Grape powder attenuates the negative effects of GLP-1 receptor antagonism by exendin-3 (9-39) in a normoglycemic mouse model

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Abstract (academic)

Prediabetes is a condition affecting 35% of US adults and about 50% of US adults age 65+. Foods rich in polyphenols, including flavanols and other flavonoids, have been studied for their putative beneficial effects on many different health conditions including type 2 diabetes mellitus and prediabetes. Studies have shown that some flavanols increase glucagon-like peptide 1 (GLP-1) levels. GLP-1 is a feeding hormone that increases insulin secretion after carbohydrate consumption and increased GLP-1 levels may be responsible for some of the beneficial effects on glycemic control after flavanol consumption. The present study explored the effects of grape powder consumption on metrics of glycemic health in normoglycemic and prediabetic C57BL/6J mice; additionally, the mechanism of action of grape powder was investigated. Grape powder significantly reduced ($p<0.01$) blood glucose levels following oral glucose gavage after GLP-1 receptor antagonism by exendin-3 (9-39) compared to sugar-matched control; indicating that it was able to attenuate the hyperglycemic effects of GLP-1 receptor antagonism. Grape powder was employed in acute (1.6 g grape powder/kg bodyweight) and long-term high fat diet (grape powder incorporated into treatment diets at 5% w/w) feeding studies in normoglycemic and prediabetic (diet-induced obesity) mice; grape powder did not improve glycemic control in these studies versus sugar-matched control. The mechanisms by which grape powder ameliorates the deleterious effects of GLP-1 receptor antagonism warrants further study.

Grape powder attenuates the negative effects of GLP-1 receptor antagonism by exendin-3 (9-39) in a normoglycemic mouse model

Thomas Carl Haufe

Abstract (public)

Prediabetes is a condition affecting 35% of US adults and about 50% of US adults age 65 and over; it describes a state of impaired glucose control or impaired glucose tolerance and is a major risk factor for development of T2DM. Polyphenols are plant secondary metabolites (often pigments) which have been studied for their potential beneficial effects on many different health conditions including T2DM and prediabetes. Some studies have suggested that polyphenols may improve signalling of hormones in the body that control blood glucose levels, including insulin and GLP-1. This study explored the effects of grape powder consumption on measures of glycemic control in several different mouse models. Additionally, we explored the mechanism of action by which grape powder was able to impart beneficial effects. Freeze dried grape powder was used as an experimental substitute for fresh, whole grapes. Grape powder was employed in one-term (1.6 g grape powder/kg bodyweight) and long-term high fat diet (5% w/w of the diet) feeding studies in normoglycemic and prediabetic (diet-induced obesity) mice; grape powder did not improve glycemic control in these studies versus sugar-matched control. Grape consumption significantly reduced ($p<0.01$) blood glucose levels following after antagonism of GLP-1 receptors in mice by exendin-3 (9-39) compared to sugar-matched control; indicating that it was able to reduce the negative hyperglycemic effects of GLP-1 receptor antagonism. This research indicates that grape consumption may be able to restore normal signalling of feeding hormones under specific circumstances.

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AUC, area under the curve; DPP4, dipeptidyl peptidase-4; FDGP, freeze-dried grape powder; GLP-1, glucagon-like peptide 1; GLP1R, glucagon-like peptide 1 receptor; GTT, glucose tolerance test; HF, high-fat; i.p.; intraperitoneal; ITT, insulin tolerance test; NG, normoglycemic; OGTT, oral glucose tolerance test; PD, prediabetic; SF, standard-fat; T2DM, Type 2 Diabetes Mellitus;

Chapter 1. Introduction

Prediabetes is a condition affecting 35% of adults in the United States and about 50% of adults aged 65 years or older. This condition is marked by impaired glucose tolerance and/or impaired fasting glucose and is a major risk factor for type 2 diabetes mellitus (T2DM); additionally, chronic prediabetes is a significant risk factor for cardiovascular, retinal, kidney and other diseases. Thus it is of central importance to public health to find effective strategies to control prediabetes to prevent a progression into T2DM. Grapes are a potent source of flavonoids, including procyanidins, which have shown potential to act as anti-diabetic compounds. In order to effectively utilize grapes as a method of managing prediabetes, it is necessary to elucidate the mechanisms by which grapes improve glycemic control. Studies have shown that grapes (and grape procyanidins) improve insulin sensitivity, lower blood glucose levels, and improve action of pancreatic β cells, among other activities. The next progression in this research is to determine the mechanisms by which grape constituents exert these effects. GLP-1 is a feeding hormone released as a response to food intake; GLP-1 has insulinotropic properties that are part of healthy glucose homeostasis. Recent work has shown that grape flavan-3-ols and other polyphenolic compounds increase circulating GLP-1. Additionally, grapes have been shown to inhibit DPP4, which is an enzyme that rapidly inactivates GLP-1. This same mechanism of DPP4 inhibition is employed by pharmaceuticals called gliptins, which increase active GLP-1 levels via this mechanism resulting in lower blood glucose levels. However, the exact mechanisms of action by which grape polyphenols are able to promote normoglycemia are yet to be determined.

The central objective of this project is to analyze the efficacy of grape powder in restoration of glycemic control in normoglycemic and prediabetic mouse models, and to explore

the pathways through which grape powder polyphenols are able to improve glycemic control. Therefore we submit the following central hypothesis for this work: grapes are able to improve glycemic control by stimulating the increase of postprandial circulating GLP-1 levels through mechanisms in the gut.

Specific Aims

Aim 1: Determine if beneficial effects of acute grape consumption are mediated through mechanisms located in the gut, specifically through the GLP-1 signaling pathway.

Hypothesis: Grape constituents exhibit glycemic control activities through mechanisms in the gut such as increasing GLP-1 levels to stimulate insulin production.

Aim 2: Determine if acute and/or long-term grape consumption can be effectively utilized as strategies to improve glycemic control in normoglycemic and prediabetic mouse models

Hypothesis: Grape consumption (both acute and chronic consumption) improves glucose tolerance and results in an increase of circulating GLP-1 and insulin in normoglycemic and prediabetic mouse models.

Chapter 2. Literature Review

Prediabetes

Background

Prediabetes is a condition affecting 35% of adults from the United States as well as 50% of U.S. adults 65 years or older [1]. Additionally, hyperglycemia (chronic elevated blood glucose levels) is a condition affecting about 40% of the population of the United States[2]. The World Health Organization defines prediabetes as impaired fasting glucose (IFG) with fasting blood glucose concentrations ranging between 110 mg/dL and 126 mg/L and/or impaired glucose tolerance (IGT, measured by oral glucose tolerance test; individuals with impaired glucose tolerance have blood glucose levels between 140-199 mg/dL two hours after a 75 gram glucose load)[3]. Prediabetes is a major risk factor for developing diabetes: 5-10% of people with prediabetes will progress to diabetes every year[4]. Additionally, chronic hyperglycemia (as seen in prediabetes) is a risk factor for cardiovascular disease, kidney disease, microvascular complications that may damage the retina and other organs, among other negative health effects[4, 5]. An American Diabetic Association expert panel estimated that 70% of people with prediabetes would eventually see the condition progress into diabetes[4]. Risk factors for Type 2 Diabetes Mellitus (T2DM) include: age over 45, obesity, physical inactivity, high blood glucose levels, family history of diabetes, high blood pressure, high blood lipid levels and some ethnic backgrounds are at increased risk[6]. Prediabetes and T2DM are associated with many harmful physiological conditions: patients with prediabetes have had damage to their eyes, kidneys, limbs, circulatory system, among other complications, as a result of the disease[4]. Discovering effective management strategies for prediabetes and hyperglycemia will be an increasingly important goal for public health in the U.S. and abroad. From 2007-2009, 18.9% of U.S. adults

(aged 65 and above) were diagnosed with diabetes[7], while ~8.9% of the total U.S. population had T2DM (and 27% of Americans with T2DM had not yet been diagnosed with the disease, according to CDC statistics).

Pathogenesis and pathophysiology

The pathogenesis (study of the cause of a disease) and the pathophysiology (analysis of the physiological changes caused by a disease) are quite interrelated in the case of prediabetes and T2DM. It is often difficult to tell if symptoms of T2DM were a cause of the disease or if they are present as a manifestation of T2DM[5]. This disease is caused by a variety of factors in most cases (nutrition, genetics, etc.); rarely, there are genetic polymorphisms directly/solely responsible for the development of T2DM[5]. Obesity is a main risk factor predicting the development of T2DM; the correlation between obesity and chance of T2DM diagnosis increases with age[8-10].

The progression of normoglycemia into T2DM is primarily characterized by increased hyperglycemia, increased gluconeogenesis, increased hepatic glyconeogenesis, insulin resistance and a series of changes in pancreatic β-cell function (resulting in impaired insulin secretion); T2DM is a systemic disorder that can negatively affect many different organs and systems in the body. Weir and Bonner-Weir proposed a five stage progression of T2DM in 2004 based on the changes seen in pancreatic β-cell mass[11]. These five stages provide a good overview of how pancreatic β-cells change throughout the progression of the disease. The stages are as follows:

0) During normoglycemia the pancreatic β-cells have normal mass
1) Insulin secretion and β-cell mass increase in order to achieve normoglycemia, as moderate insulin resistance is present

2) Blood glucose levels rise as β-cells begin to lose the ability to secrete insulin in response to chronic elevated glucose levels; this stage represents the "prediabetic" stage and people may stay in this stage for years before progressing
3) Blood glucose levels begin rising rapidly as β-cells begin to fail to control the elevated glucose levels
4) T2DM stage in which β-cell mass has been decreased by 50%, most individuals with T2DM remain in this stage
5) Severe loss of β-cell function where subjects will need insulin for survival, this usually only occurs in individuals with Type 1 Diabetes[11]

Prediabetes is diagnosed using several different biomarkers including fasting glucose levels as well as glucose tolerance levels; the exact cutoff values for these markers differ between public health organizations. For the present research, it is important to know that prediabetes is characterized as impaired fasting glucose and/or impaired glucose tolerance (some organizations also consider elevated glycated hemoglobin levels, HbA1c, indicative of prediabetes[3]). Impairment in the insulin signaling processes is one of the prominent changes seen during prediabetes: this includes insulin resistance in skeletal muscle and hepatic tissue (as well as decreased insulin secretion by pancreatic β-cells as seen above)[12]. Defective insulin signaling plays a major role in the progression from normoglycemia to prediabetes and eventually T2DM as defective insulin signaling causes dysregulation of blood glucose levels resulting in chronic hyperglycemia.

Treatment

Several different approaches are taken to prevent the progression of prediabetes into T2DM including pharmacological, lifestyle, and surgical measures. Stevens et al. found in their 2015 meta-analysis that a combination of pharmacological and lifestyle intervention (changing diet and exercise habits) is an effective strategy to prevent progression to T2DM[13]. Obese patients with prediabetes or T2DM are strongly encouraged to reduce their caloric intake and to increase their activity level[13]. During late-stage T2DM when pancreatic β-cells begin to fail, insulin administration via intravenous injection is a common method of controlling blood sugar levels to prevent hyperglycemia-induced damage to the kidneys, eyes, and other organs[14]. These organs are at risk when insulted with hyperglycemic conditions over extended periods of time. Additionally, acute hyperglycemia may result in what is known as a "hyperglycemic crisis" resulting in diabetic ketoacidosis or hyperglycemia hyperosmolar state[14]. Insulin (along with many other medications) are prescribed and administered to T2DM patients in an attempt to avoid these events.

Metformin is a medicine that is commonly prescribed to T2DM patients in the United States[5]. Metformin is prescribed to control blood glucose levels and encourage weight loss. Metformin is a biguanide compound that reduces gluconeogenesis in the liver, increases insulin sensitivity in the liver, and modulates other pathways in peripheral tissue[5].

GLP-1 receptor agonists are another class of pharmaceuticals that are increasingly employed as a method of increasing insulin secretion to help control blood glucose levels[15]. DPP4 inhibitors (gliptins) are also prescribed to T2DM patients as DPP4 inhibitors increase active GLP-1 levels, which results in an increase in insulin secretion. These pharmaceuticals exploit the incretin effects as a method of controlling blood glucose levels. Mechanisms of action

of these pharmaceuticals will be discussed later in this chapter in the "Glucagon-like peptide-1" section.

Bariatric surgery is another method used in obese patients as a method of reducing body mass and has been shown to be very effective in resolving T2DM in affected patients as well as preventing T2DM in subjects for years after the surgery. There are many variations of bariatric surgery that are outside the scope of this literature review but it is important to note that the surgery results in weight loss primarily through decreased stomach size that results in lowered food intake and decrease in the intestinal surface area that is capable of absorbing nutrients. Bariatric surgery has been shown to be extremely effective in ameliorating or reducing T2DM, hyperlipidemia, sleep apnea, and hypertension[16]. The efficacy of bariatric surgery in resolution of T2DM in surgery patients is astounding, although the chance of T2DM resolution is dependent on the type of bariatric surgery performed. From their landmark 2004 meta-analysis of bariatric surgery and obesity comorbidities, Buchwald et al. found that T2DM was resolved in 76.8% of patients (all surgery types), resolved or improved in 86.0% of patients (all surgery types), and resolved in 98.9% of patients who had received the duodenal switch procedure[16]. In a small clinical study, Guidone et al. found that: T2DM was resolved, insulin secretion was normalized, and pancreatic β-cell function was restored only one week after duodenal switch surgery[17]. Additionally, incretin levels (including GLP-1) and the efficacy of the incretin effect are restored one month after gastric bypass[18]. Bariatric surgery is a very effective method of resolving T2DM in affected patients as well as preventing T2DM onset in at-risk patients.

Complications of prediabetes

As mentioned before, prediabetes is an intermediate phase between normoglycemia and T2DM. Along with the risk of progression into T2DM, there are cardiovascular risk factors usually associated with prediabetes[19]. Insulin resistance leads to dysregulation of lipid levels (dyslipidemia); chronic dyslipidemia paired with chronic hyperglycemia results in an impaired physiological state marked by macrovascular and microvascular complications[20]. These complications include the possibility of cardiovascular events, hypertension, development of cardiovascular disease, and microvascular diseases (which affects the eyes, liver, neurons, kidney, etc.)[20]. Diabetic retinopathy, which is the most frequent cause of new blindness for adults aged 20-74, is a disease marked by increase retina vascular permeability and eventual failure of retina vascular system and it is primarily caused by chronic hyperglycemia[21].

Dietary intervention

The Diabetes Prevention Program was a large intervention study completed in the late 1990s[22]. This program gathered 3,234 individuals displaying prediabetes (elevated fasting glucose and impaired glucose tolerance as determined by OGTT) from diverse ethnic backgrounds and assigned them to one of three treatment groups: placebo, metformin, or lifestyle intervention (combination of dietary changes and increased physical exercise). The incidences of diabetes per 100 individuals were 11.0, 7.8, and 4.8 cases for placebo, metformin, and lifestyle intervention, respectively. This study shows that both metformin and lifestyle intervention are capable methods of preventing the onset of diabetes in prediabetic individuals of diverse ethnic backgrounds. Lifestyle intervention reduced the risk of diabetes by 58% compared to placebo in this study, indicating that lifestyle changes (diet and exercise) are effective in preventing the onset of diabetes in at-risk individuals[22]. A similar study was conducted in Finland (Finnish

Diabetes Prevention Study) and similar observations were noted[23]. In the Finnish study lifestyle changes (diet and exercise) resulted in a 58% reduction of the onset of T2DM in at-risk, middle aged participants (n=522)[23]. Compared to pharmaceutical interventions, lifestyle changes are an attractive method of treating prediabetes as lifestyle interventions are cost effective and are not associated with deleterious side effects as seen in some pharmaceuticals. However, lifestyle changes can be difficult for patients to maintain over extended periods of time, and the positive health results associated with lifestyle changes are contingent on sustained behaviors.

Mouse models of prediabetes and T2DM

The present thesis employed C57BL/6J mice for all studies. The acute and long-term studies use "normoglycemic" and "prediabetic" C57BL/6J mice. The prediabetic mice have the same genetic makeup but they are fed a high fat diet from birth (60% calories from fat) and quickly begin displaying a prediabetic phenotype (hyperglycemia, impaired glucose tolerance, hyperlipidemia, obesity, elevated fasting insulin levels[24]). These studies used C57BL/6J mice from Jackson Labs (Bar Harbor, ME) known as "diet-induced obesity" mice, which are fed a high fat diet from Research Diets (New Brunswick, NJ). Table 1 shows the composition of the high fat diet that is fed to mice to induce prediabetes in the diet-induced obesity model. The main sources of fat are lard (supplies ~54% of calories of the diet and is ~32% w/w) and soybean oil (~5.5% of calories of the diet and 3.2% w/w).

Table 1. Composition of high fat diet (D12492, Research Diets) used to induce T2DM phenotype in the C57BL/6J diet-induced obesity mouse model

Ingredient	**grams**	**kcal**
Protein	26	20
Carbohydrate	26	20
Fat	35	60
Total		100
kcal/gram	5.2	
Ingredient	**gm**	**kcal**
Casein, 80 Mesh	200	800
L-Cystine	3	12
Corn Starch	0	0
Maltodextrin 10	125	500
Sucrose	68.8	275
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard	245	2205
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Yellow Dye #5	0	0
FD&C Red Dye #40	0	0
FD&C Blue Dye #1	0.05	0
TOTAL	773.85	4057

The C57BL/6J mouse is often used as a model for metabolic syndrome/type 2 diabetes mellitus because *ad libitum* consumption of high fat diet by this mouse results in the development of hyperglycemia, obesity, and hyperinsulinemia; these symptoms are not present in C57BL/6J mice after consumption of a low fat diet[24]. The fat source has been shown to affect development of T2DM in this model; saturated fats such as lard are often used as the main source of lipids in the C57BL/6J strain as this has been repeatedly proven to cause T2DM in this

strain[25-27]. There are animal models based on administration of chemicals that induce a diabetic phenotype in mice, such as streptozotocin administration; this compound is toxic to pancreatic β-cells and this results in defective insulin secretion and other complications[28]. There are numerous other animal models (usually mouse or rat) that have been explored in the literature, including genetic variants (mono- and polygenic) that develop T2DM as a result of a modified gene expression (defective pancreatic β-cells or non-functioning leptin receptors, for example)[29]. Additionally, high-sucrose and high-fructose feeding models have been used as alternative methods of T2DM induction in mice[25]. Development of T2DM is more severe in C57BL/6J mice when fed a high fat versus when fed a standard fat diet with sucrose incorporated in the drinking water supply at 30%[30]. High-fructose and high-sucrose diets do not cause the dramatic increase in body mass in C57BL/6J mice seen in high-fat feeding; however, long term feeding with high-sucrose and high-fructose diets in this strain causes glucose intolerance, hyperlipidemia, insulin resistance, liver damage, and other deleterious effects[31, 32]. The high fat feeding model was employed in the long-term feeding study of this thesis because it emulates the phenotypical progression of prediabetes in humans and it allows the researchers to observe effects of grape supplementation in the context of diet-induced prediabetes. In future work, it may be valuable to study the effects of chronic grape consumption in a high-sucrose or high-fructose feeding model to determine if grape polyphenols are effective in ameliorating the prediabetic phenotype induced by these diets.

Polyphenols

Polyphenols are plant secondary metabolites that are ubiquitously found across the plant kingdom[33]. There have been over 8000 unique polyphenols identified in the plant kingdom[34], and these compounds serve a variety of roles in plants including pigmentation. Structurally, polyphenols are compounds with more than one phenol group including all derivatives and compounds conjugated with sugars, acids, lipids, etc., as well as those polyphenols polymerized with other polyphenols[35]. There are many classes of compounds within the polyphenol family, including: stilbenes, phenolic acids, xanthones and flavonoids. Polyphenols range from single molecules to large polymerized complexes [33]. It has been argued that polyphenols can be categorized into two main categories: flavonoids and non-flavonoids[36]. Flavonoids are the most widely distributed class of polyphenols within the plant kingdom[33, 37, 38]. Subclasses of flavonoids include: flavonones, flavonoids, isoflavones, anthocyanidins, proanthocyanidins[33]. Flavonoids and the subclasses of flavonoids have been extensively studied in the literature in epidemiological studies, cell studies, animal studies, human clinical studies, color research, sensory analyses, identification and quantification studies, and more.

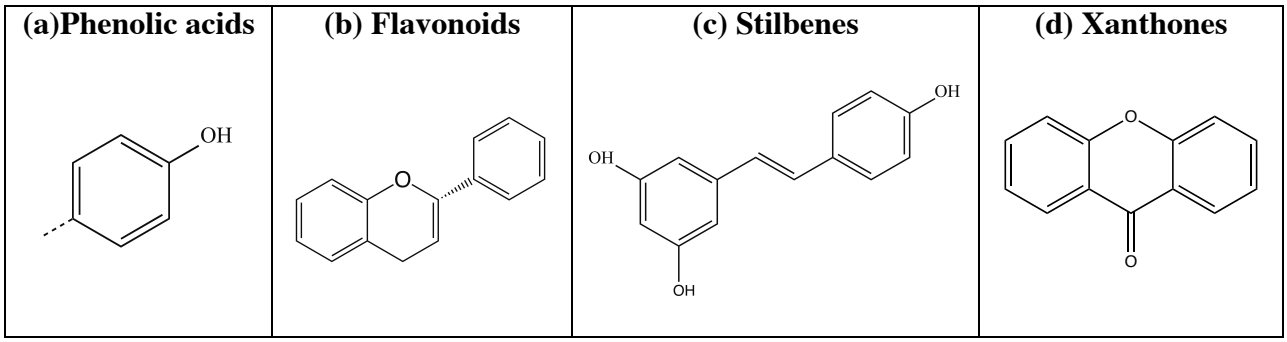


Figure 1. Base structures of major polyphenolic classes

Base structures of four main classes of polyphenols; (a) phenolic acids (b) flavonoids (c) stilbenes and (d) xanthones. Grapes contain polyphenols from all four classes shown in this figure. Adapted from [33, 39].

Polyphenols in epidemiological studies

Polyphenols were originally thought to be only "anti-nutrients" as they can bind to certain macronutrients and reduce digestibility. However, polyphenols have been increasingly studied in recent years due to their beneficial antioxidant properties and potential anti-cancer activity, among other activities[33, 35]. Epidemiological studies provide motivation for investigators to further research the potential of these compounds to potential therapeutic benefits for various diseases. These studies have shown an inverse correlation between polyphenol consumption and cardiovascular risk[40]. A survey of participants of several large study groups (including the Nurses' Health Study) found a significant reduced risk of type 2 diabetes with dietary anthocyanin consumption, but no significant associations were found with the consumption of other flavonoid subclasses[41]. Cohort studies and randomized trials have shown beneficial effects of anthocyanidins (from berries) and flavan-3-ols (from green tea and cocoa) on T2DM[42].

Flavonoids and other phenolic compounds are being investigated for their potential viability to be used as "functional foods," which are foods that impart health benefits (in addition

to sustenance) usually in references to conditions such as diabetes, cancer, inflammation, and cardiovascular disease[43]. In their 2013 meta-analysis, Liu et al. concluded that consumption of dietary flavonoids is associated with a significant decrease in risk for type 2 diabetes[44]. It should be noted that there are conflicting data in the epidemiological literature in which some studies find that increased consumption of a specific class of polyphenols reduces risk of T2DM while other studies do not find this reduced risk with the same class of polyphenols; however, the body of support evidence is sufficiently large that researchers continue to investigate this connection between polyphenols and T2DM in epidemiological studies, human clinical trials, animal work, and cell culture experiments.

Epidemiological data show lower incidence of T2DM among those who consume higher levels of dietary polyphenols; specifically, studies have linked consumption of flavan-3-ols and anthocyanins (both of which are found in grapes[45]) to lower incidence of T2DM[42, 46-49]. A 2013 analysis of three prospective longitudinal cohort studies found that consumption of three servings of grapes/raisins per week was associated with a significantly reduced pooled hazard ratio (0.88 ± 0.05) of developing T2DM[50].

Grape polyphenols

Grapes contain a variety of polyphenolic compounds; HPLC-TOF MS analysis of grape powder has shown high levels of catechins, anthocyanins, procyanidins and stilbenes, including resveratrol, malvidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, quercetin 3-*O*-glucoside, procyanidins B2, among others[51]. Several studies have shown that polyphenol profiles are different across different grape varieties[39, 52]. All four mouse studies in this thesis employed a freeze-dried grape powder provided by the California Table Grape Commission. Polyphenolic composition has been previously reported in the literature and the following compounds have

been detected and quantified in this grape powder: catechin, epicatechin, peonidin, cyanidin, malvidin, quercetin, kaempferol, resveratrol, oleanolic acid, procyanidin B2, delphinidin, rutin, as well as glucosides and other conjugates of these polyphenols[53-55].

Potential mechanisms of action of polyphenols

There are a variety of proposed mechanisms of actions through which polyphenols are able to exert anti-diabetic activities. Polyphenols have been found to inhibit uptake of carbohydrates in the gut and lower blood glucose levels partially through inhibition of carbohydrate digestive enzymes such as amylase, sucrase and glucosidase[56]. Munir et al. wrote a review article in 2013 that analyzed the potential mechanisms by which polyphenols improved insulin resistance[57]. Defective insulin signaling pathways are one of the causes of insulin resistance and ingestion of food polyphenols can help ameliorate defective signaling pathways in individuals with insulin resistance.[57] Munir et al. argue that it is not the "antioxidant" properties of food polyphenols that ameliorate defective insulin signaling pathways; rather, ingested polyphenols that reach circulation act as minor prooxidants that encourage correct pathway signaling in these defective pathways[57].

Flavonoids, including flavan-3-ols and proanthocyanidins, have been shown to have beneficial effects in treating the risk factors of metabolic syndrome[58]. Grapes and grape extracts may be beneficial in the treatment of T2DM due to their low glycemic load and glycemic index as well as their high levels of polyphenols[59]. Grape polyphenols appear to act through multiple mechanisms to exert their beneficial effects. A human study in patients with type 2 diabetes supplemented patients with grape seed extract (GSE) at 600 mg/day over a 4 week period resulting in lowered inflammatory biomarkers indicating a potential ameliorative effect[60].

Proanthocyanidin extracts of grape seed fed to rats for 16 weeks was been shown to alleviate oxidative stress in skeletal muscle, reduce endoplasmic reticulum stress in skeletal muscle, decrease plasma glucose levels and decrease insulin resistance[61]. Several studies have shown that grape polyphenols improve β-cell function in several diabetic animal models[62, 63]. Grape seed extract procyanidins decreased pancreatic β-cell triglyceride levels in rats which improved function of β-cells and normalized insulin levels compared to the control rats fed a cafeteria diet[63]. Another study showed that grape seed proanthocyanidins improved β-cell function in diabetic rats by ameliorating endoplasmic reticulum stress in pancreatic β-cells which allowed for better insulin production and secretion[62]. Grape seed extract has also been shown to improve insulin signaling by increasing expression of insulin and adiponectin receptors in skeletal muscle of rats fed a high fructose diet[64]. Another study showed that a 25 mg/kg bodyweight per day supplementation of grape seed proanthocyanidin extract (GPSE) improved glucose homeostasis in rats; however, efficacy of this treatment varied with different methods of induction of insulin resistance[58].

In vitro studies have shown that GSPE extract also can act as an insulinomimetic agent and phosphorylate insulin receptors which stimulates glucose uptake[65]; however, the relevancy of this work might be limited because of the low levels of bioavailability of procyanidins (discussed below). Another group of studies showed that GSPE supplementation in rats increased expression and activity of proteins related to cellular respiration in brown adipose tissue and skeletal muscle mitochondria, indicating increased glycosidic metabolism[66, 67].

Absorption and bioavailability of polyphenols

It is important to consider the bioavailability and metabolism of polyphenols to better analyze their putative beneficial effects. Polyphenolic compounds are absorbed and metabolized

differently depending on their native structure, conjugated compounds, degree of polymerization, gut microbial flora, among other factors[68]. There is often a discrepancy between *in vitro* studies that examine the effects of polyphenols on a specific cell line compared to what happens when these compounds are administered *in vivo*. This is partially due to the fact that the dose of polyphenols that reaches cellular targets *in vivo* may be much smaller than doses applied to cell lines during *in vitro* studies. Thus it is important to analyze the bioavailability of polyphenols when designing and analyzing studies involving these compounds.

Polyphenols are normally only absorbed in the small intestine when they are present as aglycones (native polyphenolic compounds without conjugation)[69]. Thus polyphenols conjugated with glycosides, sulfates, esters or other polyphenols must be cleaved into their aglycones in order to be absorbed into the small intestine. Polyphenols are often hydrolyzed into their aglycones by intestinal enzymes as well as gut microflora; however, gut microflora may degrade the aglycones into simple phenols or other products after cleavage from conjugates, further reducing the bioavailability of polyphenols[70].

Polyphenol bioavailability varies drastically depending on the class of compound, conjugation, stomach/intestinal conditions, food matrix, and many other factors[70]. In order to better synthesize, interpret, and understand research involving these compounds, there have been many studies performed on the absorption of different polyphenols. Several studies have been performed to determine if biomarkers such as plasma concentration can be used to estimate intake of polyphenols and vice versa[71-76]. By extension, this research also highlights the differences in bioavailability between individuals. Erlund et al. performed a crossover intervention study in 2002 involving 5-week feeding periods of a strictly controlled diet containing either low or high levels of fruits and vegetables. The investigators found that levels

of quercetin, naringenin and hesperetin in serum could not be correlated with fruit and vegetable consumption level[71]. Another study compared the polyphenols as calculated from 7 day recorded food intake records versus quantification of selected polyphenols from a plasma sample (measured by HPLC-PDA)[72]. The authors found a slight correlation between expected plasma levels (based on values calculated from the food intake data) and measured plasma levels; however, this correlation was not high enough to use reproducibly as a method of estimating total polyphenol intake over the 7 day period[72]. The plasma samples were slightly more accurate at predicting short-term polyphenol intake but the correlation was not particularly strong. There have been many other similar studies performed for selected phenolic compounds; there are varying degrees of correlation between ingested polyphenols and serum/urine concentrations depending on many factors[74, 76].

A 2003 study analyzed the bioavailability of polyphenols in rats after consumption of grape seed extract: the monomers were the most highly absorbed as determined by analysis of serum levels and excretion in urine[69]. High molecular weight polyphenolic compounds from the grape seed extract were administered (without the monomers) but these were not detected in serum, indicating poor bioavailability of proanthocyanidin polymers[69]. In a separate study, rats were administered increasing doses of grape seed proanthocyanidins extract (GSPE) and serum levels were quantified over two hours[77]. Greater amounts of proanthocyanidins were detected in the serum as the dosage increased and some dimers were detected in serum at the higher doses; the trimer was not detected at any of the doses (highest dose was 1000 mg GSPE/kg bodyweight)[77]. A 2009 study by Ferruzzi et al. found a large increase in bioavailability of catechins, gallic acid, and some metabolites of these compounds after repeated dosing compared

to an acute dose (they also found accumulation of some polyphenols in brain tissue at the higher doses)[78].

There have also been human clinical bioavailability studies performed in order to better understand human bioavailability of phenolics in different grape products. Catechin and gallic acid were measured in human plasma after consumption of 300 mL grape juice; these compounds were detected in plasma in the nanomolar range 30 minutes after consumption of the juice[79]. Stalmach et al. found in their 2012 paper[80] that anthocyanins and anthocyanin metabolites were present at much higher concentrations than flavan-3-ols after administration of 350 mL Concord grape juice. There are many other bioavailability studies in the literature focusing on absorption of polyphenols after ingestion of grape juice[80-86]. Generally, anthocyanins are more bioavailable than other classes of polyphenols in these studies; however, bioavailability of anthocyanins is usually less than 1% as detected in plasma.

Studies have found that intestinal microfloral metabolism of polyphenols increases bioavailability of polyphenols by converting them in the gut to more readily absorbable forms. Intestinal microflora may be responsible for a large fraction of polyphenols that make it into circulation[81]. Additionally, in bioavailability pharmacokinetic figures there are often two peaks in detected polyphenols: an initial peak soon after ingestion that corresponds to absorption in the small intestine, and a later peak (hours after ingestion) that is partially caused by absorption after microbial catabolism/metabolism of polyphenols in the colon[80] (it is important to note that this peak is partially caused by recycling of polyphenols through enterohepatic circulation system).

Discrepancies usually exist when comparing bioavailability/metabolism experiments between each other, whether comparing different compounds, subjects, food matrices, metabolites, etc. It is difficult to compare absolute values between studies; however, trends are

present that can guide researchers in moving forwards (for example, certain classes of compounds are generally more bioavailable than others regardless of the food matrix). Additionally, the translation of animal bioavailability data into human bioavailability often is not direct[33].

Previous work using freeze-dried grape powder

The work presented in this thesis was funded by the California Table Grape Commission. The California Table Grape Commission represents the interests of grape farmers and producers by providing financial support for the advancement of research regarding beneficial effects of whole grape consumption. CTGC provides a standardized grape powder to its grantees that is formulated with red, green and black grapes to represent average varietal consumption based on consumer data[87]. This freeze-dried grape powder is made from seeded and seedless whole grapes; the grapes are ground under dry ice and subsequently lyophilized. 26 grams of freeze-dried grape powder represents one normal serving (126 grams) of fresh grapes[87]. The use of this powder made from whole grapes simulates whole grape consumption; the use of this standardized powder increases reproducibility and allows for comparison and communication of results across different labs.

There have been numerous studies published in the literature which utilized the same freeze-dried grape powder (provided by CTGC/National Food Lab) that is used in the present study[53, 55, 88-94] (some of these papers have been mentioned in other sections of this literature review). A recent paper (published online in Feb/March 2016) from the McIntosh lab at UNC-Greensboro employed CTGC grape powder in several long term high fat feeding studies using C57BL/6J mice[95]. This study featured a 16 week feeding period with the following diets: low fat, high fat, high fat plus grape powder (5% w/w), high fat plus polyphenol-rich fraction of a grape

powder polyphenol extraction, high fat plus polyphenol-poor solids from the grape powder (this fraction includes the non-sugar, non-acid, non-polyphenol components of the grape powder), and a high fat plus polyphenol-rich fraction as well as the polyphenol-poor fraction. Their results agreed with the results seen in the present thesis; the grape powder diet did not improve measures of glycemic health, body weight, body fat, among other metrics compared to the basal high fat diet. Beneficial results were seen in the feeding group with the polyphenol-rich fraction and the polyphenol rich plus polyphenol-poor fraction. This paper will be further discussed (in Chapter 4) in relation to the results seen in study 4 of this project.

Glucagon-like peptide-1

Glucagon-like peptide-1 amide (7-36) is a peptide incretin hormone (30 amino acid residues) which is primarily released by L-cells in the gut as a response to feeding[96, 97]. GLP-1 is also secreted by pancreatic α-cells under certain physiological conditions[98]. GLP-1 stimulates secretion of insulin and inhibits secretion of glucagon via GLP-1 receptors, some of which are located in the pancreas[96]. Dipeptidyl peptidase IV (DPP4) is an enzyme found in the gut which degrades GLP-1; inhibition of DPP4 has been used in pharmaceutical agents (called gliptins) as a method of increasing GLP-1 levels, thus increasing insulin levels and reducing blood glucose levels[99]. Interestingly, flavonoids have been shown to have some similar properties to these gliptins: intestinal activity of DPP4 was decreased after consumption of grape seed procyanidins in several rat models[100].

GLP-1 is also active in neural pathways regulating appetite, satiety, and food intake[96]. GLP-1 is active at specific receptors (glucagon-like peptide-1 receptors or GLP-1R) before it is quickly degraded by dipeptidyl peptidase IV (DPP4)[101]. Different agonists of GLP-1R (such as Exenatide) have been developed to serve as treatments for individuals with T2DM as they partially mimic the action of GLP-1 via both gastrointestinal and neuronal pathways and promote reduced food intake and reduced bodyweight[102].

As mentioned earlier, GLP-1 is included in the class of hormones known as "incretins," which are hormones that potentiate postprandial insulin secretion. Glucose-dependent insulinotropic polypeptide (also known as gastric inhibitory polypeptide or GIP) is another important incretin. Carbohydrates as well as other dietary components such as lipids have been shown to stimulate GLP-1 secretion[96]; it is not yet well known how individual food components (proteins, complex vs. simple carbohydrates, lipids, etc.) and the ratios of these components in a meal affect postprandial GLP-1 secretion[103]. The increased insulin secretion is achieved by activation of GLP-1 receptors located on pancreatic β cells[104]. This increased insulin secretion assists in the regulation of glucose homeostasis; glucose homeostasis is often problematic in individuals with T2DM, thus higher incretin activity may be beneficial in preventing or ameliorating glucose dysregulation[104]. The incretin pathway is an important part of human regulation of glucose homeostasis: in human clinical trials, the insulin response has been found to be up to two to three times greater (depending on the dose) after oral administration of glucose compared to intravenous glucose administration[105]. The higher insulin secretion after oral glucose consumption should not be attributed wholly to action of incretins (as there are likely other pathways also partially responsible); regardless, it is likely that they play a major role in this increased insulin secretion. GLP-1 achieves this incretin effect by binding to GLP-1 receptors

located on pancreatic β cells, which activates the cell and triggers cAMP formation ultimately resulting in insulin secretion[96]. This incretin response is not wholly attributable to GLP-1 however it is has been shown that GLP-1 along with GIP are arguably the two most important hormones of the incretin pathway[105].

GLP-1 is secreted by intestinal L-cells concentrated in the jejunum[101]; microvilli of these L-cells extend into the lumen of the gastrointestinal (GI) tract where they are able to detect nutrients travelling through the GI tract and respond with secretion of GLP-1[96]. Interestingly, inhibition of sodium glucose dependent transport proteins in the small intestine (such as SGLT1) as well as inhibition of "gut gustatory receptors" (specifically sweet taste receptors located in the gut such as α-gustducin) in the small intestine have both been shown to severely reduce GLP-1 secretion after meal intake, indicating that these proteins may serve as "sensors" for activating L-cell secretion of GLP-1[96, 106].

GLP-1 has a diverse range of pathway targets and physiological effects, which is remarkable due to the fact that the half-life of the active form of GLP-1 is only 1.5-2 minutes as it is rapidly degraded by DPP4[96, 107]. GLP-1 is active in numerous pathways, two important pathways of action include: the "incretin" pathway as mentioned above as well as a separate, but related, neuronal pathway[97, 107, 108]. Activation of the neuronal GLP-1 pathway results in activities such as decreasing the rate of gastric emptying (known as the "ileal brake effect") and reducing secretion rates of digestive enzymes (such as chyme), both of which result in slower digestion of nutrients and lower motility of food through the GI tract[101]. Additionally, activation of this neuronal pathway results in reduction of food intake and promotion of satiety[97, 107, 109]. These effects are achieved when GLP-1 activates neuronal GLP-1 receptors proximal to the L cells and a feedback loop is initiated via the vagal nerve[107]. The GLP-1 targets (called sensory afferent

neurons) are located in the intestines, liver and the hepatoportal region; activation of these neurons initiates responses in the hypothalamus that travel back to the GI tract via the vagal nerve and result in the ileal brake effect and reduction of pancreatic digestive enzymes[107]. This is a simplification of the processes modulated by the GLP-1 pathway, but it is sufficient in order to understand the basis of the work in this thesis.

Status of GLP-1 signaling during prediabetes and T2DM

Postprandial GLP-1 secretion and the corresponding incretin insulin secretion are often (but not always) decreased in individuals with prediabetes or T2DM[18, 110-123]. The relationship between the pathogenesis of T2DM and incretin pathways is a complicated one, and there appear to be differences between individuals[111]. Holst et al. mention that during T2DM, postprandial GLP-1 secretion is reduced and postprandial GIP secretion is not reduced; however, the insulinotropic ability (ability of incretins to stimulate insulin secretion) of GLP-1 is somewhat intact while insulinotropic ability of GIP appears to stunted during T2DM[111]. This is part of the rational behind using gliptins (DPP4 inhibitors) as well as GLP-1R agonists as treatments for regulation of glucose levels in T2DM;[102] i.e. the insulinotropic ability of the GLP-1 pathway appears to be mostly intact so pharmaceutical agents target this pathway as one method of regulating glucose levels.

Fritsche et al. designed a study to compare the insulin secretory response to intravenous GLP-1 administration in normoglycemic subjects versus subjects with impaired glucose tolerance[123]. IGT subjects displayed significantly lower levels of insulin secretion compared to normoglycemic subjects after administration of GLP-1. This demonstrates the compromised insulinotropic strength of GLP-1 in cases of prediabetes. Studies have shown that plasma GLP-1

levels do not increase after a meal/glucose challenge in T2DM patients[112, 118]; in addition to the stunted incretin effect, lower GLP-1 secretion could negatively affect the neural GLP-1 pathways related to satiety and food intake. Interestingly, GLP-1/GIP secretion and the corresponding incretin effect are remarkably improved in T2DM patients one month after gastric bypass surgery compared to pre-surgery levels[18].

GLP-1 modification by ingestion of various polyphenolic compounds

There have been a multitude of recent papers demonstrating the ability of various polyphenols to stimulate and increase GLP-1 levels in humans and rodent models[124-129]. Interestingly, a 2013 paper by Yamashita et al. found that cinnamtannin A2 (a tetrameric procyanidin) increased GLP-1 levels in a mouse model[124]; this indicates that the beneficial effects elicited by some procyanidins are not dependent upon absorption as previous work has shown that procyanidin polymers are not absorbed in the small intestine[77]. Additionally, recent papers have shown that grape components inhibit DPP4 activity, which reduces cleavage of active GLP-1 by DPP4[100, 130]. A 2014 paper demonstrated an increase in active GLP-1 after OGTT in rats after consumption of GSPE versus vehicle[129]; the authors partially attributed this increase in active GLP-1 to inhibition of DPP4 by grape polyphenols. However, in this same study, GSPE administration resulted in significantly higher GLP-1 levels than a pharmaceutical DPP4 inhibitor (Vildagliptin), which indicates that DPP4 inhibition is only one of multiple pathways that result in increased GLP-1 levels. A 2016 paper found that resveratrol reversed the negative effects on glucose-stimulated insulin secretion caused by lipopolysaccharide administration (reflecting the secretion of lipopolysaccharides by gut bacteria, which leads to increased gut permeability and systemic inflammation)[131].

Conclusions

These properties of grape polyphenols indicate a possibility of grape to be used as a part of a dietary strategy to restore normoglycemia and to improve defective insulin signaling. Further work is needed to determine the mechanisms through which grape flavonoids modulate GLP-1 secretion. The following studies were completed to increase understanding of the role of GLP-1 in glycemic control mediated by consumption of grape products. Additionally, acute and chronic grape consumption studies were executed to determine if the GLP-1 promoting activities of grape polyphenols were able to improve glycemic control in normoglycemic and prediabetic mouse models.

Chapter 3. Exploration of Grape Powder Mechanisms of Action in Glycemic Control

Study 1: Effect of oral grape consumption with concurrent OGTT on glycemic response after GLP-1 receptor antagonism

Introduction

The goal of study 1 is to determine if acute dysglycemia caused by GLP-1 receptor antagonism can be reversed by oral administration of grape powder in a normoglycemic mouse model. A GLP-1 antagonist called exendin-3 (9-39) amide will be used in this study to inhibit the action of GLP-1. Exendin-3 has been shown to be an antagonist of GLP-1 receptors which has the capability of displacing GLP-1 from the enzyme active site at 70%[132]. Exendin-3 (9-39) mimics 7 of 8 COOH-terminal amino acid residues found in GLP-1 but does not conserve the NH_3-terminal amino acid residues, indicating that the NH_3-terminal residues are necessary for agonist activity (as exendin-3 (9-39) antagonizes the GLP-1 receptor)[132]. Exendin-3 (9-39) has been used in numerous studies in humans and animals to further understand effects of dysregulation of the GLP-1 signaling pathway[133-135]. As discussed above, ingestion of grape polyphenols have been shown to increase GLP-1 secretion; this study will determine how antagonizing GLP-1 receptors affects the pathway. Mice will be given I.P. injections of exendin-3 (9-39) or vehicle control, followed by intragastric gavage of grape powder solution (or sugar-matched control) in order to observe the effect of GLP-1 receptor antagonism on OGTT. Increased blood glucose AUCs after OGTT in the exendin-3 (9-39) and sugar-matched control

group versus the vehicle and sugar-matched control group will demonstrate that antagonism of GLP-1 receptors leads to a dysregulation of glycemic control.

Materials and Methods

Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee at Virginia Tech (protocol FST 14-146). A graphical representation of study 1 can be found below in **Figure 2**. Male normoglycemic C57BL/6J mice (*N*=32, 11 weeks, Jackson Labs, Bar Harbor, ME) were obtained and acclimatized for 2 weeks to vivarium (Integrated Life Sciences Building vivarium, Blacksburg, VA) conditions (2 mice per cage, 12 h light/dark period, 30-70% relative humidity, 20-26 °C). Mice were allowed access to standard regular fat diet (10% kcal from fat, D12450J, Research Diets, New Brunswich, NJ) and water *ad libitum* during acclimatization. After the 2-week acclimatization period, mice were randomized into treatment groups by weight (mice were ranked in order of ascending weight, then assigned to treatment group) to equalize any differential effects of bodyweight.

Preparation of treatments

The freeze-dried grape powder (FDGP) solution was made using California Table Grape Commission grape powder produced at the National Food Lab. 4.65 grams of freeze-dried grape powder was slowly added to 50 mL saline solution (0.9% w/v NaCl) under continuous stirring. After grape powder was added to the saline, the solution was homogenized with a Polytron for 30 seconds. 3.5 mL aliquots of this solution were pipetted to individual test tubes and frozen at -80° C until day of procedures. 500 μg of exendin-3 (9-39) amide (Santa Cruz Biotechnology,

Dallas, TX) was dissolved in 46.65 mL of saline solution for a final solution concentration of 10.95 μg/mL in order to deliver a dose of 25 nmol/kg bodyweight with an approximate I.P. injection volume of ~175 μL (depending on the bodyweight of the mouse).

Mouse procedures

Mice were assigned to treatment groups with a 2x2 design: GLP-1 receptor antagonist exendin-3 (9-39) and vehicle (saline) (n=15/16 per group); these treatment groups were further split into freeze-dried grape powder (FDGP) (n=8) or sugar-matched control (n=7/8) groups. The exendin-3 (9-39) and vehicle were administered via injection into the intraperitoneal cavity (abdominal body cavity) as an alternative to intravenous injection (I.V. injection was not possible in the present study). The grape powder solution and sugar-matched control were administered via intragastric gavage to simulate oral ingestion. Intragastric gavage is a common method used in animal research as a method of instantaneously oral administration of a specific dose of treatment or food.

In this study, all treatment groups were subjected to an oral glucose tolerance test (OGTT) by adding 0.5 g glucose/kg bodyweight to the treatment solution. This glucose load (0.5 g glucose/kg bodyweight) was chosen instead of a standard 1 or 2 g glucose/kg bodyweight for OGTT because the grape/sugar-matched control treatment adds an additional glucose load of 0.72 g glucose/kg bodyweight and an additional fructose load of 0.72 g glucose/kg bodyweight.

Mice were fasted for 12 hours followed by intraperitoneal (i.p.) injection: exendin-3 (9-39) amide (25 nmol/kg bodyweight), or saline vehicle (equal volume, roughly ~175 µL). Immediately following injection, mice were administered the assigned treatment via intragastric gavage: suspension of FDGP (1.6 g/kg body weight) plus glucose (0.5 g/kg bodyweight) in

saline, or sugar-matched control (1.44 g/kg bodyweight) plus glucose (0.5 g/kg bodyweight) in saline. Blood glucose levels were quantified at baseline (0 min) and 10, 30, 60, 90, and 120 min post-gavage via blood expressed from the tail tip using a handheld glucometer (OnePlus Touch UltraMini, Milpitas, CA) and OneTouch Ultra Blue glucose test strips. Blood (~50 μL) was also collected via the tail at 10 and 30 min in 400 μL micro serum separation tubes (Fisher Scientific, Pittsburgh, PA), clotted at room temperature for 2 h, and centrifuged at 17,000 x *g* for 10 min at room temperature. Separated serum was pipetted from these tubes into microfuge tubes containing 5 μL 100X Thermo HALT protease inhibitor (Fisher Scientific, Waltham, MA). Serum samples were stored at –80 °C. The serum samples were later analyzed with total GLP-1 ELISA assay (Millipore, Billerica, MA) as per manufacturer instructions.

Statistics

The blood glucose data provides several different sets of valuable data: blood glucose time series, area under the curve (AUC, which is the total area underneath the blood glucose time series plot), blood glucose excursion (the difference between baseline blood glucose and the maximum blood glucose level reached), C_{max} and t_{max} which are peak blood glucose concentration and time of peak blood glucose concentration, respectively. Standard error of the mean will be used to calculate error bars for these metrics. Alpha level of 0.05 will be used to establish significant difference for these data. These data will be treated with two-way analysis of variance (ANOVA) using Tukey's Honest Significant Difference to compare cell means. Relevant comparisons will be subjected to these statistical analyses and non-relevant comparisons will be ignored.

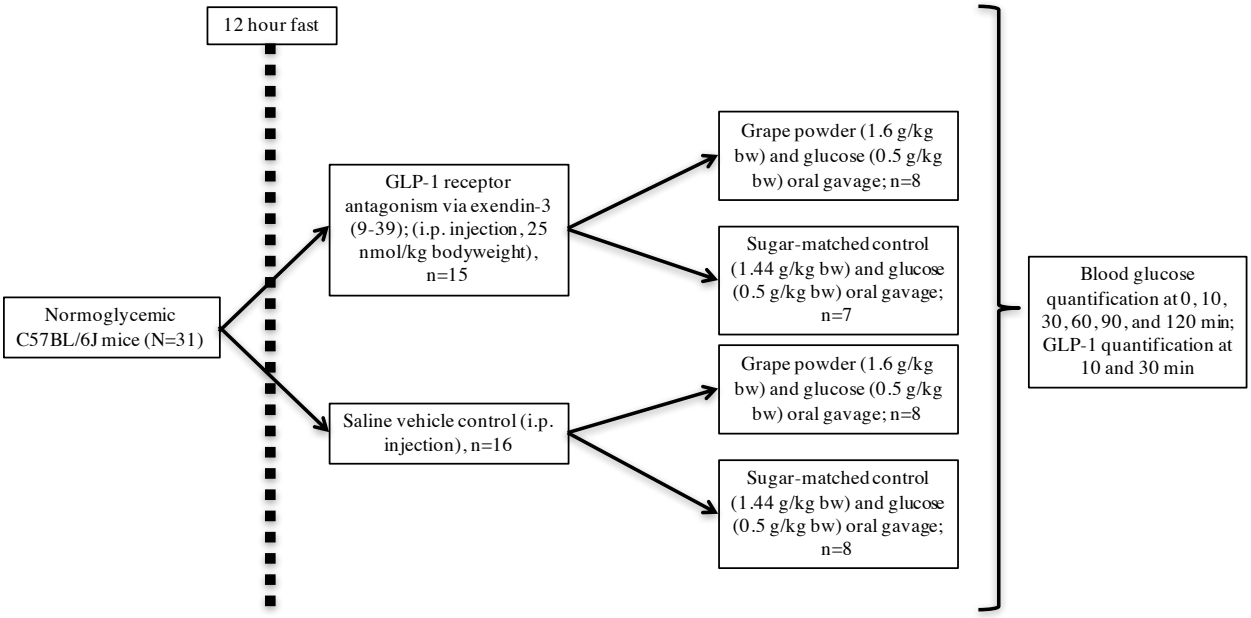


Figure 2. Experimental design of study 1

Normoglycemic mice (N=32) were split into two treatment groups: GLP-1 receptor antagonism via potent GLP-1 receptor antagonist exendin-3 (9-39) (*n*=15) and control (saline vehicle) (*n*=16). Each treatment group was further split into FDGP/OGTT (*n*=8) or sugar-matched control/OGTT (*n*=7/8). Blood glucose levels were quantified at 0, 10, 30, 60, 90, and 120 min; additionally, 10 and 30 min serum total GLP-1 levels were quantified at 10 and 30 minutes.

Results

The goal of study 1 was to determine how grape powder would alter glycemic response to OGTT under antagonism of GLP-1 receptors. A GLP-1 antagonist, exendin-3 (9-39), was used in this study to inhibit GLP-1 pathway via binding GLP-1 receptor sites; exendin-3 has been shown to be an antagonist of GLP-1 receptors that has the capability of displacing GLP-1 at 70%[132]. Mice treated with exendin-3 (9-39) and the sugar-matched control displayed significantly increased blood glucose AUC ($p<0.01$) versus the saline/sugar-matched control group, indicating that antagonizing GLP-1 receptors did have a severe negative effect on glycemic control in this mouse model. The grape powder reversed the deleterious effects of GLP-1 receptor antagonism, as the blood glucose AUC for the exendin-3 (9-39)/grape group was significantly lower ($p<0.01$) than the exendin-3 (9-39)/sugar-matched control group.

Blood glucose profiles

Blood glucose time series after OGTT for study 1 are shown in **Figure 3**. In this experiment, blood glucose levels were monitored over 120 min; however it was determined that more time was needed in order for blood glucose levels to return to baseline. It is important to have a complete capture of the glycemic response in order to observe efficiency of blood glucose clearance. Normally, when glucose tolerance tests are performed intravenously, 120 minutes is sufficient time to observe the blood glucose spike, plateau, and return to baseline; however, due to oral administration of glucose, this was not sufficient time for the blood glucose levels to return to baseline. This was addressed for subsequent studies (studies 2-4) in which blood glucose levels were monitored for 180 minutes.

Figure 3A shows the glycemic responses of all four treatments on the same graph; for ease of comparison, **Figures 3B-E** compare blood glucose profiles from different pairs of the treatments. Blood glucose profiles from the sugar match treatment (+saline or + GLP-1 antagonist) are shown in **Figures 3B**. Administration of exendin-3 (9-39) clearly inhibited blood glucose clearance, as identical peak levels were reached (10 min), but the levels in the +GLP-1 antagonist group were higher from 60-120 min. Interestingly, in the grape powder group, administration of exendin-3 (9-39) did not result in a significant rise in blood glucose levels compared to saline (**Figure 3C**). Oral administration of grape powder reversed the deleterious effect of GLP-1 antagonism by exendin-3 (9-39) compared to oral administration of the sugar-matched control (**Figure 3E**). However, despite the reversal of GLP-1 antagonism by grape powder, grape did not significantly lower blood glucose levels versus sugar-matched when both were co-administered with saline control (**Figure 3D**).

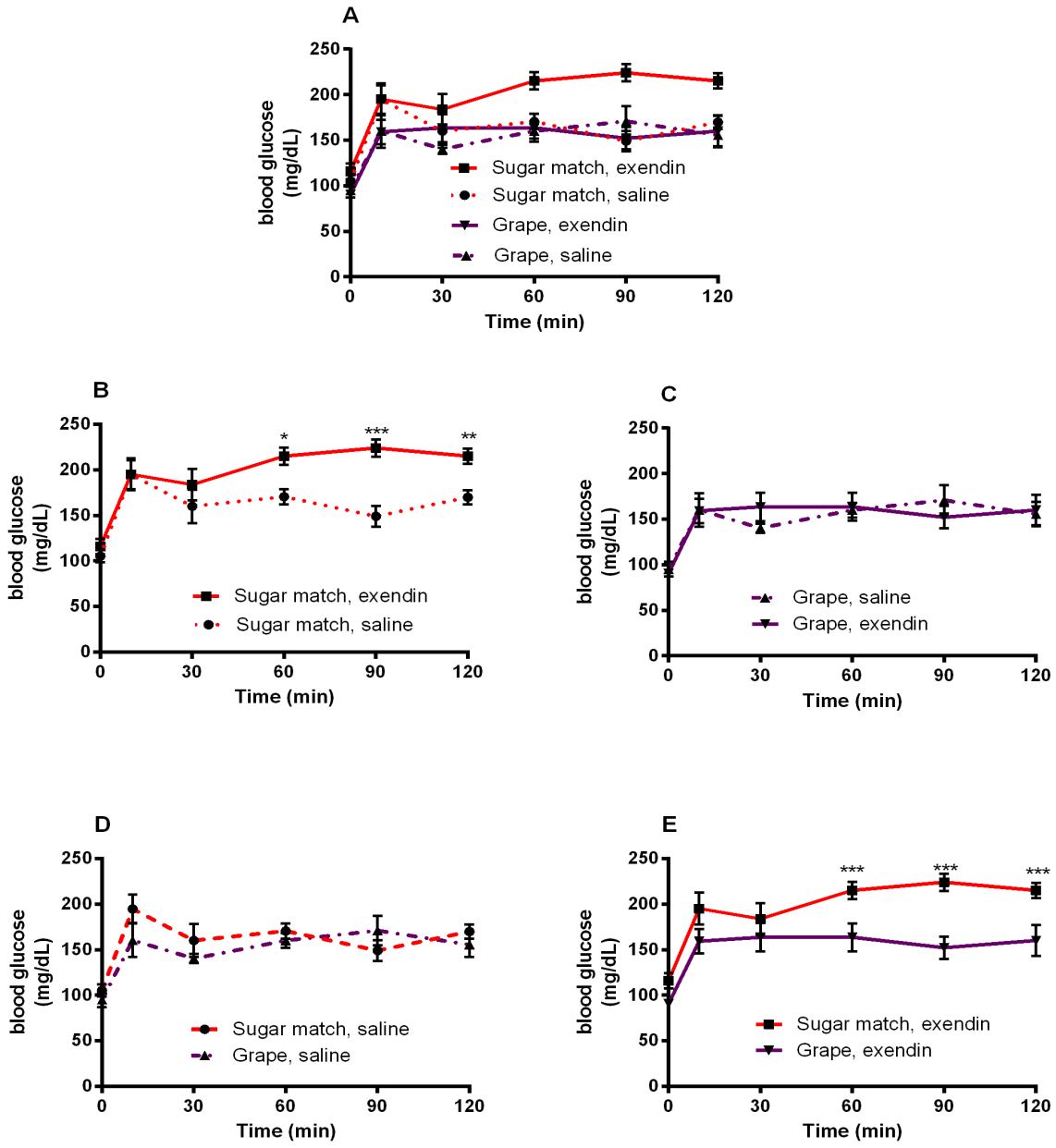


Figure 3. Blood glucose time series after GLP-1 receptor antagonism and grape powder administration

Study 1: blood glucose response curves after intraperitoneal administration of GLP-1 receptor antagonist [exendin-3 (9-39) amide] (or vehicle, saline) followed by intragastric gavage of grape powder or sugar-matched control. (A) All treatments, (B) sugar match: exendin-3 vs. vehicle, (C) grape powder: exendin-3 vs. vehicle, (D) vehicle: grape powder vs. sugar match, (E) exendin-3, grape powder vs. sugar match. Values are mean ± SEM (n=8). *=p<0.05 **=p<0.01 ***=p<0.001 indicate significant difference between two treatment means at the specified time point as indicated by two-way ANOVA with Tukey's HSD post-hoc test (significance is indicated only on graphs with paired curves for ease of interpretation).

AUC/excursion/single time point data

The area under the curve (AUC) for each glucose time series was determined by calculating the total area under the mean blood glucose time curve for each treatment group (**Figure 4A**). This measure represents the total glycemic response as it accounts for the peak concentration in blood glucose as well as the clearance of glucose from circulation after OGTT. AUC values in **Figure 4A** generally reflected the patterns of the blood glucose curves seen in **Figure 3**. Within the sugar-matched treatment, GLP-1 receptor antagonism significantly raised the blood glucose AUC versus saline control ($p=0.01$). The impact of GLP-1 receptor antagonism was alleviated by the grape treatment compared to sugar match ($p=0.01$). Interestingly, within the grape treatment, the AUC was not elevated by exendin-3 (9-39) compared to saline control; grape appeared to block or alleviate GLP-1 receptor antagonism by exendin-3 (9-39) but did not improve glycemic response under normal GLP-1 signaling conditions. In addition to AUC values, blood glucose levels at specific time points, excursions from baseline, and changes between specific time points were determined (**Figure 4B-G**). Grape treatments caused a significant reduction in blood glucose levels at 10 minute versus sugar-matched control, regardless of whether exendin-3 (9-39) or saline was administered (**Figure 3E**). No significant interactions were observed in the other time points, although GLP-1 receptor antagonism exerted a borderline significant ($p=0.079$) effect on the change in blood glucose between 10-30 min, regardless of whether sugar match or grape treatment was administered (**Figure 4G**).

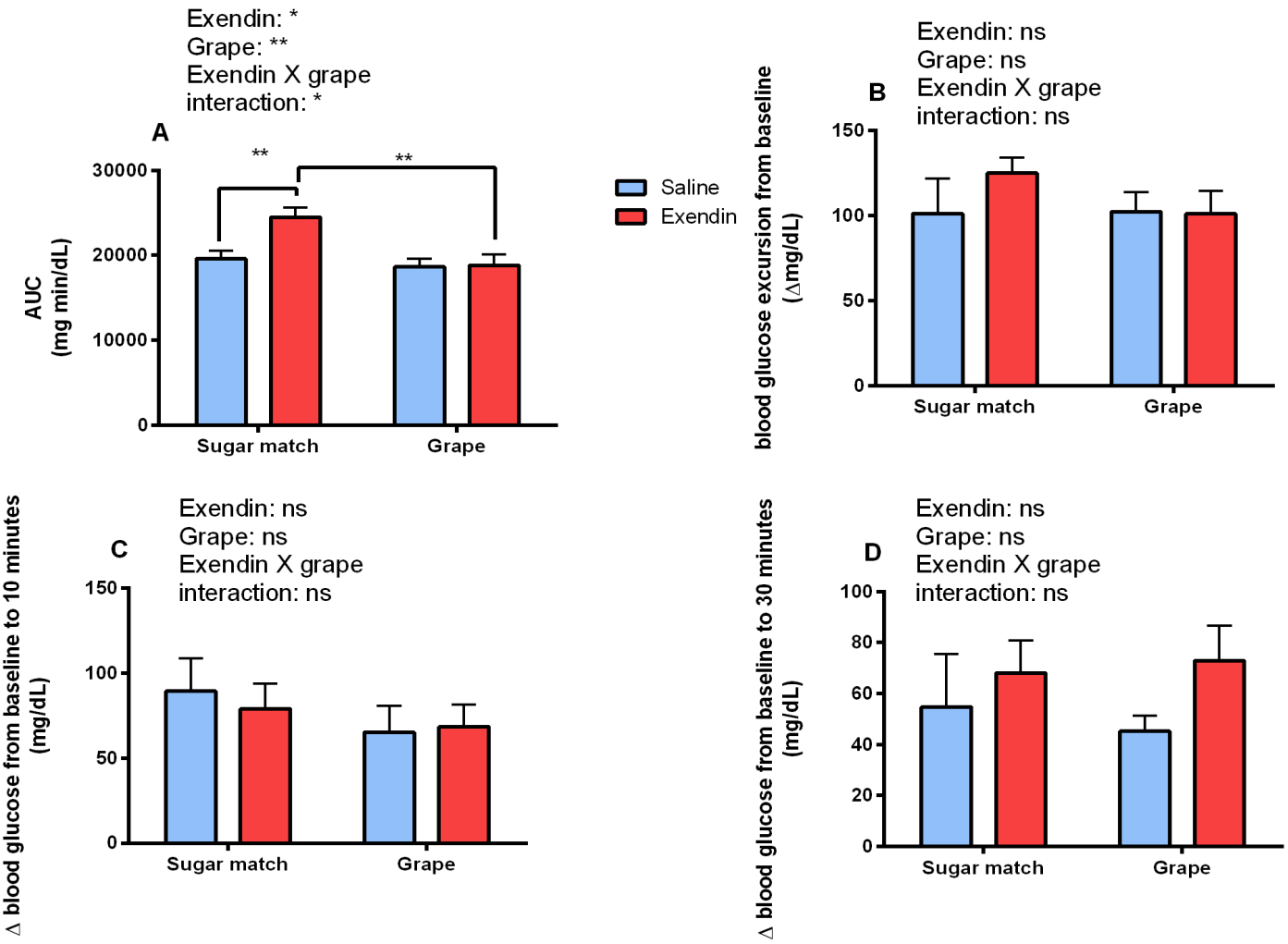


Figure 4 A-D (see caption below)

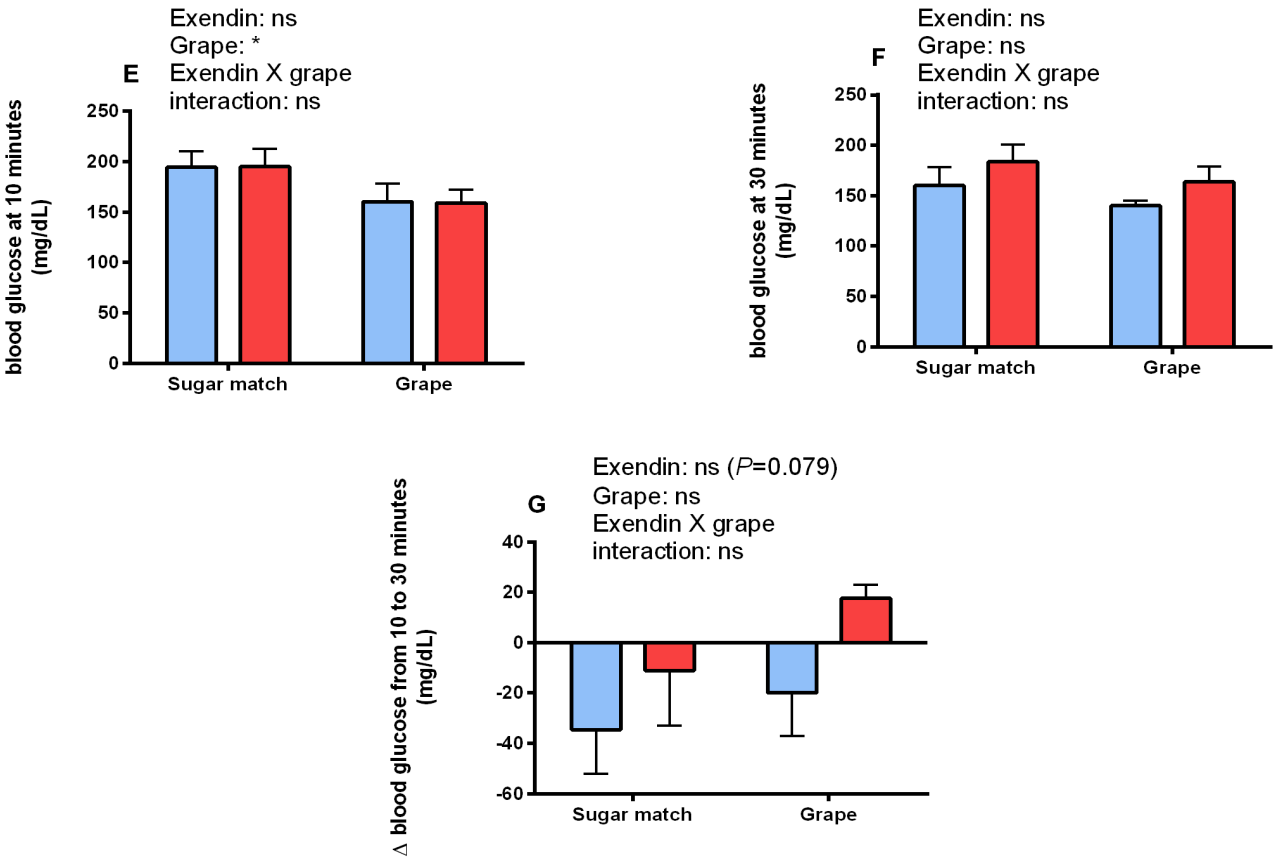


Figure 4. Blood glucose AUC and related metrics after GLP-1 receptor antagonism and grape administration

Glycemic responses parameters after intraperitoneal administration of GLP-1 receptor antagonist [exendin-3 (9-39)] or vehicle followed by intragastric gavage of grape powder or sugar-matched control. (A) Blood glucose area under the curve (AUC), (B) blood glucose excursion (maximum value minus baseline value), (C) baseline to 10 min change in blood glucose, (D) baseline to 30 min change in blood glucose, (E) blood glucose level at 10 min, (F) blood glucose level at 30 min, (G) change in blood glucose from 10 to 30 min. Values are mean ± SEM (n=8). Legends above individual graphs indicate treatment main effects as determined by two-way ANOVA. *p<0.05 **p<0.01 ***p<0.001 indicate significant difference between treatment means as indicated by two-way ANOVA with Tukey's HSD post-hoc test.

Serum GLP-1 quantification

GLP-1 levels were quantified at 10 and 30 min after treatments in study 1 (**Figure 5**). There were no significant differences observed between any of the treatments; at the 30 min time point the exendin-3 (9-39) with grape treatment had lower serum GLP-1 than the saline vehicle with grape treatment. We had planned on quantifying GLP-1 and insulin at 10 and 30 minutes after treatment; unfortunately, there were difficulties extracting the necessary volume of blood from the tail snip during the procedures and only the GLP-1 ELISA was processed.

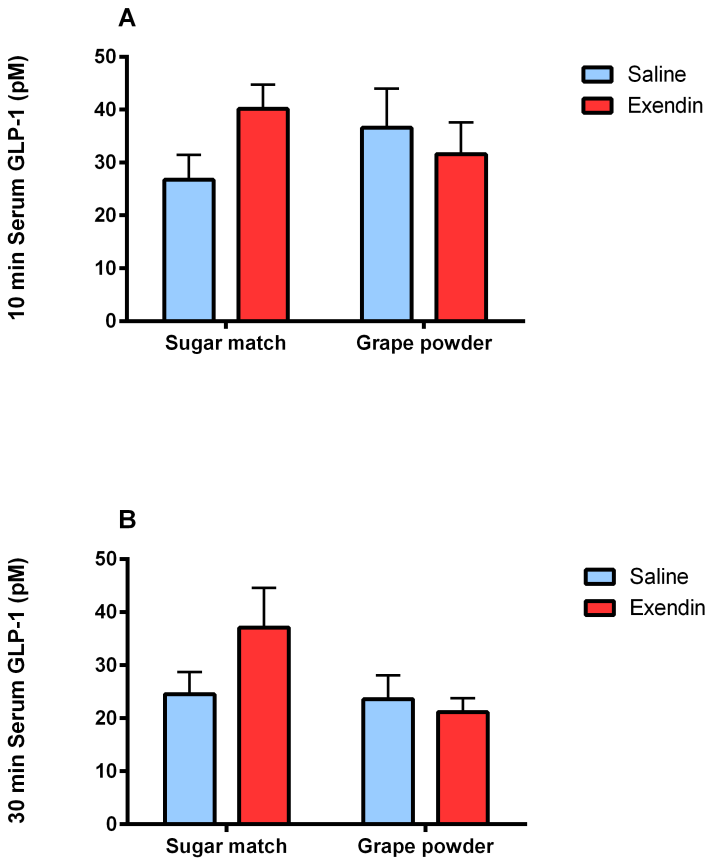


Figure 5. Serum GLP-1 levels after grape treatment during GLP-1R antagonism

Serum insulin and GLP-1 levels as quantified by ELISA assays. (A) serum GLP-1 levels at 10 min after exendin-3 (9-39) antagonism of GLP-1 receptors and subsequent grape powder vs. sugar-matched control administration, (B) serum GLP-1 levels at 30 min in Study 1 after exendin-3 (9-39) antagonism of GLP-1 receptors and subsequent grape powder vs. sugar-matched control administration. Values are mean ± SEM; significance (α=0.05) was tested with two-way ANOVA with Tukey's HSD post-hoc test.

Discussion

The present study was designed to explore the mechanisms of enhanced glycemic control by grape powder constituents. To the best of our knowledge, this is the first study to build upon the known GLP-1 promoting activities of grape extracts and demonstrate that whole grapes beneficially modulates GLP-1 levels or function[100, 128, 129, 136]. Initially, we hypothesized that acute administration of grape powder would significantly reduce glycemic response compared to sugar-matched control when the GLP-1 signalling pathway was not disturbed; however, grape powder and sugar-matched control displayed similar blood glucose AUCs after OGTT (Study 3). Regardless, study 1 was designed to observe the impact of grape consumption on glycemic response when the GLP-1 signalling pathway was blocked. Grape administration actually reversed the deleterious glycemic effects of GLP-1 receptor antagonism (**Figures 3 and 4**). This has interesting potential implications as grape powder constituents may be capable of improving impaired GLP-1 signalling and, by extension, ameliorating dysglycemia induced by impaired GLP-1 signalling.

Exendin-3 (9-39) was employed in study 1 to inhibit GLP-1 signalling in order to cause a dysregulation of post-prandial incretin action and subsequent insulin secretion; insulin secretion by pancreatic β-cells in response to food intake is largely dependent on the insulinotropic activities of incretins GLP-1 and GIP[137]. As a proof of concept, GLP-1 receptor antagonism by exendin-3 (9-39) caused a significant ($p<0.01$) increase in blood glucose AUC in the sugar-matched group after OGTT compared with the saline vehicle control (**Figure 3B**). However, when grape powder was co-administered with OGTT after GLP-1 receptor antagonism by exendin-3 (9-39), blood glucose concentrations and AUC

were significantly reduced (**Figure 4A**) and glycemic control was normalized. This is a novel finding that suggests that grape constituents potentially modulate GLP-1 signalling pathways and can be exploited to improve glucose homeostasis. However, given the limited scope and narrow focus of this study, our data cannot be immediately translated to human relevancy; this beneficial effect of grape consumption may be limited to specific cases where defective GLP-1 signalling contributes to hyperglycemia.

The acute grape powder administration may have achieved this effect by increasing GLP-1 secretion, thereby displacing the GLP-1 receptor antagonist from the active binding sites. A previous study illustrated that grape seed procyanidin extract significantly increases active GLP-1 levels after oral glucose load[128]. This increased GLP-1 secretion may have displaced the GLP1R antagonist via increased competition between active GLP-1 and the antagonist at GLP-1 receptor binding sites in order to restore normal incretin activity[132, 135].

However, alternative mechanisms could also be responsible for the observed phenomenon. First, grape power constituents are capable of inhibition of DPP4, which leads to increased GLP-1 levels. Previous work has shown that acute administration of grape seed procyanidin extract reduces intestinal DPP4 activity and expression in healthy and diet-induced obese rats[100, 128]; however, a conflicting study showed an increase of intestinal DPP4 protein expression in rats treated with grape seed procyanindin extract[128]. Second, grape power constituents could directly stimulate the GLP-1 receptor, mimicking the action of GLP-1. Finally, grape power constituents could act via other pathways to compensate for blunted GLP-1 signalling; for example, grape consumption may have encouraged uptake of blood glucose via an unrelated pathway. In a cell study, exendin-3

(9-39) administration partially inhibited insulin secretion in both GIP receptor and GLP-1 receptor transfected cells, indicating that it may antagonize receptors in both of these incretin pathways so grape powder administration may have reversed antagonism of both of these receptors; however, conflicting data from a human clinical study show exendin-3 (9-39) did not affect GIP-dependent insulin secretion[133, 135]. It is quite possible that the results seen in this work are due to a combination of these (among other) mechanisms. These potential mechanisms warrant further experiments to elucidate the mechanism of action of grape polyphenols in order to facilitate exploitation of grapes for improved glucose homeostasis.

Interestingly, grape powder only protected from the deleterious effects of GLP-1 receptor antagonism; grape did not lower the blood glucose AUC compared to sugar match when vehicle was administered instead of exendin-3 (9-39) (i.e. during normal GLP-1 signaling). This finding suggests that grape constituents (at dosages used in the present study) exert protective effects by compensating for impaired GLP-1 signalling; when GLP-1 signalling was not impaired, no protection was observed. A previous study showed that a 1 g/kg dose of grape seed procyanidin extract was able to significantly decrease glucose levels 20 minutes in rats after 2 g/kg glucose load[128]; indicating that higher dosages of grape procyanidins display protection against hyperglycemia. The present study used grape powder (1.6 g/kg) instead of grape extract, delivering a ~1000-fold lower dosage of flavanols (0.95 mg/kg) at which this protective effect was not seen. While the 1 g/kg grape seed procyanidin extract reduced blood glucose levels following OGTT, this is an equivalent human dose of 81.1 mg/kg (4.86 g for a 60 kg human), which is not translatable to humans through normal grape consumption[138]. Therefore, the present

dose (~0.077 mg/kg polyphenols, or 4.62 mg for a 60 kg human) is likely more representative of the effects likely to be observed in humans after normal grape consumption.

Therefore, the benefits of grape consumption may be highly context-dependent (e.g. during morbid obesity when there is no measurable post-prandial GLP-1 secretion[96]). Data show that control of the GLP-1 pathway may be altered in T2DM: GLP-1 secretion is significantly reduced in T2DM patients compared with healthy controls in response to mixed-meal challenge[139]. Mice with a null (non-functioning) mutation in the exon region of the GLP-1 receptor gene exhibit elevated blood glucose following oral and intraperitoneal glucose tolerance test[140]. Our results agree with these previous data that impaired GLP-1 signalling will negatively affect glucose homeostasis. GLP-1 is able to modulate glucose homeostasis through multiple pathways including gastric emptying, satiety, glucagon suppression, and stimulation of insulin release after/during meal consumption (the incretin effect)[141]. Therefore, grape consumption has the potential to improve glycemic control in humans with T2DM. It should be noted that grape consumption could be harmful in some T2DM patients if the addition of grapes to the diet results in an increase of simple carbohydrate intake. Further investigation into the exact mechanism by which grape compounds were able to leverage this beneficial effect on restoration of glycemic control after GLP1R antagonism may provide insight into the applicable scenarios in which grape consumption could be utilized as a therapeutic agent in humans.

Study 2: Effects on glycemic response of intragastric versus intraperitoneal administration of grape powder in a normoglycemic mouse model

Introduction

In study 2, grape powder solutions were administered via differing routes (i.p. injection versus intragastric gavage) followed by an oral glucose tolerance test. As mentioned in the literature review, polyphenols have been shown to demonstrate beneficial effects through mechanisms in the gut as well as mechanisms in peripheral tissue (such as skeletal muscle). Administering grape powder solution in mice via intraperitoneal tissue is analogous to administering grape powder solution intravenously in humans. Thus the reader may question the rationale behind performing this experiment if it is not translatable in humans; in response to this, the purpose of this experiment was to observe if beneficial effects of grape consumption are leveraged via mechanisms in the gut or mechanisms in peripheral tissue. A better understanding of the mechanisms of action of grape polyphenols can lead to more effective treatment strategies in the future.

Blood glucose levels were monitored for 3 hours after the treatment to observe changes in glycemic response after grape powder was administered. GLP-1 levels were quantified at +10 and +30 minutes post-treatment. As in study 1, grape powder treatments were controlled by sugar-matched treatments (1:1 fructose to glucose) equivalent to 90% (by weight) of the grape powder to control for the high sugar content of the grape powder. The purpose of this study is to determine if grape mediates its glycemic control action via mechanisms located in the gut or through mechanisms located in circulation.

Materials and Methods

Mice

Mice (N=29) from study 1 were given a 2-week rest and recovery period before the initiation of study 2; during this recovery period the standard diet (D12450J) and water were provided *ad libitum*. Mice were fasted for 12 hours prior to treatment, and re-randomized into a 2x2 design: FDGP aqueous suspension (1.6 g/kg bodyweight) (*n*=15) versus sugar-matched aqueous suspension control group (1.44 g/kg bodyweight) (*n*=14); these treatment groups were further divided by route of administration: i.p. administration (*n*=7) versus oral administration (*n*=7/8).

Preparation of Treatments

Treatment solutions required for study 4 included the following: grape powder solution (1.6 g/kg bodyweight) for i.p. injection; glucose solution (0.5 g/kg bodyweight) for OGTT; grape powder (1.6 g/kg bodyweight) and glucose (0.5 g/kg bodyweight) solution for intragastric gavage; sugar-matched solution (1.44 g/kg bodyweight) for I.P. injections; and sugar matched solution (1.44 g/kg bodyweight) mixed with glucose (0.5 g/kg bodyweight) for OGTT. Treatment solutions were prepared in the same manner as in study 1 (see above). For the two i.p. injection groups, the mice were first administered their grape or sugar-matched i.p. injection and then were immediately gavaged with the OGTT glucose solution. For the two oral administration groups, the mice were administered a single gavage that contained glucose for OGTT and either grape or sugar-matched control. Blood glucose levels were quantified as in study 1 (blood drawn via tail snip and measured with glucometer) with an additional time point added at 180 min to

ensure the entire glycemic response was captured. Blood collections were taken at 10 and 30 min and serum was obtained as described above.

Euthanasia

Immediately following this study, mice were euthanized according to American Veterinary Medical Association Guidelines on Euthanasia, as well as local IACUC guidelines. Carbon dioxide was used as the euthanizing agent.

Statistics

The blood glucose data provides several different sets of valuable data: blood glucose time series, area under the curve (AUC, which is the total area underneath the blood glucose level versus time plot), blood glucose excursion (the difference between baseline blood glucose and the maximum blood glucose level reached), C_{max} and t_{max} which are peak blood glucose concentration and time of peak blood glucose concentration, respectively. Standard error of the mean will be used to calculate error bars for these metrics. Alpha level of 0.05 will be used to establish significant difference for these data. These data will be treated with two-way analysis of variance (ANOVA) using Tukey's Honest Significant Difference comparison to compare cell means. Relevant comparisons will be subjected to these statistical analyses where non-relevant comparisons will be ignored.

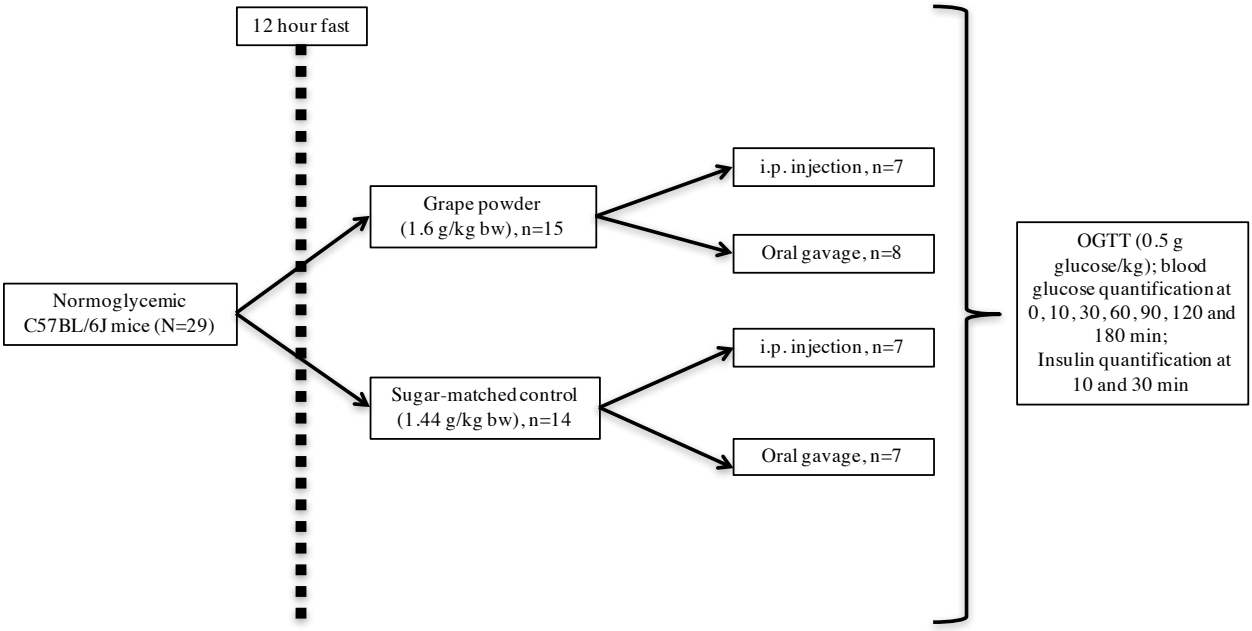


Figure 6. Experimental design of study 2

Effect of grape treatment via differing routes of administration. The grape treatment group (n=15) was split into an i.p. injection group (n=7) and an oral gavage group (n=8); the sugar control group was split into an i.p. injection group (n=7) and an oral gavage group (n=7). All groups were subjected to OGTT and subsequent monitoring of blood glucose levels for +3 hours, and GLP-1 was quantified at +10 and +30 minutes

Results

The purpose of study 2 was to determine the effects of grape powder on glycemic control via mechanisms located in the gut versus mechanisms located in circulation/peripheral tissue. This was accomplished by administering grape powder suspension via differing routes (i.p. injection versus intragastric gavage) followed by an oral glucose tolerance test. Blood glucose levels were monitored for 3 hours after the treatment and blood glucose time series were plotted and blood glucose AUCs were calculated. Blood glucose time series can be seen in **Figure 7** and blood glucose AUCs can be seen in **Figure 8**. Within the sugar-matched control treatments, the i.p. administration resulted in a faster spike in blood glucose as well as faster blood glucose clearance while the oral administration resulted in a slower rise of blood glucose as well as a slower blood glucose clearance. The grape i.p. treatment resulted in a significant ($p<0.01$) increase in blood glucose AUC compared to all other treatments. The i.p. administration of grape powder solution resulted in a significantly increase in blood glucose which was sustained for two hours until it approached baseline at 180 min.

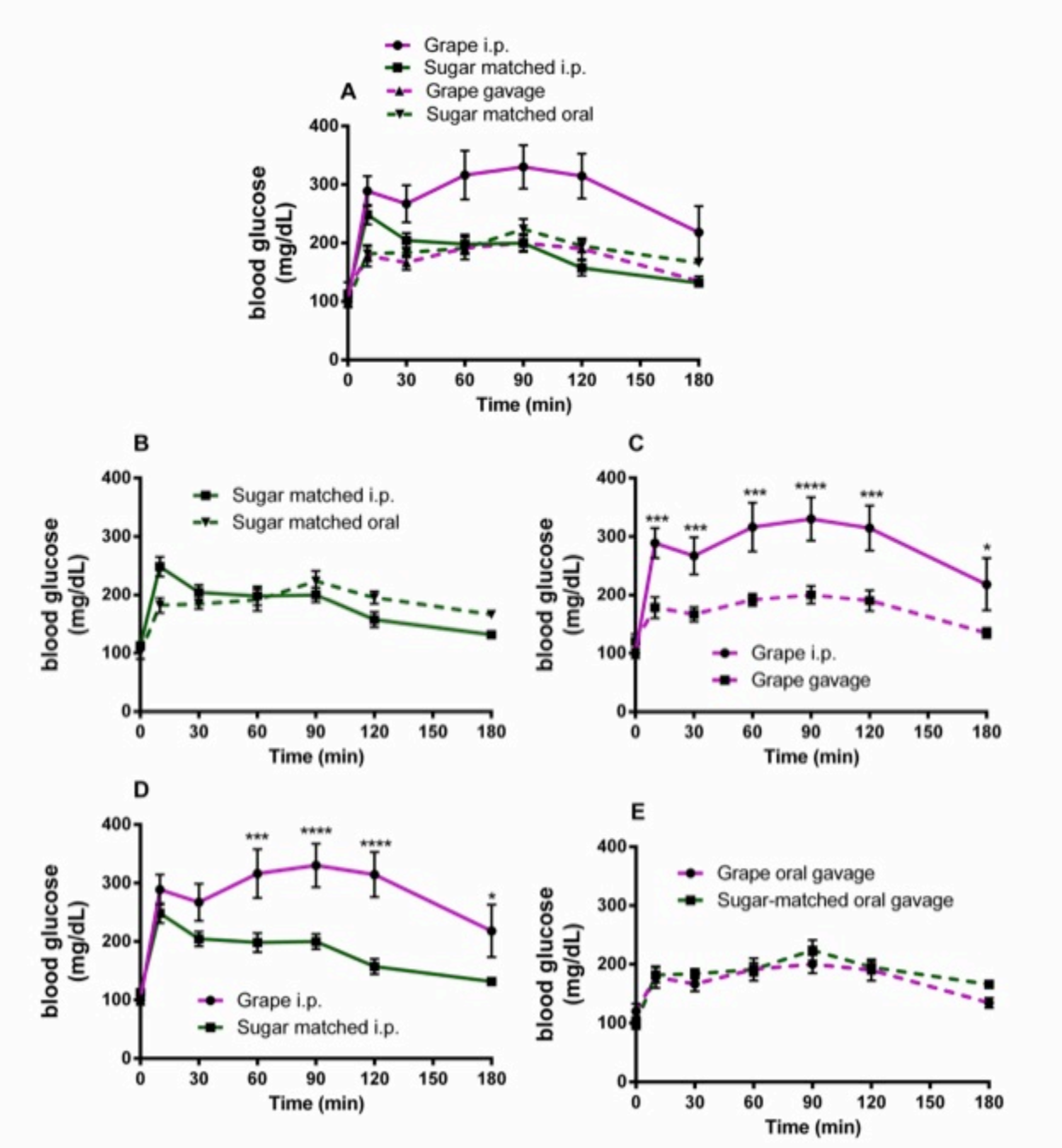


Figure 7. Blood glucose time series after i.p. versus oral grape treatment

Blood glucose response curves after oral glucose tolerance test following administration of grape powder or sugar matched control via interperitoneal (i.p.) injection or intragastric gavage. (A) All treatments, (B) sugar match: treatments, i.p vs. oral, (C) grape powder, i.p vs. oral, (D) i.p., grape powder vs. sugar (e) oral, grape powder vs. sugar match. Values are mean ± SEM (n=8). *p<0.05 **p<0.01 ***p<0.001 indicate significant difference between two treatment means at the specified time point as indicated by two-way ANOVA with Tukey's HSD post-hoc test (significance is indicated only on graphs with paired curves for ease of interpretation).

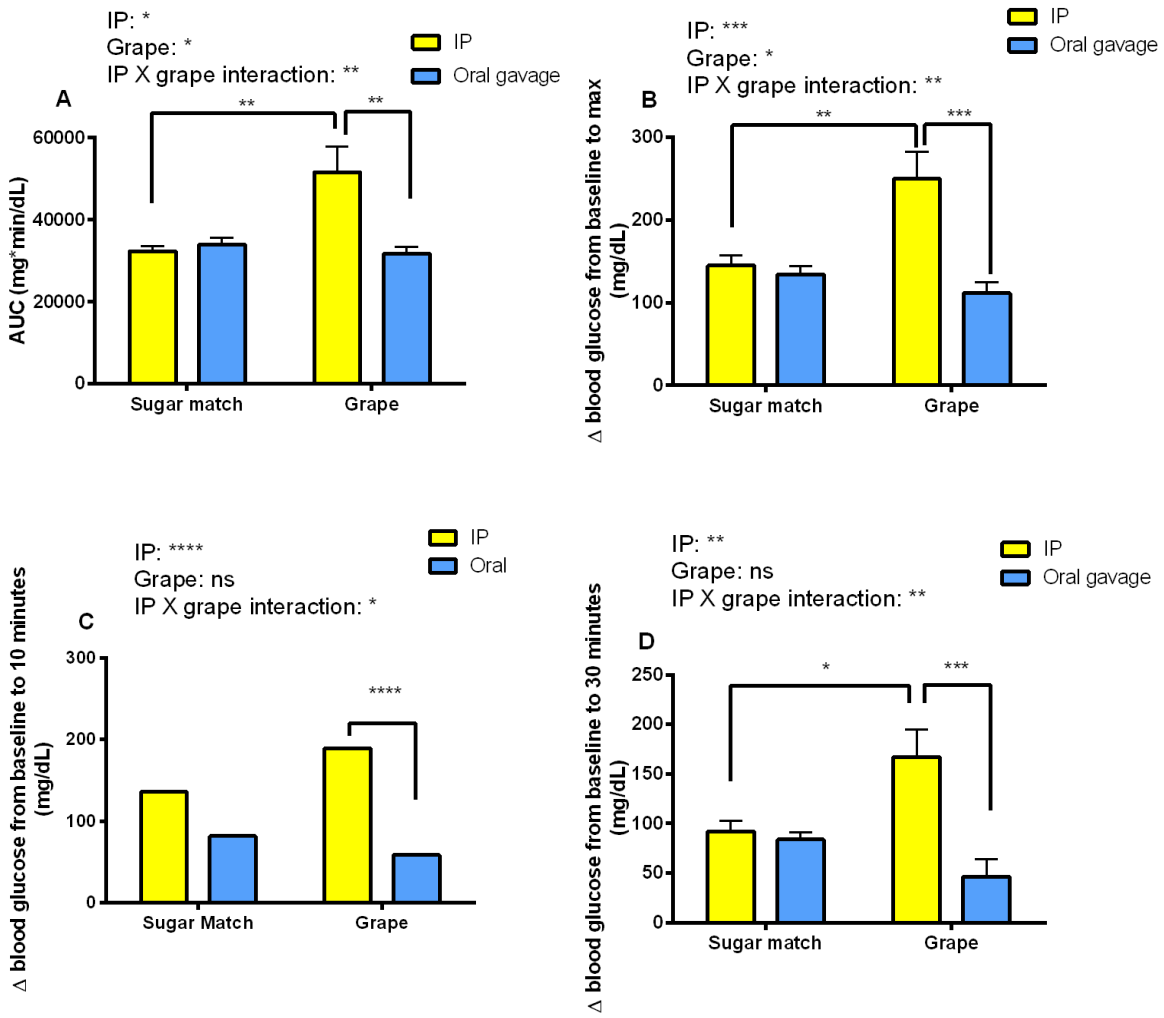


Figure 8 A-D (see caption below)

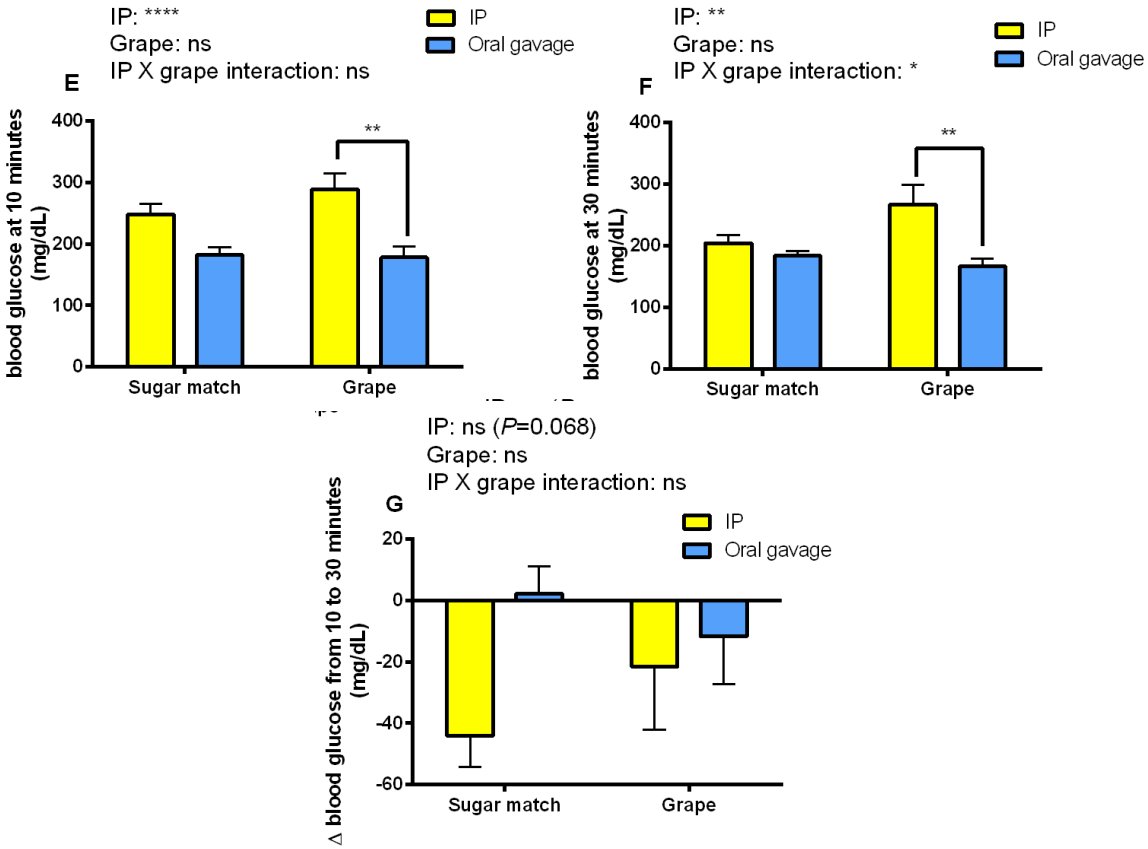


Figure 8. Blood glucose AUC and related metrics after i.p. versus oral grape treatment

Glycemic response parameters after oral glucose tolerance test following administration of grape powder or sugar matched control via interperitoneal (i.p.) injection or intragastric gavage. (A) blood glucose area under the curve (AUC), (B) blood glucose excursion (maximum value minus baseline value), (C) baseline to 10 min change in blood glucose, (D) baseline to 30 min change in blood glucose, (E) blood glucose level at 10 minute, (F) blood glucose level at 30 min, (G) change in blood glucose from 10 to 30 min. Values are mean ± SEM (n=8). Legends above individual graphs indicate treatment main effects as determined by two-way ANOVA. *p<0.05 **p<0.01 ***p<0.001 indicate significant difference (α=0.05) between treatment means as indicated by two-way ANOVA with Tukey's HSD post-hoc test.

Serum GLP-1 and insulin quantification

Serum GLP-1 and serum insulin levels were quantified at 10 and 30 minutes after treatment (**Figure 5C-F)**. Serum GLP-1 at the 30 min time point slightly, but not significantly, increased in the grape treatment versus the sugar treatment (**Figure 5D**); however, due to the small number of data points in these sets (n=3 and n=6 for sugar-matched control and grape powder, respectively), it is difficult to establish significance of these data. The limited number of data points per treatment group was a results of the difficult in extracting a sufficient volume of blood via tail snip.

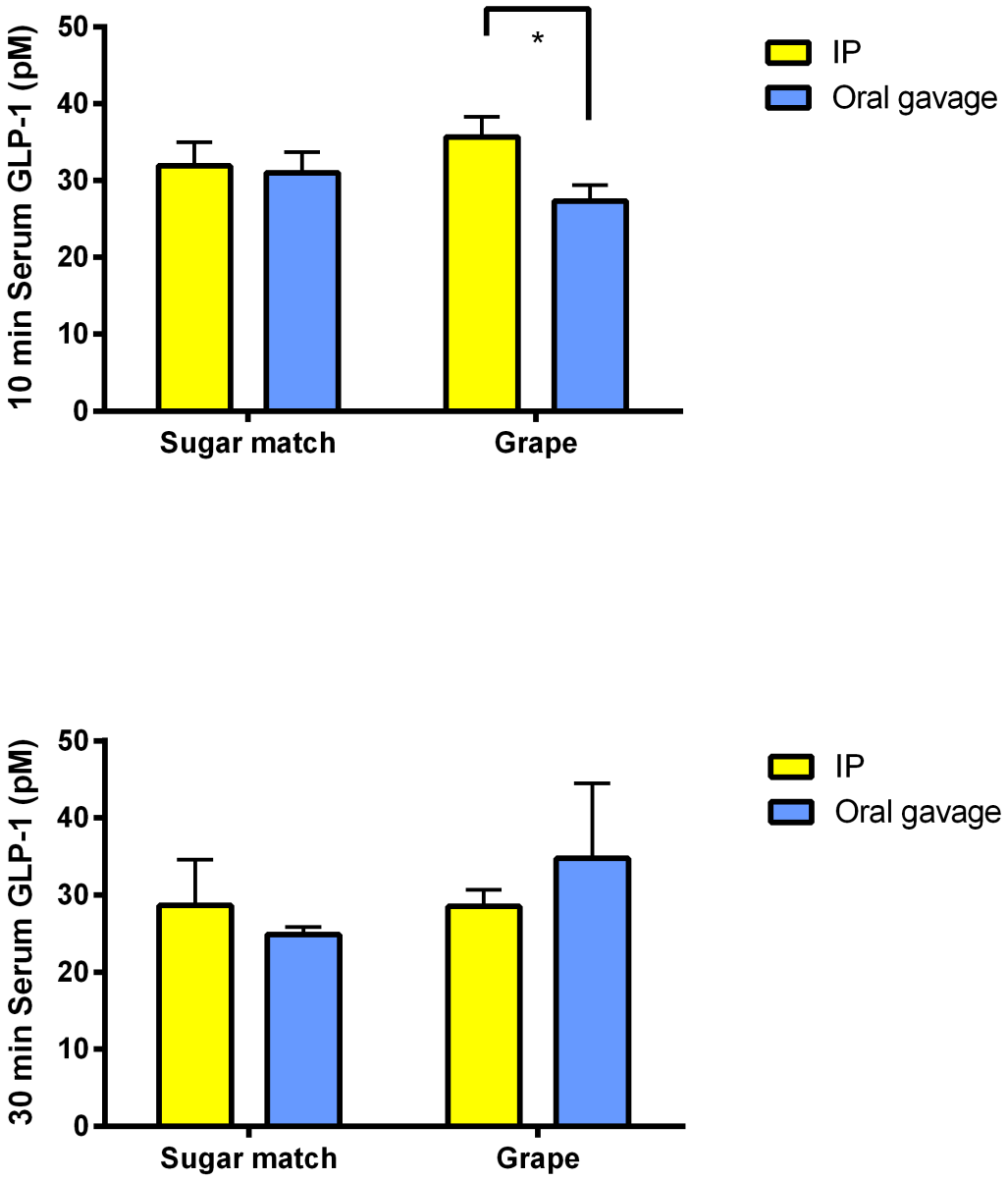


Figure 9. Serum GLP-1 levels after i.p. versus oral administration of grape powder solution

Serum insulin and GLP-1 levels as quantified by ELISA assays. Serum GLP-1 levels at 10 min in i.p. versus oral administration of grape powder or sugar-matched control and serum GLP-1 levels at 30 min in i.p. versus oral administration of grape powder or sugar-matched control. Values are mean ± SEM; significance (α=0.05) was tested with two-way ANOVA with Tukey's HSD post-hoc test.

Discussion

Intraperitoneal injection of grape powder solution resulted in a significantly increased blood glucose AUC after OGTT compared to sugar-matched control intraperitoneal injection. The results of this study are difficult to interpret: the grape powder i.p. injection should not have resulted in higher blood glucose AUC compared to the sugar-matched control i.p. administration/OGTT, as both treatments are delivering the same dose of simple carbohydrates. These results indicate that i.p. administration of grape powder resulted in an unforeseen adverse physiological reaction that caused highly elevated blood glucose levels for 3 hours post-OGTT. This can possibly be attributed to insolubility of the grape powder in the i.p. injection vehicle, which is therefore not representative of physiological delivery to the i.p. cavity and bloodstream where only bioavailable compounds would be delivered. The presence of these insoluble compounds in the intraperitoneal cavity may have potentially stimulated liver gluconeogenesis or suppressed insulin secretion. Regardless of the cause of these observations, the purpose of this study was to determine if grape powder promotes normoglycemia through mechanisms in the gut or through mechanisms in peripheral tissue and it was not possible to answer this question with the observed data. This study could be repeated by administering soluble, bioavailable grape polyphenols instead of grape powder, which might avoid the adverse glycemic response seen here and allow for insight into efficacy of grape polyphenols in the digestive tract versus in peripheral tissue.

Chapter 4. Investigation into anti-diabetic effects of acute and chronic grape powder consumption in normoglycemic and prediabetic models

Study 3: Impact of acute oral grape power administration on glycemic response in normoglycemic and prediabetic mice

Introduction

The goal of study 3 was to determine if acute consumption of freeze-dried grape powder (FDGP) affects post-OGTT blood glucose levels, insulin and GLP-1 levels in the context for normoglycemia as well as in the context of prediabetes. Normoglycemic and prediabetic mice were used in this study to observe the acute effects of grape consumption in both mouse models. The mice of the prediabetic group display a phenotype of elevated fasting blood glucose levels as well as impaired tolerance to oral glucose consumption (see Chapter 2 for more information on this mouse model of prediabetes caused by diet-induced obesity). Both groups were gavaged with a grape powder/glucose solution in order to determine how grape consumption affects blood glucose response to oral glucose tolerance test. A sugar solution (1:1 fructose to glucose) was used as a sugar-matched control for the grape powder as the freeze-dried grape powder contains a high amount of sugar (about 90% by weight, 1:1 glucose/fructose).

Materials and Methods

Mice

Prediabetic (PD) male C57BL/6J mice (*n*=16, 11 weeks old, diet-induced obesity, Jackson Labs, Bar Harbor, ME) and normoglycemic (NG) male C57BL/6J mice (*n*=32, 11 weeks old, Jackson Lab, Bar Harbor, ME) were obtained and acclimated to vivarium conditions for 2 weeks (2 mice/cage, 12h light/dark cycle, 30-70% relative humidity, 20-26 °C). During this time, the PD mice were fed a 60% kcal from fat diet while the NG mice were fed a 10% kcal from fat diet (with sucrose matched to the 60% kcal from fat diet at 6.78% kcal from sucrose); they were allowed to consume their respective diets and drink water *ad libitum*.

Preparation of Treatments

The grape treatment in this study was delivered at a dosage of 1.6 g grape powder/kg bodyweight. This dosage was selected to represent 1/3 of the daily consumption of grape powder in study 4 (which incorporated grape powder at 5% w/w into the diet). Glucose powder is also added to this solution at 0.5 g/kg bodyweight to deliver the oral glucose tolerance test. The control groups in this study received a sugar-matched control of 1:1 fructose to glucose at 1.44 g/kg bodyweight (i.e. 0.72 g fructose/kg bodyweight *and* 0.72 g glucose/kg bodyweight). This value was calculated by multiplying the grape powder concentration (1.6 g/kg) by 90% as the freeze-dried grape powder solution is about 90% sugars by weight.

Mouse procedures

Mice from each phenotype (*n*=16 per phenotype, note that *n*=16 of the NG mice were not used for study 3 as they were maintained on the basal standard fat diet until the commencement

of study 4) were then randomized by weight as above. Mice from each phenotype were separated into two treatment groups: grape powder or sugar-matched control (*n*=8 per phenotype/treatment group). Mice were fasted for 12 h, after which the treatments were delivered via intragastric gavage: grape (1.6 g grape powder/kg bodyweight) plus glucose (0.5 g/kg bodyweight) suspended in saline or sugar-matched control (1.44 g sugar mixture/kg mouse) plus glucose (0.5 g/kg bodyweight) suspended in saline. Blood glucose levels were quantified for 180 minutes as in study 2; blood collections were taken at 10 and 30 min followed by serum separation as above.

Statistical analyses

The blood glucose data provides several different sets of valuable data: area under the curve (AUC, which is the total area underneath the blood glucose time series plot), blood glucose excursion (the difference between baseline blood glucose and the maximum blood glucose level reached), C_{max} and t_{max} which are peak blood glucose concentration and time of peak blood glucose concentration, respectively. Student's t-test was used to compare data of treatment versus control within each phenotype (normoglycemic versus prediabetic). Standard error of the mean will be used to calculate error bars for these metrics. Alpha level of 0.05 will be used to establish significant difference for these data.

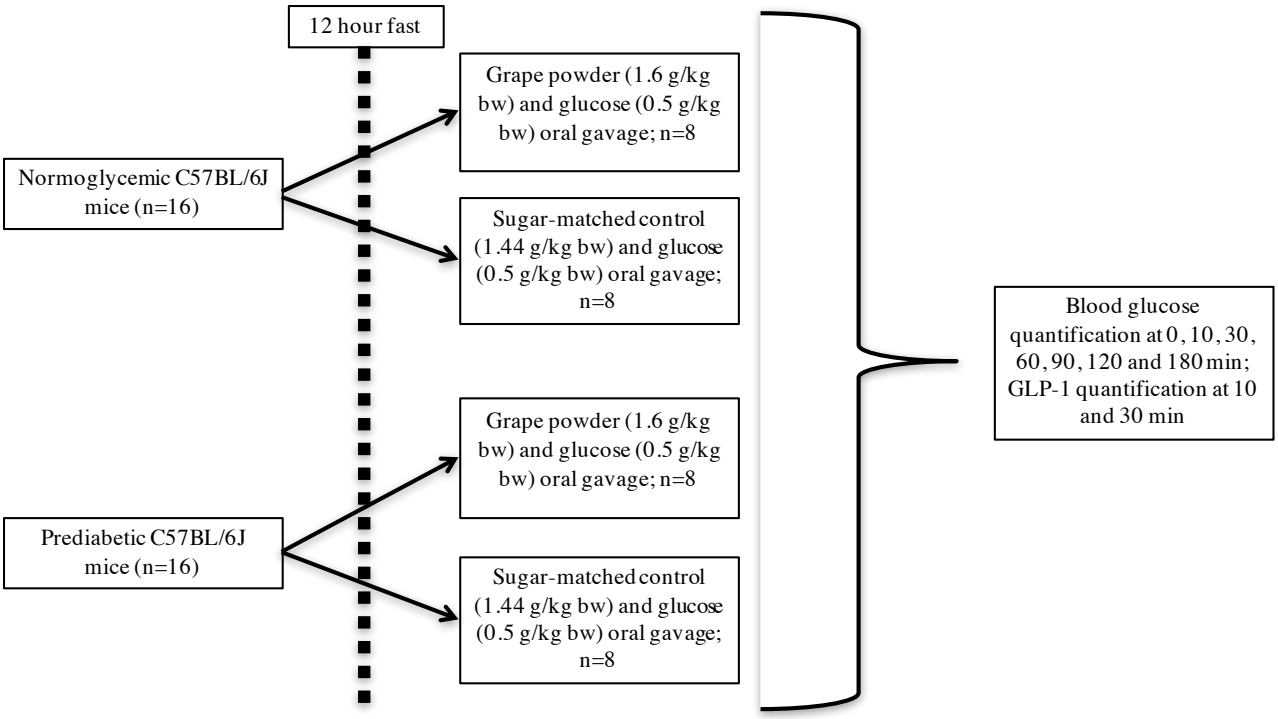


Figure 10. Experimental design of study 3

Prediabetic (*n*=16) and normoglycemic (*n*=16) were administered grape powder solution with OGTT (*n*=8 per phenotype) or sugar-matched control with OGTT (*n*=8 per phenotype); blood glucose was monitored for +3 hours and blood draws were taken at +10 min and +30 min for GLP-1 quantification

Results

The goal of study 3 was to observe and analyze how acute (i.e. a single dose) consumption of freeze-dried grape powder affects blood glucose levels after OGTT in the contexts of normoglycemia and prediabetes. Normoglycemic and prediabetic mice were used for this study to observe the acute effects of grape consumption in both physiological states. The mice of the prediabetic group displayed a phenotype of elevated fasting blood glucose levels as well as impaired tolerance to oral glucose consumption. Acute grape consumption did not result in decreased blood glucose levels in prediabetic or normoglycemic mice after OGTT.

Glycemic responses of the acute grape powder administration can been seen in **Figure 6A-E**. Blood glucose time series paired by treatment or phenotype can be seen in **Figure 6B-E.** Predictably, the PD mice displayed significantly higher blood glucose levels throughout the excursions as compared to NG mice (**Figure 6D-E**). Blood glucose AUC and excursion values can be seen in **Figure 6F-G**; no significant differences were found between grape powder and sugar-matched treatments in these observations.

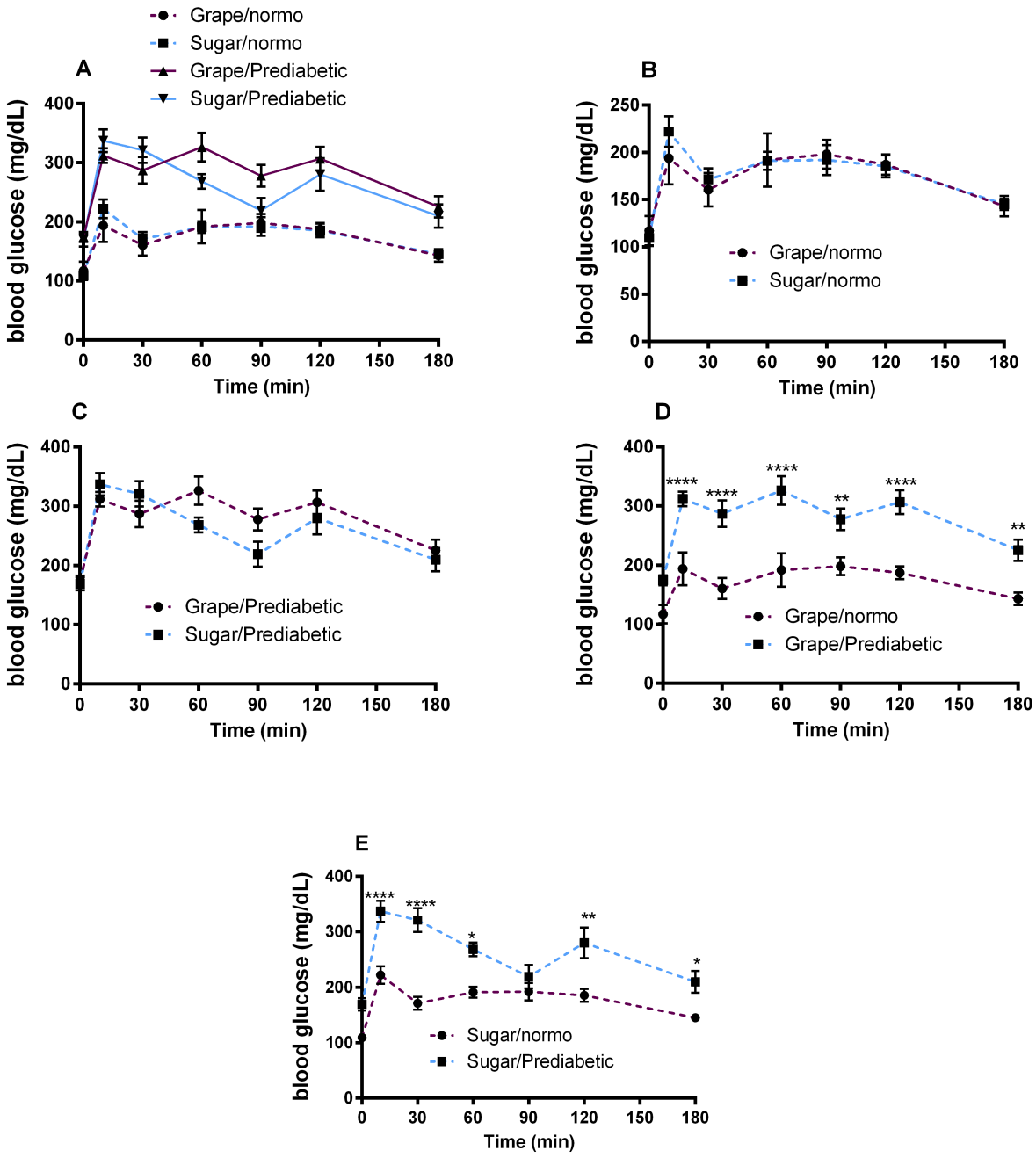


Figure 11 A-E (see caption below)

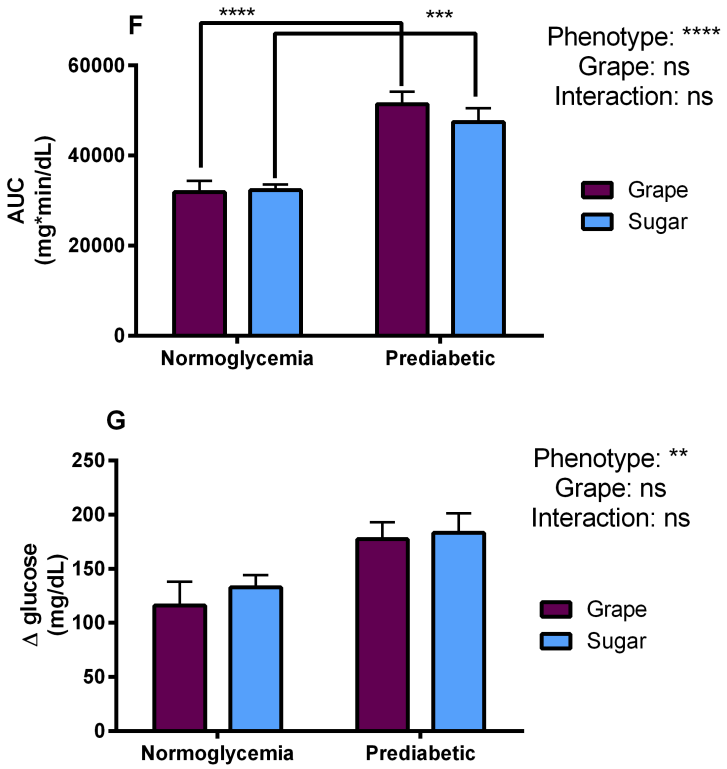


Figure 11. Blood glucose time series and AUC after acute grape treatment in prediabetic and normoglycemic mice

Blood glucose response curves and glycemic response parameters after oral glucose tolerance test with oral administration of grape powder compared to sugar matched control via oral in prediabetic vs. normoglycemic mice. (A) Blood glucose profiles for all treatments, (B) blood glucose profiles for normoglycemic mice: grape powder vs. sugar match, (C) blood glucose profiles for prediabetic mice: grape powder vs. sugar match, (D) blood glucose profiles for grape powder: normoglycemic vs. prediabetic mice, (E) sugar-match: normoglycemic vs. prediabetic mice, (F) blood glucose area under the curve (AUC), (G) blood glucose excursion (maximum value minus baseline value). Values are mean ± SEM (n=8). For B-E, *=p<0.05 **=p<0.01 ***=p<0.001 indicate significant difference between two treatment means at the specified time point as indicated by two-way ANOVA with Tukey's HSD post-hoc test (significance is indicated only on graphs with paired curves for ease of interpretation). For F-G, legends above individual graphs indicate treatment main effects as determined by two-way ANOVA. *p<0.05 **p<0.01 ***p<0.001 indicate significant difference between treatment means as indicated by two-way ANOVA with Tukey's HSD post-hoc test.

Discussion

The purpose of study 3 was to determine if a single dose of grape powder (roughly equivalent to one serving of grapes) was able to decrease blood glucose levels after OGTT as compared to the sugar-matched control. This experiment was performed in normoglycemic and prediabetic mice. As seen in **Figure 11**, there were no significant differences in blood glucose AUC in either the normoglycemic or prediabetic mice. The observations seen in the normoglycemic mice were not surprising as the same results were seen after oral administration of grape powder in study 2. As discussed in the literature review, consumption of grape polyphenol has been shown to significantly reduce blood glucose levels after OGTT in multiple animal studies; however, these studies delivered higher doses of polyphenols (usually proanthocyanidin extracts) than the doses used in this study. This beneficial effect was not seen in our data likely due to the fact that a very small dose of polyphenols was delivered in comparison to the amount of sugars delivered. There was a high amount of sugars delivered in both the treatment and the sugar-matched control groups; thus it is not surprising that the polyphenolic compounds of the grape were not able to lessen the glycemic spike. Compared to whole grape consumption, the sugars in grape powder are likely absorbed more quickly as they are freely available for absorption in the small intestine after gavage whereas in whole grapes the grapes must be further digested before the sugars can be absorbed. It should be noted that mice received the same dose of grape powder in this study as they did in study 1, where the grape powder was able to reverse the effects of GLP-1 receptor antagonism. This dose was not capable of increasing GLP-1 levels to the extent of an improved glycemic response in the prediabetic treatment group of this study, which further demonstrates that the anti-diabetic activities of grape powder seem to be very context-dependent.

Study 4: Impact of long-term grape powder consumption on glycemic control in prediabetic and normoglycemic mice

Introduction

Study 4 was a long-term feeding study featuring an 8-week feeding period with grape powder or sugar-matched control incorporated into high fat diets (60% kcal from fat) in both prediabetic (PD) and normoglycemic (NG) models. The treatment diet had grape powder incorporated at 5% (w/w) and the sugar-matched control diet incorporated fructose and glucose at 4.5% (w/w, to match the amount of sugar present in the grape powder). After 8 weeks of feeding, insulin tolerance test (ITT) and glucose tolerance test (GTT) were performed to determine how chronic grape feeding affects glycemic response and insulin sensitivity. Additionally, bodyweight, body fat percentages, and feed intake were monitored throughout the study to determine the effect of grape consumption on these metrics.

There were 6 treatment groups in this study: normoglycemic/grape/high-fat diet (n=8); normoglycemic/sugar-matched/high-fat diet (n=8); prediabetic/grape/high-fat diet (n=8); prediabetic/sugar-matched/high-fat diet (n=8); normoglycemic/basal high-fat diet (n=8); normoglycemic basal standard-fat diet (n=8). Before the 8-week feeding period began, the normoglycemic group had been fed 10% kcal from fat diet since birth (standard-fat diet), whereas the prediabetic group had been fed 60% kcal from fat diet since birth. The purposes of this study were to (1) determine if grape powder was able ameliorate prediabetes (or some symptoms of prediabetes) in mice with the preexisting condition; (2) determine if grape powder was able prevent the onset of prediabetes caused by high-fat feeding in normoglycemic mice. Also, this study differs from the other 3 studies because the GTT at the conclusion of the feeding

period was *not* co-administered with grape in order to observe beneficial effects of *chronic* grape consumption rather than effects of *acute* grape consumption.

Materials and Methods

Mice and treatment diets

Mice from study 3 were given a 2-week rest/recovery period under the vivarium and diet conditions described above; PD mice were fed HF basal diet (D12492) and NG mice were fed SF basal diet (D12450J) until the initiation of the 8-week feeding period. The *n*=16 NG mice that had not been used in study 3 were maintained on the SF basal diet (D12450J) during this time. At the beginning of the long-term feeding study, mice were randomized to treatment diets as seen in **Figure 12**. PD mice were switched to high-fat diets w/grape treatment (HF/GR) or high-fat diets w/sugar-matched control (HF/SM) (incorporated at 5% and 4.5% w/w, respectively). NG mice were switched to the following diets: HF, HF/SM, HF/GR, and SF. The basal HF and SF diet treatment groups were included as baseline references to compare against the treatment and control groups. See **Table 3** for macronutrient and ingredient composition of all treatment diets. C57BL/6J mice have been shown to consume about 10-15% of their body weight every day[142] so grape powder consumption is expected to equal about 0.75% of mouse bodyweight per day. For all groups, there were *n*=8 mice per treatment group at the start of the study and mice were housed two per cage; several pairs of mice had to be separated into separate cages due to injuries sustained from fighting.

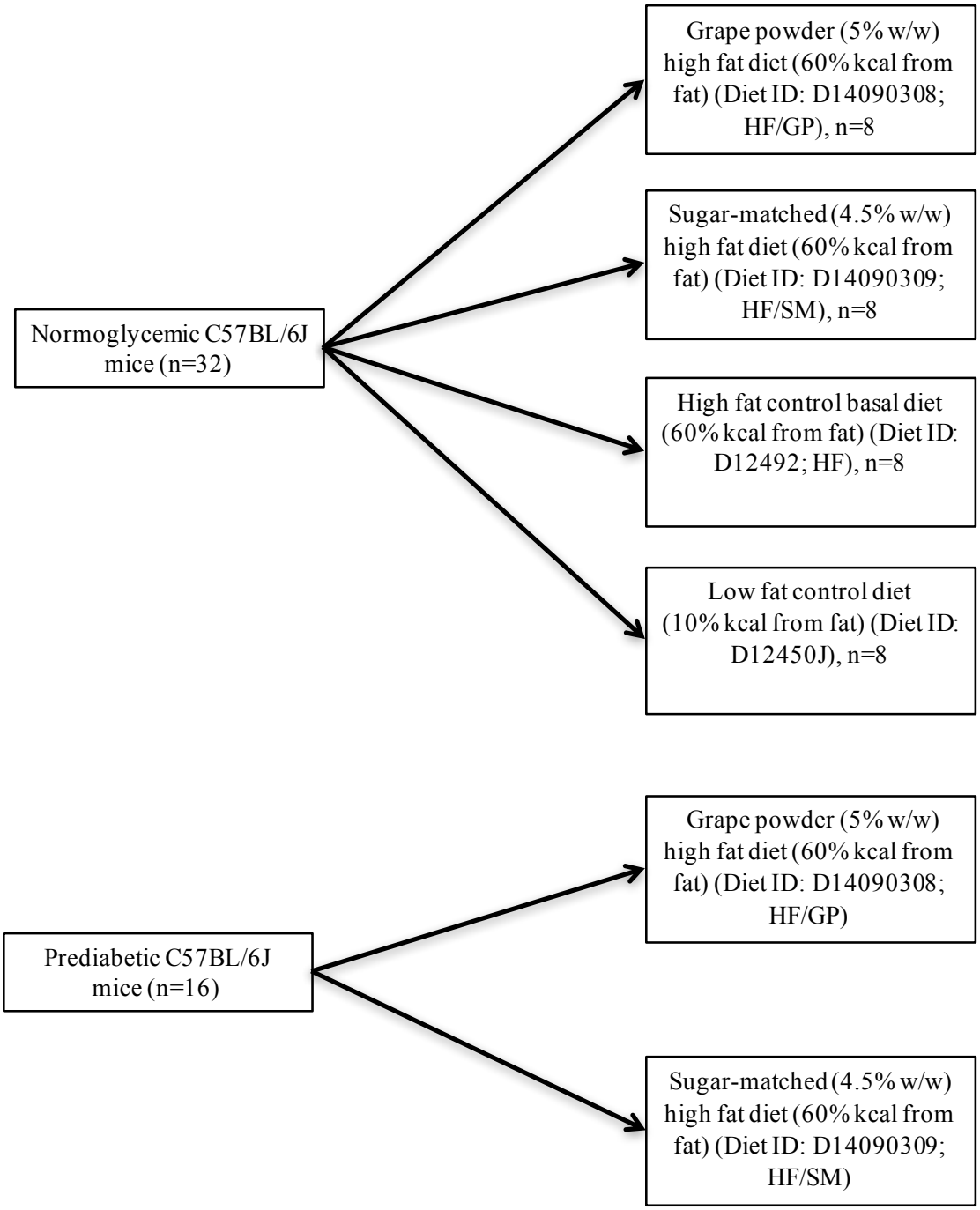


Figure 12. Experimental design for study 4

Long term grape powder feeding study experimental design. Normoglycemic mice (n=16) will be fed high fat diets incorporated with treatment (grape, n=8) and control (sugar match, n=8) for 8 weeks. Prediabetic mice (n=16) will be subjected to the sae treatments. After 8 weeks of feeding, all mice will be subjected to GTT, 1-week recovery, and ITT.

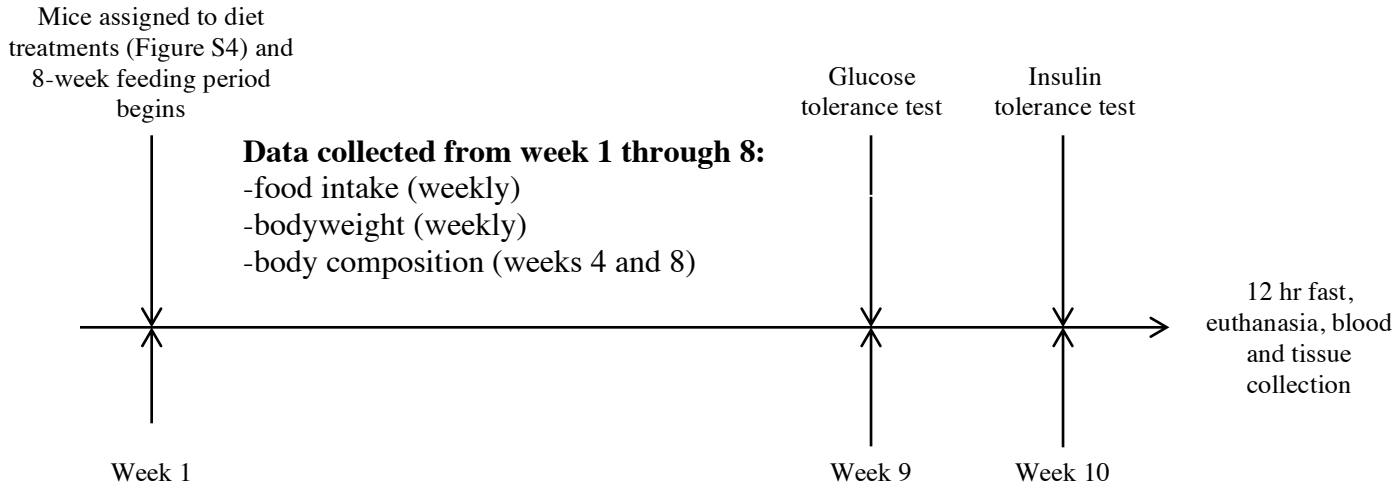


Figure 13. Study 4 treatment timeline

Feed was replaced twice per week, as oxidative rancidity of lipids in HF diets may alter consumption patterns due to sensory characteristics and/or introduce toxic lipid oxidation products into the diet; food consumption was measured as the difference between weight of food added to the cage and weight of food removed from the cage during the feed changes. Mouse bodyweight was recorded weekly. Body composition scans were completed during weeks 4 and 8 (Bruker LR90 NMR minispec, Billerica, MA).

Table 2. Composition of treatment diets in study 4

Diet ID	D12450J		D12492		D14090308		D14090309	
Composition	**Standard fat**		**High fat**		**High fat - grape**		**High fat - sugar**	
	grams	**kcal**	**grams**	**kcal**	**grams**	**kcal**	**grams**	**kcal**
Protein	19	20	26	20	26	20	26	20
Carbohydrate	67	70	26	20	27	20	26	20
Fat	4	10	35	60	35	60	35	60
Total	90	100	87	100	88	100	87	100
kcal/gram	3.8		5.2		5.2		5.2	
Ingredient	grams	kcal	grams	kcal	grams	kcal	grams	kcal
Casein, 80 Mesh	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	3	12
Corn Starch	506.2	2025	0	0	0	0	0	0
Maltodextrin 10	125	500	125	500	125	500	125	500
Sucrose	68.8	275	68.8	275	33.96	136	33.96	136
Cellulose, BW200	50	0	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225	25	225
Lard	20	180	245	2205	245	2205	245	2205
Mineral Mix S10026	10	0	10	0	10	0	10	0
Dicalcium Phosphate	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0	16.5	0	16.5	0
Grape Powder	0	0	0	0	38.7	139	0	0
Glucose/Fructose Mixture	0	0	0	0	0	0	34.83	139
Vitamin Mix V10001	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0
FD&C Yellow Dye #5	0.04	0	0	0	0	0	0.025	0
FD&C Red Dye #40	0	0	0	0	0.025	0	0.025	0
FD&C Blue Dye #1	0.01	0	0.05	0	0.025	0	0	0
TOTAL	1055.05	4057	773.85	4057	777.71	4057	773.84	4057

Glucose tolerance test and insulin tolerance test

After the 8 week feeding period, a glucose tolerance test (GTT) was performed in all mice. Note that this study employed an intraperitoneal glucose tolerance test while the other three studies in this project employed an oral glucose tolerance test. Mice were fasted for 12 h,

followed by i.p. injection of a 20% (w/v) glucose solution (Sigma Aldrich, St. Louis, MO) in saline to provide 1 g glucose/kg bodyweight. Baseline, 30, 60, 90 and 120 min blood glucose levels were measured as described above (blood glucose was not measured at 180 minutes because glucose clearance is quicker in i.p. GTT than OGTT). Following a 1-week recovery period (water and treatment diets *ad libitum*), an i.p. insulin tolerance test (ITT) was performed. Mice were fasted for 4 h followed by i.p. injection of insulin (Humulin R, Cardinal Health, Dublin OH) in saline to provide 0.65 U/kg bodyweight. Baseline, 15, 30, 45 and 60 min blood glucose levels were quantified as described above; any mice displaying symptoms of hypoglycemia were administered an i.p. injection of 20% glucose (w/v) to restore normal blood glucose levels and no further data points were collected in these mice.

Euthanasia

Following the successful conclusion of studies 3 and 4, mice were fasted for 12 hours and euthanized according to American Veterinary Medical Association Guidelines on Euthanasia, as well as local IACUC guidelines. Mice were euthanized with CO_2 immediately followed by a blood collection via cardiac puncture. Serum was prepared as described above (10 µL 100x HALT protease inhibitor was added to serum separation tubes, blood was allowed to clot, then spun at 17,000 *x g* for 10 minutes) and stored at –80 °C. Small intestine, colon and liver samples were excised from the mice, rinsed with cold 1X phosphate-buffered saline (intestinal tissues only, VWR, Radnor, PA), placed in Trizol reagent (Qiagen, Valencia, CA), snap frozen in liquid nitrogen, and stored at –80 °C.

ELISA assays

ELISA assay kits were used to quantify GLP-1 (Multi Species Total GLP-1 ELISA, EZGLP1T-36K, Millipore, Billerica, MA) and GIP (murine total GIP ELISA, EZRMGIP-55K, Millipore, Billerica, MA) in duplicate (where possible) according to manufacturer instructions.

Statistics

The blood glucose data provided several different sets of valuable data: area under the curve (AUC, which is the total area underneath the blood glucose level versus time plot), blood glucose excursion (the difference between baseline blood glucose and the maximum blood glucose level reached), C_{max} and t_{max} which are peak blood glucose concentration and time of peak blood glucose concentration, respectively. Weight gain and body composition were compared between the different treatment groups. These data were treated with student's t-test to compare effects of treatment versus control within each phenotype. Standard error of the mean will be used to calculate error bars for these metrics. Alpha level of 0.05 will be used to establish significant difference for these data.

Results

Study 4 was the long-term feeding study featuring an 8-week feeding period with grape powder or sugar-matched control incorporated into high fat diets (60% kcal from fat) in both prediabetic and normoglycemic models. After 8 weeks of feeding, insulin tolerance (ITT) and glucose tolerance (GTT) tests were performed (separated by a one week recovery period) to determine how chronic grape feeding affects glycemic response and insulin sensitivity.

Blood glucose profiles and AUCs for the GTT and ITT can be found in **Figure 14A-D**. As seen in study 3, the PD mice had significantly higher blood glucose levels throughout the time series but there was not a significant difference within the PD group between the grape powder and sugar-matched diets in the glucose tolerance test. In both GTT and ITT, the LF group displayed better glycemic control than all high fat diet groups (**Figure 14C-D**). Weight and fat gains can be seen in **Figure 14E-H.** All mice fed any high-fat diet (NG *and* PD groups) experienced similar weight gain over the course of the study, regardless of the addition of grape or sugar to the diet (**Figure 14E-H**); the LF group gained less weight and fat during the study. Chronic grape powder consumption did not result in significant changes in glycemic control, insulin sensitivity, or bodyweight.

A blood collection was performed in all mice immediately post-sacrifice after a 12 hr fast. **Figure 15** shows fasting (a) GLP-1 levels and (b) GIP levels. Grape powder increased, although not significantly, the fasting serum GLP-1 versus sugar matched control in both prediabetic and normoglycemic mice.

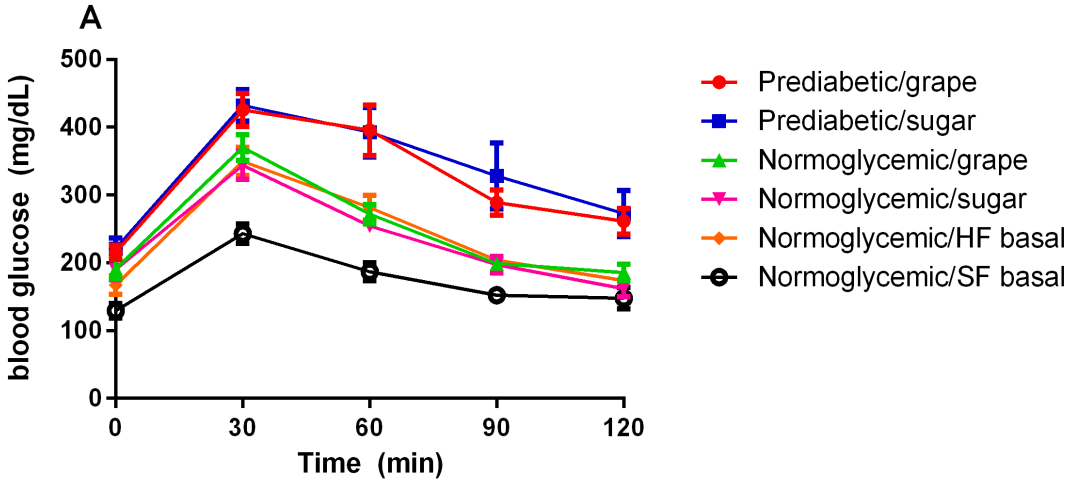


Figure 14 (a) Glucose tolerance test time series

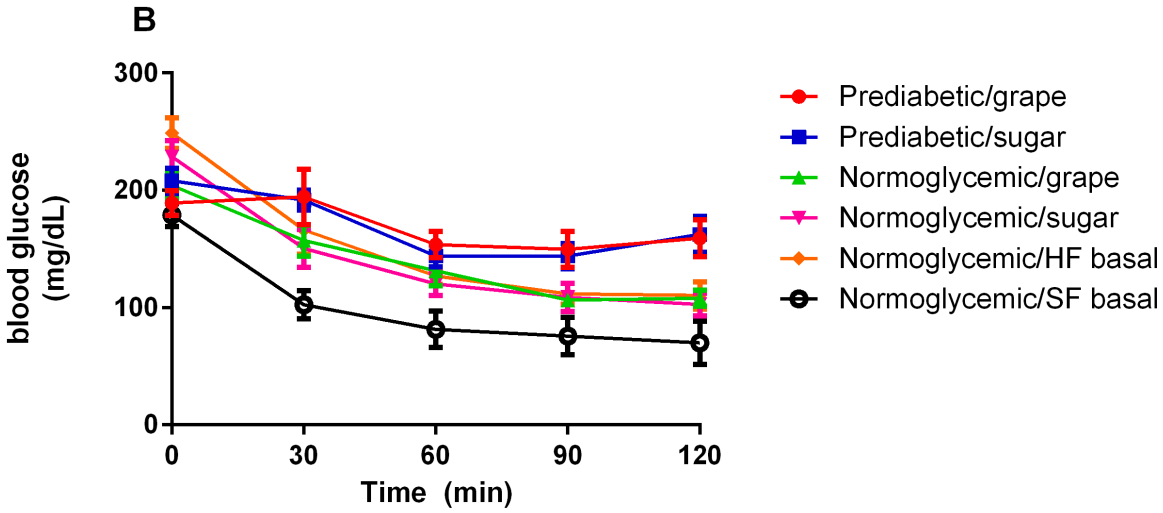


Figure 14 (b). Insulin tolerance test time series

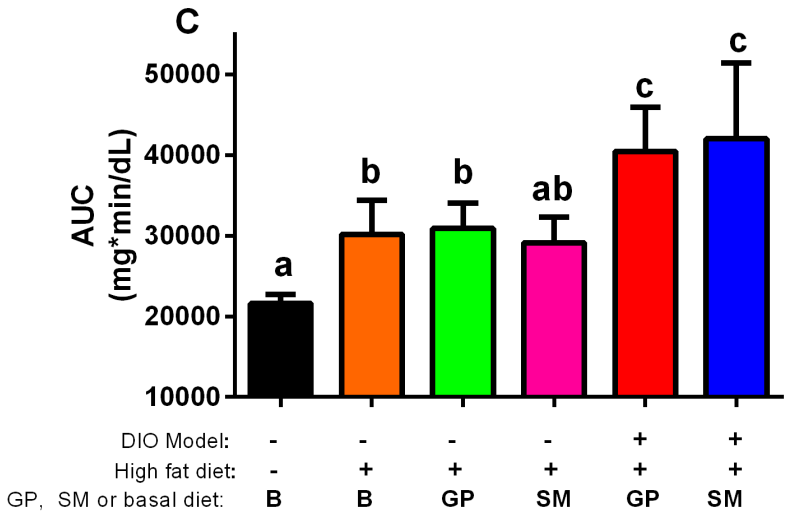


Figure 14 (c). Blood glucose AUCs after GTT

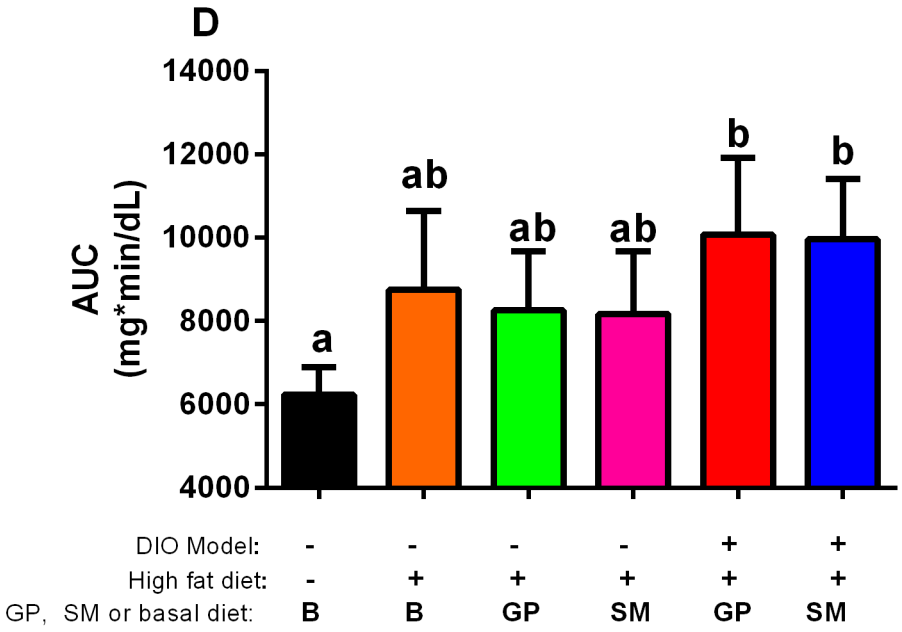
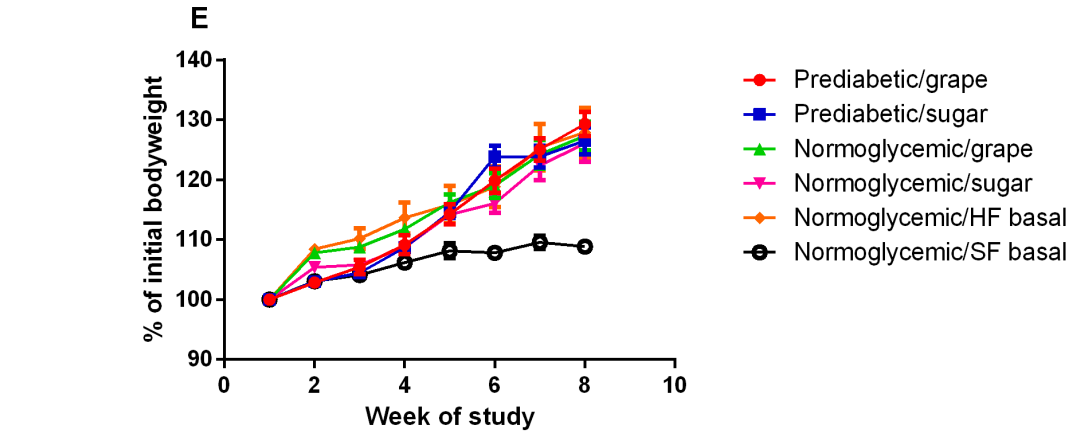
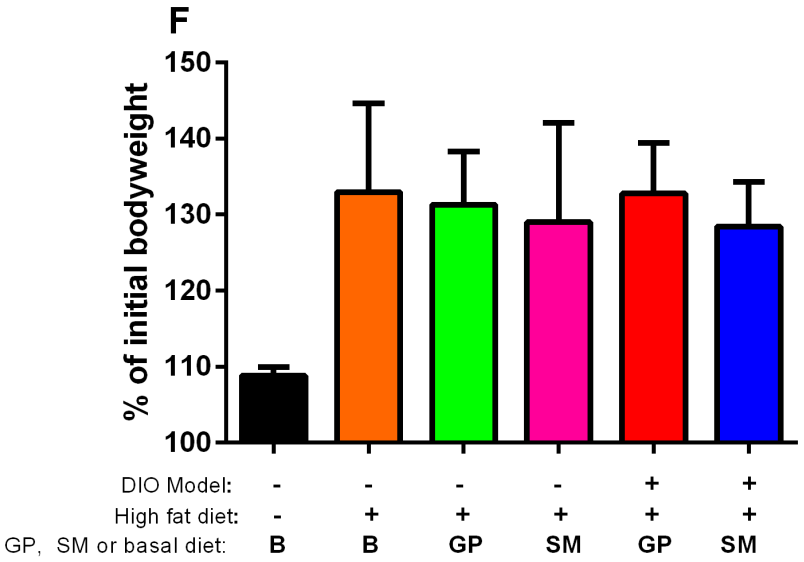
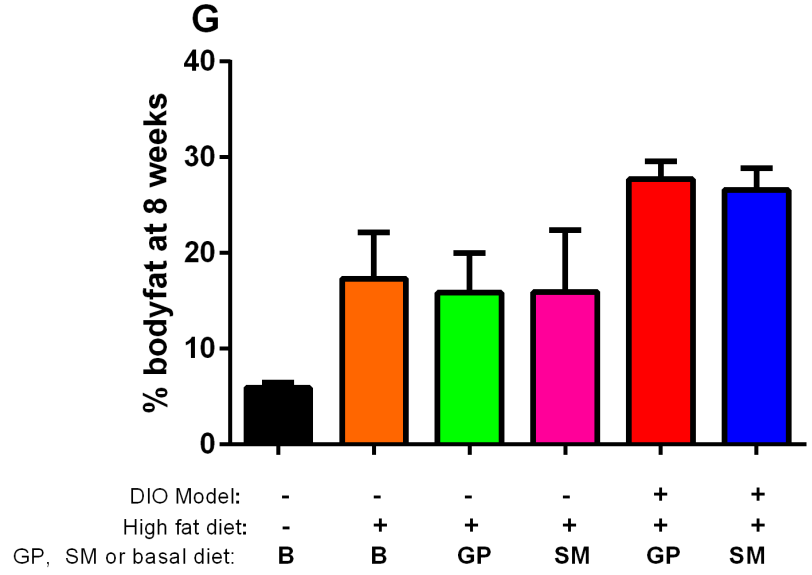


Figure 14 (d). Blood glucose AUCs after ITT


E
% of initial bodyweight
140
130
120
110
100
90
0
2
4
6
8
10
Week of study
Prediabetic/grape
Prediabetic/sugar
Normoglycemic/grape
Normoglycemic/sugar
Normoglycemic/HF basal
Normoglycemic/SF basal


F
% of initial bodyweight
150
140
130
120
110
100
DIO Model: - - - - + +
High fat diet: - + + + + +
GP, SM or basal diet: B B GP SM GP SM


G
% bodyfat at 8 weeks
40
30
20
10
0
DIO Model: - - - - + +
High fat diet: - + + + + +
GP, SM or basal diet: B B GP SM GP SM

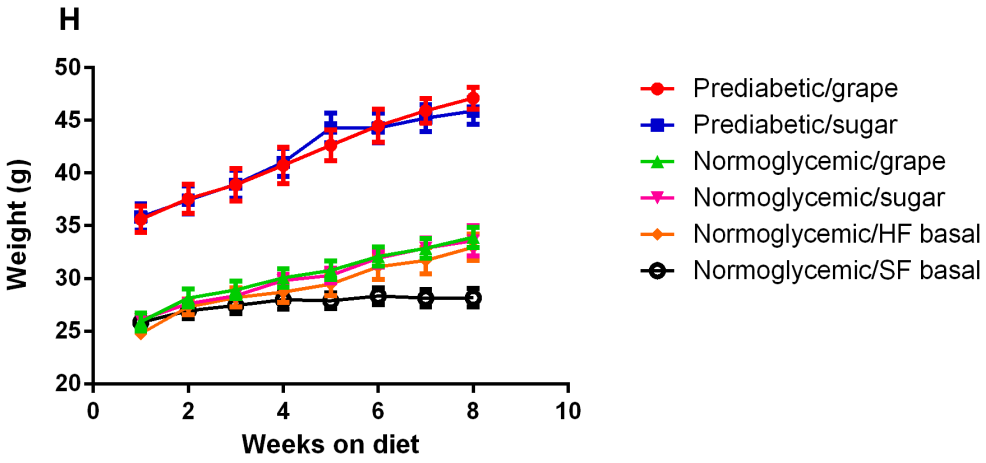


Figure 14. Glycemic control and bodyweight measurements after 8-week feeding study

Physiological changes and outcomes during the long-term feeding study. (A) i.p. glucose tolerance test (GTT) blood glucose time series, (B) i.p. insulin tolerance test (ITT) blood glucose time series, (C) GTT blood glucose area under the curve (AUC), (D) ITT blood glucose AUC, (E) weight gain over time as a percentage of initial bodyweight, (F) total weight gain compared by initial bodyweight at the beginning of the study versus the end of the study, (G) body fat content at week 8, (H) changes in bodyweight during the 8-week feeding period. Values are mean ± SEM (*n*=7/8).

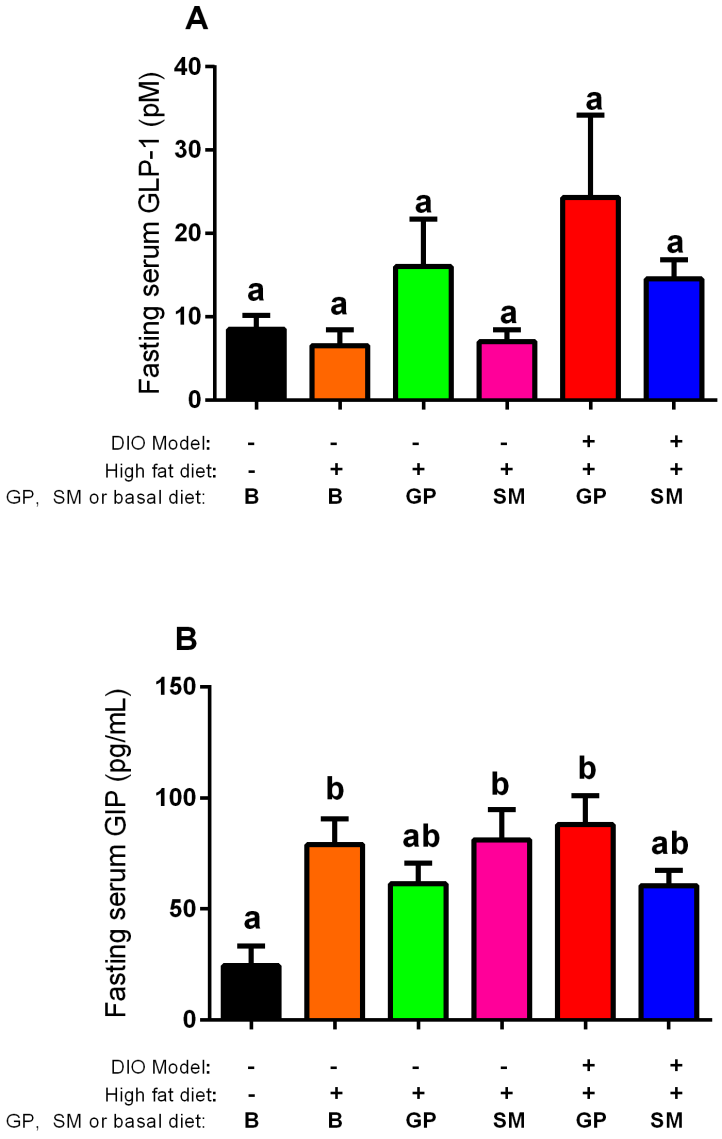


Figure 15. Fasting serum GLP-1 and GIP levels after long-term grape feeding

Study 4: fasting serum levels of (A) GLP-1 (B) GIP; blood samples were taken via cardiac puncture immediately after sacrifice following a 12 hr fast; values are mean ± SEM. These hormone levels were measured via ELISA assays. Values with different superscripts are significantly different as indicated by one-way ANOVA with Tukey's HSD post-hoc test ($p<0.05$).

Discussion

This study was designed to show the efficacy of grape powder to ameliorate prediabetes in mice with the preexisting condition; additionally this work was designed to show if the grape powder can prevent the onset of prediabetes in normoglycemic mice after switching to a high fat diet. The test diet had grape powder incorporated at 5% (w/w) to emulate a level of grape consumption that is obtainable through normal eating patterns. Study 4 differed from the other 3 studies in that the GTT was not co-administered with grape powder in order to isolate beneficial effects of chronic grape consumption rather than effects of acute grape consumption.

Long-term consumption of grape powder did not result in significantly improved glycemic control after GTT or ITT in prediabetic and normoglycemic as hypothesized. Additionally, normoglycemic and prediabetic mice in the grape treatment group did not have lower bodyweight compared to their controls. Although these results were unexpected, it does add value to the project as a whole especially in consideration to the results seen in study 1. Study 1 showed that grape powder reversed deleterious effects of GLP-1 receptor antagonism, but these beneficial effects did not translate to the long-term feeding study. A 2004 study employed the same high-fat feeding model with female C57BL/6J mice and demonstrated the efficacy of a DPP4 inhibitor that was able to normalize glucose tolerance and increase insulin secretion[143]. Grape polyphenols have been shown to be an inhibitor of DPP4 *in vivo*[144], regardless, beneficial effects were not observed in the present study. It is possible that the high fat and simple carbohydrate load of the treatment diets outweighed any potential beneficial effects of the grape powder polyphenols in the diet. Additionally, it is possible higher doses of grape powder

polyphenols may be necessary in order to significantly normalize glucose tolerance and to reduce bodyweight and body fat. Collins et al. published a 2016 paper featuring a 12-week high-fat feeding study in male C57BL/6J mice using the same grape powder that was employed in our study; they found no significant improvements in the high-fat/grape powder (5% w/w) treatment group which agrees with the results seen in our study. However, one high-fat treatment diet included a polyphenol-rich fraction extracted from the grape powder and consumption of this treatment diet resulted in significantly improved glucose tolerance and lower body fat percentage[95]. This indicates that grape powder polyphenols are capable of improving glucose tolerance and other symptoms of T2DM, but these benefits are dependent on other dietary components.

Given the results of study 1, a more focused approach to studying the effects of long term grape consumption in mice with blunted GLP-1 signalling may show beneficial effects (assuming that grape constituents ameliorated the negative effects of GLP-1R antagonism by increasing GLP-1 secretion). Study 1 showed that grape powder has the ability to improve GLP-1 signalling under specific conditions. High fat feeding (diet-induced obesity C57BL/6J mouse model) is an effective method of inducing insulin resistance, impaired glucose tolerance, fasting hyperglycemia and obesity[24, 145, 146], which is a characteristic symptom of prediabetes[143]. It has an advantage to monogenetic variant models when studying novel therapeutic dietary options as it is a closer representation of the complex nature of diet-induced obesity/prediabetes in humans, whereas monogenic models may only target one organ or metabolic pathway[145, 147]; C57BL/6J mice fed a high-fat diet compared with the same mice fed a standard fat diet (10% kcal from fat) display significantly elevated insulin levels (hyperinsulinemia), significantly elevated blood

glucose levels, and significantly reduced tolerance to oral glucose tolerance test[147]. C57BL/6J mice display a "thrifty genotype" in which they appear to store fats extremely efficiently compared to carbohydrates, which makes a high-fat diet a good way to induce obesity in these mice[146]. When this strain of mice is fed a high-fat diet, fat is the primary cause of inducing hyperglycemia and hyperinsulinemia[146]. However, this model may not have induced defective GLP-1 signalling, which study 1 suggests is the pathway through which grape constituents exert their effects. There are conflicting data regarding the effect of high fat feeding on GLP-1 secreting intestinal L-cells: one study found a decrease in the function of intestinal L-cells after 16 weeks of high fat feeding in mice[148], a conflicting study shows an increase in GLP-1 positive intestinal L-cells in obese humans and mice fed a high fat diet[149]. A better model of defective GLP-1 signalling will allow for more focused exploration into the mechanisms and specific situations in which grape constituents may rectify defective GLP-1 signalling. Perhaps repeating these experiments with a GLP-1 receptor knockout mouse model would determine if grape constituents displayed their beneficial effect via ameliorating impaired GLP-1 signalling or via other pathways.

Chapter 5. Brief analysis of grape powder

Introduction

Homogenized, freeze-dried grape powder (FDGP, provided by the California Table Grape Commission, Fresno, CA via The National Food Lab, Livermore, CA) was made from whole, ripe, seeded, and seedless red, green, and black California table grapes and this powder was employed in all four studies. The grape varieties used in this powder are matched to consumer consumption patterns, and 23 g of powder is equivalent to 1 serving of fresh grapes (3/4 cup or 126 g). FDGP is used in place of fresh, whole grapes in order for ease of administering treatments and experimental reproducibility. FGDP is made by freezing and grinding with food-grade dry ice, freeze-drying, and re-grinding; Good Manufacturing Practices for food products were employed throughout processing. FDGP was maintained at 0 °C when not in use. Per the California Table Grape Commission, FDGP contains ~90% sugar (w/w) (1:1 fructose: glucose). A brief characterization of the grape powder was performed to gain a further understanding of the treatments administered throughout these four studies.

Materials and Methods

Grape powder was subjected to several analyses including characterization of the polyphenol profile and proximate analysis. The 4-dimethylaminocinnamaldehyde (DMAC) colorimetric method was used to quantify total flavan-3-ol content, using a method adapted from analysis of procyanidins in cranberry powder[150]. Total phenolics were assessed (whole FDGP and polyphenol extract were both assessed) using the Folin-Ciocalteu reagent[151]. Anthocyanin

content of the grape powder was assessed via a slightly modified spectrophotometry method[152]. A sample size of *n*=4 was employed for all assays.

A modified polyphenol extraction was performed per California Table Grape Commission guidelines[87] to isolate polyphenolic compounds for analysis. FDGP (100.27 g) was dissolved in 600 mL Milli-Q water and stirred for 1.5 h with a magnetic stir plate at 400 RPM. This suspension was centrifuged for 20 min (612 x *g* at 10 °C) on a Beckman JA-14 rotor and the supernatant was collected and refrigerated. 300 mL methanol (Sigma-Aldrich, St. Louis, MO) was added to the pellet to further extract any polyphenols; the suspension was stirred, centrifuged and the supernatant collected (under the same conditions as above). The pellet was submitted to a final extraction with 300 mL of 70% acetone, 28% water, 2% glacial acetic acid (v/v/v); this suspension was agitated with a Polytron (Brinkmann Instruments, Rexdale, Canada) for 1 min, sonicated for 30 s at 40% with a tip ultrasonicator (Fisher Scientific, Pittsburgh, PA). This solution was centrifuged and the supernatant was pooled with the previous supernatants. Volatile solvents were removed from pooled supernatants by rotary vacuum evaporation in a Rotovap (IKA RV10 Basic, Staufen, Germany, 45 °C), and the dried sample was refrigerated.

An open chromatography column (5 cm x 60 cm) was prepared with 500 g Diaion HP-20 (Sigma-Aldrich, St. Louis, MO) stationary phase per manufacturer instructions, and equilibrated with ~8 L distilled water (10x column bed volume). The FDGP extract was slowly loaded onto the column over a period of 20 min. The column was first eluted with water (~3 L water); this cloudy eluent containing sugars and other polar compounds was discarded. Next, the column was eluted with ~2 L methanol followed by ~500 mL acetone to completely elute all desired polyphenolic compounds off the column; all eluents were collected and pooled (see **Figure S1**). The pooled eluents were dried by rotary evaporation as described above in order to remove

volatile solvents from the solution. The remaining aqueous solution was lyophilized at –50 °C (Labconco FreeZone 1, Kansas City, MO). The final dry extract yield of the FDGP (0.8787 g, 0.876% yield) was stored at –80 °C.

Standard methods were employed for proximate analysis of FDGP. Moisture content was determined via drying in a vacuum oven at 70 °C for 3 h under a vacuum of 23 inHg (gauge pressure); moisture was calculated by weight difference. Ash content was measured via dry ashing (ashed at 600 °C for 24 h), and calculated by weight difference. Lipid content was determined by Soxhlet. Crude protein content was determined by Kjeldahl (N x 6.25). A sample size of *n*=4 was used for proximate analysis. Carbohydrate content (on a wet weight basis, wwb) was determined by difference:

$$\%\ \boldsymbol{carbohydrate}\ (\boldsymbol{wwb}) = \mathbf{100}\% - [\%\ \boldsymbol{moisture} + \%\ \boldsymbol{ash} + \%\ \boldsymbol{lipid} + \%\ \boldsymbol{protein}](\boldsymbol{wwb})$$

Results

Composition of grape powder

The composition of the grape powder (flavanol, anthocyanin and polyphenol contents; concentrations of individual flavonoids; proximate composition) is shown in **Table 1**. The grape powder contained 93.4% carbohydrates by weight, which roughly agrees with the sugar content specified by the California Table Grape Commission (~90% sugar). The total polyphenol content was calculated as 0.324% (324 mg gallic acid equivalents/100 g grape powder); while previously published data indicated 580 mg total phenols/100 g FDGP[88]. The content of total flavanols, as measured by DMAC, was 0.0594% (59.44 mg procyanidin B2 equivalents/100 g grape powder),

while previous data indicated 410 mg flavanols/100 g grape powder[88]. Based on our data, the acute dose of 1.6 g/kg is equivalent to 0.950 mg/kg total flavanols; the chronic dose of 5% (w/w) in the diet corresponds to 0.00297% total flavanols in the diet. For the long term study, this means that the normoglycemic grape treatment group received 2.67 mg total flavanols/kg/d based on daily feed consumption (~90 g/kg/d), and the prediabetic treatment group received 2.08 mg total flavanols/kg/d based on daily feed consumption (~70 g/kg/d).

Table 3. Composition of freeze dried grape powder

Component	**Amount per 100 g (±SEM)**
Protein (g)	3.39 ± 0.74
Carbohydrates (g)	92.36
Fat (g)	0.14 ± .03
Moisture (g)	1.46 ± 0.066
Ash (g)	2.65 ± 0.036
Total polyphenols[a] (mg)	323.6 ± 8.09
Total flavanols[b] (mg)	59.44 ± 0.86
Total anthocyanins[c] (mg)	9.62 ± 0.14

[a]gallic acid equivalents, as measured by Folin-Ciocalteu
[b]procyanidin B2 equivalents, as measured by DMAC
[c]cyandin-3-glucoside equivalents, as measured by the pH-shift assay

Chapter 6. Conclusions

The present study reports a novel phenomenon: that grape constituents reversed dysfunctional GLP-1 signalling. However, the data also raise significant questions regarding the context in which grape consumption may effectively improve glycemic control. Grapes appears to have the potential to improve glucose homeostasis and overcome blunted GLP-1 signalling; however, more investigation is required in order to identify the precise biological mechanisms involved, determine the specific disease contexts in which this protection occurs, and create experimental conditions that can effectively mimic these disease contexts.

The benefits of grapes appear promising, and the hypotheses raised by the present study warrant further experiments. Perhaps we need to find a more appropriate model of impaired GLP-1 response/signalling in order to study this phenomenon. To test whether grape acts through other mechanisms to circumvent blunted GLP-1 response, we could employ GLP-1 and/or GLP-1 receptor knockout mice. High-sugar diets may be employed to test the effects of long-term grape consumption in a different obesity model. Grape powder consumption may be extremely beneficial as insulinemia progresses after long-term high fat feeding, when increased incretin effect by GLP-1 may improve insulin secretion at the end of beta cell life.

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