate in the ER Grp78 activates ER stress receptors PERK, ATF6 and IRE1 via dissociation. The first stress receptor, PERK, blocks general protein synthesis and initiates translation of ATF4 through phosphorylation of eIF2-α. ATF4, a transcription factor (TF), translocates to the nucleus and induces genes required for the restoration of ER homeostasis. The second stress receptor, ATF6, is activated by limited proteolysis after translocation to the golgi apparatus. ATF6, which is a TF and a stress receptor, then activates transcription of the TF XBP1. The final stress receptor, IRE1, activates XBP1 by splicing its mRNA. Spliced XBP1 translocates to the nucleus and controls the transcription of chaperones and proteins involved in protein degradation. These receptors work in a sequential fashion to reduce ER stress and restore homeostasis by preventing protein synthesis and promoting protein degradation. [Source: Szegezdi E, Logue SE, Gorman AM, Samali A: Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 2006, 7:880-885.]
1.7 Complementary and Alternative Medicine

38 percent of adults and 12 percent of children in America use some type of alternative therapy to treat chronic diseases and maintain their health.

Complementary and alternative medicine (CAM) use differs among different demographics within this population [25]. A National Health Interview survey found that CAM use differs between gender, age, education level and ethnicity (Table 2).

Less than one percent of these individuals use complementary and alternative medicine (CAM) to treat diabetes [26]. However, diabetic individuals are sixty percent more likely to use an alternative therapy than the rest of the population [27]. Individuals afflicted with other chronic conditions do not show this increased preference for CAM, demonstrating a higher interest for alternative medicine in the diabetic community.

The CAM therapies currently utilized by patients with diabetes include prayer or spiritual healing, herbal treatments, chiropractic care, massage, special diets and homeopathy (Table 3) [28]. There are also numerous natural products which are used as alternative medicines, including botanicals such as bitter melon, garlic, Aloe vera and fenugreek, as well as supplements which including magnesium, chromium and omega-3 fatty acids (Table 4) [29]. Given the potential side effects and decreasing effectiveness of available therapies, additional treatments are increasingly sought by patients with T2D, as indicated by the high interest in CAM within the diabetic community [26]. A therapy targeting upstream of insulin receptors has the potential
to prevent insulin resistance before complications associated with insulin resistance arise.

**Table 2.** Demographic distribution of alternative medicine use. The age, gender, ethnic and educational distribution of a population of diabetic individuals using complementary or alternative medicine. Data was obtained from a National Health Interview Survey. [Adapted from: Garrow D, Egede LE: Association between complementary and alternative medicine use, preventive care practices, and use of conventional medical services among adults with diabetes. *Diabetes care* 2006, 29:15-19.]

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Sub-Category</th>
<th>Percent of Study Population (n=1,148)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>18 – 34</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>35 – 49</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td>50 – 64</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>65 and older</td>
<td>52.2</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>47.8</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>52.2</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Hispanic</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>White</td>
<td>72.2</td>
</tr>
<tr>
<td></td>
<td>Black</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>5.2</td>
</tr>
<tr>
<td>Education</td>
<td>Less than high school</td>
<td>20.9</td>
</tr>
<tr>
<td></td>
<td>High school graduate</td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td>More than high school graduate</td>
<td>48.1</td>
</tr>
</tbody>
</table>
Table 3. Types of alternative therapy used by diabetics. Overview of the frequency and the different types of complementary and alternative therapy used by adults with diabetes. Data was obtained from a National Health Interview Survey. [Adapted from: Pagan JA, Tanguma J: Health care affordability and complementary and alternative medicine utilization by adults with diabetes. *Diabetes care* 2007, 30:2030-2031.]

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Percent of Study Population (n=2,142)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall use</td>
<td>70.48</td>
</tr>
<tr>
<td>Prayer and spiritual healing</td>
<td>61.34</td>
</tr>
<tr>
<td>Herbal treatments</td>
<td>15.09</td>
</tr>
<tr>
<td>Relaxation</td>
<td>11.79</td>
</tr>
<tr>
<td>Chiropractic care</td>
<td>6.57</td>
</tr>
<tr>
<td>Massage</td>
<td>3.15</td>
</tr>
<tr>
<td>Special diets</td>
<td>3.12</td>
</tr>
<tr>
<td>Megavitamins</td>
<td>2.91</td>
</tr>
<tr>
<td>Yoga/tai chi/qigong</td>
<td>1.88</td>
</tr>
<tr>
<td>Homeopathy</td>
<td>1.50</td>
</tr>
<tr>
<td>Acupuncture</td>
<td>1.33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type</th>
<th>Therapy</th>
<th>Hypothesized Effect(s) on Glucose Metabolism</th>
<th>Reported Adverse Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botanicals</td>
<td><em>Allium sativum</em> (garlic)</td>
<td>Insulin secretagogue</td>
<td>Blood thinning</td>
</tr>
<tr>
<td></td>
<td><em>Aloe vera</em></td>
<td>Insulin secretagogue</td>
<td>1. Abdominal pain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Diarrhea and loss of</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>electrolytes</td>
</tr>
<tr>
<td></td>
<td><em>Coccinia indica</em> (ivy gourd)</td>
<td>Insulin mimetic</td>
<td>None reported</td>
</tr>
<tr>
<td></td>
<td><em>Momordica charantia</em> (bitter melon)</td>
<td>1. Insulin mimetic</td>
<td>1. Glucose-6-phosphate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Decreased hepatic glucose production</td>
<td>deficiency</td>
</tr>
<tr>
<td></td>
<td><em>Opuntia streptacantha</em> (prickly pear cactus)</td>
<td>Decreased hepatic glucose production</td>
<td>2. Contraindicated in pregnancy</td>
</tr>
<tr>
<td></td>
<td><em>Panex ginseng</em>, <em>Panex quiquefolius</em> (ginseng)</td>
<td>1. Insulin mimetic</td>
<td>1. Medication interference</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Alters hepatic glucose metabolism</td>
<td>2. Estrogenic effects</td>
</tr>
<tr>
<td></td>
<td><em>Trigonella foenum graecum</em> (fenugreek)</td>
<td>1. Insulin secretagogue</td>
<td>3. Hypertension</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Decreased carbohydrate absorption</td>
<td>4. Insomnia</td>
</tr>
<tr>
<td>Supplements</td>
<td>Alpha-lipoic acid</td>
<td>Increased insulin sensitivity</td>
<td>Changes in thyroid function</td>
</tr>
<tr>
<td></td>
<td>Chromium</td>
<td>Increased insulin sensitivity</td>
<td>Minimal</td>
</tr>
<tr>
<td></td>
<td>Magnesium</td>
<td>1. Insulin secretagogue</td>
<td>1. Diarrhea, abdominal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Increased insulin sensitivity</td>
<td>cramping</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Toxicity in individuals with renal failure</td>
</tr>
<tr>
<td></td>
<td>Omega-3 fatty acids</td>
<td>Slight increase in blood glucose</td>
<td>1. Increased risk of bleeding</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. May increase LDL</td>
</tr>
<tr>
<td></td>
<td>Vanadium</td>
<td>Insulin mimetic</td>
<td>Prolonged high doses may cause renal toxicity</td>
</tr>
</tbody>
</table>
1.8 Bitter Melon

*Momordica charantia*, commonly known as bitter melon (BM), is a vegetable that has been traditionally used in Ayurvedic and Chinese medicine to treat diabetes and associated complications (Figure 9) [30]. Bitter melon, also known as karela, bitter gourd and balsam pear, is also used in South America, Africa and Asia to treat T2D. Previous studies have demonstrated a reduction in obesity, insulin resistance and diabetes in *in vitro*, *in vivo* and clinical models. This study is aimed at determining the mechanism behind BM’s efficacy as an anti-diabetic treatment in which BM prevents hepatic insulin resistance in HFD mouse model that has been previously shown to induce obesity, insulin resistance and T2D.

![Unripe bitter melon growing on a vine.](image)

**Figure 9.** Unripe bitter melon growing on a vine.

1.9 Bitter Melon Pharmacognosy

Multiple phytochemicals have been isolated from bitter melon in the last 50 years, including alkaloids, glycosides and polypeptides (Table 5) [31]. Currently, there are
over 200 compounds which been isolated from BM and come from all different parts of the plant, including leaves, stems, roots, pericarp and seeds [32]. Many of these compounds have been isolated through different fractionation techniques, including saponin, methanolic and water fractionations. A significant amount of these phytochemicals have been found to have an effect on lipids, blood glucose and insulin levels (Table 6). Although some compounds have not been investigated for their effect, their structure has been determined via NMR or mass spectroscopic analysis (Table 5). The most commonly investigated phytochemicals are the polypeptides and glycosides. Charantin is a glucoside that was isolated from bitter melon in the 1960s and was initially shown to decrease blood sugar in rabbits when delivered orally or intravenously [33]. Vicine, another glucoside, was isolated from bitter melon seeds and shown to lower blood glucose in rats [34].

In 1974, a polypeptide was isolated from bitter melon and named p-insulin or polypeptide-p [35]. This polypeptide has been shown to have insulin-like properties in both animal and human studies. There are some amino acid differences between p-insulin and animal insulin, though they have a similar hypoglycemic effect.

Our lab has isolated polyphenols through a methanolic extraction of freeze-dried BMJ [36]. The polyphenol which was most abundant in this methanolic fraction was catechin. Catechins isolated from other plants have been shown to decrease glucose transport from the stomach and into the small intestine and decrease blood glucose levels [37, 38]. In another study, a methanolic fraction of BM contained triterpenoids which enhanced cellular insulin signaling [39].
More recent studies have fractionated freeze-dried BM into lipid, hydrophilic and saponin fractions [40]. All three fractions were found to have anti-diabetic effects in mice, though the lipid and saponin fractions had more pronounced effect. Additional studies have found saponin fractions of BM to be more effective in stimulating insulin secretion than other BM extracts [41].

In addition to novel phytochemicals, BM is an antioxidant and contains significant amounts of iron, beta-carotene, potassium, calcium, phosphorus, dietary fiber and vitamins C and B1 to B3 [42].
Table 5. Chemicals isolated from different parts of bitter melon.

<table>
<thead>
<tr>
<th>Author(s), [Reference(s)]</th>
<th>Country</th>
<th>Fruit Part and/or Fraction</th>
<th>Chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lotlikar and Rao, [33]</td>
<td>India</td>
<td>Leaves and fruit</td>
<td>Charantin [glucoside]</td>
</tr>
<tr>
<td>Khanna et al., [35]</td>
<td>India</td>
<td>Fruit and seeds</td>
<td>Polypeptide-P [protein]</td>
</tr>
<tr>
<td>Handa et al., [34]</td>
<td>India</td>
<td>Seeds</td>
<td>Vicine [glucoside]</td>
</tr>
<tr>
<td>Nakamura et al., [43]</td>
<td>Japan</td>
<td>Methanolic extract from whole fruit</td>
<td>Karavilagenins A, B, and C [triterpene]</td>
</tr>
<tr>
<td>Nakamura et al., [43]</td>
<td>Japan</td>
<td>Methanolic extract from whole fruit</td>
<td>Karaviloside I – V [glycosides]</td>
</tr>
<tr>
<td>Li et al., [44]</td>
<td>China</td>
<td>Ethanol extract from whole fruit juice, without seeds</td>
<td>Momordicoside M, N and O [triterpenoid saponins]</td>
</tr>
<tr>
<td>Chen et al., [45]</td>
<td>China</td>
<td>Air dried roots</td>
<td>Kuguacins A-E [cucurbitacins]</td>
</tr>
<tr>
<td>Liu et al., [46]</td>
<td>China</td>
<td>Methanolic extract from whole, dried fruit</td>
<td>Steroidal glycoside</td>
</tr>
<tr>
<td>Liu et al., [46]</td>
<td>China</td>
<td>Methanolic extract from whole, dried fruit</td>
<td>Triterpenes (3)</td>
</tr>
<tr>
<td>Nerurkar et al., [36]</td>
<td>USA</td>
<td>Whole fruit, Methanolic fraction</td>
<td>Catechin [polyphenol]</td>
</tr>
</tbody>
</table>
Table 6. Bitter melon pharmacognosy. Summary of the uses of different parts of bitter melon along with identified phytochemicals.

<table>
<thead>
<tr>
<th>Author(s), [Reference(s)]</th>
<th>Country</th>
<th>Fruit Part</th>
<th>Chemical or Fraction</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lotlikar and Rao, [33]</td>
<td>India</td>
<td>Leaves and fruit</td>
<td>Charantin [glucoside]</td>
<td>Lowers blood glucose.</td>
</tr>
<tr>
<td>Handa et al., [34]</td>
<td>India</td>
<td>Seeds</td>
<td>Vicine [glucoside]</td>
<td>Lowers blood glucose.</td>
</tr>
<tr>
<td>Khanna et al., [35]</td>
<td>India</td>
<td>Fruit and seeds</td>
<td>Polypeptide-P [protein]</td>
<td>Lowers blood glucose.</td>
</tr>
<tr>
<td>Cheng et al., [39]</td>
<td>Taiwan</td>
<td>Fruit, seed and stem</td>
<td>Methanol extract</td>
<td>Increased glucose uptake by cells.</td>
</tr>
<tr>
<td>Klomann et al., [40]</td>
<td>Germany</td>
<td>Fractions obtained from whole fruit</td>
<td>Saponin and lipid fractions</td>
<td>Reduction of lipid peroxidation of adipose tissue. Increase in insulin sensitivity.</td>
</tr>
<tr>
<td>Keller et al., [41]</td>
<td>USA</td>
<td>Whole fruit</td>
<td>Saponin fraction</td>
<td>Stimulated insulin secretion <em>in vitro</em>.</td>
</tr>
<tr>
<td>Nerurkar <em>et al.</em> and Igarashi <em>et al.</em>, [36, 38]</td>
<td>USA</td>
<td>Whole fruit</td>
<td>Methanol Fraction - Catechin</td>
<td>Lowers blood glucose.</td>
</tr>
</tbody>
</table>
1.10  Bitter Melon In Vitro Studies

*In vitro* studies of BM have shown a variety of beneficial effects in regard to obesity, insulin resistance and T2D. Several studies have used different cell lines to determine the hypoglycemic effect of BM. Given the importance of glucose transport in patients with diabetes, one study used a rat L6 myoblast cell line. These cells were treated with either an aqueous or chloroform BMJ extract. Through reverse-transcriptase analysis of mRNA, BM was found to augment glucose transport through increased expression of the proteins GLUT4, peroxisome proliferators-activated receptor-γ (PPARγ) and phosphatidylinositol 3-kinase (PI3K). Glucose transport is a crucial step in glucose utilization and this up-regulation demonstrates a molecular mechanism for the attenuation via BM of insulin resistance [47].

A separate study examined the effects of BM in mouse myoblast, C2C12, and adipose, 3T3-L1, cell lines. Prior to BM treatment, these cell lines were made insulin resistant and then treated with a methanol BM extract. Through Western blotting analysis, BM was found to activate the protein adenosine monophosphate-activated protein kinase (AMPK), which is important in the tyrosine phosphorylation of IRS-1, and thus necessary for maintenance of insulin signaling levels [39].

In addition to increases in glucose transport and insulin signaling, BM was also able to increase insulin secretion during a study using a mouse insulinoma cell line [41]. This cell line was chosen as it most closely resembled pancreatic islet function and so was optimum for determining levels of insulin secretion. The saponin-rich fraction of
a BM ethanol extract was found to proportionally increase insulin secretion as the concentration of extract increased [41].

HepG2 cells are a model for human hepatocytes as they are a human liver carcinoma line. Our lab has shown that BMJ inhibits cellular triglyceride, cholesterol levels and apoB secretion in HepG2 cells, this showing an effect on cellular processes within hepatic cells [48, 49]. Given this information, the mechanism through which BM is able to decrease glucose levels in hepatic tissue may be via insulin secretion. The decrease in apoB and triglyceride levels is also an indicator of decreased risk for CVD.

Our lab has also shown that BM decreases lipid accumulation in human adipocytes, demonstrating a potential application as a weight loss therapy to prevent or control T2D, in addition to glycemic considerations [50]. These in vitro studies have demonstrated that BM may be effective in attenuating both insulin resistance and obesity.
Table 7. Summary of bitter melon *in vivo* studies. Bitter melon *in vitro* cell culture studies involving obesity, lipid metabolism, glucose metabolism and insulin signaling.

<table>
<thead>
<tr>
<th>Author(s), [Reference]</th>
<th>Country</th>
<th>Fruit part or Extract</th>
<th>Cell Line(s)</th>
<th>Effects</th>
</tr>
</thead>
</table>
| Nerurkar et al., [48, 49] | USA     | Juice from whole fruit, without seeds | HepG2 cells: human liver carcinoma cell line | 1. Inhibition of apolipoprotein B secretion  
2. Inhibition of triglyceride synthesis and secretion |
| Cheng et al., [39] | Taiwan  | Methanolic extract from whole fruit with seeds and stem | FL83B (mouse liver) and C2C12 (mouse myoblast) cell lines; induced insulin resistance | 1. Increased glucose uptake by cells  
2. Enhanced insulin signaling via IRS-1 tyrosine phosphorylation |
| Kumar et al., [47] | India   | Chloroform and aqueous extracts from whole fruit without seeds | L6 muscle cells: Rat myoblast cell line | 1. Increased glucose uptake  
2. Increased GLUT4 transcription  
3. Increased PPARγ and PI3K transcription |
| Nerurkar et al., [50] | USA     | Whole fruit juice without seeds | Human preadipocytes | 1. Reduction in lipid content  
2. Reduction in adipogenic TFs  
3. Increased lipolysis |
| Keller et al., [41] | USA     | Whole fruit, Saponin fraction | MIN6 β-cells: Mouse insulinoma pancreatic cell line | Concentration-dependent increased insulin secretion |
1.11 Bitter Melon *In Vivo* Studies

In order to test the efficacy of BM in an animal model, many *in vivo* studies have demonstrated the anti-diabetic effect of BM (Table 8). Rodent studies have shown that bitter melon decreases obesity and T2D. Mice previously fed a high-fat diet have shown a significant decrease in obesity when fed BM over a period of 4 weeks through a decrease in weight of white adipose tissue and visceral fat [51, 52]. Lowered blood glucose levels, decreased insulin resistance and reduced hyperglycemia were also observed in these mice [51, 53]. BM has also increased, though not normalized, liver glycogen levels in diabetic mice [53]. This increased rate of glycogenesis may be responsible for the decrease in blood glucose of mice on a BM supplemented diet. It is also possible that these anti-diabetic effects were due to interactions with glucose insulin transporter 4 (GLUT4) [54]. Additionally, cataracts, neuropathy and gastropathy are diabetes associated conditions and were found to be delayed or decreased in rats supplemented with bitter melon [55].

Studies from our laboratory have shown that mice fed a HFD for a period of 16 weeks are considered obese, insulin resistant and to have T2D via measurements of body weight, BMI, and insulin and glucose tolerance. These mice can be used in studies to examine physiological and molecular effects of insulin resistance. We have shown that BM attenuates metabolic stress and diabetes-associated effects of a HFD in mice (Figure 10) [36, 56]. We have also shown that HFD fed mice showed a decrease in the activation of insulin receptors and IRS-1 proteins in hepatic tissue through a lack of tyrosine phosphorylation, indicating a decrease in insulin signaling and an increase
in insulin resistance [56]. However, with the addition of BM, these mice showed tyrosine phosphorylation rates significantly higher than that of control mice. This data indicates that BM not only restores insulin-signaling levels, but also increases them beyond normal expression.

This past year, two in vivo studies have shown that BM decreases insulin resistance, one in peripheral tissue and one in hepatic tissue [57]. The hepatic study, in rat liver, showed a decreased in hepatic triacylglycerol (TAG) levels through increased levels of TAG synthesis and enhanced fatty acid oxidation.

Our lab has also shown BM to be a benefit to other obesity-associated conditions. In addition to insulin resistance, CVD disease has shown a strong correlation to obesity. High levels of apolipoprotein B (apoB) cause arterial plaques and can lead to vascular and heart disease [58]. Thus, lowered levels of apoB are an indication of a reduced risk of heart disease. Our lab has shown that BM lowers plasma apolipoprotein B-100 and apolipoprotein B-48 levels in HFD fed mice [56]. Bitter melon has also been shown to protect the brain from oxidative stress and neuroinflammation in mice. These mice also decreased in body weight and displayed a normalization of systematic inflammation and plasma antioxidants [36].

Overall, our lab has been able to show that BM attenuate obesity, metabolic stress and insulin resistance. However, it is not yet known if the molecular pathway connecting obesity and metabolic stress to insulin signaling is through the endoplasmic reticulum. In this study, liver tissue was extracted from the mice in which BM had
been shown to decrease or normalize insulin resistance levels in order to further examine the mechanism for hepatic insulin resistance attenuation via BM.

Figure 10. Effects of bitter melon juice (BM) on plasma glucose tolerance (A) and insulin tolerance (B) in mice fed control rodent chow (♦), high-fat-diet (HFD; ■) and HFD+BM (▲). Values are means with standard deviations depicted by vertical bars (n=6). Mean values were significantly different from those of the control and HFD+BM group: *P<0.05. [Source: Nerurkar PV, Lee YK, Motosue M, Adeli K, Nerurkar VR: *Momordica charantia* (bitter melon) reduces plasma apolipoprotein B-100 and increases hepatic insulin receptor substrate and phosphoinositide-3 kinase interactions. *Br J Nutr* 2008, 100:751-759.]
Table 8. Summary of bitter melon *in vivo* studies which have shown an effect on insulin resistance in the last 5 years.

<table>
<thead>
<tr>
<th>Author(s), [Reference]</th>
<th>Country</th>
<th>Fruit Part or Extract</th>
<th>Animal Model</th>
<th>Feeding Method</th>
<th>Effects</th>
</tr>
</thead>
</table>
| Shih et al., [51]      | Taiwan   | Organic solvent extraction | Male C57BL/6J Mice            | Extract given orally               | 1. Fat weight decrease  
2. Lowered blood glucose  
3. Lowered insulin resistance  
4. Decreased hyperglycemia |
| Nerurkar et al., [56]  | USA      | Whole fruit without seeds, juiced | Female C57BL/6 Mice           | Juice freeze dried and added to chow | 1. Decreased insulin resistance  
2. Increased insulin signaling  
3. Decreased risk of CVD       |
| Shih et al., [54]      | Taiwan   | Organic solvent extraction | Male Sprague–Dawley Rats      | Extract given orally               | 1. Decrease in FFA levels  
2. Improved hyperglycemia  
3. Increased expression of PPARγ  
4. Increased GLUT4 expression |
| Hossain et al., [53]   | Bangladesh | Methanolic extract  | Male Evans Rats, diabetes induced | Intraperitoneal (body cavity) injection | 1. Lowered blood glucose  
2. Increased hepatic glycogenesis |
| Wang et al., [57]      | USA      | Prepared BM extract purchased | Male C57BL/6 Mice              | Extract added to chow              | 1. Decreases in weight, plasma glucose, insulin and leptin levels  
2. Improved glucose metabolism and insulin signaling |
| Senanayake et al., [59] | Japan   | Methanolic fraction   | Male Sprague–Dawley Rats      | Extract added to chow              | 1. Decreased hepatic triacylglycerol synthesis  
2. Enhanced fatty acid oxidation |
1.12 Bitter Melon Clinical Studies

The mechanism through which BM is able to decrease diabetes-associated complications is not yet known, yet many clinical studies have already studied the effects of BM in human. High blood glucose levels have been shown to decrease with bitter melon supplements in human subjects participating in pre-clinical studies (Table 9). Generally, studies using bitter melon fruit juice, or the whole fruit, have been found to be more successful than those using commercially available supplements with prepared extracts [60-62].

Current studies have shown a reduction in blood glucose levels with the addition of BM, but do not show a statistical significance (Table 9) [60, 63]. One such study, on 40 type II diabetics over a 6-month trial, was randomized and double-blind, but used a commercial capsule containing compounds isolated from BM, rather than the whole fruit. Although there were small decreases in blood glucose, cholesterol and BMI, none of these decreases were statistically significant [60].

A pilot study on five healthy, but overweight, males found no statistical difference between control conditions and an acute dose of freeze dried BMJ [63]. Both of these studies had small sample sizes or a flaw in the study design that resulted in a lack of statistical significance, rather than the efficacy of BM as a therapy. Hypoglycemic effects found in numerous extracts and compounds isolated from BM support that the whole fruit is more beneficial therapeutically than single compounds.
BM has been studied for years. Studies in the 1980s were not as scientifically rigorous as current investigations, but do demonstrate that BM is an effective therapy. One study, involving 18 newly diagnosed diabetic subjects, were given an acute dose of 100mL of BM juice (BMJ) made from the whole fruit minus seeds. A significant decrease in blood glucose was observed in 13 of 18 patients [61].

In another study, 9 Asian non-insulin-dependent diabetic patients were treated with BMJ in two different trials. The first trial was an oral glucose test after an acute dose of 50 mL of BMJ. This treatment was shown to decrease blood glucose levels. The second treatment required the subjects to eat .23kg of fried BM each day for 11 weeks. The trial with the fried BM did show a decrease in blood glucose levels, although the decrease was less severe than the BMJ [62].

In an attempt to compare BM to a current oral diabetes medication, one study gave participants differing concentrations of dried BMJ or metformin, a biguanide insulin sensitizer diabetic medication. The study participants included 143 patients who were between the ages of 35 – 70, were healthy and not previously on a diabetes related medication. The doses of BMJ were 500mg, 1000mg or 2000mg/day, or a 1000mg tablet of metformin a day. Fructosamine levels were measured in each patient at the beginning and end of each study period to determine levels of glycemic control, as fructosamine levels are a good indication of blood glucose levels over a short period of time. The 500mg and 1000mg doses of BM were not found to significantly change hypoglycemic control, while the 2000mg dose was found to have a statistically significant effect. However, this effect was less pronounced than that of metformin in lowering fructosamine levels [64].
Given the significant effect of BM on T2D in *in vitro, in vivo* and pre-clinical studies it is apparent that this fruit has the potential for therapeutic application. Despite the well-documented effects of BM to improve diabetes, the mechanism of BM’s anti-diabetic effect is still unknown. As there is no current therapy for T2D that focuses on managing diabetes via ER stress, we will investigate if BM’s anti-diabetic effect is involved in this pathway. Managing T2D via ER stress has the potential be more effective than current therapies, as it targets a molecular mechanism upstream of insulin signaling. It is important to target mechanisms more closely related to the mechanisms that cause hepatic insulin resistance, as these therapies may reduce additional T2D associated complications which are downstream of insulin signaling. This study will allow for greater understanding of how BM counteracts a high fat diet (HFD)-associated T2D for future therapeutic and clinical applications.
Table 9. Summary of recent and/or significant clinical bitter melon studies.

<table>
<thead>
<tr>
<th>Author(s), [Reference]</th>
<th>Country</th>
<th>Fruit part or Extract</th>
<th>Preparation Method and Quantity</th>
<th>Human Subjects</th>
<th>End-Point: Effects</th>
<th>Statistically Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dans et al., [60]</td>
<td>Philippines</td>
<td>Commercially available Charantia Ampalaya Capsules®</td>
<td>Two capsules, three times a day; 3 month trial.</td>
<td>T2D patients, 7%&lt;A1c levels&lt;9%, randomized, double blind, (n=40)</td>
<td>A1c levels: Decrease in A1c</td>
<td>No</td>
</tr>
<tr>
<td>Kasbia et al., [63]</td>
<td>Canada</td>
<td>Whole fruit juice</td>
<td>Freeze-dried BM in capsules; acute dose of 50mg or 100mg/kg of body weight</td>
<td>Healthy, overweight, male subjects, (n=5)</td>
<td>Oral glucose tolerance test: no change</td>
<td>No</td>
</tr>
<tr>
<td>Fuangchan et al., [64]</td>
<td>Thailand</td>
<td>Dried whole fruit without seeds</td>
<td>Capsules: 500mg, 1000mg or 2000mg/day; 4 week trial</td>
<td>T2D patients, randomized, double blind, ages 35 – 70, (n=143)</td>
<td>Fructosamine levels via a Calorimetric Test: Improved glucose tolerance.</td>
<td>Yes, at the 2000mg/day dosage</td>
</tr>
<tr>
<td>Leatherdale et al., [62]</td>
<td>Great Britain</td>
<td>Whole fruit without seeds</td>
<td>1. 0.23 kg fried BM per day; 11 week trial 2. Acute dose of 50 mL BMJ</td>
<td>T2D patients, average weight, (n=9)</td>
<td>50g oral glucose tolerance test: Improved glucose tolerance.</td>
<td>Yes</td>
</tr>
<tr>
<td>Welihinda et al., [61]</td>
<td>Sri Lanka</td>
<td>Whole fruit without seeds</td>
<td>Acute dose of 100 mL BMJ</td>
<td>T2D patients without complications or on medication, (n=18)</td>
<td>50g oral glucose tolerance test and blood sugar estimation: Improved glucose tolerance.</td>
<td>Yes, in 13/18 participants</td>
</tr>
</tbody>
</table>
CHAPTER 2

2 Hypothesis and Aims

BM has been shown to alleviate hepatic insulin resistance in numerous in vivo, in vitro and clinical studies. However, the mechanism through which BM decreases insulin resistance is not well understood. Our laboratory has shown that BM is able to attenuate obesity, metabolic stress and insulin resistance. Given the recent research on ER stress connecting obesity and insulin resistance, we will investigate if ER stress attenuation is the mechanism through which BM is able to decrease insulin resistance.

2.1 Hypothesis

Bitter melon will alleviate obesity-associated insulin resistance by regulating ER stress in mice fed a HFD, specifically via the PERK pathway (Figure 11).

2.2 Aims and Objectives

The specific goal of our study is to determine the effects of bitter melon on high-fat-diet induced ER stress in mice.

Objective: Determine if BM can attenuate ER stress in HFD-mice.

Approach: Western blots were performed on liver tissue obtained from mice fed a control diet, HFD and a HFD supplemented with BM.
Figure 11. Hypothesized mechanism by which BM attenuates ER stress. [Adapted from: Szegezdi E, Logue SE, Gorman AM, Samali A: Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 2006, 7:880-885]
CHAPTER 3

3 Materials and Methods

3.1 Bitter Melon Preparation

Chinese variety of young bitter melon was obtained fresh, washed and deseeded (Figure 12). Bitter melon juice (BMJ) was extracted using a household juicer and centrifuged at 4500 rpm at 4°C for 30 minutes [56]. Supernatant BMJ was lyophilized at 45°C for 72 hours and stored at -80°C until used for feeding studies and manually mixed into HFD mouse chow for feeding mice [36].

Figure 12. Chinese bitter melon. The physical appearance of unripe Chinese bitter melon (A) before preparation and (B) in cross section, showing the seeds with pericarp.
3.2 Animal Studies

Details of animal experiments have been previously published. Frozen tissues from these published studies were used to address current experiments [36]. The University of Hawaii Institutional Animal Care and Use Committee approved all procedures. In brief, 24 male and female four to six week old C57BL/6 mice were randomized into three groups and were fed their respective diets for a period of 16 weeks: 1) control diet (11% kcal), 2) HFD (58% kcal), and 3) HFD + 1.5% freeze dried BMJ (w/w) [56]. Mice were housed individually and allowed to drink water and eat *ab libitum*. Mouse weight and food intake was measured daily, with water intake measured weekly. Mice were anesthetized and sacrificed after 16 weeks and their livers were excised for further study. Livers were snap frozen using liquid nitrogen and frozen at –80°C. Liver tissue homogenates were then used for Western blotting to detect the proteins of interest: Grp78, eIF2α-P and ATF4 (Figure 13).
Figure 13. Study Design. C57BL/6 mice were randomized into 3 groups: control, high-fat-diet (HFD) and high-fat-diet + bitter melon (HFD+BM).

3.3 Protein Preparation

Whole cell proteins were extracted from frozen liver tissue by preparing a 5% homogenate in ice-cold buffer containing 10mM Tris-Hcl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 mM NaF, 150 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 mM PMSF and protease inhibitor (Roche Diagnostics Corporation, Indianapolis, IN) using a PRO 200 Laboratory Homogenizer (Pro Scientific). The homogenates were centrifuged at 12,000 RPM at 4°C for 20 minutes
and supernatants stored at -80°C. Protein concentrations were determined using a Bradford protein assay (BioRad Laboratories, Hercules, CA, USA) [65].

### 3.4 Western Blotting

Samples containing 75µg of proteins were separated on an 8% SDS-PAGE gel to examine the levels of eIF2α-P and ATF4 proteins. 10 µg of protein were used to examine the Grp78 levels and also separated on an 8% gel. Protein was transferred to a nitrocellulose membrane, blocked for 1 hour in 1% BSA and probed with primary antibodies eIF2α-P, CREB-2 (ATF4) or Grp78 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. Blots were then probed with a secondary antibody for 1 – 2 hours with goat anti-rabbit (eIF2α-P, ATF4) or donkey anti-goat (GRP-78) (Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by enhanced chemiluminescence detection. Blots were stripped using 1X Re-Blot (Millipore, Billerica, MA) and re-probed with β-actin (Sigma, St. Louis, MO) to compare protein concentration in each lane. Band intensity was determined using a densitometry scan and expressed as a percentage of the control (β-actin).

### 3.5 Data Analysis

Western blots were analyzed via densitometry scans and band intensity normalized to control. Statistical analysis was conducted using GraphPad Prism 5. Data sets are expressed as mean values ± SEM. Animal groups were compared using a one-way ANOVA and considered significant with P-values < 0.05.
4 Results

4.1 ER Stress Protein Expression

ER stress proteins Grp78, phosphorylated eIF2α and ATF4 exhibited increased levels of expression in HFD conditions. With the addition of BM to HFD, Grp78 and ATF4 expression was seen to normalize and expression of eIF2α-P decreased significantly. This attenuated expression indicates BM successfully inhibited the UPR pathway.
4.1.1 Grp-78

Feeding of HFD significantly increased Grp78 protein levels by 200% (p<0.05) as compared to untreated controls, while BMJ normalized the protein in HFD-fed mice (Figure 14).

Figure 14. Ten (10) µg of total hepatic proteins were separated on 4-12% gradient gel and probed overnight with anti-mouse Grp78 antibody. Blots were later stripped and probed for β-actin. Bar graph shows data expressed as a percentage of control values, set at 100%. Values are means ± SE (n = 6). Mean values with common letters do not differ (p<0.05).
4.1.2 Phosphorylated eIF2-α

Feeding of HFD significantly increased phosphorylated eIF2α by 200% (p<0.05) as compared to untreated controls, while BMJ normalized the protein in HFD-fed mice (Figure 15).

**Figure 15.** Seventy-five (75) µg of total hepatic proteins were separated on 4-12% gradient gel and probed overnight with phosphorylated anti-mouse eIF2α antibody and later stripped and incubated with anti-mouse eIF2α antibody. Blots were later stripped and probed for β-actin. Bar graph shows data expressed as a percentage of control values, set at 100%. Values are means ± SE (n = 6). Mean values with common letters do not differ (p<0.05).
4.1.3 ATF4

Feeding of HFD significantly increased ATF4 protein levels by 150% (p<0.05) as compared to untreated controls, while BMJ normalized the protein in HFD-fed mice (Figure 16).

Figure 16. Seventy-five (75) µg of total hepatic proteins were separated on 8% SDS-PAGE polyacrylamide gel and probed overnight with anti-mouse ATF4/CREB-2 antibody. Blots were later stripped and probed for β-actin. Bar graph shows data expressed as a percentage of control values, set at 100%. Values are means ± SE (n = 6). Mean values with common letters do not differ (p<0·05).
CHAPTER 5

5 Discussion

5.1 ER Stress Protein Expression

Obesity causes inflammatory and metabolic stress through an energy imbalance and increases in adipocytes and lipids. Obese or overweight individuals show an increase in blood glucose, lipids and ROS, which leads to cellular and ER stress. Furthermore, increases in glucose levels have been shown to exacerbate fatty-acid induced ER stress and apoptosis [66]. These increased levels in ER stress result in an increased expression of the proteins PERK, Grp78 and eIF2α-P [22]. This expression pattern is parallel to expression of ER stress proteins Grp78, eIF2α-P and ATF4 in the current study of the effects of a HFD on mouse hepatic tissue.

The importance of Grp78, eIF2α-P and ATF4 in the development of insulin resistance has been demonstrated previously in numerous studies. Blocking phosphorylation of eIF2α in mice resulted in a severe diabetic phenotype impaired glucose tolerance and controlled insulin secretion in pancreatic β-cells, demonstrating the importance of this protein in maintaining cell homeostasis. This phenotype was confirmed by a successful transgene rescue of eIF2α-P [67]. Conversely, mice heterozygous for Grp78, when compared to WT, had normalized levels of eIF2α-P and ATF4 proteins. These mice had increased insulin sensitivity when exposed to a HFD due to a reduction in gene expression [68]. Both of these studies demonstrate
the importance of the ER stress proteins Grp78, eIF2α-P and ATF4 in the insulin resistance pathway through their expression patterns.

5.2 Attenuated Endoplasmic Reticulum Stress by Bitter Melon

Investigating the effect of BM on ER stress is important to harnessing this vegetable as a potential therapy to combat hepatic insulin resistance, as well as other downstream mechanisms. Previous studies have demonstrated the effectiveness of BM as an anti-diabetic therapy through in vivo, in vitro and clinical studies. Our lab has previously shown that BM decreases lipid accumulation in human adiposities, decreases metabolic stress, up-regulates IRS proteins and increases hepatic insulin signaling levels, all of which can help to prevent or control insulin resistance and T2D (Figure 18) [36, 50, 56]. However, the mechanism through which BM was able to do so has not been previously evaluated. It was hypothesized that BM would alleviate obesity associated insulin resistance through ER stress regulation under HFD conditions (Figure 11). Western blot analysis of the PERK pathway proteins Grp78, phosphorylated eIF2α and ATF4 have shown that ER stress is induced in HFD conditions but normalizes when a HFD is supplemented with BM (Figure 14, Figure 15, Figure 16). There have previously been no studies on the effect of bitter melon on ER stress. Through investigation of the PERK pathway this study has shown that BM does attenuate HFD induced ER stress in hepatic mouse tissue (Figure 17). This data illustrates the mechanism through which BM has been able to alleviate obesity associated metabolic syndromes.
Through this attenuation of ER stress BM has been able to prevent or decrease many health conditions that are affected by downstream molecules. Given that the PERK pathway is parallel to the ER stress response that results in insulin insensitivity, and eventually T2D, it is apparent that BM’s success in treating T2D at least partially through attenuating the ER stress response (Figure 18). Adding BM to an individual’s diet is a feasible method to reduce ER stress and insulin resistance in the human population.

5.3 Other Alternative Therapies

Given the knowledge that ER stress links obesity to hepatic insulin resistance, research has begun to focus on therapies for the prevention or attenuation of ER stress [69, 70]. In addition to the current study of BM on the attenuation of ER stress, additional research has examined the effects of other natural substances on this pathway. However, no other products have shown the same potential as BM as a diabetes therapy. Grapes are a powerful antioxidant and are able to prevent apoptosis of hepatic cells [69]. Apoptosis can be prevented through various pathways, with ER stress attenuation demonstrating a potential for an anti-diabetes therapy. This study found that although the grapes did attenuate apoptosis in liver cells they were unable to affect apoptosis via ER stress and instead prevented apoptosis through mitochondrial and oxidative stress pathways. Given this knowledge, BM has the potential to be more successful as an anti-diabetic therapy than grapes as the insulin resistance pathway goes through the ER.
Conversely, chromium has been found to alleviate insulin resistance and glucose intolerance via ER stress through increased expression of PERK, eIF2α-P and IRE-1, a protein expression pattern similar to BM [70]. However, there were no changes in serum lipid levels, which has been previously shown in BM by our lab, and so chromium does not have the weight loss potential previously shown by BM. This inability of chromium to affect lipid levels suggests that BM acts upstream of chromium and thus is a more powerful therapy. These other two natural products may have health benefits, but BM appears to be the most beneficial to attenuating obesity, ER stress and hepatic insulin resistance.

5.4 Additional Health Benefits of Bitter Melon ER Stress Attenuation

If BM attenuated insulin resistance directly, rather than through attenuation of ER stress, BM could not alleviate additional medical complications downstream of the UPR. This ability of BM to decrease expression of ER stress proteins can help in understanding the mechanism behind additional therapeutic effects. BM has been shown to have therapeutic potential on life threatening health conditions, such as cancer and HIV [71]. However, the connection between these conditions and BM has not previously been linked to ER stress.

BM successfully inhibits proliferation in multiple types of cancer cell lines, including breast, skin, pancreatic, prostate and leukemia [72-76]. Specifically, our lab has shown that BM inhibits proliferation and promotes targeted apoptosis of breast cancer MCF-7 and MDA-MB-231 cells, while not affecting normal cell growth [73]. The mechanism by which BM was able to negatively impact these cancer cells may have
been influenced by ER stress attenuation. Studies have shown that the PERK pathway investigated in this study is necessary for optimal tumor growth [77]. Additionally, overexpression of ER chaperone proteins, such as Grp78, is associated with cancer resistance to chemotherapy [78]. Tumor cells have been shown to become more sensitive to extracellular stress, such as hypoxia, when they have compromised PERK signaling. The attenuation of the UPR and the PERK pathway that was have shown in this study may sensitize cancer cells to stress. This increased sensitivity to stress may be partially responsible for the decreased rates of proliferation and increased rates of apoptosis.

Various phytochemicals isolated from BM, including glycosides and proteins, have been shown to inhibit human immunodeficiency virus (HIV) infection and replication [45, 79]. Upregulation of ATF4, a transcription factor attenuated by BM in this study, has recently been shown to enhance HIV replication [80]. Therefore, the ability of BM to inhibit HIV may be through attenuation of ER stress, and thus ATF4.

This study has helped elucidate the mechanism of BM’s ability to attenuate cancer, HIV and CVD, as all of these health complications are effected by ER stress. Any molecular mechanisms that are downstream of the UPR are candidates for BM therapy.

5.5 Global Attenuation of Obesity via Bitter Melon

During this study BM has been shown to alleviate hepatic ER stress, and thus can be speculated to attenuate all downstream pathways as well. Our lab has previously shown BM to reduce adiposity, lipid accumulation, insulin levels, blood glucose and
oxidative stress [36]. Our lab has also shown that BM decreases levels of apoB in HepG2 cells [49, 56]. This decreased level of apoB is associated with a decreased risk for CVD, a complication that is also correlated with obesity. Adiposity, lipid accumulation and oxidative stress are upstream mechanisms of ER stress and have been shown to decrease with the addition of BM. However, it was not known if the effect of BM on insulin signaling was directly to insulin receptors or through the ER. This comprehensive attenuation demonstrates that BM is able to compensate for hepatic nutrient overload prior to symptom development. When an individual is exposed to a HFD increases in lipids, glucose and ROS cause an increase in ER stress. However, BM has been shown to reduce hepatic lipid levels and attenuate ER stress. Furthermore, the ER stress pathway may be responsible for the ability of BM to increase of insulin signaling above control levels. ER stress has been seen to affect additional cellular functions, rather than just insulin signaling, and thus knowing BM’s effect on ER stress is important.
**Figure 17.** Results summary. Under conditions of a high-fat-diet, the unfolded protein response (UPR) pathways are attenuated by BM. BM has been shown to inhibit the UPR by targeting the dissociation of Grp78 from the stress receptors, and thus all downstream proteins. [Adapted from: Szegezdi E, Logue SE, Gorman AM, Samali A: Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 2006, 7:880-885.]
Figure 18. Bitter melon attenuates ER stress. Our lab has previously shown that bitter melon decreases obesity and metabolic stress, while increasing insulin signaling. This study has shown that this increase in insulin signaling is accomplished, at least partially, through an attenuation of ER stress. [Adapted from: Muoio DM, Newgard CB: Insulin Resistance Takes a Trip Through the ER. Science 2004, 306:425-426.]
CHAPTER 6

6 Conclusion

The increasing prevalence of obesity and associated disorders throughout the world have created a need for the development of alternative and natural therapies. Obesity increases the potential of an individual to develop different syndromes and diseases, including CVD and T2D. Recent discoveries have shown that ER stress links obesity and associated complications. These complications can range from insulin resistance to atherosclerosis [81].

More specifically, insulin resistance can become a serious complication as it frequently goes undiagnosed. These conditions can be life threatening and are difficult to treat. The connection between obesity and T2D led to this investigation on developing new therapies for diabetes prevention.

This study has shown that BM does attenuate ER stress in hepatic mouse tissue, thus demonstrating the mechanism for the previously demonstrated increases in insulin signaling and subsequent prevention of insulin resistance.

6.1 Significance

Determining the relationship between obesity and associated disorders, such as T2D, can help elucidate methods to decreasing global obesity problem. This study has increased the understanding of how BM helps decrease obesity and associated symptoms while confirming its effectiveness. This increased knowledge will improve the accessibility of BM as both an alternative medicine as well as a possible
pharmaceutical therapy. Despite the traditional use of BM in Eastern countries, increased understanding of its pharmacognosy will help to apply its usefulness globally.

6.2 Future Studies

Given the previous studies in our lab demonstrating the effectiveness of BM as a therapy we will be further investigating its effects in a pre-clinical study. Various BM supplements are commercially available, although none have been proven effective or safe through clinical trials (Figure 19). Previous clinical studies have not been able to show statistical significance when using BM as an insulin therapy due to their study design and sample size. In this study we will obtain blood work from overweight individuals who are fed BM over a months time. In this way we plan to investigate how BM effects molecular pathways within individuals who are at risk for T2D.

![Commercially available bitter melon supplements. This is a sampling of the available supplements that are currently available within the United States.](image)
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