ABSTRACT

Title of Document: EFFECTS OF GRAPE POMACE ON METABOLIC SYNDROME: DIABETES AND OBESITY

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Diabetes and obesity are twin epidemics that are closely linked to each other. In the United States, diabetes currently affects approximately 29.1 million adults and children. The estimated economic cost of treating diabetic patients and their related complications reached $245 billion in the US in 2012. Additionally, the prevalence of obesity is increasing during the recent decades. The National Health and Nutrition Examination Survey 2011-2012 reported that more than two-thirds of US adults (68.5%) are overweight, and more than one-third (34.9%) were obese.

Both diabetes and obesity are currently considered diseases. Although they cannot be completely cured, diabetes and obesity can sometimes be prevented by increasing physical activity and eating healthy foods. Producing healthy foods or healthy agro-produced supplements would be the first line of defense against such diseases. In search of plant products that can be used for preventative medicine, we recently discovered that grape pomace, the by-product

from the waste of the wine and juice industries, has great potential to prevent diabetes and obesity. The fundamental goal of this research is to elucidate the molecular mechanism(s) of the grape pomace extract's (GPE) preventive functions on diabetes and obesity, and to provide scientific evidence to guide its use in developing functional foods for diabetes and obesity prevention. We hypothesize that GPE may prevent diabetes and obesity through altering the expression of genes on the signaling or metabolic pathways that lead to diabetes and obesity manifestation. This research project targeted on 4 specific objectives: 1) to characterize the action of GPE in reducing postprandial hyperglycemia through inhibition of alpha-glucosidase; 2) to understand the mode of molecular action of GPE in control of diabetes; 3) to understand the mode of molecular action of GPE in control of obesity; 4) to examine the effects of GPE on diabetes and obesity at genome wide.

Throughout these research activities, we provided molecular evidence toward understanding the mode action of GPE in preventing diabetes and obesity. Such knowledge will provide guidance for future studies in developing GPE as an alternative therapeutic for the control of diabetes and obesity. Moreover this study may also lead to food industry applications in producing functional foods for diabetic and obese populations.

EFFECTS OF GRAPE POMACE ON METABOLIC SYNDROME: DIABETES AND OBESITY

By

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Dedication

I would like to dedicate my dissertation work to my family. A special feeling of gratitude to my beloved husband Dr. Shuxin Ren for his inspiring support and enlightening help with my research.

I also dedicate this dissertation to my loving parents, Professors Changping Li and Xiumin Liang, whose words of encouragement and financial support were keys to my study. Last but not least, to my lovely son Kevin Ren for his understanding and never wavering love.

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List of Abbreviation

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BMI	Body mass index
CART	Cocaine and amphetamine regulatory peptide
CNS	Central neural system
DPPH	2,2-diphenyl-1-picrylhydrazyl
FAS	Fatty acid synthase
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon like peptide-2
GLUT4	Glucose transporter type 4
HDL	High-density lipoprotein
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprote lipase
LPS	Lipopolysaccharide
NPY	Nucleus peptide Y
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
POMC	Pro-opiomelanocortin
PPARα	Peroxisome proliferator-activated receptor alpha
Peroxisome	Peptide YY
Peroxisome	Sterol Regulatory Element-Binding Protein
Peroxisome	Tumor necrosis factor
Peroxisome	Very low-density lipoprotein

Introduction

The metabolic syndrome refers to a cluster of risk factors leading to an increased risk of heart disease and other health problems. High blood glucose (diabetes) and obesity are two of the major factors used as diagnostics of metabolic syndrome. In fact, diabetes and obesity are considered twin epidemics, closely linked to each other. In the United States, diabetes currently affects approximately 29.1 million adults and children. The estimated economic cost for treating diabetic patients and their related complications reached $245 billion in the U.S. in 2012. The prevalence of obesity has also dramatically increased during recent decades. The National Health and Nutrition Examination Survey 2011-2012 reported that more than two-thirds of U.S. adults (68.5%) are overweight, and more than one-third (34.9%) are obese. Both diabetes and obesity are currently considered diseases. Although they cannot be completely cured, diabetes and obesity can both be prevented by increasing physical activity and eating healthy foods. Therefore, producing healthy foods or healthy agro-produced supplements would be the first line of defense in preventing such diseases.

The grape, *Vitis vinifera*, is one of civilization's oldest domesticated fruit crops and has become one of the most economically important crops adopted by many cultures. Health benefits of grapes and grape products, such as wine, are well documented in the literature. In recent research, grape pomace, the by-products of the waste of the wine and juice industries, was discovered to have great potential to prevent diabetes and obesity. The fundamental goal of this research is to elucidate the molecular mechanism(s) of grape pomace extract (GPE) in preventing against

diabetes and obesity and to provide scientific evidence to guide its use in developing functional foods for the prevention of diabetes and obesity. We hypothesize that GPE may help to prevent diabetes and obesity through altering the expression of genes, either known or novel, in the signaling or metabolic pathways leading to the manifestation of diabetes and obesity. The specific objectives for the current study were: 1) to characterize the action of GPE in reducing postprandial hyperglycemia through the inhibition of alpha-glucosidase; 2) to understand the mode of molecular action of GPE in the control of diabetes; 3) to understand the mode of molecular action of GPE in the control of obesity; 4) to examine the effects of GPE on genome-wide transcriptome expression using the RNA-sequencing approach.

Throughout these designated research activities, we provided molecular evidence toward understanding the mode action of GPE in preventing diabetes and obesity. Such knowledge could provide guidance for future studies in developing GPE as an alternative therapeutic for the control of diabetes and obesity. Moreover this study may also lead to food industry applications in producing functional foods for diabetic and obese populations.

Detailed outlines for each specific objective, including methods and anticipated findings, were described as follow.

Specific Objective 1: to characterize the action of GPE in reducing postprandial hyperglycemia through inhibition of alpha-glucosidase.

In this objective, GPE was extracted from grape pomace and used in an in-vitro yeast alpha-glucosidase inhibition assay to understand the kinetics of GPE-

affected alpha-glucosidase inhibition. Additionally, the extracted GPEs were also gavaged to STZ-induced diabetic mice for 2h OGTT tests to examine the acute effects of GPE on the control of blood glucose.

Through these experiments, we hypothesized that GPE could inhibit yeast alpha-glucosidase activity in vitro. This inhibition is dosage-dependent and through competing the active reaction site(s) with substrates. We also hope to demonstrate that GPE can reduce acute blood glucose level in mice when it is consumed with starch.

Specific Objective 2: to understand the mode of molecular action of GPE in control of diabetes.

Long term effects of GPE on treatment and prevention of diabetes were examined using a mouse model. For diabetes treatment experiments, mice were first STZ treated to induce diabetes. For the diabetes prevention experiment, mice were fed a high fat diet (HFD).

All mice, including STZ treated or non-STZ-treated mice, were fed with HFD supplemented with (GPE treatment) or without (control) GPE for 12 weeks. Body weight, food intake, and blood glucose were monitored weekly. After the feeding experiment, blood and other tissues (pancreas, intestine, and liver) were collected and subjected to various examinations, including HbA1c and peptide hormones using ELISA and gene expression changes at RNA level using real time RT-PCR.

No significant effects of GPE were found in STZ-induced diabetic mice, indicating that GPE may not be used for diabetic treatment. However, GPE can

significantly reduce blood glucose in non-STZ-treated mice with decreasing accumulation of GHbA1c. Glucose homeostasis-related peptide hormones such as GLP-1, glucagon, DPP-4, and insulin were significantly altered by GPE. Furthermore, GPE significantly downregulated the expression of insulin, glucagon and several gut hormones, as well as those regulating systemic inflammation. These results provide insights into the molecular mechanisms directing the effects of GPE in the prevention of diabetes.

Specific Objective 3: to understand the mode of molecular action of GPE in control of obesity.

To understand the role of GPE in the control of obesity, we first examined the effects of GPE on adipose cell differentiation using 3T3-L1 cell line. Adipocyte differentiation and lipid accumulation was monitored and quantified by Oil-Red-O staining. Gene expression changes related to adipocyte differentiation were monitored using the RT-PCR approach. To further examine the role of GPE in obesity prevention, we then designed a long term feeding experiment using mouse model. All mice were fed with HFD supplemented with or without GPE for 12 weeks. Body weight, food intake, and fasting glucose were monitored weekly. After the mice were sacrificed, animal adipocytes was analyzed using the histo-chemistry approach. Tissues of liver, heart, kidney, pancreas and white adipose were collected for RNA expression analysis. The ARC and LHA regions of the hypothalamus tissue were also dissected and used to examine the expression of genes related to food intake.

GPE significantly affected adipogenesis in 3T3-L1 cells, as indicated by decreased oil accumulation. GPE also altered gene expression patterns involved in

the adipogenesis transcriptional cascade in 3T3-L1 cells. GPE reduced body weight and inhibited adiposity process in the mouse feeding experiment. Although no significant changes in gene expressions related to food intake in ARC and LHA regions were observed, GPE did attenuate the expressions of CEBPα, SREBF1 and PPARγ. GPE also significantly reduced systematic inflammation through the inhibition of PAI-1 and the increasing expression of adiponectin.

Specific Objective 4: to examine the effects of GPE on genome-wide transcriptome expression using the RNA-sequencing approach.

GPE plays important roles in prevention of diabetes and obesity. Several key genes regulating the secretion of peptide hormones related to glucose homeostasis and those to adipocyte differentiation were significantly altered by GPE. However, it is not clear how GPE affects gene expression changes on a genome-wide scale. Using the RNA-seq approach, in this objective, we intended to examine the role of GPE in tuning genome-wide gene expressions which lead to prevention against diabetes and obesity. Total RNAs were isolated from the livers of mice treated or untreated with GPE and subjected to high throughput transcriptome sequencing using RNA-seq techniques. Data were bioinformatically analyzed to identify those that are up- or down-regulated by GPE.

Overall, more than 36 million clean reads were obtained from each sample, and approximately 95% of reads were mapped to the mouse reference genome. Over 15,000 genes were identified that were expressed in the mouse. Only 181 genes were identified with altered expression by GPE. Most of the GPE upregulated genes were involved in the metabolism process to accelerate energy expenditure and solute

transport. However, GPE significantly downregulated a large number of genes related to immune responses, oxidative stress responses, and inflammation biomarkers.

Finally, due to limited resources at VSU, all animal feeding experiments were conducted through collaborations or paid service at the University of Georgia (Dr. Tai L. Guo's research group) or the China Agriculture University (Dr. Xiangdong Li's research group), respectively. The project was approved by the respective IACUCs of their institutions. Animal care, handling, feeding, and animal sacrifice were handled by staff in their research teams. At no time was any animal handled by the author of this dissertation. However, all molecular experiments conducted after sacrifice of the mice used for these experiments and those not involving animal feedings were conducted by the author alone.

Chapter 1: Literature Review

1.1 Metabolic syndrome and its prevention

Metabolic syndrome is a cluster of risk factors that includes obesity; high triglycerides, blood pressure, and blood glucose and low high-density cholesterol. It increases the risk for heart disease and other health problems. Metabolic syndrome is caused by the imbalance of energy utilization and storage and can increase the risk of development of coronary heart disease, predominantly heart attack, stroke, kidney failure, gout, cancer, amputations and blindness (Eckel et al., 2005; Isaacs & Vagnini, 2006), and it is associated with morbidity and all-cause mortality (Malik et al., 2004; Lakka et al., 2002; Hu et al., 2005). The prevalence of metabolic syndrome has been increasing worldwide in recent decades. According to the 2003-2006 National Health and Nutrition Examination Survey, in the United States, the prevalence of metabolic syndrome is approximately 34% of the adult population (Ford et al., 2005; Ford et al., 2010).

Among the factors that can cause metabolic syndrome, genetics play a key role in controlling its development. People who have inherited the genetic tendency towards insulin resistance are candidates to develop metabolic syndrome. On the other hand, increased consumption of a high calorie diets and decreased physical activity have accelerated the prevalence of metabolic syndrome. Clinical and epidemiological evidence suggests that lifestyle factors, especially diet, are essential in controlling metabolic syndrome (Grundy et al., 2005). Therapies adopting a

healthy lifestyle approach become the first-line of intervention on its prevention. A healthy lifestyle includes a balanced variety of foods and daily exercise. Consuming a healthy diet rich in fruits and vegetables is highly recommended, and it may reduce the risk of cancer and other chronic diseases (CDC State Indicator Report on Fruits and Vegetables 2013). Maintaining a healthy weight, waist measurement and body mass index (BMI) by increasing physical activity is another important lifestyle choice. Research has highlighted the potential therapeutic effects and nutraceutical properties found in natural products. Positive effects were revealed from food-based nutritional compounds for maintaining healthy body weight, lowering blood glucose, correcting lipid panels and decreasing the blood pressure (Ford et al., 2003). The anti-inflammatory and antioxidant effects of nutraceuticals have specific applications in preventing oxidative stress, which may play a role in the pathophysiology of diabetes, obesity and cardiovascular disease (Oberly, 1988; Rao, 2002). In a word, "An ounce of prevention is worth a pound of cure".

1.2 Diabetes

Diabetes mellitus is a group of metabolic syndromes with a hallmark of hyperglycemia- increased blood glucose levels. It is caused by 1) interruption of the homeostasis of carbohydrate, protein and lipid metabolism, 2) lack of insulin secretion, or 3) increase of insulin resistance. Diabetes mellitus currently affects 200 million people globally, approximately 5 % of the adult population. Diabetes is ranked as the 6th leading cause of death in the U.S. Diabetes effects young people as well as aging people. About 215,000 people younger than 20 years had diabetes (type

1 or type 2) in the U.S. in 2010. Approximately 25.8 million adults and children in the U.S. suffer from diabetes according to CDC's National Diabetes Fact Sheet 2012. This number is expected to be over 40 million (Stephens et al., 2006) or more by 2025, given that more than 86 million American adults were diagnosed as pre-diabetes in 2012 (National Diabetes Statistics Report, 2014).

The number of diabetic patients has dramatically increased in recent decades; the prevalence of diabetes was boosted by the increased consumption of refined sugar and processed foods with high calories, along with decreased physical activity (Stephens et al., 2006). The prevalence of diabetes is increasing dramatically worldwide. Growth is expected to occur in developing countries along with the consequences of population ageing, increasing urbanization, unhealthy diets, obesity and sedentary lifestyles.

Insulin is a principal peptide hormone, produced by beta cells of the pancreas, that regulates the uptake of glucose from the blood. Based on the insulin secretion and response to insulin, diabetes has been classified into three categories: Type 1 Diabetes: the failure of sufficient insulin production from β cells of pancreas causes of high blood sugar. It is an insulin-dependent diabetes, also called juvenile diabetes with early onset. Type 2 diabetes mellitus or "adult-onset diabetes" results from insulin resistance, a condition in which cells fail to use insulin properly, referred to as non-insulin-dependent diabetes, which is more than ninety percent of all cases of diabetes. The third type of diabetes is gestational diabetes caused by the hormonal changes (Biessels et al., 2004) during pregnancy, which lead to insulin insufficiency

or insulin resistance. Deficiency of insulin or the insensitivity of its receptors plays a critical role in all forms of diabetes mellitus (Bell et al., 1980).

The serious long-term complications from diabetes are the major cause of hospitalization and death. Diabetes can cause retinopathy, cardiomyopathy, renal failure, altered immune functions, peripheral neuropathy, and intestinal dysfunction (Biessels et al., 2004; Martinez-Tellez et al., 2005). The economic cost of treating diabetic patients, and their related complications, reached $245 billion in 2012. Although long-term complications of diabetes develop gradually, untreated diabetes can cause many complications, even disability and death. Acute complications were common in Type I diabetes patients, including symptoms such as diabetic ketoacidosis and nonketotic hyperosmolar coma. Type 2 diabetes is the most common form of diabetes, affecting 90% to 95% of diabetes patients. Improperly treated diabetes can cause serious long-term complications. Over time, the high glucose levels in the blood can damage the nerves and small blood vessels of the eyes, kidneys, and heart. Complications include diabetic retinopathy, nerve damage, stroke, cardiovascular disease, blindness, kidney failure, and macro and micro-vascular damage, nephropathy, impaired immune functions, and lower limb amputation. (Martinez-Tellez et al., 2005).

1.2.1 Diabetes treatment

Diabetes is treatable and preventable. Conventional anti-diabetic drugs play a key role in diabetes treatment. However, side effects from these drugs can be problematic. Side effects include: 1) unwanted weight gain; 2) acidosis, caused by

buildup of acidic metformin byproducts in the blood stream; 3) several dangerous and life threatening symptoms such as malaise, fatigue, nausea and weakness; and 4) some anti-diabetes drugs, such as acarbose, sulfonylureas, metformin or thiazolidinediones, can cause gastrointestinal disturbances, upset stomach, nausea, diarrhea, constipation and increased flatulence.

Fortunately, diabetes is preventable. Many plant species have shown promising effects in the management of diabetes. Medicinal plants, as well as agricultural by-products, are inherently safe because of their natural origin, and many have been traditionally used since ancient times by physicians to treat a great variety of human diseases.

The existing conventional diabetes drugs, along with their molecular targets and sites of action, have been extensively studied (Prabhakar et al., 2011). Different diabetes drugs target different organs, such as liver, pancreas, small intestine, adipose tissue and muscle (Eurich et al., 2007). Based on each drug's mode of action, they can be divided into two groups: 1) Insulin secretagogues (sulfonylureas, meglitinides, phenylalanine derivatives insulin) which stimulate insulin release by pancreatic beta cells; and 2) Insulin sensitizers (biguanides, metformin, thiazolidinediones) that reduce insulin resistance by activating PPAR-γ in fat and muscle to increase the peripheral glucose uptake and act on liver to decrease glucose production (Prabhakar et al., 2011).

1.2.2. Carbohydrate metabolism and Alpha-glucosidase inhibitors

Diabetes is, in part, related to the amount of carbohydrates in the diet.

Complex carbohydrates are digested primarily in the lumen of the small intestine by α-amylase to yield linear oligosaccharides and branched isomaltose oligosaccharides. Both of these can be further subjected to hydrolysis to release glucose and fructose by α-glucosidase and be absorbed into the bloodstream (Casirola et al., 2006). Alpha-glucosidase is a key membrane-bound intestinal enzyme presented in the brush-border of the small intestine mucosa. It inhibits glucoside hydrolase activity by controlling the delay of the carbohydrate absorption and lowering the postprandial glycaemia. α-glucosidase has been used widely as a target by the commercial antidiabetic medicines (for example acarbose, miglitol, and voglibose) in the regulation of blood glucose levels and treatment of type 2 diabetes (Yamazaki et al., 2007; Van et al., 2006; Casirola et al., 2006; Van et al., 2005; Nakamura et al., 2005; Fujisawa et al., 2005). Studies of the anti-hyperglycemic effects of α-glucosidase inhibitors have demonstrated its efficacy in reducing postprandial blood glucose levels, improving glycated haemoglobin (HbA1c) levels, and attenuating pancreatic islet damage (Negishi et al., 1996; Yamazaki et al., 2007). It attenuates postprandial hyperinsulinemia, increases the sensitivity of insulin receptor thus improve insulin sensitivity (Yamazaki et al., 2007), stimulating secretion and prolonging the effect of the key incretin hormone glucagon-like peptide-1 (GLP-1) (Yusuke et al., 2009), and it can delay or prevent the long-term complications of the type 2 diabetes (Lee et al., 1982). The effect of chronic alpha-glycosidase inhibition on diabetic nephropathy in the db/db mouse is well established. Alpha-glucosidase inhibitors like acarbose can be used as a monotherapy and in combination therapy with other commercial diabetes drugs. Synergistic effects

have been reported with the combination of acarbose and DPP-4 inhibitors (inhibitors of dipeptidyl peptidase 4 reduce glucagon and blood glucose levels) (Aoki et al., 2012; Narita et al., 2012; Qualmann et al., 1995). Even though glucose lowering drugs were effectively used to manage blood glucose levels under normal range, it is very challenging to find a medicine that has no side effects. Side effects and other disadvantages of conventional antidiabetic drugs are very common. Gastrointestinal disturbances, upset stomach, nausea, diarrhea, constipation and increased flatulence caused by commercial α-glucosidase inhibitors have been reported (Prabhakar et al., 2011), as well as stomach pain and bloating, liver problems, skin reactions, and swelling due to fluid build-up

1.2.3. Medicinal Plants: An Alternative Tool to Manage Diabetes

An alternative to these synthetic conventional diabetes drugs is the use of many plant species that have shown promising effects in the management of diabetes. Medicinal plants have been traditionally used since ancient times by physicians to treat a great variety of human diseases. Currently, over 800 different natural medicines are widely used in several traditional medical systems to prevent diabetes (Casirola et al., 2006). Oriental countries such as China, Korea and India have long histories of using medicinal plants for the management of diabetes. Several medicinal plants have been investigated for their beneficial effects in the treatment of different types of diabetes. In addition, thousands of herb formulas and dietary supplements are available on the market for diabetic patients to use (Prabhakar et al., 2011). Dietary phytochemicals work through various metabolic pathways to reduce postprandial hyperglycemia. Plants with hypoglycemic activity have been the subject

of numerous studies; many of these natural medicines regulate blood sugar levels through a variety of mechanisms. In some cases, their effects are similar to conventional medicines. Multiple beneficial activities include regulating carbohydrate metabolism, preventing ß-cells apoptosis (which is one of the key point on triggering diabetes), increasing insulin releasing activity, and improving glucose uptake and utilization.

1.3 Obesity

Obesity is another complex disorder involving an excess of body fat accumulation. Obesity is defined by BMI (Body Mass Index), which is a measurement obtained by dividing a person's weight by the square of the person's height, by the World Health Organization 2006 Classification. People are classified as obese when their BMI exceeds 30 kg/m2. Obesity is a complex medical condition with a negative impact on health, leading to reduced mortality and mobility and augmented health problems (Haslam and James, 2005). The prevalence of obesity has been increasing during the recent decades. Obesity rates have more than doubled in adults and children since the 1970's (National Center for Health Statistics, 2009). The National Health and Nutrition Examination Survey 2011-2012, reported that more than two-thirds of U.S. adults (68.5%) are overweight, and over one-third (34.9%) of adults were obese (Ogden et al., 2014). The trend for increased prevalence of obesity has persisted, particularly for adolescents and men (Ogden et al., 2004). The onset of obesity is happening earlier with 31.8% of children and adolescents being overweight or obese in the U.S. (Ogden et al., 2013).

Obesity, particularly excessive abdominal obesity, plays a key role in developing metabolic syndrome. It increases the risk of many common health problems. Numerous complications associated with obesity include the dramatic increase of diabetes mellitus (Colditz et al., 1995; Chan et al., 1994) , hypertension (Sjostrom et al., 2004; Huang et al., 1998), cardiovascular disorders (Suk et al., 2003; Millionis et al., 2004; Song et al., 2004), cancers (Calle et al., 2003; Danaei et al., 2005), dyslipidaemia (Datillo and Kris-Etherton, 1992), non-alcoholic fatty liver disease (NAFLD) (Malnick et al., 2003; Hamaguchi et al., 2005), osteoarthritis (Hart et al., 1993; Cicuttini et al., 1996) and psychological disorders (Gortmaker et al., 1993). The predominant treatment for obesity is lifestyle modification, increasing physical activity and improving lifestyle choices to maintain a healthy body weight and waist measurement. For certain patients, drugs or bariatric (weight-loss) surgery are recommended. The total medical cost related to managing obesity and related health problems is a burden on the public health system. The financial cost of combatting the current prevalence of obesity in the U.S. has increased annually. Based on the current trends in the growth of obesity, total healthcare costs attributable to obesity and obesity-related diseases will increase by as much as $66 billion annually. By the year 2030, this annual cost will reach $861 to $957 billion, and will account for 16% to 18% of U.S. health expenditures (F as in Fat: How Obesity Threatens America's Future 2012).

1.4 Close link between diabetes and obesity

There has been a rapid increase in obesity prevalence worldwide during the past decades. The number of diabetes patients has increased by 40%, particularly the

number of individuals diagnosed with obesity and developing diabetes later. Diabetes and obesity have become major public health problems, with serious implications in industrialized countries as well as in developing nations. Smyth and Heron (2005) have reported that there is increased interest among researchers in the link between diabetes and obesity, the twin epidemics. A connection between obesity and diabetes was investigated and discovered that obese individuals had more than a 10-fold increase in chances of developing type 2 diabetes compared to normal weight individuals (Must et al., 1999; Field et al., 2001). Obesity-derived cytokines and insulin resistance are the main factors involved in the development of type 2 diabetes. Several other factors, including glucose toxicity, lipotoxicity and beta cell failure (Stumvoll et al., 2005) also contribute to the development of type 2 diabetes. Numerous studies have revealed that insulin resistance plays a key role between obesity and diabetes (Jung and Choi, 2014) Obesity is characterized by increased storage of fatty acids in an expanded adipose tissue mass. It is closely associated with the development of insulin resistance. Individuals with insulin resistance are not sensitive to insulin, and cells in the body are unable to use insulin effectively. With the reduced response to insulin, pancreatic cells increase their production of insulin to combat the increased levels of blood glucose (Shanik et al., 2008). Meanwhile, insulin-resistance stimulates pancreatic β-cells proliferation, which leads to pancreatic β-cell deterioration and apoptosis, decreased insulin secretion and eventually to the development of diabetes (Dandona et al., 2005; Chetboun et al., 2012; Butler et al., 2003).

1.5 Chronic inflammation, obesity and insulin resistance

1.5.1. Adipose tissue is an endocrine organ

During the past two decades, the complex nature of adipose tissue stands out and has become an area of intense investigation. Fat tissue, or adipose, was formerly considered a passive energy storage element and related to lipid metabolism. For many years, it was believed that the main function of adipose was thought to store energy in the form of triglycerides during energy consumption, releasing fatty acids when energy expenditure exceeds energy intake. Another biological function of fat tissue was considered to act as a cushion and insulate the body to prevent heat lost. However, in 1994 with the identification and characterization of leptin, a 16-kDa polypeptide with structural homology to cytokine, adipose tissue was consequently confirmed to be a complex and highly active metabolic and endocrine organ (Kershaw and Filer, 2004; Zhang et al., 1994; Ahima et al., 2000; Fruhbeck et al., 2001). Adipose tissue secretes numerous peptide hormones and receptors which have neuroendocrine functions, including effectively communicating with the brain and peripheral tissues to modulate glucose homeostasis, influencing energy metabolism, regulating appetite and insulin resistance and controlling immune function (Matsuzawa et al., 1999; Funahashi et al., 1999). When immune cells, such as macrophages (Bornstein et al., 2000; Xu et al., 2003; Weisburg et al., 2003) and T lymphocytes (Kintscher et al., 2008; Rausch et al., 2007; Wu et al., 2007), infiltrate into adipose tissue in obese mice and humans, the infiltrated adipose tissue immune cells trigger insulin resistance by promoting a proinflammatory environment within the adipose tissue. This discovery has led to the second revolution in the field of

adipose tissue biology (Bonnie 2008). Inflammation and insulin resistance presented in adipose tissue link obesity and diabetes together.

1.5.2. Adipose-derived hormones -- adipokines

Adipose tissue contains numerous cell types such as adipocytes, immune cells, endothelia cells and fibroblasts. Abundant peptide hormones that are involved in signaling transduction pathways, are secreted and released into systemic circulation by adipose tissue. These adipocyte-specific proteins are known as adipokines (Kwon and Pessin, 2013). Functioning as classic circulating hormones, they have been shown to have a variety of local, peripheral, and central effects and to play a primary role in communication with other organs including liver, muscle, brain, intestine and immune system (Kwon and Pessin, 2013). Over the last two decades several research teams have explored the linkage of adipokines with obesity, type 2 diabetes and cardiovascular disease. Increased risk of developing diabetes has been observed in obese individuals (BMI≥30 kg/m2), compared to those with normal body weight (BMI<25 kg/m2) (Luft et al., 2013), even though the molecular mechanisms of the association of obesity and type 2 diabetes are still unclear. Inflammatory responses in adipose tissue have been shown to be a major factor explaining the mechanism of obesity-associated type 2 diabetes. Human and rodent animal models have been used to investigate how this low-grade chronic systemic inflammation contributes to the association between obesity and diabetes. Mild obesity-induced inflammation has been reported in liver and muscle, and significant immune cells infiltration and inflammation has been observed in adipose tissue (Kwon and Pessin, 2013; Duncan et al., 2003; Hotamisligil, 2006; Luft et al., 2013;

Shoelson et al., 2006; Schenk et al., 2008; Ouchi et al., 2011). Based on these studies, metabolism and immunity are undistinguishably linked to each other (Odegaard and Chawla, 2013).

Adipose tissue secreted adipokines play important roles in regulating a variety of complex processes, including fat metabolism, feeding behavior, energy and glucose homeostasis, vascular tone and insulin sensitivity (Rondinone 2006). Chronic low-grade inflammation is a key feature of obesity that is correlated with insulin resistance and type 2 diabetes (Kim et al., 2009). The inflammatory function of adipokines plays a crucial role in mediating obesity-induced insulin resistance.

Adipose-derived hormones include tumor necrosis factor (TNF)-alpha, resistin, IL-6, IL-8, acylation-stimulating protein (ASP), angiotensinogen, plasminogen activator inhibitor-1 (PAI-1), leptin and adiponectin. Adipokines have been categorized into two groups: pro- and anti-inflammatory adipokines.

1.5.3. Pro-inflammatory adipokines

In 1994, Friedman et al. reported a novel gene called leptin (Lepob). Leptin was the first adipocyte-derived hormone to be discovered. Leptin receptor (Leprdb) was reported in 1995 and 1996; the db gene encodes the leptin receptor and is expressed in the hypothalamus, where it helps to regulate hunger and body weight gain (Tartaglia et al., 1995; Chan et al., 1994; Hua et al., 1996). Leptin regulates food intake and energy expenditure predominantly through hypothalamic pathways by acting on receptors in the hypothalamus (Williams et al., 2009). Leptin was named from the Greek “lepto” meaning “thin”. It was initially thought to be as a signal to

the brain to decrease food intake and lower body weight (Zhang, 1994). Humans and rodents that are leptin deficient Lepob/ Lepob and leptin receptor deficient Leprdb/Leprdb express the lack of a functional leptin or leptin receptor and exhibit a remarkable hyperphagia, obesity and insulin resistance. In Lepob/ Lepob mice, obesity was reduced and insulin sensitivity was restored by exogenous administration of leptin (Kwon and Pessin, 2013). However, the concept of leptin as an anti-obesity hormone has been questioned due to the phenomenon that the circulating levels of leptin and leptin resistance were increased in obese rodents and humans. The relationship between plasma leptin and adiposity in normal weight and overweight women were reported by Havel et al. Leptin concentration is directly associated with adiposity, and plasma leptin levels increased with weight gain and decreased with weight loss (Havel et al., 1996), while adipocytes secreted leptin in a positive linear correlation to total body adipose tissue mass. Thus, leptin is now viewed as signal of energy deficiency rather than a weight loss hormone.

In addition to its effects on energy homeostasis, the biological function of leptin is to regulate neuroendocrine function (Galic et al., 2010; Howard et al., 2004; Ahima and Flier, 2000; Friedman and Halaas, 1998). Blüher and Mantzoros reported that certain leptin levels are required to activate the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroid axes in men (Blüher and Mantzoros, 2004). Several other important endocrine effects of leptin were reported, including regulation of immune function by changing the cytokine production by immune cells, modulation of fetal and maternal metabolism, stimulation of endothelial cell growth, and the acceleration of beta islet cells proliferation. On the other hand, leptin interacts

with other hormones and energy regulators, such as insulin, glucagon, insulin-like growth factor, growth hormone, glucocorticoids and cytokines, which are also involved in numerous biological metabolisms (Kershaw and Flier, 2004; Margetic et al., 2002).

Numerous adipose-secreted factors have recently been described as potential mediators that link obesity-derived chronic inflammation with insulin resistance (Figure 1-1). One such adipokine is inflammatory regulator interleukin-6 (IL-6) (Kim et al., 2009). IL-6 is a cytokine secreted by abundant cell types including adipocytes, adipose stromal cells and muscle cells (Kern et al., 2001; Crichton et al., 1996). Approximate 1/3 of the IL-6 was produced by adipose tissue, and circulation of IL-6 concentrations was positively correlated with obesity. IL-6 impaired glucose tolerance and insulin resistance (Fernandez-Real and Recart, 2003), and decreased expression and circulating concentrations of IL-6 were detected with weight loss in obese and diabetes individuals (Kramer et al., 2006; Hotamisligil et al., 1995; Kern et al., 2001; Lazar, 2005). IL-6 appears to play a central role in regulating adipocytes and hepatocytes in the insulin signaling pathway. Senn et al., 2003 reported that IL-6 is the key pro-inflammatory cytokines. By inducing SOCS3, a suppressor of cytokine signaling and a negative regulator of insulin signaling, the cascade genes expression including insulin-induced insulin receptor, IRS-1 tyrosine phosphorylation, p85 binding and down-stream PKB/Akt phosphorylation was impaired in liver cells (Galic et al., 2010; Senn et al., 2003). On the other hand, Matthews et al (2010) conducted a test to determine whether complete deletion of IL-6 in mice, an IL-6(-/-), results in induced obesity, hepatosteatosis, inflammation

and insulin resistance. IL-6 deficient mice displayed mature-onset obesity, hepatosteatosis, liver inflammation and insulin resistance when compared with control mice on a standard chow diet. A process related to defects in mitochondrial metabolism was found in IL-6 deficient mice; IL-6 deficiency exacerbates high fat diet-induced hepatic insulin resistance and inflammation (Matthews et al., 2010). To investigate the possible mechanism of the anti-obesity effect of IL-6, Wallenius et al., 2002) injected rats centrally and peripherally with IL-6 at low doses. IL-6 treatment increased energy expenditure and reversed insulin resistance, indicating that IL-6 exerts anti-obesity effects in rodents (Wallenius et al., 2002). Furthermore, IL-6 was reported to promote fatty acids oxidation and glucose uptake in skeletal muscle (Galic et al 2010; Carey et al., 2006; Kelly et al., 2004). These discoveries shed a different light on the role of IL-6 in obesity and insulin resistance.

Another adipocyte secretory protein involved in insulin resistance is Tumor Necrosis Factor α (TNFα), which was the first adipose derived factor associated in the induction of insulin resistance in obesity and type 2 diabetes. TNFα is a paracrine/autocrine factor highly expressed in adipose tissues that is associated with obesity, chronic inflammation and type 2 diabetes. The direct effects of TNFα on the functions of adipose tissue have been studied (Ruan and Lodish 2013). The expression levels of TNFα were increased in both obese animals and human subjects. A positive association between adiposity and insulin resistance was reported. A number of studies have demonstrated that TNFα is implicated in the pathogenesis of obesity and in alterations in gene expression in metabolically important tissues such as adipocyte and liver (Ruan and Lodish 2013; Ruan et al., 2002). TNFα induces

genes implicated in inflammatory responses such as chemokines, cytokines, and their receptors in adipose tissue. It represses genes involved in glucose uptake and fatty acid metabolism in liver, increases plasma concentrations of free fatty acids and exhibits repressive effects on gene expression in skeletal muscle. TNFα also inhibits insulin signaling and attenuates insulin sensitivity in adipocytes (Hotamisligil 2003; Fernandez-Real and Ricart 2003). Over-expression of TNFα in adipose tissue will affect several downstream mediators in insulin signaling pathways. These include the increase of activation of serine kinase, which in turn increases serine phosphorylation of IRS1 and 2 (Insulin receptor Substrate 1 and 2), decreases the sensitivity of insulin receptors, thus leading to a critical decrease in insulin sensitivity.

More recently, the finding of other adipocyte-specific factors was reported, such as resistin, CCL2, chemokine (C-C motif) ligand 2 which has also been referred to as monocyte chemotactic protein 1 (MCP1), plasminogen-activator inhibitor type 1 (PAI-1), angiopoietin-like protein 2 and chemerin. Diet-induced obesity triggered immune cell infiltration into adipose tissue, and pro-inflammatory adipokines were secreted, circulating adipokines, leading to inflammation and prompting the development of insulin resistance and type 2 diabetes.

1.5.4. Anti-inflammatory adipokines- adiponectin

Adipose tissue is an active endocrine organ that secretes numerous adipokines, the majority of which are pro-inflammatory factors that lead to the development of insulin resistance and type 2 diabetes. However, the discovery of

adiponectin, the 247 amino acids peptide produced by adipose most abundant gene transcript 1 (apM1), shed light on the anti-inflamatory function of the adipokines (Maeda et al., 1996). Adiponectin was induced in differentiation from pre-adipocytes to adipocytes. It is entirely synthesized in white adipose tissue and secreted into the bloodstream. The circulating concentration is relatively high at approximately 0.01% of all plasma protein (about 5-10 μg/mL). Chandran et al. have demonstrated that plasma adiponectin levels have a strong and consistent inverse association with both insulin resistance and inflammatory states. Adiponectin plays an important role in the regulation of glucose metabolism in human and animal models (Chandran et al., 2003). Adiponectin levels were increased with the administration of insulin-sensitizing drugs (Kershaw and Filer 2004; Chandran et al., 2003; Diez et al., 2003). Adiponectin has potent anti-inflammatory properties, and levels of adiponectin are decreased in patients with obesity, type 2 diabetes and atherosclerosis. Furthermore, circulating levels of adiponectin were significantly increased with weight loss (Vázquez-Vela 2008; Coppola et al., 2008; Yatagai et al., 2003).

The mechanisms for adiponectin's metabolic effects have been fully described in various organs, in both rodent and human models. The administration of adiponectin has been shown to regulate insulin signaling by increasing insulin-induced tyrosine phosphorylation of the insulin receptor in skeletal muscle and improving whole-body insulin sensitivity (Yamauchi et al., 2001; Stefan et al., 2002). In the liver, adiponectin enhances insulin sensitivity by increasing tissue fat oxidation and reducing circulating fatty acid levels and intracellular triglyceride contents. Also, inhibited gluconeogenesis and increased glucose uptake were observed in liver. In

vascular endothelial cells, adiponectin suppresses monocyte adhesion by inhibiting the expression of adhesion molecules, thereby reducing cytokine production from macrophages so as to decrease the inflammatory environments occuring during the early phases of atherosclerosis (Diez et al., 2003; Chandran et al., 2003). Taken together, adiponectin is a unique adipokine positively linking insulin resistance with obesity and diabetes.

Understanding the mechanism of signaling pathways by which adipokines are controlled in target tissues such as skeletal muscle and liver may also expose novel therapies for obesity-related diseases (Galic et al., 2010).

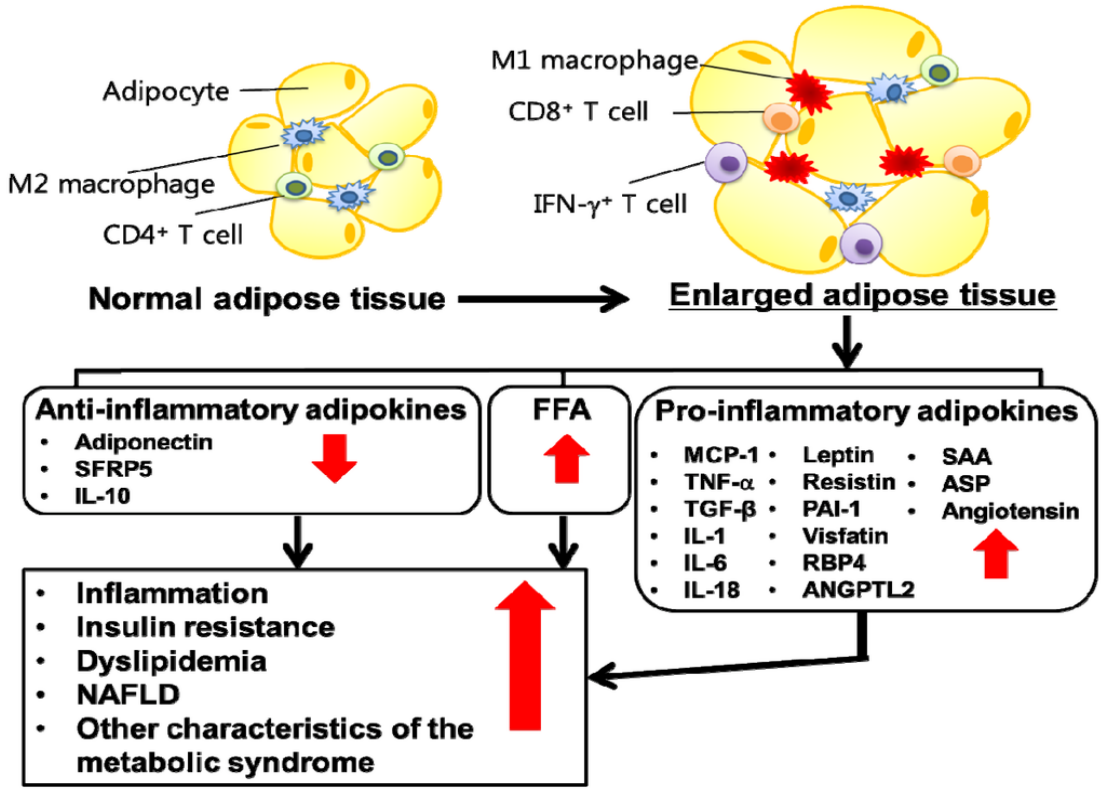


Figure 1- 1 Sexretion of inflammatory adipokines from adipose tissue in obese state. In obese state, the enlarged adipose tissue leads to dysregulated secretion of adipokines and increased release of free fatty acids. The free fatty acids and pro-inflammatory adipokines get to metabolic tissues, including skeletal muscle and liver, and modify inflammatory responses as well as glucose and lipid metabolism, thereby contributing to metabolic syndrome. In addition, obesity induces a phenotypic switch in adipose tissue from anti-inflammatory (M2) to pro-inflammatory (M1) macrophages. On the other hand, the adipose production of insulin-sensitizing adipokines with anti-inflammatory properties, such as adiponectin, is decreased in the obese state. - See more at: http://www.mdpi.com/1422-0067/15/4/6184/htm#sthash.0787EcKY.dpuf

1.6 Network connection of multiple peripheral gastrointestinal hormones and hypothalamic central neuron peptide hormones to control energy balancing

Obesity is a complex disorder involving an excess of body fat accumulation. It has been officially recognized as a chronic disease by the American Medical Association (AMA).

When energy intake exceeds energy expenditure, obesity will develop. Food

intake and energy expenditure are the key factors contributing to adiposity and obesity. Maintenance of energy homeostasis and body weight involves the regulation of the coordinated network of peripheral gastrointestinal hormones and hypothalamic neuropeptides. During the past two decades, the complex nature of adipose tissue has become an area of intense investigation. The discovery of leptin, a 16-kDa polypeptide with structural homology to cytokine, has led to the redefinition of adipose tissue as a complex and highly active metabolic and endocrine organ (Kershaw and Filer, 2004; Zhang et al., 1994; Ahima et al., 2000; Fruhbeck et al., 2001). Numerous studies have indicated that leptin has an anorexigenic effect and informs the brain of energy status (Aydin et al., 2008). Energy homeostasis is controlled by a complex neuroendocrine system consisting of peripheral signals (such as leptin), and central signals, in particular, neuropeptides. Several neuropeptides with anorexigenic (POMC, CART, and CRH) as well as orexigenic (NPY, AgRP, and MCH) actions are involved in this complex controlling system (Hillebrand et al., 2002).

The hypothalamus and its circuits that play a central role in controlling feeding behavior and regulating short-term and long-term food intake behavior (Clifford et al., 2002; Williams et al., 2001) are shown in Figure 1-2. Numerous neuropeptides produced by the hypothalamic arcuate nucleus (ARC), particularly neuropeptide Y (NPY) and agouti-related protein (AgRP), are involved in maintaining food intake and body weight control. NPY is a central stimulator of food intake (Chamorro et al., 2002). Ninety per cent of NPY neurons co-express with

AgRP (Schwartz et al., 2000). Leptin and insulin inhibit food intake and increase energy expenditure through inhibition of orexigenic NPY/AgRP neurons and stimulation of anorexigenic pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons in the ARC. NPY/AgRP neurons simultaneously activate an anabolic pathway and inactivate a catabolic pathway (Shi et al., 2009; Baskin et al., 1999; Kristensen et al., 1998; Cowley et al., 2001).

Control of food intake involves not only the hypothalamus in the central nervous system (CNS), but also the gastrointestinal (GI) tract signaling system, pancreas and liver (Figure 2-2). Numerous peripheral peptide hormones play a key role in regulating food intake and energy homeostasis (Naslund et al., 2007). Many of the peptides involved in controlling food intake and satiety signals in the hypothalamus are also present in the enteroendocrine cells of the mucosa of GI tract; neuropeptides in the CNS that influence food intake are influenced by peptide signals from the gut. In the neuronal network, satiety signals from the gut were closely linked with hypothalamic centers for controlling food intake and eating behavior. Multiple gastrointestinal peptides that influence food intake are connected to the central neuron system controlling food intake. Ghrelin, secreted by endocrine cells in the stomach, is an orexigenic hormone that stimulates growth hormone release and enhances feeding and weight gain to regulate energy homeostasis (Tschöp et al., 2000). Ghrelin acts on the hypothalamus to stimulate feeding. It was considered an appetite-stimulating hormone and was implicated in both short- and long-term appetite and body weight regulation. When acting as an orexigenic peptide, ghrelin stimulates food intake by activating NPY/AgRP neurons and balancing inhibitory

signals received from insulin, leptin and peptide YY (Hillebrand et al., 2002; Kojima et al., 1999; Tschöp et al., 1999; Wren et al., 2001). In addition, high-fat diet (HFD) feeding caused ghrelin resistance in arcuate neuropeptide Y (NPY)/Agouti related peptide neurons (Briggs, 2011), and the hypothalamic circuitry became resistant to ghrelin. Anorexic peripheral peptides also play important roles in regulating food intake and energy homeostasis. Cholecystokinin (CCK) is the most studied gut peptide regulating food intake (Ritter, 1999). It is secreted by cells in the duodenum and jejunum when they are exposed to food, and it induces satiety in the CNS. Glucagon-like peptides (GLP-1 and GLP-2) are peptide hormones that stimulate the secretion of insulin. GLP is released from the lower gastrointestinal tract following food intake and plays dual roles in the regulation of blood glucose concentrations through its concurrent insulin tropic and glucagonostatic actions (glucose-lowering effect) (Näslund et al., 1999; Ørskov, 1999). Numerous anorexic peripheral peptides such as Peptide YY, amylin, insulin, bombesin, glucagon, and GIP, are all known to be anorexigenic and are recognized as physiologic regulators of food intake contributing to the control of obesity (Arora 2006). This multiple-hormone-controlled energy homeostasis has become a central target for the design of pharmacological agents to prevent and treat obesity.

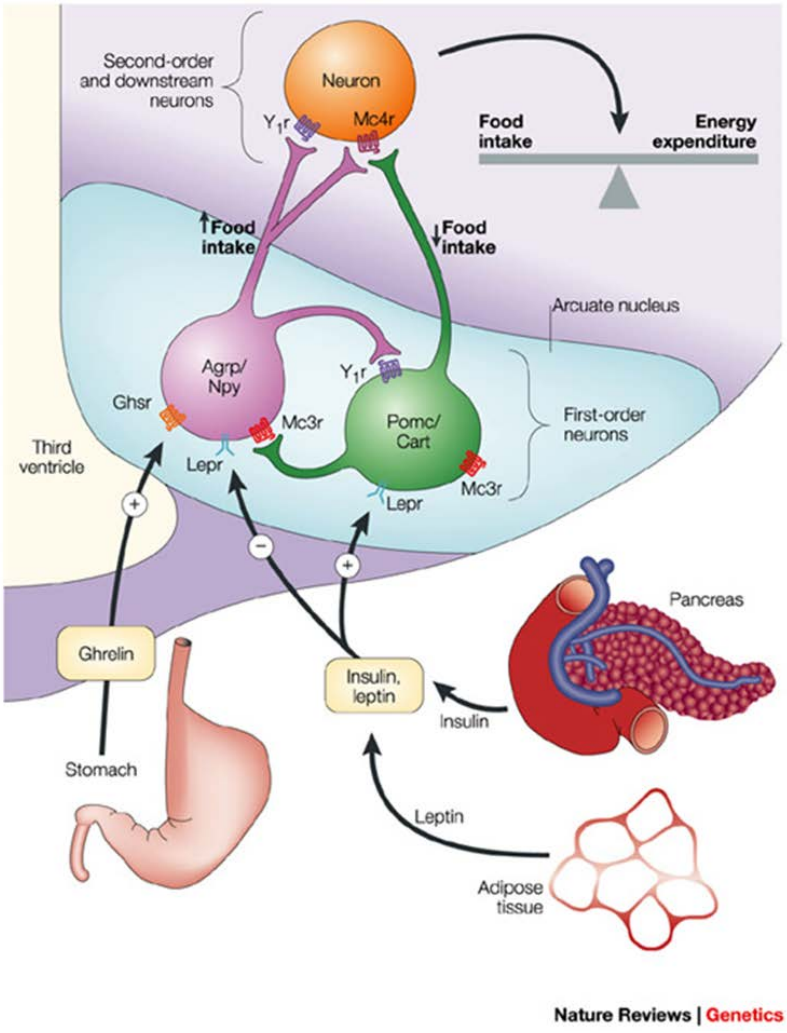


Figure 1- 2 Control of energy homeostasis by arcuate nucleus neurons.There are two sets of neurons in the arcuate nucleus — Agrp/Npy and Pomc/Cart neurons — that are regulated by circulating hormones. Agrp (agouti-related protein) and Npy (neuropeptide Y) are neuropeptides that stimulate food intake and decrease energy expenditure, whereas the alpha-melanocyte stimulating hormone (a post-translational derivative of proopiomelanocortin, Pomc) and Cart (cocaine- and amphetamine-regulated transcript) are neuropeptides that inhibit food intake and increase energy expenditure. Insulin and leptin are hormones that circulate in proportion to body adipose stores; they inhibit Agrp/Npy neurons and stimulate adjacent Pomc/Cart neurons. Lower insulin and leptin levels are therefore predicted to activate Agrp/Npy neurons, while inhibiting Pomc/Cart neurons. Ghrelin is a circulating peptide secreted from the stomach that can activate Agrp/Npy neurons, thereby stimulating food intake; this provides a potential molecular mechanism for integrating long-term energy balance signals with short-term meal pattern signals. Ghsr, growth hormone secretagogue receptor; Lepr, leptin receptor; Mc3r/Mc4r, melanocortin 3/4 receptor; Y1r, neuropeptide Y1 receptor. Adopt from Nature Reviews Genetics 3, 589-600.

1.7 Adipogenesis, a highly orchestrated multistep process of the transcriptional network

Adipogenesis is the process of the differentiation of fibroblast like pre-adipocytes into the well-established matured spherical adipocytes which contains lipids (Lefterova and Lazar, 2009). Take the advantage of the in vitro model of 3T3-L1 and 3T3-F442A, the pre-adipocyte cell lines, the adipogenesis has been precisely illustrated (Rosen et al., 2000; Gregoire, 2001). A highly orchestrated multistep process of the transcriptional network was involved in adipogenesis (Ali et al., 2013). Several transcription factors are sequentially activated in adipogenesis transcriptional cascade. Remarkably CCAAT/enhancer binding proteins (C/EBPβ, C/EBPδ and C/EBPα) gene family, peroxisome proliferator activated receptor-γ (PPAR-γ) and Sterol regulatory element-binding transcription factor 1 (SREBF1), have been shown to have significant roles in promoting adipogenesis (White and Stephens, 2009). These factors are essential to promote the terminal or mature adipocyte phenotype. Differentiation of pre-adipocyte cells into adipocytes involves a series of events including growth-arrest, mitotic clonal expansion, and terminal differentiation and mature adipocytes (Sun et al., 2009; Lefterova and Lazar, 2009). In response to hormonal stimuli such as cortisol and insulin, C/EBPβ and C/EBPδ are induced immediately which in turn activate PPARγ and C/EBPα (Farmer, 2006). PPARγ and C/EBPα form a positive feedback loop to induce the expression of each other and regulate the adipocyte differentiation (Rosen et al., 2002). PPARγ and C/EBPα in turn to induce the expression of genes that are necessary for regulating fatty acid storage and glucose metabolism (Rosen, E.D. and MacDougald, 2006). They are highly expressed in adipose tissue and are key factors controlling the down-stream adipocyte

differentiation and adipocyte-specific gene expression (Lefterova et al., 2008), such as lipoprotein lipase (LPL), which is considered as the early stage marker for the adipocyte differentiation, and adipocyte fatty acid–binding protein (aP2), which is an intermediate sign of adipocyte differentiation (Rosen and Spiegelman , 2000; (Bernlohr et al., 1984; Katz et al., 1999; Spiegelman et al., 1983; MacDougald et al., 1995). Sterol regulatory element-binding proteins (SREBPs) are also involved in adipocyte differentiation. SREBPs are a family of transcription factors that regulate lipid homeostasis. SREBPs are induced very early during adipocyte differentiation and stimulate the expression of PPARγ, and induce the fundamental coordinator of the adipocyte differentiation process (Eberlé et al., 2004; Shao and Espenshade, 2012; Saladin et al., 1999).

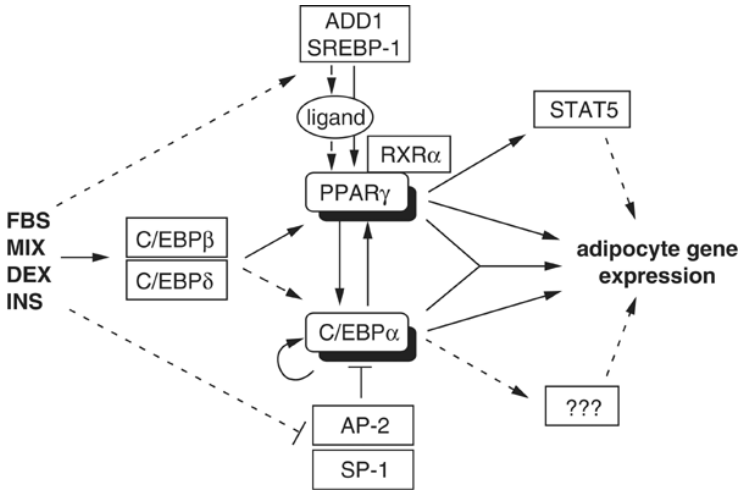


Figure 1- 3 Transcriptional events in adipocyte differentiation. A summary (present view) of the molecular processes of adipocyte differentiation, focusing only on transcriptional events. Direct or indirect transcriptional events are indicated by solid lines. Broken lines represent interactions that are less well understood. Specific transcription factors are denoted with square boxes; unknown factors are indicated with question marks. Abbreviations: ADD1, adipocyte determination- and differentiation-dependent factor 1; C-EBPalpha, CCAAT-enhancer binding protein-alpha; RXR, retinoid X receptor; SREBP, sterol regulatory element binding proteins; STAT, signal transducers and activators of transcription. Adapted from Morrison and Farmer. Picture was adopted from International Journal of Obesity (2003) 27, 147–161.

1.8 Health benefits of grape

1.8.1 Grape and grape pomace

The grape (Vitis vinifera) is one of the oldest fruit crops domesticated. Cultivation of grapes began 6,000–8,000 years ago in the Near East, and is one of the most economically important plants adopted by all cultures. Several phenolic compounds that can improve the human health are found in grapes. The well-known "French Paradox" identifies the fact that the epidemiological observation of despite high intake of dietary cholesterol and saturated fat, but low incidence of coronary heart disease (CHD) death rates are present in the Mediterranean region (Renaud et al., 1992). The routine consumption of red wine was considered as the factor lowering the development of coronary heart disease. This attracted more attention of scientists to define the chemical composition in grapes. A large number of different natural grape products are widely used in the nutritional products market, and the beneficial effects of using flavonoids and poly-phenolic compounds from grape and grape-derived products have been well studied. The bioactivities of grape polyphenols and the health promoting effects of different varieties of grape have been documented.

1.8.2. Bioactivites of phenolic compounds in grape products.

Numerous phytochemicals are found in grapes. Among these, phenolic compounds are the most abundant and widely studied. Different phenolic compounds are present in grape skin, seeds, fruit stems, as well as juice and wine

product. The phenolic compounds mainly include anthocyanins, flavanols, resveratrol and phenolic acids (Xia et al., 2010; Dopico-Garcia et al., 2008; Novaka et al., 2008; Spacil et al., 2008). Anthocyanins are pigments that mainly exist in grape skins. Red grape skin has more total polyphenols compared to white grape due to the lack of anthocyanins in white grape varieties. Flavan-3-ols are more abundant in white varieties. Flavonoids are widely distributed in grapes, especially in seeds and stems. Georgiev et al summarized the most common flavonoids found in grapes: they are anthocyanins (3-O-monoglucosides or 3,5-O-diglucosides of malvidin, cyanidin, peonidin, delphinidin, pelargonidin and petunidin, as well as their acetyl-, p-coumaroyl- and/or caffeoyl-esters), flavonols (3-O-glycosides of quercetin, kaempferol, myricetin, laricitrin, isorhamnetin and syringetin), flavanols [(+)-catechin, (−)-epicatechin, (−)-epicatechin-3-O-gallate], dihydroflavonols (astilbin and engeletin) and proanthocyanidins [principally contain (+)-catechins, (−)-epicatechin and procyanidin polymers] (Georgiev et al., 2014; Chacona et al., 2009; Bagchi et al., 2000; Cantos et al., 2002). There are different phenolic compounds in different parts of the grape. The distribution and occurrence of polyphenols in grape are different.

During 2014, more than 7.8 million tons of grapes were grown commercially in the U.S. (http://www.agmrc.org/commodities__products/fruits/grapes-profile/), and more than 60% of these were used to make wine or juice. Grape pomace is the main byproduct of wine and juice production and is primarily composed of skin, seed, pericarp, and fruit-stem; it comprises approximately 25% of grape weight during grape processing. Grape pomace is currently used as fodder or fertilizer, or it

may be thrown away at a cost. The complex phytochemicals especially polyphenols in the seed and skin of grape pomace attracted more scientists to study the potential of turning the by-product waste to nutraceuticals and value adding products. On the other hand, grape pomace contains mixtures of flavonoid structures; different degrees of polymerization will occur during its secondary metabolism. This makes it difficult to establish which of the molecules are active. Furthermore, it is likely that the beneficial effects on human health may not be due to one molecule but a combination of effects of different phytochemicals (Pedrielli et al., 2002; Montagut et al., 2010).

1.8.3. Antioxidants of grapes

Numerous studies have shown that phenolic compounds from grape exerts positive benefits on human health; many of these compounds have been shown to have potent antioxidant activities. Significant antioxidant activity has been observed from pomace and seed flour extract of grapes (Ruberto et al., 2007; Parry et al., 2006). Grape seed extract may be useful for the prevention of certain metabolic syndromes and cardiovascular disease. Grape and grape products also possess other important properties including anti-radiation, anti-mutagenic, anti-inflammatory, anti-bacterial, and other beneficial effects (Bagchi et al., 2003; Halliwel et al., 1992; Belleville, 2002; Sun et al., 2002).

1.8.4. Protect against cardiovascular disease

Grape derived products (especially grape seed extract) are commercially available as over counter medicine from many pharmacies and grocery stores.

Abundant animal model and preclinical evidence suggests that grape seed extract has beneficial effects on the cardiovascular system. The polyphenol resveratrol is found in grape seed. Resveratrol has been identified as the potential cause of beneficial properties in red wine (Tomé-Carneiro et al., 2012; Bertelli et al., 1998). The beneficial effects of resveratrol were also reported as decreasing insulin resistance, reducing ischemic heart disease, preventing heart failure and avoiding hypertension (Petrovski et al., 2011; Bertelli et al., 1998). The polyphenols in grapes can reduce phosphodiesterase-5 activity, decrease the risk of cardiovascular disease (Dell et al., 2005), reduce platelet adhesion and aggregation and generate superoxide anion (Olaset al., 2008).

1.8.5. Protect against metabolic syndrome: Diabetes and obesity

Metabolic syndrome, especially diabetes and obesity are the most prevalent health problem affecting one third of U.S. population. Numerous flavonoid polyphenolics, including catechin, anthocyanin, isoflavones, quercetin, rutin are presented in grapes extracts. The mixture of these flavonoids structures in grape extracts were reported to have anti hyperglycemic effects. Abundant evidences suggest that flavonoids found in grapes play a key role in preventing the development of diabetes and obesity by acting as multi-target modulators with reducing the oxidative stress and lower the degree of systematic inflammation (Tsuda et al., 2012; Chuang et al., 2011), and improve the insulin resistance. Chuang et al (2012) screened different varieties of red, green, and blue-purple seeded and seedless California grapes to test the effects of freeze-dried grape powder and grape powder extracts on glucose tolerance and inflammation in obese mice, and found that grape

powder acutely improves glucose tolerance and chronically reduces inflammation in obese mice. The polyphenols in the grape have demonstrated dramatic inhibitory effects in streptozotocin-induced diabetic rat models (Al-Awwadi et al., 2004; Al-Awwadi and Bornet, 2004); reducing body growth, food intake and hyperglycemia, and increasing insulin sensitivity. Procyanidin, quercetin, resveratrol and proanthocyanidin derived from grape products have been widely studied. The benefit efficacy of stimulating glucose uptake in adipocyte cells, decreasing lipid peroxidation, increasing pancreatic glutathione levels, protecting pancreas β-cell function and protecting against β-cell loss, have been fully reported (El-Alfy et al., 2005; Baur et al., 2006; Lagouge et al., 2006). Studies on Carols Muscadine also demonstrated the inhibitory and anti-diabetic effects of the extracts on α-glucosidase and pancreatic lipase were investigated (You et al., 2011; Montaguta et al., 2010; Moreno et al., 2003). Muscadine possessed the strongest anti-diabetic activity in the EtOH fractions. Competitive mode of action was shown in their enzymatic inhibitions.

Diabetes and obesity could be manageable through a combination of diet, exercise and appropriate medications to lower adipogenesis. Many plant-based products show promising effects on the management and prevention of diabetes and obesity. Therefore, producing healthy foods or healthy agro-produced supplements would be the first line of defense against such metabolic syndrome. In search of such products, we recently discovered that grape pomace has great potential to prevent diabetes and obesity. Water extracts of grape pomace (GPE) significantly reduced blood glucose levels and decreased body weight when fed to mice. However, neither

the long term effects of GPE on diabetes and obesity control nor the molecular mechanisms involved has yet been fully elucidated. Such information is critical for its practical use in prevention and management of diabetes and obesity. Understanding the molecular mechanism(s) underlying GPE's role in preventing diabetes and obesity is important to guide its use in developing functional foods. The research plan proposed here is to define the long term effect(s) of GPE in preventing diabetes and obesity and unveil the possible molecular mechanism(s) of such prevention properties, focusing on different hormone peptides and the interrelationship among the central and peripheral neuropeptides.

1.9 Summary and hypothesis

Grape pomace, the by-product from the wine and juice industry, may have great potential beneficial health effects in preventing diabetes and obesity. However, the molecular mechanisms of such preventative properties are largely unknown. This research project was designed to elucidate such mechanisms at the physiological and molecular levels to provide evidence on grape pomace's roles in preventing diabetes and obesity. The designated research activities will target four specific objectives: 1) to characterize the action of grape pomace extract (GPE) in reducing postprandial hyperglycemia through inhibition of alpha-glucosidase; 2) to understand the mode of molecular action of GPE in control of diabetes; 3) using a 3T3-L1 pre-adipocyte cells and mouse model to examine the effects of long term GPE administration on reducing obesity through regulation of adipogenesis; and 4) to examine the effects of GPE on blood glucose and body weight control in genome wide with RNA-seq, whole transcriptome shotgun sequencing technology. Throughout these designated

research activities, we will provide molecular evidence toward understanding the mode action of GPE in preventing diabetes and obesity. Such knowledge will provide guidance for future studies in identifying bio-activate compounds from GPE to develop alternative medicines for the control of diabetes and obesity. More importantly, this study may lead to food industry applications in producing functional foods for diabetes and obesity populations.

The fundamental goal of this research is to begin to elucidate the molecular mechanism(s) of the GPE's preventive functions on the metabolic syndrome, diabetes and obesity, and to provide scientific evidence to guide its application for the use as functional food or alternative medicine. We hypothesize that GPE may prevent metabolic syndrome including diabetes and obesity through alternating the expression of genes, known or novel, on the signaling or metabolic pathways that lead to reduction of diabetes and obesity. The project focused on examining such molecular mechanism(s) and hypothesis that support the incorporation of GPE as an alternative therapy for controlling postprandial blood sugar, regulate insulin resistance and preventing diabetes and obesity. Four specific objectives, as described following, will be addressed for this rpoject.

Specific Objective 1: To characterize the action of GPE in reducing postprandial hyperglycemia through inhibition of alpha-glucosidase. GPE is rich in phytochemicals which could lower the blood glucose; however the fundamental mechanism remains unclear. Grape products may lower blood glucose through α-Glucosidase activity inhibition. This hypothesis will be tested using both an in vitro α-Glucosidase activity assay and an in vivo induced diabetes mouse model. Yeast α-

Glucosidase will be used to monitor GPE's inhibitory effects and the inhibitory kinetic assays will be conducted to determine the inhibition mode of GPE against a-glucosidase activity. For the in vivo assay, the STZ-induced diabetes mice will be orally administrated starches with or without GPE supplementation at 400 mg/kg body weight. Postprandial blood glucose will be monitored to determine the effects of GPE on suppression of postprandial hyperglycemia.

Specific Objective 2: To understand the mode of molecular action of GPE in control of diabetes.

It is well known that diabetes occurrence is due to the disturbance of glucose metabolism and homeostasis. Peptide hormones play important roles in regulating glucose metabolism and homeostasis. However, how the GPE will affect these regulatory systems is currently unknown. This objective will examine the effects of GPE in regulating these key processes using mouse model. Our hypothesis is that the prolonged oral administration of GPE will regulate glucose homeostasis in small intestine and pancreas in mouse. Mouse glycated hemoglobin (HbA1C) and molecular markers involved in peptide hormones (Ins, Lep, Adipo, Gip, Glp-1, Gcg, PAI-1 and Resis) will be analyzed at the transcriptional level to decode the effects of GPE in regulating these pathways. In addition, a shorter list of these markers (insulin, leptin, and adiponectin, ghrelin, GIP, GLP-1, glucagon, PAI-1, Resis) will also be tested in plasma at the protein level using ELISA assays. The results will be compared to elucidate the GPE's regulation mechanisms at both transcriptional and translational levels.

Specific Objective 3: To understand the mode of molecular action of GPE in

control of obesity. Consumption of a high fat diet can lead to obesity and insulin resistance (Qatanani and Lazar, 2007). Our current study approved that oral administration of GPE can alleviate obesity resulting from prolonged consumption of western-style diet. However, the mechanism(s) of this effect remain(s) unknown. In this objective, we will first focus on: 1) using adipocyte precursor cell model 3T3-L1, we will also examine the role of GPE in regulating genes expression involved in adipocyte differentiation, a major process to induce full maturation into adipocytes and generate fat mass; 2) the possible roles of GPE in regulating inflammation using a mouse model. The genes examined will include Adipoq, Cebpa, Pparg, Ppargc1a, (PGC-1a), Cebpα, Cebpβ, aP2, FAS, SREBP-1c, LPL, PGC-1α and PGC-1β; and 3) to examine the long term effects of GPE administration in reducing obesity through regulation of central neuropeptides.

Energy homeostasis is controlled by a complex neuroendocrine system consisting of peripheral signals, such as leptin and CCK, and central signals, in particular, neuropeptides. Several neuropeptides with anorexigenic (POMC, CART, and CRH) and orexigenic (NPY, AgRP, and MCH) actions are involved in this complex controlling system (Hillebrand et al., 2002). NPY/AgRP neurons exist as neuronal targets of leptin in the ARC where they are concentrated in the ventromedial ARC and regulate food intake. This objective will focus on investigating the expression of these hypothalamic neuropeptide to test if GPE can alter their expression and hence regulate energy homeostasis.

To test the hypothesis that GPE can regulate energy balancing, reduce body weight and acute blood glucose levels through controlling the central neuron system,

a 12-week mouse feeding experiment will be conducted to investigate GPE's long term effect(s) on food intake, blood glucose control, as well as lipid accumulation.

Specific Objective 4: By taking advantage of high throughput RNA sequencing technology, we will elucidate gene expression profiles at a genomic level caused by GPE treatment under high fat diet condition. Our specific focus will be on glucose and lipid metabolisms and their related signal transduction pathways.

1.10 Significant of research

Grape products have been known to be abundant in various bioactive compounds. Known beneficial effects of bioactive compounds from grape products include: functional foods (dietary fiber + polyphenols), food processing (biosurfactants), cosmetics (grapeseed oil + antioxidants), pharmaceutical/biomedical (pullulan) and supplements (grape pomace powder) (Dwywe et al., 2014). The annual production of grape pomace (along with its multitude of applications) create an opportunity to discover an unexploited market with great commercial potential in the prevention of chronic diseases such as diabetes and obesity.

Chapter 2: Grape Pomace Aqueous Extract Inhibits α-Glucosidase in vitro and Suppresses Postprandial Hyperglycemia in vivo

2.1 Abstract

Diabetes is a group of metabolic diseases, the serious long-term complications of which are a major cause of hospitalization and death. Postprandial hyperglycemia is a direct and independent risk factor for type 2 diabetes. Intestinal α-glucosidases are key enzymes in controlling carbohydrate digestion and glucose absorption. Recently, there has been growing interests in using food-based α-glucosidase inhibitors as alternative treatment for improving postprandial hyperglycemia and controlling blood glucose.

A specific grape pomace (Cabernet Franc), the by-product from wine and juice manufacturing was prepared in water. An in vitro yeast α-glucosidase inhibition assay, as well as the measurement of the kinetics of GPE-affected yeast α-glucosidases inhibition were conducted. Total carbohydrates were evaluated by HPLC. Mice were treated with streptozocin to induce diabetes. Oral GPE treatment and starch challenge were performed on STZ-induced diabetic mice to determine whether the administration of GPE can moderate postprandial hyperglycemia through the inhibition of intestinal a-glucosidases.

Grape pomace water extract (GPE) caused a dose-dependent reduction of yeast α-glucosidase activity with an IC50 value of 508.4μg equivalents/mL. Yeast α-glucosidase activity was reduced by 87.1% when treated with 1mg equivalent/mL GPE for 30 min. The enzyme kinetic study revealed that the GPE was a competitive

inhibitor of α-glucosidase. When consumed, GPE significantly decreased blood glucose by 43.6±7.5% in diabetic mice.

The results in this current study strongly support the potential use of GPE as an alternative to commercial α-glucosidase inhibitors used in diabetes prevention and treatment.

2.2 Introduction

Diabetes mellitus is a group of metabolic syndromes with a hallmark characteristic of hyperglycemia - increased blood glucose. The prevalence of diabetes is increasing dramatically worldwide, boosted by the increased consumption of refined sugar and processed foods with high calories, as well as by decreased physical activity (Stephens et al., 2006). Diabetes affects young as well as aging people. Based on the National Diabetes Statistics Report, 2014, approximately 29.1 million adults and children in the U.S. suffer from diabetes. This number is expected to rise to over 40 million (Stephens et al., 2006; National Diabetes Statistics Report, 2014). The serious long-term complications from diabetes are major causes of hospitalization and death. The economic cost of treating diabetes and its related complications reached $245 billion in 2012. Although long-term complications of diabetes develop gradually, untreated diabetes can cause many complications and disabilities, even death. Over time, hyperglycemia can damage the nerves and small blood vessels of the eyes, kidneys, and heart. Furthermore, improperly treated diabetes can cause serious long-term complications, including diabetic retinopathy, nerve damage, stroke, cardiovascular disease, blindness, kidney failure, and macro and micro-vascular damage, nephropathy, impaired immune functions, and lower limb amputation (Biessels et al., 2004; Martinez-

Tellez et al., 2005).

Diabetes is sometimes treatable and preventable. Conventional anti-diabetic drugs play a key role in diabetes treatment. However, side effects and other disadvantages of conventional anti-diabetic drugs are very common. These include unwanted weight gain (Prabhakar et al., 2011), stomach pain and bloating, liver problems, skin reactions, and swelling due to fluid build-up. Other symptoms such as malaise, fatigue, nausea, and weakness have also been reported. Medicinal plants, as well as agricultural by-products, have shown promising effects in the management of diabetes. Many have been traditionally used since ancient times to treat a great variety of human diseases. Several medicinal plants have been investigated for their beneficial effects in the treatment of different types of diabetes (Prabhakar et al., 2011). Bitter melon (Mormodicacharantia) has long been used in China and India as a vegetable and is reported to help control blood glucose levels. Hypoglycemic effects of bitter melon have been demonstrated in cell culture, animal models and human clinical studies. It's powerful insulin lowering properties are currently being considered as an effective treatment for diabetes (McCarty, 2004; Krawinkel and Keding 2006). Panax Ginseng was used as a holly panacea in oriental cultures for thousands of years, especially for its healthy glucose level maintenance property. Chinese or Korean ginseng has the highest therapeutic potency among all ginseng varieties. Several studies have found that Korean red ginseng significantly stimulates insulin release from isolated rat pancreatic islets, decreases serum levels of glucose, and glycosylates hemoglobin A1c (HbA1c) in streptozotocin (STZ)-induced diabetic rats in a dosage-dependent manner (Kim 2008; Anoja et al., 2002; Hui et al., 2009).

Diabetes is, in part, related to the amount of carbohydrate in the diet. Complex carbohydrates are digested primarily in the lumen of the small intestine by α-amylase to yield linear oligosaccharides and branched isomaltose. These are then further hydrolyzed by α-glucosidase to release glucose and fructose which will be absorbed into the bloodstream (Casirola et al., 2006). Alpha-glucosidase is a key intestinal enzyme presented in the brush-border of the small intestine mucosa. It controls the rate of carbohydrate absorption and adjusts postprandial glycaemia. Alpha-glucosidase has been used widely as a target by several commercial antidiabetic medicines (for example acarbose, miglitol, and voglibose) in the regulation of blood glucose and treatment of type 2 diabetes (Yamazaki et al., 2007; Van et al., 2006; Casirola et al., 2006; Van et al., 2005; Nakamura et al., 2005; Fujisawa et al., 2005, Yamamoto and Otsuki 2006; Chiasson et al., 2004).

Studies of the anti-hyperglycemic effects of α-glucosidase inhibitors have demonstrated its efficacy in reducing postprandial blood glucose, improving glycated haemoglobin (HbA1c), and attenuating pancreatic islet damage (Negishi et al., 1996; Yamazaki et al., 2007). Alpha-glucosidase inhibitors diminish postprandial hyperinsulinemia and improve insulin sensitivity through increasing insulin receptor sensitivity (Yamazaki et al., 2007). α-glucosidase inhibitors stimulate insulin secretion by prolonging the effect of the key incretin hormone glucagon-like peptide-1 (GLP-1) (Yusuke et al., 2009), and by preventing the long-term complications of type 2 diabetes (Lee et al., 1982). The alpha-glucosidase inhibitor acarbose can be used as a monotherapy. It also works well in combination therapy with other commercially available anti-diabetic drugs. Synergistic effects have been reported

with the combination of acarbose and DPP-4 inhibitors (inhibitors of dipeptidyl peptidase 4 reduce glucagon and blood glucose levels) (Aoki et al., 2012; Narita et al., 2012; Qualmann et al., 1995). Even though glucose lowering drugs were effectively used to manage blood glucose, it is very challenging to find a medicine that has no side effects.

Cultivation of grapes began 6,000 to 8,000 years ago in the Near East. It is one of the oldest fruit crops that has been domesticated and adopted by all cultures. Several phenolic compounds that can improve human health are found in grapes. The well-known “French Paradox” refers to the epidemiological observation of the high intake of dietary cholesterol and saturated fat and the low incidence of coronary heart disease (CHD) and related deaths present in the Mediterranean region (Renaud et al., 1992). The routine consumption of red wine is considered to be the factor lowering the development of coronary heart disease. This attracted more attention from scientists seeking to define the chemical composition of grapes. A large number of different natural grape products are widely accepted in the nutritional products market. The beneficial effects of using flavonoids and poly-phenolic compounds from grape and grape-derived products have been well studied (Quiñones et al., 2013, Kim et al., 2014). Different phenolic compounds are present in grape skin, seeds, and fruit stems, as well as juice and wine products. The phenolic compounds mainly include anthocyanins, flavanols, resveratrol, and phenolic acids (Xia et al., 2010; Dopico-Garcia et al., 2008; Novaka et al., 2008; Spacil et al., 2008; Montaguta et al., 2010; Al-Awwadi et al., 2004). Anthocyanins are pigments that mainly exist in grape skins. Red grape has more total polyphenols compared to white grape due to the lack of

anthocyanins in white grape varieties. On the other hand, flavan-3-ols are more abundant in white varieties.

During 2012, more than 7.3 million tons of grapes were grown commercially in the U.S. (http://www.agmrc.org/commodities products/fruits/grapes-profile/), and over 60% of these were used to make wine or juice. Grape pomace is the main byproduct of wine and juice production and is primarily composed of skin, seed, pericarp, and fruit-stem; it comprises approximately 25% of grape weight during grape processing. Grape pomace is currently used as either fodder or fertilizer, or it may be discarded at a cost. The complex phytochemicals, especially polyphenols, in the seed and skin of grape pomace have promoted the study of the potential of turning this waste by-product into nutraceuticals and value-added products. Grape pomace contains mixtures of flavonoid structures, and different degrees of polymerization will occur during its secondary metabolism. This makes it difficult to establish which of the molecules are active. Furthermore, it is likely that the beneficial effects on human health may not be due to one molecule but a combination of effects of different phytochemicals (Pedrielli et al., 2002; Montagut et al., 2010).

Grape pomace may have great potential health benefits in preventing diabetes. However, the molecular mechanisms of such preventative properties are largely unknown. In our previous study (Parry et al., 2011), grape pomace from variety Tinta Cao was investigated. High antioxidant activities, inhibition of the proliferation of HT-29 and Caco-2 colon cancer cells through the triggering of apoptosis pathway, and phytochemical compositions have been reported. In our current study, the anti-diabetic properties of grape pomaces were investigated. The in

vitro action of GPE in reducing postprandial hyperglycemia through inhibition of alpha-glucosidase was examined. In vivo studies of both diabetic mice and healthy human subjects were conducted. GPE's inhibitory effect on postprandial hyperglycemia and the effects of GPE on acute insulin secretion and effects on lipid metabolism in human pilot trials were observed.

2.3 Materials and methods

Cabernet Franc grape pomace samples (fermented for 2 weeks) were obtained from Chrysalis Vineyards, Virginia in 2011. Dulbecco's Modified Eagle's Medium (DMEM), antibiotic/ antimycotic, fetal bovine serum (FBS), and 0.25% trypsin with 0.9 mM EDTA were purchased from Invitrogen (Carlsbad, CA). IEC-6 cell lines were purchased from American Type Culture Collection (Rockville, MD). Acarbose and p-Nitrophenyl- β- D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO).

Sample preparation and extractions

Grape pomace samples were air dried under low pressure. Dried pomace was milled to 40 mesh size using a Scienceware Bel Art Micromill, (Pequannock, NJ). The milled grape pomace was then extracted with water (1:10 m/v) by shaking for 2 hours at ambient temperature. Following extraction, the mixture was centrifuged at 4,000 rpm at 20oC for 30min. Supernatants were collected and freeze dried in the bench top freeze drier (AdVantage 2.0 BenchTop Freeze Dryer / Lyophilizer, SP

Scientific) to obtain GPE.

In vitro yeast α-glucosidase inhibition assay

Yeast α-glucosidase (Sigma Aldrich Chemical Co, USA) was dissolved in phosphate buffer (pH 6.8) at a concentration of (1 U/mL). p-Nitrophenyl- β- D-glucopyranoside (pNPG) (Sigma Aldrich Chemical Co., U.S.A.) was used as the substrate for the yeast α-glucosidase activity assay. An inhibition assay was conducted according to the protocol described by Hogan et al, (2010) with a minor modification. In brief, the mixture of 80µl sample solution with 20µl enzyme solution (1 U/mL) was first incubated for 3 minutes at 37oC. After incubation, 100µl of 4 mM p-nitrophenyl-α-d-glucopyranoside (pNPG) solution in phosphate buffer (pH 6.8) was added to the reaction and then incubated for additional 30 minutes at 37oC. The α-glucosidase activity was quantified by measuring the p-nitrophenol release from pNPG at 405 nm wave-length. The phosphate buffer (pH 6.8) was used as control. All measurements were taken in triplicate. The inhibition of Alpha-glucosidase activity was expressed as percentage of the control using the following formula:

% Inhibition= [Abs (Control)-Abs (Sample)]/Abs (Control) X100

Kinetics of GPE-affected yeast α-glucosidases inhibition

To further determine the time and dose effects of GPE on yeast a-glucosidase inhibition, a series of enzymatic reactions with different dosages of GPE (from 0 to 1000 µg/ml) were conducted in a 96-well plate. Reactions were dynamically measured at 405 nm for 75 min with a 5-minute interval. The combinations of

different concentrations of substrate pNPG (0.4, 0.5, 1, 2 mM) and different concentrations of the GPE (0, 100, 200, 300, 400, and 500 μg/ml) were tested. The double reciprocal Lineweaver–Burke plots were prepared to illustrate the inhibition pattern. The IC50 value is defined as the concentration of an inhibitor required to inhibit 50% of the α-glucosidase activity, which is a measure of the inhibitor's relative effectiveness. Acarbose was used as a positive control.

Determination of total carbohydrate

Total carbohydrate was determined by the Phenol-Sulfuric Acid Method described by Nielson (Nielson et al., 2010). Trehalose was used for standardization. Individual sugar content was measured using HPLC according to Bogdanov (Bogdanov, et al., 1997) on a high-pressure SHIMADZU liquid chromatograph equipped with LC-10ATVP liquid chromatograph pumps, DGU-14A degasser, CTO-10AVP column thermostat, RID-10A refractometric detector, POL-LAB CHROMA 2001 software and SUPELCOSIL LC-NH2), 25 cm x 4.6 mm, 5 μm chromatograph column. 20 μl samples were injected onto the column and separation was conducted at 30°C with the mobile phase made up of acetonitrile: water (8:2) at a flow rate of 1.3 ml/min. The identification of sugars in GPE was conducted by comparing retention times of individual sugars in the reference vs. tested solution. The quantitative assays were made of the following carbohydrates: fructose, glucose, sucrose, turanose, maltose, isomaltose, and trehalose.

STZ induction of diabetic mice

Six-week old male C57BL/6NCr mice, (National Cancer Institute, Frederick,

MD, USA) were housed in clean cages at 12-hour light dark cycle at 20°C to 22°C with 2 mice/cage with food access ad libitum. The animals were used after a 2-week acclimatization period before starting the experiment. The mice were maintained on rodent feed (Harlan Tekland Global Diets 2018 rodent diet containing 60% of calories from carbohydrate, 23% of calories from protein, and 17% of calories from fat; digestible energy of 3.4 Kcal/g, Madison WI, USA) with continuous access to tap water for the duration of the experiment. Animal husbandry, care, and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia. Streptozotocin was dissolved in ice-cold 100mM sodium citrate buffer (pH 4.5) and injected intraperitoneally immediately at a dose of 150mg/kg body weight. Fasting blood glucose (FBG) was monitored on day 7 post injection. Values higher than 126 mg/dL were considered to be diabetic as previously described by Hogan (Hagon et al., 2010) and assigned to one of 3 groups (n = 10).

Oral GPE treatment and starch challenge

Diabetic mice were fasted for 14-hours in freshly cleaned cages with free access to water before the experiment. Basic Corn Starch, (Cargill incorporated, Cargill Gel # 03420) was suspended in 0.1 M sodium citrate buffer (pH 4.5) with 25 mg/ml and vortexed vigorously. GPE was dissolved in starch-sodium citrate solution (250 mg/ml). Mice in the control group were given 0.2 mL of starch sodium citrate solution by oral gavage while the GPE treatment group mice were administered with the mixture of GPE and starch suspension 0.2 ml. Acarbose (Sigma Aldrich Chemical Co, USA) was suspended in the same starch solution and fed by oral gavage at 75 mg/kg BW in the positive control group.

Samples of approximately 5 μL of whole blood were collected from the tail vein of each mouse at 7 time points 0, 15, 30, 45, 60, 90, and 120 minutes after treatment. Blood glucose was measured with a blood glucometer and accompanying test strips (ACCU-CHEK Meter®, Roche Diagnostics, Kalamazoo, MI). The area under the glucose tolerance curve (AUC0-120 min) was calculated using SPSS software. The total anti-hyperglycemic response (AUC0-120 min) was expressed as percentage of inhibition compared with the control group.

Statistical Analysis

Tests were conducted in triplicate with data reported as mean ± standard deviation. Analysis of variance (ANOVA) and Tukey's post hoc analyses were used to determine differences among group means. Pearson Correlation Coefficients were used to determine correlations among means. Statistical significance was defined as P value less than 0.05.

2.5 Results

In vitro yeast α-Glucosidase inhibition by GPE

To examine whether GPE can inhibit α-glucosidase activity, enzymatic inhibitory assays were performed. The inhibitory effects of GPE on yeast α-glucosidase activity at different concentrations were observed at different reaction times (Fig. 2-1). A dose-dependent pattern of the α-glucosidase inhibition was found (Fig. 2-1a). Inhibitory action of GPE against yeast α-glucosidase started to show when the enzymatic reaction was treated with 200μg/ml GPE, and at

400μg/ml GPE level, 37.9±8.1% of the α-glucosidase activity was inhibited. An IC50 value of 508.4μg/mL was calculated. Yeast α-glucosidase activity was reduced by 87.1±1.0% after 30 min reaction at 1mg mL. Furthermore, GPE produced a dose-dependent pattern of yeast α- glucosidase inhibition (Fig. 2-1a). To determine the GPE's mode action on α-glucosidase, a Lineweaver-Burk plot was performed. A strong competitive inhibition was observed indicating that GPE inhibits α-glucosidase through binding to the active site on the enzyme and preventing further substrate binding.

Enzymatic inhibition of α-Glucosidase was used for screening the potential capacity of GPE on controlling blood glucose through down regulation of carbohydrate digestion. To determine the GPE's mode action towards α-glucosidase, a Lineweaver-Burk double reciprocal plot was performed (Fig. 2-1c). The maximum velocity (Vmax) of the reaction was unchanged, and this was confirmed by the intersecting point for different doses of GPE occurring on the 1/v axis (1/vmax). The enzyme kinetic study revealed that GPE contains active compounds that bind to the catalytic site of the enzyme α-glucosidase and prevent further binding of the substrate to the enzyme.

Determination of total carbohydrates

The phenol-sulfuric acid method was used to determine total carbohydrate in GPE. This colorimetric method detected virtually all classes of carbohydrates in a sample, including mono-, di-, oligo-, and polysaccharides. 48.3± 0.5 mg/g of total carbohydrate were detected. Individual sugar content assayed by HPLC identified 17.5 mg/g glucose, 21.0 mg/g fructose, 1.5 mg/g sucrose, 8.0 mg/g trehalose, and

0.17 mg/g rafinose. These results indicated that GPE contained very little carbohydrate and would not be expected to affect postprandial blood glucose significantly.

Effects of the grape pomace treatment on postprandial blood glucose by oral administration of GPE in STZ-treated mice.

Changes in postprandial blood glucose were measured after carbohydrate load to the fasted diabetic mice with or without co-administration of GPE. The commercial anti-diabetic drug acarbose was used as positive control. Blood glucose increased by 49.6±7.1 mg/dl after 30 min administration of corn starch to the control group. Only a 29.3±6.2 mg/dl blood glucose increase was observed in corn starch with 250 mg/kg bw GPE group. The highest inhibitory effect was observed at the 60-min time point with 53.1% of inhibition by GPE compared to the control group (Fig. 2-2a). A similar result was observed in the group of mice gavaged with corn starch and acarbose (75 mg/kg bw). GPE also exerted a significant decrease ($p<0.05$) of the increment in blood glucose; AUC0-120 min (Area Under the Curve for 120 min) (Fig. 2-2b). When corn starch was loaded, blood glucose AUC0-120 min in the GPE and acarbose groups was reduced 47.7% and 43.4% respectively. No significant difference between GPE and acarbose tested groups was observed.

2.6 Discussion

In the present study we examined the properties of GPE on α-glucosidase activity inhibition. Yeast α-glucosidase was used as a model for investigating the

potential capacities of GPE on α-glucosidase activity in vitro. GPE also exhibited inhibitory effects on mammalian α-glucosidase activity and suppressed postprandial hyperglycemia in STZ-induced diabetic mice in vivo. Similar results were reported by Hogan (Hogan et al. 2010). This prompted us to investigate whether grape pomace can be used to prevent diabetes through α-glucosidase inhibition in humans by lowering plasma glucose, prolonging glucose absorption, and suppressing peak levels of postprandial blood glucose for the future study.

Complex carbohydrates cannot be absorbed in the small intestinal lumen and transported into blood circulation until the polysaccharides are digested and broken down into monosaccharides. Small intestinal alpha-glucosidase plays a key role in carbohydrate metabolism. Alpha-glucosidase inhibition has been used widely as a good model for investigating the effects of antidiabetic compunds. Alpha-glucosidase inhibition can delay the carbohydrate digestion and glucose absorption, eventually lowering postprandial hyperglycemia. Pre-diabetes occurs when a patient's blood glucose is higher than normal but not yet high enough to be diagnosed as diabetes (between 100 and 125 mg/dL). Pre-diabetes leads to a higher risk for developing type 2 diabetes. 15% to 30% of people with pre-diabetes will develop type 2 diabetes within five years without lifestyle changes to improve their health. Recently, there has been a growing interest in food based α-glucosidase inhibitors, which provide a potential therapeutic approach in the prevention of diabetes among healthy people and in the management of pre-diabetic patients and type 2 diabetes. Our results suggest that grape pomace, a food based byproduct, could be an excellent candidate as an anti-diabetic treatment. Few publications report the bioactive compounds in different grape

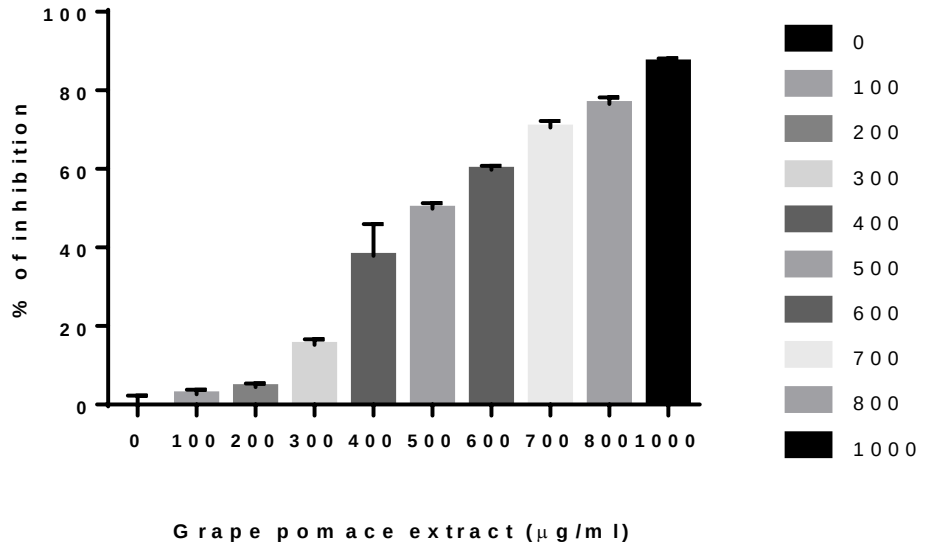
varieties, and the phenolic contents are the major contributors for the natural α-glucosidase inhibitors (Horgan et al., 2010). In our previous study with Tinta Cao pomace extract (Parry et al., 2011), high antioxidant activities were determined using an oxygen radical absorption capacity (ORAC) assay along with DPPH• and ABTS•+ scavenging capacities. Phytochemical compositions including total phenolic content (TPC); individual phenolic acids were also studied, and the results demonstrated that grape pomace extract has the potential to be a bioactive food ingredient. Similar results were reported with grape products (Gonzalez-Paramas, et al., 2004; Zhang et al., 2011). Catechin, quercetin and ellagic acid were reported to exhibit very strong anti-diabetic activities in muscadine against α-glucosidase (Yu et al., 2012).

The goal of controlling diabetes is to manage blood glucose so that it stays within healthy levels. Dietary change is recommended to control type 2 diabetes in patients with or without insulin intervention. Numerous studies using cultured cell lines and rodents have been reported for the potential diabetic-prevention effects of grapes, grape skin, grape seeds, and grape polyphenol extracts. However, clinical trials addressing the effect of grape pomace on regulating blood glucose are limited. The effects of grape pomace water extract on improvement of postprandial hyperglycemia in vivo with human subjects will be tested. Alpha-glucosidase inhibitors such as Acarbose and Miglitol were widely used for managing diabetes; however, there were strong side effects associated with taking these drugs (Fujisawa et al., 2005). Alternative to these synthetic conventional diabetes medicines, natural products have shown promising effects on diabetes management. Thousands of herb formulas and dietary supplements are available on the market for diabetic patients

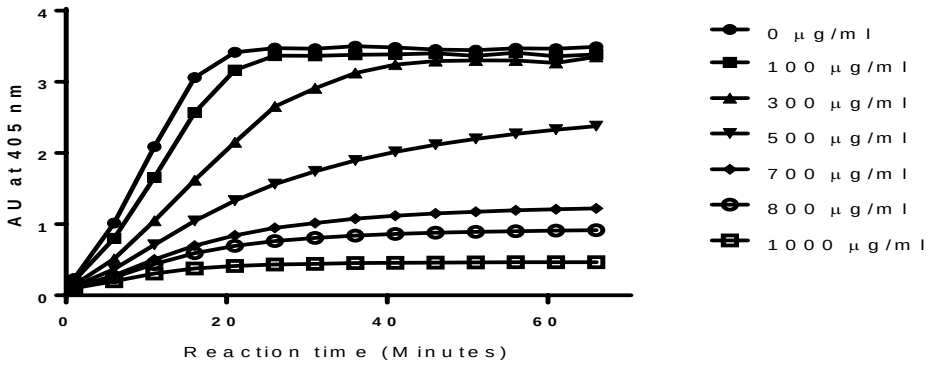
(Prabhakar et al., 2011; Casirola et al., 2006). Dietary phytochemicals work through various metabolic pathways to reduce postprandial hyperglycemia. Plant products with hypoglycemic activity have been the subject of numerous studies. These natural medicines regulate blood sugar through a variety of mechanisms including carbohydrate metabolism alternation, ß-cells apoptosis prevention (which is one of the key points in triggering diabetes), insulin releasing activity growth, and glucose uptake improvement and utilization. Understanding the long term effects of grape pomace on regulating carbohydrate metabolism and the molecular mechanism(s) involved is an important aspect and should be the focus of future studies.

2.6 Conclusion

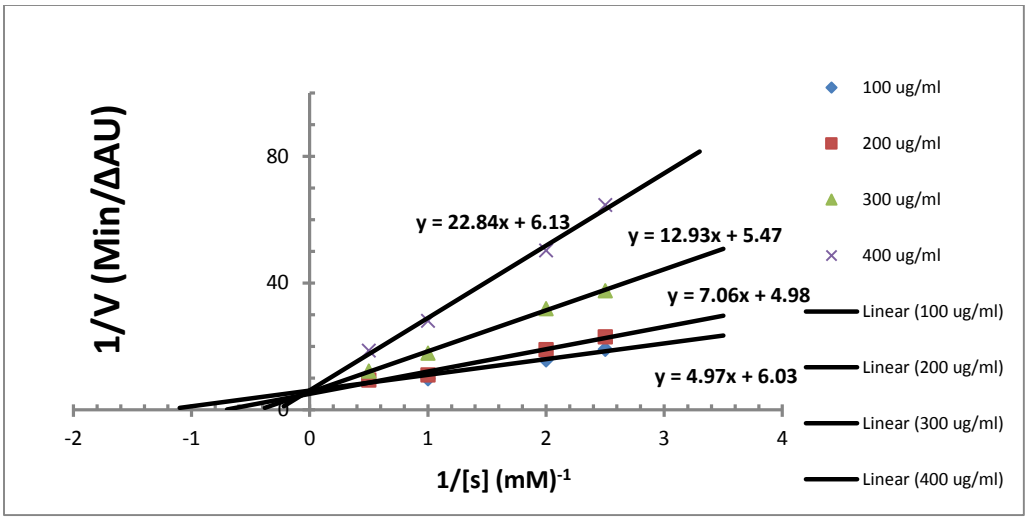
Grape pomace suppressed postprandial hyperglycemia through inhibitory effects on α-glucosidase activity. GPE also reduced acute blood glucose in vivo in STZ induced-diabetic mice. This study provides basic knowledge and information on the potential use of GPE in functional foods for preventing diabetes and possibly obesity as well. The long term effects of GPE on control of blood glucose and lipid metabolism warrants further investigation.


100
80
60
40
20
0
% of inhibition
0 100 200 300 400 500 600 700 8001000
Grape pomace extract (µg/ml)
0
100
200
300
400
500
600
700
800
1000

(A)

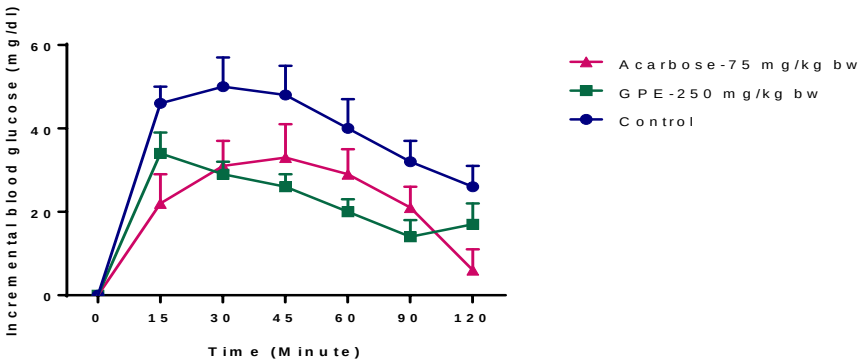

4
3
2
1
0
AU at 405 nm
0
20
40
60
Reaction time (Minutes)
0 µg/ml
100 µg/ml
300 µg/ml
500 µg/ml
700 µg/ml
800 µg/ml
1000 µg/ml

(B)

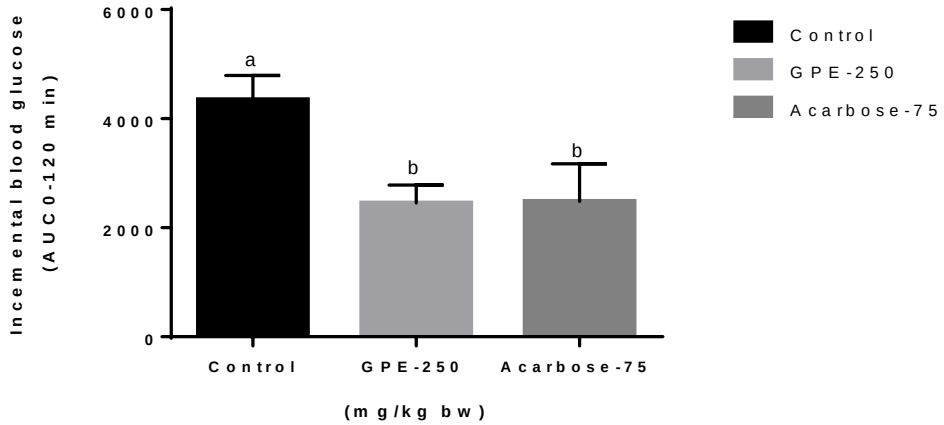


(C)

Figure 2- 1 Kinetics studies of Cabernet Franc pomace extract on yeast α-glucosidase inhibition at different concentrations.a. Cabernet Franc pomace extract exerted a dose-dependent pattern on yeast α-glucosidase inhibition at 37ºC for 30 min. b. Time course responses during 66 minutes of different concentration of the pomace extracts on the enzyme activity; GPE exhibits a consentration-dependent manner on yeast α-glucosidase inhibition. c. Lineweaver-Burk plots of kinetic analysis of yeast α-glucosidase inhibition at different concentration of substrate pNPG (0.4, 0.5, 1, 2 mM)) with different concentrations (100-400 µg/ml) of Cabernet Franc pomace extract.



(A)



(B)

Figure 2- 2 Changes in postprandial blood glucose level (incremental AUC0-120 min) affected by administration of GPE in STZ-induced diabetic mice. a. Modified 2h OGTT (Oral Glucose Tolerant Test) shows the glycemic response curve in diabetic mice after corn starch challenge. b. Increment of the blood glucose $AUC_{0\text{-}120}$ min (Area Under the Curve for 120 min) after administration of GPE. Results are expressed as Mean± SEM. *: $p < 0.05$ (n = 10)

Chapter 3: Grape Pomace Aqueous Extract (GPE) Prevents Western High Fat Diet-induced Diabetes and Attenuates Systemic Inflammation

3.1 Abstract

Diabetes mellitus is a group of metabolic syndromes with a hallmark of hyperglycemia. Serious long-term complications from diabetes are a major cause of hospitalization and death. We recently discovered that grape pomace, the by-product from the waste of the wine and juice industries, has great potential to prevent diabetes. In this study, we examined the potential use of grape pomace in controlling high blood glucose in response to Cabernet Franc Grape pomace aqueous extract (GPE). Both streptozotocin (STZ)-induced diabetic mice and non-STZ treated mice were fed with high fat diet that was supplemented with 2.4 g/kg GPE for 12 weeks. GPE had no significant effect on STZ-induced diabetic mice. However, GPE significantly reduced blood glucose by 16.1% ($p<0.05\%$) in non-STZ treated HFD-fed mice following a 10-week feeding period when compared with high fat diet controls. GPE reduction of hyperglycemia also promoted a significant reduction of GHbA1c accumulation. Circulating peptide hormones related to glucose homeostasis, including GLP-1, glucagon, DPP-4 and insulin, were drastically altered by GPE. Moreover, GPE attenuated the expressions of insulin, glucagon, and several gut hormones at the mRNA level. In addition, GPE significantly down regulated 5 biomarker genes for systematic inflammation. Taken together, our results highlight a role of GPE as a potential alternative approach to control diabetes epidemic.

3.2. Introduction

Grape pomace is the main by-product of wine and juice production. It is primarily composed of skin, seed, pericarp, and fruit-stem; and it comprises approximately 25% of grape weight during grape processing. The complex phytochemicals (especially polyphenols) in grape pomace have attracted many scientists to study the potential of turning the by-product waste to a nutraceutical and value added product. Numerous phenolic compounds, especially flavonoids, are widely distributed in grapes. Many of these have been shown to have beneficial health effects. The most common phenolic compounds found in grapes with bioactivities are anthocyanins, flavonols, flavanols, dihydroflavonols and proanthocyanidins (Georgiev et al., 2014; Chacona et al., 2009; Bagchi et al., 2000; Cantos et al., 2002). Grape derived products are commercially available as over-the-counter medicines from many pharmacies and grocery stores. Animal and preclinical studies suggest that grape seed extract has preventive effects on certain metabolic syndromes and protects the cardiovascular system (Georgiev et al., 2014). Furthermore, resveratrol is a major polyphenol that is thought to be a potential contributor of several beneficial properties of red wine (Tomé-Carneiro et al., 2012; Bertelli et al., 1998). Resveratrol decreases insulin resistance, prevents heart failure, and prevents hypertension (Petrovski et al., 2011; Bertelli et al., 1998). It decreases the risk of cardiovascular disease (Dell et al., 2005), and reduces platelet adhesion, aggregation and generation of superoxide anion (Olaset al., 2008).

Diabetes is a group of metabolic syndromes with a hallmark of hyperglycemia. It is caused by an interruption in carbohydrate, protein and lipid metabolism. Lack of insulin secretion and increase of insulin resistance (Stumvoll et al., 2005). The network

of insulin, glucagon and multiple gastrointestinal hormones play key roles in the regulation of blood glucose homeostasis (Aronoff et al., 2004). Glucagon-like peptides (GLP-1 and GLP-2) and glucose-dependent insulinotropic peptide (GIP) are the major incretins that stimulate the secretion of insulin (Zunz and La Barre, 1929). GLPs are released from the lower gastrointestinal tract following food intake and play dual roles in the regulation of blood glucose concentrations through their concurrent insulin tropic and glucagonostatic actions (glucose-lowering effect) (Näslund et al., 1999; Ørskov, 1999). GIP is recognized as a physiologic regulator of food intake contributing to the control of obesity (Arora, 2006). Energy balance and glucose homeostasis have become central targets for the design of pharmacological agents needed to prevent and treat diabetes.

Abundant evidence suggest that grape flavonoids play a key role in preventing the development of diabetes by acting as multi-target modulators that reduce oxidative stress, lower the degree of systematic inflammation (Tsuda et al., 2012; Chuang et al., 2011), and improve insulin resistance and anti-hyperglycemic effects. In obese mice, grape powder has been shown to acutely improve glucose tolerance and chronically reduce inflammation (Chuang et al., 2012). In streptozotocin (STZ)-induced diabetic rat models (Al-Awwadi et al., 2004; Al-Awwadi and Bornet, 2004), polyphenols in the grape could reduce body weight, food intake and hyperglycemia, and increase insulin sensitivity. Grape pomace byproducts from the Norton grape have been reported to contain significant amount of antioxidants (Hogan et al., 2010), and consumption of Norton grape pomace for 3 months exerted an anti-inflammatory effect in a diet induced mouse obesity model (Hogan et al., 2011).

Diabetes can be manageable through a combination of diet, exercise and appropriate medications. Many plant-based products show promising effects on the management and prevention of diabetes. Producing healthy foods or healthy agro-produced supplements could be a first line of defense against diabetes. However, the molecular mechanisms of such preventative properties are largely unknown. In our previous study (Parry et al., 2011), the grape pomace from the variety Tinta Cao was investigated. High antioxidant activities, and inhibition of the proliferation of HT-29 and Caco-2 colon cancer cells through triggering apoptosis were demonstrated. The in vitro action of grape pomace aqueous extract (GPE) in reducing postprandial hyperglycemia through inhibition of alpha-glucosidase was also demonstrated. In addition, in vivo studies with diabetic mice and healthy human subjects revealed GPE's effect on postprandial hyperglycemia, acute insulin secretion and lipid metabolism (Li et al., Submitted). However, neither the long-term effects of GPE on diabetes nor the molecular mechanisms involved have yet been fully elucidated. Such information is critical for its practical use in the prevention and management of diabetes. In our current study, the anti-diabetic properties of grape pomaces were investigated.

3.3. Materials and Methods

Sample Preparation and Extractions

Cabernet Franc grape pomace samples (fermented for 2 weeks) were obtained from Chrysalis Vineyards, Virginia in 2011. Grape pomace was air dried under low pressure and milled to 40 mesh size using a Scienceware Bel Art Micromill (Pequannock, NJ). The milled grape pomace was then extracted with water (1:10 m/v)

with shaking for 2 hours at ambient temperature. Following extraction, the mixture was centrifuged at 4,000 rpm at 20^{o}C for 30 min. Supernatants were collected and freeze dried in the bench top freeze drier (AdVantage 2.0 BenchTop Freeze Dryer / Lyophilizer, SP Scientific) to obtain GPE.

STZ and High Fat Diet (HFD)-induction of Hyperglycemia in Mice

Approximately sixteen-week old male C57BL/6NCr mice (National Cancer Institute, Frederick, MD, USA) were housed in clean cages at 12-hour light dark cycle at 20°C to 22°C with 5 mice/cage. Animal husbandry, care, and experimental procedures were approved by the Institutional Animal Care and Use Committee at University of Georgia. Streptozotocin was dissolved in ice-cold 100 mM sodium citrate buffer (pH 4.5) and injected intraperitoneally at a dose of 150 mg/kg body weight. A total of 24 STZ-induced diabetic mice with fasting blood glucose higher than 120 mg/dL were used. 12 mice without STZ induction served as STZ treatment control. All 36 mice were maintained on rodent chow (8728C Teklad Certified Rodent Diet, Harlan Laboratories, Inc, Frederick, MD) until the experiment started. Two groups of STZ-induced mice (n=12 in each group) and non-STZ mice (n=6 in each group) were fed with either 1) high fat diet (D04011601), or 2) high fat diet mixed with GPE at 2.4 g/kg diet (D12121001). The composition of the animal diet is summarized in Table 3-1. Mice were fed with their respective diets for 12 weeks with water available *ad libitum*. Approximately 5 μL of whole blood was collected from the tail vein of each mouse once a week. Blood glucose was measured with a blood glucometer and accompanying test strips (ACCU-CHEK Meter®, Roche Diagnostics, Kalamazoo, MI). Body weight

was measured weekly.

Animals and Tissue Collection

At the end of the feeding study, diet was removed 12 h prior to sacrifice. Mice were anesthetized with CO_2, and blood was collected by cardiac puncture into glass tubes for serum isolation. Seperate plastic vials that had been previously rinsed with potassium EDTA solution were used for HbA1c measuraments. The serum was separated after centrifugation at 1,000x g for 15 min at 4°C and stored for later use. Small intestine and pancreas were collected, immediately frozen in liquid nitrogen and stored in -80^{o}C for analysis.

Mouse Glycated Hemoglobin (HbA1C) Determination

Glycosylated (or glycated) hemoglobin (hemoglobin A1c, Hb1c, or HbA1c, A1C) is a form of hemoglobin used primarily to identify the average plasma glucose concentration over prolonged periods of time. It is commonly used as a golden rule for measurement of blood glucose management in type 2 diabetes. HbA1c was measured with a mouse glycated hemoglobin A1c (GHbA1c) ELISA Kit (Biotrend, CA, USA). Briefly, the microtiter plate was pre-coated with an antibody specific to GHbA1c. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for GHbA1c and Avidin conjugated to Horse-radish Peroxidase (HRP), and incubated for 60 minutes. The TMB (3,3'5, 5' tetramethyl-benzidine) substrate solution was then added to each well following several washes. The enzyme-substrate reaction was terminated by the

addition of a sulphuric acid solution and the color change was measured at a wavelength of 450 nm ± 2 nm using a spectrophotometer. The concentration of GHbA1c in the samples was then determined by comparing the O.D. of the samples to the standard curve.

RNA Isolation and Gene Expression Analysis

Total RNA was extracted from small intestine and pancreas with RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of RNA were determined by measuring the absorbance in a Nano drop spectrophotometer. Total RNA was treated with DNase I at room temperature for 15 min to remove genomic DNA contamination. RT2 First Strand Kit from Qiagen (Qiagen, Hilden, Germany) was used to synthesize first strand complementary DNA (cDNA). The gene expression levels were analyzed by Quantitative real-time RT-PCR conducted on the Bio-Rad CFX-96 Real-Time PCR System using RT2 SYBR Green Master mix (Bio-Rad Laboratories, Hercules, CA). The customized Mouse Diabetes RT² Profiler™ PCR Array containing 84 genes related to the onset, development, and progression of diabetes was used to examine the expression patterns of the selected genes. The marker genes for diabetes and glucose homeostasis, inflammatory mediators tested are listed in Table 3-2. Hot-Start DNA Taq Polymerase was activated by heating at 95°C for 10 min and real time PCR was conducted for 40 cycles (15 s for 95°C, 1 min for 60°C). All results were obtained from at least three independent biological repeats. Data were analyzed using the ΔΔCT method. Actin gene was used as the house-keeping gene for expression calculation.

Gene Expression Analysis at Protein Levels

To further verify the expression changes at mRNA level, Bio-Plex Pro Diabetes Assays were conducted to get insight for the selected genes' expressions at the translational level using a Mouse Diabetes Multiplex magnetic bead–based multiplex assay kit by following the manufacturer's instruction (Bio-Rad, Hercules, CA). Plates were run on a Bio-Plex MAGPIX™ Multiplex Reader with Bio-Plex Manager™ MP Software (Luminex, Austin, TX). Each biomarker concentration was calculated as pg/ml. The levels of sensitivity in this panel were 0.64, 4.31, 0.59, 0.5, 68.29, 5.07, 2.98 and 184.89 pg/ml for ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1 and resistin, respectively.

Statistical analysis

All data collected, including blood glucose, ELISA and HbA1c, were expressed as mean ± SE, and analyzed using one-way ANOVA followed by Bonferroni's multiple comparison post-hoc tests. Statistical significance was defined as $p \leq 0.05$.

3.4. Results

Effect of Different Dietary Treatments on Blood Glucose and Body Weight

STZ-induced diabetic mice and non-STZ treated mice were used to examine the efficacy of GPE on diabetes treatment and prevention. Figure 3-1 shows the effects of long-term consumption of GPE incorporated in a high fat diet on blood glucose. In STZ treated groups, there was no significant difference on blood glucose between the HFD fed and HFD+GPE fed mice. In non-STZ treated groups, at early treatment stages (first

4 weeks), there was no significant difference on blood glucose between HFD and HFD+GPE fed mice. However, after week 10, hyperglycemia was successfully induced by consumption of HFD diet with average blood glucose higher than 180 mg/dl. Importantly, consumption of GPE significantly inhibited such blood glucose increase induced by HFD. The blood glucose in HFD+GPE treatment group was 12.6%, 15.1% and 10.1% lower than that of HFD fed alone at week 8, 10 and 12 respectively ($p=0.07$, $p< 0.01$, $p< 0.05$, respectively). Furthermore, blood glucose of the HFD+GPE group did not differ actoss the 12-week experimental period. For body weight, no significant difference was observed among all groups during the 12-week feeding period (data not shown).

Effect of Different Dietary Treatments on Glycated Hemoglobin, HbA1C

HbA1C was measured at the end of the experiment (Figure 3-2). We observed a significant decrease (32.8% at $p< 0.05$) in non-STZ treated mice fed with HFD+GPE compared to those fed with HFD only. No significant difference was observed between STZ-induced diabetic groups. As the level of plasma glucose increased, the fraction of glycated hemoglobin also increased.

Effect of GPE on peptide hormones related to glucose homeostasis

Circulating peptide hormones such as glucagon, insulin, PAI-1 and resistin are important biomarkers for glucose homeostasis. We quantitatively examined the amount of these hormones in serum using the Bio-Plex Pro Diabetes Assay. GPE consumption did not affect these peptide hormone levels in STZ-induced mice. No significant differences were observed between STZ-induced diabetic mice fed with HFD and those

fed with HFD+GPE. However, between non-STZ treated mouse groups, GPE significantly reduced serum levels of glucagon, PAI-1, insulin and resistin (Figure 3-3).

RNA expression profile of the peptide hormones regulating glucose homeostasis

The mRNA expression profiles of the peptide hormones which potentially regulate glucose metabolism were also examined in non-STZ treated groups. These genes encoded peptide hormones, including glucagon, insulin, PAI-1, dipeptidylpeptidase-4 (DPP-4), GLP-1 and GIP. The expressions of glucagon, insulin and resistin were examined using pancreas RNA while PAI-1, DPP-4, GLP-1 and GIP were examined using small intestine RNA. As shown in Figure 3-4, long-term consumption of GPE downregulated the expression of glucagon (39.1%, $P<0.05$) and insulin (53.5% at $P=0.07$ level) in pancreas. GPE also downregulated PAI-1 (71.6%, $P<0.05$) and DPP-4 (46.7%, $p<0.01$) in small intestine. DPP-4 promotes the degradation of GLP-1 and GIP and enhances the release of glucagon (Kim and Egan, 2008). Consistently, GLP-1 expression in small intestine was increased 44.7% ($P<0.05$) following consumption of GPE (Figure 3-4), while no significant change was observed for GIP expression (data not shown).

GPE reduced inflammation cytokines expression

Obesity is often considered as a systematic inflammation. During obesity development and its progression to hyperglycemia, various inflammation cytokines are expressed. To investigate the effect of GPE on systematic inflammation, we examined

the expression changes of several inflammation mediators in the small intestine (Figure 3-5). Real-time RT-PCR showed that dietary GPE supplementation significantly suppressed the expression of inflammation cytokines TNF-α, INF-γ and IL-12β (Fig. 3-6) by 63.3%, 38.4% and 64.8% respectively ($P<0.05$). Resistin was 31.7% down, ($P<0.05$) in pancreas. PAI-1 was also significantly downregulated (71.6% down, $P<0.01$) by GPE consumption. No significant changes in IL-6 were observed.

3.5. Discussion

A growing body of evidence suggests that the consumption of grapes and/or grape derived food products rich in polyphenols promotes a variety of health effects. Grape products show beneficial effects in preventing the development of metabolic syndromes, particularly diabetes, obesity and heart disease (El-Alfy et al., 2005; Kim et al., 2014; Kim et al., 2015; Pezzuto 2008; Seeram 2008). In our previous studies (Li et al, submitted), grape pomace water extract showed a dose-dependent inhibition of α-glucosidase activity and significantly decreased blood glucose in diabetic mice and normal human subjects within 3 hours of GPE consumption. In addition, GPE delayed carbohydrate digestion, absorption and transportat to the blood stream. These results prompted us to examine the long-term effects of GPE in regulating carbohydrate metabolism and to elucidate the molecular mechanism(s) involved.

In this study, we found that GPE did not play a critical role on STZ-induced diabetic mice during the 12-week feeding period, although there was a slight reduction in blood glucose at week 8. In our previous studies using a modified 2h Oral glucose Tolerant Test, STZ-induced diabetic mice gavaged with GPE-corn starch suspension resulted in a significant decrease in postprandial blood glucose compared with the STZ-

induced mice only gavaged with corn starch (Li et al., submitted). Taken together, our results suggest that GPE could suppress acute blood glucose but long-term consumption of GPE would not help treat diabetes. Interestingly, when we fed GPE to non-STZ treated mice supplied with HFD, we found that GPE had significant effects on preventing HFD-induced hyperglycemia. Circulating peptide hormones in the blood and HbA1c were also dramatically altered by GPE. Further, RNA expression of several peptide hormones related to glucose homeostasis were significantly changed by GPE. In addition, biomarker genes for systematic inflammation were also altered by GPE. These results suggest that daily consumption of GPE might prevent hyperglycemia caused by high fat diet. Similar evidence was obtained by Laight et al., (2009) who reported that consumption of grape seed extract for 4 weeks significantly improved markers of inflammation and glycaemia in obese Type 2 diabetic subjects with 32 patients involved in a double blind randomized placebo controlled trial.

To elucidate the molecular mechanisms involved in blood glucose lowering properties of the GPE, we further evaluated the effects of GPE on the glucose homeostasis. Glucose homeostasis was primarily governed by the two counteracting hormones: the glucose-elevating hormone glucagon and the glucose-lowering hormone insulin (Aronoff et al., 2004). Disruption of the equilibration by augmented secretion of glucagon and decreased secretion of insulin typically promotes commenced postprandial hyperglycemia (Yabe et al., 2015; Unger & Cherrington, 2012). Incretins, such as GLP-1, GIP and DPP-4, stimulate insulin secretion from pancreatic β cells (Yabe et al., 2015; Drucker, 2013; Holst, 2007; Seino & Yabe, 2013); GLP-1 inhibits glucagon release from the alpha cells of the islets of Langerhans, while GIP enhances

glucagon secretion (Yabe et al., 2015; Christensen et al., 2014; Christensen et al., 2011; Drucker, 2013; Holst, 2007; Mentis et al., 2011; Seino & Yabe, 2013; Taminato et al., 1977). Both GLP-1 and GIP are rapidly degraded by the enzyme DPP-4 (McIntosh et al., 2005; Behme et al., 1995; Dupre et al., 1995). Mice fed diet that was supplemented with GPE significantly decreased glucagon expression in this study. On the other hand, GLP-1 expression was significantly increased, which is consistent with the observation that DPP-4 expression was significantly decreased by GPE consumption. Numerous studies are in agreement with these findings. It has been reported that grape seed extract enriched in procyanidins (GSPE) improves glycemia by affecting the insulin release and decreasing glucose levels in Wistar female rats, increasing the active form of GLP-1 and inhibiting intestinal DPP-4 activity (González-Abuín et al., 2014; González-Abuín et al., 2014; González-Abuín et al., 2012).

Accumulating evidence indicates that grape polyphenols work in many different ways to decrease blood glucose (Zhang et al., 2011), improve insulin resistance (Baur et al., 2006; Sun et al., 2007; Lagouge et al., 2006), suppress hyperinsulinemia (Hokayem et al, 2013), and reduce blood pressure and lipid concentration (Perez-Jimenez et al., 2008; Vislocky et al., 2010; Schini-Kerth et al., 2010; Barona et al., 2012). Consistently, our results showed that the plasma glucose and insulin mRNA expression was significantly decreased in the non-STZ treated mice fed with GPE. Consumption of a high fat diet can increase body fat deposition, diminish glucose uptake and consumption in skeletal muscle, and consequently increase insulin resistance (Lovejoy et al., 2001; Mayer-Davis et al., 1997; Flanagan et al., 2008; Tanaka et al., 2007). Adipose tissue also increases the amount of adipocytes and

produces several hormones and cytokines that affect glucose homeostasis and fat metabolism (Ahima & Flier, 2000; Trujillo & Scherer, 2006). For example, resistin, a cysteine-rich adipose-derived peptide hormone, potentially links obesity and insulin resistance (Barnes & Miner, 2009). In rodents, exposure to resistin results in decreased response to insulin (Rangwala et al., 2004). Conversely, acute administration of resistin impairs glucose tolerance and insulin action (Kim et al., 2001). Our study demonstrated that GPE had a decreasing effect on circulating and mRNA expression level of resistin. This might contribute to an improvement of glucose metabolism and insulin resistance.

Additional evidence indicate that excessive fatty acid consumption in high fat diets results in excessive lipid accumulation in adipose and peripheral tissues, and subsequent chronic low-grade systemic inflammation (Ouchi et al., 2011). Chuang et al. (2011) reported that grape powder extract rich in phenolic phytochemicals possess anti-oxidant and anti-inflammatory properties. It attenuated TNFα-mediated inflammation through inhibiting the expression of IL-6, IL-1β, IL-8, MCP-1, COX-2 and TLR-2 and improves insulin resistance in primary cultures of newly differentiated human adipocytes. Increased expression of TNF-α mRNA in white adipose tissue in obese humans has a strong positive correlation with the level of hyperinsulinemia and can lead to insulin resistance (Hotamisligil et al., 1995; Hotamisligil et al., 1993). In our study, the mRNA expression levels of inflammation cytokine/ adipokines genes including PAI-1, TNF-α, INF-γ and IL-12β in small intestine were significantly suppressed by GPE consumption while IL-6 showed no significant difference. Similar to our findings, HFD-induced obese mice supplemented with the Norton grape pomace (250mg/kg body weight for 12 weeks) exhibited less oxidative stress (Hogan et al.,

2010), and that one-year consumption of grape extract downregulated the expression of key pro-inflammatory cytokines (Tome-Carneiro et al., 2013).

3.6. Conclusion

Our main goal was to investigate the effects of a dietary grape pomace extract supplement on the prevention of hyperglycemia and diabetes. This study provides important evidence that long-term consumption of GPE can: 1) lower the blood glucose and improved HbA1c. 2) down-regulate the expression of several cytokines involved in chronic low-grade inflammation triggered by high fat diet, and 3) attenuate insulin resistance in HFD-induced diabetes mice, but not in STZ-induced diabetic mice. It is likely that GPE has more beneficial effects on diabetes prevention instead of its treatment.

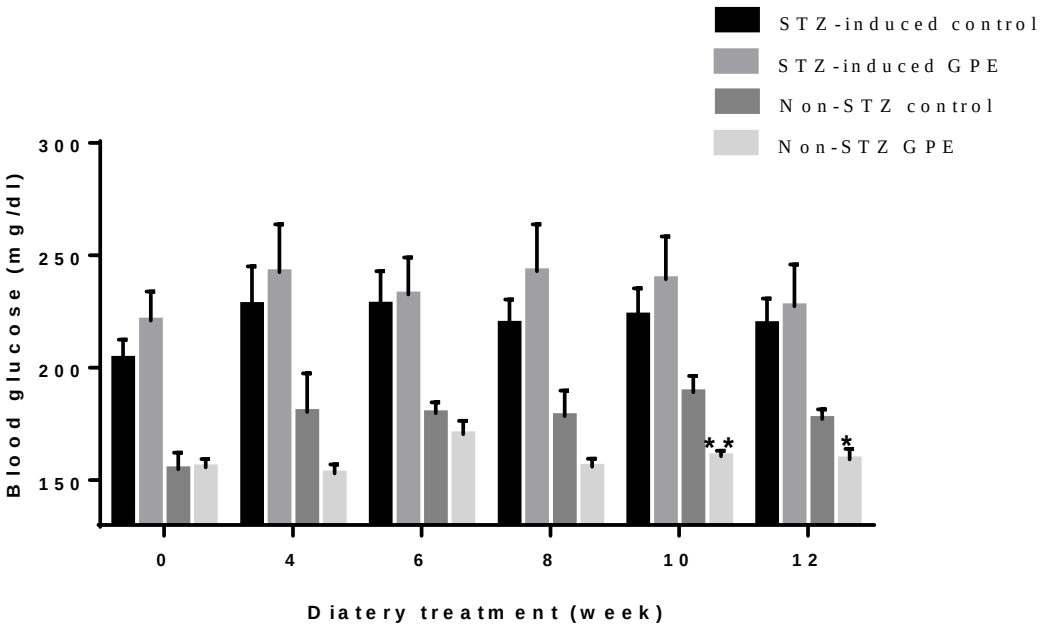


Figure 3- 1 Effect of two different dietary treatments (with or without GPE) on blood glucose in STZ-treated and non-STZ mice. Blood glucose was expressed as the mean ± SE (n=6-20). Means of blood glucose at each time point with asterisk indicated significant difference. *, $p<0.05$; **, $p<0.01$.

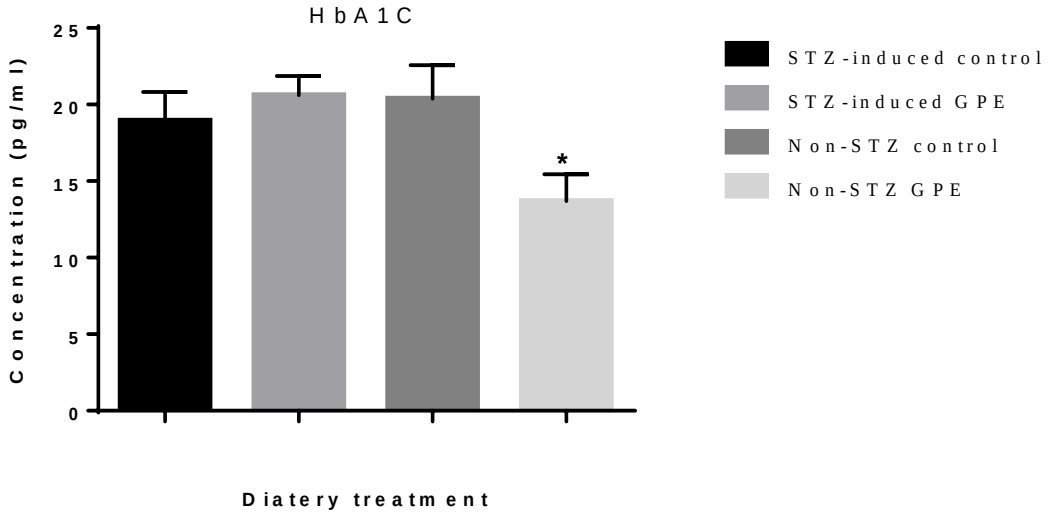
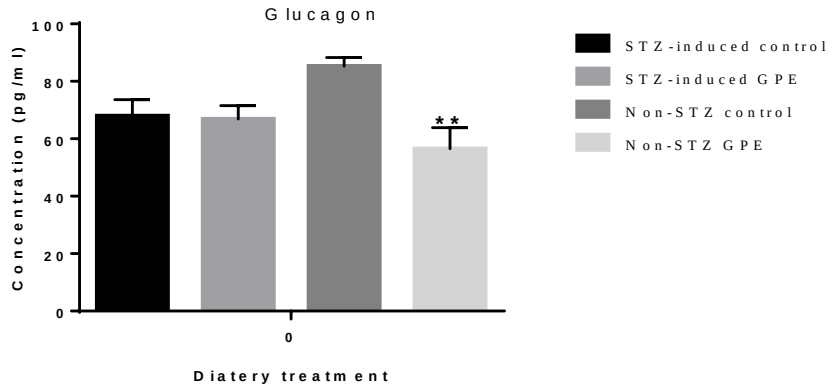
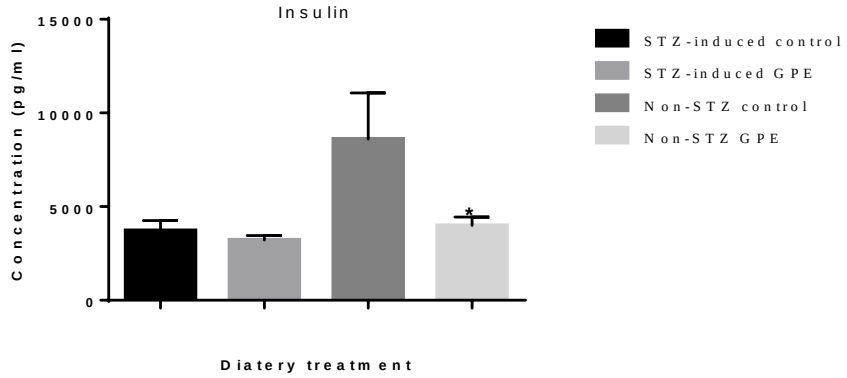


Figure 3- 2 Effect of two different dietary treatments (with or without GPE) on glycated hemoglobin, HbA1C in STZ-treated and non-STZ mice. Glycated hemoglobin A1C was expressed as the mean ± SE (n=6-20). Mean HbA1C for each group with * indicated significant difference from the corresponding control, $p<0.05$.


Glucagon
Concentration (pg/ml)
100
80
60
40
20
0
**
0
Diatery treatment
STZ-induced control
STZ-induced GPE
Non-STZ control
Non-STZ GPE

(A)


Insulin
Concentration (pg/ml)
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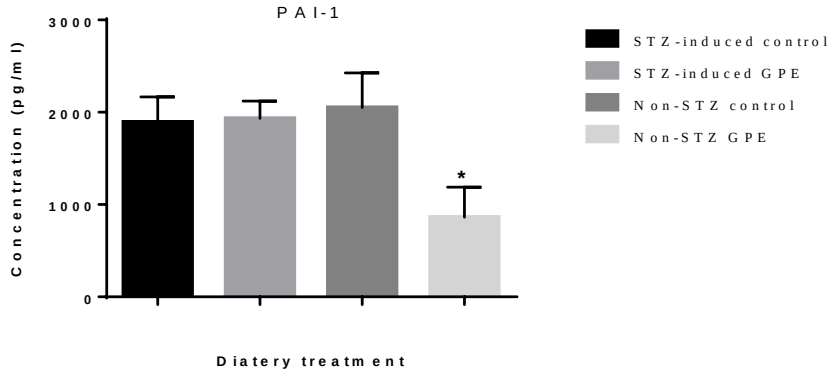
5000
0
*
Diatery treatment
STZ-induced control
STZ-induced GPE
Non-STZ control
Non-STZ GPE

(B)


PAI-1
Concentration (pg/ml)
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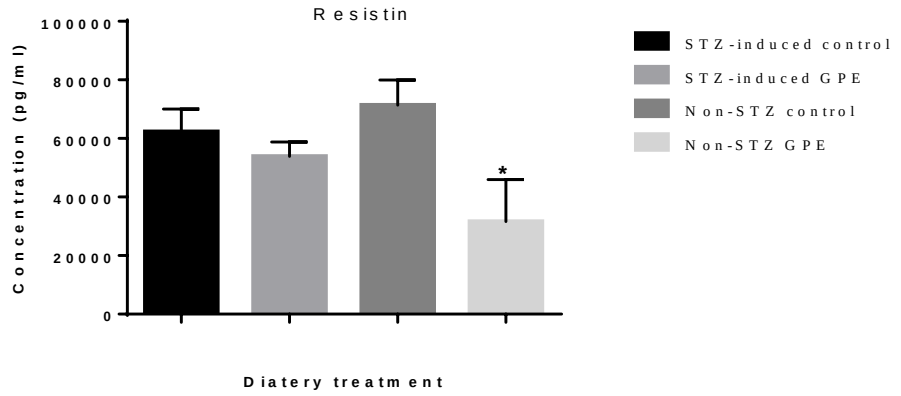
2000
1000
0
*
Diatery treatment
STZ-induced control
STZ-induced GPE
Non-STZ control
Non-STZ GPE

(C)



(D)

Figure 3- 3 Effect of GPE on serum levels of insulin, glucagon, PAI1, resistin. Results were expressed as relative expression levels (mean ± SEM, n=6-12). Significant difference between control and GPE treatment was marked with asterisk ($p<0.05$).

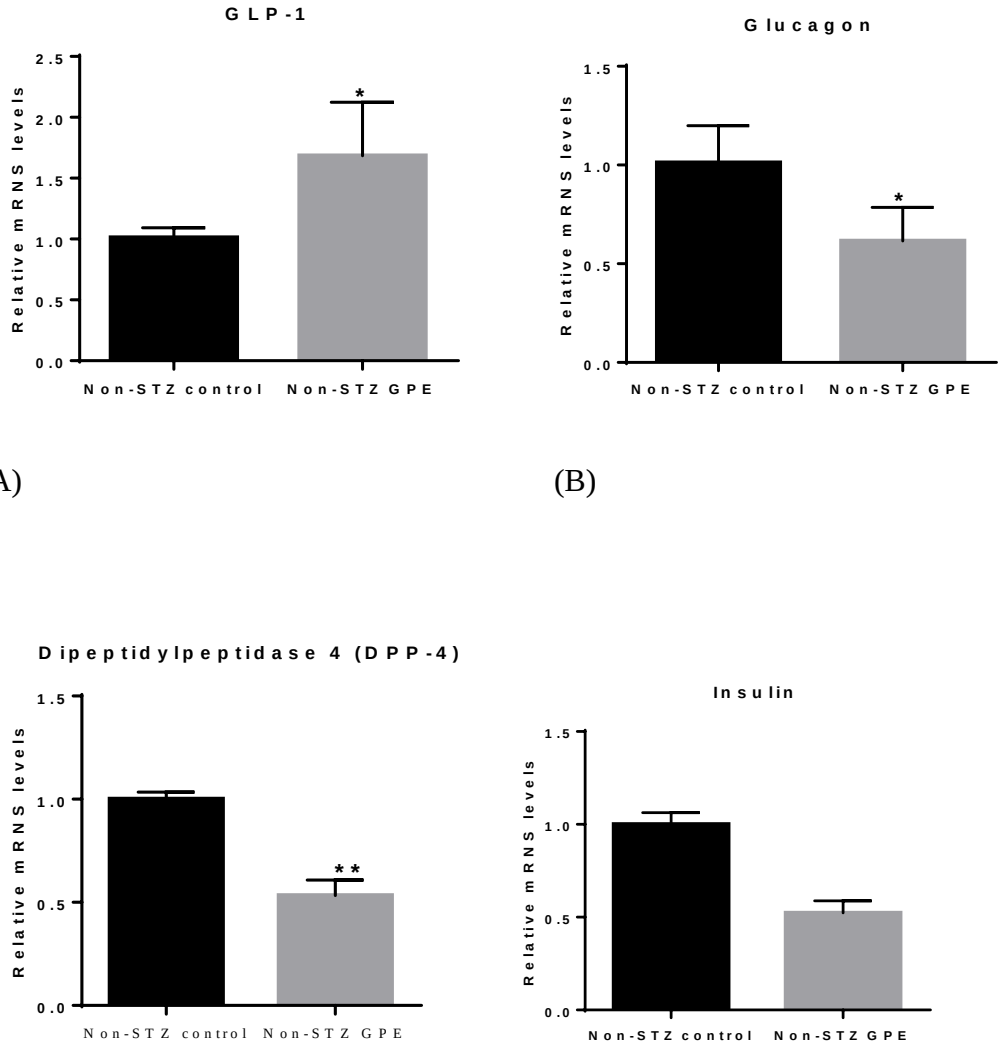
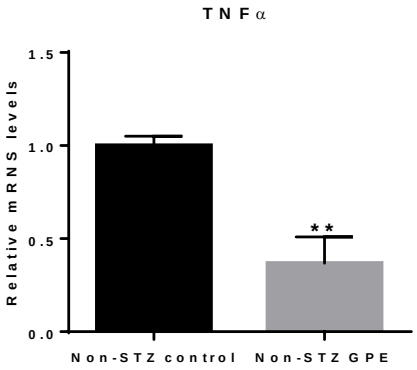
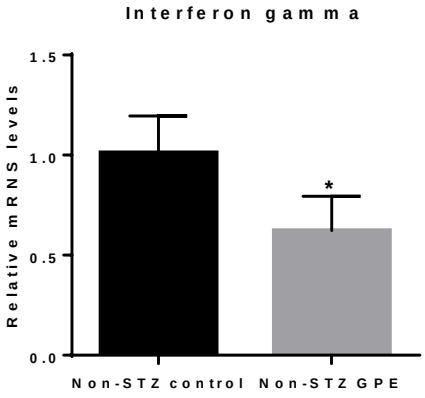


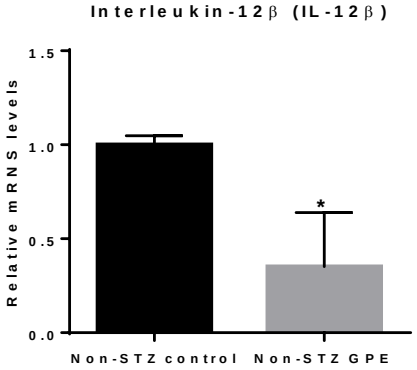
Figure 3- 4 GPE affected peptide hormones which regulate blood glucose homeostasis in non-diabetic mice.A-D: GLP-1, glucagon, DPP-4 and insulin gene. Pancreas and small intestine were harvested from animals on different diets, total mRNA was extracted, and the mRNA level was determined using real-time PCR. Results are expressed as relative expression levels (mean ± SEM, n = 6) to control. Columns marked with * are significantly different from each other at $p \leq 0.05$.


TNFα
Relative mRNS levels
1.5
1.0
0.5
0.0
**
Non-STZ control
Non-STZ GPE

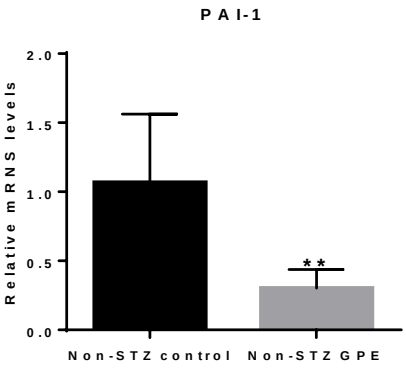
(A)


Interferon gamma
Relative mRNS levels
1.5
1.0
0.5
0.0
*
Non-STZ control
Non-STZ GPE

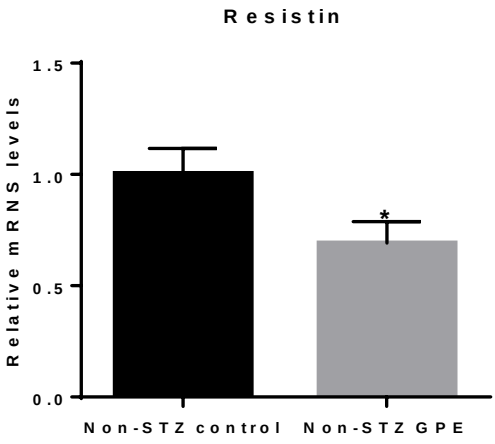
(B)


Interleukin-12β (IL-12β)
Relative mRNS levels
1.5
1.0
0.5
0.0
*
Non-STZ control
Non-STZ GPE

(C)


PAI-1
Relative mRNS levels
2.0
1.5
1.0
0.5
0.0
**
Non-STZ control
Non-STZ GPE

(D)


Resistin
Relative mRNS levels
1.5
1.0
0.5
0.0
*
Non-STZ control
Non-STZ GPE

(E)

Figure 3- 5 Effects of GPE on inflammatory mediator genes in small intestine and pancreas.Pancreas and small intestine were harvested from animals on different diets, total mRNA was extracted, and the mRNA level was determined using real-time PCR. Results are expressed as relative expression levels (mean ± SEM, n = 6) to control. Columns marked with * are significantly different from each other at $p \leq 0.05$.

Table 3-1. Macronutrient and micronutrient content of the mouse diets.

	HFD		**HFD+GPE**	
Product #	**D04011601**		**D12121001**	
%	gm	*kcal*	gm	*kcal*
Protein	24	*20*	24	*20*
Carbohydrate	41	*35*	41	*35*
Fat	24	*45*	24	*45*
Total		*100*		*100*
kcal/gm	4.7		4.7	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein, 80 Mesh	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*
Corn Starch	0	*0*	0	*0*
Maltodextrin 10	100	*400*	100	*400*
Sucrose	245.	*982*	245.	*982*
Cellulose, BW200	50	*0*	50	*0*
Soybean Oil	25	*225*	25	*225*
Lard	177.	*1598*	177.	*1598*
Mineral Mix S10026	10	*0*	10	*0*
Mineral Mix S10026B	0	*0*	0	*0*
DiCalcium Phosphate	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
Grape Extract	0	*0*	2.07	*0*
FD&C Yellow Dye #5	0.05	*0*	0	*0*
FD&C Red Dye #40	0	*0*	0	*0*
FD&C Blue Dye #1	0	*0*	0.05	*0*
Total	**858.1**	***4057***	**860.2**	***4057***
Grape Extract (g/kg)	**0**		**2.4**	

Table 3-2. List of genes encoding different peptide hormones related to glucose homeostasis and inflammatory cytokines.

Peptide hormones related to glucose homeostasis: GLP-1, glucagon, insulin, GLP-1, DPP-4, GIP
Inflammatory mediators: PAI-1, resistin, IL-12β, IL-6, TNFα, INF-γ

Chapter 4: Anti-obesity Activity of Grape Pomace Aqueous Extract on Suppressing Adipogenesis in Murine 3T3-L1 Adipocytes and Reducing Obesity in High Fat Diet Induced Mice

4.1 Abstract

Obesity is a key factor leading to metabolic syndromes that are characterized by an increase in body fat accumulation. We have recently reported that grape pomace extract can prevent obesity. In this study, we examined the potential role of grape pomace extract in controlling adipogenesis and body weight. Cabernet Franc Grape pomace aqueous extract (GPE) was used in the experiments. 3T3-L1 pre-adipocyte cells were induced into adipocyte in culture medium with or without GPE. GPE significantly affected adipogenesis in 3T3-L1 cells supported by decreased oil accumulation and changes in gene expression patterns involved in the adipogenesis transcriptional cascade. In a second experiment, C57BLK/6J mice were fed a high fat diet supplemented with or without 2.4g/kg GPE for 12 weeks. GPE reduced body weight compared to the control group, and inhibited adiposity by promoting a significant reduction in abdominal fat accumulation. In addition, GPE attenuated the expression of CEBPα, SREBF1 and PPARγ and reduced systematic inflammation through inhibition of PAI-1 expression and increase of the anti-inflammatory adipokine adiponectin. Our results highlight the role of GPE as an alternative approach to preventing obesity occurrence and development.

4.2 Introduction

Obesity significantly increases the risk of several life-threatening diseases, such as heart disease, type 2 diabetes and cancer. In 2013, the American Medical Association officially classified obesity as a disease (http://www.ama-assn.org). Obesity is dramatically increasing globally, with more than 1.9 billion adults (39%) overweight. Of these, over 600 million (13%) people worldwide are suffering from obesity according to World Health Organization (WHO report 2014, http://www.who.int/mediacentre/factsheets /fs311/en/). In the United States, based on published data by CDC, more than one-third of adults (34.9% or 78.6 million) are obese (Ogden et al., 2014).

Overweight and obesity are defined as excessive fat accumulation in the body. It is caused by the interruption of the equilibrium of energy intake and expenditure (Spiegelman and Flier, 2001; Panico and Iannuzzi, 2004; Kopelman, 2000). Obesity is a major factor associated with serious health disorders such as type 2 diabetes. The infiltration of inflammatory cells into adipose tissue triggers a low-grade systematic inflammation and eventually impairs insulin sensitivity and develops into type 2 diabetes (Hotamisligil and Erbay, 2008; Nath et al., 2006; Mokdad et al., 2003). Obesity has also been associated with several other disorders, such as hypertension (Dorresteijn et al., 2012), atherosclerosis (Ouimet 2013), and cardiovascular disease (Must 1999). In addition, obesity is closely associated with cancers of the colon, breast, endometrium, kidney, and esophagus (adenocarcinoma) (Calle and Thun, 2004).

Adipose tissue, until recently has been considered as an inactive reservoir for

energy storage. With the discovery and characterization of leptin, the 16-kDa polypeptide with structural homology to cytokine, adipose tissue was re-discovered and consequently recognized as a complex and highly active metabolic and endocrine organ (Kershaw and Filer, 2004; Zhang et al., 1994; Ahima et al., 2000; Fruhbeck et al., 2001). Adipose tissue plays an important role in the regulation of whole body fatty acid homeostasis (Galic et al., 2010) by secreting numerous bioactive peptide hormones, named "adipokines". Adipokines, such as leptin, adiponectin, plasminogen activator inhibitor-1 (PAI-1) and resistin regulate metabolism from both the local (autocrine/paracrine) and systemic (endocrine) level (Kershaw and Filer, 2004). Adipose tissue expresses numerous receptors for traditional endocrine hormones, nuclear hormone receptors, cytokine receptors and catecholamine receptors. Those receptors connect the response of the traditional hormone system to the network of the central neuron system (Kershaw and Filer, 2004). The hormones and receptors secreted by adipose tissue effectively communicate with the brain and peripheral tissues to modulate glucose homeostasis, influence energy metabolism, regulate appetite, adjust insulin resistance and control immune function (Matsuzawa et al., 1999; Funahashi et al., 1999).

Adipogenesis is the process where fibroblasts like pre-adipocytes differentiate into the well-established matured spherical adipocytes, which contain lipids (Lefterova and Lazar, 2009). An in vitro model of 3T3-L1 and 3T3-F442A, the pre-adipocyte cell lines, has been developed to study adipogenesis (Rosen et al., 2000; Gregoire, 2001). A highly orchestrated multistep process of the transcriptional network is involved in adipogenesis (Ali et al., 2013). Several transcription factors are sequentially activated in adipogenesis transcriptional cascade. Remarkably

CCAAT/enhancer binding proteins (C/EBPβ, C/EBPδ and C/EBPα) gene family, peroxisome proliferator activated receptor-γ (PPAR-γ) and Sterol regulatory element-binding transcription factor 1 (SREBF1), have been shown to have significant roles in promoting adipogenesis (White and Stephens, 2009). These factors are essential in promoting the terminal or mature adipocyte phenotype. Differentiation of pre-adipocyte cells into adipocytes involves a series of events including growth-arrest, mitotic clonal expansion, and terminal differentiation and mature adipocytes (Sun et al., 2009; Lefterova and Lazar, 2009). In response to hormonal stimuli such as cortisol and insulin, C/EBPβ and C/EBPδ are induced immediately which in turn activate PPARγ and C/EBPα (Farmer, 2006). PPARγ and C/EBPα form a positive feedback loop to induce the expression of each other and regulate the adipocyte differentiation (Rosen et al., 2002). PPARγ and C/EBPα in turn induce the expression of genes that are necessary for regulating fatty acid storage and glucose metabolism (Rosen, E.D. and MacDougald, 2006). They are highly expressed in adipose tissue and are key factors controlling the down-stream adipocyte differentiation and adipocyte-specific gene expression (Lefterova et al., 2008), such as lipoprotein lipase (LPL), which is considered as the early stage marker for the adipocyte differentiation, and adipocyte fatty acid–binding protein (aP2), which is an intermediate sign of adipocyte differentiation (Rosen and Spiegelman , 2000; (Bernlohr et al., 1984; Katz et al., 1999; Spiegelman et al., 1983; MacDougald et al., 1995). Sterol regulatory element-binding proteins (SREBPs) are also involved in adipocyte differentiation. SREBPs are a family of transcription factors that regulate lipid homeostasis. SREBPs are induced very early during adipocyte differentiation and stimulate the expression of PPARγ,

and induce the fundamental coordinator of the adipocyte differentiation process (Eberlé et al., 2004; Shao and Espenshade, 2012; Saladin et al., 1999).

The prevalence of obesity has increased worldwide and negatively impacted human health. Systemic chronic inflammation due to adipogenesis is major factor for obesity. The main causes of obesity are thought to be related to diet (high fat consumption) and lifestyle (lack of physical activities). The existing literatures indicated that prolonged consumption of a high fat diet can lead to obesity and insulin resistance. Thus, understanding adipogenesis has been a major focus of researchers. New therapeutic intervention strategies to combat obesity involve identifying factors that control fat accumulation. Bioactive food components have been well studied in their ability to prevent obesity. The grape (Vitis vinifera) is one of the world's oldest domesticated fruit crops, as well as one of the most economically important fruit plants worldwide. A large variety of natural grape products are widely used as nutritional products. The bioactivities of grape polyphenols and the health promoting effects of different varieties of grape have been documented. Numerous bioactivities of phenolic compounds in grape products, especially flavonoids are widely distributed in grapes. Many of these have potent positive effects modulating human health (Georgiev et al., 2014; Chacona et al., 2009; Bagchi et al., 2000; Cantos et al., 2002). Flavonoids, for example, anthocyanins, flavonols, flavanols, dihydroflavonols and proanthocyanidins are the most common phenolic compounds found in grapes. Phenolic compounds from grapes also exert positive benefits on human health. Some of the more important benefits reported include anti-radiation, anti-mutagenic, anti-inflammatory and anti-bacterial effects (Bagchi et al., 2003; Halliwel et al., 1992;

Belleville, 2002; Sun et al., 2002). These beneficial effects have also been pharmacologically documented as decreasing insulin resistance, reducing ischemic heart disease, preventing heart failure and avoiding hypertension (Petrovski et al., 2011; Bertelli et al., 1998).

Grape pomace, the by-product from the wine and juice industry, may have potential beneficial effects in preventing diabetes and obesity. In our previous studies, we have reported that oral administration of Grape Pomace Aqueous Extract (GPE) can attenuate diabetes through inhibiting small intestine α-glucosidase activity and decreasing post-prandial hyperglycemia resulted from the prolonged consumption of western-style diet. However, the mechanism(s) of the effect of GPE on adipogenesis and obesity remains unknown. In the present study, using adipocyte precursor cell model 3T3-L1, we examined the role of GPE in regulating expression of genes that are involved in adipocyte differentiation. In a second study, we investigated the possible roles of GPE in regulating obesity, inflammation and insulin resistance in a high-fat-diet fed mouse model.

4.3 Materials and methods

Materials

Cabernet Franc grape pomace samples (fermented for 2 weeks) were obtained from Chrysalis Vineyards, Virginia in 2011. Dulbecco's Modified Eagle's Medium (DMEM), antibiotic/ antimycotic, fetal bovine serum (FBS), and 0.25% trypsin with 0.9 mM EDTA were purchased from Invitrogen (Carlsbad, CA). 3T3-L1 cell line was purchased from the American Type Culture Collection (Rockville, MD).

Adipogenesis Assay Kit (Cell-Based), Adiponectin Mouse ELISA kit and Glycerol-3-Phosphate Dehydrogenase (G3PDH) Colorimetric Assay Kits were purchased from Abcam, (Cambridge, MA).

Sample Preparation and Extractions

Grape pomace samples were air dried under low pressure. Dried pomace was milled to 40 mesh size using a Scienceware Bel Art Micromill, (Pequannock, NJ). The milled grape pomace was then extracted with water (1:10 m/v) by shaking for 2 hours at ambient temperature. Following extraction, the mixture was centrifuged at 4,000 rpm at 20^{o}C for 30 min. Supernatants were collected and freeze dried in the bench top freeze drier (AdVantage 2.0 BenchTop Freeze Dryer / Lyophilizer, SP Scientific) to obtain GPE.

Cell viability assay

GPE extracted using water was re-dissolved in 10% DMSO. The 10% DMSO GPE mixtures were then examined for cytotoxicity on 3T3-L1 cells. Cells are propagated in T-75 flasks and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate (ATCC® 30-2002™) with 10% fetal bovine serum and antibiotic/antimycotic. Cells were seeded at 5x10^{4}/well in 96-well plate at 37^{o}C in a humidified atmosphere with 5% CO_2 supplied. Cells were treated with GPE at 0-4000μg/ml concentrations. Culture medium with 0.1% DMSO was used as negative control. Following 24 h exposure, cell viability was determined by the ATPlite™ Luminescence Assay System (PerkinElmer, Massachusetts, USA) according to the manufacturer's direction. Absorbance at 570 nm will be directly

correlated to live cell number. All measurements were taken in triplicate with PerkinElmer VICTOR^3 V Multilabel Readers 96-well plate reader (Massachusetts, USA).

Cell culture differentiation

Adipocyte differentiation of 3T3-L1 cells was measured with a cell-based adipogenesis assay kit following manufacture's instructions. In brief, cells were cultured in 12-well plates to 100% confluence for 2 days and then transferred to differentiation medium (DM) containing 10 μg/ml insulin (Sigma, St. Louis, MO), 0.5 μM dexamethasone (Sigma, St. Louis, MO), and 0.8 mM isobutylmethyl xanthine (IBMX). GPE were added in differentiation medium at 0, 200, 400, 800μg/ml. After a 3-day induction, the medium is changed to DMEM with 10% fetal bovine serum containing 10 μg/ml insulin for differentiation and DMEM with 10% fetal bovine serum for control group at 37 °C, 95% O_2, and 5% CO_2 for 4 days. Fresh medium was changed every other day.

Oil Red O staining

Seven days after adipocyte differentiation, an oil red staining assay was conducted. Culture medium was remove from the wells, and 75 μL diluted Lipid Droplets Assay Fixative was added to each well and incubate for 15 minutes. Wells were washed with 100 μL Wash Solution two times for five minutes each. The plate was dried completely and then 75 μL oil Red O Working Solution was added to all wells and incubated for 20 minutes. Oil Red O Solution was then removed and the cells were washed with distilled water several times until the water contained no visible pink color. After Oil Red-O stain, microscope images were taken to visualize

pink to red oil droplets staining in differentiated cells. Cells were photographed using a phase-contrast microscope (Olympus CKX41, Tokyo, Japan) in combination with a digital camera at 100X magnification. Differentiated cells were dried completely and 400µL of dye extraction solution was added to each well and gently mixed for 15-30 min. 250µl extraction solution with cells were added to 96-well plate and read the absorbance at 490-520 nm with a PerkinElmer VICTOR^3 V Multilabel Readers 96-well plate reader (Massachusetts, USA).

Colorimetric Glycerol-3-Phosphate Dehydrogenase (G3PDH) Assay

3T3-L1 adipocyte cells were plated in six-well plates with a density of 2 x 10^6 cells per well. Two days after, differentiation medium (DM) was added to the cells. GPE was added at various concentrations, and cells were incubated at 37ºC with 5% humidified CO_2 for 3 additional days. Abcam colorimetric Glycerol-3-Phosphate Dehydrogenase (G3PDH) Assay was performed following manufacture's protocol (Abcam, Cambridge, MA). In brief, the 3T3-L1 adipocytes were harvested 7 days after the initiation of differentiation with various concentrations of GPE. Cells were carefully washed twice with ice-cold PBS and collected with a scraper. Cells were spun down briefly and suspended in 200µL ice cold GPDH Assay Buffer. 50µL of sample and positive control were added to each well and mixed thoroughly with 50µL of the Reaction Mix containing 46µL of GPDH Assay Buffer, 2µL of GPDH Substrate and 2 µL of GPDH Probe. Reactions were incubated for 20-60 min at 37ºC and measured OD at 450 nm. Enzyme activity (%) was expressed as a ratio of the experimental condition relative to the control (which was set at 100% activity).

RNA isolation and gene expression analysis with quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

For gene expression analysis, highly sensitive and reliable real-time RT-PCR's were performed. Total RNA was extracted from 3T3-L1 adipocytes, which had been treated with various concentrations of GPE for 7 days, with RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of RNA was determined by measuring absorbance in a Nano drop spectrophotometer. RNA was treated with DNaseI at room temperature for 15 min to remove genomic DNA contamination. RT^2 First Strand Kit from Qiagen (Qiagen, Hilden, Germany) was used to synthesis first strand complementary DNA (cDNA). Gene expression were analyzed by Quantitative real-time RT-PCR conducted on the Bio-Rad CFX-96 Real-Time PCR System using RT^2 SYBR Green Master mix (Bio-Rad Laboratories, Hercules, CA).

The Mouse Adipogenesis RT^2 Profiler™ PCR Array containing 84 genes related to the differentiation and maintenance of mature adipocytes was used to examine the expression patterns of the selected genes. The marker genes for adipogenesis are listed in Table 4-1. Genes encoding adipokines that were tested are listed in Table 4-2. Hot-Start DNA Taq Polymerase was activated by heating at 95°C for 10 min and real time PCR was conducted for 40 cycles (15 s for 95°C, 1 min for 60°C). All results were obtained from at least three independent biological repeats. Data were analyzed using the $\Delta\Delta^{CT}$ method. The expression of all genes was normalized using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control.

Gene expression analysis at protein levels

Fully differentiated adipocytes (at 7 days after differentiation induction) were treated with the 0, 400 and 800 µg/ml of GPE for 72 h, and adiponectin concentrations in cell culture supernatants were determined. A quantitative adiponectin enzyme-linked immunosorbent assay (ELISA) was performed using a commercial Mouse adiponectin ELISA kit (Abcam, Cambridge, MA) according to the manufacturer's protocols.

Animal experiments

Mouse food preparation:

Rodent chow and western style high fat diet (HFD) was purchased from Research Diets, INC (New Brunswick, NJ). For GPE containing HFD diet, 2.4 grams of GPE was mixed with 1 kilogram of HFD by the same company. All mouse food was sterilized by irradiation to minimize bacterial contamination. Macronutrient and selected micronutrient content in the mouse diet is summarized in Table 4-3.

Animals:

6-week male C57BLK/6J mice, were housed in clean cages at 12-hour light dark cycle at 20°C to 22°C, with 4 mice/cage. The mice were maintained on rodent feed with continuous access to tap water so as to acclimatize to the experimental conditions for 1 week prior to being assigned to experimental groups.

Experimental group assignment:

Each mouse was assigned to one of the following groups: 1) group-1 mice fed with regular rat chow and served as control (n=14); 2) group-2 mice fed with HFD (n=14); 3) group-3 mice fed with HFD + GPE (n=14). All mice were fed with the respective diets for 12 weeks with water available ad libitum.

Food intake, body weight and fasting glucose measurements during the experiment:

During the 12-week feeding experiment, food intake and body weight were recorded every week. Fasting blood glucose levels was also monitored weekly using a blood glucometer and the accompanying test strips (ACCU-CHEK Meter®, Roche Diagnostics, Kalamazoo, MI). Plasma insulin was monitored monthly at week 0, 4, 8 and 12. A Mouse insulin ELISA kit from BioRad was used to quantify insulin following the manufacturer's procedure.

Animal sacrifice and tissue collection:

Mice were anesthetized with CO2 at the end of feeding study. Blood samples were collected and the plasma was separated. Liver, heart, kidney, white adipose tissue and pancreas were dissected, weighed and immediately frozen in liquid nitrogen and stored in -80oC. The ARC and LHA regions of the hypothalamus were dissected under a binocular microscope and immediately frozen in liquid nitrogen.

Histological analysis of animal adipocytes

Freshly isolated epididymal WAT sections were fixed in 10% formalin for 24 hours followed by embedding the tissues in paraffin. The paraffin-embedded samples were sectioned and stained with hematoxylin-eosin following the procedures used by An et al., 2010.

RNA isolation and real-time RT-PCR in hypothalamus:

The ARC and LHA regions of the hypothalamus tissue were subjected to total RNA extraction with RNeasy Plus Universal Mini Kit (Qiagen USA). DNase I treated RNA samples were used to synthesis first strand complementary DNA (cDNA) using RT2 First Strand Kit from Qiagen (Qiagen USA). We used ARC tissue to test the NPY, AgRP, POMC, CART, and MC4R, and LHA tissue to test the MCH through quantitative real-time RT-PCR (qRT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes was used as an internal control. All data were analyzed using the ΔΔCT method.

4.4. Results

Effects of GPE on cell viability, adipocyte differentiation and triglyceride accumulation

The ATPlite™ Luminescence Assay was performed to determine the effect of GPE on 3T3-L1 cell viability. 3T3-L1 cells were exposed to various concentrations of GPE. As shown in Fig 4-1, no significant effect on viability was observed at GPE concentrations up to 1000 µg/ml after 72h incubation with GPE.

To investigate the effect of GPE on attenuation adipocyte differentiation and preventing lipid accumulation, 3T3-L1 adipocytes were incubated with differentiation medium supplemented with different concentrations of GPE. The total amount of lipid accumulation was examined by Oil-Red-O staining. Shown in Fig 4-2A, GPE significantly reduced lipid accumulation, as indicated by decreasing Oil Red-O staining. Triglyceride content in 3T3-L1 adipocytes was drastically decreased at 400 µg/ml and 800 µg/ml GPE concentrations ($p<0.01$) to 79.7% and 68.2% respectively as compared to control cells (Fig. 4-2B).

Glycerol-3-phosphate dehydrogenase (GPDH) is an enzyme that links carbohydrate metabolism and lipid metabolism. GPDH activity was verified to determine the effect of GPE on 3T3-L1 pre-adipocytes differentiation. GPDH activity in 3T3-L1 adipocytes was significantly decreased by GPE at 400 µg/ml and 800 µg/ml to 84.4% ($p<0.05$) and 79.8% ($p<0.01$) respectively. In contrast, no significant effect on GPDH activity was showed in control treatment (Fig. 4-3).

Effect of GPE on 3T3-L1 adipocyte differentiation and the expression level of key adipogenesis genes

Real-time RT-PCR was performed to examine the effect of GPE on the gene expression of adipogenic transcription factors in 3T3 L1 pre-adipocytes. Shown in Fig. 4-4, 3T3-L1 pre-adipocyte cells incubated with 800 µg/ml GPE exhibited markedly decreased mRNA expression of C/EBPα, C/EBPβ, PPARα, PPARγ, PGC-1α, and SREBP1 (Fig. 4-4A-F) when compared with control ($p<0.01$). The expression of most these genes did not significantly differ between 400 µg/ml and 800 µg/ml GPE treatments. Only C/EBPβ showed dosage dependent inhibition where 800 µg/ml GPE inhibited significantly more expression than that of 400 µg/ml GPE.

Finally we also investigated the effect of GPE on the expression of adipocyte-related genes that are known to participate in the adipogenesis. Shown in Fig 4-4. G-J, LPL and ap2, were significantly decreased by 400 and 800 µg/ml of GPE treatments ($p<0.05$). The expression of the lipase gene was not inhibited until 800ug/ml GPE was added. No significant changes were detected on FAS, an important fatty acid biosynthesis enzyme (Fig. 4-4. G-J).

Effect of GPE on the expression of adipocytokines in 3T3-L1 pre-adipocyte

Inflammatory cytokines released from adipose tissue are a major cause of systemic low-grade inflammation. To understand the molecular mechanism underlying the anti-inflammatory effect of GPE, adipocytokines during 3T3-L1 adipocyte differentiation were investigated. The expression level of the key adipokines including angiopoietin, adiponectin, leptin, resistin, PAI1 (Fig. 4-5). These were examined by real-time RT-PCR. The expression of angiopoietin, resistin and PAI1 was significantly reduced ($p<0.05$ or $p<0.01$) after treatment with 800 µg/ml GPE (Fig 4-5A, B and E). 400 µg/ml GPE inhibited the expression of resistin ($p<0.01$). No changes were observed on the expression of leptin (Fig. 4-5C). An effect of GPE on adiponectin, was also observed. 800 µg/ml GPE significantly increased adiponectin expression in the fully differentiated adipocytes with a 1.8-fold increase compared to the control treatment.

Effect of GPE on the secretion of adiponectin during 3T3-L1 adipocyte differentiation

To understand the molecular mechanisms responsible for the GPE-induced upregulation of adiponectin expression, fully differentiated adipocytes were treated with 0, 200, 400 µg/mL and 800 µg/mL GPE, and the concentrations of adiponectin in cell culture supernatants were determined with ELISA kit. Shown in Fig 6, GPE significantly increased the secretion of adiponectin in a dose-dependent manner (Fig. 4-6). Significant differences were observed at GPE concentrations of 400 and 800 µg/mL with 20.6% and 50.6%, respectively ($p<0.05$).

Effect of GPE on body weight, abdominal fat weight and adiposity in HFD induced obese mice.

We also conducted an animal study to evaluate the effect of GPE on fat metabolism and its potential capacity of being an anti-obesity treatment. As shown in Figure 4-7A, the HFD and HFD+GPE dietary treatments significantly increased body weight compared to the normal diet control group. However, significantly decreased body weight was shown in animals that were fed an HFD with GPE compared with HFD only. A significant difference between the HFD+ GPE group and the HFD group started at week 6 ($P < 0.05$), and was constantly maintained until the end of the experiment. No difference in food intake was observed between the HFD+ GPE group and the HFD group (data not show). To test whether body weight loss was caused by decreased adiposity, mice were sacrificed and abdominal white adipose tissue was dissected and weighed (Fig. 4-7B). Abdominal white adipose tissue was significantly reduced in the HFD+GPE group ($p<0.01$) with 26.6% less than in the HFD group. Abnormalities of epididymal white adipose tissue in the HFD group were observed in the histology WAT stained with hematoxylin and eosin (Fig. 4-7C). The size of the adipocytes in HFD+GPE group was markedly decreased when compared with that of the HFD group.

Effects of GPE on blood glucose and organ weight in HFD induced obese mice.

Blood glucose and organ weight (heart, liver and pancreas) were measured at week-12 and summarized in Table 4-2. A significant increase in blood glucose was

observed in the HFD fed group when compared with normal diet ($p<0.01$). However, HFD supplemented with GPE shown no difference in blood glucose after fed for 12 weeks in comparison to normal diet ($p<0.05$), but decreased by 23.3% compared with HFD alone. No difference in liver and pancreas weights were observed between HFD and HFD+GPE. However, HFD increased the weight of the heart compared with normal diet and HFD+GPE.

Effect of GPE on neuropeptides produced by the hypothalamic arcuate nucleus (ARC) in HFD induced obese mice.

Neuropeptide Y (NPY)/agouti-related protein (AGRP) neurons, and the pro-opiomelanocortin (POMC)/ cocaine and amphetamine related transcript (CART) neurons in the arcuate nucleus of the hypothalamus play a central role in controlling energy homeostasis and feeding behavior. We examined the mRNA expression of AGRP, NPY, POMC and CART to determine the effects of GPE on the hypothalamic gene expression (Fig. 4-8). No significant difference was observed for NPY among mice fed with different diets (Fig. 4-8B). Compared to the normal diet, the high-fat diet significantly increased AGRP expression by a factor of 3.5. Animals fed the GPE supplemented diet showed a similar increase in AGRP as mice fed a high-fat diet (Fig. 4-8A). POMC, was up-regulated 92.2% and 74.5% in high-fat diets with or without GPE supplementation as compared to normal diet (Fig. 4-8C). CART expression exhibited no differences between the HFD diets and HFD supplemented with GPE (Fig. 4-8D).

Relative expression of genes related to adipogenesis and lipogenesis in HFD induced obese mice.

To determine whether reduced adiposity was associated with reduced adipogenesis through the multiple transcription factors, C/EBPs, PPARs and SREBPs transcription cascade and the down-stream genes involved in fatty acid metabolism were evaluated. As shown in Fig. 4-9, no significant difference was observed for SREBP1 between normal diet and HFD diet, as well as between normal diet and HFD+GPE. However, 44.7% less expression of SREBP was observed in the HFD+ GPE group when compared with the HFD alone group (Fig. 4-9A). In mice fed the HFD CEBPα expression in adipose tissue was not different compared with the group fed the normal diet, but GPE supplementation reduced adipose CEBPα by 2-fold compared with HFD alone (Fig. 4-10B). PPARγ was significantly higher (42.6% increased) in mice fed with HFD as compared to mice on the normal diets. GPE consumption reduced its expression to 1.6-fold compared to mice fed with HFD only. High fat diet fed mice recorded significant increase ($p<0.05$) in mRNA expression of ap2 ($p<0.05$), and GPE supplementation significantly decreased ap2 gene expression by 75% (Fig. 4-9D). However, the expression of FAS, LPL, and lipase were not affected by the diet (data not show).

GPE attenuated the inflammatory cytokines/adipokines expression in adipose tissue.

Inflammatory cytokines/adipokines released from adipose tissue are key factors in promoting systematic inflammation, insulin resistance and obesity. To investigate the effect of GPE on systematic inflammation, we examined the

expression changes on several inflammation cytokines/adipokines in white adipose tissue (Fig. 4-10). Real-time RT-PCR showed that dietary supplement of HFD with GPE significantly suppressed the expression of inflammation factors PAI-1 (Fig. 4-10A) by 48.0% comparing with HFD alone ($P<0.05$). There was no difference observed in comparison with a normal diet control. Leptin expression was not affected by GPE consumption (Fig. 4-10C). No significant changes on resistin, IFNγ, TNFα, and angiopoietin2 were observed (data not show). Adiponectin levels were also reduced in association with insulin resistance. GPE consumption caused a significant increase in the adiponectin (~1.6 fold increase, $p<0.05$) compared to HFD (Fig. 4-10C).

4.5. Discussion

Grape has been reported to have various biological activities and health benefits. As a nutraceutical product, its anti-cancer, anti-inflammatory and anti-diabetes properties have been well documented (Bagchi et al., 2003; Belleville, 2002; Sun et al., 2002, Hogan et al., 2010, Zhang et al., 2011). We have reported that GPE inhibited post prandial hyperglycemia through inhibition of α-glucosidase activity in STZ induced diabetic mice and healthy human subjects (submitted data). Additionally, GPE significantly decreased both postprandial and random blood glucose levels in mice fed 12 weeks of HFD supplemented with GPE. GPE regulates glucose homeostasis hormones and attenuates low-grade systemic inflammation. Nevertheless, the anti-obesity effects of GPE had not been explored. In the present study, we investigated the anti-obesity effects of GPE using 3T3-L1 adipocyte cells and HFD induced obese mice. This research was designed to elucidate the physiological and molecular mechanisms of grape pomace's roles in preventing

obesity using an in vitro cell-based study and high fat induced obese mice model. We evaluated GPE for its putative effects on reducing adipocyte differentiation, decreasing fatty acid accumulation and G3PDH activity, controlling body weight, obesity-related biochemical parameters, and regulating adipose tissue gene expression in both 3T3-L1 pre-adipocyte and high-fat diet–induced obese mice. Our data clearly demonstrate when treated with GPE, maturation and lipid accumulation in the 3T3-L1 cells were inhibited; consumption of GPE in high-fat diet induced obese mice exhibited noticeable attenuation of weight gain, adiposity and abdominal fat-pad weight. GPE also inhibited transcriptional factors expression and the down-stream genes in adipogenesis and fatty acid synthesis and metabolism. The inflammatory cytokines such as PAI1 were also decreased. On the contrary, adiponectin, the anti-inflammatory adipokine, was increased in mice fed with HFD supplemented with GPE. These results indicate that GPE exerts anti-obesity effects.

Our data showed that GPE significantly decreased the mRNA levels of the inflammatory adipokines in both 3T3-L1 pre-adipocyte differentiation and high fat diet induced obese mice. This was in agreement with previous reported studies. Taey et al. reported that PAI-1, plasminogen activator inhibitor-1 plays a key role in obesity, diabetes and cardiovascular disease (Taey et al., 2005) and contributes directly to the complications of obesity, such as type 2 diabetes, pathogenesis of atherothrombosis and cardiovascular diseases. Elevation of PAI-1 can also be attributed to the accumulation of visceral fat (Kruithof, 1988; Skurk et al., 2004; Primrose et al., 1992). Direct inhibition of PAI-1 shows beneficial effects on obesity and insulin resistance. Arçari et al. found that yerba maté extract (Ilex paraguariensis) reduced the expression

of PAI-1 in high-fat diet–induced obese mice and exhibited anti-obesity effects (Arçari et al., 2009).

Adiponectin is the most abundantly adipokine secreted by fat tissue. Biological functions of adiponectin and its close connection with obesity has been reported. Circulating levels of adiponectin are negatively correlated with obesity, insulin resistance, and coronary artery disease (CAD). Plasma adiponectin is relevant in the prediction of insulin resistance and metabolic syndrome (Hara et al., 2006; Eglit et al., 2013). In addition, the anti-inflammatory capacity of adiponectin has become a major focus of research. Adiponectin plays a causal role in protecting against inflammation related diseases such as atherosclerosis, CVDs, and insulin resistance (Surmi et al., 2008, Villarreal-Molina and Antuna-Puente, 2012; Ouchi et al., 2007). Adiponectin targets inflammatory cells to stimulate the expression of the anti-inflammatory cytokines and suppress the genes involved in inflammatory signaling pathway, such as NF-κB, TNFα (Ouchi and Walsh, 2007; Nigro et al., Shibata et al., 2004). The present study showed that adiponectin expression in white adipose tissue was significantly increased in mice administrated GPE with high fat diet for 12 weeks. Similar results were revealed in the 3T3-L1 pre-adipocytes. Both mRNA expression and protein secretion in the differentiating 3T3-L1 cells are dramatically increased after being treated with GPE. Beneficial effects of grape products on serum adiponectin and inflammation were also reported by Tomé-Carneiro et al., with a 75 stable-CAD patients involved triple-blind, randomized, placebo-controlled, 3-arm pilot clinical trial, (Tomé-Carneiro et al., 2013). Grape resveratrol extract exhibited an increase of the anti-inflammatory serum adiponectin ($p = 0.01$) and a decrease of

the plasminogen activator inhibitor type 1 (PAI-1) ($p = 0.05$). These results indicated that grape and its derivations have potential anti-inflammatory effects and attenuate obesity related metabolic syndrome.

Obesity is caused by the imbalance of energy intake and energy expenditure. Irregular adipocyte differentiation, lipogenesis, and excess amount of lipid droplets accumulated in the adipocyte all contribute to obesity. In the present study, GPE reduced oil droplets in adipocytes, depleted fat storage and reduced the triglycerol content and inhibited the activity of H3PDH activity in in-vitro pre-adipocytes (Fig 4-2 and 4-3). GPE also decreased body weight, reduced blood glucose, attenuated abdominal fat weight, and affected white adipocyte morphology in high fat induced obese mice (Fig 4-7. And Table 4-2). The molecular mechanisms of GPE on the regulation of adipocyte differentiation were evaluated. Adipogenesis is a highly coordinated multistep process regulated by transcriptional activators synergistically interacted with the transcriptional regulators, such as PPARs, SREBPs and C/EBPs. They are sequentially activated in adipogenesis transcriptional cascade (Ali et al., 2013). In the present study the mRNA expression of PPAR-γ was upregulated in visceral adipose tissue from mice fed the high-fat diet. The results presented in this study show that treatment with GPE recovered PPAR-γ expression to normal levels. Furthermore, GPE also decreased CEBPα and SREBF1 expression to attenuate adipogenesis in high fat induced mice.

Our results indicate that GPE has potential anti-obesity effect through reduction of adipogenesis and lipogenesis. However, genome-wide studies of how

GPE regulate obesity remained to be conducted. Genome-wide transcriptome analysis should be conducted to provide insights and answers to many remaining questions.

4.6. Conclusion

Our primary goal was to investigate the effects of a dietary grape pomace extract supplement on the prevention of obesity. This study provides preliminary evidence that long-term consumption of GPE can: 1) lower the body weight. 2) down-regulate the expression of several cytokines involved in chronic low-grade inflammation triggered by high fat diet, and 3) attenuate adipogenesis both *in vitro* and *in vivo* in 3T3-L1 pre-adipocytes and high fat diet induced obese mice. GPE shows beneficial effects on anti-obesity.

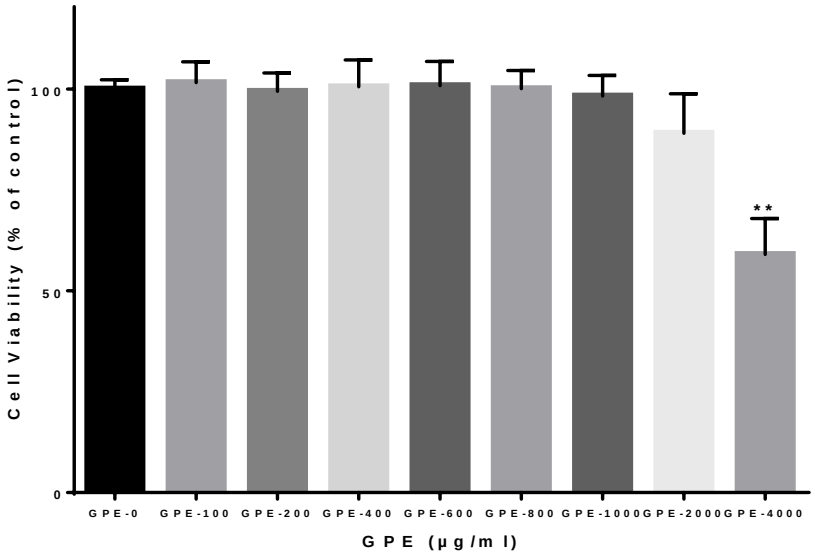
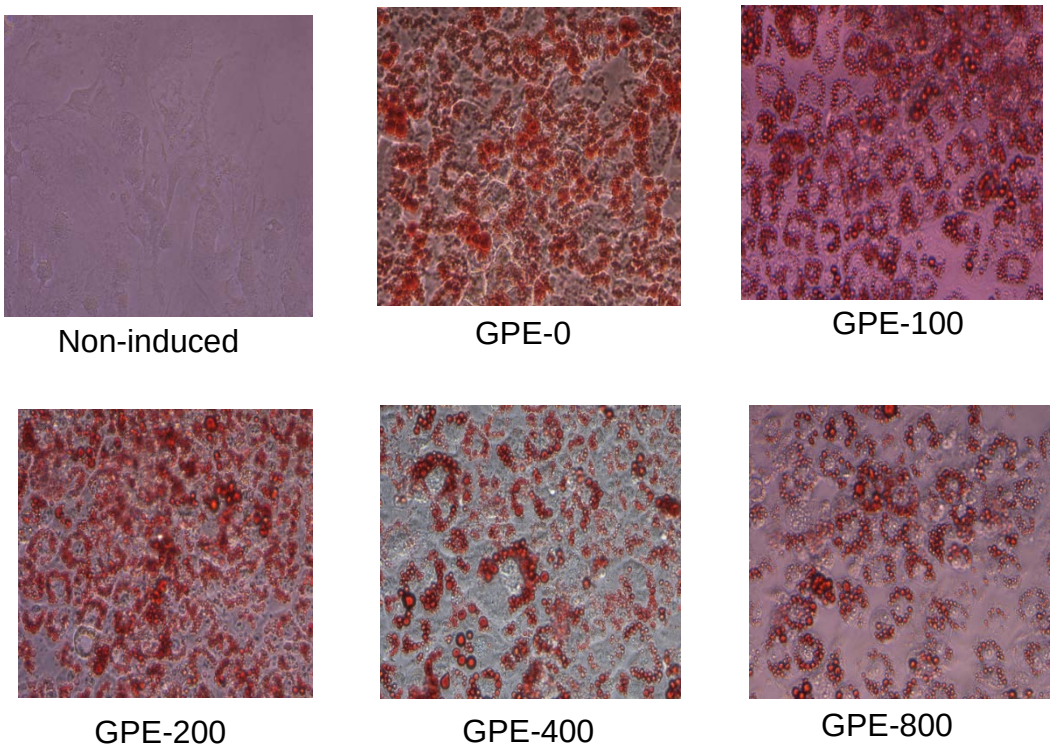
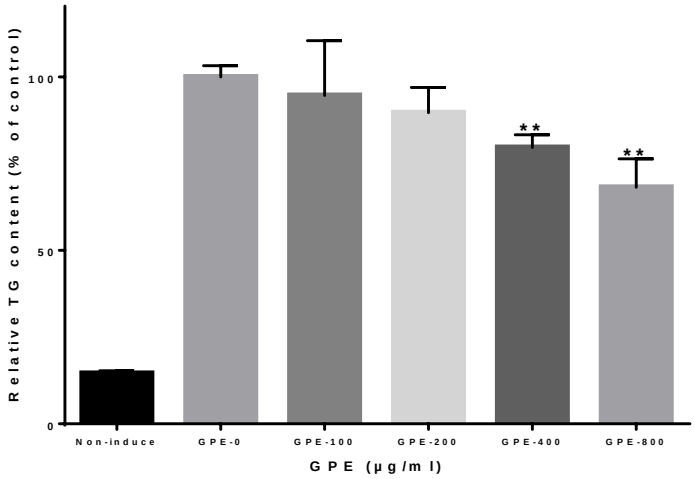


Figure 4- 1 Effect of GPE on pre-adipocyte cell 3T3-L1 viability.3T3-L1 pre-adipocytes were treated with GPE at various concentrations (0, 100, 200, 400, 600, 800, 1000, 2000, 4000 µg/mL) for 24 h. The results represent mean ± SD (n=4). *:$p<0.05$, **: $p< 0.01$.



(A)



(B)

Figure 4- 2 Effect of GPE on inhibiting 3T3-L1 adipocyte differentiation.3T3-L1 cells were differentiated using differentiation media with or without hormones for 3 days under different GPE treatments: 0, 100, 200, 400, 800µg/mL. (A) Intracellular lipids

stained with Oil Red-O. (B) Triglyceride content quantified by measuring absorbance. Non, undifferentiated cells; DM, differentiated medium. The results represent mean ± SD (n=3). *****: $p<0.05$, ******: $p< 0.01$.

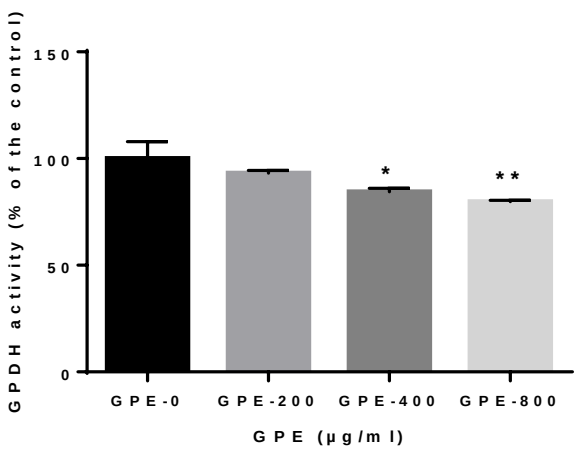
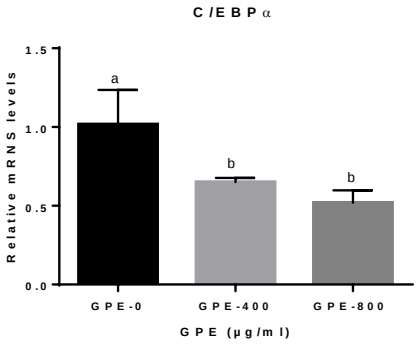
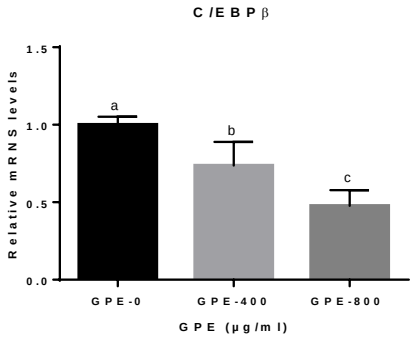


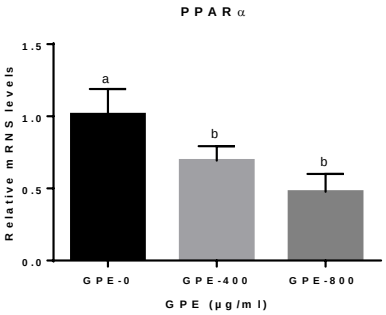
Figure 4- 3 Effect of GPE on Glycerol-3-phosphate dehydrogenase (GPDH) activity during 3T3-L1 adipocyte differentiation.3T3-L1 cells were stimulated with differentiation media with hormones for 3 days under different GPE concentrations: 0, 200, 400, 800µg/mL. The results represent mean ± SD (n=3). *****: $p<0.05$, ******: $p< 0.01$.


C/EBPα
Relative mRNS levels
1.5
1.0
0.5
0.0
a
b
b
GPE-0
GPE-400
GPE-800
GPE (µg/ml)

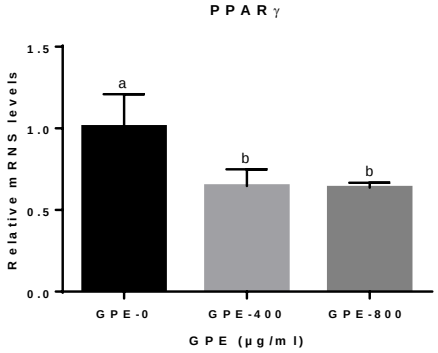
(A)


C/EBPβ
Relative mRNS levels
1.5
1.0
0.5
0.0
a
b
c
GPE-0
GPE-400
GPE-800
GPE (µg/ml)

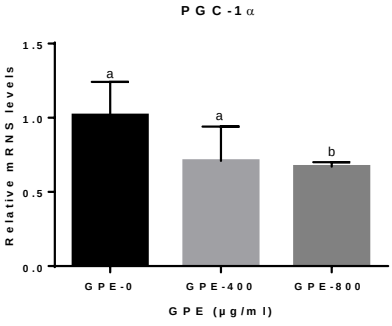
(B)


PPARα
Relative mRNS levels
1.5
1.0
0.5
0.0
a
b
b
GPE-0
GPE-400
GPE-800
GPE (µg/ml)

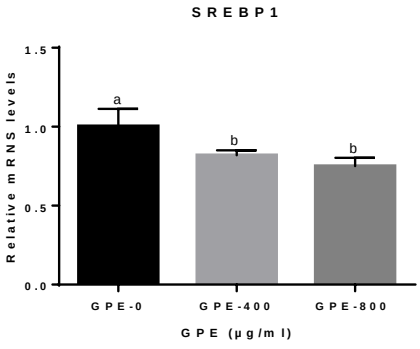
(C)


PPARγ
Relative mRNS levels
1.5
1.0
0.5
0.0
a
b
b
GPE-0
GPE-400
GPE-800
GPE (µg/ml)

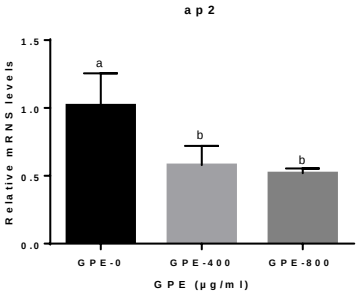
(D)


PGC-1α
Relative mRNS levels
1.5
1.0
0.5
0.0
a
a
b
GPE-0
GPE-400
GPE-800
GPE (µg/ml)

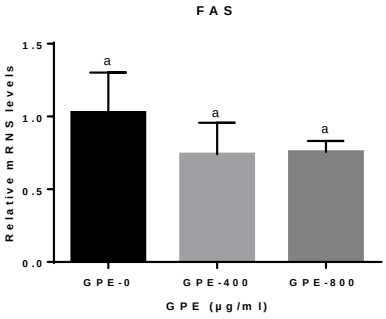
(E)


SREBP1
Relative mRNS levels
1.5
1.0
0.5
0.0
a
b
b
GPE-0
GPE-400
GPE-800
GPE (µg/ml)

(F)


ap2
Relative mRNS levels
1.5
1.0
0.5
0.0
a
b
b
GPE-0
GPE-400
GPE-800
GPE (µg/ml)

(G)


FAS
Relative mRNS levels
1.5
1.0
0.5
0.0
a
a
a
GPE-0
GPE-400
GPE-800
GPE (µg/ml)

(H)

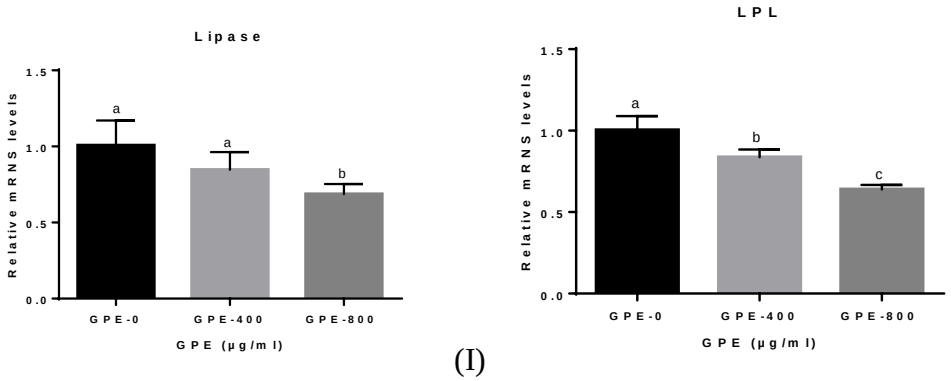


Figure 4- 4 Effect of GPE on adipogenesis gene expression. A-J: C/EBPα, C/EBPβ, PPARα, PPARγ, PGC-1α, SREBP1, ap2, FAS, and Lipase gene in 3T3-L1 adipocytes were investigated using real-time RT-PCR. 3T3-L1 cells were stimulated to differentiation with hormones for 3 days in different GPE concentrations: 0, 400, 800µg/mL. The results represent mean ± SD (n=3). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

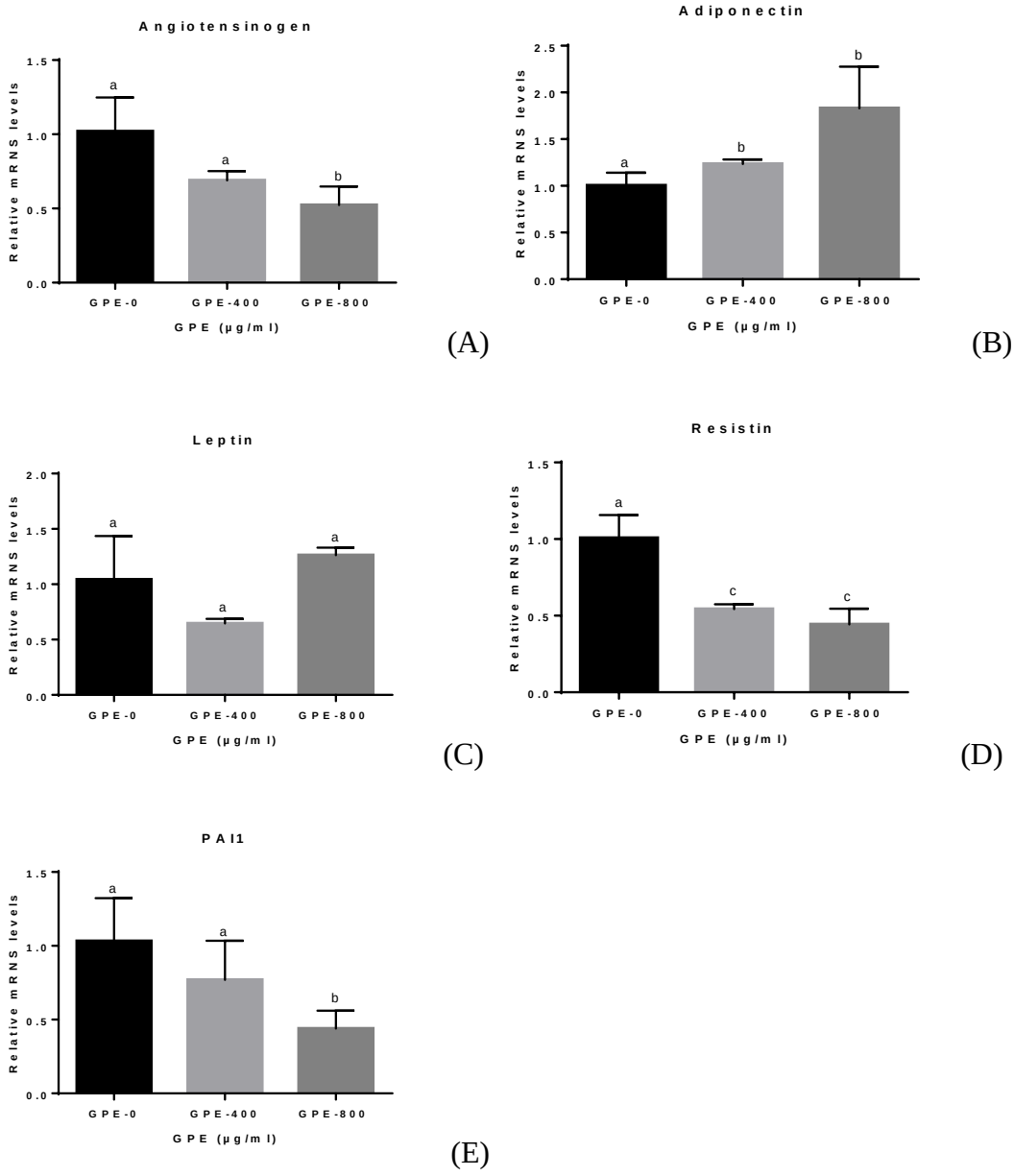


Figure 4-5 Effect of GPE on adipokines gene expression.A-E: angiotensinogen, adiponectin, leptin, resistin, and PAI1 in 3T3-L1 adipocytes were investigated using real-time RT-PCR. 3T3-L1 cells were stimulated to differentiation with hormones for 3 days in different GPE concentrations: 0, 400, 800µg/mL. The results represent mean ± SEM (n=3). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

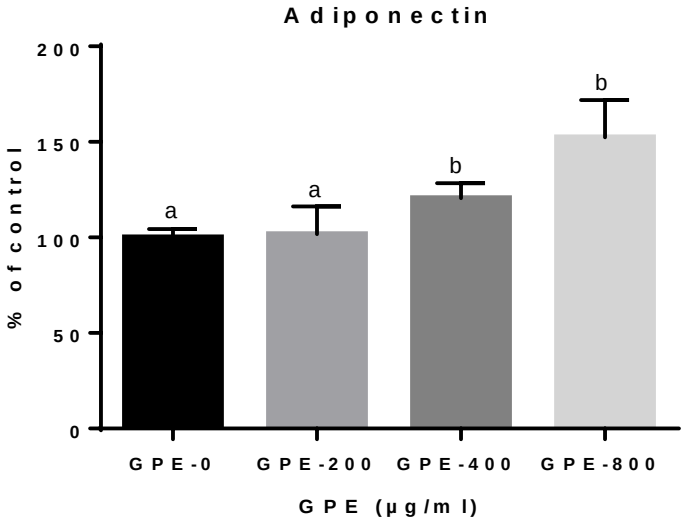
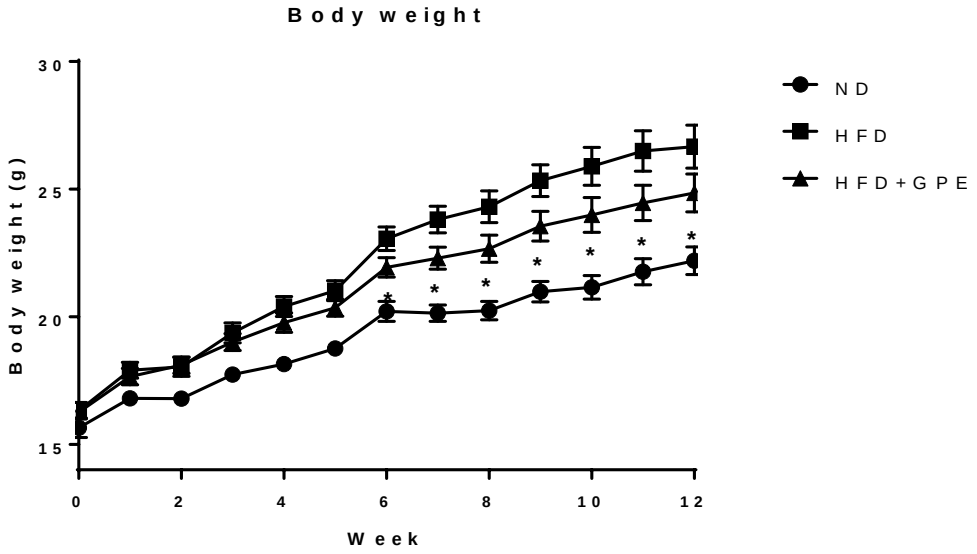
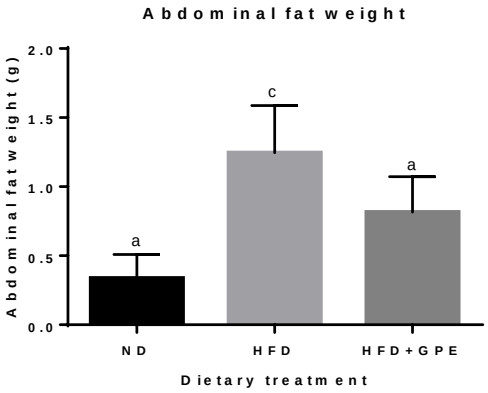


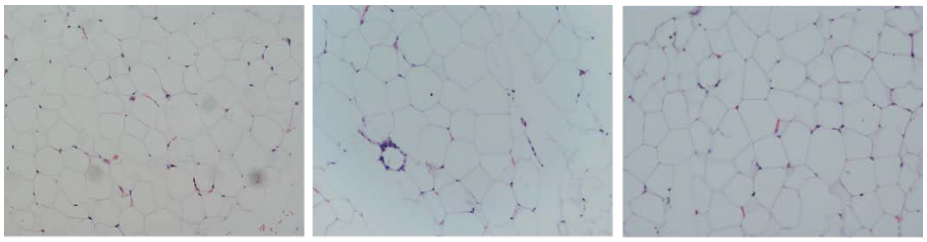
Figure 4- 6 Effect of GPE on protein secretion in the cultured media.3T3-L1 cells were stimulated to differentiation with hormones for 3 days in different GPE concentrations: 0, 200, 400, 800µg/mL. The results represent mean ± SEM (n=3). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.


Body weight
Body weight (g)
30
25
20
15
0
2
4
6
8
10
12
Week
ND
HFD
HFD+GPE
*

(A)


Abdominal fat weight
Abdominal fat weight (g)
2.0
1.5
1.0
0.5
0.0
a
c
a
ND
HFD
HFD+GPE
Dietary treatment

(B)



(C)

Figure 4- 7 GPE reduced body weight, abdominal fat weight and the size of white adipocytes in HFD-induced obese mice.8-week-old C57BLK/6J male mice were randomly divided into three dietary groups fed on ND (normal diet), HFD (high fat diet), HFD+ GPE (high fat diet supplemented with GPE) for 12 weeks (n=14 per group). (A) Body weight of the mice during the 12-week treatment. The results represent mean ± SEM (n=14). (B) Abdominal weight of the mice at 12^{th} week treatment. The results represent mean ± SD (n=14). Columns marked with different letters are significantly different from each other at $p \leq 0.05$. (C) Histology of white adipose tissue in HFD-induced obese mice. Representative epididymal WAT freshly isolated from each mouse group was fixed in 4% formalin and embedded in paraffin. Sections with 8 mm thick were stained with hematoxylin and eosin (magnification x200).

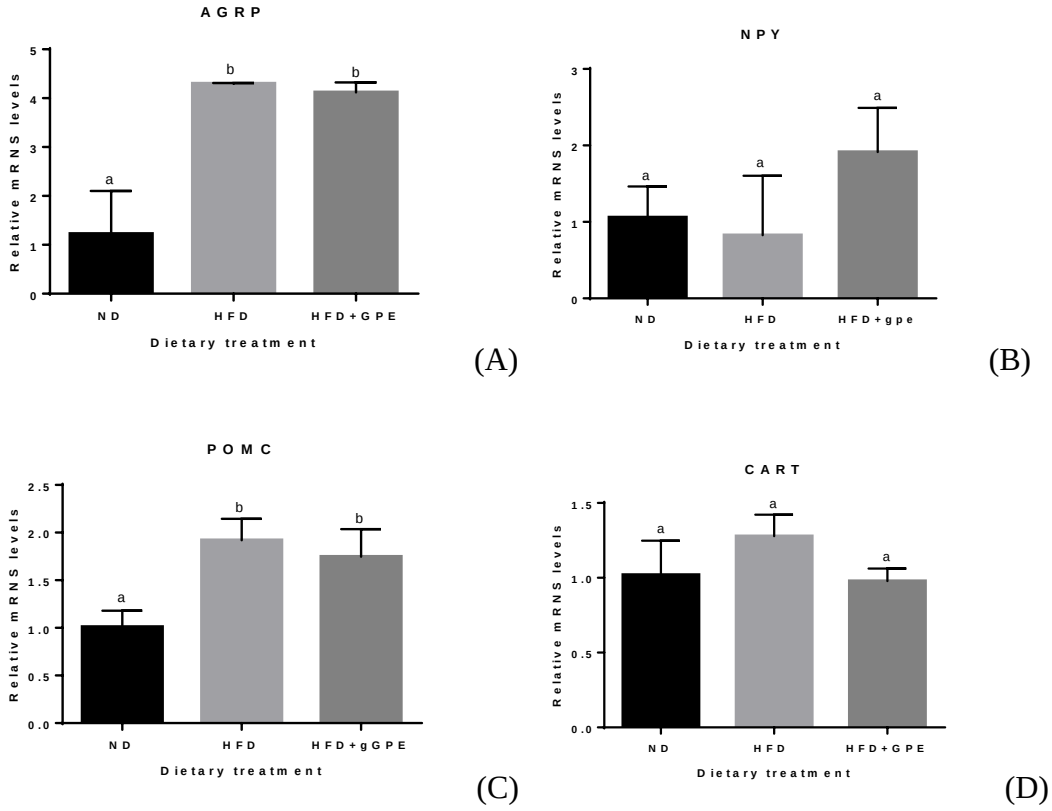


Figure 4-8 Expression of several hypothalamic neuropeptide genes in the hypothalamus.ARC region of hypothalamus were dissected from mice on different diets. Total RNA was extracted and the expression was determined using Real Time PCR. A-D: AGRP, NPY, POMC and CART gene. The results represent mean ± SEM (n=6). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

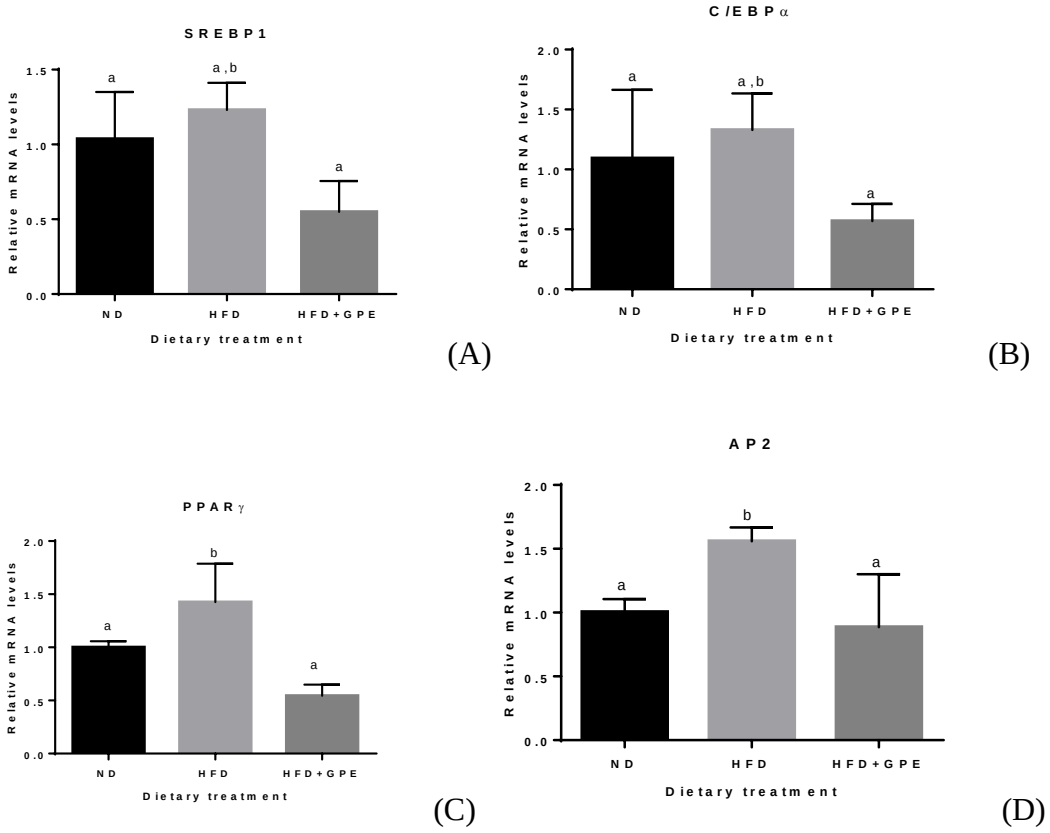


Figure 4-9 Effects of GPE on adipogenesis related transcription factors. White adipocyte tissue were harvested from animals on different diets. A-D: SREBP1, C/EBPα, PPARγ and ap2 gene. Results were expressed as relative expression levels (mean ± SEM, n = 6) to normal diet. Columns marked with different letters are significantly different from each other at $p \leq 0.0$.

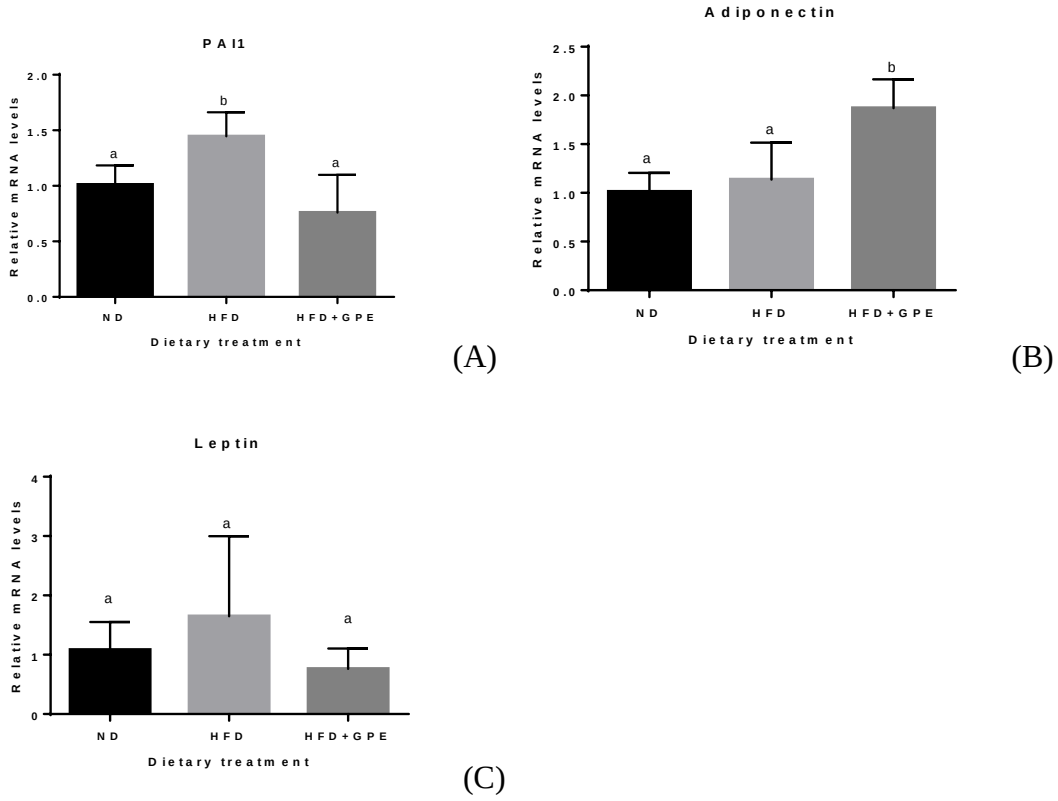


Figure 4-10 Effects of GPE on inflammatory cytokine/adipokine in adipose tissue.WATs were harvested from animals on different diets. A-C: PAI-1, adiponectin and leptin. Results were expressed as relative expression levels (mean ± SEM, n = 6) to normal diet. Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

Table 4-1. The list of genes encoding different peptide hormones related to glucose homeostasis.

Adipogenesis regulation genes: Cebpb, Cebpd
PPARγ Targets: Adiponectin, Cebpa, FAS, Fabp4, Lipe, Lpl, Pparg, Ppargc1α (Pgc-1α), Ppargc1β (Pgc-1β), Srebf1

Table 4-2. The list of genes which are adipokines secreted in adipose tissue

Anti-inflammation adipokines: adiponectin
Pro-inflammation adipokines: leptin, angiotensinogen, resistin, PAI-1

Table 4-3. Macronutrient and selected micronutrient content in the mouse diet.

Rodent Diet with 60 kcal% Fat and 2.4 g Grape Extract /kg

Product #	**D12492**		**D12492+GPE**	
%	gm	*kcal*	gm	*kcal*
Protein	26.2	*20*	26.2	*20*
Carbohydrate	26.3	*20*	26.3	*20*
Fat	34.9	*60*	34.9	*60*
Total		*100*		*100*
kcal/gm	5.2		5.24	
Ingredient	**gm**	***kcal***	**gm**	***kcal***
Casein, 80 Mesh	200	*800*	200	*800*
L-Cystine	3	*12*	3	*12*
Corn Starch	0	*0*	0	*0*
Maltodextrin 10	125	*500*	125	*500*
Sucrose	68.8	*275*	68.8	*275*
Cellulose, BW200	50	*0*	50	*0*
Soybean Oil	25	*225*	25	*225*
Lard	245	*2205*	245	*2205*
Mineral Mix S10026	10	*0*	10	*0*
Mineral Mix S10026B	0	*0*	0	*0*
DiCalcium Phosphate	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*
Grape Extract	0	*0*	2.4	*0*
FD&C Yellow Dye #5	0.05	*0*	0	*0*
FD&C Red Dye #40	0	*0*	0	*0*
FD&C Blue Dye #1	0	*0*	0.05	*0*
Total	**773.8**	***4057***	**773.8**	***4057***
Grape Extract (g/kg)	**0**		**2.4**	

Table 4-4. Effects of GPE on blood glucose, food intake, body weight, organ weight on 12th week of treatment.

	DN	HFD	HFD+ GPE
Blood glucose	136.80±3.95[a]	179.09±4.30[b]	136.35±1.78[a]
Food intake (g/day)	0.41±0.15[a]	0.31±0.16[b]	0.30±0.12[b]
Heart weight	0.16±0.003[a]	0.22±0.015[b]	0.17±0.008[a]
Liver weight	1.25±0.045[a]	1.81±0.15[b]	1.40±0.056[b]
Pancreas weight	0.24±0.022[a]	0.34±0.037[a]	0.30±0.041[a]

Chapter 5: Effects of Grape Pomace Extract on preventing Western Pattern Diet Induced Obesity Revealed by Hepatic RNA-sequencing Transcriptome Profile

5.1. Abstract

Obesity and diabetes are two of the leading risk factors causing health challenges worldwide. Western pattern diet (WPD) with its high fat and high sugar content is well known to cause obesity and diabetes. Recently, we and others have argued that plant-based products could provide a great potential in preventing WPD induced obesity and diabetes. Our previous studies and others' have demonstrated that grape pomace extract (GPE) can reduce blood glucose and eliminate inflammation caused by high fat diet. In the present study, we investigated the role of GPE in the control of body weight. In particular, we wanted to better characterize the molecular basis of how GPE could prevent obesity in mice fed a high fat diet by using an RNA-sequencing (RNA-seq) approach. GPE marginally, but consistently increased body weight gain and significantly reduced circulating leptin. Hepatic transcriptome profiling analysis indicated that GPE significantly modulated expression of 181 genes. Further analysis indicated that GPE upregulated many genes involved in metabolic processes and those associated with energy expenditure and solute carrier transport. Additionally, a large number of genes related to immune responses, oxidative stress responses and inflammation biomarkers were significantly down regulated by GPE. Taken together, our results suggest that GPE can prevent obesity development through enhancing metabolism, accelerating energy expenditure, and reducing various stresses caused by WPD consumption. This study provides a molecular basis of GPE's

potential as a functional food ingredient to prevent WPD-induced obesity development.

5.2. Introduction

Obesity is one of the leading risk factors for a number of diseases that have collectively been referred to as "metabolic syndrome". These include heart disease, diabetes and hypertension (Pi-Sunyer 2009 (ref27). In 2013, the American Medical Association officially classified obesity as a disease (http://www.ama-assn.org). According to the World Health Organization's estimate, globally, more than 39% of adults aged 18 years and older are over-weight, and of these, more than half a billion are obese (www.who.int). In the United States, newly released statistics by the Centers for Disease Control and Prevention (CDC) indicated that 69.0% of Americans aged 20 and older are over-weight and almost half of them are obese (CDC Statistics 2011-2012 data).

Obesity and diabetes are considered to be twin epidemics (Smyth & Heron, 2006). They are two of the most serious public health challenges in developed countries, as well as in developing countries. In addition to genetic factors, the main causes for obesity are thought to be related to diet (high fat and high carbohydrate consumption) and lifestyle (lack of physical activity). It is believed that prolonged consumption of a western pattern diet (WPD), typically a high fat and high carbohydrate diet, can lead to obesity and insulin resistance (Newberry et al., 2006; Prada et al., 2005).

Due to its great challenge to human health, molecular mechanisms that regulate obesity development are being intensively investigated. Adipose tissue,

traditionally considered to merely being used as the body's energy storage tissues, has been re-examined and discovered to play important roles in secreting numerous protein hormones and receptors. These include TNF-alpha, IL-6, leptin and adiponectin. These hormones and receptors effectively communicate with the brain and peripheral tissues to modulate glucose homeostasis, influence energy metabolism, regulate appetite and insulin resistance and control immune function (Matsuzawa et al., 1999; Funahashi et al., 1999). It is also well known that PPAR genes, a transcription factor family regulating glucose, lipid, and cholesterol metabolism in response to fatty acids, are highly expressed in adipose tissue and are key factors controlling adipocyte differentiation and adipocyte-specific gene expression (Jeong et al., 2012). In addition, systemic chronic inflammation due to adipogenesis was also found as a major factor promoting obesity (Xu et al., 2003; Arkan et al., 2005).

Long term high fat diet consumption significantly contributes to the development of both diabetes and obesity in both humans and rodents (Astrup et al., 1994; Lin et al., 2000). At the molecular level, high fat diets cause genome-wide gene expression changes. Several genes encoding enzymes or signaling components related to lipid and glucose metabolism are significantly altered by high fat diet (Murase et al., 2001; Yu et al., 2000; Maquoi et al., 2005; Croce et al., 2007;). Using C57BL/6J mouse as human obesity model, Kim et al. (2004) examined genome-wide hepatic gene expression profiles affected by high fat diet by using a microarray analysis. Compared to a low fat diet (that was used as the control diet), high fat diet consumption significantly altered the expression of 97 hepatic genes involved in metabolism, inflammation responses, apoptosis and cell cycle, and transport (Kim et

al., 2004). These studies provided important insights on signals and molecules involved in the development of high fat diet induced obesity.

Some forms of obesity are thought to be preventable through diet and lifestyle changes (Bray & Popkin, 1998; Leonard et al.,. 2001). In recent years, many researchers have focused on the identification of plant-based natural products which may play potential roles in preventing or delaying the development of obesity and diabetes (Prior et al., 2008; Decorde et al., 2009; Prior et al., 2010). One group of such natural products (including polyphenols, flavonoids and resveratrol) are derived from grapes and other fruits and vegetables. Flavonoids in grapes play a key role in preventing the development of diabetes by acting as multi-target modulators that reduce oxidative stress, lower the degree of systematic inflammation (Tsuda et al., 2012; Chuang et al., 2011), and improve the insulin resistance and other anti-hyperglycemic effects. In obese mice, grape powder has been shown to acutely improve glucose tolerance and chronically reduce inflammation (Chuang et al., 2012). Grape pomace from the Norton variety have been reported to contain significant amount of antioxidants (Hogan et al., 2010), and consumption of Norton grape pomace for 3 months exerted an anti-inflammatory effect in a high fat diet induced mouse obesity model (Hogan et al., 2011).

Previously we investigated the properties of GPE from a Tinta Cao grape variety. GPE had high antioxidant activities, and significantly inhibited the proliferation of HT-29 and Caco-2 colon cancer cells through triggering apoptosis (Parry et al., 2011). The in vitro action of GPE in reducing postprandial hyperglycemia through inhibition of alpha-glucosidase has also been demonstrated (Li et al, 2015a,

submitted). In-vivo studies with STZ-induced diabetic mice as well as with healthy human subjects have been conducted. GPE's acute effect on postprandial hyperglycemia, acute insulin secretion and lipid metabolism were noted (Li et al., 2015a Submitted). Furthermore, we examined several long term effects of GPE consumption on blood glucose regulation, as well as on several circulating peptide hormones. Although GPE did not affect blood glucose in STZ-induced diabetic mice, it significantly reduced blood glucose in WPD induced obese mice. In addition, GPE consumption significantly altered peptide hormones in circulation, such as insulin, GLP-1 and DPP-4.

The liver is an essential organ for lipid and carbohydrate metabolism (Carey et al., 1983). It also plays a critical role in maintaining energy balance. Energy imbalance over a prolonged period can cause obesity. Therefore, in the current study, we focused on examination of hepatic gene expression profiles affected by long term consumption of GPE in a WPD induced obesity mouse model using RNA-seq approach. The present study was conducted to provide genome-wide molecular insights on how GPE balances the effects of WPD on induction of obesity, and prevents and delays the onset of obesity development caused by diets.

5.3. Materials and Methods

Sample preparation and extractions

Grape pomace fruits were received from Chrysalis Vineyards, Virginia. GPE water extraction was prepared as previously described (Li et al., 2015 submitted).

Animals and diet preparation

Animal husbandry, care, and experimental procedures in this study were approved by the Institutional Animal Care and Use Committee at the University of Georgia. Twelve-week old male C57BL/6NCr mice (National Cancer Institute, Frederick, MD, USA) were housed in clean cages at 12-hour light dark cycle at 20°C to 22°C with five mice per cage. After two-week acclimation in the facility with standard rodent chow (8728C Teklad Certified Rodent Diet, Harlan Laboratories, Inc, Frederick, MD), twelve mice were randomly assigned to one of two dietary groups for a 12-week feeding experiment (n=6). Group 1 was fed with WPD diet (45% fat kcal, 35% carbohydrate, 20% protein); while the other group was fed with same WPD diet supplemented with GPE at 2.4 g/kg diet. All mice were fed with the respective diet for 12 weeks with water available ad libitum. Food intake and body weight were recorded every week.

Tissue collection and sample analysis

After completion of the experiments, diet was removed 12 h prior to sacrifice. Mice were anesthetized with CO2, and blood was collected by cardiac puncture with syringes previously rinsed with potassium EDTA solution (15% w/v). The plasma was separated by centrifugation at 1,000x g for 15 min at 4°C and transferred the serum to a clean polypropylene tube and stored at -80°C until analyzed. Liver tissues were collected and immediately frozen in liquid nitrogen and stored in -80°C for subsequent analysis.

For collected serum, plasma leptin and ghrelin were measured using ELISA assay kit (Bio-Rad Laboratories, Hercules, CA). All assays were conducted according to the manufacturer's protocols.

RNA isolation, library construction and RNA-seq sequencing

Total RNA was extracted from liver samples using RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA integrity was monitored using a Nanodrop 2000 Spectrophotometer and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only those samples with RIN larger than 7.0 were used for RNA-seq library construction. Total RNA samples of two randomly chosen mice from each treatment were subject to RNA-seq analysis. Messenger RNAs (mRNA) was isolated with oligo (dT) selection through Dynal magnetic beads. The pair-end libraries were constructed using the TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) following the manufacture's instructions. Briefly, the mRNA was fragmented by exposure to divalent cations at 94°C and the fragmented mRNA was converted into double stranded cDNA. The cDNA ends were polished, the 3′-hydroxls extended with A bases, and ligated to Illumina-specific adapter-primers. The adaptor ligated DNA was amplified by 15 cycles of PCR followed by purification using PCR purification kit (Qiagen, Hilden, Germany) to obtain the final library for sequencing. The DNA yield and fragment insert size distribution of the library was measured using an Agilent Bio-analyzer. Library quantifications were performed by qPCR assays using the KAPA Library Quant Kit™ following the manufacturer's instructions. The constructed libraries were loaded on an Illumina flow cell and were sequenced on the HiSeq2000 sequencing instrument using 101PE pair-ends protocol. Primary data analysis and base calling was performed by the Illumina instrument software.

Bioinformatics analyses and data mining

RNA-seq mapping and expression analyses were performed using the Tuxedo pipeline (Trapnell et al., 2012), and using iPlant cyberinfrastructure resources as described previously (Goff et al., 2011; Mandadi and Scholthof, 2015). Briefly, raw RNA-seq reads were subjected to quality-based filtering and trimming using the FASTQ-quality trimmer and FASTQ-quality filter programs (hannonlab.cshl.edu/fastx_toolkit/index.html). Filtered RNA-seq reads were mapped to the mouse reference genome (NCBIm37) using the TopHat2 for paired-end reads program. The resulting alignments were used to assemble and quantify transcripts, and also were merged with the reference genome using Cufflinks2 and Cuffmerge2 programs, respectively. Normalization of expression was performed using the fragments per kilobase transcript per million mapped fragment (FPKM) metric. Differential gene expression analysis was performed using the Cuffdiff2 program (Trapnell et al., 2013). Statistically significant changes in gene expression were identified after FDR corrections (FDR<0.05).

Global quality assessment of the RNA-seq data was performed by density plot, box plot and scatter plot representation using the CummeRbund program (Trapnell et al., 2012). Statistically significant (FDR<0.05) differences in gene expression were visualized using volcano plots. Visualization of transcript assemblies in the genomic context was performed using the Integrative Genomics Viewer tool (Broad Institute) (Robinson et al., 2011).

Gene ontology (GO)-based functional annotation of the differentially expressed genes was performed using Blast2Go PRO software (Conesa and Gotz,

2008). The assembled transcript sequences were used to perform BLASTX (E-value: 1e-5) analysis against the reference mouse genome sequences, followed by mapping and annotation of the significant hits, according to the recommended instructions (Conesa and Gotz, 2008). Additionally, gene set enrichment analyses (GSEA) and directed acyclic graphs (DAG) of the enriched GO terms in biological process, molecular function and cellular component were performed using WEBbased GEne SeT AnaLysis Toolkit (WebGestalt) (Wang et al., 2013). The Hypergeometric statistical method and FDR correction, using the Benjamini & Hochberg (1995) method, was used for GSEA analysis (Benjamini and Hochberg, 1995)

Statistical analysis

All data were presented as means ±SEM. The differences between two groups were analyzed by t-test using Prism 6 (Graphpad). Differences were considered significant when the P value was ≤ 0.05.

5.4. Result

Effects of GPE on body weight gain and food intake in WPD-induced obesity mice

We monitored body weight and food intake weekly during the feeding experiment. Before the start of the feeding program, the average body weight of the control group was 29.8g, the treatment group (WPD+GPE) weighed 29.8g. Although there was no statistically significant difference between the treatments, the body weight for WPD group was always slightly heavier than that of WPD+GPE feeding group, and reached 43.00g and 40.98g for WPD and WPD+GPE feeding groups respectively at the end of 12-week experiment. Total body weight gain during 12-

week experiment was 13.2g for WPD group, and 11.2g for WPD+GPE feeding group. This difference represented 17.8% less gain on body weight by consumption of GPE.

Food intake was also measured weekly. As shown in Table 5-1, mice fed GPE consumed significantly more food at weeks 5-8 and the 12th week than that fed with WPD only. Overall, on average, 246.1g of food was consumed by each mouse in GPE feeding group during the 12-week experiment, while only 216.0g was consumed for its counterpart in WPD group representing a 14.0% more food consumed by mice feeding with GPE.

Although no statistic difference were detected for both body weight gain and food intake, such trends of changes indicated a potential positive role of GPE in control of body weight and therefore obesity development.

Effects of GPE on serum peptide hormones related to obesity development

To further investigate the role of GPE in control of obesity development, we examined the changes of two obesity related serum peptide hormones. Ghrelin is known as a hunger hormone and plays a significant role in regulating the energy distribution and use efficiency (Inui et al., 2004; Burger & Berner, 2014). Blood ghrelin level was not significantly altered by GPE consumption when compared to the control group (Figure 5-1). However, Leptin, another hormone that regulates energy balance (Friedman, 2010), was significantly altered by GPE feeding. As shown in Figure 5-1, mice from the GPE feeding group had significantly reduced leptin in the blood (95.5 ng/ml in GPE feeding group versus 28,9pg/ml in the control group).

GPE alters hepatic gene expression profiles

We previously studied the effects of GPE on diabetes control and found that GPE can significantly reduce blood glucose and GHbA1C. In addition, GPE attenuated the expression of several peptide hormones related to glucose homeostasis (Li et al., 2015, submitted). Furthermore, we demonstrated in this study that GPE reduced body weight gain under western pattern diet condition and significantly inhibited the secretion of leptin in the circulating system. These results promoted us to further examine the role of GPE on regulating genome-wide transcriptome expression profiles. We conducted high throughput RNA sequencing using liver tissues from both mice fed with WPD and WPD+GPE. Two randomly chosen independent mice from each group were used as biological replicates and subjected to RNA-seq.

After filtering and trimming short prematurely terminated sequences, low quality sequences and ambiguous bases, a total of more than 36 million high quality reads (left and right ends) from each library (WPD-1, WPD-2, GPE-1 and GPE-2) were generated (Table 5-2). These reads were mapped to the mouse reference genome, and transcript assembly and gene expression analysis were conducted, using the Tuxedo RNA-seq data analyses pipeline (Trapnell et al., 2010, 2012, 2013; Mandadi and Scholthof, 2015) (Figure 5-2A). Approximately 95% reads from each library were successfully mapped to the mouse reference genome. Of the mapped sequences, 73% to 86% were unique, and mapped only once to a genomic locus in the mouse reference genome. Global inspection of the gene expression results was further performed using the CummeRbund program (Trapnell et al., 2012). Pairwise scatter matrix plots, density and box plots of the gene expressions revealed normal

distribution of the data, and no systematic biases (Figures 5-2A, 2B and 2C) (Dillies et al., 2013). Volcano matrix plots showed that a significant (FDR<0.05) number of transcripts were up- or down-regulated by GPE (Figures 2D).

In total, we detected 15,759 genes from WPD only group with 1,384 genes uniquely expressed, and 15,276 genes from WPD+GPE feeding group with 901 genes uniquely expressed (Figure 5-3A). Using FPKM≥1 as cut off, these numbers were reduced to 11,168 (with 685 unique genes) and 11,118 (with 635 unique genes) respectively (Figure 5-3B). Furthermore, we identified 286 (2.6%) genes from the WPD group, and 270 (2.4%) genes from the WPD+GPE feeding group as previously unannotated genes that were expressed at FPKM≥1. Fourteen of these genes were significantly differently expressed between WPD group and WPD+GPE group at FDR value less than 0.05. BLASTX analysis against the publicly available reference sequence databases indicated that a few genes were unannotated within any gene models or genome annotations. Others had matches to putative orthologs in other species. For example, these genes encode endonuclease reverse transcriptase (XLOC_027768), NAD-dependent protein deacylase sirtuin (XLOC_033674), GTP-Rho binding proteins (XLOC_019482 and XLOC_007568) and uncharacterized hypothetical proteins (XLOC_023889, XLOC_025814, XLCO_028520, and XLOC_030807).

Differential gene expression analysis between WPD and WPD+GPE groups was conducted using Cuffdiff2. At FDR<0.05 correction, we identified a total of 181 genes that showed significant difference between the two feeding groups. Of these, 94 genes were significantly down regulated by GPE consumption, and 87 genes were

significantly upregulated by GPE. The hierarchical clustering (HCL) and the heat-map analysis clearly showed that samples within the treatment grouped in one cluster distinct from control samples (Figure 5-3C). Moreover, four genes (Cyp2a4, Gsta1, Sprr1a and XLOC-033998) were completely suppressed by GPE feeding while another 4 genes (Moxd 1, Crybb3, XLOC_006742 and XLOC_021098) were uniquely induced by GPE feeding. When using FDR<0.10 as cut off, we identified ~240 genes with significantly different expressions between the groups. The list of these genes with their FPKM values, fold changes and p and FDR-adjusted p values (q values) are presented in Table 5-5 (upregulated genes) and Table 5-6 (down regulated genes). All the genes with significant changes in expression levels in response to GPE treatment at FDR<0.05 level were further analyzed and classified according to their functions using Blast2Go PRO software.

Annotation and classification of differentially expressed genes into functional categories

The Gene Ontology (GO) classification was conducted for all genes exhibiting a significant change in transcript levels using Blast2Go PRO software and against the reference mouse genome sequences as background. All genes were classified according to their biological process and molecular functions. Of the up-regulated genes that are classified based on biological functions, approximately 54% were involved in metabolic process including lipid and carbohydrate metabolisms and small molecule metabolism (Table 5-3). For the molecular function category, 39% were dedicated to catalytic activity (Table 5-4). However, in the genes that were down-regulated by GPE, 27.7% of genes were involved in negative regulation of

biological process, and 26.6% involved in responses to chemical stimulus. A total of 21.3% of genes were involved in immune responses or innate immune responses (Table 5-3). Small amount of genes (3.1%) involved in lipoprotein transport were also down regulated by GPE. When categorized by molecular function, 61.7% of these genes were involved in binding, and of these, more than half (56.9%) were involved in ion binding. Proteins involved in growth factor binding (5.3%) were also down regulated by GPE (Table 5-4).

Genes related to metabolism and energy expenditure were up regulated by GPE

Several genes upregulated by GPE were involved in metabolism and energy expenditure. Many genes controlling metabolism were downregulated by HFD in liver when compared to regular diet (Kim et al., 2004). However, in our experiment (using diets that were high fat and high sugar) when supplemented with GPE, these genes appeared to be upregulated back to the level comparable to that of regular diet condition. These genes included Fabp5 with 1.5 fold change, Serpina4-ps1 with 2.9 fold change, Slc22a7 with 2.8 fold change, 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (Papss2, with 1.5 fold change), elongation of very long chain fatty acids (Elovl3 with 1.1 fold change), cystathionine beta-synthase (Cbs with 1.3 fold change), carbamoyl-phosphate synthetase 1 (Cps1 with 2 fold change), Lipin1 with 1 fold change and at least 6 cytochrome P450 (with 1 to 1.6 fold changes).

Interestingly, genes involved in energy expenditure were also upregulated by GPE. The expression changes for some of these genes are presented individually as FPKM plots (Figure 5-4). Among the GPE altered genes, 6 major urinary proteins (Mup1, Mup 9, Mup11, Mup 12, Mup16, and Mup17) were significantly up regulated

by GPE with at least 2-fold changes (Figure 5-4). Although MUP proteins were originally identified in urine and played key roles in chemical signaling, recent studies suggested that circulating MUP proteins regulate glucose and lipid metabolism. Most members of this gene family are not yet well studied, however, MUP1 has been extensively investigated. It increases energy expenditure and serves as a regulator for glucose and lipid metabolism (Hui et al., 2009; Zhou et al., 2009). Additionally, ENHO (energy homeostasis associated gene) was also significantly up regulated by GPE (Figure 5-4). ENHO encodes a peptide, adropin which is a newly discovered, liver-secreted hormone, and is believed to regulate glucose and lipid metabolism (Kumar et al., 2008).

Another gene family that appeared to be significantly upregulated by GPE is the solute carriers (SLC), which play important roles in transporting organic molecules and inorganic ions in and out of cells through cell membrane. We identified six SLC genes that were significantly altered by GPE. Four of them are upregulated and include Slc22a7, Slc7a2, Slco1a1 and Slco2a1 (Figure 5-4). Furthermore, four serpin peptidase inhibitors (Serpina11, Serpina1e, Serpina4-ps1 and Serpine2) were also significantly up regulated by GPE in liver tissues (Figure 5-4).

Genes related to immune responses and stress responses were down regulated by GPE

In contrast to the GPE up regulated genes, most genes down regulated by GPE were involved in immune responses and include biomarker genes for inflammation. For example, PPARγ, an important gene in regulating diabetes and obesity, was significantly down regulated by GPE, as noted with a 2.7 fold change (Figure 5-5). In

HFD induced obese mice, PPARγ was highly expressed. PPARγ promotes adipogensis and leads to synthesis and deposit of excess fat tissue. GPE consumption significantly reduced PPARγ expression that is induced by HFD suggesting the role of GPE in preventing adipogensis and fat tissue formation. Aox1 gene, a potential target of PPARα was also significantly down regulated by GPE. CD36 was another down regulated gene by GPE. CD36 is responsible for the transport of long chain fatty acids into the adipose tissue (Coburn et al., 2000) and induced by HFD (Kim et al., 2004). Both PPARγ and CD36 are also involved in lipoprotein transport. Another lipoprotein transporter identified as being down regulated by GPE was Unc119. Additionally, several hepatic genes associated with stress responses were significantly down regulated by GPE. These genes include transcription factor myc, heat shock protein 1 (Hspb1), glutamate-cysteine ligase (catalytic subunit) (Gclc), metallothionein 1 (Mt1), and glutathione S-transferases (GSTs). Many of these genes were induced by HFD (Kim et al., 2004). The reduction in expression of these genes by GPE suggests that GPE significantly reduced the stress response caused by high fat contained diets.

5.5. Discussion

It is well known that western pattern diet induces obesity and diabetes in both mice and humans. We and others have demonstrated that grape and grape derived products have the potential to prevent or delay obesity development caused by western pattern diet (Décordé et al., 2009). The purpose of this study was to examine hepatic gene expression profiles affected by GPE in a WPD induced obese mouse model, and to provide molecular mechanisms that drive GPE's action in prevention of obesity

development. After a 12-week feeding experiment, mice fed with WPD supplemented with 2.4g/kg GPE, gained 21% less in body weight. Interestingly, food intake analysis indicated that mice consuming GPE ate 13% more food than HFD consuming mice. The peptide hormone grehlin, a known factor in controlling food intake, did not show any difference between two treatments. However, leptin, another peptide hormone regulating obesity development, was significantly reduced in circulating system by GPE supplementation. Kang et al., (2011) also reported that feeding mice a mixture of red grape extract, soy isoflavone and L-carnitine improved high fat diet induced obesity through down regulation of plasma leptin. Taken together, our results and others strongly suggest that GPE may play positive roles in preventing increased body weight and obesity.

The liver plays an essential role in maintaining energy balance. In general, obesity can be caused by energy imbalance over a prolonged time. Previously, the effects of HFD on transcriptional changes in liver were investigated in both the early stage of HFD intake (Gregorie et al., 2002) and a long term HFD feeding stage using microarray technology (Kim et al., 2004, 2005; Gu et al, 2009; Xie et al., 2010). Most of these studies identified approximately 100 genes (from 86 to 130 genes) that were significantly altered by HFD when compared to standard diet. In our current study, using the RNA-seq approach, we examined the role of GPE on regulating hepatic transcriptome profiles under high fat and high sugar conditions. We used a more stringent high fat diet, with 45% of calories derived from fat when compared to other studies with 35% of calories derived from fat (Kim et al, 2004, 2005). In addition, another 35% of the calories in our diet were derived from sugar. In total, more than

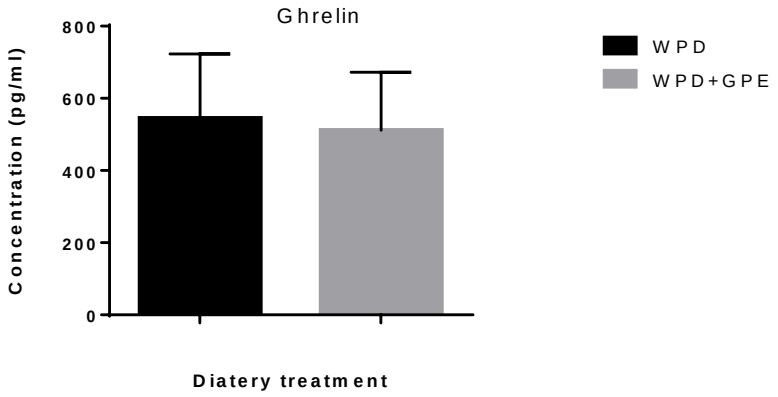
15,000 genes were detected in both WPD-fed and WPD+GPE fed mice. Of these, approximately 180 genes were significantly up- or down- regulated by GPE after FDR correction (<0.05). Based on our literature review, we found that ~50% of these genes were previously identified as either being induced or suppressed by high fat diet or were found in obese mice and humans, even though the specific roles for most of these genes in obesity development are not yet understood. Surprisingly, expression of many of these genes was adjusted by GPE supplementation. Expression levels were closer to that under the standard diet condition. Furthermore, about 45% of identified genes in the current research were previously unidentified in relation to obesity or diabetes.

GO analysis showed that most of the up regulated genes were involved in metabolism. Solute carrier genes and serpin peptidase inhibitors were also upregulated by GPE. Interestingly, we also found that several hepatic genes that are involved in energy expenditure were significantly upregulated by GPE. These include several members of MUP family and ENHO. Upregulation of these genes is related to metabolism and energy expenditure by GPE and could enhance the efficient use of the excess energy derived from WPD, and in that way could prevent the deposit of unused energy into adipose tissue. On the other hand, those that were down regulated by GPE were involved in immune and stress responses. Accordingly, many immune responsive genes and stress defending genes were up regulated by high fat diet (Kim et al., 2004, 2005). Obesity causes systemic chronic inflammation, hence HFD leads to significant induction of biomarker genes related to inflammation (Xu et al., 2003; Arkan et al., 2005). Supplementation of GPE, along with WPD, significantly

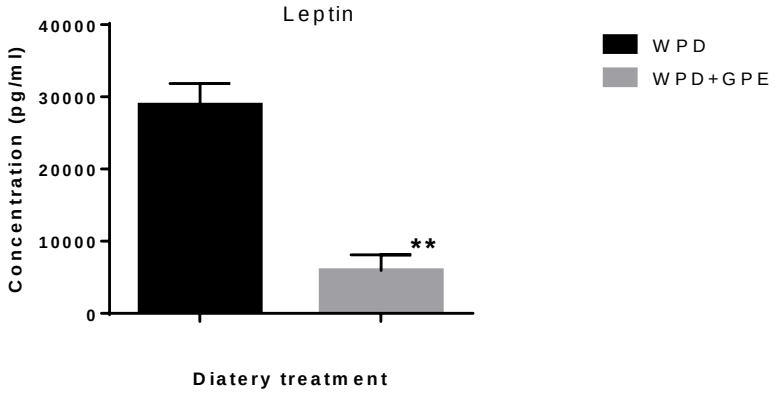
downregulated expression of inflammation biomarkers, which are commonly induced by HFD. These included PPARγ, cd36, cd74 for immune responses; MTs and GSts for stress responses; and postn, gpc1 and saa2 for inflammation. Downregulation of these genes by GPE suggests that GPE can reduce stresses caused by WPD and delays obesity development under WPD condition.

5.6. Conclusion

Our hepatic transcriptome analysis has shown that GPE supplementation could enhance energy expenditure through upregulation of metabolism related genes and genes controlling energy homeostasis. Furthermore, GPE reduces various WPD-induced stress responses and obesity-induced inflammation. This study provides insights at the molecular level into understanding GPE's actions in preventing and delaying the development of obesity by a western pattern diet. Our previous research found that, when consumed with white bread, GPE can significantly reduce acute blood glucose level (Li et al., 2015). Taken together, our studies indicate that GPE may be a potential functional food ingredient that can potentially prevent or delay the onset of both diabetes and obesity.

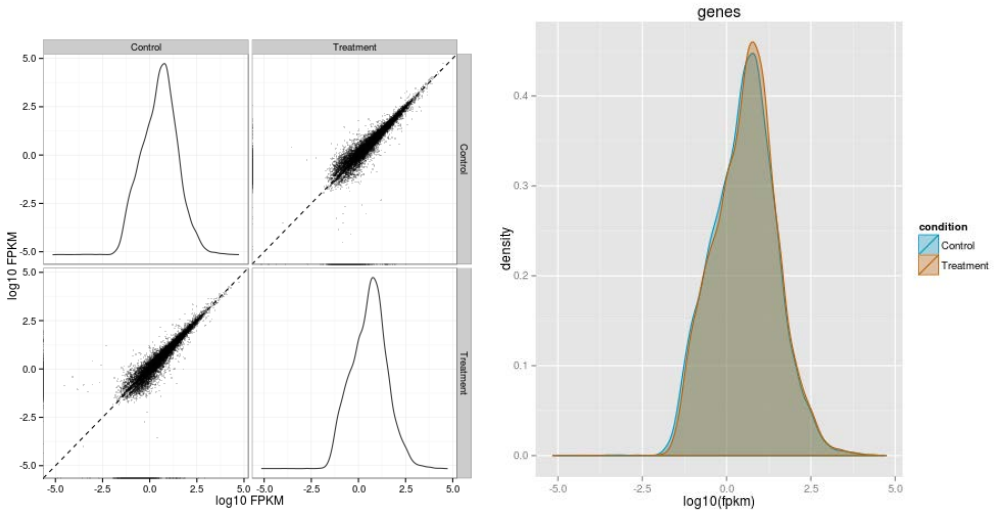


A

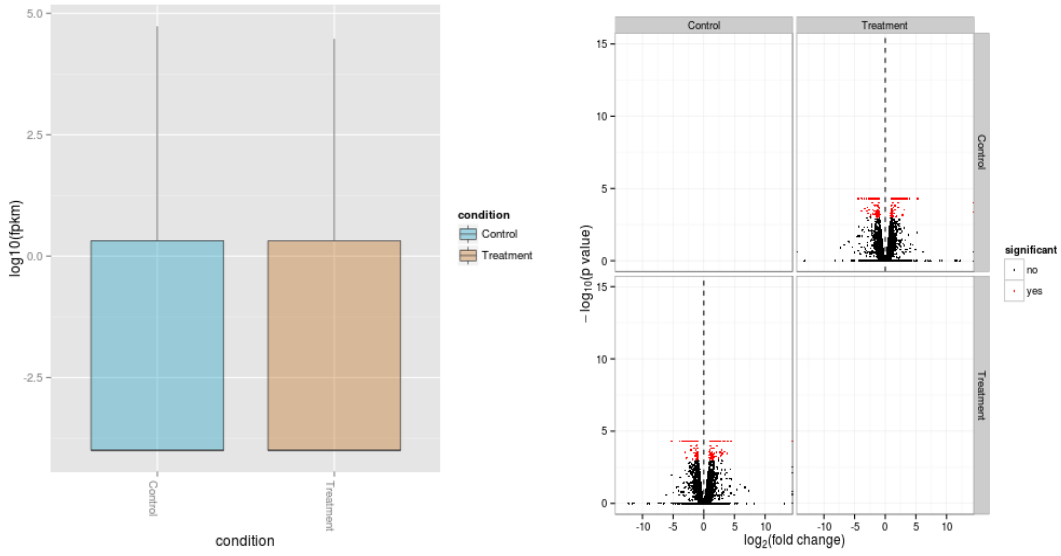


B

Figure 5- 1 Circulated peptide hormone expression in WPD and WPD+GPE treated groups.A. Ghrelin expression showed no difference between treatments; B. Leptin expression was significantly reduced by GPE supplementation.

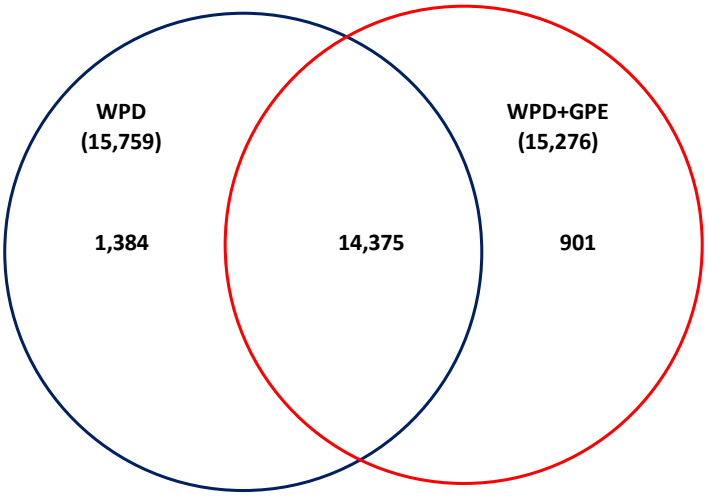


(A) (B)

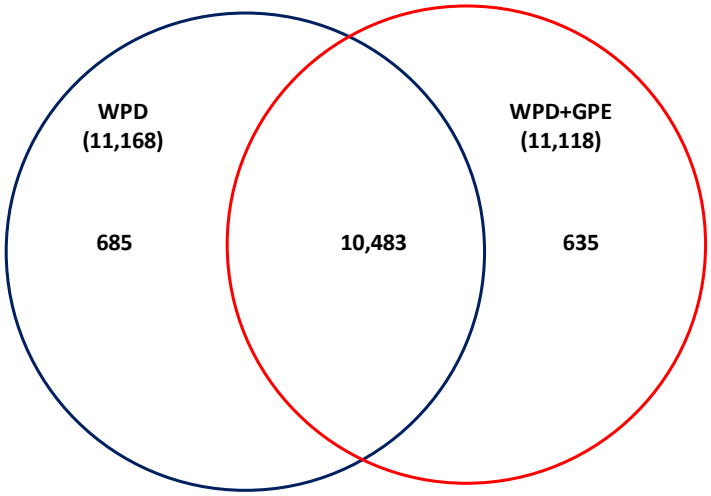


(C) (D)

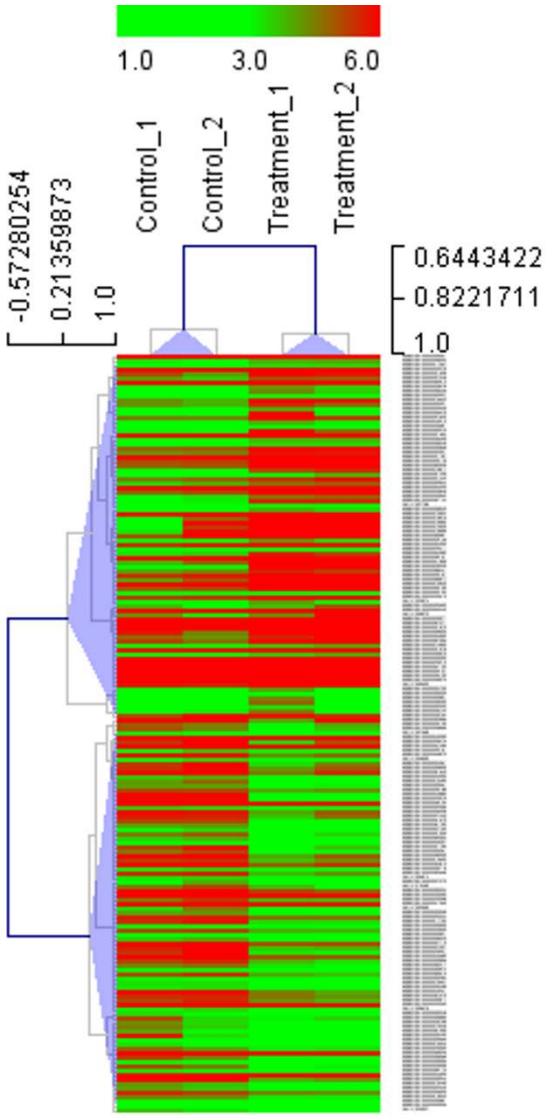
Figure 5- 2 Global analysis of gene expression in WPD and WPD+GPE treated mice showed normal distribution of the expression data and differentially expressed genes.A. Expression scatter matrix, B. Expression density plots, C. Expression box plots, and D. Expression fold change volcano matrix


WPD
(15,759)
WPD+GPE
(15,276)
1,384
14,375
901

(A)

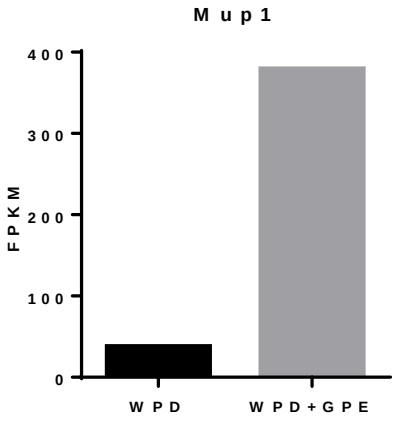

WPD
(11,168)
WPD+GPE
(11,118)
685
10,483
635

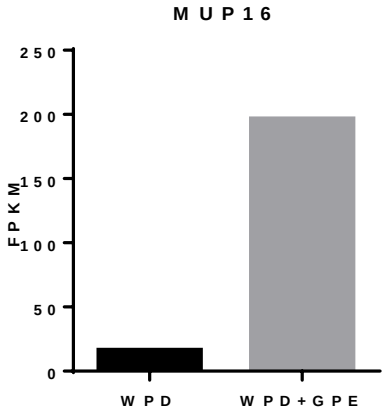
(B)

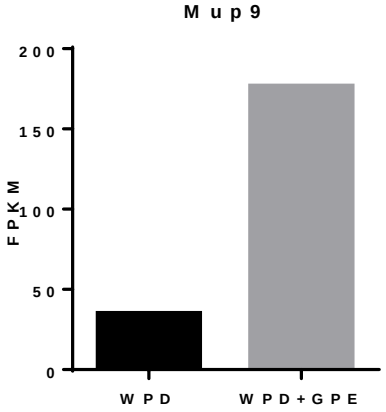


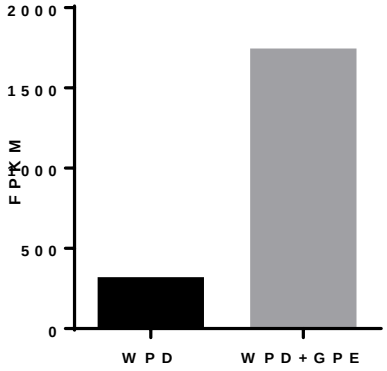
(C)

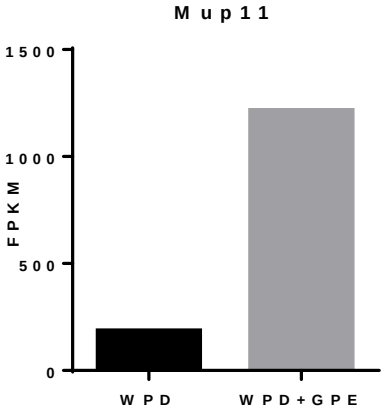
Figure 5- 3 Venn diagrams of the expressed genes and hierarchical cluster analysis of differentially expressed genes between WPD and WPD+GPE treatments. A. Venn diagram showing the distribution of all genes expressed in WPD group and WPD+GPE group. B. Venn diagram showing the distribution of genes with FPKM>1 in WPD group and WPD+GPE group. C. Cluster analysis of all genes with significant differences in their expression between the groups. Control group represents those fed with WPD only, treatment group represents mice with WPD+GPE feeding.

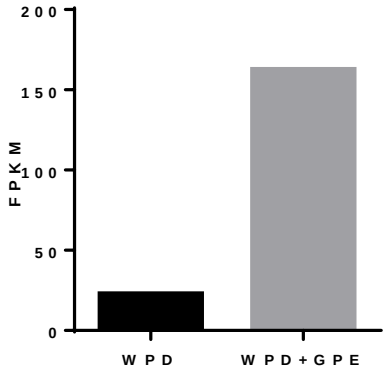

Mup1
400
300
200
100
0
FPKM
WPD
WPD+GPE


MUP16
250
200
150
100
50
0
FPKM
WPD
WPD+GPE


Mup9
200
150
100
50
0
FPKM
WPD
WPD+GPE


Mup 17
2000
1500
1000
500
0
FPKM
WPD
WPD+GPE


Mup11
1500
1000
500
0
FPKM
WPD
WPD+GPE


MUP12
200
150
100
50
0
FPKM
WPD
WPD+GPE

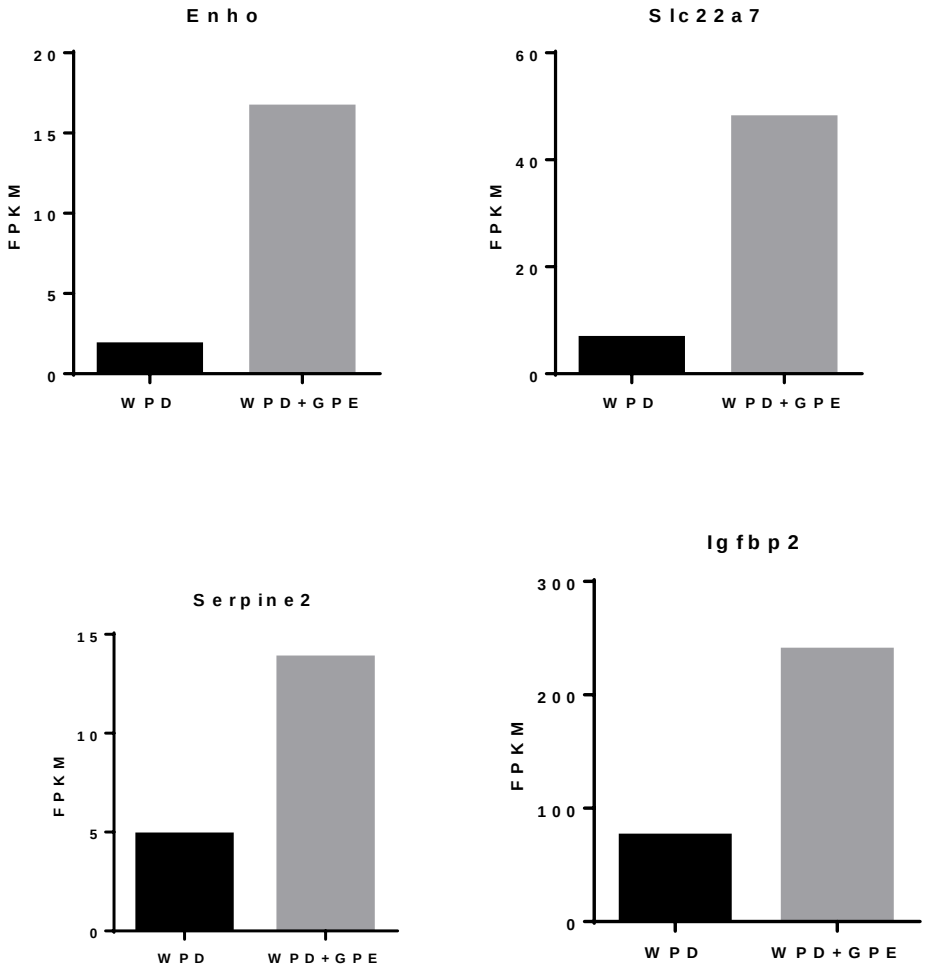
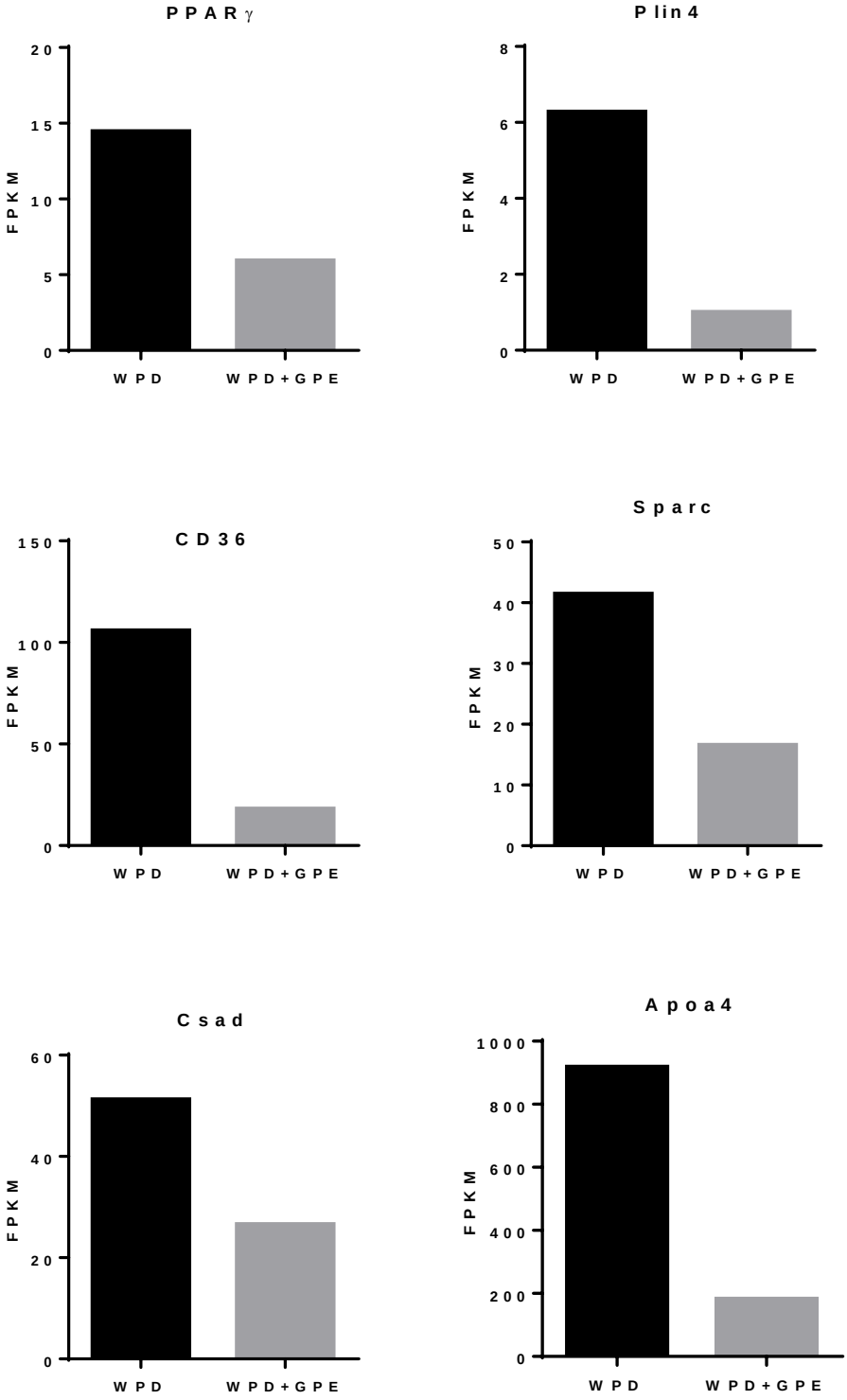


Figure 5-4 Expression plots of selected upregulated genes in GPE supplementation.Levels of expression (FPKM) of each individual gene is plotted to reveal the difference between WPD fed and WPD+GPE fed mice.


PPARγ
FPKM
20
15
10
5
0
WPD
WPD+GPE
Plin4
FPKM
8
6
4
2
0
WPD
WPD+GPE
CD36
FPKM
150
100
50
0
WPD
WPD+GPE
Sparc
FPKM
50
40
30
20
10
0
WPD
WPD+GPE
Csad
FPKM
60
40
20
0
WPD
WPD+GPE
Apoa4
FPKM
1000
800
600
400
200
0
WPD
WPD+GPE

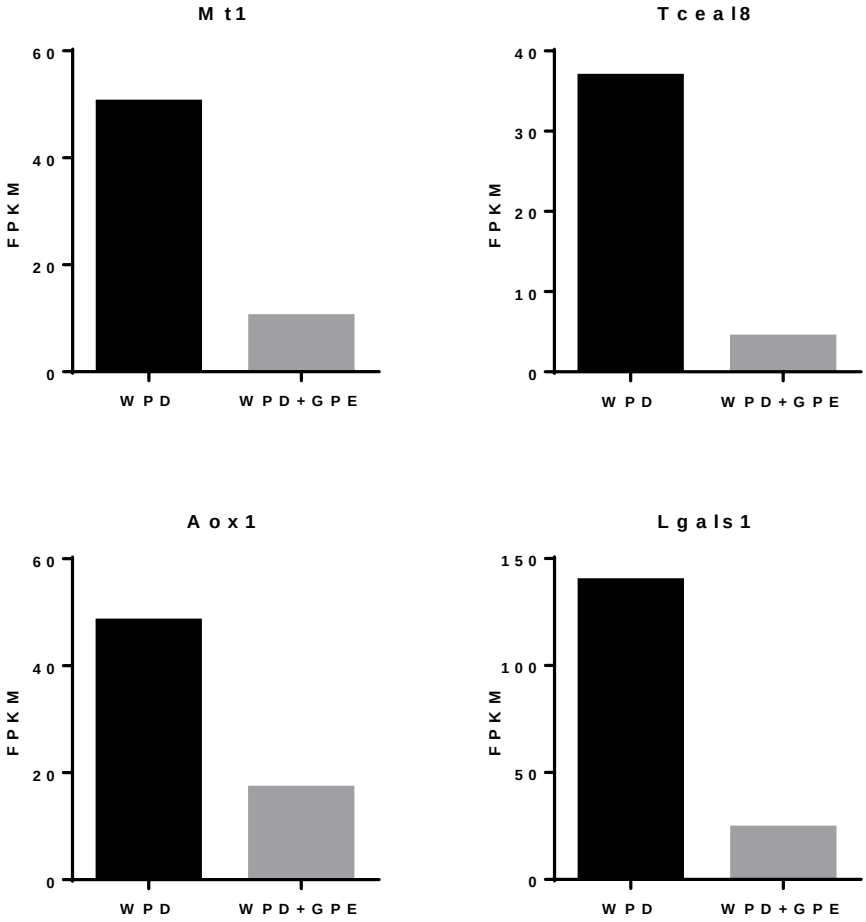


Figure 5-5 Expression plots of selected downregulated genes in GPE supplementation.Levels of expression (FPKM) of each individual gene is plotted to reveal the difference between WPD fed and WPD+GPE fed mice.

Table 5-1. Body weight gain and food intake during 12-week experiment in WPD and WPD+GPE mice

Week	WPD Body Weight (g)	WPD+GPE Body Weight(g)	WPD Food Consuming (g)	WPD+GPE Food Consuming(g)
0	29.82±0.77	29.79±0.72		
1	33.59±1.14	32.56±1.24	3.74±0.19	3.97±0.2
2	34.6±1.59	33.1±1.22	2.59±0.13	2.79±0.06
3	36.53±1.59	34.08±1.47	2.83±0.13	2.9±0.14
4	35.74±1.64	33.18+1.47	2.53±0.06	2.74±0.10
5	37.87±1.71	35.46+1.56	2.51±0.10	2.89±0.13*
6	38.28±1.57	36.35+1.48	2.33±0.02	2.71±0.14*
7	39.09±1.52	36.58+1.89	2.36±0.09	2.85±0.09**
8	39.79±1.59	38.16±2.33	2.45±0.08	3.02±0.12**
9	39.68±1.62	38.16±2.28	2.02±0.09	2.28±0.20
10	40.58±1.54	38.68±1.98	2.43±0.03	2.78±0.18
11	41.97±1.53	39.46±2.17	2.52±0.20	3.22±0.29
12	43± 1.49	40.98±2.04	2.54±0.06	3.01±0.18*
Total weight gain (g)	13.18	11.19		
Total Food Consumed(g)			215.95	246.12
Difference (%)	1.99 (17.8%)		30.17 (14.0%)	

Table 5-2. Statistics of RNA-seq mapping analysis.

	WPD-1	WPD-2	WPD+GPE-1	WPD+GPE-2
Trimmed reads				
Left	18,145,626	19,501,734	18,446,697	19,181,364
Right	18,113,614	19,469,121	18,418,621	19,152,427
Mapped				
Unique left (%)	14,836,086(81.8)	15,188,107(77.9)	13,997,103(75.9)	14,058,494(73.3)
Nonunique left (%)	2,517,763(13.9)	3,283,928(16.8)	3,568,483(19.3)	4,256,496(22.2)
Unique right (%)	14,796,700(81.7)	15,150,906(77.8)	13,965,442(75.8)	14,036,156(73.3)
Nonunique right (%)	2,504,632(13.8)	3,268,023(16.8)	3,553,152(19.3)	4,242,932(22.2)
Overall alignment	95.55%	94.65%	95.15%	95.45%
Total aligned pairs	16,628,026	17,537,998	16,762,308	17,539,443

Table 5-3. GO enrichment analysis of differentially expressed genes in biological process category.

GO term	Description	No. in input list	No. in Ref	P value	FDR
Genes in Group A					
GO:0044281	small molecule metabolic process	23	1821	6.57E-09	4.15E-06
GO:0006082	organic acid metabolic process	14	731	7.76E-08	2.45E-05
GO:0019752	carboxylic acid metabolic process	13	675	2.26E-07	4.75E-05
GO:0043436	oxoacid metabolic process	13	717	4.49E-07	7.08E-05
GO:1901606	alpha-amino acid catabolic process	5	57	1.14E-06	0.0001
GO:0009063	cellular amino acid catabolic process	5	67	2.56E-06	0.0003
GO:0046395	carboxylic acid catabolic process	6	148	8.57E-06	0.0007
GO:0016054	organic acid catabolic process	6	148	8.57E-06	0.0007
GO:0008152	metabolic process	47	8894	1.14E-05	0.0008
GO:0044282	small molecule catabolic process	6	196	4.20E-05	0.0021
Genes in Group B					
GO:0070887	cellular response to chemical stimulus	21	1053	1.49E-10	1.21E-07
GO:0042221	response to chemical stimulus	25	2020	3.62E-08	1.47E-05
GO:0055114	oxidation-reduction process	15	919	1.20E-06	0.0002
GO:0010035	response to inorganic substance	9	279	9.08E-07	0.0002
GO:0006950	response to stress	22	2088	4.54E-06	0.0007
GO:0006955	immune response	12	669	6.15E-06	0.0008
GO:0048519	negative regulation of biological process	26	2902	9.40E-06	0.0011
GO:0071229	cellular response to acid	4	38	1.16E-05	0.0012
GO:0045087	innate immune response	8	299	1.49E-05	0.0013
GO:0042953	lipoprotein transport	3	14	1.72E-05	0.0014

- Genes in Group A are upregulated by GPE supplementation; and genes in group B are down regulated by GPE supplementation

Table 5-4. GO enrichment analysis of differentially expressed genes in molecular function category

GO term	Description	No. in input list	No. in Ref	P value	FDR
Genes in Group A					
GO:0030170	pyridoxal phosphate binding	5	51	7.22E-07	3.84E-05
GO:0009055	electron carrier activity	6	108	1.56E-06	3.84E-05
GO:0008483	transaminase activity	4	24	1.12E-06	3.84E-05
GO:0016769	transferase activity, transferring nitrogenous groups	4	25	1.33E-06	3.84E-05
GO:0070279	vitamin B6 binding	5	51	7.22E-07	3.84E-05
GO:0020037	heme binding	6	114	2.15E-06	4.41E-05
GO:0031406	carboxylic acid binding	7	189	3.07E-06	4.90E-05
GO:0046906	tetrapyrrole binding	6	122	3.19E-06	4.90E-05
GO:0004497	monooxygenase activity	6	143	7.97E-06	0.0001
GO:0003824	catalytic activity	34	5105	8.72E-06	0.0001
Genes in Group B					
GO:0050840	extracellular matrix binding	5	38	2.39E-07	3.01E-05
GO:0005488	binding	58	10781	6.83E-06	0.0004
GO:0016491	oxidoreductase activity	12	737	1.18E-05	0.0005
GO:0019838	growth factor binding	5	107	4.17E-05	0.0013
GO:0043236	laminin binding	3	22	6.51E-05	0.0016
GO:0004364	glutathione transferase activity	3	26	0.0001	0.0021
GO:0043167	ion binding	33	5208	0.0003	0.0054
GO:0046983	protein dimerization activity	11	934	0.0005	0.0063
GO:0008289	lipid binding	9	648	0.0005	0.0063
GO:0005518	collagen binding	3	41	0.0004	0.0063

- Genes in Group A are upregulated by GPE supplementation; and genes in group B are down regulated by GPE supplementation

Table 5-5. List of significantly upregulated genes by GPE under WPD condition

Gene Name	FPKM-WPD	FPKM-GPE	log2 (fold change)	p_value	q_value
Moxd1	0	36.0572	inf	5.00E-05	0.004307
Crybb3	0	3.9232	inf	5.00E-05	0.004307
XLOC_021098	0	9.05402	inf	0.0001	0.008061
XLOC_006742	0	98.1434	inf	0.0004	0.023908
Hsd3b5	3.61628	141.493	5.29008	5.00E-05	0.004307
Cbx8	3.15135	48.0516	3.93054	5.00E-05	0.004307
Mup16	16.359	196.478	3.58621	5.00E-05	0.004307
Serpina1e	194.381	2187.37	3.49224	5.00E-05	0.004307
XLOC_033674	1.06501	11.8183	3.47207	5.00E-05	0.004307
Mup1	37.6937	379.341	3.3311	5.00E-05	0.004307
Enho	1.81	16.6186	3.19874	5.00E-05	0.004307
Scara5	0.463545	3.92739	3.08279	0.0003	0.019818
Selenbp2	25.056	197.383	2.97777	5.00E-05	0.004307
Serpina4-ps1	8.05187	60.2661	2.90395	5.00E-05	0.004307
Slc22a7	6.6424	47.8653	2.84921	5.00E-05	0.004307
Rabl2	1.66147	11.8461	2.83388	0.0007	0.036608
Mup12	22.9421	162.792	2.82697	5.00E-05	0.004307
Mup11	186.086	1215.61	2.70764	5.00E-05	0.004307
C8b	30.4408	176.799	2.53803	5.00E-05	0.004307
Slco1a1	21.6119	125.337	2.53592	5.00E-05	0.004307
Mup17	306.03	1731.28	2.50009	5.00E-05	0.004307
Ighm	7.3934	39.6005	2.42121	5.00E-05	0.004307
XLOC_014487	1.50612	7.91347	2.39348	0.0016	0.069249

Mup9	35.1333	176.757	2.33086	5.00E-05	0.004307
XLOC_027770	2.0418	9.88298	2.2751	0.00145	0.063699
Slc43a1	0.89391	4.3248	2.27443	0.00265	0.09824
Zbtb49	0.901191	4.14683	2.20211	0.0027	0.098843
Egfr	32.2418	142.533	2.1443	0.0002	0.014286
Upp2	38.5061	167.763	2.12327	5.00E-05	0.004307
Acmsd	2.57294	10.8568	2.07711	0.0002	0.014286
Lrrc16a	2.69661	11.2614	2.06217	0.0001	0.008061
Marco	3.6366	15.1593	2.05954	5.00E-05	0.004307
Cps1	178.254	727.483	2.02898	5.00E-05	0.004307
XLOC_015037	1.74251	7.02461	2.01125	0.00195	0.079318
Igkc	14.2835	56.6227	1.98703	5.00E-05	0.004307
Acpp	1.62289	6.28508	1.95336	5.00E-05	0.004307
Lifr	11.3699	43.8454	1.9472	5.00E-05	0.004307
Socs2	1.54613	5.45279	1.81834	0.0024	0.093303
Cadm4	1.24048	4.36957	1.81659	0.00255	0.095338
Hamp	1274.89	4474.75	1.81143	5.00E-05	0.004307
Avpr1a	1.60847	5.61163	1.80273	0.00085	0.042675
XLOC_027768	2.43088	8.4263	1.79342	0.0009	0.044175
Prhoxnb	17.3853	60.2084	1.79209	5.00E-05	0.004307
XLOC_008927	2.03094	6.82999	1.74973	0.00195	0.079318
Pold3	6.59681	21.5652	1.70886	0.00255	0.095338
XLOC_026364	1.78265	5.81716	1.70629	0.0024	0.093303
Igfbp2	75.4467	239.364	1.66568	5.00E-05	0.004307
Susd4	4.71087	14.6646	1.63827	5.00E-05	0.004307
Tat	240.842	747.02	1.63306	0.00015	0.011264
Cyp2c70	106.006	325.516	1.61858	5.00E-05	0.004307
Cyp7b1	41.6257	126.977	1.60902	5.00E-05	0.004307
Tnrc18	1.37261	4.16457	1.60125	0.00245	0.094411
Trim14	9.04926	27.148	1.58497	0.00015	0.011264
Fabp5	23.34	69.309	1.57024	5.00E-05	0.004307

Mpv17l	4.89329	14.5088	1.56805	0.0003	0.019818
Ces3b	68.2837	197.388	1.53142	5.00E-05	0.004307
Cyp1a2	76.905	221.594	1.52677	5.00E-05	0.004307
Emr1	3.51451	10.076	1.51952	5.00E-05	0.004307
Tgm1	1.63912	4.64967	1.5042	0.0013	0.057979
Serpine2	4.87296	13.8167	1.50355	5.00E-05	0.004307
Cyp2c54	33.0642	93.1461	1.49422	5.00E-05	0.004307
Camkk2	1.35482	3.7647	1.47444	0.0018	0.075669
Fgl1	207.214	573.246	1.46804	5.00E-05	0.004307
Papss2	23.4493	64.827	1.46705	5.00E-05	0.004307
Eapp	7.75341	20.8587	1.42774	0.00255	0.095337
Camk1d	6.06286	15.6069	1.36412	5.00E-05	0.004307
Tfdp2	1.83488	4.719	1.36279	0.0005	0.02816
Cela1	20.7264	52.2742	1.33463	0.00035	0.021809
Nnmt	86.5215	217.402	1.32923	5.00E-05	0.004307
Rnase4	249.116	623.428	1.32341	5.00E-05	0.004307
Cbs	133.045	328.714	1.30492	0.0002	0.014286
Cdh1	2.73557	6.74993	1.30303	0.0005	0.02816
Ablim3	1.5797	3.83855	1.28091	0.00165	0.070717
Got1	72.0253	173.019	1.26435	5.00E-05	0.004307
Ankrd33b	2.12592	5.10462	1.26372	0.00085	0.042675
Cyp4f14	49.6096	116.851	1.23598	5.00E-05	0.004307
Agxt	77.9814	183.563	1.23507	5.00E-05	0.004307
Tnfaip2	5.04012	11.8391	1.23204	0.0026	0.096795
Ccbl1	32.4535	75.9433	1.22655	5.00E-05	0.004307
Anubl1	5.2151	12.0711	1.21079	0.0005	0.02816
Nudt7	310.368	717.806	1.20961	0.0001	0.008061
Gabbr2	4.03488	9.23289	1.19426	0.0025	0.095338
Rdh11	18.9883	43.1703	1.18493	0.0001	0.008061
Dicer1	2.52702	5.71979	1.17853	0.0017	0.072156
Pde9a	10.3118	23.3092	1.1766	0.0013	0.057979

Setd3	23.9092	53.8866	1.17236	0.00115	0.053744
Slco2a1	5.17976	11.598	1.16291	5.00E-05	0.004307
XLOC_028520	52.2106	116.183	1.15398	5.00E-05	0.004307
BC029214	10.3873	23.019	1.14801	0.0019	0.078373
Slc7a2	26.5029	58.6511	1.14601	0.00035	0.021809
Gprc5c	15.0448	33.2781	1.14531	0.0017	0.072156
2810007J24Rik	271.66	599.196	1.14122	0.0006	0.032743
Gpcpd1	14.7812	32.2696	1.12641	0.0001	0.008061
Elovl3	50.2301	109.576	1.12531	0.00035	0.021809
Gnat1	16.1289	34.9645	1.11624	0.00125	0.057201
Casp3	5.11642	11.0782	1.11451	0.002	0.080977
C6	47.8015	103.33	1.11213	5.00E-05	0.004307
Fam188a	15.4096	33.278	1.11074	0.0027	0.098843
Eif4ebp2	17.1322	36.5717	1.09401	5.00E-05	0.004307
Cml1	108.524	229.996	1.0836	5.00E-05	0.004307
Prodh	51.4369	108.798	1.08078	0.00125	0.057201
Nfix	7.8389	16.4177	1.06653	0.0011	0.051682
Pstpip2	6.94864	14.5424	1.06546	0.0009	0.044175
Serpina11	31.1668	64.7905	1.05577	0.0003	0.019818
C9	134.528	279.541	1.05515	0.0022	0.086678
Cyp2c44	73.232	151.388	1.0477	5.00E-05	0.004307
Pdia5	13.2052	27.1489	1.03979	0.00025	0.017433
Kcnk5	5.60549	11.4846	1.03479	0.0013	0.057979
Hes6	69.1855	141.515	1.03242	0.00015	0.011264
Mafb	7.36383	15.0254	1.02887	0.00045	0.026358
Il13ra1	5.56609	11.2701	1.01776	0.0008	0.041346
Lpin1	11.5968	23.1468	0.99708	0.0004	0.023908
Als2	8.11245	16.0915	0.988088	0.00065	0.034612
Sdc3	8.2911	16.3595	0.98049	0.001	0.048541
Slc29a1	62.7708	122.298	0.962232	0.0018	0.075669
F7	26.5526	50.4188	0.925108	0.0007	0.036608
Cpn2	35.6567	67.0016	0.910021	0.0005	0.02816
F11	13.4264	24.9979	0.896728	0.00205	0.081499
Tmem86b	55.3413	102.926	0.895173	0.00105	0.050412
Atg9a	11.5721	21.3438	0.883167	0.0011	0.051682
Cyp4a12b	15.4838	28.503	0.880351	0.00205	0.081499
Mthfd1	37.7002	69.3482	0.879289	0.0011	0.051682
Igfals	28.2896	50.3251	0.831005	0.0019	0.078373

Aadat	31.6905	56.3069	0.829262	0.00255	0.095338
Slc6a6	9.2793	16.327	0.815174	0.00185	0.077034

Table 5-6. List of significantly downregulated genes by GPE under WPD condition

Gene Name	FPKM-WPD	FPKM-GPE	log2 (fold_change)	p_value	q_value
Cyp2b9	73.0556	3.48953	-4.38789	5.00E-05	0.004307
Cidec	59.7329	4.15838	-3.84443	5.00E-05	0.004307
XLOC_030807	11.396	0.806843	-3.8201	0.00035	0.021809
Slc22a29	6.87116	0.655381	-3.39015	5.00E-05	0.004307
Plin4	6.27789	0.607294	-3.36981	0.00025	0.017433
Wdr67	8.40753	0.963578	-3.12521	5.00E-05	0.004307
Tceal8	36.8431	4.33769	-3.08639	5.00E-05	0.004307
Ttc39a	3.81451	0.469118	-3.02348	0.00035	0.021809
Themis	3.01026	0.381673	-2.97948	0.0011	0.051682
Myc	6.47278	0.855089	-2.92024	0.0002	0.014286
Pnldc1	23.0335	3.27144	-2.81573	0.0005	0.02816
Ramp2	11.3924	1.6343	-2.80133	0.00165	0.070717
Uap1l1	30.3376	4.61341	-2.7172	0.0003	0.019818
Igfbp1	39.5268	6.10681	-2.69434	5.00E-05	0.004307
Anxa2	53.3494	8.66955	-2.62144	5.00E-05	0.004307
Cd36	105.851	18.0294	-2.55362	5.00E-05	0.004307
Cd63	29.8768	5.10886	-2.54795	0.0003	0.019818
Lgals1	139.644	24.1011	-2.53458	5.00E-05	0.004307
Cgref1	10.2737	1.90823	-2.42864	0.00085	0.042675
Cfd	17.6928	3.40088	-2.37918	0.0007	0.036608
Gm10680	1948.71	377.857	-2.36661	0.00105	0.050412
Apoa4	918.231	181.889	-2.3358	5.00E-05	0.004307
Mt1	50.4376	10.3475	-2.28521	5.00E-05	0.004307
Cyp3a11	866.007	177.775	-2.28432	5.00E-05	0.004307
H2-Q1	10.2868	2.13004	-2.27185	5.00E-05	0.004307
Wfdc2	49.6306	10.6468	-2.22081	5.00E-05	0.004307
S100a11	61.1705	13.5342	-2.17622	5.00E-05	0.004307
Gm11419	8.96552	2.015	-2.15361	5.00E-05	0.004307
Raet1d	16.1388	3.65446	-2.1428	0.0001	0.008061
Col1a2	5.57628	1.26349	-2.14189	5.00E-05	0.004307
Unc119	17.4467	3.97793	-2.13286	5.00E-05	0.004307
Col3a1	14.6281	3.35332	-2.12508	5.00E-05	0.004307
Postn	9.07662	2.08395	-2.12284	5.00E-05	0.004307
Tubb2a	108.361	25.1149	-2.10923	5.00E-05	0.004307
Spon2	10.9116	2.57511	-2.08315	5.00E-05	0.004307
Limk1	5.16549	1.24166	-2.05664	0.00055	0.030584
Osbpl3	5.90132	1.43485	-2.04014	0.0003	0.019818
4-Sep	10.1363	2.51407	-2.01144	5.00E-05	0.004307
Cyp2c38	30.1022	7.49422	-2.00602	5.00E-05	0.004307

Spp1	124.633	33.6478	-1.88911	5.00E-05	0.004307
XLOC_026575	10.3609	2.91741	-1.82838	5.00E-05	0.004307
Anxa5	128.165	36.1193	-1.82716	5.00E-05	0.004307
2010003K11Rik	65.8868	19.2202	-1.77736	5.00E-05	0.004307
XLOC_023889	17.3503	5.14681	-1.75321	0.0006	0.032743
St6gal1	42.1959	12.5805	-1.74591	0.00035	0.021809
Ifi27l2b	28.0596	8.47923	-1.72649	0.00015	0.011264
Hspb1	54.3618	16.5179	-1.71857	5.00E-05	0.004307
Ccnd1	17.5741	5.44684	-1.68996	5.00E-05	0.004307
XLOC_025814	10.3863	3.23401	-1.68329	5.00E-05	0.004307
S100a10	305.285	97.807	-1.64215	5.00E-05	0.004307
Gadd45g	15.7716	5.11409	-1.62478	0.0013	0.057979
Tubb6	7.95814	2.58134	-1.62431	0.00135	0.059905
Lyve1	5.86838	1.91127	-1.61843	0.00185	0.077034
Mgst3	27.2076	8.93182	-1.60698	0.00015	0.011264
Cyp2j9	5.86434	1.93339	-1.60084	0.0027	0.098843
Fam126a	5.08877	1.69807	-1.58343	0.00065	0.034612
Gpr98	9.53986	3.26245	-1.54801	0.00055	0.030584
Aox1	48.3686	17.1363	-1.49701	5.00E-05	0.004307
Rgs16	62.1802	22.116	-1.49136	5.00E-05	0.004307
Pcolce	10.8077	3.8482	-1.48981	0.0009	0.044175
Prss8	15.8814	5.66085	-1.48824	0.00255	0.095338
Fabp4	41.2702	14.7232	-1.48701	0.0004	0.023908
XLOC_019482	31.0554	11.1694	-1.47529	5.00E-05	0.004307
Acnat2	23.7726	8.61251	-1.46479	5.00E-05	0.004307
Gpc1	13.9353	5.10096	-1.4499	5.00E-05	0.004307
Tm4sf1	8.1777	2.99996	-1.44675	0.00245	0.094411
Abcc3	118.257	44.1636	-1.42099	0.00085	0.042675
Cyp3a59	11.7773	4.41253	-1.41632	0.0004	0.023908
Nid1	3.36952	1.2712	-1.40636	0.00035	0.021809
Vnn1	50.0463	19.2761	-1.37645	5.00E-05	0.004307
Aifm2	12.5959	4.94253	-1.34964	0.00095	0.046371
Pyroxd2	13.494	5.32754	-1.34078	5.00E-05	0.004307
Sparc	41.4332	16.5735	-1.32191	5.00E-05	0.004307
9130409I23Rik	15.3932	6.27387	-1.29486	0.0005	0.02816
Hsd17b6	58.9736	24.1439	-1.28841	5.00E-05	0.004307
Pparg	14.4594	5.93214	-1.28538	0.0003	0.019818
Reep5	6.91942	2.87242	-1.26838	0.0013	0.057979
Rbp1	6.65631	2.77371	-1.26291	0.0023	0.090213
Kdsr	6.34172	2.65333	-1.25707	0.00015	0.011264
Plk3	24.6551	10.319	-1.25658	5.00E-05	0.004307
Gstm1	1307.3	548.699	-1.2525	0.00015	0.011264
Chkb	40.8391	17.2272	-1.24526	0.00075	0.038991

Abhd2	23.8715	10.1769	-1.22999	5.00E-05	0.004307
Vcam1	5.56274	2.38051	-1.22453	0.00195	0.079318
Srxn1	36.6311	15.704	-1.22194	0.0006	0.032743
Acss3	8.165	3.50868	-1.21853	0.00125	0.057201
Cxcl9	8.43835	3.72329	-1.18038	0.0015	0.065567
Kctd2	20.492	9.15258	-1.16281	0.0004	0.023908
Tpm1	32.5309	14.6819	-1.14777	5.00E-05	0.004307
Hsd17b13	694.382	313.444	-1.14752	0.00015	0.011264
Slc16a7	26.8333	12.251	-1.13113	5.00E-05	0.004307
Rcan1	16.8597	7.81532	-1.1092	0.00045	0.026358
Gclc	146.543	68.1511	-1.10451	0.0001	0.008061
Cd9	22.535	10.6991	-1.07468	0.0021	0.083111
Lcn2	51.7583	24.6078	-1.07267	0.00065	0.034612
Orm2	73.0595	35.3577	-1.04705	0.00085	0.042675
Mfge8	13.1544	6.37522	-1.045	0.0025	0.095338
Gstm2	67.7112	32.8547	-1.04329	0.0002	0.014286
XLOC_007568	7.8437	3.86274	-1.02191	0.0009	0.044175
1500017E21Rik	44.6667	22.1219	-1.01372	5.00E-05	0.004307
Cd74	74.2839	36.9092	-1.00907	0.0002	0.014286
Krt8	176.855	90.4303	-0.96769	0.00035	0.021809
Gas6	29.9915	15.3573	-0.96562	0.00125	0.057201
Saa2	327.926	168.145	-0.96366	0.0004	0.023908
Hexa	42.0673	21.587	-0.96254	0.00045	0.026358
Adora1	7.67779	3.94294	-0.96142	0.00205	0.081499
Mme	9.46745	4.89563	-0.95148	0.00145	0.063699
Csad	51.2336	26.5667	-0.94747	0.00025	0.017433
Mapre3	34.781	18.2388	-0.93129	0.00065	0.034612
Vnn3	30.9468	16.5149	-0.90602	0.0016	0.069249
Aqp8	261.857	141.749	-0.88544	0.00205	0.081499
Sprr1a	12.5782	0	inf	5.00E-05	0.004307
Gsta1	11.0491	0	inf	5.00E-05	0.004307
Cyp2a4	10.2681	0	inf	5.00E-05	0.004307
XLOC_033998	8.65451	0	inf	5.00E-05	0.004307

Summary

It is promising and essential to investigate grape pomace's health promoting potential as an important value adding products. Our main goal was to investigate the effects of a dietary grape pomace extract supplement on the prevention of diabetes and obesity. We demonstrated that GPE may prevent metabolic syndrome including diabetes and obesity through alternating the expression of genes, on the signaling or metabolic pathways that lead to reduction of diabetes and obesity. This current stusy was focused on examining such molecular mechanism(s) that support the incorporation of GPE as an alternative therapy for controlling postprandial blood sugar, regulate insulin resistance and preventing diabetes and obesity.

Grape pomace can suppress postprandial hyperglycemia through inhibitory effects on α-Glucosidase activity in vitro. It also reduces acute blood glucose in vivo in induced-diabetic mice. qRT- PCR and ELISA tests confirmed that grape pomace extracts affected insulin signaling pathway at both RNA and protein levels. GPE could correct insulin resistance, decrease HbA1c and blood glucose levels, and consequently prevent type 2 diabetic through the regulation of glucose metabolism as well as glucose homeostasis at both transcriptional and translational levels. Also, grape pomace extracts translational regulation of the adipokine, cytokine/chemokine, inflammatory factors, lower systematic inflammation status, decreased the adipogenesis and lowering the fatty acids accumulation. High throughput RNA sequencing technology elucidated gene expression profiles at genomics level caused by GPE treatment under high fat diet condition. Genes involved in glucose and lipid

metabolisms and the related signal transduction pathways were altered.

This project provided scientific evidence for the health beneficial effects of the grape pomace extract, and may promote the use of GPE as healthy dietary component and enhance food and agriculture economy.

Future perspective

Isolate and identify the active compound(s) of GPE effect in diabetes and obesity and GPE effect on microbiome in the GI tract may be investigated in future studies.

GPE has been studied for its effect and ability to regulate glucose homeostasis, and influence energy balance and control body weight through attenuation of the adipogenesis. Additional analyses should be conducted to isolate, purify and identify the active compound(s) of GPE to gain more understanding of GPE effects metabolic syndrome.

The gut microbiome can be a factor involved in the control of host energy metabolism and systematic inflammation status. PGE will be further tested on the gut microbiota composition in rat models under high fat diet and its association with metabolic biomarkers. Fecal samples and colon sections can be collected from animals and determined by PCR-DGGE profiles followed by sequencing of selected bands from DGGE gels, the total number and types of microbes in GI tract can be determined. The associations between the gut microbiota and appetite-regulating hormones that may be important in terms of satiety and host metabolism.

Molecular mechanism of GPE's anti-diabetes, anti-obesity and anti-inflammatory effect and GPE' long-term effect on metabolic disorder induced by western style diet may be investigated in future studies.

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Projects:

Prevention property of grape pomace on metabolic syndrome (obesity and diabetes): Effects and molecular actions.

1) The research aims to prevention of metabolic syndrome through basic molecular and preclinical studies.
2) Antioxidant capacity estimation and in vitro cytotoxicity, antiproliferative, apoptotic activities study of variety of pro-drugs.

Research Specialist II Department of Anatomy & Neurobiology, Virginia Commonwealth University Medical Campus, 2007- 2008.

Projects:

Investigating the role of microglia, the resident innate immune cell in the brain, in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease

1). Define how microglia both initiate neuron damage and fuel progressive neurotoxicity

2). Identify the triggers of deleterious microglial activation towards the development of novel therapeutic compounds capable of halting the progression of neurodegenerative diseases

Research Associate Department of Biochemistry, Texas A&M University, 2006- 2007

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Characterization of *Drosophila* Sialyltransferase at molecular and genetic levels to dissect the roles of sialylation in *Drosophila* central nervous system development.

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Research Associate Department of Entomology, Texas A&M University, 2002-2006

Projects:

Molecular study of the symbiotic bacteria from fire ant midgut.

1). Isolation, biochemical and molecular characterization of bacteria from the red imported fire ant midgut.

2). Genetic modification of the bacteria by Mariner transposon Himar1 mediated gene integration.

3). Transcriptional profiling and protein localization in the red imported fire ant.

Graduate Research Assistant Department of Entomology, Texas A&M University, 2000-2002

Projects:

Identification and biodiversity study of sorghum green bug biotypes.

1). Identification of molecular marker and biodiversity study of green bug using AFLP technique.

2). Construction and characterization of Bacterial Artificial Chromosome (BAC) library of green bug.

Achievements:

1). Studied the biodiversity relationship of the green bug and separated a sorghum

related green bug group.

2). Developed a green bug Megabase DNA isolation protocol and constructed a Green bug BAC library.

3). One paper was published based on master thesis.

Graduate Research Assistant Department of Forest, Texas A&M University, 1999-2000

Projects:

Genetic modification of cotton by Agrobacterium-mediated transformation and identification of the integrated junction sequence in the plant genome.

Achievements:

1). Developed a rapid and high yield miniprep DNA isolation protocol for cotton

2). Published one paper.

Research Assistant Institute of Developmental Biology, Chinese Academy of Sciences, Beijing, China 1994-1996

Teaching Experience

Teaching Assistant, Department of Biochemistry, Texas A&M University, 2001-2002

Molecular genetics laboratory course, trained undergraduate students in biochemistry and genetics majors on molecular biology techniques such as cloning, protein expression, SDS-PAGE, non-radioactive labeling Southern blotting, PCR and Western blots.

Teaching Assistant, Department of Nutrition and Food Science, University of Maryland 2011-2012

Food chemistry laboratory course, trained undergraduate students in Food and nutrition Science major on food chemistry techniques such as HPLC, GC, enzymatic activity, water activity, lipid and carbohydrate characterization.

Teaching Assistant, Department of Nutrition and Food Science, University of Maryland 2014-2015

Food Science and Technology course, trained undergraduate students in Food and nutrition Science major on basic concept of Food Science and Technology.

Honors and Awards

1) Student Research Day of Department of Nutrition and Food Science, poster comtetition second place, 2015

2) Travel grant from Molecular and Environmental Plant Sciences program, 2000

3) Academic Excellence Scholarship of Molecular and Environmental Plant Sciences program, 2001

4) Elected Member of The Honor Society of Phi Kappa Phi at TAMU, 2001.

Professional Affiliations

The American Society of Plant Biologists 2000-2001

International Food Technologists, 2013-present

Publications

1. **Li, H.**, Luo, J., Hemphill, K.J., Wamg, J and Gould, J. (2001) A Rapid and high yielding DNA miniprep for Cotton (Gossypium spp.) ***Plant Mol. Biol. Rep*. 19:1-5.**
2. Zhu-Salzman, K., **Li, H.**, Klein, P.E and Gorena, R.L. (2003) Using high throughput amplified fragment length polymorphism to distinguish sorghum greenbug (Homoptera: Aphididae) biotypes. ***Agr. Forest Entomol.* 5: 311-315.**
3. **Li, H.**, Medina, F., Bradleigh, S., Vinson and Coates, C. (2005) Isolation, Characterization and Molecular Identification of Bacteria from the Red Imported Fire Ant (Solenopsis invicta) Midgut. ***J. Invertebr. Pathol*. (89): 203-209.**
4. Medina F[#], **Li H[#]**, Vinson S, Coates C (2009) Genetic transformation of midgut bacteria from the red imported fire ant (Solenopsis invicta). ***Curr Microbiol.*** 58(5):478-82**. #Authors have contributed equally to this work.**
5. Repnikova E, Koles K, Nakamura M, Pitts J, **Li H,** Ambavane A, Zoran M, Panin V. (2010) Sialyltransferase regulates nervous system function in Drosophila. **J Neurosci.** 30(18):6466-76.
6. Parry J[#], **Li H[#]**, Liu J, Zhou K, Zhang L and Ren S, (2011) Antioxidant Activity, Antiproliferation of Colon Cancer Cells, and Chemical Composition of Grape

Pomace," ***Food and Nutrition Sciences*****. 2(6):** 530-540. **#Authors have contributed equally to this work.**

7. **Li H** and Parry J, (2011) Phytochemical Compositions, Antioxidant Properties, and Colon Cancer Antiproliferation Effects of Turkish and Oregon Hazelnut," ***Food and Nutrition Sciences,*** 2 (10): 1142-1149.
8. Xu Y, Sismour E, Parry J, Hanna M, and **Li H.** (2012). Nutritional composition and antioxidant activity in hazelnut shells from US-grown cultivars**. International Journal of Food Science & Technology. 47 (5):** 940–946.
9. Ren S, Weeda S, **Li H**, Whitehead B, Guo Y, Atalay A, Parry J. (2012) Salt tolerance in soybean WF-7 is partially regulated by ABA and ROS signaling and involves withholding toxic Cl- ions from aerial tissues. ***Plant Cell Rep.*** 22;31(8):1527-33.
10. Pao S, Hagens BE, Kim C, Wildeus S, Ettinger MR, Wilson MD, Watts BD, Whitley NC, Porto-Fett ACS, Schwarzn JG, Kaseloo P, Ren S, Long W, **Li H**, Luchansky JB. (2013) Prevalence and molecular analyses of Campylobacter jejuni and Salmonella spp. in co-grazing small ruminants and wild-living birds. ***Livestock Science***. 160: 163–171.
11. Li X, **Li H**, Jia L, Li X, Nafis Rahman N. (2015) Oestrogen action and male fertility: experimental and clinical findings. ***Cellular and Molecular Life Sciences.*** 72: 3915-3930.
12. **Li H**, Guo TL, Parry J, Pao S, Zhou K, Castonguay T. Grape Pomace Aqueous Extract Inhibits α-Glucosidase in vitro and Suppresses Postprandial Hyperglycemia in vivo. Submmited.
13. **Li H**, Parry J,Weeda S, Ren S , Pao S, Castonguay L. Guo TL. Grape Pomace Aqueous Extract (GPE) Prevents Western High Fat Diet-induced Diabetes and Attenuates Systemic Inflammation. Submmited.
14. **Li H**, Tai L. Guo, Parry J, Weeda S, Ren S , Castonguay T. Effects of Grape Pomace Extract (GPE) on preventing Western Pattern Diet (WPD) Induced Obesity Revealed by Hepatic RNA-seq Transcriptome Profile. Submitted.

14. Kim C, Nartea T, Pao S, **Li H**, Jordan K, Xu Y, Stein R, Sismour E. Microbial Quality

of Shiitake Mushrooms (Lentinula edodes) Acquired from Internet and Local Retail Markets. Submitted.

Book chapter

Parry J and **Li H** (2012) Cereals and Pulses: Nutraceutical Properties and Health Benefits. John Wiley & Sons, Inc. Chapter 5.

Presentations

1. Luo, J-H., **Li, H** and Gould, J. 2000. Cotton Transformation for Disease Resistance. San Diego, CA. Plant Biology 2000.
2. **Li, H**, Medina, F., Bradleigh, S., Vinson and Coates, C. 2005. Toward Fire Ant Biological Control I: Isolation and Molecular Characterization of Bacteria from the Red Imported Fire Ant (Solenopsis invicta) Midgut. Gulfport, MS. Annual Red Imported Fire Ant Conference 2005.
3. **Li, H**, Medina, F., Bradleigh, S., Vinson and Coates, C. 2005. Toward Fire Ant Biological Control II: Genetic Engineering of Midgut Bacteria From The Red Imported Fire Ant (Solenopsis invicta). Gulfport, MS. Annual Red Imported Fire Ant Conference 2005.
4. Medina, Freder., **Li, H**. Bradleigh, S., Vinson and Coates, C. 2006. Bacterial microbiology of the red imported fire ant, Solenopsis invicta Büren midgut. Indianapolis, IN. Entomological Society of America Annual Meeting 2006.
5. Parry, J.W., Liu, J.R., **Li, H** and N. Smith. Fatty acid composition and antioxidant activities of chardonnay grape pomace. Atlanta, GA. Association of Research Directors Annual Meeting 2009.
6. Smith, N., J.R. Liu, **Li, H**., and J.W. Parry. Cytotoxic and antiproliferative effect of chardonnay grape pomace extracts on Caco-2 and HT-29 human colon cancer cells.Atlanta, GA. Association of Research Directors Annual Meeting 2009.
7. Parry, J.W., **Li, H**. Antioxidant properties and cancer antiproliferation effects of chardonnay grape pomace extracted by three solvent systems. Anaheim, CA. Institute of Food Technologists Annual Meeting 2009.

8. Parry JW, Liu JR, **Li H** 2009. Antioxidant properties and cancer antiproliferation effects of chardonnay grape pomace extracted by three solvent systems. Institute of Food Technologists, June 6-10, Anaheim/OC, CA
9. Parry JW, Liu JR, **Li H**, Smith N 2009. Fatty acid composition and antioxidant activities of chardonnay grape pomace. The 2nd Annual Graduate Research Symposia, Virginia State University, Petersburg, VA – April 18.
10. Smith N, Liu JR, **Li H**, Parry JW 2009. Cytotoxic and antiproliferative effect of chardonnay grape pomace extracts on Caco-2 and HT-29 human colon cancer cells. The 2nd Annual Graduate Research Symposia, Virginia State University, Petersburg, VA – April 18.
11. Parry JW, Liu JR, **Li H**, Smith N 2009. Fatty acid composition and antioxidant activities of chardonnay grape pomace. The 15th meeting of the Association of Research Directors, Atlanta, GA, March 28 – April 1.
12. Smith N, Liu JR, **H Li**, Parry JW 2009. Cytotoxic and antiproliferative effect of chardonnay grape pomace extracts on Caco-2 and HT-29 human colon cancer cells. The 15th meeting of the Association of Research Directors, Atlanta, GA, March 28 – April 1.
13. **Li, H**., Liu, J.R., Zhou K., Ren, S., Parry, J.W. Antioxidant activity, and cancer antiproliferation effects of grape pomace extracts. Chicago, IL. Institute of Food Technologists Annual Meeting 2010.
14. Parry, J.W., Liu, J.R., Zhou K., **Li, H**. Chemical composition, antioxidant activity, and cancer antiproliferation effects of apple and tomato pomaces. Chicago, IL. Institute of Food Technologists Annual Meeting 2010.
15. Parry JW, Liu J, **Li H**, Zhou K, Zhang L. 2010. Chemical composition, antioxidant activity, and cancer antiproliferation effects of grape, apple, and tomato pomaces. The 240th ACS National Meeting in Boston, MA, Aug 22-26, Society Committee on Education. # 21616.
16. Parry JW, Liu JR, **Li H**, Zhou, K 2010. Chemical composition, antioxidant activity, and cancer antiproliferation effects of apple and tomato pomaces. Institute of Food Technologists, July 17 – 21, Chicago, IL.

17. Taliaferro B, Mohrman M, **Li H**, Bhardwaj H, Parry JW 2010. Changes in chemical composition and antioxidant activities in milled flaxseed periodically exposed to oxygen. Spring 2010 Graduate Research Symposia, Virginia State University, Petersburg, VA April 15.
18. **Li H**, Liu J-R, Parry JW. 2011. Phytochemical compositions, antioxidant properties, and colon cancer antiproliferation effects of Turkish and Oregon Hazelnut. Institute of Food Technologists, June 11 – 14, New Orleans, LA.
19. Parry JW, Liu J-R, **Li H**. 2011.Chemical Composition, Antioxidant Activity, and Cancer Antiproliferation Effects of Apple and Tomato Pomaces. Institute of Food Technologists, June 11 – 14, New Orleans, LA.
20. Taliaferro B, **Li H**, Parry JW. 2011. Changes in Chemical Composition and Antioxidant Activities in Milled Flaxseed Periodically Exposed to Oxygen. . Institute of Food Technologists, June 11 – 14, New Orleans, LA.
21. Parry JW, Liu J-R, **Li H**. 2011.Chemical Composition, Antioxidant Activity, and Cancer Antiproliferation Effects of Apple and Tomato Pomaces. Association of Research Directors of 1890 Land Grant Institutions, Atlanta, GA, April 9 – 13.
22. Taliaferro B, **Li H**, Parry JW. 2011. Changes in Chemical Composition and Antioxidant Activities in Milled Flaxseed Periodically Exposed to Oxygen. Association of Research Directors of 1890 Land Grant Institutions, Atlanta, GA, April 9 – 13.
23. **Li H**, Parry JW. 2012. Reduction of Alpha-Gucosidase Activity by Chardonnay, Tinta Cao, and Cabernet Franc Grape Pomace Extracted Using Water and Fermented at Different Brix Levels. Institute of Food Technologists, June 25 – June 28. Las Vegas, NV.
24. Parry JW, **Li H**, Rutto L, Brandt M. 2012. Stinging Nettle: Effects on Colon Cancer Cell Proliferation and Anti-diabetic Properties. Institute of Food Technologists, June 25 – June 28. Las Vegas, NV.
25. Parry JW, **Li H**, Rutto L, Brandt M. 2012. Antioxidant Capacities of Chardonnay, Tinta Cao, and Cabernet Franc Grapes Fermented at Different Brix Levels. Institute of Food Technologists, June 25 – June 28. Las Vegas, NV.

26. **Li H**, Rutto L, Brandt M, and Parry JW 2012. Reduction of α-Glucosidase Activity by Chardonnay, Tinta Cao, and Cabernet Franc Grape Pomace Fermented at Different Brix Levels. 2012 IFT Annual Meeting, June 25 - 28, Las Vegas NV.
27. **Li H**, Shi H, Yu L, Guo T, Yan H,. Parry JP 2013. Cabernet Franc Grape Pomace Inhibits α-Glucosidase in vitro and Suppresses Postprandial Hyperglycemia in Diabetic Mice. IFT Annual Meeting, July 13 – 16, 2013, Chicago, IL.
28. Parry JW, Zhang B, Tadesse M, **Li H**, Liu JR, Coral L 2014. Antioxidant Activity of Elite Large-Seeded Soybean Breeding Lines in Va. IFT Annual Meeting, June 21 – 24, New Orleans, LA
29. Parry J, **Li H**, Moore JC, Yu L 2014. The Effect of 50% Acetone Extracts From Selected Fruit Seeds and Spices on the Proliferation of Human HT-29 Colon Cancer Cells. IFT Annual Meeting, June 21 – 24, New Orleans, LA
30. Parry J, **Li H**, Moore JC, Yu L 2014. The Effect of Different Solvent Extractions of Mushroom and Sophora Flavescens (Ku Shen) Root on the Antiproliferation Effects of HT-29 Human Colon Cancer Cells. IFT Annual Meeting, June 21 – 24, New Orleans, LA.
31. **Li H**, Guo T, Yu L, Parry JP 2014. Cabernet Franc Grape Pomace Suppresses Postprandial Hyperglycemia in High Fat Feeding Mice. IFT Annual Meeting, June 21 – 24, New Orleans, LA.
32. **Li H,** Guo TL, Parry J, Castonguay. 2015. Grape Pomace Aqueous Extract Reduces Blood Glucose in vivo. Mid-Atlantic Diabetes Research Symposium, September 25, Bethesda, MD