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Obesity is rapidly increasing worldwide among all age groups. Insulin resistance or type 2 diabetes is one of several debilitating health problems associated with obesity. An emerging feature of obesity and type 2 diabetes is their linkage with chronic, low-grade inflammation that begins in white adipose tissue (WAT) and eventually becomes systemic. One potential dietary strategy to reduce chronic inflammation is consumption of fruits and vegetables rich in polyphenols, including grape products, which possess anti-oxidant and anti-inflammatory properties. Notably, several clinical and animal studies have shown that supplementation with grape products like grape juice, grape powder or extracts, and red wine reduced oxidative damage and inflammation. However, the suppressive effects of grape powder on adipocyte-derived inflammation and insulin resistance remains uncertain. Additionally, the bioavailability of grape polyphenols and their ability to lower inflammation and insulin resistance in vitro and in a diet-induced obese animal model are unclear.

Therefore, the specific aims of this research were to determine the extent to which 1) grape powder extract (GPE) and several of its polyphenols decrease tumor necrosis factor alpha (TNFα)-mediated inflammation and insulin resistance and their mechanisms of action in primary cultures of human adipocytes (Aim 1), and 2) grape powder polyphenols are absorbed and reduce markers of inflammation and insulin resistance in high fat-fed obese mice (Aim 2). In Aim 1, GPE and quercetin, an abundant polyphenol

in GPE, attenuated TNFα-induced a) expression of inflammatory genes, b) activation of inflammatory mitogen-activated protein kinases (MAPKs) and transcription factors nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1), c) expression or abundance of two negative regulators of insulin sensitivity, and d) suppression of insulin-stimulated glucose uptake. Taken together, these data demonstrate that GPE and quercetin attenuate TNFα-mediated inflammation and insulin resistance in primary cultures of human adipocytes, possibly by suppressing the activation of inflammatory MAPKs and transcription factors that cause insulin resistance. In Aim 2, it was found that a) quercetin 3-*O*-glucoside was one of the most abundant polyphenols detected in the sera of mice gavaged with GPE, b) high fat-fed mice supplemented with quercetin-rich grape powder had improved glucose disposal rates acutely and reduced markers of inflammation in the sera and WAT chronically, and c) quercetin 3-*O*-glucoside reduced several markers of inflammation in primary cultures of human adipocytes. Collectively, these findings are expected to contribute critical insights for the development of dietary strategies using grape products for the control of obesity-related conditions including inflammation and insulin resistance or type 2 diabetes. However, clinical trials are needed to determine the extent to which these findings can be reproduced in humans.

GRAPE POLYPHENOLS ATTENUATE INFLAMMATION AND INSULIN RESISTANCE IN HUMAN ADIPOCYTES AND OBESE MICE

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Overview

Obesity is the most prevalent nutrition-related health problem worldwide, with 1.5 billion adults and 43 million children under the age of five classified as overweight and more than 500 million people classified as obese (1). Obesity is caused by a positive energy balance due to overconsumption of calories relative to energy expenditure that leads to the expansion of white adipose tissue (WAT) mass. Enlarged WAT is associated with a progressive infiltration and accumulation of macrophages that contribute to a chronic, low grade inflammation characterized by increased pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα; 2) or interleukin-6 (IL-6; 3) and chemokines such as monocyte chemoattractant protein-1 (MCP-1; 4). These inflammatory adipokines that activate mitogen-activated protein kinase (MAPK) signaling pathways, including extracellular signal-related kinase (ERK) or c-Jun-NH2 terminal kinase (JNK) and their downstream transcription factor nuclear factor-kappa B (NF-κB) or activator protein-1 (AP-1), respectively, involve in the development of obesity-mediated insulin resistance or type 2 diabetes (Reviewed in 5, 6).

Polyphenol-rich grapes are one of the most widely consumed fruits in the world. Early epidemiologic research reported that consumption of red wine correlated with a lower risk of cardiovascular diseases, referred to as the “French Paradox” (7, 8). Recent

studies have demonstrated cardioprotective effects of grapes and their by-products (e.g., red wine, grape juice, powder, and extracts) due to their abundant content of polyphenols (e.g., quercetin and resveratrol), which possess anti-oxidant and anti-inflammatory properties (reviewed in 9, 10). However, the inhibitory effect of grapes and their polyphenols on obesity-mediated inflammation and insulin resistance remains uncertain. Specifically, the ability of grape polyphenols to attenuate inflammation and insulin resistance triggered by TNFα and their mechanisms of action in primary human adipocytes are unknown. Additionally, the bioavailability of grape polyphenols and their ability to reduce inflammation and insulin resistance in a diet-induced obese animal model are unclear. Elucidating these questions will provide valuable information for the development of dietary strategies for the control of obesity-associated inflammation and insulin resistance.

Central Hypothesis and Specific Objectives

The central hypothesis for the proposed research is that 1) grape powder extract (GPE) and two of its polyphenols, quercetin and resveratrol, attenuate TNFα-mediated inflammation and insulin resistance, possibly by suppressing the activation of MAPKs (i.e., ERK and JNK), NF-κB, and AP-1, in primary human adipocyte cultures, and 2) several grape powder polyphenols are bioavailable in mice, and improve glucose disposal rates and reduce markers of chronic inflammation in high fat-fed obese mice.

In order to test this hypothesis, the following two specific aims were investigated: Aim 1) determine the extent to which GPE and several of its polyphenols decrease

TNFα-mediated inflammation and insulin resistance and their mechanisms of action in primary cultures of human adipocytes, and Aim 2) determine the extent to which grape powder polyphenols are absorbed and reduce markers of inflammation and insulin resistance in high fat-fed obese mice.

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CHAPTER II

REVIEW OF LITERATURE

Background and Significance

Obesity, Inflammation, and Metabolic Diseases

Factors Influencing White Adipose Tissue Growth, Development, and Overexpansion that Lead to Obesity

Obesity is a global health issue, with more than 500 million people classified as obese and 1.5 billion overweight, including 43 million children under the age of five (http://www.who.int/mediacentre/factsheets/fs311/en/index.html). In the United States, obesity is rapidly increasing among all age groups. Currently, in all but two states at least 20% of the population is classified as obese (http://www.cdc.gov/obesity/data/trends.html) Diets high in calories, especially from sugars, saturated fatty acids (SFAs), and long-chain omega-6 polyunsaturated fatty acids (PUFAs), and sedentary lifestyles lacking physical activity contribute significantly to this obesity epidemic. Endogenous and exogenous factors that enhance preadipocyte proliferation or adipocyte hypertrophy have the capacity to increase white adipose tissue (WAT) mass and the development of obesity (reviewed in 66, 116).

Metabolic Consequences of Expanding White Adipose Tissue and Its Impact on Liver, Muscle, and Pancreas

The rapid rise in obesity is accompanied by a similar increase in cardiovascular disease (CVD), hypertension, and insulin resistance or type 2 diabetes (http://www.who.int/mediacentre/factsheets/fs311/en/index.html). For instance, ~80% of people with type 2 diabetes are overweight or obese (13), suggesting a strong positive relationship between the two diseases (http://www.cdc.gov/nccdphp/dnpa/obesity/consequences.htm). Obese patients with type 2 diabetes have elevated levels of tumor necrosis factor alpha (TNFα) in their blood (133), WAT (50), and muscle (97). Furthermore, impaired glucose disposal is positively correlated to TNFα expression (97, 104, 133). Metabolic endotoxemia [e.g., elevated lipopolysaccharide (LPS) level in circulation] is also associated with obesity (14).

This cluster of obesity-related, metabolic diseases is known as the metabolic syndrome. One emerging feature of the metabolic syndrome is its linkage with chronic inflammation in WAT that becomes systemic. It is characterized by engorged adipocyte death (107), increased cytokine/chemokine production and inflammatory signaling, and recruitment of leukocytes (reviewed in 48). Thus, excess WAT is an overactive endocrine organ secreting an array of inflammatory adipokines that contribute to the metabolic syndrome (reviewed in 44). Furthermore, as WAT mass increases, its lipid- and glucose-buffering capacity decreases. This results in elevated blood levels of free fatty acids (FFAs), very-low-density lipoprotein (VLDL) (hyperlipidemia), glucose (hyperglycemia), and insulin (hyperinsulinemia), as well as ectopic lipid accumulation in skeletal and

cardiac muscle, liver (steatosis), and pancreas (**Figure 2.1**). Understanding the mediators of this inflammation and their mechanisms of action is essential in order to develop effective strategies to prevent chronic inflammatory signaling from WAT.

Pro-inflammatory Role of Free Fatty Acids and Adipokines

Free fatty acids, especially SFAs, contribute significantly to chronic inflammation (reviewed in 58). For example, palmitate activates protein kinase C (PKC) signaling, generates ceramide and reactive oxygen species (ROS), and increases oxidative or endoplasmic reticulum (ER) stress, all of which are associated with chronic inflammation or insulin resistance. Dietary constituents like SFA may also alter gut microflora, leading to elevated blood levels of LPS or TNFα that cause endotoxemia (14).

Tumor necrosis factor alpha, LPS, SFA, or FFA instigate inflammation and insulin resistance by triggering ROS-mediated oxidative or ER stress and serine/threonine kinase phosphorylation signaling that active inflammatory mitogen-activated protein kinases (MAPKs), including extracellular signal-related kinase (ERK) and c-Jun-NH2-terminal kinase (JNK), IκBα kinase (IKK), and transcription factors nuclear factor-kappa B (NF-κB) and activating protein (AP)-1 (reviewed in 45). Collectively, these proteins induce inflammatory gene transcription, antagonize peroxisome proliferator-activated receptor gamma (PPARγ) activity, or directly impair insulin receptor substrate (IRS)-1 signaling, leading to insulin resistance. Furthermore, engorged adipocytes release chemokines such as monocyte chemoattractant protein (MCP)-1 that attract monocytes and stimulate their recruitment and differentiation into macrophages (MΦ) via

chemotaxis (reviewed in 77). Monocyte chemoattractant protein-1 has also been reported to convert alternatively activated MΦ (M2) into classically activated MΦ (M1), further increasing inflammation and insulin resistance (**Figure 2.2**). Such an inflammatory scenario in perivascular WAT can lead to plaque formation and smooth muscle migration and proliferation in the vascular endothelium and wall, thereby increasing the development of CVD (reviewed in 95).

Thus, a positive energy balance expands WAT, generating adipokines such as TNFα and MCP-1 as well as FFA that recruit and activate MΦ and activate inflammatory cascades. These responses antagonize PPARγ activity, leading to decreased glucose and FA uptake and metabolism, causing ectopic lipid accumulation (lipodystrophy), hyperglycemia, and hyperlipidemia. Proposed mechanisms by which these inflammatory signals reduce PPARγ activity include (*a*) decreased PPARγ mRNA levels, (*b*) increased phosphorylation of serine residue 112 of PPARγ by ERK, leading to PPARγ ubiquination and proteosome degradation, (*c*) decreased PPARγ DNA binding, and (*d*) decreased PPARγ transcriptional activity (**Figure 2.3**) (reviewed in 129). Discovery of dietary strategies, including the consumption of polyphenol-rich grapes or grape products, to reduce obesity-related chronic inflammation could potentially attenuate metabolic diseases.

Types and Abundance of Phenolic Phytochemicals in Grapes

Types of Phenolic Phytochemicals in Grapes and Grape Products

Grapes and their by-products are consumed worldwide. There are more than 50 varieties of seeded and seedless grapes in black, blue, blue-black, golden, red, green, purple, and white colors. Common grape products include table grapes, wine, raisins, juices, and preservatives. The average annual consumption of fresh grapes in the United States is about eight pounds per person (http://www.ers.usda.gov/Data/FoodConsumption) Most of the grapes consumed in the United States are grown in California. Table grapes contain essential nutrients such as water, carbohydrates, proteins, fats, vitamins, minerals, and fiber (reviewed in 127) and nonessential compounds including phytochemicals. Phenolic phytochemicals in grapes (**Table 2.1**) possess biological activities that have been reported to promote health (reviewed in 125). Some of the early health benefits of consuming red wine (e.g., decreased the risk of CVD), referred to as the "French Paradox" (92, 103), have been attributed to its phenolic phytochemicals including resveratrol. Ironically, the resveratrol content of grapes and red wine is relatively low compared to other polyphenols (**Table 2.1**).

The predominant phenolic phytochemicals in grapes are flavonoids such as flavonols, flavan-3-ols (monomers, oligomers, or polymers), and anthocyanidins/ anthocyanins, and to a much lesser extent nonflavonoids such as stilbenes, phenolic acids/hydroxybenzoates, and hydroxycinnamates (**Tables 2.1** and **2.2**; 15, 17, 18, 29, 30, 41, 43, 56, 71, 78, 84, 85, 88, 90). Quercetin is the major flavonol in grapes (**Figure 2.4A**) and usually occurs as *O*-glucosides in the D-glucose isoform such as quercetin 3-*O*-

glucoside (**Figure 2.4B**). Grape flavan-3-ols are mainly represented by monomeric catechins such as (+)-catechin (**Figure 2.4C**) and oligomeric procyanidins, also known as proanthocyanidins or condensed tannins, such as procyanidin B2 (**Figure 2.4D**). Anthocyanins (i.e., anthocyanidins with sugar groups) in grapes are primarily 3-*O*-glucosides such as malvidin (**Figure 2.4E**) and malvidin 3-*O*-glucoside (**Figure 2.4F**), which are found in red grapes. *Trans*-resveratrol (**Figure 2.4G**) is probably the most well-known and studied stilbene found in grapes and red wine. Phenolic acids, also known as hydroxybenzoates, are commonly represented by gallic acid (**Figure 2.4H**) in grapes. In grapes or grape products, hydroxycinnamates usually undergo esterification with tartaric acid such as caffeic acids (**Figure 2.4I**) and its tartaric acid esters such as caftaric acid (**Figure 2.4J**).

The phenolic phytochemical content of grapes varies due to grape type, color, and ripeness, and climatic, geographical, and cultural factors. In general, flavonols and anthocyanidins/anthocyanins represent colored phenols and flavan-3-ols, phenolic acids/hydroxybenzoates, and hydroxycinnamates represent noncolored phenols in grapes or grape products (**Table 2.2**). These polyphenols are present mainly in skins and seeds and contribute to the astringency, bitterness, and color of the grapes and wine. Polyphenols also impact the quality of the wine produced and potential health benefits associated with wine consumption.

Relatively High Abundance of Anthocyanidins/Anthocyanins and Flavonols in Grapes

Grapes are particularly rich in anthocyanidins/anthocyanins (e.g., malvidin, cyanidin, and peonidin) and to a lesser extent flavonols (e.g., quercetin) (**Tables 2.1** and **2.2**). The average of daily anthocyanidin/anthocyanin and flavonol intake from all foods in the United States is approximately 12.5 mg (124) and 20.0 mg (68, 99) per person, respectively. Average adult plasma levels of flavonols such as quercetin and isorhamnetin, a metabolite of quercetin, are approximately 53.9 nM and 3.0 nM, respectively (34). However, anthocyanidins/anthocyanins such as malvidin 3-*O*-glucoside were undetectable in the plasma of adult subjects (12, 40). Also, variation in plasma levels of polyphenols or their metabolites between individuals is high at baseline and after intervention (34, 75).

The bioavailability of these anthocyanidins/anthocyanins and flavonols has been reported to be poor based on in vitro (11, 30, 37, 130) and in vivo studies (reviewed in 32, 96). However, phenolic phytochemicals may be rapidly metabolized by cells and therefore difficult to detect within the circulation or cells following consumption or supplementation (105). Also, despite having relatively low abundance and poor bioavailability, some polyphenols have potent biological actions (i.e., resveratrol; 5, 6, 62). Because of the relative high abundance and biological activity of grape anthocyanidins/anthocyanins and flavonols, the following sections focus on potential mechanisms by which they reduce chronic inflammation associated with obesity and related metabolic diseases.

Mechanisms by Which Anthocyanidins/Anthocyanins and Flavonols, Major Phenolic Phytochemicals in Grapes, Reduce Chronic Inflammation

Acting as an Antioxidant or Increasing Antioxidant Gene or Protein Expression

Reactive oxygen species and reactive nitrogen species (RNS) can be generated by enzyme systems such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide synthase (NOS), respectively, or transition metals, and are notorious mediators of oxidative stress and inflammation. Reactive oxygen species trigger redox-sensitive kinases such as apoptosis signal-regulating kinase 1 (ASK1) that activate downstream MAPKs, NF-κB, and AP-1, which in turn induce inflammatory gene expression. Phenolic phytochemicals have a strong antioxidant potential owing to the abundance of hydroxyl groups associated with their aromatic rings. Phenolic phytochemicals also have the capacity to increase the levels of anti-inflammatory genes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and heme oxygenase (HO)-1 via activation of the transcription factor nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) (reviewed in 91). Thus, polyphenols have an inherent capacity to reduce ROS and other free radicals, thereby preventing their activation of oxidative stress and inflammation. Consistent with this antioxidant potential, Sprague-Dawley rats consuming a high-fructose diet supplemented with a grape skin extract (21 mg/kg body weight for six weeks) were protected against ROS production, possibly due to reduced levels of NADPH oxidase, thereby preventing cardiac hypertrophy and hypertension induced by a high-fructose diet (2).

In vitro, pretreatment with freeze-dried grape powder (300 μg/ml) restored glutathione (GSH) content in human hepatoma cells (Huh7 cells) and primary mouse hepatocytes treated with hydrogen peroxide (H2O2) (126). The flavonols quercetin and kaempferol (5-50 μM) decreased the levels of oxidized GSH, peroxides, superoxide anions, and nitric oxide in parenchymal liver cells (Chang liver cells) treated with a mixture of inflammatory cytokines (28). Quercetin pretreatment (25 μM) of primary cultures of rat neurons exposed to H2O2 increased GSH levels, the nuclear translocation of Nrf2, and the expression of γ-glutamate-cysteine ligase catalytic subunit (GCLC), the rate-limiting enzyme for GSH synthesis, compared with those treated with H2O2 only (3). However, cells treated with doses of quercetin higher than 25 μM for 24 hours showed signs of cytotoxicity.

Quercetin and isorhamnetin (10 μM) pretreatment of murine macrophages (RAW264.7 cells) treated with LPS decreased the expression of inflammatory genes and inducible NOS and increased the protein levels of HO-1 (10). However, cells treated with doses of these flavonols equal to or greater than 25 μM for 24 hours showed signs of cytotoxicity. Quercetin pretreatment (10-50 μM) of immortalized human keratinocytes (HaCaT cells) treated with H2O2 increased mitochondrial membrane potential and cell viability and reduced the percentage of apoptotic cells compared with controls (122). Quercetin pretreatment (10 and 100 μM) of human hepatoma cells treated with H2O2 or CuSO4 (to initiate the Fenton reaction for the production of hydroxyl radicals) acutely decreased ROS production (60). However, long term treatment with high levels of quercetin (100 μM) decreased cell viability, suggesting pro-oxidant actions of quercetin

at high levels. Pretreatment with the anthocyanin cyanidin 3-glucoside (10-40 μM) of murine adipocytes (3T3-L1 cells) exposed to H2O2 or TNFα decreased ROS production and insulin resistance compared with controls (46). Quercetin and rutin (1-25 μM), a quercetin metabolite, blocked oxidized low-density lipoprotein (LDL)-mediated apoptosis of human umbilical vein endothelial cells via modulation of Janus kinase/signal transducers and activators of the transcription signaling pathway (23). Rutin, but not quercetin, inhibited JNK and p38 signaling by decreasing the activation of ASK1, a redox-sensitive kinase that triggers inflammatory MAPK signaling.

Collectively, these data suggest that grape polyphenols or their metabolites have the capacity to protect cells against oxidative damage by (*a*) neutralizing ROS and RNS or transition metals that produce ROS, RNS, and oxidize GSH, (*b*) decreasing the activity of enzymes such as NAPDH oxidase and NOS that produce ROS and RNS, respectively, (*c*) suppressing inflammatory signaling cascades including ASK1 and downstream MAPK, and (*d*) activating transcription factors such as Nrf2 that induce the transcription of antioxidant enzymes such as GPx, SOD, HO-1, and GCLC (**Figure 2.5**). However, high levels of these polyphenols may be cytotoxic.

Attenuating Endoplasmic Reticulum Stress Signaling

The development of obesity and the metabolic syndrome has been linked to ER stress and inflammation (reviewed in 49). Endoplasmic reticulum stress activates the unfolded protein response (UPR), which involves activation of three ER membrane-associated proteins: (*a*) double-stranded RNA-activated protein kinase (PKR)-like

eukaryotic initiation factor 2α (eIF2α) kinase (PERK), (*b*) inositol requiring enzyme 1 (IRE1), and (*c*) activating transcription factor 6 (ATF6). Studies on flavonols suggest that kaempferol (10 μM) or quercetin (25-150 μM) attenuate ER stress in a rat cardiac muscle cell line (H9c2 cells) and isolated rat hearts (59) or in human colon cancer cell lines (Caco-2 and LS180 cells) (76), respectively, by blocking PERK-mediated eIF2α phosphorylation, IRE1-mediated X-box-binding protein 1 activation, and ATF6 expression (**Figure 2.5**). In contrast, freeze-dried grape powder (300 μg/ml) did not prevent ER stress-mediated apoptosis of human hepatoma cell line (Huh7 cell) and primary mouse hepatocytes (126). Research investigating the inhibitory effects of anthocyanidins/anthocyanins on ER stress is lacking, and the preventive effects of grape anthocyanidins/anthocyanins and flavonols on ER stress and chronic inflammation are unknown.

Blocking Pro-Inflammatory Cytokines or Endotoxin-Mediated Kinases and Transcription Factors Involved in Metabolic Diseases

Anthocyanins and flavonols modulate inflammation and insulin resistance associated with obesity. For example, Zucker fatty rats fed a high-fat diet supplemented with an anthocyanin-rich, 1% tart cherry powder (w/w) for 12 weeks had lower WAT and plasma levels of TNFα and interleukin (IL)-6 and WAT NF-κB activity compared with controls (101). Dahl salt-sensitive, hypertensive rats supplemented with 3% grape powder in the diet (w/w) for 18 weeks had lower plasma levels of TNFα and IL-6, decreased cardiac tissue oxidative damage (102), and reduced expression of inflammatory genes

and NF-κB DNA binding activity in cardiac tissue (100) compared with high-sugar controls (3% fructose:glucose; 1:1). C57BL/6J mice fed a high-fat diet supplemented for eight weeks with an anthocyanin-rich, 4% blueberry powder (w/w) had lower indices of inflammation (e.g., decreased gene expression of markers of WAT MΦ and inflammation) and oxidative stress and greater insulin sensitivity compared with control mice (33). C57BL/6J mice fed a high-fat diet supplemented with 0.8% quercetin in the diet (w/w) for eight weeks had lower plasma levels of interferon γ, IL-1α, and IL-4 compared with controls (106). Finally, Zucker fatty rats receiving a daily dose of quercetin (2 or 10 mg/kg body weight, o.p.) for 10 weeks had lower inflammatory markers, improved insulin sensitivity and blood lipid profiles, and decreased blood pressure compared with placebo controls (94).

In vitro, cyanidin 3-glucoside (10-40 μM) prevented TNFα-mediated JNK activation, IRS-1 phosphorylation at serine residue 307, and insulin resistance in 3T3-L1 adipocytes (46). Notably, grape seed flavan-3-ols/procyanidins (50 and 100 mg/liter) modulated LPS- and TNFα-induced inflammatory signal and gene expression in human MΦ (differentiated THP-1 monocytes) and Simpson-Golabi-Behmel Syndrome adipocytes, respectively (19). Moreover, oligomerized grape seed polyphenols (10 and 20 μg/ml) attenuated activation of inflammatory signaling and production of inflammatory cytokines in a murine cell line of adipocytes (HW mouse white adipocytes) co-cultured with a murine MΦ cell line (RAW264 cells) (98). Consistent with these data, our lab demonstrated that grape powder extract (GPE; 30-100 μg/ml), made from grape powder provided by the California Table Grape Commission, attenuated inflammatory gene

expression (i.e., IL-6, IL-1β, IL-8, and MCP-1) in primary human adipocytes induced by conditioned media collected from LPS-treated MΦ (differentiated U937 monocytes) (81). Furthermore, quercetin (3-30 μM), the most abundant polyphenol in GPE (81), prevented inflammation in human MΦ and primary human adipocytes treated with MΦ conditioned media (82). We showed that pretreatment of human MΦ with quercetin (3-30 μM) prevented MΦ-mediated insulin resistance in primary human adipocytes (82). We also demonstrated that GPE (10-60 μg/ml) and quercetin (3-60 μM) attenuated TNFα-mediated inflammation and insulin resistance in primary human adipocytes by blocking activation of ERK, JNK, NF-κB, and AP-1 signaling and negative regulators of insulin signaling (e.g., protein tyrosine phosphatase (PTP)-1B and phosphorylation of serine residue 307 on IRS) (25, 26). Furthermore, the quercetin content of the cells increased within one hour of treatment with 30 μM quercetin, suggesting that quercetin is rapidly taken up by human adipocytes (26). No signs of cytotoxicity were observed for these doses of GPE or quercetin.

Quercetin (1-5 μM) suppressed carcinogen-mediated inflammation by directly binding to MEK, which prevents activation of inflammatory MEK/MAPK signaling in JB6 P+ cells, a JB6 promotion-sensitive mouse skin epidermal cell line (65). Moreover, kaempferol (1 and 3μM) inhibited TNFα-mediated inflammation by directly blocking nuclear translocation and DNA binding of NF-κB and AP-1 in A549 cells, an alveolar epithelial cell carcinoma cell line (21). Taken together, these data suggest that grape polyphenols, especially anthocyanins and flavonols, inhibit pro-inflammatory cytokine- and metabolic endotoxin-triggered activation of inflammatory MEK/MAPK, NF-κB, and

AP-1 signaling, which increase inflammatory gene expression (e.g., TNFα, IL-6, IL-8, IL-1β, and MCP-1) and negative regulators of insulin signaling (**Figure 2.6**). Therefore, grape polyphenols may be useful in preventing inflammatory-mediated insulin resistance and other related metabolic diseases.

Suppressing Inflammatory- or Inducing Metabolic-Gene Expression via Increasing Histone Deacetylase Activity

Sirtuins (SIRT), class III histone deacetylases, are nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases. Sirtuins deacetylate not only histones, but also nonhistone proteins including transcription factors by transferring an acetyl group from the targeted protein to NAD+, generating deacetylated histones or nonhistone proteins, nicotinamide, and *O*-acetyl-ADP-ribose. This NAD+ dependency contributes to the role that SIRT play in the regulation of chromatin structure and gene expression, cell survival, and energy homeostasis. In a SIRT1 in vitro screening assay (51), several polyphenols such as *trans*-resveratrol and quercetin (100 μM) were shown to activate SIRT1.

In vitro (0.01-10 μM) and in vivo (2.5-400 mg/kg body weight/day) studies have reported that resveratrol activated SIRT1, thereby (*a*) decreasing inflammatory gene expression by deacetylation/inactivation of NF-κB (39, 79, 128, 132) and (*b*) improving insulin sensitivity by deacetylation/activation of PPARγ coactivator-1 alpha (PGC-1α) (62, 111), a regulator of PPAR activity (reviewed in 20). Activation of PPARα or PPARβ/δ by PGC-1α also enhances energy expenditure (reviewed in 38, 110). Consistent

with these data, quercetin supplementation (12.5 and 25 mg/kg body weight/day) for seven days increased SIRT1 and PGC-1α expression and mitochondrial biogenesis in male ICR mice (31). These findings suggest that resveratrol and quercetin prevent inflammation by activating SIRT1, which deacetylates NF-κB (inactive form) and PGC-1α (active form), thereby suppressing inflammatory- and inducing metabolic-gene expression, mitochondrial biogenesis, and oxidative phosphorylation via activation of PPAR (**Figure 2.7**). However, more research is needed on the regulation of SIRT1 by grape polyphenols to determine which specific anthocyanidins and flavonols are involved.

Activating Transcription Factors that Antagonize Chronic Inflammation

The PPARs (i.e., PPARα, PPARβ/δ, and PPARγ) are ligand-dependent, nuclear transcription factors that regulate energy homeostasis, glucose and lipid metabolism, and immune response (reviewed in 7). Activation of PPARs has been reported to suppress inflammatory gene expression by directly interfering with transcriptional activation of NF-κB or AP-1 (reviewed in 93). For example, upon activation by ligand binding, PPARγ can be SUMOylated by binding to SUMO1, a small ubiquitin-like modifier. The SUMOylated PPARγ subsequently binds to nuclear receptor corepressors, which interferes with clearance of the corepressor complex of NF-κB, thereby transrepressing LPS-mediated NF-κB activation (83).

Several studies reported that anthocyanin-rich cherries or berries increase the level of PPARγ gene, protein, or activity. For example, the supplementation with 1% tart cherry in the diet (w/w) for 12 weeks or 3% grape powder in the diet (w/w) for 18 weeks

suppressed inflammation and improved metabolic diseases by increasing PPARγ gene expression in Zucker fatty rats (101) or by increasing PPARα/γ mRNA levels and protein activity in Dahl salt-sensitive, hypertensive rats (100), respectively. Consistent with these data, our lab (26) and others (36) showed that by increasing PPARγ activation, quercetin or kaempferol (3-60 μM) attenuated inflammation or insulin resistance in adipocytes. These findings suggest that grape polyphenols increase the expression or activation of PPARs that antagonize inflammatory transcription factors, thereby blocking inflammation and the development of metabolic diseases (**Figures 2.6** and **2.7**).

Implications and Conclusions

Potential Health Benefits of Consuming Grapes or Grape Products

Cardiovascular Disease

Inflammatory mediators released from excess WAT cause endothelial dysfunction, plaque initiation and progression, and plaque rupture, leading to CVD (reviewed in 63). Supplementation with quercetin (64 mg/kg body weight/day for 10 and 20 weeks) attenuated inflammation and endothelial dysfunction in a mouse model of atherosclerosis (ApoE-/- knockout mice) (69). Randomized, clinical trials with proanthocyanidin-rich cocoa powder (40 g cocoa powder in 500 ml skim milk/day for four weeks) (73) and anthocyanin- and flavonol-rich bilberry juice (330 ml/day for four weeks) (57) showed decreased plasma markers of inflammation in adults with high CVD risk who consumed these polyphenol-rich products.

Consumption of red grape juice (50 ml concentrate twice per day for two weeks) reduced serum markers of inflammation and oxidative stress/oxidized-LDL and improved dyslipidemia in hemodialysis patients (16). Consumption of raisins (one cup per day for six weeks) reduced systolic blood pressure and inflammatory cytokines and improved lipid profiles in men and postmenopausal women (89). Furthermore, consumption of grape seed flavan-3-ols procyanidins (0.32 mg/g diet/day for 19 weeks) attenuated inflammatory markers in liver, WAT, and circulation in high-fat-fed, obese male Zucker fatty rats (114). Also, consumption of whole table grape powder (3% in the diet, w/w, for 18 weeks) attenuated systolic blood pressure, cardiac hypertrophy, and diastolic dysfunction in Dahl salt-sensitive hypertensive rats (102). Finally, Pérez-Jiménez & Saura-Calixto (86) reviewed 75 trials including human and animal studies that investigated the relationship between consumption of grapes or grape products and their effects on lowering risk factors for CVD including (*a*) inflammation, (*b*) oxidative stress/LDL oxidation, (*c*) endothelial dysfunction, (*d*) platelet aggregation, (*e*) dyslipidemia, and (*f*) hypertension. They concluded from these studies that grapes and grape products have antihypertensive, antihyperlipidemic, antiatherosclerotic, and antioxidant effects. Therefore, supplementation with grapes or grape products rich in polyphenols may be a useful dietary strategy for the attenuation of CVD associated with obesity and inflammation.

Insulin Resistance or Diabetes

Obesity associated, low-grade inflammation causes insulin resistance (**Figures 2.1** and **2.2)**. Insulin resistance is a prediabetic state characterized by decreased tissue sensitivity to insulin and hyperinsulinemia. These chronic perturbations in glucose disposal lead to the development of noninsulin-dependent diabetes (type 2) and eventually pancreatic beta cell failure, resulting in insulin-dependent diabetes (type 1) (reviewed in 108). Elevated inflammatory TNFα, LPS, SFA, or FFA contribute to obesity-associated insulin resistance as shown in **Figure 2.2**.

Grape polyphenols such as flavan-3-ols/procyanidins or stilbenes/resveratrol improved glucose intolerance in type 1 diabetic rat models (1, 22, 35, 87, 109). Resveratrol (0.04% in the diet w/w for 12-48 weeks, or 2.5-400 mg/kg body weight/day for 15-16 weeks) improved glucose and lipid homeostasis in high-fat-fed C57BL/6J obese mice (6, 62, 111). Consumption of 150 ml of muscadine grape wine or dealcoholized grape wine per day for 28 days improved fasting blood glucose and insulin levels in subjects with type 2 diabetes (4). Overall, results suggest that grapes or grape products are good candidates for dietetic management of type 2 diabetes because of their abundant polyphenol content and low glycemic index (134). However, clinical studies investigating the effect of grape consumption on insulin resistance or type 2 diabetes are limited.

Cancer

Obesity-associated inflammation contributes to cancer initiation or progression (reviewed in 27, 52, 80). García-Lafuente et al. (42) summarized the potential anti-inflammatory mechanisms of flavonoids found in fruits and vegetables that are linked to the prevention or treatment of cancer. For example, resveratrol prevents certain cancers due to its ability to inactivate inflammatory signaling, including protein kinases such as MEK/MAPK and transcription factors such as NF-κB and AP-1, and to modulate carcinogenic signaling by down-regulation of cyclooxygenase (COX)-2 and iNOS (47, 54, 72, 113; reviewed in 8, 112, 117).

Interestingly, the anticarcinogenic effects of red wine extract may be due to not only resveratrol but to flavonols as well (65). For example, flavonol/quercetin (9, 24, 64, 67, 123) and anthocyanidin/delphinidin (53, 61, 120, 131) exert anti-inflammatory and anticancer activity. However, most studies used pharmacological doses of these polyphenols. Future studies are needed to investigate the relationship between physiologically relevant doses of polyphenols from grapes and grape products and their ability to prevent or treat certain types of cancer.

Neurodegenerative Diseases linked to Obesity

Grapes and grape products have been reported to have antiaging and antineurodegenerative effects due to their antioxidant properties (reviewed in 55). Moreover, rats fed 6.5 ml/kg body weight per day white or red wine or 2.5 mg/kg body weight per day resveratrol for two weeks had higher expression levels of genes and

proteins related to longevity (74). Consumption of red wine containing 0.2 mg/liter of resveratrol for seven months attenuated the development of Alzheimer's disease (AD) in Tg2576 AD transgenic mice due to reduced aggregation of brain amyloid beta-protein (119). Grape powder or extract also has been reported to modulate the pathogenesis of neurodegenerative disease (70, 115, 118, 121). However, the extent to which polyphenols in grapes other than resveratrol extend lifespan or prevent neurodegenerative diseases is unknown, as are their mechanisms of action.

Potential Risks of Excess Consumption of Grapes or Grape Products

Overconsumption of grapes or grape products could lead to (*a*) excess weight gain due to the high sugar content of grapes, (*b*) immunosuppression due to the anti-inflammatory actions of polyphenols, (*c*) impairment of micronutrient absorption or metabolism, and (*d*) alcohol toxicity associated with excess wine consumption. Of these four, the deleterious effects of excess alcohol consumption (e.g., gastrointestinal cancers, cirrhosis, pancreatitis, malabsorption, nonsteroidal anti-inflammatory drug interactions, impaired judgment, alcoholism, mania) are well-documented risks. Thus, moderation is the key with regard to consuming grapes or their by-products, especially alcoholic beverages made from grapes. Detrimental effects of consuming high levels of supplements made from grapes or their by-products are not well reported in the literature.

Conclusions

Potential mechanisms by which polyphenol-rich grape products reduce chronic inflammation and metabolic diseases associated with obesity are summarized in **Figure 2.8**. Chapter III will address the hypothesis that GPE attenuates TNFα-mediated inflammation and insulin resistance, possibly by suppressing the activation of ERK, JNK, NF-κB, and AP-1, in primary cultures of newly differentiated human adipocytes. Chapter IV will address the hypothesis that quercetin and resveratrol, two of important grape polyphenols, are absorbed and attenuate TNFα-mediated inflammation and insulin resistance, possibly by decreasing ERK, JNK, NF-κB, and AP-1 activity, and increasing PPARγ expression and activity, in primary human adipocyte cultures. Chapter V will address the hypothesis that several grape powder polyphenols are bioavailable in mice, and improve glucose disposal rates and reduce markers of chronic inflammation in high fat-fed obese mice.

Table 2.1. The content of polyphenols analyzed in freeze-dried table grape powder (GP)[a]

Polyphenols	Compounds	Content (mg/kg GP)
Anthocyanins	Malvidin	145.2
	Cyanidin	125.0
	Peonidin	31.7
Flavonols	Quercetin	32.6
	Isorhamnetin	6.8
	Kaempferol	5.6
Flavan-3-ols	Catechin	19.7
	Epicatechin	12.6
Stilbenes	Resveratrol	1.75

[a]Data are from California Table Grape Commission Information Sheet (2005), http://agbioresearch.msu.edu/rfp/ca_grape2009.pdf.

Table 2.2. Phenolic phytochemicals found in grapes and grape products

Resource	Phenolic phytochemicals	References
Whole grapes	Flavonol glycosides (quercetin, kaempferol, myricetin, laricitrin, isorhamnetin, syringetin), anthocyanins[a] (malvidin, peonidin, petunidin, cyanidin, delphinidin, pelargonidin), flavan-3-ols (catechin, epicatechin), phenolic acids (protocatechuic acid, gallic acid), hydroxycinnammates (caftaric acid, coutaric acid, fertaric acid), stilbenes (*trans*-resveratrol)	(15, 17, 78)
Grape seed	Flavan-3-ols (catechin, catechin gallate, epicatechin, epicatechin gallate, procyanidin gallate, B1, B2, other dimers, trimers, and tetramers)	(18, 84)
Grape skin	Flavonol glycosides (quercetin, kaempferol, myricetin, laricitrin, isorhamnetin, syringetin), anthocyanins[a] (malvidin, peonidin, petunidin, cyanidin, delphinidin, pelargonidin), flavan-3-ols (catechin, epicatechin, gallocatechin, procyanidin B1, B2, B4, other dimers, C1, other trimers, and tetramers), phenolic acids (protocatechuic acid, gallic acid), hydroxycinnammates (caftaric acid, coutaric acid, fertaric acid), stilbenes (*trans*-resveratrol, *cis*-resveratrol, resveratrol dimmers and tetramers), flavanonol glycosides (taxifolin)	(15, 17, 18, 84)
Grape stem	Flavonols (quercetin glucoside), flavan-3-ols (catechin, catechin gallate, epicatechin, procyanidin B1 and B2), hydroxycinnammates (caffeic acid, coumaric acid), stilbenes (*trans*-resveratrol, *trans*-resveratrol glucoside, resveratrol dimers, trimers, and tetramers), flavanonols (taxifolin glucoside)	(71, 90)
Grape leaf	Flavonols (quercetin, rutin, kaempferol, myricetin), flavan-3-ols (catechin), phenolic acids (gallic acid, ellagic acid), stilbenes (resveratrol), flavanones (naringin)	(29, 84)
Red grape juice	Flavonols (quercetin glucoside, rutin, myricetin), anthocyanins[a] (malvidin, peonidin, petunidin, cyanidin, delphinidin), flavan-3-ols (catechin, procyanidin B2)	(30)
Red wine	Flavonol glycosides (quercetin, kaempferol, myricetin, isorhamnetin), anthocyanins[a] (malvidin, peonidin, petunidin, cyanidin, delphinidin), flavan-3-ols (catechin, catechin gallate, epicatechin, epicatechin gallate, procyanidin B1, B2, B4, and trimers), phenolic acids (protocatechuic acid, gallic acid, ellagic acid, vanillic acid, syringic acid), hydroxycinnammates (caffeic acid, caftaric acid, coutaric acid, ferulic acid, fertaric acid, coumaric acid, sinapic acid), stilbenes (*trans*-resveratrol, *trans*-resveratrol glucoside)	(41, 43, 85, 88)
Raisin	Flavonol glycosides (quercetin, kaempferol), phenolic acids (protocatechuic acid), hydroxycinnammates (caftaric acid, coutaric acid)	(56)

[a]Anthocyanidins/anthocyanins are detected only in red grapes.

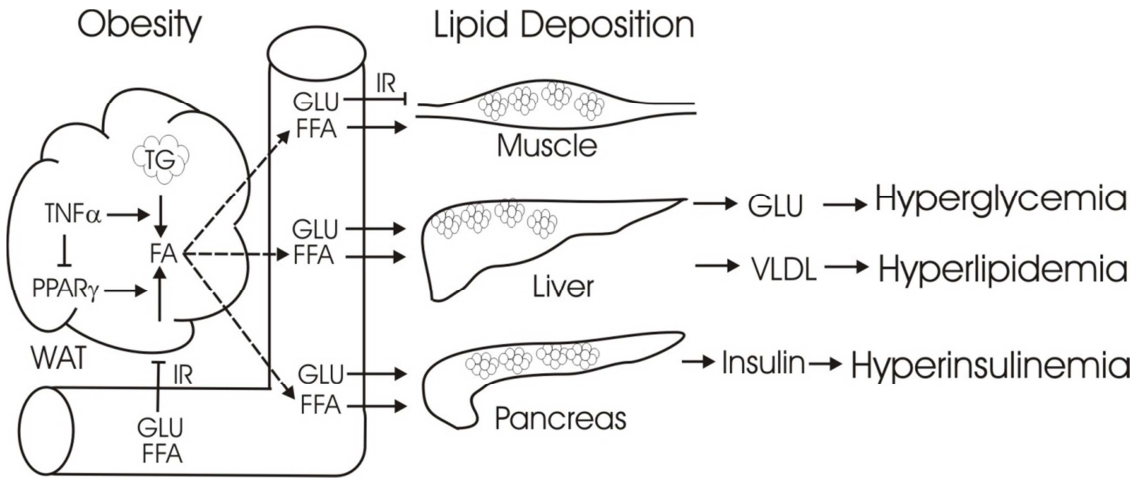

Figure 2.1. Decreased lipid- and glucose-buffering capacity of adipocytes leads to hyperglycemia, hyperlipidemia, and hyperinsulinemia. Nutrient overload in white adipose tissue (WAT) leads to insulin resistance [IR; i.e., decreased free fatty acids (FFAs) and glucose (GLU) uptake] coupled with increased secretion of FFAs and pro-inflammatory adipokines such as tumor necrosis factor alpha (TNFα). Elevated FFAs are deposited ectopically in muscle, liver, and pancreas, leading to hyperglycemia, hyperlipidemia, and hyperinsulinemia, respectively. TG, triglyceride; PPARγ, peroxisome proliferator-activated receptor gamma; VLDL, very low-density lipoprotein

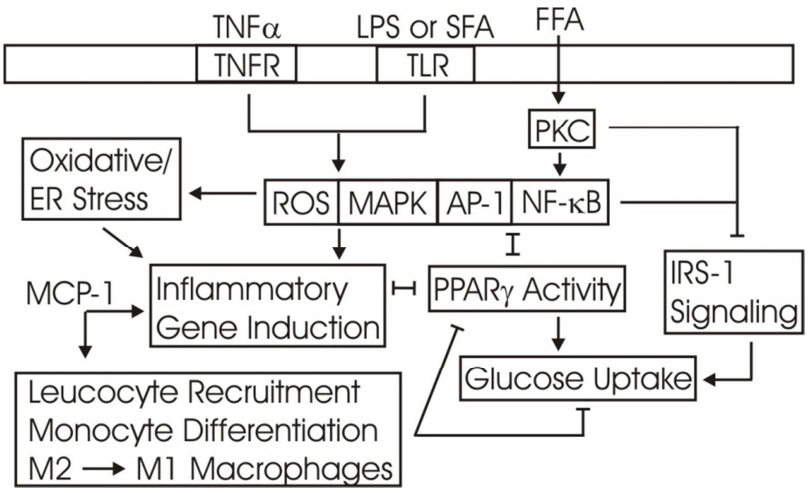

Figure 2.2. Mechanisms by which tumor necrosis factor alpha (TNFα), lipopolysaccharide (LPS), saturated fatty acids (SFAs), and free fatty acids (FFAs) promote inflammation and insulin resistance. Elevated levels of TNFα, LPS, SFA, or FFA activate their cognate cell surface receptors or diffuse into the cell, thereby activating protein kinases such as protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs) or enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that produce reactive oxygen species (ROS), thereby triggering oxidative or endoplasmic reticulum (ER) stress signals or activating inflammatory transcription factors such as nuclear factor-kappa B (NF-κB) and activating protein (AP)-1. Together, these inflammatory signals induce inflammatory gene expression, enhance leukocyte recruitment and differentiation including activating monocytes into classically activated M1-macrophages (MΦ), antagonize peroxisome proliferator-activated receptor gamma (PPARγ) activity, and impair insulin receptor substrate (IRS)-1 signaling, leading to insulin resistance. MCP-1, monocyte chemoattractant protein-1; TLR, toll-like receptor; TNFR, TNFα receptor

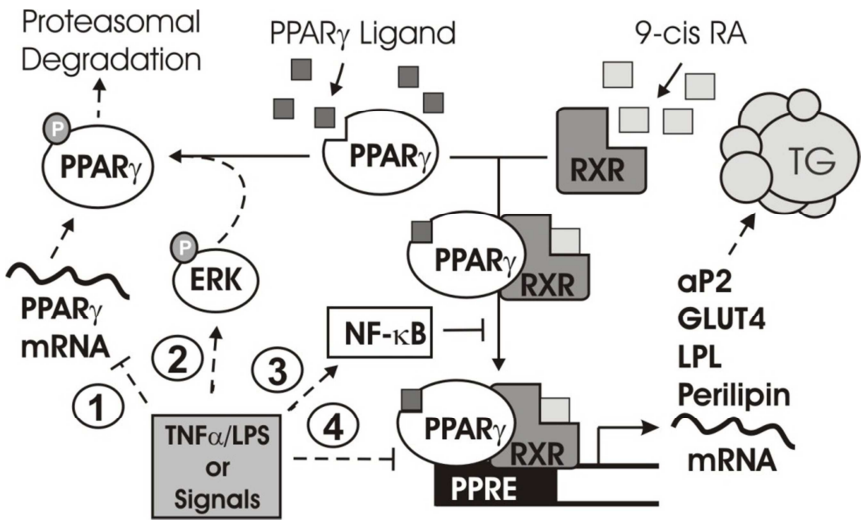

Figure 2.3. Mechanisms by which tumor necrosis factor alpha (TNFα) or lipopolysaccharide (LPS) antagonizes peroxisome proliferator-activated receptor gamma (PPARγ) activity. Tumor necrosis factor α and LPS or their signals antagonize PPARγ activity by (*a*) decreasing PPARγ mRNA levels, (*b*) phosphorylation (P) of PPARγ on serine 112 by extracellular signal-related kinase (ERK), leading to PPARγ ubiquination and proteosomal degradation, (*c*) activating nuclear factor-kappa B (NF-κB), which antagonizes PPARγ activity and its DNA binding capacity, or (*d*) activating nuclear corepressors, which decrease PPARγ transcriptional activity and target gene expression. Collectively, this leads to insulin resistance in white adipose tissue, thereby elevating circulating free fatty acids (lipodystrophy) and glucose (hyperglycemia) that promote ectopic lipid deposition in liver, skeletal muscle, heart, and pancreas. 9-cis RA, 9-cis retinoic acid; aP2, adipocyte fatty acid-binding protein; GLUT4, insulin-dependent glucose transporter 4; LPL, lipoprotein lipase; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor, TG, triglyceride

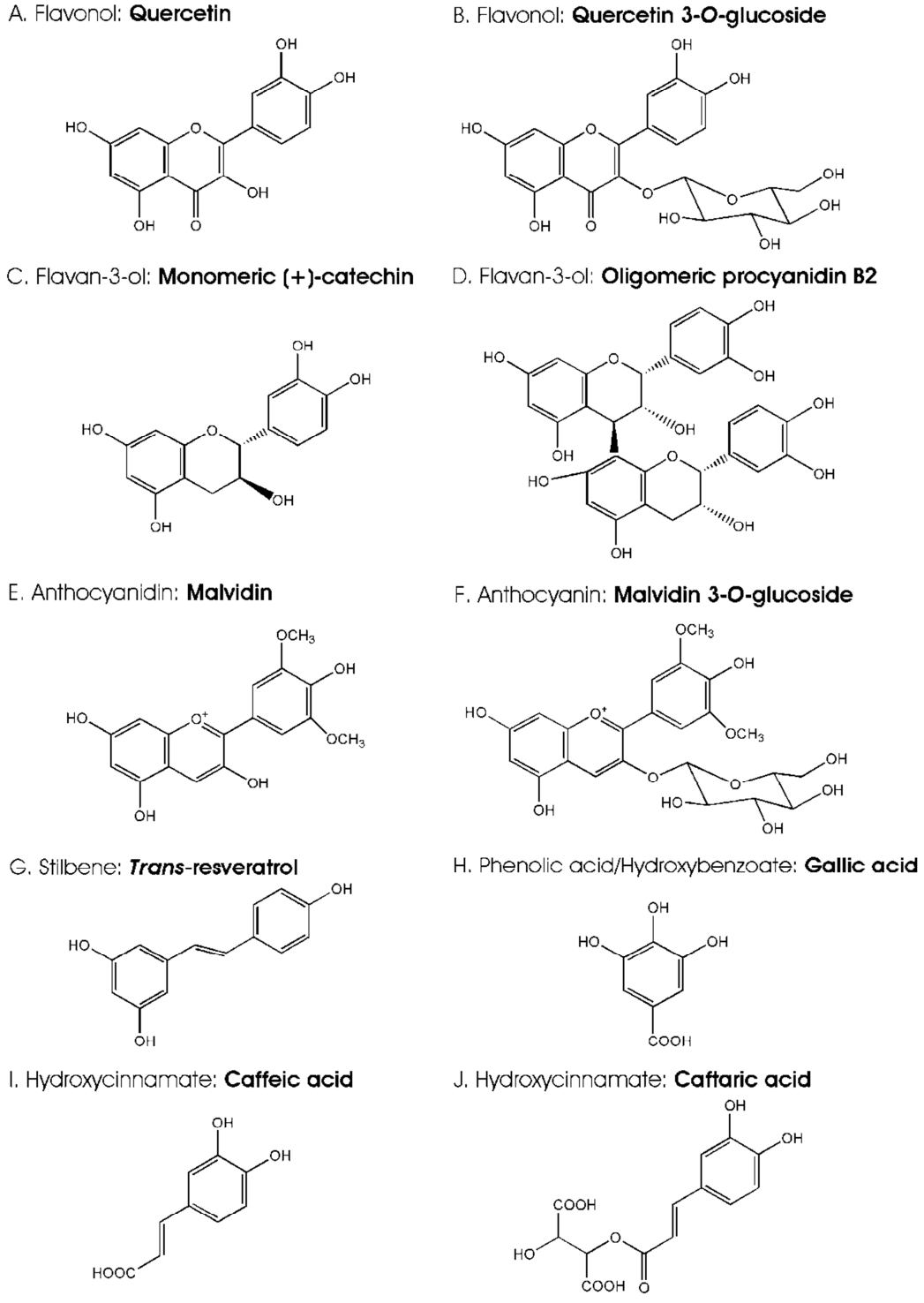

Figure 2.4. Structures of polyphenols commonly found in grape products.

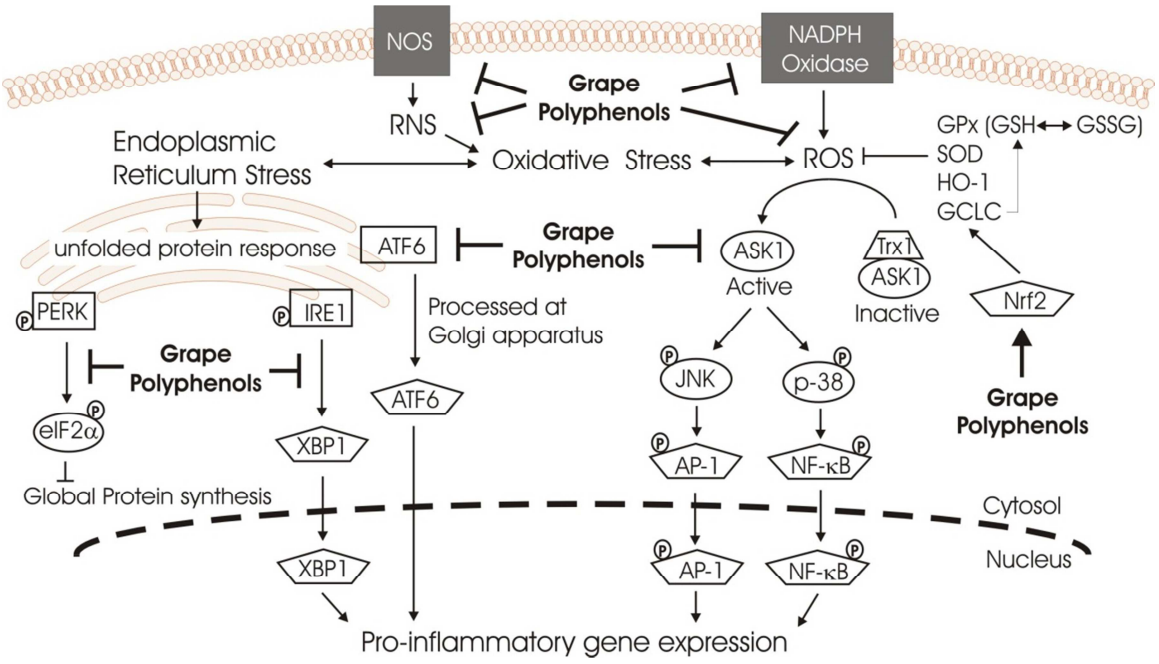

Figure 2.5. Potential mechanisms by which grape polyphenols prevent chronic inflammation associated with obesity. Grape polyphenols may attenuate oxidative or endoplasmic reticulum (ER) stress-mediated inflammation associated with obesity by (*a*) inhibiting the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or nitric oxide synthase (NOS) that generate reactive oxygen species (ROS) or reactive nitrogen species (RNS), respectively, (*b*) acting as a classic antioxidant by directly scavenging ROS and RNS, (*c*) preventing ROS-mediated activation of apoptosis signal-regulated kinase 1 (ASK1), a redox-sensitive, inflammatory signaling pathway, (*d*) increasing the expression of antioxidant genes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), heme oxygenase (HO)-1, and γ-glutamate-cysteine ligase catalytic subunit (GCLC) via activation of transcription factor nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2), or (*e*) blocking ER stress-mediated unfolded protein response including double-stranded RNA-activated protein kinase (PKR)-like eukaryotic initiation factor 2α (eIF2α) kinase (PERK)-mediated eIF2α, inositol requiring enzyme 1 (IRE1)-mediated X-box binding protein 1 (XBP1), and activating transcription factor 6 (ATF6) signaling pathways that trigger proinflammatory gene expression. AP-1, activating protein-1; GSH, reduced glutathione; GSSG, oxidized glutathione; JNK, c-Jun-NH2 terminal kinase; NF-κB, nuclear factor-kappa B; P, phosphorylated; Trx1, thioredoxin-1

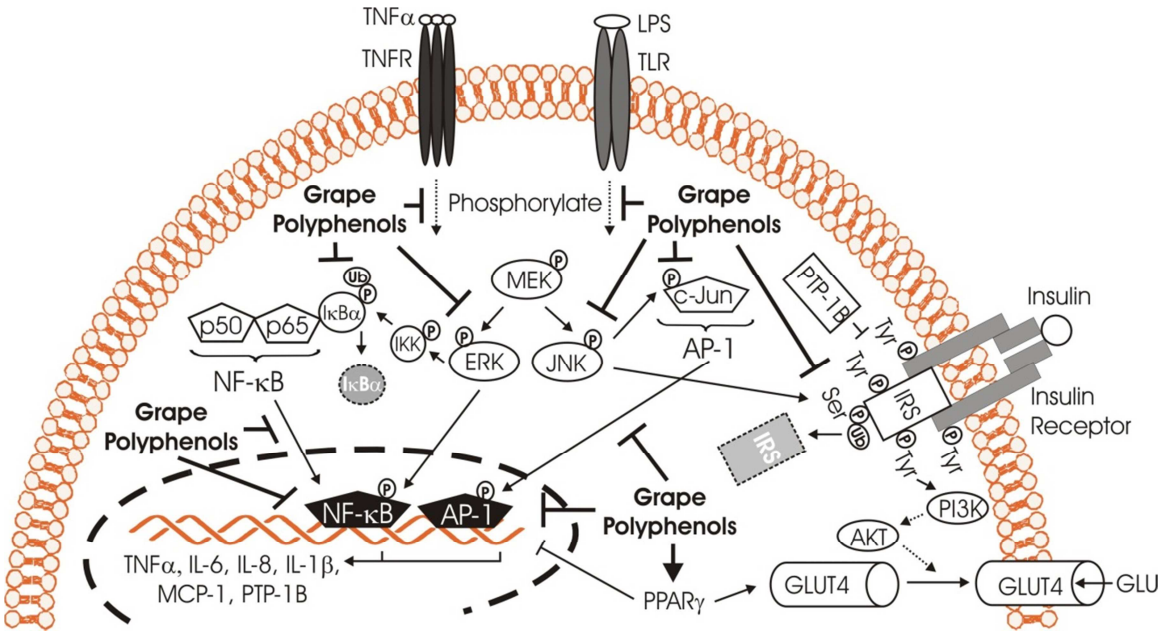

Figure 2.6. Grape polyphenols may prevent inflammation and insulin resistance associated with obesity by blocking activation of mitogen-activated protein kinases (MAPKs), nuclear factor-kappa B (NF-κB), and activating protein (AP)-1. Grape polyphenols may prevent inflammatory tumor necrosis factor alpha (TNFα)/TNF receptor (TNFR) or lipopolysaccharide (LPS)/toll-like receptor (TLR) signaling to MAPK kinases (MEK) and their downstream, extracellular signal-related kinase (ERK) and c-Jun-NH2 terminal kinase (JNK). This would attenuate the activation of inflammatory transcription factors NF-κB and AP-1, which are potent inducers of inflammatory gene expression [e.g., TNFα, interleukin (IL)-6, IL-8, IL-1β, and monocyte chemoattractant protein (MCP)-1] and negative regulators of insulin signaling [e.g., phosphorylation of serine residue 307 on insulin receptor substrate (IRS) and protein tyrosine phosphatase (PTP)-1B], and which suppress insulin signaling necessary for insulin-dependent glucose transporter 4 (GLUT4) translocation to the plasma membrane. AKT, AKT/protein kinase B; GLU, glucose; IKK, IκBα kinase; PI3 K, phosphatidylinositol 3-kinases; P, phosphorylated; PPAR, peroxisome proliferator-activated receptor; Ser, serine; Tyr, tyrosine; Ub, ubiquinated degradation

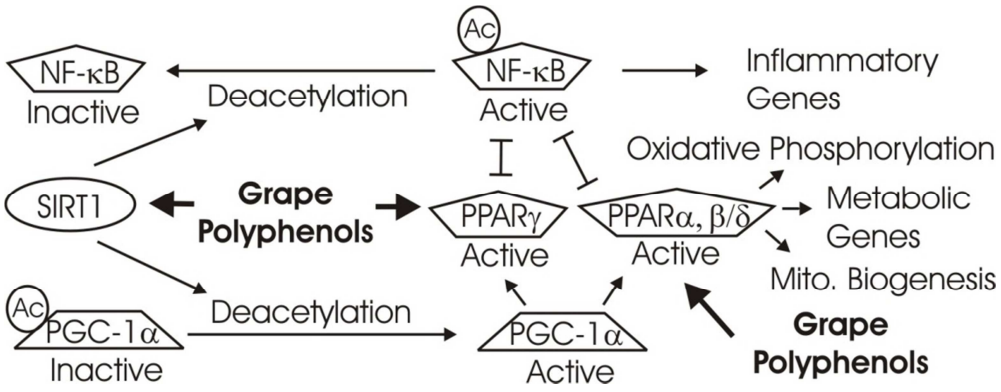

Figure 2.7. Grape polyphenols may activate sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor (PPAR) that antagonize inflammation and improve the metabolic diseases associated with obesity. Grape polyphenols may prevent inflammation and improve metabolic diseases associated with obesity by activating SIRT1. SIRT1 has been reported to (*a*) deacetylate/inactivate nuclear factor-kappa B (NF-κB), resulting in the suppression of inflammatory gene expression, and (*b*) deacetylate/activate PPARγ coactivator-1 alpha (PGC-1α), resulting in the activation of PPARs. Activation of PPARα and PPARβ/δ increases oxidative phosphorylation, metabolic gene expression, and mitochondrial (Mito) biogenesis. Activation of PPARγ may antagonize NF-κB transcriptional activation of inflammatory genes, thereby attenuating inflammation associated with obesity.

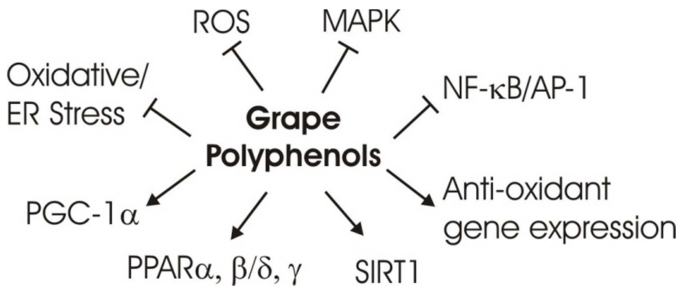

Figure 2.8. Summary of potential mechanisms by which polyphenol-rich grape products reduce chronic inflammation and metabolic diseases associated with obesity. AP-1, activating protein-1; ER, endoplasmic reticulum; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; PPAR, peroxisome proliferator-activated receptor; PGC-1, PPARγ coactivator-1; ROS, reactive oxygen species; SIRT, sirtuins

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CHAPTER III

GRAPE POWDER EXTRACT ATTENUATES TUMOR NECROSIS FACTOR α-MEDIATED INFLAMMATION AND INSULIN RESISTANCE IN PRIMARY CULTURES OF HUMAN ADIPOCYTES

Abstract

Grapes are rich in phenolic phytochemicals that possess anti-oxidant and anti-inflammatory properties. However, the ability of grape powder extract (GPE) to prevent inflammation and insulin resistance in human adipocytes caused by tumor necrosis factor α (TNFα), a cytokine elevated in plasma and white adipose tissue (WAT) of obese, diabetic individuals, is unknown. Therefore, we examined the effects of GPE on markers of inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes treated with TNFα. We found that GPE attenuated TNFα-induced expression of inflammatory genes including interleukin (IL)-6, IL-1β, IL-8, monocyte chemoattractant protein (MCP)-1, cyclooxygenase (COX)-2 and Toll-like receptor (TLR)-2. GPE attenuated TNFα-mediated activation of extracellular signal-related kinase (ERK) and c-Jun NH2-terminal kinase (JNK) and activator protein-1 (AP-1, i.e., c-Jun). GPE also attenuated TNFα-mediated IκBα degradation and nuclear factor-kappa B (NF-κB) activity. Finally, GPE prevented TNFα-induced expression of protein tyrosine phosphatase (PTP)-1B and phosphorylation of serine residue 307 of insulin receptor substrate-1 (IRS-1), which are negative regulators of insulin sensitivity, and suppression of insulin-stimulated glucose uptake. Taken together, these data demonstrate that GPE

attenuates TNFα-mediated inflammation and insulin resistance in human adipocytes, possibly by suppressing the activation of ERK, JNK, c-Jun and NF-κB.

Introduction

Obesity is associated with low-grade, chronic inflammation of white adipose tissue (WAT) characterized by increased production of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) [1]. The inflammatory effects of TNFα involve the activation of nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) [2-4]. Several studies have shown that activation of these transcription factors was responsible for increasing the expression of inflammatory cytokines such as interleukin (IL)-6, IL-1β, IL-8, and monocyte chemoattractant protein (MCP)-1 [3,5] and inflammatory proteins such as Toll-like receptor (TLR)-2 [6] and cyclooxygenase (COX)-2, which contributes to inflammatory prostaglandin production [7].

Obesity-associated inflammation plays an important role in the development of insulin resistance. Several studies reported that deletions of inflammatory cytokine genes including TNFα, IL-6 or MCP-1 protect against the development of insulin resistance and hyperglycemia in obese mice [8-11]. Moreover, inhibition of COX-2 activation in rats [12] and disruption of TLR expression in mice [13] protect against obesity-induced inflammation and insulin resistance. Furthermore, TNFα-activated mitogen-activated protein kinase (MAPK) signaling pathways, including extracellular signal-related kinase (ERK) and c-Jun-NH2 terminal kinase (JNK), contribute to the development of insulin resistance [14-17]. Insulin receptor substrate 1 (IRS-1) is one of the targets for ERK and

JNK that phosphorylate serine residues of IRS-1, which impairs the ability of the insulin receptor to phosphorylate tyrosine residues of IRS-1 [18], thereby reducing insulin action [17]. Finally, TNFα activates NF-κB, which in turn enhances the gene expression of protein tyrosine phosphatase (PTP)-1B, a negative regulator of insulin signaling, by dephosphorylating tyrosine residues on IRS-1 [19-21].

Grapes are rich in phenolic phytochemicals and are one of the most widely consumed fruits in the world. Human [22] and animal [23-25] studies have shown that lyophilized grape powder has cardioprotective effects due to its abundant content of polyphenols that possess anti-oxidative and anti-inflammatory properties. Furthermore, several polyphenols in grapes such as resveratrol [26] and quercetin [27,28] reduce inflammation or insulin resistance in rodent models. However, the protective effects of grapes or their byproducts on inflammation and insulin resistance in WAT are unclear, and the mechanisms by which grape products protect against inflammation and insulin resistance in human adipocytes are unknown.

Therefore, we investigated the extent to which grape powder extract (GPE) attenuated TNFα-mediated inflammation and insulin resistance in primary cultures of human adipocytes. We hypothesized that GPE attenuated TNFα-mediated induction of inflammatory genes and insulin resistance by suppressing the activation of MAPK, AP-1, or NF-κB.

Materials and Methods

Preparation of GPE

Lyophilized grape powder, obtained from red, green and blue-purple seeded and seedless California grapes, was acquired from the California Table Grape Commission (CTGC). The CTGC grape powder has been reported to contain several types of polyphenols including anthocyanins, monomeric flavanols, flavonols and stilbenes [25]. The powder was extracted to remove the sugars (90% w/w) at the University of North Carolina at Greensboro (UNCG) using a Diaion HP-20 anion resin column and eluted from the column using methanol and subsequently lyophilized to become GPE. GPE was dissolved in dimethyl sulfoxide (DMSO) to make the concentration of 100 mg/ml as the stock solutions stored at -20°C. Stock solutions were diluted immediately before use.

Materials

All cell culture ware were purchased from Fisher Scientific (Norcross, GA, USA). Adipocyte medium was purchased from Zen Bio, Inc. (Research Triangle Park, NC, USA). Tri-Reagent was purchased from Molecular Research Center (Cincinnati, OH, USA). Gene-specific primers were purchased from Applied Biosystems (Foster City, CA, USA). Goat polyclonal antibody for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho (Thr183/Tyr185) and -total JNK, anti-phospho (Thr202/Tyr204) and -total ERK, anti-phospho (Ser63) and -total c-Jun, anti-phospho (Ser307) and -total IRS-1, and anti-IκBα rabbit polyclonal antibodies were purchased from Cell Signaling Technologies

(Beverly, MA, USA). Immunoblotting buffers and precast gels were purchased from Invitrogen (Carlsbad, CA, USA). Western Lightning Plus Chemiluminescence Substrate was purchased from Perkin Elmer Life Science (Boston, MA, USA). The Nucleofector and Dual Glo luciferase kits were obtained from Amaxa (Cologne, Germany) and Promega (Madison, WI, USA), respectively. All other reagents and chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

Primary cultures of human adipocytes

Abdominal WAT was obtained from nondiabetic, Caucasian and African-American females, between the ages of 20 and 50 years old with a body mass index less than 32.0 during abdominoplasty. Approval was obtained from the Institutional Review Board at UNCG and the Moses Cone Memorial Hospital in Greensboro, NC, USA. Tissue was digested using collagenase, and stromal vascular (SV) cells were isolated, proliferated and induced to differentiate in adipocyte medium (AM-1, Zen Bio, Inc., RTP, NC, USA) plus 250 μmol/L isobutylmethylxanthine and 1 μmol/L of the thiazolidinedione rosiglitazone (BRL 49653, a gift from Dr. Per Sauerberg at Nova Nordisk A/S, Copenhagen, Denmark) for 3 days. Cultures were then grown in AM-1 only for 3-9 days as previously described [29]. Cultures containing ~50% preadipocytes and ~50% adipocytes, based on visual observations, were treated between Days 6 and 12 of differentiation. Each experiment was repeated at least twice at different times using a mixture of cells from two to three subjects unless otherwise indicated.

RNA isolation and real-time qPCR

Primary human SV cells were seeded in 35-mm dishes at 0.4×106 per dish and differentiated for 6 days. On Day 6, media was changed. Twenty-four hours later, cultures were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and subsequently treated with 0.5 ng/ml TNFα for 3 h. This 3-h treatment with TNFα was chosen based on the results of a pilot dose and time-course study (data not shown). Following treatment, cultures were harvested and total RNA was isolated using TRI-Reagent according to the manufacturer's protocol. For real-time qPCR, 2.0 μg total RNA was converted into first-strand cDNA using Applied Biosystems High-Capacity cDNA Archive Kit. Real-time qPCR was performed in an Applied Biosystems 7500 FAST Real Time PCR System using Taqman Gene Expression Assays. To account for possible variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample, and data were normalized accordingly.

Immunoblotting

Primary human SV cells were seeded in 35-mm dishes at 0.4×10^6 per dish and differentiated for 6 days. On Day 6, media was changed. Twenty-four hours later, cultures were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 0.5 ng/ml TNFα for 15 min. This 15-min treatment with TNFα was chosen based on the results of a pilot dose and time-course study (data not shown). Immunoblotting was conducted as previously described [30].

Transient transfections of human adipocytes

For measuring NF-κB activity, primary cultures of human adipocytes were transiently transfected with the NF-κB responsive luciferase (luc) reporter construct pNF-κB (Strategene, La Jolla, CA, USA) using the Amaxa Nucleofector system from Lonza Inc. (Walkersville, MD, USA). Briefly, on Day 6 of differentiation, 1.2×10^6 cells from a 60-mm plate were trypsinized and resuspended in 100 μl of Amaxa Nucleofector solution (cell line nucleofector kit V) and mixed with 1 μg of pNF-κB and 25 ng pRL-CMV for each sample. Electroporation was performed using the Amaxa Nucleofector Device (V-33 Nucleofector program). Cells were replated in 96-well plates after 10 min of recovery in calcium-free DMEM media. Following 24 h, transfected cells were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 100 ng/ml TNFα for 24 h. This 24-h treatment with TNFα was chosen based on the results of a pilot dose and time-course study (data not shown). Firefly luciferase activity was measured using the Promega Dual-Glo luciferase kit and normalized to renilla luciferase activity from the co-transfected control pRL-CMV vector. All luciferase data will be presented in relative light units as the ratio of firefly luciferase to renilla luciferase activity.

2-[3H]Deoxy-glucose uptake

Primary human SV cells were seeded in 12-well plates at 1.6×10^5 per well and differentiated for 12 days. On Day 12, media was changed to serum-free low glucose (5 mmol/L) and insulin (20 pmol/L)-containing media. Twenty-four hours later, cultures

were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 5 ng/ml TNFα for 24 h. This 24-h treatment with TNFα was chosen based on the results of a pilot dose and time-course study (data not shown). Culture media was removed and replaced with 0.5 ml of HBSS buffer containing 100 nmol/L human insulin for 10 min. After insulin preincubation, 2-[3H]deoxy-glucose (2-DOG) was added to a final concentration of 0.5 μCi per well and incubated at 37°C for 90 min. Basal and insulin-stimulated 2-DOG uptake were measured as described previously [30].

Statistical analysis

Statistical analyses were performed for data in **Figures 3.1, 3.3B** and **3.4A** by testing the main effects of TNFα (− or +), GPE alone (0, 10, 30 or 60 μg/ml) and their interaction (TNFα×GPE) using two-way ANOVA (SPSS version 16.0 for Windows, SAS Institute, Cary, NC, USA). Statistical analyses were performed for data in **Figure 3.4C** by using one way ANOVA (SPSS version 16.0 for Windows). Tukey's HSD tests were used to compute individual pairwise comparisons of means ($P<.05$). Data are expressed as means±S.E.M.

Results

GPE blocks TNFα-induced inflammatory gene expression

To investigate the protective effects of GPE on TNFα-induced inflammation in human adipocytes, we pretreated primary cultures of newly differentiated human adipocytes with varying doses of GPE for 1 h followed by 3 h of TNFα treatment to

induce inflammatory gene expression. GPE decreased TNFα-induced expression of IL-6, IL-1β, IL-8, MCP-1, COX-2 and TLR-2 in a dose-dependent manner (**Figure 3.1**). No visible signs of GPE cytotoxicity were noted (e.g., no floating cells, no abnormal changes in cell morphology of the monolayer). These data demonstrate that GPE blocks TNFα-induced inflammatory gene expression in primary cultures of newly differentiated human adipocytes.

GPE prevents TNFα-mediated ERK and JNK activation

Because the phosphorylation of MAPK plays an important role in activating transcription factors that induce inflammatory gene expression, we investigated the impact of GPE on MAPK phosphorylation. We found that 1-h pretreatment of cultures of human adipocytes with GPE blocked basal and TNFα-mediated phosphorylation of ERK and JNK in a dose-dependent manner (**Figure 3.2**). These data demonstrate that GPE prevents TNFα activation of ERK and JNK in primary cultures of newly differentiated human adipocytes.

GPE decreases TNFα-mediated c-Jun and NF-κB activation

Given the important role that AP-1 and NF-κB play in the transcriptional activation of inflammatory genes, we examined the preventive effects of GPE on AP-1 and NF-κB activation. Pretreatment of the cultures with GPE for 1 h decreased TNFα-mediated phosphorylation of c-Jun, a component of AP-1 and a downstream target of JNK (**Figure 3.3A**). Similarly, GPE blocked basal and TNFα-induced IκBα degradation

(**Figure 3.3A**). Consistent with these data, GPE attenuated TNFα-stimulated NF-κB reporter activity in a dose-dependent manner (**Figure 3.3B**). Taken together, these data demonstrate that GPE decreases TNFα-mediated c-Jun phosphorylation and NF-κB activity in primary cultures of newly differentiated human adipocytes.

GPE prevents TNFα-mediated insulin resistance

Increased activation of MAPK, AP-1 and NF-κB, and inflammatory gene expression cause insulin resistance. This causal relationship has been linked to increased TNFα levels in plasma and WAT of obese individuals [1]. Thus, we examined the ability of GPE to prevent TNFα-mediated insulin resistance in adipocytes, using increased PTP-1B expression and phosphorylation serine residue 307 of IRS-1 (p-Ser307-IRS-1), which are negative regulators of insulin sensitivity, and decreased insulin-stimulated 2-DOG uptake as indicators of insulin resistance. GPE decreased TNFα-mediated PTP-1B expression (**Figure 3.4A**) and p-Ser307-IRS-1 (**Figure 3.4B**), and increased insulin-stimulated 2-DOG uptake (**Figure 3.4C**) in a dose-dependent manner. Collectively, these data demonstrate that GPE prevents TNFα-mediated insulin resistance in primary cultures of newly differentiated human adipocytes, possibly by inhibiting upstream mediators of inflammation and their downstream negative regulators of insulin sensitivity.

Discussion

In this study, we demonstrated the protective effects of GPE on TNFα activation of upstream proteins and induction of inflammatory genes and negative regulators of

insulin sensitivity in primary cultures of newly differentiated human adipocytes. We demonstrated that (1) GPE attenuated TNFα-induced expression of IL-6, IL-1β, IL-8, MCP-1, COX-2 and TLR-2 (**Figure 3.1**); (2) GPE attenuated TNFα activation of ERK and JNK (**Figure 3.2**), MAPK linked to the activation of NF-κB and c-Jun, a component of AP-1; (3) GPE prevented TNFα activation of c-Jun and attenuated TNFα-mediated IκBα degradation and NF-κB activity (**Figure 3.3**); and (4) GPE prevented TNFα-induced expression of PTP-1B and phosphorylation of serine residue 307 of IRS-1, which are negative regulators of insulin sensitivity, and suppression of insulin-stimulated glucose uptake (**Figure 3.4**). Taken together, these findings are the first to demonstrate that GPE inhibits TNFα-mediated activation of ERK and JNK and transcription factors AP-1 and NF-κB that induce inflammatory genes known to cause insulin resistance in human adipocytes.

Based on these data, we propose the following scenario by which GPE reduces TNFα-mediated inflammation and insulin resistance **(Figure 3.5**). We speculate that GPE initially attenuates TNFα-mediated activation of MAPK, AP-1 and NF-κB at 15 min. This GPE-mediated decrease in MAPK, AP-1 and NF-κB activation, in turn, attenuates TNFα-induced expression of inflammatory genes (i.e., IL-6, IL-1β, IL-8, MCP-1, COX-2 and TLR-2) at 3 h. This results in decreased autocrine/paracrine activation of the inflammatory cascade, including attenuation of negative regulators of insulin sensitivity (i.e., PTP-1B and p-Ser307-IRS-1).

TNFα was identified as one of the first links between obesity-associated inflammation and insulin resistance [8]. Elevated production of TNFα by WAT was

reported in obese humans and is positively correlated with impaired glucose disposal [1]. However, mechanism(s) by which TNFα signals to its downstream targets in human adipocytes is (are) unclear. The activation of MAPK, AP-1 and NF-κB signaling by TNFα has been reported to cause inflammation and insulin resistance in several cell lines [2,16,18]. Consistent with these studies, we found that TNFα activates ERK, JNK, c-Jun and NF-κB, which together increase inflammatory gene expression and induce insulin resistance in primary cultures of newly differentiated human adipocytes.

Several studies have examined the anti-inflammatory properties of grape byproducts rich in phytochemicals such as wine, grape juice and grape seeds [31]. For example, Sakurai et al. [32] demonstrated that oligomerized grape seed polyphenols attenuated inflammation in cocultures of adipocytes and macrophages via their anti-oxidative properties. Lee et al. [33] demonstrated that red wine extract (RWE) inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced transformation of JB6 promotion-sensitive mouse skin epidermal (JB6 P+) cells by blocking the activation of Raf/MEK/ERK/p90RSK signaling pathway and subsequently suppressing the activation of AP- 1 and NF-κB transcription factors. Similarly, our data showed that GPE attenuated TNFα-mediated inflammation and insulin resistance, in part, by suppressing the activation of ERK, JNK, c-Jun and NF-κB.

We also demonstrate that GPE effectively prevents TNFα-mediated insulin resistance in human adipocytes. Notably, GPE attenuated TNFα-induced expression of PTP-1B and phosphorylation of serine residue 307 of IRS-1, which are negative regulators of insulin sensitivity. Phosphorylation of serine residues of IRS-1 is one of the

most common mechanisms by which MAPK and IκB kinase-β, a central coordinator of inflammatory responses through activation of NF-κB, decrease insulin-stimulated glucose uptake [34]. The activation of MAPK by TNFα increased the phosphorylation of serine residues on IRS-1 [18], which impairs the ability of the insulin receptor to phosphorylate tyrosine residues of IRS-1, thereby reducing insulin action [17]. PTP-1B, an NF-κB target gene [19,20], negatively regulates insulin sensitivity by dephosphorylating tyrosine residues of IRS-1 [21]. Thus, TNFα-induced PTP-1B gene expression [35] contributes to insulin resistance. Here, we demonstrate that GPE prevents TNFα-mediated insulin resistance in primary cultures of newly differentiated human adipocytes, possibly by inhibiting the activation of upstream mediators of inflammation such as ERK, JNK, c-Jun or NF-κB and their downstream negative regulators of insulin sensitivity such as PTP-1B expression and phosphorylation of serine residue 307 of IRS-1.

Lee et al. [33] found that the anticarcinogenic effects of RWE may be due to the higher content of the flavonol quercetin, as compared to the phytoalexin resveratrol. It has been reported that quercetin has anti-inflammatory properties. For example, quercetin supplementation of high fat-fed mice [27] or obese Zucker rats [28] reduced circulating markers of inflammation. In vitro, quercetin attenuated differentiation and markers of inflammation in murine 3T3-L1 adipocytes [36] and suppressed TPA-mediated activation of MEK/ERK, AP-1 and NF-κB in murine skin epidermal (JB6 P+) cells [33]. Notably, we found using reverse-phase HPLC that one of the major components in GPE was quercetin glucosides (9.2%) (unpublished data). However, quercetin glucosides may not exist in circulation, because β-glucosidase hydrolysis of quercetin glucosides occurs

when GPE polyphenols are absorbed across the intestine and pass through the liver [37,38]. Also, the concentrations of quercetin found in circulation vary markedly, ranging from 0.1 to 10 μM [39,40]. Therefore, the doses of 10, 30 and 60 μg/ml GPE will contain ~3, 9 and 18 μM quercetin (molecular weight, 302.2), respectively. Thus, the 10 and 30 μg/ml GPE may provide physiological levels of quercetin for those consuming a diet rich in grape products.

The other components we found in GPE were catechins (4.2%), gallic acid (1.4%) and resveratrol (0.53%) (unpublished data). Resveratrol is a potential anti-inflammatory polyphenol in GPE. Several animal studies reported that resveratrol prevents insulin resistance and adiposity in mice fed a high-fat diet [26,41,42]. These studies suggest that resveratrol shifts excess calories away from storage in WAT and towards oxidation in muscle and brown adipose tissue. In vitro, resveratrol has been shown to reduce inflammation in murine 3T3-L1 adipocytes [43], enhance glucose transport in muscle [44], reduce oxidative stress in human lung epithelial cells [45,46], reduce ER stress in mouse macrophages [47] and decrease TNFα-mediated NF-κB activation in hepatocytes [48] and coronary arterial endothelial cells [49]. Studies are underway in our laboratory to determine the extent to which quercetin, resveratrol and other polyphenol candidates in GPE prevent inflammation and insulin resistance in primary cultures of human adipocytes.

Collectively, these data demonstrate that GPE attenuates TNFα-mediated inflammation and insulin resistance by suppressing the activation of ERK, JNK, c-Jun, and NF-κB that induce the expression of inflammatory genes and negative regulators of

insulin sensitivity in primary cultures of newly differentiated human adipocytes. In vivo studies are needed to determine the ability of GPE to recapitulate these in vitro findings in WAT, because of the extensive metabolism of polyphenols in vivo [50].

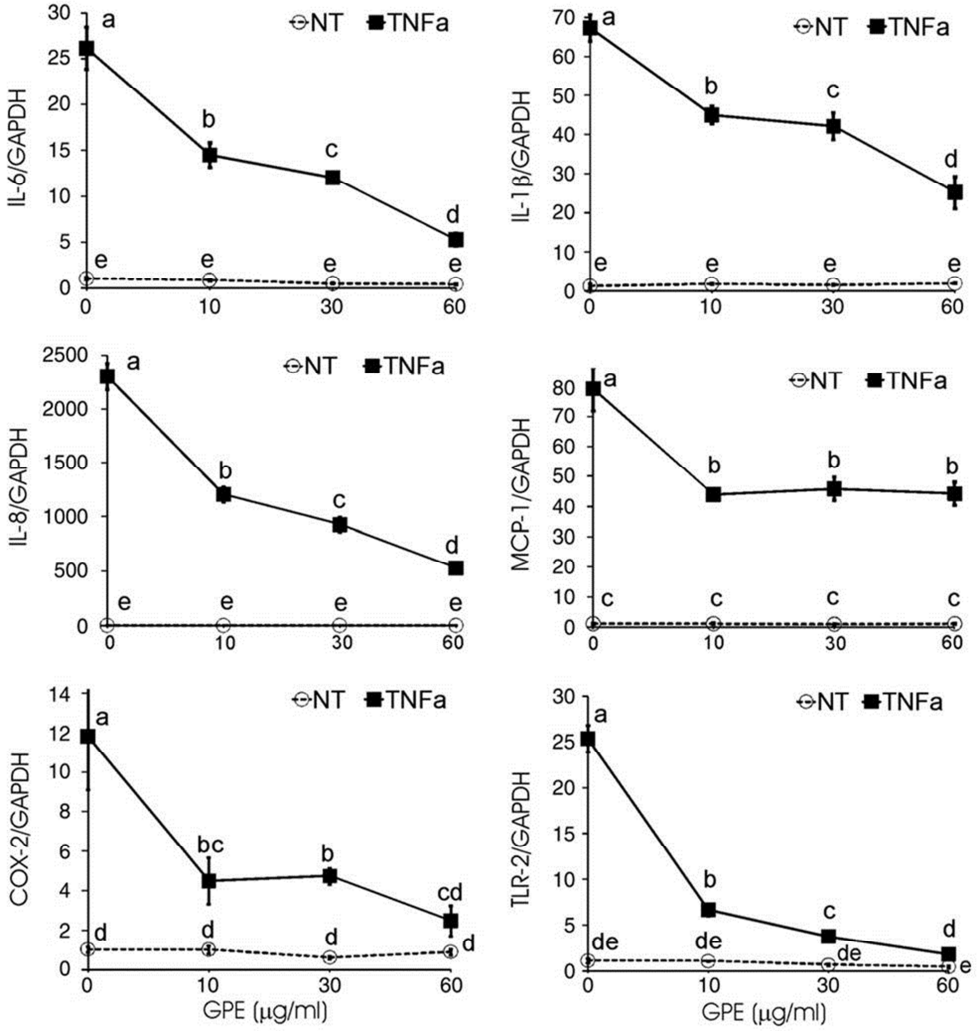

Figure 3.1. GPE blocks TNFα-induced inflammatory gene expression. Cultures of newly differentiated human adipocytes were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 0.5 ng/ml TNFα for 3 h. Subsequently, cultures were harvested for the determination of mRNA levels of IL-6, IL-1β, IL-8, MCP-1, COX-2 and TLR-2 by real-time qPCR. Data are representative of three independent experiments. Values are means±S.E.M., n=3. Means without a common letter differ, P<.05.

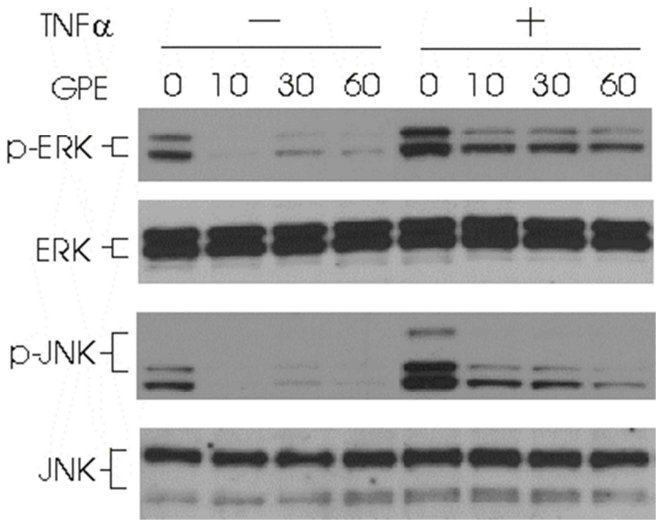

Figure 3.2. GPE prevents TNFα activation of ERK and JNK. Cultures of newly differentiated human adipocytes were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 0.5 ng/ml TNFα for 15 min. Subsequently, cultures were harvested for the determination of the protein levels of p-ERK, ERK, p-JNK and JNK by immunoblotting. Data are representative of three independent experiments.

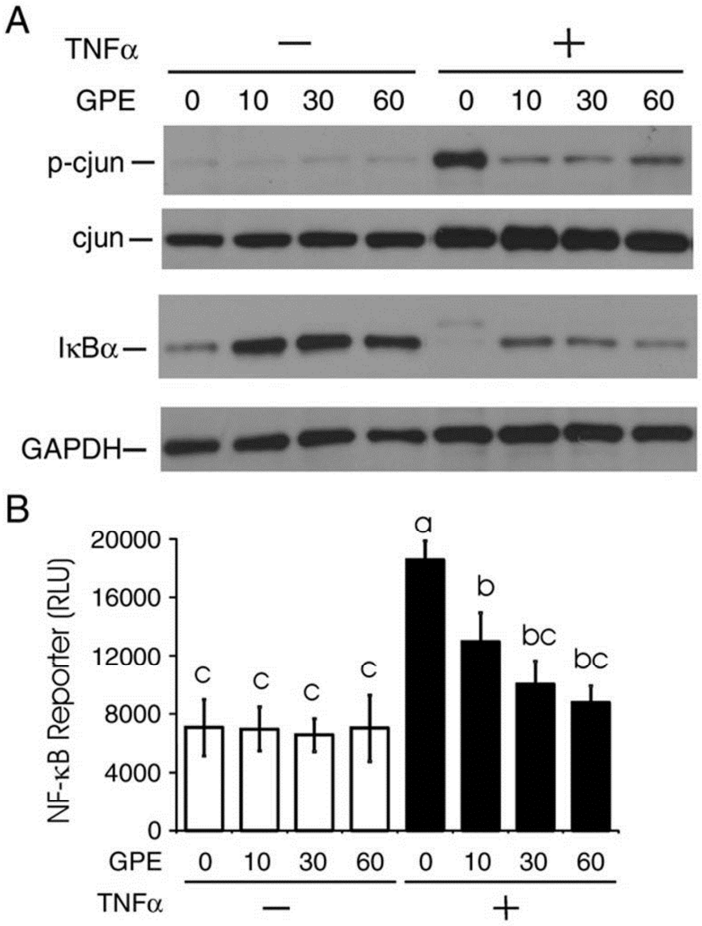

Figure 3.3. GPE decreases TNFα-mediated c-Jun activation, IκBα degradation and NF-κB transcriptional activity. (A) Cultures of newly differentiated human adipocytes were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 0.5 ng/ml TNFα for 15 min. Subsequently, cultures were harvested for the determination of the protein levels of p-c-Jun, c-Jun, IκBα and GAPDH by immunoblotting. **(B)** Cultures were transfected on Day 6 with pNF-κB luc and pRL-CMV. Twenty four hours later, transfected cells were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 100 ng/ml TNFα for 24 h. **(A)** Data are representative of three independent experiments. **(B)** Values are means±S.E.M., n=6. Means without a common letter differ, P<.05.

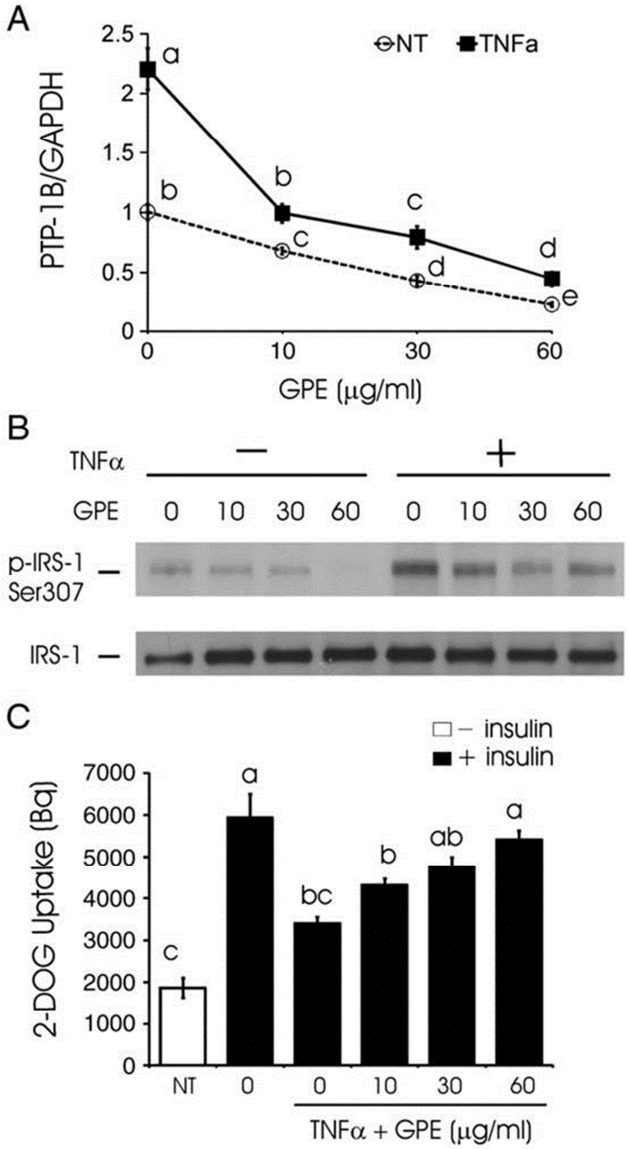

Figure 3.4. GPE prevents TNFα-mediated insulin resistance. (A) Cultures of newly differentiated human adipocytes were pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 0.5 ng/ml TNFα for 3 h to determine the mRNA level of PTP-1B by real-time qPCR, or **(B)** for 15 min to determine the protein level of p-Ser307-IRS-1 by immunoblotting. **(C)** Cultures were incubated with serum free low glucose media for 24 h and then pretreated with DMSO vehicle (0) or with 10, 30 or 60 μg/ml GPE for 1 h and then treated with 5 ng/ml TNFα for 24 h. Insulin-stimulated 2-DOG uptake was measured after a 100-min incubation with 100 nmol/L insulin. **(A,B)** Data are representative of two independent experiments. **(A)** Values are means±S.E.M., n=3. **(C)** Data are representative of two independent experiments. Values are means±S.E.M., n=4. Means without a common letter differ, P<.05.

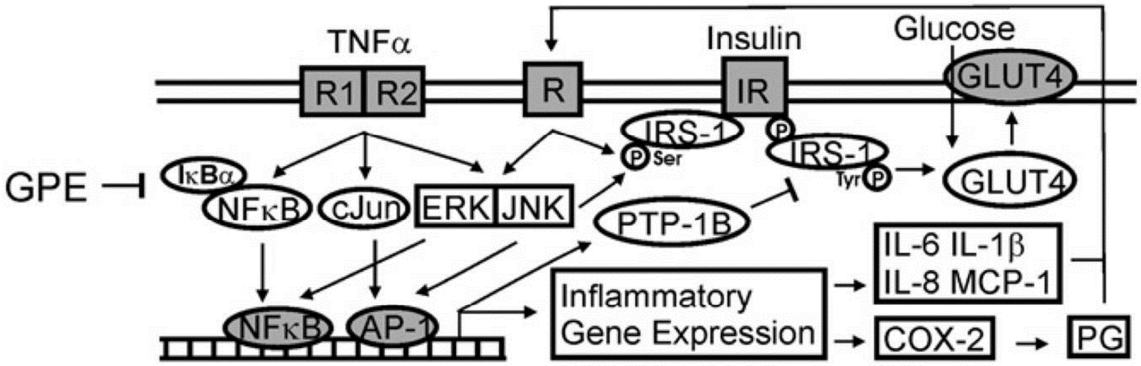

Figure 3.5. Working model on how GPE prevents TNFα-mediated inflammation and insulin resistance. We propose that GPE initially blocks the activation of MAPK, AP-1 and NF-κB. This prevents them from inducing inflammatory gene expression, thereby blocking autocrine/paracrine signals associated with propagating the inflammatory cascade. By preventing this inflammatory cascade, GPE decreases TNFα-mediated IRS-1-serine phosphorylation and increases IRS-1-tyrosine phosphorylation. Increased IRS-1 tyrosine phosphorylation increases GLUT4 translocation to the plasma membrane, thereby facilitating glucose uptake.

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CHAPTER IV

QUERCETIN IS EQUALLY OR MORE EFFECTIVE THAN RESVERATROL IN ATTENUATING TUMOR NECROSIS FACTOR-α–MEDIATED INFLAMMATION AND INSULIN RESISTANCE IN PRIMARY HUMAN ADIPOCYTES

Abstract

Background: Quercetin and *trans*-resveratrol (*trans*-RSV) are plant polyphenols reported to reduce inflammation or insulin resistance associated with obesity. Recently, we showed that grape powder extract, which contains quercetin and *trans*-RSV, attenuates markers of inflammation in human adipocytes and macrophages and insulin resistance in human adipocytes. However, we do not know how quercetin and *trans*-RSV individually affected these outcomes.

Objective: The aim of this study was to examine the extent to which quercetin and *trans*-RSV prevented inflammation or insulin resistance in primary cultures of human adipocytes treated with tumor necrosis factor-α (TNF-α), an inflammatory cytokine elevated in the plasma and adipose tissue of obese, diabetic individuals.

Design: Cultures of human adipocytes were pretreated with quercetin and *trans*-RSV followed by treatment with TNF-α. Subsequently, gene and protein markers of inflammation and insulin resistance were measured.

Results: Quercetin, and to a lesser extent *trans*-RSV, attenuated the TNF-α–induced expression of inflammatory genes such as interleukin (IL)-6, IL-1β, IL-8, and monocyte chemoattractant protein-1 (MCP-1) and the secretion of IL-6, IL-8, and MCP-1.

Quercetin attenuated TNF-α–mediated phosphorylation of extracellular signal-related kinase and c-Jun-NH2 terminal kinase, whereas *trans*-RSV attenuated only c-Jun-NH2 terminal kinase phosphorylation. Quercetin and *trans*-RSV attenuated TNF-α–mediated phosphorylation of c-Jun and degradation of inhibitory κB protein. Quercetin, but not *trans*-RSV, decreased TNF-α–induced nuclear factor-κB transcriptional activity. Quercetin and *trans*-RSV attenuated the TNF-α–mediated suppression of peroxisome proliferator-activated receptor γ (PPARγ) and PPARγ target genes and of PPARγ protein concentrations and transcriptional activity. Quercetin prevented the TNF-α–mediated serine phosphorylation of insulin receptor substrate-1 and protein tyrosine phosphatase-1B gene expression and the suppression of insulin-stimulated glucose uptake, whereas *trans*-RSV prevented only the TNF-α–mediated serine phosphorylation of insulin receptor substrate-1.

Conclusion: These data suggest that quercetin is equally or more effective than *trans*-RSV in attenuating TNF-α–mediated inflammation and insulin resistance in primary human adipocytes.

Introduction

Obesity or excess white adipose tissue (WAT) is associated with increased production of proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α), which contribute to chronic, low-grade inflammation (1, 2). Hotamisligil et al (3) first reported that the overexpression of TNF-α was intimately involved in the development of inflammation-associated insulin resistance in the WAT of

obese mice. To date, numerous articles support this seminal finding and extend our understanding of how obesity increases TNF-α production in WAT, which causes local and systemic inflammation and insulin resistance (4-6).

TNF-α causes inflammation by triggering a serine/threonine kinase phosphorylation cascade that activates mitogen-activated protein kinases (MAPK) such as extracellular signal-related kinase (ERK) and c-Jun-NH2 terminal kinase (JNK) and transcription factors such as nuclear factor-κB (NF-κB) and activator protein (AP)-1 (7, 8). Together, these proteins indirectly cause insulin resistance by inducing the transcription of inflammatory genes and antagonizing peroxisome proliferator-activated receptor γ (PPARγ) activity (6) or directly impair insulin signaling via decreasing insulin receptor substrate (IRS)-1/2 signaling (9). In addition, TNF-α–mediated activation of NF-κB enhances the expression of protein tyrosine phosphatase (PTP)-1B, a negative regulator of insulin signaling that dephosphorylates tyrosine residues on IRS-1 (10-12). Thus, TNF-α can cause insulin resistance by increasing the serine phosphorylation of IRS-1/2 via JNK and decreasing the tyrosine phosphorylation of IRS-1/2 via PTP-1B.

Quercetin is a major flavonol abundantly found in plant products such as capers, lovage, apples, onions, and grapes that possesses antioxidative and antiinflammatory properties (13, 14). Recently, we showed that grape powder extract (GPE), which is rich in quercetin and to a lesser extent in *trans*-resveratrol (*trans*-RSV), attenuates markers of inflammation in human adipocytes (15) and macrophages (16) and insulin resistance in human adipocytes (15). Consistent with these in vitro data, quercetin reduced inflammation or insulin resistance in rodent models of obesity (17, 18). *Trans*-RSV is

another polyphenol (ie, a phytoalexin) found in GPE (16) that has antioxidative and antiinflammatory properties (19). In vitro, *trans*-RSV was recently reported to reduce inflammation via its suppression of ERK and NF-κB activity in a human mast cell line (20). In vivo, *trans*-RSV has been reported to reduce hyperglycemia or hyperlipidemia in obese mice fed a high-fat diet (21, 22).

However, the protective effects of quercetin and *trans*-RSV against inflammation and insulin resistance and their mechanism of action in primary human adipocytes are unclear. Furthermore, we do not know which polyphenols in our GPE attenuated inflammation or insulin resistance (15, 16). Therefore, we investigated the extent to which quercetin and *trans*-RSV attenuated TNF-α–mediated inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes.

Materials and Methods

Materials

All cell culture ware was purchased from Fisher Scientific (Norcross, GA). Adipocyte medium (AM-1) was purchased from Zen Bio Inc (Research Triangle Park, NC). Tri-Reagent was purchased from Molecular Research Center (Cincinnati, OH). Gene-specific primers were purchased from Applied Biosystems (Foster City, CA). Goat polyclonal antibody for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mouse monoclonal antibody for anti-PPARγ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho (Thr202/Tyr204) and -total ERK, anti-phospho (Thr183/Tyr185) and -total JNK, anti-phospho (Ser63) and -total c-Jun, anti-

phospho (Ser307) and -total IRS-1, and anti-inhibitory κB protein (IκBα) rabbit polyclonal antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Immunoblotting buffers and precast gels were purchased from Invitrogen (Carlsbad, CA). Western Lightning Plus Chemiluminescence Substrate and 2-[3H]deoxyglucose (2-DOG; 25–50 Ci/mmol, no. NET54900) were purchased from Perkin-Elmer Life Science (Boston, MA). *Trans*-RSV (no. 70675, ≥ 98% purity) was purchased from Caymen Chemicals (Ann Arbor, MI). Quercetin (no.Q0125-dihydrate, ≥ 98% purity) and all other reagents or chemicals were purchased from Sigma Chemical Co (St Louis, MO) unless otherwise stated.

Primary cultures of newly differentiated human adipocytes

Abdominal WAT was obtained from nondiabetic, white, and African American women [age: 20-50 y; body mass index (in kg/m^2), <32.0] during abdominoplasty. Approval was obtained from the Institutional Review Board at University of North Carolina-Greensboro and the Moses Cone Memorial Hospital in Greensboro, NC. Tissue was digested by using collagenase, and stromal vascular (SV) cells were isolated, proliferated, and induced to differentiate in adipocyte media (AM-1) plus 250 μmol isobutylmethylxanthine/L and 1 μmol thiazolidinedione rosiglitazone/L (BRL 49653; a gift from Per Sauerberg, Novo Nordisk A/S, Copenhagen, Denmark) for 3 d. Cultures were then grown in AM-1 only for 3-9 d. Cultures containing ~50% preadipocytes and ~50% adipocytes, based on visual observations, were treated between day 6 and 12 of

differentiation. Each experiment was repeated at least twice at different times by using a mixture of cells from 3 subjects unless otherwise indicated.

Analysis of quercetin and RSV accumulation in primary cultures of human adipocytes

Primary human SV cells were seeded in 100-mm dishes at 3.2 x 10^6 per dish and differentiated for 6 d. On day 6, media were changed. Twenty-four hours later, cultures were treated with 30 μmol quercetin/L or *trans*-RSV/L. At 0, 1, 3, 8, and 24 h of incubation, cultures were washed with Hank's Buffered Salt Solution (HBSS) twice and treated on ice with 150 μL lysis buffer (Cell Signaling Technologies) for 20 min and subsequently harvested. The protein content in cell lysates was measured by using bicinchoninic acid protein assay purchased from Pierce Biotechnology (Rockford, IL). Then, lysates were incubated with 100 μL of 0.1 mol ascorbic acid/L, 50 μL of 0.78 mol sodium acetate buffer/L (pH 4.8), and 50 μL β-glucuronidase (~100,000 units β-glucuronidase/L, ≤1000 units sulfatase/mL) from Helix pomatia at 37 ºC for 8 h. The mixture was extracted twice with 0.5 mL ethyl acetate, and the pooled supernatant fluid was concentrated to dryness by using a Thermo Electron Savant SC110 SpeedVac system (Waltham, MA). The sample was reconstituted in 150 mL methanol:water (50:50, vol:vol), filtered through a 0.45-μm membrane purchased from Millipore (Bedford, MA) and then subjected to liquid chromatography interfaced with the time of flight mass spectroscopy (LC-TOFMS) analysis as described (23).

RNA isolation and real-time quantitative polymerase chain reaction

Primary human SV cells were seeded in 35-mm dishes at 0.4 x 10^6 per dish and differentiated for 6 d. On day 6, the media were changed. Twenty-four hours later, cultures were pretreated with dimethyl sulfoxide (DMSO) vehicle (0) or 10, 30, or 60 μmol quercetin/L or *trans*-RSV/L for 1 h and subsequently treated with or without 0.5 ng TNF-α/mL for 3 h. This 3-h treatment with TNF-α was chosen on the basis of the results of a pilot dose and time course study showing significant induction of inflammatory genes at 3 h (data not shown). After treatment, conditioned culture media were harvested to determine inflammatory adipokine secretion as described in the next section. Next, cultures were harvested and total RNA was isolated by using Tri-Reagent according to the manufacturer's protocol. For real-time quantitative polymerase chain reaction (qPCR), 2.0 μg total RNA was converted into first-strand cDNA by using a high capacity cDNA Archive Kit (Applied Biosystems). qPCR was performed in an Applied Biosystems 7500 FAST Real Time PCR System by using Taqman Gene Expression Assays. To account for possible variations related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample, and the data were normalized accordingly.

Analysis of inflammatory adipokine secretion

The conditioned culture media were collected as described in the previous section (RNA isolation and real-time quantitative polymerase chain reaction) from cells pretreated with DMSO vehicle (0) or 10, 30, or 60 μmol quercetin/L or *trans*-RSV/L for

1 h and subsequently treated with or without 0.5 ng TNF-α/mL for 3 h. The concentrations of IL-6, IL-8, IL-1β, and monocyte chemoattractant protein (MCP)-1 were determined in the conditioned culture media by using the BioPlex Suspension Array System from Bio-Rad (Hercules, CA) following the manufacturer's protocol as described (16).

Immunoblotting

Primary human SV cells were seeded in 35-mm dishes at 0.4 x 10^6 per dish and differentiated for 6 d. On day 6, the media were changed. Twenty-four hours later, cultures were pretreated with DMSO vehicle (0) or 10, 30, or 60 μmol quercetin/L or *trans*-RSV/L for 1 h and then treated with or without 0.5 ng TNF-α/mL for 15 min. This 15-min treatment with TNF-α was chosen based on the results of a pilot dose and time course study showing significant activation of MAPK, c-Jun, and NF-κB at 15 min (data not shown). Immunoblotting was conducted as previously described (24).

Transient transfections

For measuring NF-κB activity, primary cultures of human adipocytes were transiently transfected with the NF-κB responsive luciferase reporter construct, pNF-κB-luc, purchased from Stratagene (La Jolla, CA), by using the Amaxa nucleofector system purchased from Lonza (Walkersville, MD) as previously described (15). Briefly, on day 6 of differentiation, ~1.2 x 10^6 adipocyte cultures from a 60-mm plate were trypsinized and resuspended in 100 μL Amaxa nucleofector solution (ie, cell line nucleofector kit V) and

mixed with 1 μg pNF-κB-luc and 25 ng plasmid renilla luciferase-cytomegalovirus (pRLCMV) for each sample. Electroporation was performed by using Amaxa nucleofector device (V-33 nucleofector program). Cells were replanted in a 96-well plate after a 10-min recovery in calcium-free Dulbecco's modified Eagle's medium (DMEM). After 24 h, transfected cells were pretreated with DMSO vehicle (0) or 3, 10, or 30 μmol quercetin/L or *trans*-RSV/L for 1 h and then treated with or without 100 ng TNF-α/mL for 24 h. This 24-h treatment with TNF-α was chosen based on the results of a pilot dose and time course study showing significant activation of pNF-κB-luc at 24 h (data not shown). For the measurement of PPARγ activity, primary cultures of human adipocytes were transiently transfected with the multimerized PPAR response element (PPRE) luciferase reporter construct, PPRE-X3-TK-luc (a gift from Susanne Mandrup, University of Southern Denmark), by using the Amaxa nucleofector system from Lonza. On day 12 of differentiation, ~1.2 x 10^6 adipocyte cultures from a 60-mm plate were trypsinized and resuspended in 100 μL Amaxa nucleofector solution (ie, cell line nucleofector kit V) and mixed with 2 μg PPRE-X3-TK-luc and 25 ng pRL-CMV for each sample. Electroporation was performed by using the Amaxa nucleofector device (V-33 nucleofector program). After a 10-min recovery in calcium-free DMEM, transfected cells were treated with DMSO vehicle (0) or 10 ng TNF-α/mL with or without 3, 10, or 30 μmol quercetin/L or *trans*-RSV/L and replated in a 96-well plate. After 24 h of the treatment, 0.1 μmol BRL 49653/L, a PPARγ ligand, was added to activate the PPRE luciferase reporter for 24 h. This dose and duration of TNF-α or BRL 49653 treatments were selected based on the results of a pilot dose and time course study showing

significant suppression of PPRE-X3-TK-luc by TNF-α and significant activation of PPRE-X3-TK-luc by BRL 49653 at 24 h (data not shown). Firefly luciferase activity was measured by using the Dual-Glo luciferase kit purchased from Promega (Madison, WI) and normalized to renilla luciferase activity from the co-transfected control pRL-CMV vector. All luciferase data were presented in relative light units as the ratio of firefly luciferase to renilla luciferase activity.

Insulin-stimulated 2-DOG uptake

Primary human SV cells were seeded in 12-well plates at 1.6 x 10^5 per well and differentiated for 12 d. On day 12, media were changed to serum free low glucose (5 mmol/L) DMEM. Twenty four hours later, cultures were pretreated with DMSO vehicle (0) or 3, 10, or 30 μmol quercetin/L or *trans*-RSV/L for 1 h and then treated with or without 5 ng TNF-α/mL for 24 h. This 24-h treatment with TNF-α was chosen based on the results of a pilot dose and time course study showing significant suppression of 2-DOG at 24 h (data not shown). Culture media were removed and replaced with 0.5 mL HBSS with or without 100 nmol human insulin/L for 10 min. After insulin preincubation, 4 nmol 2-DOG/L (0.5 μCi per well) was added to each well and incubated at 37 ºC for 90 min. Basal and insulin-stimulated 2-DOG uptakes were measured as described previously (24).

Statistical analysis

Statistical analyses were performed by using one-factor analysis of variance (ANOVA) (JMP version 6.03; SAS Institute, Cary, NC) or by testing the main effects of TNF-α alone, quercetin alone, or *trans*-RSV alone and their interaction by using 2-factor ANOVA (JMP version 6.03). Tukey's honestly significant difference tests were used to compute individual pairwise comparisons of means ($P < 0.05$). Data are expressed as means ± SEMs.

Results

Quercetin or *trans*-RSV is taken up by primary human adipocytes

The polyphenol content of cultures treated with 30 μmol quercetin/L or *trans*-RSV/L for 0, 1, 3, 8, and 24 h is shown in **Figure 4.1**. Intracellular quercetin and *trans*-RSV contents were highest after 1 h of incubation with either polyphenol and then declined thereafter. Thus, it appears that quercetin and *trans*-RSV are rapidly taken up by adipocytes. However, it is not possible to determine the relative rates of absorption of quercetin or *trans*-RSV from these data, because the quercetin or *trans*-RSV could be rapidly interacting with cellular targets, thereby being transformed into other types of quercetin or *trans*-RSV metabolites or species that we did not have standards for measuring. Indeed, we detected some unknown peaks by LCTOFMS after treatment of quercetin or *trans*-RSV in primary human adipocytes.

Quercetin, and to a lesser extent *trans*-RSV, attenuate TNF-α–mediated inflammatory gene expression and protein secretion

To investigate the protective effects of quercetin and *trans*-RSV on TNF-α–induced inflammation in human adipocytes, cultures were treated with 10, 30, or 60 μmol quercetin/L or *trans*-RSV/L for 1 h and then treated with 0.5 ng TNF-α/mL for 3 h to induce inflammatory gene expression and protein secretion. Quercetin decreased TNF-α–induced IL-6, IL-1β, IL-8, and MCP-1 gene expression (**Figure 4.2A**) and IL-6, IL-8, and MCP-1 secretion (**Figure 4.2B**) in a dose-dependent manner. IL-1β protein in the media was undetectable in all of the treatments. *Trans*-RSV also reduced inflammatory gene expression (**Figure 4.2C**), but to a relatively lesser extent than quercetin for IL-6, IL-8, and IL-1β. Furthermore, only the lowest dose of *trans*-RSV decreased TNF-α–mediated IL-6 and IL-8 protein secretion, and only higher doses of *trans*-RSV decreased MCP-1 protein secretion (**Figure 4.2D**). No visible signs of quercetin or *trans*-RSV cytotoxicity were noted (eg, no floating cells, no visual differences in the number of attached cells or protein concentrations, and no abnormal changes in cell morphology).

Quercetin decreases TNF-α–mediated ERK and JNK activation to a greater extent than *trans*-RSV

We investigated the effect of quercetin and *trans*-RSV on ERK and JNK phosphorylation, because the phosphorylation of MAPK plays a role in activating transcription factors that induce inflammatory gene expression. Pretreatment of the cultures with 10, 30, or 60 μmol/L quercetin decreased TNF-α–mediated phosphorylation

of ERK and JNK (**Figure 4.3A**). In contrast, only the 60 μmol/L concentration of *trans*-RSV decreased TNF-α–mediated phosphorylation of JNK, and *trans*-RSV had no effect on ERK phosphorylation (**Figure 4.3B**).

Quercetin and *trans*-RSV attenuate TNF-α–mediated c-Jun phosphorylation and IκBα degradation

Next, we examined the ability of quercetin and *trans*-RSV to prevent the activation of c-Jun, a component of AP-1, and NF-κB, because of their ability to induce the transcription of inflammatory genes. Pretreatment with quercetin (**Figure 4.4A**) and *trans*-RSV (**Figure 4.4B**) suppressed the TNF-α–mediated phosphorylation of c-Jun and degradation of IκBα. Notably, quercetin (**Figure 4.4C**), but not *trans*-RSV (**Figure 4.4D**), decreased basal and TNF-α–stimulated NF-κB reporter activity.

Quercetin and *trans*-RSV attenuate TNF-α–mediated suppression of mRNA concentrations of PPARγ target genes and PPARγ mRNA, protein, and activity levels

Given the important role that PPARγ plays in regulating inflammation and insulin resistance, we examined the effect of quercetin and *trans*-RSV on the mRNA concentrations of several PPARγ target genes and on PPARγ mRNA, protein, and activity levels in TNF-α–treated cultures. Pretreatment with quercetin attenuated TNF-α–mediated suppression of mRNA concentrations of PPARγ and PPARγ target gene, adipocyte fatty acid-binding protein (aP2), but not adiponectin (**Figure 4.5A**). Consistent

with these data, quercetin attenuated the TNF-α–mediated suppression of PPARγ protein concentration (**Figure 4.5B**). Pretreatment with *trans*-RSV attenuated TNF-α–mediated suppression of mRNA concentrations of PPARγ, aP2, and adiponectin (**Figure 4.5C**) and the protein concentration of PPARγ (**Figure 4.5D**) in a dose-dependent manner. Finally, quercetin, and to a greater extent *trans*-RSV, blocked TNF-α–mediated suppression of the activity of a PPRE reporter construct (**Figure 4.5E**).

Quercetin prevents TNF-α–mediated insulin resistance

Last, we examined the ability of quercetin and *trans*-RSV to prevent insulin resistance in TNF-α–treated cultures. Insulin resistance was determined by measuring the phosphorylation of serine residue 307 on IRS-1 (Ser307-IRS-1), the induction of PTP-1B gene expression, and reductions in insulin-stimulated 2-DOG uptake. Pretreatment of quercetin decreased TNF-α–mediated phosphorylation of Ser307-IRS-1 (**Figure 4.6A**) and PTP-1B gene expression (**Figure 4.6B**), and increased insulin-stimulated 2-DOG uptake (**Figure 4.6C**). Although *trans*-RSV decreased TNF-α–mediated phosphorylation of Ser307-IRS-1 (**Figure 4.6D**), it had no effect on PTP-1B expression (**Figure 4.6E**) or insulin-stimulated 2-DOG uptake (**Figure 4.6F**). Collectively, these data demonstrate that quercetin prevents TNF-α–mediated insulin resistance in primary cultures of newly differentiated human adipocytes, possibly by inhibiting upstream mediators of inflammation and their downstream negative regulators of insulin signaling.

Discussion

In this study, we reported the protective effects of quercetin, and to a lesser extent *trans*-RSV, on TNF-α–mediated activation of upstream inflammatory cascades that induce inflammatory adipokines and negative regulators of insulin signaling and antagonize PPARγ in primary cultures of newly differentiated human adipocytes. We showed that 1) quercetin and *trans*-RSV are taken up by primary human adipocytes (**Figure 4.1**); 2) quercetin, and to a lesser extent *trans*-RSV, attenuated TNF-α–induced inflammatory gene expression and protein secretion (**Figure 4.2**); 3) quercetin attenuated TNF-α–mediated activation of inflammatory MAPK (**Figure 4.3**) and transcription factors (**Figure 4.4**) to a greater extent than *trans*-RSV; 4) quercetin and *trans*-RSV attenuated TNF-α–mediated suppression of PPARγ mRNA, protein, and activity levels (**Figure 4.5**); and 5) quercetin prevented TNF-α–mediated insulin resistance (**Figure 4.6**) in primary human adipocytes. On the basis of these data, we proposed a working model in **Figure 4.7** by which quercetin and *trans*-RSV attenuated TNF-α–mediated inflammation and insulin resistance.

To our knowledge, this is the first study to show that quercetin and *trans*-RSV aglycones are taken up by primary human adipocytes (**Figure 4.1**). Consistent with our data, quercetin was detected in human hepatocarcinoma HepG2 cells after treatment of red grape juice (25) and in human intestinal Caco-2 cells after treatment of whole onion and apple peel extracts (26). These studies support speculation that significant amounts of quercetin glucosides may not exist in circulation, because of β-glucosidase hydrolysis of quercetin glucosides that occurs when they are absorbed across the intestine and pass

through the liver (26, 27). Because of these issues, we used quercetin instead of quercetin glucosides to test its ability to prevent inflammation and insulin resistance. In contrast, other researchers have questioned the significance of in vitro studies performed with unconjugated polyphenols, arguing that they may not circulate in vivo due to extensive conjugation in the intestinal wall and the liver (28). However, several in vitro studies showed that quercetin and *trans*-RSV aglycones are absorbed by passive diffusion in Caco-2 cells (29) and HepG2 cells (30), respectively. Here, we provide data showing that quercetin and *trans*-RSV aglycones are taken up by primary human adipocytes.

Several potential mechanisms by which quercetin and *trans*-RSV blocked TNF-α–mediated inflammation or insulin resistance in adipocytes include 1) interfering with TNF-α receptor (TNFR) binding, 2) suppressing TNF-α–TNFR signaling, or 3) altering the activity of proteins involved in inflammation or glucose and lipid metabolism (**Figure 4.7**). TNF-α causes inflammation and insulin resistance by activating key inflammatory pathways, including MAPK, NF-κB, and AP-1. Together, these proteins antagonize the ability of PPARγ to transcriptionally induce target genes such as the insulin-sensitive glucose transporter 4, aP2, lipoprotein lipase, perilipin, and adiponectin, proteins essential for glucose and fatty acid disposal. Proposed mechanisms by which these inflammatory signals reduce PPARγ activity include 1) decreased PPARγ mRNA or protein concentrations; 2) increased phosphorylation of serine residue 112 on PPARγ by ERK, which leads to ubiquination and proteosome degradation; and 3) decreased PPARγ DNA binding and transcriptional activity (31). Consistent with these mechanisms, we found that quercetin or *trans*-RSV attenuated TNF-α–mediated activation of MAPK

(**Figure 4.3**), AP-1, and NF-κB (**Figure 4.4**) and suppression of PPARγ (**Figure 4.5**) and insulin signaling (**Figure 4.6**). However, we do not know whether quercetin or *trans*-RSV directly silences these activators of inflammation or indirectly silences them by activating PPARγ, which is known to antagonize NF-κB and AP-1 (32). Indeed, PPARγ agonists such as thiazolidinedione reduce insulin resistance or inflammation associated with obesity. Notably, several studies have reported that polyphenols increase PPARγ expression or activity (33, 34). Thus, quercetin and *trans*-RSV may attenuate inflammation or insulin resistance by directly interfering with TNF-α signaling or indirectly by activating PPARγ.

Another potential mechanism by which quercetin or *trans*-RSV prevents inflammation and insulin resistance is via inhibition of TNF-α–mediated reactive oxygen species (ROS) production (35). ROS increases inflammatory gene expression by activating redox-sensitive proteins such as apoptosis signal-regulating kinase-1/thioredoxin (36) and redox-sensitive transcription factors such as AP-1, NF-κB, and NF-E2-related factor-2 (37). Moreover, increased ROS production by NAPDH oxidase and decreased antioxidative enzymes such as superoxide dismutase and glutathione peroxidase contribute to obesity-associated inflammation and insulin resistance (38). *Trans*-RSV has been shown to attenuate inflammation by inducing gene expression of antioxidative enzymes including heme oxygenase-1, superoxide dismutase, and glutathione peroxidase (37). Recently, Sakurai et al (39) showed that oligomerized grape seed polyphenols decrease inflammation by suppressing ROS production and NF-κB activation. Other polyphenols such as anthocyanins prevented inflammation by inhibiting

TNF-α–stimulated ROS production (40). Future studies are needed to investigate these potential mechanisms.

One notable finding of this study was that quercetin exerted relatively stronger preventive effects than did *trans*-RSV. For example, quercetin showed greater capacity than *trans*-RSV to attenuate TNF-α–mediated inflammatory adipokine expression and secretion, MAPK and NF-κB activation, and insulin resistance. Similarly, Lee et al (41) reported that red wine extract and quercetin, but not *trans*-RSV, prevented TPA-induced carcinogenesis in mouse skin epidermal cells by inhibiting MAPK signaling pathway. Notably, our previous data showed that GPE containing quercetin and *trans*-RSV prevented inflammation and insulin resistance in TNF-α–treated primary human adipocytes (15). In addition, several studies suggested that combinations of quercetin, *trans*-RSV, or other polyphenols have additive effects on antioxidative activity in human erythrocytes (42), antiproliferation in C6 rat glioma cells (43) and in MDA-MB-231 human breast cancer cells (44), and antiadipogenesis in mouse 3T3-L1 adipocytes (45, 46). However, the contribution of combined grape polyphenols such as quercetin and *trans*-RSV on prevention of inflammation and insulin resistance is unknown. Therefore, future studies will investigate 1) additive or synergistic effects of quercetin, *trans*-RSV, or other polyphenols found in our GPE on prevention of TNF-α–mediated inflammation and insulin resistance in vitro and 2) antiinflammatory and antidiabetic effects of GPE polyphenols in diet-induced obese animal models or in overweight or obese humans.

In contrast with our findings, 2 studies reported that quercetin decreased insulin-stimulated glucose uptake (47, 48). Nomura et al (47) used mature adipocytes from a

differentiated murine cell line and found that a 1-h treatment with quercetin decreased glucose uptake. Strobel et al (48) used freshly isolated rat adipocytes and found that a 30-min treatment with quercetin decreased glucose uptake. In contrast, we found that 25-h treatment with quercetin prevented TNF-α from suppressing glucose uptake. Differences in these studies and ours include the use of 1) a murine cell line or freshly isolated murine adipocytes compared with human primary adipocytes, 2) very short treatment durations with quercetin (ie, 30–60 min) compared with a longer term treatment (ie, 25 h), and 3) nonstimulated cells compared with TNF-α–stimulated cells.

Concentrations of quercetin (49-51) and RSV (52) found in the circulation after consumption of food rich in these polyphenols vary markedly, ranging from not detectable to 10 μmol/L. Furthermore, recent clinical trials showed that plasma concentrations of quercetin (53) and RSV (54) varied widely after supplementation, even though supplementation was shown to reduce inflammation in many in vivo models (17, 18, 21, 22, 49). Notably, consuming whole foods or supplements rich in phytochemicals such as quercetin and RSV provide many important health benefits. These benefits are most likely due to their ability to attenuate oxidative stress and inflammation (37), as shown in the current study and in other in vitro studies (15, 16, 19, 20, 25, 34, 39-42, 55).

In summary, these data indicate that quercetin and *trans*-RSV aglycones are taken up by primary human adipocytes and that quercetin is equally or more effective than *trans*-RSV in attenuating TNF-α–mediated inflammation and insulin resistance. Potential mechanisms for these antiinflammatory actions include direct effects (eg, suppression of the activation of ERK, JNK, c-Jun, and NF-κB, which induce inflammatory gene

expression and protein secretion) and indirect effects (eg, activation of PPARγ activity). Limitations of these in vitro studies include 1) testing mixed cell populations instead of testing each cell type separately (eg, preadipocytes and adipocytes), 2) lack of available information about the subjects providing the fat specimens, and 3) the high doses of quercetin and *trans*-RSV used. In vivo studies are needed to confirm these in vitro effects of quercetin and RSV on glucose metabolism and insulin resistance.

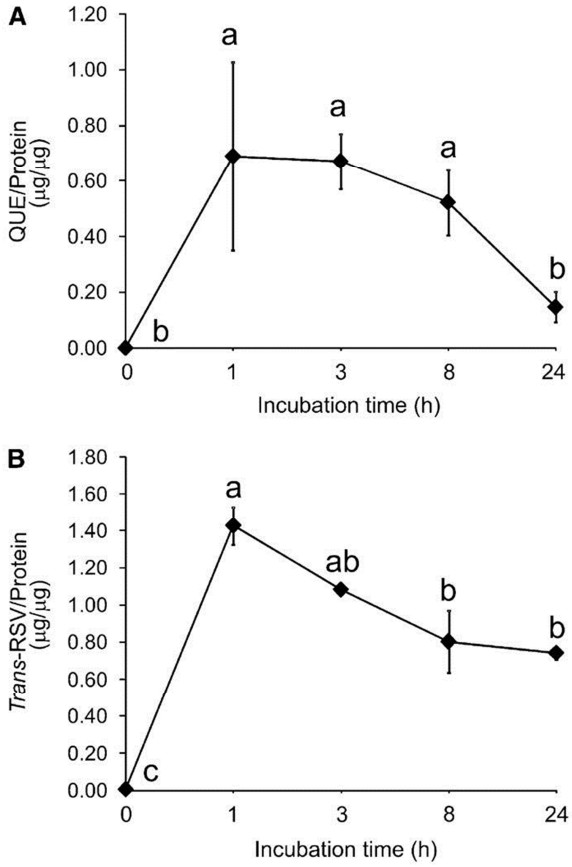

Figure 4.1. Quercetin (QUE) and *trans*-resveratrol (*Trans*-RSV) are taken up by primary human adipocytes. Cultures of newly differentiated human adipocytes (day 7) were supplemented with 30 µmol QUE/L **(A)** or *trans*-RSV/L **(B)** for 0, 1, 3, 8, and 24 h. Cultures were then harvested to determine the amount of QUE or *trans*-RSV that accumulated in adipocytes by liquid chromatography interfaced with time-of-flight mass spectroscopy analysis. All data were analyzed by using one-factor ANOVA and Tukey's honestly significant difference tests. Mean (±SEM) values with different lowercase letters are significantly different, $P < 0.05$ (n = 2-3). Data are representative of 2 independent experiments.

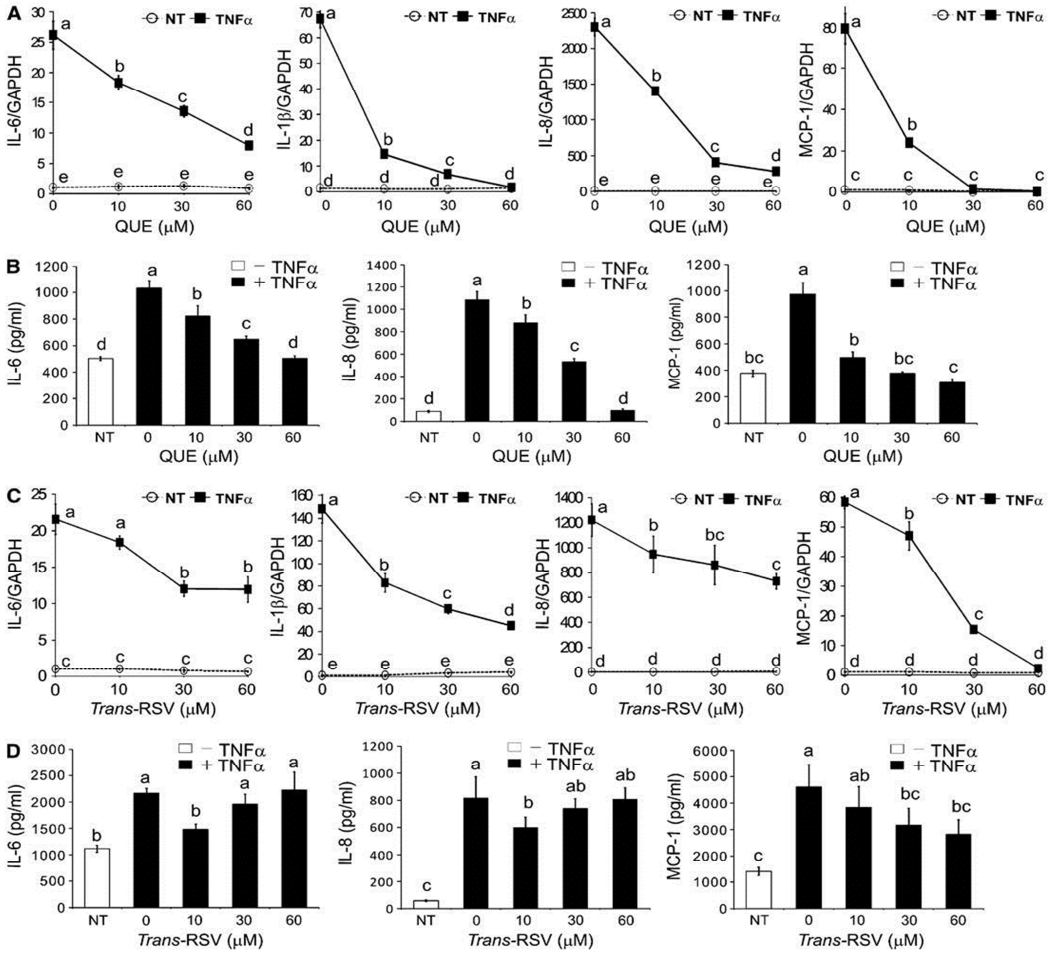

Figure 4.2. Quercetin (QUE), and to a lesser extent *trans*-resveratrol (*Trans*-RSV), attenuate tumor necrosis factor-α (TNFα)–mediated inflammatory gene expression and protein secretion. Cultures of newly differentiated human adipocytes (day 7) were pretreated with dimethyl sulfoxide vehicle (0) or 10, 30, or 60 μmol QUE/L (**A** and **B**) or *trans*-RSV/L (**C** and **D**) for 1 h and were then treated with (filled squares or filled bars) or without (open circles or open bars) 0.5 ng TNF-α/mL for 3 h. Cultures and culture media were then harvested to determine mRNA concentrations of interleukin (IL)-6, IL-1β, IL-8, and monocyte chemoattractant protein-1 (MCP-1) by quantitative polymerase chain reaction (**A** and **C**) and protein concentrations of IL-6, IL-8, and MCP-1 in media by using Bio-Rad's Multi-Plex System (Hercules, CA; **B** and **D**). Data were analyzed by using one-factor (**B** and **D**) or 2-factor (**A** and **C**) ANOVA and Tukey's honestly significant difference tests. **A** and **C**: Mean (±SEM) values with different lowercase letters are significantly different, $P < 0.05$ ($n = 3$). Data are representative of 3 independent experiments. **B** and **D**: Mean (±SEM) values with different lowercase letters are significantly different, $P < 0.05$ ($n = 4$). Data are representative of 2 independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NT, no treatment

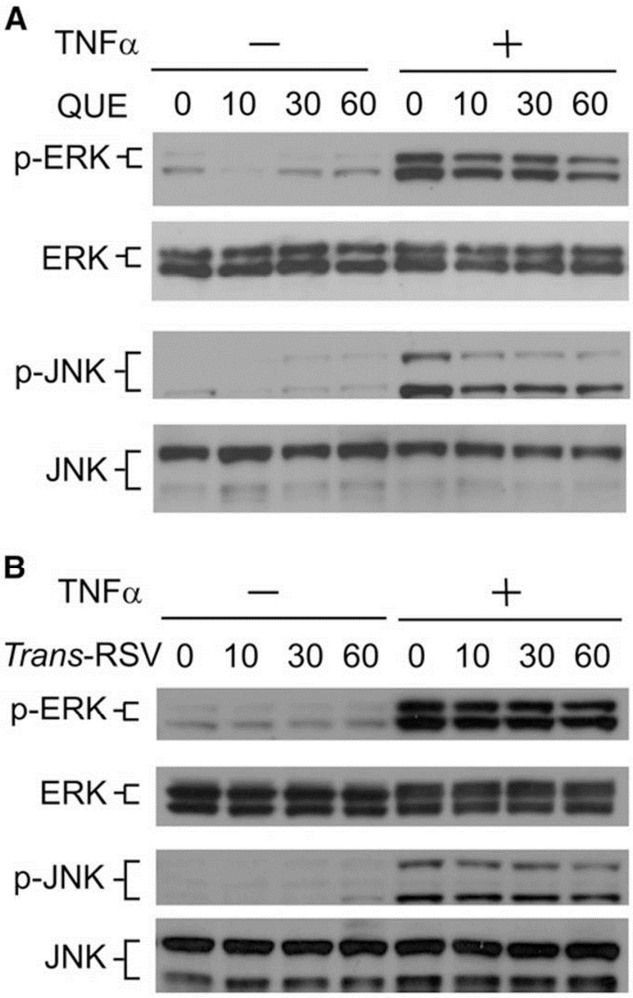

Figure 4.3. Quercetin (QUE) attenuates tumor necrosis factor-α (TNF*α*)–mediated extracellular signal-related kinase (ERK) and c-Jun-NH2 terminal kinase (JNK) activation to a greater extent than does *trans*-resveratrol (*Trans*-RSV). Cultures of newly differentiated human adipocytes (day 7) were pretreated with dimethyl sulfoxide vehicle (0) or 10, 30, or 60 μmol QUE/L (**A**) or *trans*-RSV/L (**B**) for 1 h and were then treated with (+) or without (–) 0.5 ng TNF-α/mL for 15 min. Cultures were then harvested to determine the protein concentrations of phospho (p) or total ERK and JNK by immunoblotting. Data are representative of 3 independent experiments.

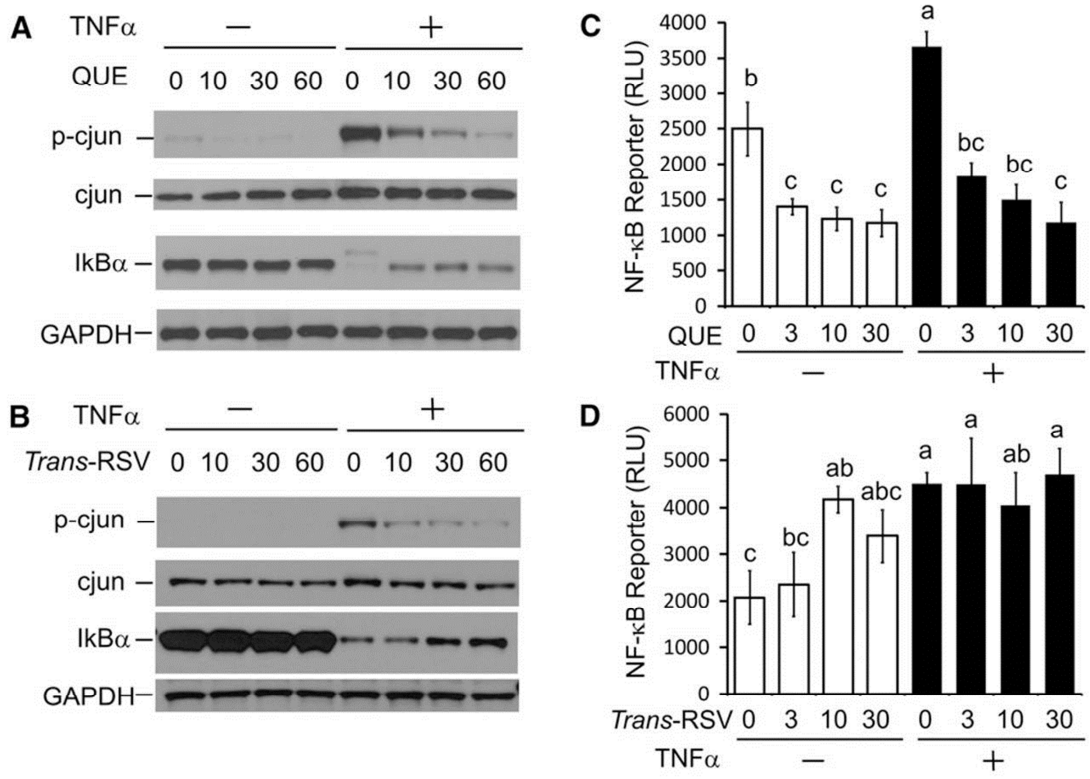

Figure 4.4. Quercetin (QUE) and *trans*-resveratrol (*Trans*-RSV) attenuate tumor necrosis factor-α (TNFα)–mediated c-Jun (cjun) activation and inhibitory κB protein (IκBα) degradation. **A** and **B**: Cultures of newly differentiated human adipocytes (day 7) were pretreated with dimethyl sulfoxide (DMSO) vehicle (0) or 10, 30, or 60 μmol QUE/L (**A**) or *trans*-RSV/L (**B**) for 1 h and were then treated with (+) or without (–) 0.5 ng TNFα/mL for 15 min. Cultures were then harvested to determine the protein concentrations of phospho (p) or total c-Jun, IκBα, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by immunoblotting. **C** and **D**: Cultures were transfected on day 6 with pNF-κB-luc and pRL-CMV. Twenty-four hours later, transfected cells were pretreated with DMSO vehicle (0) or 3, 10, or 30 μmol QUE/L (C) or *trans*-RSV/L (D) for 1 h and were then treated with (filled bars or +) or without (open bars or –) 100 ng TNF-α/mL for 24 h. Firefly luciferase activity was measured and normalized to renilla luciferase activity from the co-transfected control pRL-CMV vector. **A** and **B**: Data are representative of 3 independent experiments. **C** and **D**: Data were analyzed by using 2-factor ANOVA and Tukey's honestly significant difference tests. Mean (±SEM) values with different lowercase letters are significantly different, $P < 0.05$ ($n = 4$-6). Data are representative of 2 independent experiments. RLU, relative light unit; NF-κB, nuclear factor-κB

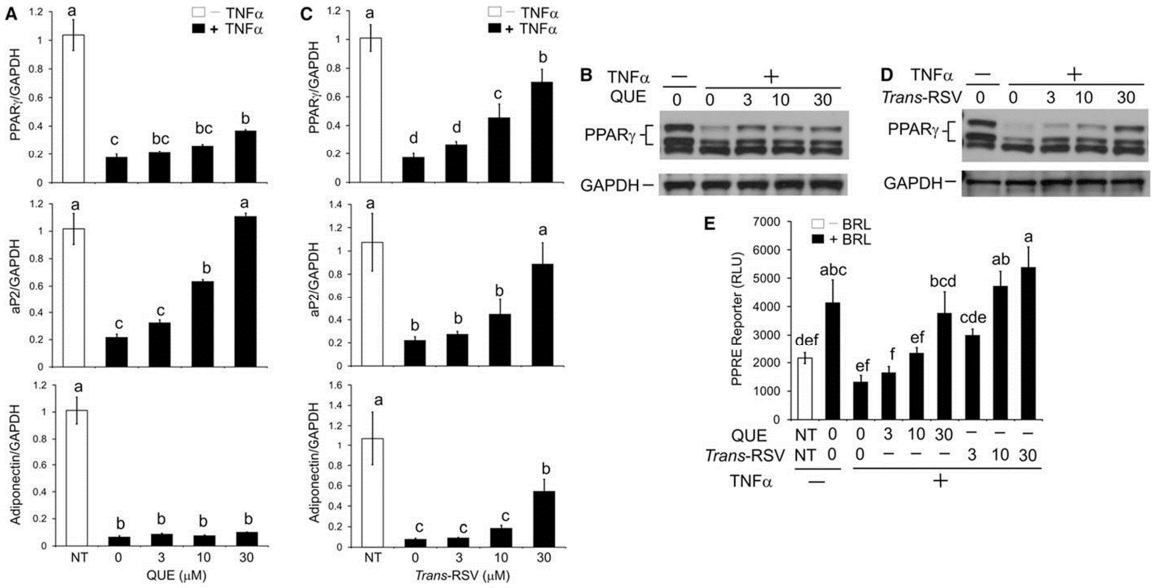

Figure 4.5. Quercetin (QUE) and *trans*-resveratrol (*Trans*-RSV) attenuate tumor necrosis factor-α (TNFα)–mediated suppression of peroxisome proliferator-activated receptor γ (PPARγ) and PPARγ target gene expression, protein concentrations, and transcriptional activity. A-D: Cultures of newly differentiated human adipocytes (day 12) were pretreated with dimethyl sulfoxide (DMSO) vehicle (0) or 3, 10, or 30 μmol QUE/L (**A** and **B**) or *trans*-RSV/L (**C** and **D**) for 1 h and were then treated with (filled bars or +) or without (open bars or **–**) 5 ng TNF-α/mL for 24 h. Cultures were then harvested to determine them RNA concentrations of PPARγ and PPARγ targets [ie, adipocyte fatty acid-binding protein (aP2) and adiponectin] by quantitative polymerase chain reaction and the protein concentrations of PPARγ by immunoblotting. **E**: Cultures were transfected on day 12 with pTK-PPRE33-luc and pRL-CMV and were then treated with DMSO vehicle (0) or 3, 10, or 30 μmol QUE/L or *trans*-RSV/L with (+) or without (**–**) 10 ng TNF-α/mL for 24 h. Cultures were then supplemented with (filled bars) or without (open bars) 0.1 μmol BRL49653/L (ie, rosiglitazone) for 24 h. Firefly luciferase activity was measured and normalized to renilla luciferase activity from the co-transfected control pRL-CMVvector. **A**, **C**, and **E**: Data were analyzed by using one-factor ANOVA and Tukey's honestly significant difference tests. **A** and **C**: Mean (±SEM) values with different lowercase letters are significantly different, $P < 0.05$ ($n = 3$). Data are representative of 3 independent experiments. **B** and **D**: Data are representative of 3 independent experiments. **E**: Mean (±SEM) values with different lowercase letters are significantly different, $P < 0.05$ ($n = 4$-6). Data are representative of 2 independent experiments. BRL 49653 was a gift from Per Sauerberg; Novo Nordisk A/S. RLU, relative light unit; NT, no treatment; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

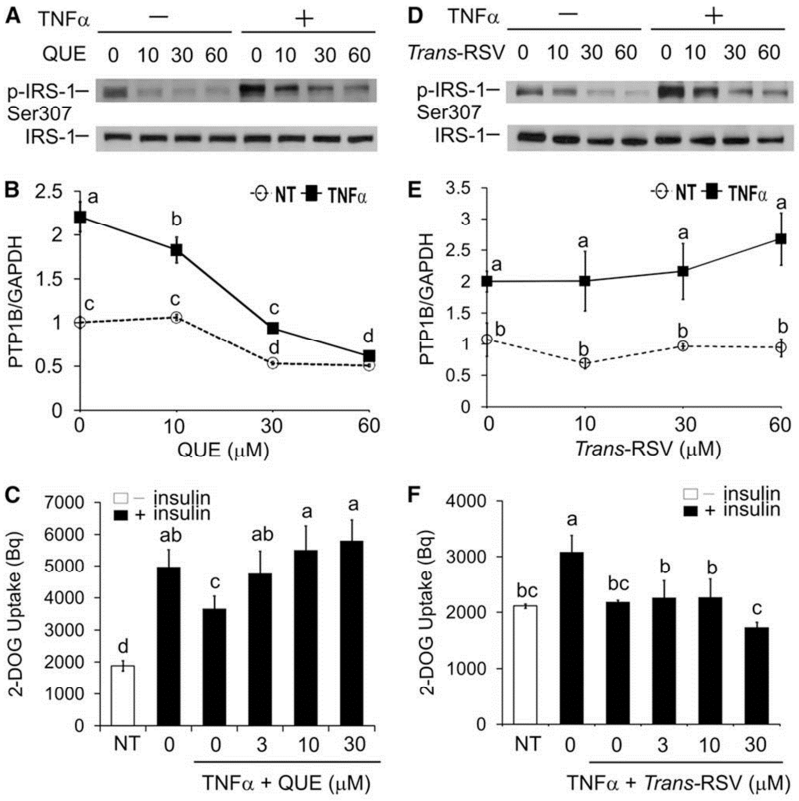

Figure 4.6. Quercetin (QUE) prevents tumor necrosis factor-α (TNFα)–mediated insulin resistance to a greater extent than does *trans*-resveratrol (*Trans*-RSV). A, **B**, **D**, and **E**: Cultures of newly differentiated human adipocytes (day 7) were pretreated with dimethyl sulfoxide (DMSO) vehicle (0) or 10, 30, or 60 μmol QUE/L (**A** and **B**) or *trans*-RSV/L (D and E) for 1 h and were then treated with (filled squares or +) or without (open circles or –) 0.5 ng TNF-α/mL for 15 min to determine the protein concentration of serine residue 307 phosphorylation of insulin receptor substrate-1 (IRS-1; p-IRS-1-Ser307) by immunoblotting (**A** and **D**) or for 3 h to determine the mRNA concentration of protein tyrosine phosphatase-1B (PTP1B) by quantitative polymerase chain reaction (**B** and **E**). **C** and **F**: Cultures (day 13) were pretreated with DMSO vehicle (0) or 3, 10, or 30 μmol QUE/L (**C**) or *trans*-RSV/L (**F**) for 1 h and were then treated with or without 5 ng TNF-α/mL for 24 h. Subsequently, insulin-stimulated 2-[3H]deoxyglucose (2-DOG) uptake was measured after a total 100 min of incubation with (filled bars) or without (open bars) 100 nmol insulin/L. **B** and **E**: Data were analyzed by using 2-factor ANOVA and Tukey's honestly significant difference tests. Means (±SEM) values with different lowercase letters are significantly different, $P < 0.05$ ($n = 3$). **C** and **F**: Data were analyzed by using one-factor ANOVA and Tukey's honestly significant difference tests. Mean (±SEM) values with different lowercase letters are significantly different, $P < 0.05$ ($n = 4$). **A**-**F**: Data are representative of 3 independent experiments. NT, no treatment; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

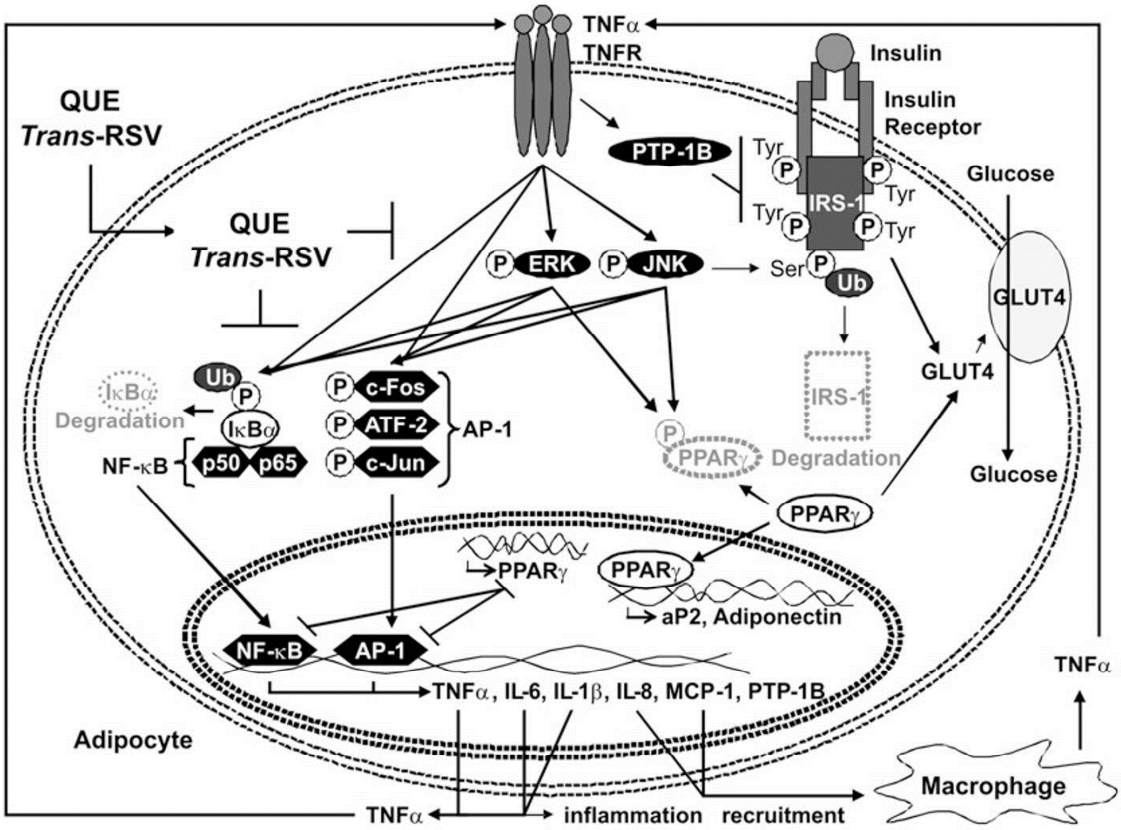

Figure 4.7. Working model on how quercetin (QUE) and *trans*-resveratrol (*Trans*-RSV) block tumor necrosis factor-α (TNFα)–mediated inflammation or insulin resistance. Treating primary cultures of newly differentiated human adipocytes with QUE or *trans*-RSV prevents TNF-α from directly activating extracellular signal-related kinase (ERK), c-Jun-NH2 terminal kinase (JNK), c-Jun, and nuclear factor-κB (NF-κB), which are potent inducers of inflammatory gene expression and protein secretion and negative regulators of insulin signaling. In addition, QUE or *trans*-RSV may indirectly prevent inflammation and insulin resistance by increasing peroxisome proliferator-activated receptor γ (PPARγ) activity, thereby antagonizing NF-κB or activator protein-1 (AP-1) transcriptional activation of inflammatory genes and negative regulators of insulin signaling. Together, these block TNF-α–mediated induction of inflammatory cascades and suppression of insulin signaling necessary for glucose transporter 4 (GLUT4) translocation to the plasma membrane. TNFR, TNF-α receptor; IRS, insulin receptor substrate; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; P, phosphorylated; Ub, ubiquinated; aP2, adipocyte-specific fatty acid binding protein; ATF-2, activating transcription factor 2; PTP-1B, protein tyrosine phosphatase-1B; IκBα, inhibitory κB protein

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CHAPTER V

BIOAVAILABILITY, GLUCOSE DISPOSAL RATE, AND ANTI-INFLAMMATORY PROPERTIES OF GRAPE POWDER IN HIGH FAT-FED OBESE MICE

Abstract

We reported that grape powder extract (GPE) from grape powder (GP) attenuated inflammation and insulin resistance in primary human adipocytes. However, we do not know if these results can be reproduced in vivo. Therefore, we examined 1) which polyphenol metabolites in GPE were bioavailable, 2) the impact of GP and GPE on insulin resistance and inflammation in high fat-fed mice, and 3) if a bioavailable polyphenol in GPE decreases markers of inflammation in human adipocytes. In experiment 1, C57BL/6J mice were gavaged with GPE and serum polyphenols were measured over time. In experiment 2, mice were fed high-fat diets supplemented with 3% GP or 0.02% GPE for 18 wk and markers of insulin resistance and inflammation were measured. In experiment 3, human adipocytes were treated with the bioavailable polyphenol quercetin 3-*O*-glucoside (Q3G) and markers of inflammation were measured. Serum Q3G increased at 1 h post-GPE gavage and decreased thereafter. GP supplementation improved glucose disposal rate at 5 wk, and decreased serum levels of TNFα and monocyte chemoattractant protein-1 (MCP-1) and mRNA levels of inflammatory genes in adipose tissue at 18 wk. In contrast, GPE had no impact on these outcomes. Q3G attenuated TNFα-mediated MCP-1 and IL-1β expression in human

adipocytes, possibly by suppressing c-Jun-NH_2 terminal kinase and c-Jun activation. In summary, 1) Q3G is a bioavailable polyphenol in GPE, 2) GP acutely improves glucose disposal rates and chronically reduces markers of inflammation in high-fat-fed obese mice, and 3) Q3G reduces several markers of inflammation in human adipocytes.

Introduction

Obesity is the most prevalent nutrition-related health problem worldwide, with 1.5 billion adults and 43 million children under the age of five classified as overweight and more than 500 million people classified as obese (1). Obesity is caused by a positive energy balance due to overconsumption of calories relative to energy expenditure that leads to the expansion of white adipose tissue (WAT) mass. Enlarged WAT is associated with a progressive infiltration and accumulation of macrophages that contribute to a chronic, low grade inflammation (2, 3). Moreover, several studies demonstrated that increased pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα; 4) or interleukin-6 (IL-6; 5, 6) and chemokines such as monocyte chemoattractant protein-1 (MCP-1; 7-9) play an important role in the development of obesity-mediated insulin resistance and type 2 diabetes.

Two strategies to suppress WAT expansion and obesity-mediated inflammation and insulin resistance are reduced caloric consumption and increased physical activity (10, 11). However, long term lifestyle changes such as caloric restriction and exercise are usually poorly maintained. Also, pharmacological and surgical interventions to treat obesity and metabolic diseases, while effective, have considerable side effects and

financial costs (12). Therefore, alternative strategies for suppressing obesity-associated inflammation and insulin resistance are needed. One potential dietary strategy is consuming grapes or grape products (e.g., wine, grape juice, powder, extract, raisins), which are rich in phenolic compounds that possess anti-oxidant and anti-inflammatory properties. Indeed, human, rodent, and cell studies (13, 14) support anti-inflammatory and anti-diabetic effects of grape products including grape powder (GP) or grape powder extract (GPE). However, the bioactive compounds in grape products are unclear, and mechanisms by which these products prevent inflammation or insulin resistance are debatable.

Recently, our group reported that GPE isolated from GP rich in quercetin obtained from the California Table Grape Commission attenuated inflammation in human macrophages (15) and adipocytes (16) and insulin resistance in primary human adipocytes (16). We also showed that quercetin aglycone repressed inflammation in human macrophages (17) and adipocytes (18) and insulin resistance in human adipocytes (18). However, we did not know whether these in vitro effects could be reproduced in vivo. Also, the polyphenol metabolite profile in circulation following the consumption of GPE or GP was unknown. Therefore, the objective of this study was to determine 1) which polyphenols in GPE were absorbed in mice, 2) the effect of GPE and GP on inflammation and insulin resistance in a diet-induced, obese mouse model, and 3) the extent to which a bioavailable grape polyphenol suppressed markers of inflammation in primary human adipocytes.

Research Design and Methods

Preparation of GP and GPE

Lyophilized GP, obtained from red, green and blue-purple seeded and seedless California grapes, was provided by the California Table Grape Commission in aluminum bags. The GP was extracted to remove the sugars (90% wt:wt) in the laboratory of Dr. Wei Jia at the University of North Carolina at Greensboro (UNCG) Center for Research Excellence in Bioactive Food Components in Kannapolis, NC. To remove the sugars, a Diaion HP-20 anion resin column was used to elute the extract from the column using methanol, and then it was lyophilized to make the final GPE (19). Then, the polyphenol compositions of GP and GPE (**Table 5.1**) were analyzed using an Agilent liquid chromatography time-of-flight mass spectrometry (HPLC-TOFMS; Agilent, Santa Clara, CA) as previously described (15).

Experiment 1-Gavaging mice with GPE

Male, 12 wk old C57BL/6J mice (*n*=32), weighing approximately 30 g, were obtained from Jackson Laboratories (Bar Harbor, ME) and housed individually in a 12 h light/12 h dark cycle, temperature-controlled room. Ethical treatment of animals was assured by the UNCG Institutional Animal Care and Use Committee. Mice were acclimated to a low-fat purified diet (#D12450B from Research Diets, New Brunswick, NJ) for 10 d containing casein as the protein source devoid of phytoestrogens or other phytochemicals. Subsequently, mice were deprived of food for 6 h and then 8 mice were gavaged with water only (1% carboxymethylcellulose sodium salt; CMC; Sigma-Aldrich,

St. Louis, MO) for the 0 h time point or 24 mice were gavaged with GPE (1 g/kg body weight, dissolved in a 1% CMC water solution; 1 g GPE per 10 mL). Mice were then anesthetized by isoflurane inhalation at 0 (water control), 1, 3, or 8 h post-GPE gavage. Blood was collected by orbital sinus puncture into a Vacutainer tube (BD Vacutainer Systems, Franklin Lakes, NJ). Serum was obtained by centrifugation at 1100 x g for 10 min at 4°C and immediately stored at -80°C prior to polyphenol profile analysis.

Analysis of serum polyphenols

Serum polyphenol levels were measured by a UPLC-MS/MS system (ACQUITY UPLC-Quattro Premier XE MS, Waters Corp., Milford, MA) as described below using a modification of the method of Day et al. (20). Briefly, each serum sample (200 µL) of mice was acidified to pH 3 with 20% aqueous formic acid (10 µL; Thermo Fisher Scientific, Fair lawn, NJ). Then, 2 g/L ascorbic acid (20 µL; Sigma-Aldrich, St. Louis, MO) was added to prevent oxidation during sample preparation and 60 mmol/L apigenin (10 µL; Chromadex, Irvine, CA) was added to the serum as internal standard (I.S.). Acetonitrile (600 µL) was used to precipitate plasma proteins and extract flavonol metabolites. The samples were vortexed (VWR digital vortex mixer, VWR International, West Chester, PA) for 30 s every 2 min over 10 min period. The mixture was centrifuged at 13,500 rpm for 20 min at 4.0 ± 0.5 °C (Microfuge 22R centrifuge, Beckman Coulter, Inc., Atlanta, GA). The supernatant was collected and the pellet was re-extracted as described above but with methanol instead of acetonitrile. The acetonitrile and methanol supernatants were combined and reduced to dryness in CentriVap Vacuum Concentrator

(Labconco, Kansas City, MO). Extracts were dissolved in methanol (100 µL) plus 1% formic acid (100 µL) and then centrifuged at 13,500 rpm for 20 min (Allegra X-15R, Beckman Coulter, Inc., Atlanta, GA). The supernatant was filtered through a 13-mm syringe filter with a 0.2 µm PTFE membrane (Millipore Corp., Billerica, MA) before LC-MS analysis.

Linearity, Recovery

A total of 68 standards were analyzed. Each standard was individually dissolved in methanol or water and prepared as stock solution at a concentration of 1 g/L. Each aliquot of standard stock solution was mixed to obtain a mixed stock solution. The resulting mixed solution was diluted at a series of concentration of 5.12, 2.56, 1.28, 0.64, 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, or 0.005 mg/L. The apigenin (I.S.) was added to the diluted solutions and the final concentration for I.S. is 150 µg/L. The calibration curve and the corresponding regression coefficients were obtained by I.S. adjustment. Quantification of polyphenols was performed by UPLC-MS/MS using a multiple reaction monitoring (MRM) mode. The concentration of each identified polyphenol was automatically calculated using the corresponding calibration curve in the MassLynx application manager, QuanLynx (Waters Corp., Milford, MA).

Experiment 2-High fat-fed obese mice supplemented with GP and GPE

C57BL/6J mice (*n*=50) were obtained from Jackson Laboratories at 5 wk of age and housed individually in a 12 h light/12 h dark cycle, temperature-controlled room.

Ethical treatment of animals was assured by the UNCG Institutional Animal Care and Use Committee. After several days of acclimation, mice were randomly assigned to one of five dietary treatments (*n*=10 mice per treatment; **Table 5.2**) for 18 wk. Diets (Research Diets) were as follows: 1) a low-fat diet (LF) containing 10% of energy from fat, 2) a high-fat control diet (HF) containing 60% of energy from fat, 3) a HF diet supplemented with 0.02% (wt:wt) GPE (HFGE), 4) another high-fat control diet with a modified sugar content (HS) to control for the amount and type of sugar in GP, and 5) a high-fat diet supplemented with 3% (wt:wt) GP (HFGP). The sugar composition in GP was ~90%, and consisted of dextrose and fructose at 50:50 ratio (wt:wt; 19). Energy from sugars including sucrose, dextrose, and fructose was adjusted in the LF, HF, and HS diets to be equivalent to energy from sucrose, dextrose, and fructose in the HFGE and HFGP. The amount of 0.02% GPE in the HFGE was based on the amount of total polyphenols in 3% HFGP. Diets were packed under inert gas in individual 2.5 kg foil bags and stored at -20°C until use. Fresh diet was provided twice per week to minimize oxidization. Mice had *ad libitum* access to both food and water. Food intake and body weight were measured weekly.

Intraperitoneal glucose tolerance tests (GTT)

GTT was performed at baseline, 5, 10, and 15 wk on non-anesthetized mice. Mice were deprived of food for 8 h and given an intraperitoneal glucose (Sigma-Aldrich) injection at a dose of 1 g/kg body weight. Blood was obtained from the tail vein and glucose levels were determined at 0, 5, 15, 30, 60 and 120 min following glucose

administration using Contour blood glucose monitoring system (Bayer Diabetes Care, Tarrytown, NY). Total GTT area under the curve (AUC) was calculated as described (21).

Analysis of serum insulin and inflammatory cytokine and chemokine levels

Fasting serum insulin levels were determined at 12 wk using commercially-available, enzyme-linked, immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Crystal Chem, Downers Grove, IL). Serum TNFα and IL-6 levels were determined at 18 wk using the Bio-Plex magnetic bead-multiplex immunoassay on the Bio-Plex 200 system according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Serum MCP-1 levels were determined at 18 wk using commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Tissue RNA analysis and real-time quantitative PCR (qPCR)

Skeletal muscle and WAT were harvested at 18 wk and total RNA from muscle was extracted using RNeasy Plus Universal Kit (Qiagen, Valencia, CA) and WAT was extracted using RNeasy Lipid Tissue Kit (Qiagen) combined with RNase-Free DNase Set (Qiagen). RNA integrity was assessed using Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). For real-time qPCR, 2 μg total RNA from mouse tissues was converted into first-strand cDNA by using a high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The qPCR was performed in a 7500 FAST Real Time PCR System by using Taqman Gene Expression Assays (Applied

Biosystems). Fold differences in gene expression were calculated as $2^{-\Delta\Delta Ct}$ using the endogenous reference gene TATA-binding protein (TBP*).*

Experiment 3-Primary cultures of human adipocytes treated with quercetin 3-*O*-glucoside (Q3G)

Abdominal WAT was obtained from nondiabetic and nonobese Caucasian, and African American women [age: 20-50 y; body mass index (BMI in kg/m^2) < 32.0] during abdominoplasty. Approval was obtained from the Institutional Review Board at UNCG and the Moses Cone Memorial Hospital in Greensboro, NC. Tissue was digested by using collagenase (Worthington, Lakewood, NJ), and stromal vascular (SV) cells were isolated, proliferated, and induced to differentiate in adipocyte media (AM-1; ZenBio, Research Triangle Park, NC) plus isobutylmethylxanthine (250 μmol/L; Sigma-Aldrich) and thiazolidinedione rosiglitazone (1 μmol/L; BRL 49653, a gift from Dr. Per Sauerberg, Novo Nordisk A/S, Copenhagen, Denmark) for 3 d. Cultures were then grown in AM-1 only for 4 d. Cultures containing ~50% preadipocytes and ~50% adipocytes, based on visual observations, were treated at day 7 of differentiation. Each experiment was repeated at least three more times using a mixture of cells from three subjects.

Cell RNA analysis and qPCR

Primary human SV cells were seeded in 35-mm dishes at 0.4×10^6 per dish and differentiated for 7 d. On day 7, human adipocyte cultures were pretreated with DMSO vehicle (0) or 1.5 nmol/L or 3, 10, or 30 μmol/L of Q3G for 1 h and subsequently treated

with or without 20 ng/L of TNFα for 3 h. Total RNA from primary human adipocytes was extracted using RNeasy Mini Kits (Qiagen). RNA integrity was estimated as described above for tissue RNA. For real-time qPCR, 1 μg total RNA was converted into first-strand cDNA by using a high-capacity cDNA Archive Kit and qPCR was conducted as described above for tissue RNA. Fold differences in gene expression were calculated as $2^{-\Delta\Delta Ct}$ using the endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunoblotting

Primary human SV cells were seeded in 35-mm dishes at 0.4×10^6 per dish and differentiated for 7 d. On day 7, primary human cultures were pretreated with DMSO vehicle (0) or 1.5 nmol/L or 10, 30, or 60 μmol/L of Q3G for 1 h and then treated with or without 20 ng/L of TNFα for 1 h. Immunoblotting was conducted as previously described (22).

Statistics

Data are expressed as the mean ± the standard error of the mean (SEM). Data were analyzed using one-way ANOVA, followed by each pair of Student's *t*-tests for multiple comparisons. Differences were considered significant if $P<0.05$. All analyses were performed using JMP version 8.0 software (SAS Institute, Cary, NC).

Results

Several quercetin metabolites rapidly appear in the serum of mice gavaged with GPE (Experiment 1)

To determine the bioavailability of grape polyphenols, mice were gavaged with 1 g of GPE per kg body weight based on work by Tsang et al. (23). GPE traveled quickly through the gastrointestinal tract, as evidenced by visual examination of the movement of purple digesta through the gastrointestinal tract from 1-8 h, and the appearance of entirely purple feces at 8 h (**Figure 5.1A**). Six polyphenols increased in circulation post-GPE gavage (**Figure 5.1B-G**). However, only Q3G (**Figure 5.1B**) was significantly (P<0.0006) increased at 1 and 3 h compared to baseline. Rutin, another metabolite of quercetin, increased at 1 h post-GPE gavage, but this increase was not significant (P=0.07).

GP supplementation of a high-fat diet improves glucose disposal rates acutely and decreases markers of inflammation chronically (Experiment 2)

In order to determine the extent to which two sources of grape polyphenols (GP, GPE) prevented insulin resistance and chronic inflammation associated with diet-induced obesity, mice were fed high-fat diets containing 3% GP (HFGP) or 0.02% GPE (HFGE) for 18 wk. The 3% GP diet was chosen based on work by Seymour et al. (24, 25) demonstrating anti-inflammatory and cardioprotective effects of 3% GP treatment for 18 wk in a Dahl Salt-Sensitive rat model of hypertension. This level of GP is equivalent to nine daily human servings of fresh grapes, assuming 1 serving = ¾ cup grapes weighing 126 g = 23 g of dried grape powder, and that a 30 g mouse eats ~3 g/d of food. The

0.02% GPE diet contained the same amount of total polyphenols as 3% GP diet. A high-fat control diet (HF) contained the same amount of fat and sucrose as the HFGE diet and second high-fat control diet, HS, contained the same amount of fat and sugars (i.e., 50:50 ratio of dextrose/fructose) as the HFGP diet (**Table 5.2**). A low-fat diet (LF) was fed as a control for the HF, HFGE, HS, and HFGP diets (**Table 5.2**).

High fat-feeding impaired glucose disposal rate at 5, 10, and 15 wk (**Figure 5.2A-C**), and decreased mRNA markers of glucose disposal in muscle and WAT at 18 wk (**Figure 5.2D-E**) and raised fasting glucose and insulin levels at 12 wk (**Table 5.3**) compared to the LF-feeding. Notably, the HFGP group had improved GTT at 30 and 60 min and a lower AUC for the GTT ($P<0.002$) compared to their HS control group at 5 wk, suggesting better glucose disposal rates (**Figure 5.2A**). However, these beneficial effects of GP were not seen at 10 wk (**Figure 5.2B**) or 15 wk (**Figure 5.2C**), compared to their HS controls. Furthermore, fasting glucose and insulin levels (**Table 5.3**) at 12 wk and the mRNA markers of glucose disposal in muscle (**Figure 5.2D**) and WAT (**Figure 5.2E**) at 18 wk were not affected by feeding GP. Surprisingly, HFGE had no effect on glucose disposal rate at any time point (**Figure 5.2A-C**) or mRNA markers of glucose disposal at 18 wk (**Figure 5.2D-E**) as well as fasting glucose and insulin levels at 12 wk (**Table 5.3**) compared to their HF control group.

Given the important role of macrophage recruitment and accumulation in WAT during the development of obesity-associated inflammation and metabolic diseases (26, 27), we sought to determine the extent to which grape polyphenols attenuated markers of macrophage recruitment/accumulation and inflammatory cytokine and chemokine levels

in circulation and mRNA levels in WAT. We isolated four WAT depots including epididymal (bilateral intra-abdominal visceral fat depots attached to the epididymis), inguinal (bilateral superficial subcutaneous fat depots between the skin and muscle fascia just anterior to the lower segment of the hind limbs), mesenteric (a glue-like visceral net located in the mesenterium of the intestines), and retroperitoneal (bilateral visceral fat depots in abdominal cavity behind the peritoneum on the dorsal side of the kidneys) WAT. We found that the wet weights of the four WAT depots and liver at 18 wk (**Table 5.3**) were lower in the LF group compared to all four high-fat groups; however, there were no significant differences between the HF control and HFGE groups (P>0.07) or the HS control and HFGP groups (P>0.1) at 18 wk (**Table 5.3**). Thus, feeding GP or GPE to HF-fed mice did not reduce WAT depot or liver weights.

Although GP and GPE did not prevent HF-induced WAT expansion, we found that HFGP group had lower serum levels of TNFα (P=0.001) and MCP-1 (P=0.03) compared to the HS controls (**Figure 5.3A**). However, there were no differences in serum TNFα (P=0.09) and MCP-1 (P=0.2) levels in the HFGE group compared to HF group at 18 wk (**Figure 5.3A**). Moreover, there were no differences in circulating interleukin (IL)-6 levels in HFGP group compared to HS control group or HFGE group compared to HF control group at 18 wk (data not shown). Consistent with serum levels, the HFGP group had lower mRNA levels of TNFα (P=0.05), MCP-1 (P=0.002), and two markers of macrophage recruitment and accumulation, CD11c (P=0.03) and F4/80 (P=0.05), in epididymal WAT compared to the HS control group (**Figure 5.3B**). In contrast, there were not differences in the expression of these markers in epididymal WAT in the HFGE

group compared to the HF control group at 18 wk (**Figure 5.3B**). Finally, the HFGP group had lower mRNA levels of IL-6 (P=0.03), MCP-1 (P=0.02), interferon gamma-induced protein 10 (IP-10; P=0.02), and F4/80 (P=0.003) in inguinal WAT compared to the HS controls (**Figure 5.3C**). In contrast, there were no significant reductions in any of these markers in inguinal WAT in the HFGE group compared to the HF controls (**Figure 5.3C**).

Q3G attenuates several inflammatory markers in primary human adipocytes treated with TNFα (Experiment 3)

To investigate the potential mechanism by which bioavailable grape polyphenols attenuate inflammation, we pretreated primary cultures of newly-differentiated human adipocyte with Q3G followed by acute treatment with TNFα, an inflammatory cytokine elevated in circulation and WAT of obese individuals (4) and in obese mice in this study. Cultures were treated with 1.5 nmol/L or 3, 10, or 30 μmol/L Q3G for 1 h and subsequently treated with 20 ng/L of TNFα for 1 h to determine activation of inflammatory mitogen-activated protein kinases including extracellular signal-related kinase (ERK) and c-Jun-NH_2 terminal kinase (JNK) and their downstream transcription factors including nuclear factor (NF)-κB and cJun, respectively, or 3 h to determine inflammatory gene expression. The 1.5 nmol/L of Q3G and 20 ng/L of TNFα were chosen on the basis of the results of Experiment 1 and 2, respectively, showing serum levels of Q3G increased to 1.5 nmol/L at 1 h post-gavage with GPE (**Figure 5.1B**) and serum levels of TNFα increased to 20 ng/L in mice fed with high-fat diets for 18 wk

(**Figure 5.3A**). We found that Q3G at 10 or 30 μmol/L, but not at lower levels, attenuated TNFα-induced MCP-1 and IL-1β gene expression (**Figure 5.4A**) and modestly decreased JNK and c-Jun phosphorylation (**Figure 5.4B**). However, Q3G did not suppress TNFα-mediated induction of other inflammatory genes (e.g., IL-6) or activation of ERK and NF-κB (data not shown).

Discussion

We previously reported that GPE extracted from GP obtained from the California Table Grape Commission attenuated markers of inflammation in human adipocytes and macrophages that were associated with improved insulin sensitivity in human adipocytes (15, 16). However, we did not know whether these in vitro effects could be reproduced in vivo. Also, the profile of phenolic metabolites in circulation following the consumption of GPE or GP was unknown, and the extent to which these phenolic metabolites impacted diet-induced insulin resistance and chronic inflammation had not yet been established. Here, we reported that Q3G levels robustly increased in the serum of mice at 1 h post-gavage with GPE and decreased thereafter. We found that high fat-fed mice supplemented with GP, but not GPE, had better glucose disposal rates at 5 wk, but not at 10 or 15 wk, compared to controls. Notably, feeding GP, but not GPE, reduced markers of inflammation in circulation and in two WAT depots at 18 wk. Finally, the bioavailable polyphenol Q3G attenuated TNFα-mediated JNK and c-Jun activation and MCP-1 and IL-1β gene expression in primary human adipocytes.

Several reports indicate that Q3G is not commonly present in the circulation, because of β-glucosidase hydrolysis of quercetin glucosides in the intestine and liver (20, 28, 29). However, we detected a robust increase in Q3G levels in the mouse serum after 1 h post-gavage with GPE. Indeed, Manach et al. (30) reviewed 97 human bioavailability studies and reported that quercetin glucosides might not be detected in plasma after oral administration at nutritionally-relevant levels. Therefore, it is possible that the robust peak in Q3G after 1 h of administration was due to the high dose administered as a bolus. As a bolus, quercetin glucosides may have passively diffused across or between enterocytes, escaping intestinal as well as hepatic β-glucosidase hydrolysis. Consistent with our data, cyanidin 3-*O*-glucoside and its metabolites such as methylated cyanidin 3-*O*-glucosides were detected in the rat plasma after 30-60 min post-gavage with cyanidin 3-*O*-glucosides (M.W. 484) at 0.9 mmol/l (0.44 g) per kg body weight (31). Interestingly, anthocyanins including cyanidin 3-*O*-glucosides or malvidin 3-*O*-glucoside were present in relatively high and varied amounts in our GPE and GP (**Table 5.1**). However, anthocyanins or their metabolites were undetectable in our mouse serum samples within 1-8 h after GPE gavage. Consistent with our data, several intervention studies reported that anthocyanins such as malvidin 3-*O*-glucoside were undetectable in the plasma of adult subjects after a single oral administration of red wine and red grape juice (32, 33). One possible explanation for this lack of detection of these metabolites is that they can be rapidly degraded or metabolized by the intestinal microflora or by freezing of urine or blood samples for storage (34).

The rapid rise in obesity is accompanied by a similar increase in insulin resistance and type 2 diabetes. For instance, ~90% of people with type 2 diabetes are overweight and ~50% are obese (35), suggesting a strong positive relationship between the two diseases. Here, we showed that mice fed a high fat diet (i.e., 60% energy from fat) supplemented with GP had improved glucose disposal rates acutely at 5 wk (**Figure 5.2A**), but not chronically at 10 wk (**Figure 5.2B**) or 15 wk (**Figure 5.2C**). Consistent with our data, consumption of muscadine grape wine or dealcoholized grape wine (150 mL/day) for 4 wk improved fasting blood glucose and insulin levels in subjects with type 2 diabetes (36). In contrast to our study, resveratrol, a polyphenol found in grapes and red wine, has been reported to improve glucose and lipid homeostasis in high fat-fed C57BL/6J obese mice during long term supplementation at 0.04% in the diet (wt:wt) for 12-48 wk or 2.5-400 mg/kg body weight/day for 15-16 wk (37-39). Overall, multiple studies suggest that grape products have anti-diabetic effects due to their abundant polyphenol content and low glycemic index (40).

An emerging feature of obese and type 2 diabetes is their linkage with chronic, low-grade inflammation that begins in WAT and eventually becomes systemic. For instance, obese patients with type 2 diabetes have elevated levels of TNFα (4) and MCP-1 (9) in their WAT and blood. Our study showed that 3% GP, but not 0.02% GPE, attenuated the levels of TNFα and MCP-1 in circulation (**Figure 5.3A**) and WAT (**Figure 5.3B**) at 18 wk. Consistent with our data, Dahl salt-sensitive, hypertensive rats supplemented with 3% GP in the diet (wt:wt) for 18 wk had lower plasma and cardiac levels of TNFα (24, 25). Nevertheless, we were surprised that GPE had no effect on

glucose intolerance acutely or on markers of inflammation chronically in obese mice, considering that the GPE diet had the same amount of total polyphenols as the GP diet. Furthermore, our in vitro studies using GPE (15, 16) and other in vivo studies using grape seed extract (41) reported inhibitory effects on inflammation or insulin resistance. This discrepancy may be due to loss of ~10% water insoluble polyphenols (e.g., quercetin aglycone) during the preparation of GPE from GP (42, 43). Indeed, quercetin aglycones were undetectable in our GPE (**Table 5.1**) as well as in circulation following GPE gavage. Thus, it suggests that whole fruits such as GP contain a greater variety of polyphenols or other phytochemicals than GPE that may play an important role in attenuating inflammation and improving glucose disposal rate in diet-induced obese mouse models.

In our cell studies, we chose to examine the direct effects of Q3G in human primary adipocytes, because of their application to human obesity versus rodent obesity. However, using a murine primary adipocytes or immortalized adipocyte cultures (i.e., 3T3-L1) might have been more applicable to our mouse studies. Sakurai et al. (44) reported that oligomerized grape seed polyphenol attenuated inflammatory TNFα and MCP-1 production in HW mouse white adipocytes co-cultured with a murine macrophage cell line (RAW264 cells). Here, we reported that Q3G attenuated inflammatory MCP-1 and IL-1β gene expression in human primary adipocyte cultures treated with TNFα (**Figure 5.4A**). It has been demonstrated that TNFα-induced MCP-1 gene expression in adipocytes or other cell types occurs via activation of the transcription factors NF-κB or AP-1 (i.e., c-Jun, c-fos, ATF), because the promoter of MCP-1 gene contains NF-κB and AP-1 binding site (45-47). Indeed, we found that Q3G reduced

TNFα-mediated phosphorylation of JNK and downstream c-Jun activation (**Figure 5.4B**), but not ERK activation or IκBα degradation (data not shown), in human primary adipocyte cultures. Further investigations examining upstream activators of TNFα-mediated inflammatory signaling are needed to understand how Q3G suppresses this pathway.

In summary, our findings suggest that feeding GP equivalent to 9 daily human servings to high fat-fed mice improves glucose disposal rates acutely and markers of inflammation chronically without affecting body fat levels. In addition, Q3G may be one of the polyphenols in GP that contributes to these beneficial outcomes. Further mouse studies feeding a high-fat diet that more closely resembles a typical western diet (i.e., 34% of energy from fat) and clinical studies with overweight or obese individuals are needed to determine if these effects of GP or other grape products can be reproduced in humans.

Table 5.1. Polyphenols in grape powder extract (GPE) and grape powder (GP)[1]

Polyphenols	GPE (*mg/kg*)[2]	GP (*mg/kg*)[2]
Catechin	403.0 ± 24.9	8.1 ± 0.2
Epigallocatechin	89.7 ± 4.7	-
Catechin gallate	102.5 ± 14.1	1.5 ± 0.1
Oleanolic acid	1118.2 ± 20.4	-
Kaempferol	46.0 ± 2.8	-
Epicatechin	136.4 ± 5.0	4.1 ± 0.1
Epicatechin gallate	230.9 ± 13.0	2.1 ± 0.1
Kaempferol 3-*O*-glucoside	2536.6 ± 22.8	8.6 ± 0.3
Rutin	2066.9 ± 28.8	8.2 ± 0.3
Quercetin 3-*O*-glucoside	15465.5 ± 213.6	49.3 ± 0.3
Trans-resveratrol	555.3 ± 152.1	-
Quercetin	N/A[3]	0.2 ± 0.01
Procyanidin B2	5885.4 ± 102.0	58.4 ± 2.7
Delphinidin 3-*O*-glucoside	1995.2 ± 34.9	32.7 ± 0.5
Cyanidin 3-O-glucoside	2541.7 ± 98.5	27.8 ± 0.3
Petunidin 3-O-glucoside	4555.1 ± 24.6	54.5 ± 1.7
Peonidin 3-O-glucoside	33804.0 ± 110.1	226.9 ± 0.7
Malvidin 3-O-glucoside	30601.3 ± 1182.6	207.3 ± 0.1
Gallic acid	-	7.9 ± 0.4
Total	100324.5	689.5

[1] 1 kg of GP yielded 4 g of GPE = 0.4 % of initial weight
[2] Data are mean ± SEM
[3] Quercetin in GPE can be detected but was under the limit of detection

Table 5.2. Compositions of a low-fat (LF), high-fat (HF), HF plus grape powder extract (HFGE), HF with modified sugar content (HS), and HF plus grape powder (HFGP) diet

	LF		HF		HFGE		HS		HFGP	
Ingredient	***g/kg***	***kJ/kg***	***g/kg***	***kJ/kg***	***g/kg***	***kJ/kg***	***g/kg***	***kJ/kg***	***g/kg***	***kJ/kg***
Casein	200	3360	200	3360	200	3360	200	3360	200	3360
L-Cystine	3	50.4	3	50.4	3	50.4	3	50.4	3	50.4
Corn starch	506.2	8505	0	0	0	0	0	0	0	0
Maltodextrin 10	125	2100	125	2100	125	2100	125	2100	125	2100
Sucrose	68.8	1155	68.8	1155	68.8	1155	45.5	764.4	45.5	764.4
Dextrose	0	0	0	0	0	0	11.65	195.7	0	0
Fructose	0	0	0	0	0	0	11.65	195.7	0	0
Cellulose, BW200	50	0	50	0	50	0	50	0	50	0
Soybean oil	25	945	25	945	25	945	25	945	25	945
Lard	20	756	245	9261	245	9261	245	9261	245	9261
Mineral mixture [1]	10	67	10	67	10	67	10	67	10	67
DiCalcium phosphate	13	0	13	0	13	0	13	0	13	0
Calcium carbonate	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0
Potassium citrate, $1{\cdot}H_2O$	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin mixture [2]	10	164	10	164	10	164	10	164	10	164
Choline bitartrate	2	0	2	0	2	0	2	0	2	0
GPE	0	0	0	0	0.15	0	0	0	0	0
GP	0	0	0	0	0	0	0	0	23	386.4
FD&C yellow dye #5	0.025	0	0	0	0	0	0.025	0	0	0
FD&C red dye #40	0.025	0	0	0	0.025	0	0	0	0.05	0
FD&C blue dye #1	0	0	0.05	0	0.025	0	0.025	0	0	0
Total	1055.05	17102.4	773.85	17102.4	774	17102.4	773.85	17103.2	773.55	17098.2
GE, %	**0**		**0**		**0.02**		**0**		**0**	
GP, %	**0**		**0**		**0**		**0**		**3**	

[1] Mineral mixture (S10026) contained the following (g/kg mineral mix): sodium chloride, 259; magnesium oxide, 41.9; magnesium sulfate, 257.6; ammonium molybdate tetrahydrate, 0.3; chromium potassium sulfate, 1.925; copper carbonate, 1.05; sodium fluoride, 0.2; potassium iodate, 0.035; ferric citrate, 21; manganese carbonate hydrate, 12.25; sodium selenite, 0.035; zinc carbonate, 5.6; sucrose, 399.105. Sucrose in the mineral mix provided 67 kJ/kg diet.

[2] Vitamin mixture (V10001) contained the following (g/kg vitamin mix): vitamin A palmitate, 0.8; vitamin D3, 1; vitamin E acetate, 10; menadione sodium bisulfate, 0.08; biotin (1%), 2; cyanocobalamin (0.1%), 1; folic acid, 0.2; nicotinic acid, 3; calcium pantothenate, 1.6; pyridoxine-HCl, 0.7; riboflavin, 0.6; thiamin HCl, 0.6; sucrose, 978.42. Sucrose in the vitamin mix provided 164 kJ/kg diet.

Table 5.3. The effect of grape powder extract (GPE) or grape powder (GP) on mice fed with a high-fat (HF) or HF with modified sugar content (HS) diet, respectively, compared to low-fat (LF) controls.

	LF		HF		HFGE		HS		HFGP	
Time points (wk)	12	18	12	18	12	18	12	18	12	18
Total body weight gain (g)	-	9.6±1.0[b]	-	22.5±1.4[a]	-	22.8±0.7[a]	-	24.0±0.8[a]	-	23.2±0.9[a]
Total food intake (kJ)	-	6786.8±376.1[b]	-	5531.1±328.9[a]	-	5392.5±457.3[a]	-	5407.2±354.1[a]	-	5681.8±450.2[a]
Adipose tissue weight (g)										
Epididymal	-	1.1±0.1[b]	-	2.8±0.2[a]	-	2.8±0.2[a]	-	2.7±0.2[a]	-	2.6±0.2[a]
Inguinal	-	0.5±0.1[b]	-	1.6±0.2[a]	-	1.9±0.1[a]	-	2.2±0.1[a]	-	2.1±0.2[a]
Mesenteric	-	0.3±0.0[b]	-	1.0±0.1[a]	-	0.9±0.1[a]	-	1.2±0.1[a]	-	1.2±0.1[a]
Retroperitoneal	-	0.4±0.1[b]	-	1.1±0.1[a]	-	1.0±0.0[a]	-	1.2±0.0[a]	-	1.3±0.1[a]
Total fat depot weight (g)	-	2.3±0.3[b]	-	6.4±0.4[a]	-	6.6±0.2[a]	-	7.2±0.4[a]	-	7.2±0.3[a]
Liver wt (g)	-	1.1±0.1[b]	-	1.7±0.2[a]	-	1.5±0.1[a]	-	1.9±0.2[a]	-	1.9±0.2[a]
Fasting glucose (mg/L)	11.0±0.3[b]	-	13.0±1.0[a]	-	13.3±0.6[a]	-	14.8±0.5[a]	-	13.8±0.6[a]	-
Fasting insulin (μg/L)	0.8±0.1[b]	-	1.7±0.3[a]	-	1.6±0.3[a]	-	2.1±0.4[a]	-	2.2±0.4[a]	-

Data are means ± SEM, n = 7-9. Means in a row without a common letter differ, $P < 0.05$.

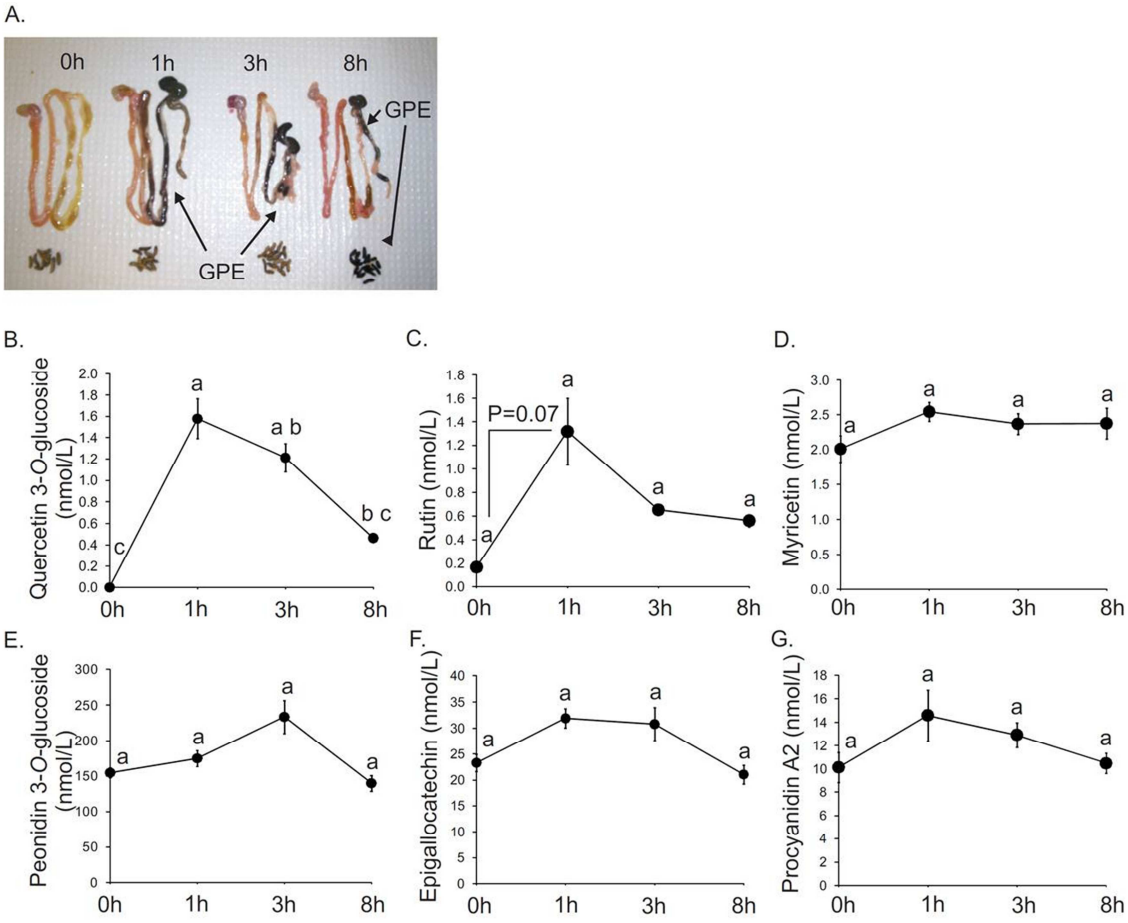

Figure 5.1. Several quercetin metabolites were detected in serum of mice following oral administration of grape powder extract (GPE). Male C57BL/6J mice were gavaged with the water vehicle (controls, *n*=8) or 1 g/kg body weight of GPE (*n*=24). Immediately, blood samples were collected from vehicle control mice (0 h). Then, blood samples were collected from mice that received the GPE bolus at 1, 3, or 8 h post-gavage. The movement and excretion of GPE (purple/dark color identified by an arrow) in the gastrointestinal tract and feces (**A**). The concentration (nmol/L) of quercetin 3-*O*-glucoside (**B**), rutin (**C**), myricetin (**D**), peonidin 3-*O*-glucoside (**E**), epigallocatechin (**F**), and procyanidin A2 (**G**) in mouse serum was measured using a UPLC-MS/MS system. Data were analyzed using one-way ANOVA and Student's t tests. Means ± SEM (*n*=8) without a common letter differ, $P<0.05$.

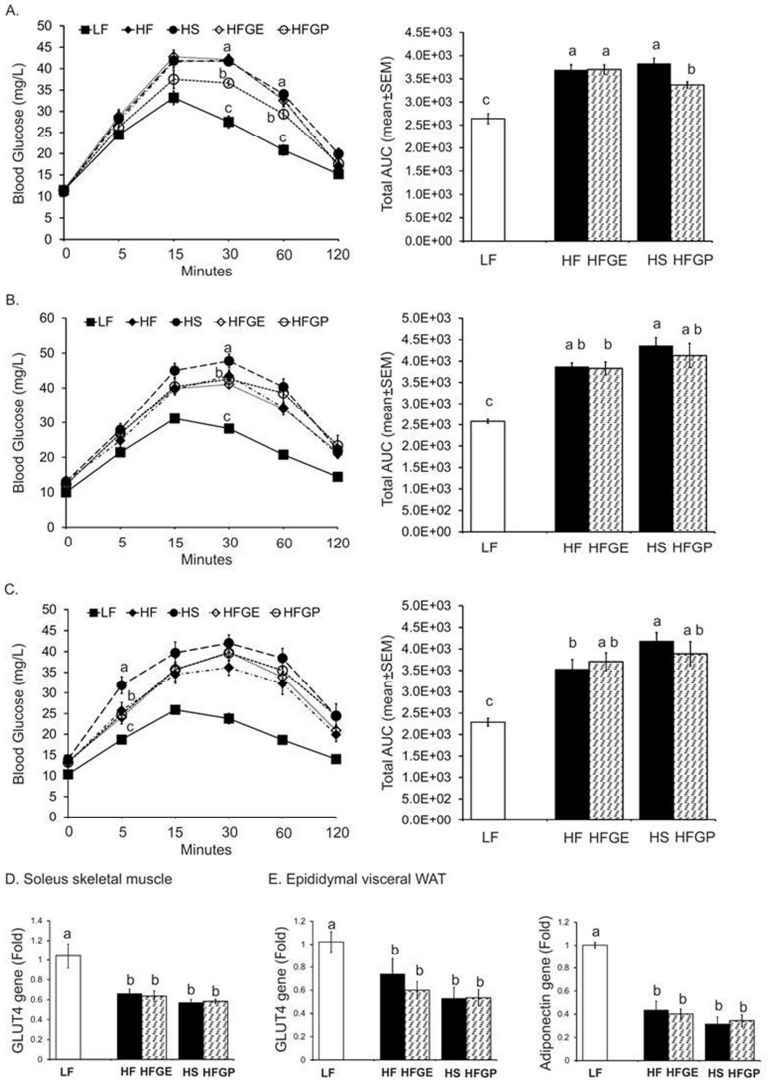

Figure 5.2. High fat-fed obese mice supplemented with grape powder (GP), but not grape powder extract (GPE), had better glucose disposal rates acutely, but not chronically. Male C57BL/6J mice were fed for 18 wk a low-fat (LF) diet, a high-fat (HF) diet, a high-fat diet plus GPE (HFGE) diet, a high-fat diet with a modified sugar (HS) content similar to the HFGP group, or a high-fat diet plus GP (HFGP) diet. A-C: Intraperitoneal glucose tolerance test (GTT) was performed and total GTT area under the curve (AUC) was calculated at 5 wk (**A**), 10 wk (**B**), and 15 wk (**C**). The mRNA levels of insulin-dependent glucose transporter 4 (GLUT4) in mouse soleus skeletal muscle at 18 wk (**D**) was measured using qPCR. The mRNA level of GLUT4 and adiponectin in mouse epididymal WAT at 18 wk (**E**) was measured using qPCR. Data were analyzed using one-way ANOVA and Student's t tests. Means ± SEM (n=7-9) without a common letter differ, P<0.05. Black squares or clear bars = LF; Black diamonds or black bars = HF; Clear diamonds or striped bars = HFGE; Black circles or black bars = HS; Clear circles or striped bars = HFGP.

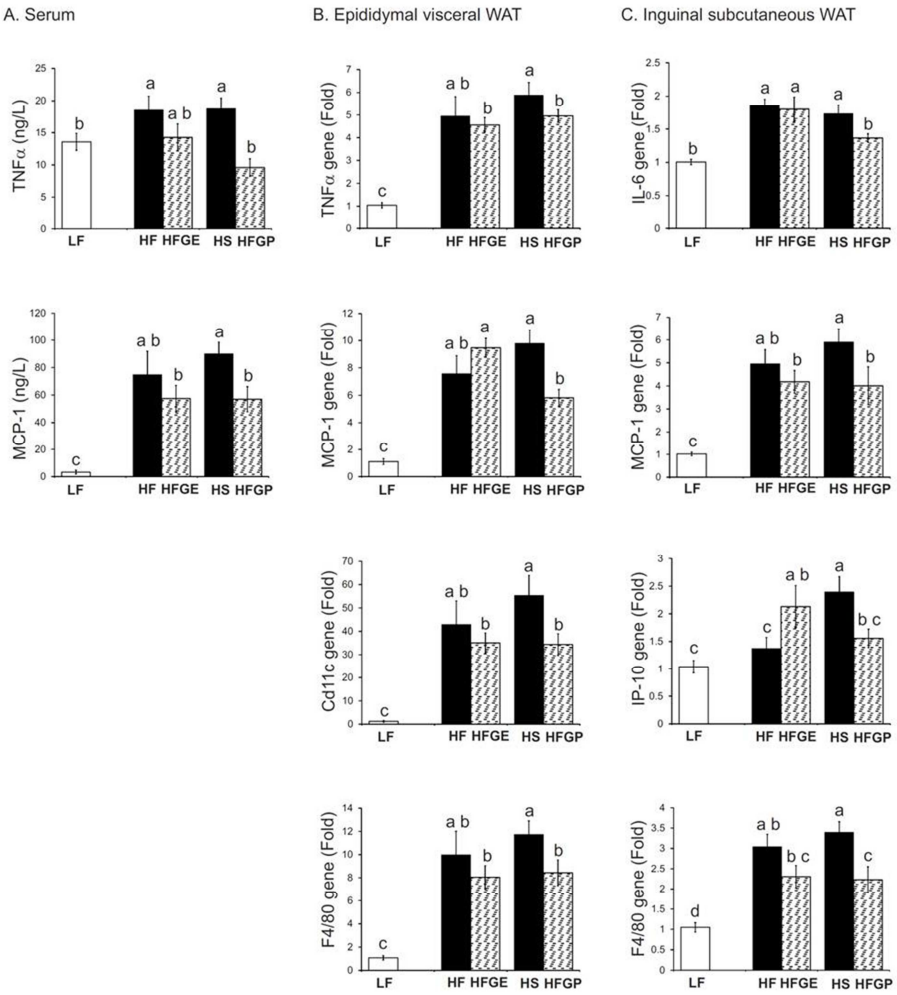

Figure 5.3. High fat-fed obese mice supplemented with grape powder (GP), but not grape powder extract (GPE), had lower systemic inflammation. Male C57BL/6J mice were fed for 18 wk a low-fat (LF) diet, a high-fat (HF) diet, a high-fat diet plus GPE (HFGE) diet, a high-fat diet with a modified sugar (HS) content similar to the HFGP group, or a high-fat diet plus GP (HFGP) diet. At 18 wk, blood was collected and serum was harvested to measure the levels of an inflammatory cytokine (e.g., TNFα) and chemokine (e.g., MCP-1). Also, WAT depots were harvested to measure the mRNA levels of inflammatory cytokines (e.g., TNFα, IL-6) and chemokines (e.g., MCP-1, IP-10) and macrophage markers (e.g., CD11c, F4/80). The concentrations (ng/L) of TNFα and MCP-1 in mouse serum (**A**) were measured using magnetic bead-multiplex immunoassay and enzyme-linked immunosorbent assay, respectively. The mRNA levels of TNFα, MCP-1, CD11c, and F4/80 in epididymal WAT (**B**) were measured using qPCR. The mRNA levels of IL-6, MCP-1, IP-10, and F4/80 in inguinal WAT (**C**) were measured using qPCR. Data were analyzed using one-way ANOVA and Student's t tests. Means ± SEM (n=7-9) without a common letter differ, P<0.05. Clear bars = LF; Black bars = HF or HS; Striped bars = HFGE or HFGP.

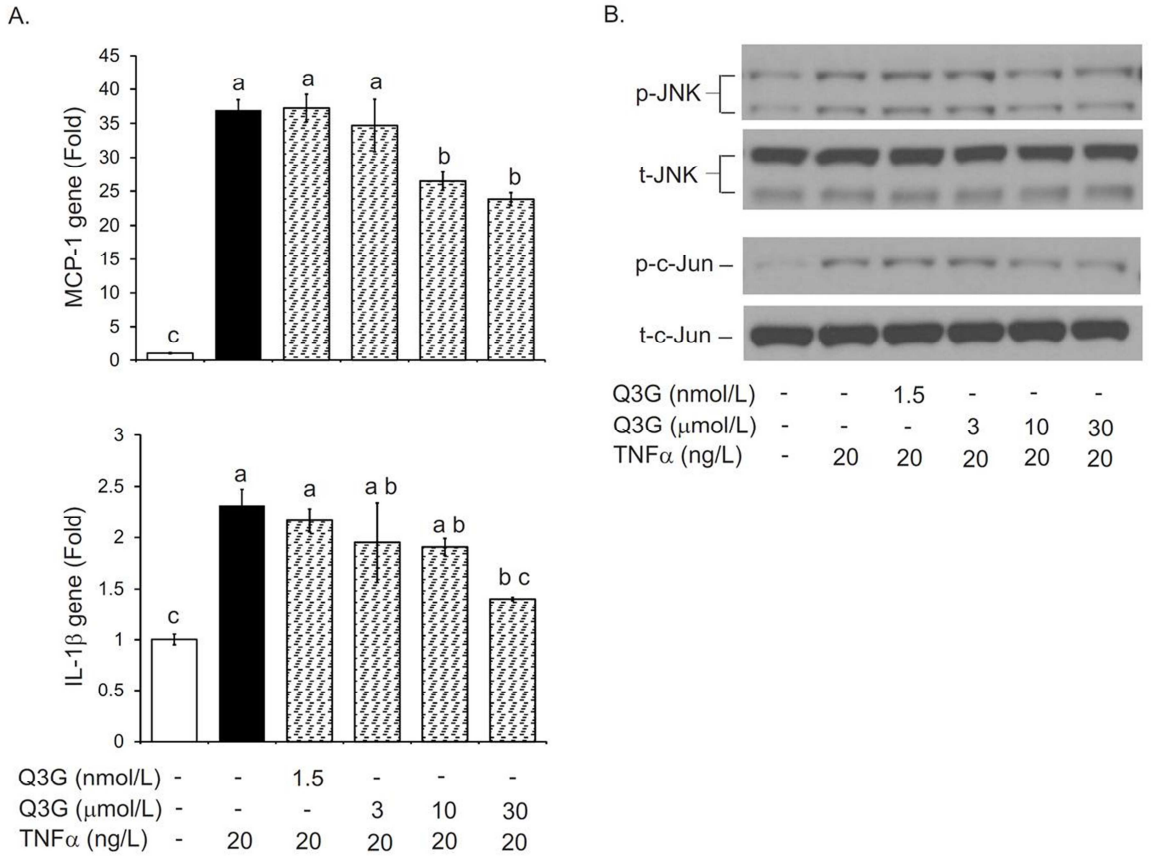

Figure 5.4. Quercetin 3-*O*-glucoside (Q3G) attenuated TNFα-mediated MCP-1 and IL-1β gene expression and JNK and cJun activation in primary human adipocytes. Cultures of newly-differentiated primary human adipocytes (d 7) were pretreated with DMSO vehicle (-), or 1.5 nmol/L, or 3, 10, or 30 μmol/L Q3G for 1 h and then treated without (-) or with 20 ng/L TNFα for 3 h to measure inflammatory gene expression or 1 h to measure inflammatory kinase and transcription factor activation. The mRNA level of MCP-1 and IL-1β (**A**) was measured using qPCR. The protein level of phosphorylated JNK, total JNK, phosphorylated c-Jun, and total c-Jun (**B**) were determined using immunoblotting. Data in panel **A** were analyzed using one-way ANOVA and Student's t tests. Means ± SEM (n=3) without a common letter differ, P<0.05. Data in panels **A** and **B** are representative of three independent experiments. Clear bars = DMSO vehicle controls; Black bars = TNFα alone; Striped bars = TNFα + Q3G.

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CHAPTER VI

EPILOGUE

Over the past three decades, the prevalence of obesity and related diseases (e.g., type 2 diabetes, atherosclerosis, and hypertension), have risen dramatically in the United States (1). Indeed, obesity is becoming a global health issue (1). One of the important mechanisms that connect obesity and insulin resistance or type 2 diabetes is the overexpansion of white adipose tissue (WAT) mass that leads to a chronic, low grade inflammation characterized by increased pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα; 2). Several strategies to suppress WAT overexpansion and obesity-mediated inflammation and insulin resistance include deceased caloric consumption and increased physical activity, or pharmacological and surgical interventions (3). However, these strategies involve long-term lifestyle changes which are usually poorly maintained. Furthermore, pharmacological and surgical approaches have potential side effects and can be expensive without a guarantee of successful long-term weight loss (3). Therefore, alternative strategies for decreasing obesity-associated inflammation and insulin resistance are needed. One potential dietary strategy is consuming grapes.

Early epidemiologic data and recent intervention studies suggest that grapes and grape products (e.g., wine, grape juice, powder, and extracts) exert cardioprotective effects due to their abundant content of polyphenols such as quercetin and resveratrol that

possess anti-oxidant and anti-inflammatory properties (4-8). However, the inhibitory effect of grapes and their polyphenols on obesity-mediated inflammation and insulin resistance remains uncertain. Specifically, the ability of grape polyphenols to attenuate inflammation and insulin resistance triggered by TNFα and their mechanisms of action in primary human adipocytes are unknown. Additionally, the bioavailability of grape polyphenols and their ability to reduce inflammation and insulin resistance in a diet-induced obese animal model are unclear. Therefore, I investigated 1) the extent to which grape powder extract (GPE) and several of its polyphenols decrease TNFα-mediated inflammation and insulin resistance and their mechanisms of action in primary cultures of human adipocytes (**CHARPTER III and IV**), and 2) the extent to which grape powder (GP) polyphenols are absorbed and reduce markers of inflammation and insulin resistance in high fat-fed obese mice (**CHARPTER V**).

Based on the results from these studies, I found that in primary cultures of newly-differentiated human adipocytes GPE, quercetin, and to a lesser extent resveratrol attenuated TNFα-induced expression of inflammatory genes including interleukin (IL)-6, IL-1β, IL-8, and monocyte chemoattractant protein (MCP)-1, possibly by decreasing TNFα-mediated activation of extracellular signal-related kinase (ERK) or c-Jun NH2-terminal kinase (JNK) and their downstream transcription factors nuclear factor-kappa B (NF-κB) or activator protein-1 (AP-1; i.e., c-Jun), respectively. Moreover, GPE and quercetin attenuated TNFα-mediated serine phosphorylation of insulin receptor substrate-1 (IRS-1) and gene expression of protein tyrosine phosphatase (PTP)-1B, which are negative regulators of insulin sensitivity, and suppression of insulin-stimulated glucose

uptake. Notably, quercetin and resveratrol attenuated TNFα-mediated suppression of peroxisome proliferator-activated receptor γ (PPARγ) and PPARγ target genes and of PPARγ protein concentrations and transcriptional activity. I also demonstrated that quercetin 3-*O*-glucoside (Q3G) was increased in the sera of mice at 1 h post-GPE gavage and decreased to baseline levels within 3-8 h. GP supplementation of high fat (HF)-fed obese mice improved glucose disposal rate at 5 wk, but not at 10 or 15 wk. Notably, HF-fed obese mice supplemented with GP had lower serum levels of TNFα and MCP-1 and mRNA levels of inflammatory-related genes (e.g., TNFα, IL-6, MCP-1, IP-10, F4/80, CD11c) in WAT at 18 wk. Surprisingly, GPE had no significant impact on these outcomes. Lastly, bioavailable Q3G attenuated TNFα-mediated MCP-1 and IL-1β gene expression in primary human adipocytes, possibly by suppressing the activation of JNK and c-Jun.

Based on these findings, I have developed the following research questions: 1) Do grape polyphenol metabolites play a role in attenuating obesity-mediated inflammation and insulin resistance?, 2) What role do grape anthocynins play in attenuating obesity-mediated inflammation and insulin resistance?, and 3) Is the gut microbiota the target of grape components and responsible for their anti-inflammatory and anti-diabetic properties?

Q1. Do grape polyphenol metabolites play a role in attenuating obesity-mediated inflammation and insulin resistance?

Polyphenols are present in grapes primarily in the glycosylated form, not in the aglycone form. After deglycosylation, polyphenols can be glucuronidated, sulfated, or

methylated in small intestines, colon, liver or kidney (9). However, our bioavailability study did not measure any glucuronidated, sulphated, or methylated polyphenols. Thus, we do not know if these polyphenol metabolites were bioavailable after gavaging GPE, or if they contributed to GP's suppression of insulin resistance at 5 wk, and markers of inflammation at 18 wk in our feeding study. However, treatment of primary human adipocytes with quercetin 3-*O*-glucuronide at 1.5 nmol/L~60 μmol/L levels did not suppress the mRNA levels of several inflammatory genes (e.g., IL-6, IL-8, IL-1β, and MCP-1) induced by TNFα (data not shown); suggesting that this quercetin metabolite is not a major anti-inflammatory polyphenol in GP. Therefore, it will be important in the future to identify bioavailable polyphenol metabolites in circulation and examine their effect on inflammation and insulin resistance in a diet-induced obese mouse model or clinical studies with overweight or obese individuals supplemented with GP.

Q2. What role do grape anthocyanins play in attenuating obesity-mediated inflammation and insulin resistance?

Our data showed that anthocyanins (e.g., malvidin 3-*O*-glucoside and peonidin 3-*O*-glucoside) were present in relatively high amounts in our GPE and GP. However, anthocyanins were undetectable in our mouse serum samples within 1-8 h after GPE gavage. Consistent with our data, several intervention studies reported that anthocyanins such as malvidin 3-*O*-glucoside or their metabolites were undetectable or at very low levels in the plasma of adult subjects after a single oral administration of red wine and red grape juice within 0.5-2.5 h (10, 11). These findings suggest that grape anthocyanins are 1) poorly absorbed from the intestine, 2) metabolized by the gut microbiota, or 3) rapidly

eliminated in the urine (12). However, in spite of the low bioavailability of grape anthocyanins, several health benefits of consuming berries (e.g. decreased obesity-mediated inflammation and insulin resistance) have been attributed to their anthocyanins (13, 14). Therefore, future studies will be needed to investigate the relationship between the low bioavailability of grape anthocyanins and their metabolites, and the high biological activity of grape anthocyanins and their metabolites in attenuating obesity-mediated inflammation and insulin resistance. For example, identifying molecular targets that interact with grape anthocyanins or their metabolites would be important.

Q3. Is gut microbiota the target of grape components and responsible for their anti-inflammatory and anti-diabetic properties?

Due to their poor absorption, grape polyphenols accumulate in the lower small intestine, cecum, and colon. Here, resident microbiota metabolizes polyphenols by deglycosylation and cleavage of ring structures, producing phenolic acids and aldehydes (15). These metabolites, in turn, can be absorbed or further interact with gut microbes. Therefore, it is conceivable that grape components like polyphenols interact with gut microbiota, affecting their populations and metabolic end products. Consistent with this concept, recent studies reported that high fat diets increase the expansion of WAT and subsequent inflammation and metabolic dysfunction in concert with changes in gut microbiota and gut barrier dysfunction (16-18). Thus, given the low bioavailability of grape polyphenols, it is possible that grape polyphenols or non-digestible components in grapes modulate gut microbiota by decreasing populations of inflammatory microbes. For examples, reductions in pro-inflammatory microbes in the gut such as sulphate-reducing

bacteria that cause mucosal barrier damage and leakage leading to endotoxemia (i.e., elevated lipopolysaccharides or LPS due to translocation of gram negative bacteria) and systemic inflammation, could be responsible for the beneficial effects of GP consumption that we observed in our high-fat feeding study. Therefore, future studies will focus on the interaction between polyphenols and other non-digestible components in grapes and changes in gut microbiota and barrier function to understand the mechanisms by which grape powder improves systemic inflammation and insulin resistance (**Figure 6.1**).

In summary, further research is needed to better understand the role of bioavailable grape polyphenols, especially anthocyanins, and their metabolites that play in attenuating obesity-mediated inflammation and insulin resistance. In addition, it is important to understand the mechanism of action of grape components in attenuating intestinal inflammation and its related metabolic diseases associated with consuming a high fat diet. The proposed studies will provide valuable information for developing dietary strategies using grape products for the control of obesity-related conditions including inflammation and insulin resistance or type 2 diabetes.

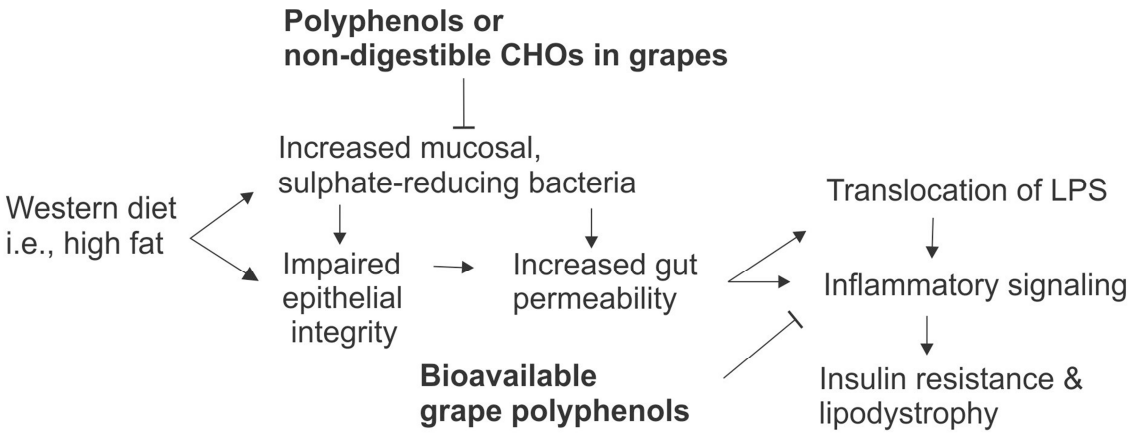

Figure 6.1. Working model on how grape powder blocks obesity-mediated inflammatory signaling, insulin resistance, and lipodystrophy.
LPS, lipopolysaccharides.

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