Resveratrol and Metformin Combination Therapy in Prevention and Treatment of Insulin Resistance

by

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A Thesis
Presented to
The University of Guelph

In partial fulfillment of requirements for the degree of Master of Science in Human Health and Nutritional Sciences

Guelph, Ontario, Canada
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Abstract

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We sought to examine the combined (COM) effects of resveratrol (RSV), a polyphenol, and metformin (MET), a commonly prescribed anti-diabetic drug, in treatment and prevention of insulin-resistance. For examination of treatment effects, mice were fed a low (LFD) or high (HFD) fat diet to induce insulin-resistance. HFD mice were then separated into control HFD, MET, RSV, and COM. COM improved glucose and insulin tolerance compared to HFD. MET improved insulin tolerance, while RSV had no independent effects. COM increased insulin-stimulated phosphorylation of Akt in triceps and scAT. To examine prevention effects, mice were separated into LFD, HFD, MET, RSV and COM at the onset of dietary interventions. MET, RSV and COM therapy prevented HFD-induced glucose intolerance and exhibited significantly greater insulin tolerance compared to HFD. COM increased Akt phosphorylation in triceps and scAT compared to HFD. RSV, MET and COM increased eWAT Akt phosphorylation compared to HFD.
Acknowledgements

To my advisor, Dr. David Wright, thank you for giving me so many opportunities to succeed and grow. Your guidance and insight into not only my project, but also science as a whole and the political aspects that go with it has been amazing. You gave me the opportunity to work on multiple projects and travel to other labs to learn additional techniques, which has helped me gain an appreciation for the importance of being able to balance multiple projects and maintain connections with other researchers. Thank you for putting up with me for 2 years and everything you have done for me in that time.

To Dr. David Dyck, thank you for your help with the design of my study and your advice throughout my Masters. I appreciate that you were always willing to stop and talk to me, whether about science or just making conversation in the hallway. I’d like to thank Dr. Lindsay Robinson for chairing my defense and giving me an understanding of inflammatory processes in her graduate course that proved very useful in my project.

I would like to thank Dr. Matthew Watt for giving me the opportunity to work in his lab and mentoring me. You taught me a lot of new techniques and gave me an appreciation for living a balanced life-style of working hard but taking time to relax. To Dr. Sandra Peters, thank you for introducing me to Dr. David Wright and helping me get set up with my Masters at Guelph. But most of all, thank you for sparking my interest in scientific research and continuing to be there for me to talk science. Thank you to Dr. Rebecca Macpherson for all of your help from my undergraduate thesis at Brock to my Masters at Guelph. You have helped me understand things in lab and are always there to talk to.

To my Guelph lab mates Will, Zac, Laura, Tara, Charlie and Laelie, thank you for all of our science talks and putting up with me. I have really enjoyed working with all of you and I don’t think I will ever again experience working with such a good group of people. A special
thanks to the A-Team (Andra, Anne and Ann), Diana, Andy and Becky for all of their help keeping me, my animals and our lab organized. I would also like to thank Jen, Tom, Dave, Pom, Maria, Ruth, Stacey, Arthe and Ruzaidi from Dr. Watt’s lab at Monash for teaching me and making it really easy for me to get comfortable in a new lab and new country. I really appreciate everything you guys did for me and I hope to see you all again soon.

To my family, thank you for always being there and supporting me in whatever I do. You have given me the confidence to pursue whatever I set my mind to. To my parents, I have modeled my drive and sense of humour off you both and I really appreciate everything you do for me. To Monika, thanks for everything you have done for me. You have really helped me get through my Masters and were always there when I needed you. I can’t wait to begin the next chapter of our lives together in Toronto.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AS160</td>
<td>Akt substrate of 160 kDa</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>COM</td>
<td>Combined metformin and resveratrol treatment</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase subunit IV</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DGAT1</td>
<td>Diacylglycerol acyltransferase-1</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>eWAT</td>
<td>Epididymal white adipose tissue</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose 6 phosphatase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GNG</td>
<td>Glyceroneogenesis</td>
</tr>
<tr>
<td>GyK</td>
<td>Glycerol kinase</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>HGP</td>
<td>Hepatic glucose production</td>
</tr>
<tr>
<td>HMB</td>
<td>Hydroxymethylbutyrate</td>
</tr>
<tr>
<td>IPGTT</td>
<td>Intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>IPITT</td>
<td>Intraperitoneal insulin tolerance test</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
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</table>
IRS  Insulin receptor substrate
ITT  Insulin tolerance test
JNK  c-Jun NH2-terminal protein kinase
LFD  Low-fat diet
MET  Metformin
NFκB Nuclear factor kappa B
OGTT Oral glucose tolerance test
p38  p38 mitogen-activated protein kinase
PDH  Pyruvate dehydrogenase
PDK  Phosphoinositide-dependent kinase
PEPCK Phosphoenolpyruvate carboxykinase
PGC-1α Peroxisome proliferator activating receptor γ co-activator 1 alpha
PI3K Phosphatidylinositol 3-kinase
PIP2 Phosphatidylinositol-4,5-bisphosphate
PIP3 Phosphatidylinositol-3,4,5-triphosphate
PKCθ Protein kinase C theta
PPARγ Peroxisome proliferator activating receptor gamma
R3G  Trans-resveratrol-3-glucuronide
R3S  Trans-resveratrol-3-sulfate
RER  Respiratory exchange ratio
RSV  Resveratrol
scAT  Subcutaneous adipose tissue
SEM  Standard error of the mean
SFA  Saturated fatty acid
SIRT1 Sirptuin 1
TAG  Triacylglycerol
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>UCP1</td>
<td>Uncoupling protein-1</td>
</tr>
<tr>
<td>VO2</td>
<td>Oxygen consumption</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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Chapter 1: Literature Review

1.1. Introduction

The incidence of type 2 diabetes is increasing in Canada, placing a rising economic burden on the health care system\(^1\). In 2013 just under 2 million Canadians over the age of 12 were diabetic, costing the healthcare system $13.1 billion\(^{1,2}\). These factors, along with the association of diabetes with cardiovascular risk factors\(^3\), establishes a need to understand the contributing factors that lead to the development of diabetes and the necessity of developing cost effective treatments. As type 2 diabetes is characterized by reduced insulin sensitivity and associated impairments in glucose homeostasis, it is imperative that we understand the mechanisms of insulin action and the means through which insulin signaling is impaired in order to explore effective treatment strategies.

Insulin is the primary hormone responsible for mediating glucose disposal, particularly in response to post-prandial increases in glucose absorption. Insulin reduces plasma glucose concentration through binding to receptors on insulin sensitive tissues and subsequently increasing glucose uptake as well as inhibiting hepatic glucose production\(^4,5\). However, insulin resistance, a major contributor in the development of type 2 diabetes, is increasingly common in Canada. Obesity\(^6\), lipotoxicity\(^7\), inflammation\(^8\) and mitochondrial dysfunction\(^9,10\) are commonly associated with insulin resistance. Therefore, many pharmacological therapies have been developed to target these players in the development of insulin resistance. Two medications are commonly prescribed: thiazolidinedione’s (TZDs), which target mitochondrial biogenesis and inflammation in adipose tissue\(^11\), and metformin (MET), which attenuates hepatic glucose production\(^12\). These medications are often prescribed in combination to target multiple contributors of impaired glucose homeostasis\(^13\). However, TZDs produce major adverse side
effects\textsuperscript{14}. Therefore, it is important to identify a safer and more effective alternative to improve insulin sensitivity. Resveratrol (RSV), a natural compound found in red grapes, represents a potential adjunct treatment for use with MET, as it has been shown to promote similar beneficial effects to TZDs in adipose tissue\textsuperscript{15–17}. This literature review aims to provide background on the development of insulin resistance, identify the mechanisms through which TZDs and MET act, and attempts to support the use of RSV as an anti-diabetic compound that could be used as a substitute for TZDs in combination with MET.

1.2. Insulin and Glucose Homeostasis

Insulin is important for regulating postprandial blood glucose homeostasis through increasing glucose uptake into various tissues, including skeletal muscle, liver and adipose tissue, as well as inhibiting hepatic glucose production (HGP)\textsuperscript{4,5}. As glucose uptake represents a major step in glucose metabolism\textsuperscript{4}, it is important to understand the processes responsible for mediating insulin action under normal conditions. Although most glucose transporters (GLUT) are expressed in plasma membranes and allow basal levels of glucose uptake at rest, such as GLUT1 in skeletal muscle, insulin sensitive tissues also contain an intracellular pool of the GLUT4 isoform, which may be localized to the plasma membrane following stimulation. Insulin regulates the translocation of GLUT4 from intracellular vesicles to the plasma membrane and increases the activity of these transporters in order to effectively increase the rate of glucose uptake into insulin sensitive tissues\textsuperscript{5,18,19}. This process involves a cascade of reactions that are initiated by the binding of insulin to the insulin receptor (IR) on the extracellular surface of cells. IRs are ligand-activated receptor tyrosine kinases, and once insulin binds a conformational change within the IR activates the kinase domain, leading to autophosphorylation and increased catalytic activity of the IR. Subsequent insulin receptor substrate (IRS) phosphorylation triggers
IRS-induced activation of phosphoinositide 3-kinase (PI3K). PI3K is a lipid kinase that converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) \(^{20,21}\). PIP3 is then responsible for recruiting Akt to the plasma membrane for subsequent phosphorylation by phosphoinositide-dependent kinase (PDK)\(^ {20,21}\). Akt in turn phosphorylates AS160, removing the inhibitory effect of AS160 on GLUT4 vesicles and allowing them to translocate to the plasma membrane to increase glucose uptake\(^ {20,22,23}\). In addition to initiating GLUT4 translocation, insulin is believed to improve the activity of GLUT4 in the plasma membrane through changes in the phosphorylation status of GLUT4\(^ {24}\).

1.3. Insulin Resistance

Although the insulin signaling pathway is highly regulated and conserved, dysfunction may occur leading to insulin resistance and eventually diabetes; a pathological condition defined by an inability to maintain plasma glucose concentrations. Insulin signaling may be effected through two possible mechanisms; (I) reducing insulin delivery to the target tissues or (II) impairing the target tissues’ response to insulin. Insulin resistance occurs when target tissues are unable to respond appropriately to physiological levels of insulin. This attenuates insulin-stimulated glucose uptake, and the inhibition of adipose tissue lipolysis and HGP\(^ {25,26}\).

Whole-body insulin sensitivity and glucose homeostasis can be measured using various methodologies\(^ {27,28}\). Insulin tolerance (ITT) and oral glucose tolerance tests (OGTT) are measures of insulin action and glucose homeostasis that may be used in human research\(^ {27,28}\). The ITT is a very simplistic measure, involving a bolus injection of insulin followed by blood glucose measures at various time-points, with faster declines in glucose concentrations representative of enhanced insulin sensitivity. The OGTT is also a simple measure, commonly used to examine the efficiency of the body at clearing a glucose load. A large glucose load is ingested and blood
glucose is measured at various time-points, with prolonged higher blood glucose indicative of glucose intolerance. The OGTT is perhaps a more physiological measure than the ITT, as it more closely mimics the dynamic glucose levels experienced post-prandially and very crudely accounts for both insulin release and action. However, glucose tolerance is not an equivalent measure to insulin sensitivity and therefore the latter cannot be measured using an OGTT alone. Similar to the measures observed in humans, in normal, healthy mice, intraperitoneal injection with glucose (IPGTT) results in an acute spike in plasma glucose followed by rapid clearance of glucose into insulin-stimulated tissues, while an insulin injection (IPITT) results in an immediate reduction in plasma glucose. Glucose intolerant mice experience a similar initial increase in plasma glucose following glucose injection, however the glucose concentration remains high for a prolonged period, as these mice are incapable of efficiently clearing glucose. In terms of insulin tolerance, insulin resistant mice experience an attenuated reduction in plasma glucose following insulin injection. Normal plasma glucose concentrations can be maintained initially in insulin resistance due to compensatory hyperinsulinemia, however type 2 diabetes will likely develop over time due to an inability of the pancreas to maintain high insulin output.

Although it may be promising to examine insulin resistance and anti-diabetic therapies in humans, rodent models are important in order to first identify potential adverse effects and allow for a greater ability to control extraneous variables, such as differences in diet, exercise and hereditary factors. Although knockout rodent models have been developed, they fail to mirror the complexity of effects occurring in human insulin resistance as they only target one factor of insulin action. Although these models have proven useful in clarifying the functions of specific proteins in insulin signaling and identifying important factors, they fail to mimic the
An alternative rodent model is that of diet-induced insulin resistance. As human obesity results from excessive caloric intake and foods high in fat content have been shown to increase the risk of diabetes, high-fat fed rodents represent an ideal model of insulin resistance\textsuperscript{32}. These models mirror the increased fat availability of foods in Western society, which is a major contributor to insulin resistance\textsuperscript{32}. However, certain rodent strains are resistant to high-fat diet effects and it is therefore important to select the appropriate rodent model for such studies\textsuperscript{32}. C57BL/6 mice represent an ideal strain for rodent high-fat diet models of insulin resistance, as they have been highly characterized and mimic the development of obesity and insulin resistance observed in humans consuming a high-fat diet\textsuperscript{32}. As such, this model has become important for examining the effects of high-fat Western diets on obesity and insulin resistance\textsuperscript{32}.

1.3.1 The Pathophysiology of Insulin Resistance

The pathophysiology of insulin resistance is multifactorial. Insulin resistance is typically initiated by consumption of a high-fat diet (HFD) and the development of obesity, followed by subsequent alterations in lipid metabolites, inflammation and mitochondrial content\textsuperscript{6,8,26,33,34}. This culmination of factors was demonstrated by Lee et al.\textsuperscript{26} using a time course HFD intervention to examine the development and progression of insulin resistance. Consumption of a 60% kcal from fat HFD induced whole-body glucose intolerance and insulin resistance in as early as 3 days in mice, with a simultaneous increase in body weight, epididymal adipose tissue mass, and adipocyte size. These detrimental changes progressively worsened over time, with maximal impairment occurring at 10 weeks. Lipid-derived metabolites may be responsible for
short-term insulin resistance, as ceramides, free fatty acids (FFAs) and diacylglycerols (DAGs) were increased in liver and skeletal muscle at 3 days, while inflammation may be responsible for insulin resistance following a long-term HFD and the development of obesity\textsuperscript{26}. However, the role of inflammation in the development of short-term HFD-induced insulin resistance remains controversial, as others have shown that adipose tissue inflammation is a key component in the development of hepatic insulin resistance following a short-term HFD\textsuperscript{35}. Although a relationship between reduced skeletal muscle and adipose tissue mitochondrial content and insulin resistance has been established, the role of mitochondrial dysfunction in insulin resistance remains controversial\textsuperscript{9,10}.

1.3.2 Lipotoxicity

Obesity and high-fat feeding are associated with lipotoxicity, induced by marked increases in circulating lipids and intracellular triacylglycerol (TAG) and DAG in liver and skeletal muscle\textsuperscript{26,36–39}. HFD and lipid infusion models have been utilized to examine the effects of lipotoxicity on whole-body and tissue specific insulin sensitivity\textsuperscript{38}. Plasma FFA concentration and skeletal muscle and liver TAG and DAG content are negatively associated with whole-body insulin sensitivity\textsuperscript{7,26,30,40}. This has been supported by reductions in insulin-stimulated glucose infusion rate and glucose disposal as well as attenuated inhibition of HGP\textsuperscript{41–43}. Interestingly, lipid infusion and HFD studies also suggest that lipid accumulation may impair insulin action in the absence of obesity and its related physiological abnormalities\textsuperscript{43}. However, others have shown that lipid infusion in healthy subjects was unable to induce whole body insulin resistance until skeletal muscle lipid content increased, indicating that accumulation of plasma FFAs, unlike cellular lipids, may not be directly causative in insulin resistance\textsuperscript{40,42,44}. Moreover, endurance trained individuals display high levels of intramuscular TAG storage, comparable to those of
diabetic individuals, while maintaining a high degree of insulin sensitivity\textsuperscript{45,46}. This association of high TAG content in insulin sensitive muscle of endurance athletes is termed the athletes paradox.

Although the association between lipid accumulation and insulin resistance is well established in diabetic individuals, the mechanisms responsible have yet to be elucidated. Various hypotheses have been proposed to ascertain the effects of lipids in liver and skeletal muscle insulin signaling. One such example is the potential for FFA to indirectly induce insulin resistance through propagation of inflammatory signaling in macrophages, resulting in pro-inflammatory molecule release, as will be discussed in detail below\textsuperscript{47}. It has also been postulated that increased fat oxidation associated with lipid accumulation, as measured by a reduced respiratory exchange ratio, impairs glucose metabolism\textsuperscript{37,42,43,48}. Within insulin resistant muscle, intracellular DAGs and TAGs are associated with reduced tissue insulin sensitivity and glucose uptake, resulting in diminished glycogen synthesis and glucose metabolism\textsuperscript{36,44,49}. Additionally, intrahepatic lipid accumulation inhibits insulin-induced suppression of HGP\textsuperscript{41,50}. These effects in muscle and liver appear to be mediated through reduced IRS-1 and Akt phosphorylation\textsuperscript{38,48–51}. Specifically, increases in FFA and DAG content activate protein kinase C theta (PKC\(\theta\)) in muscle, which in turn phosphorylates IRS-1 at serine-307\textsuperscript{38,40,49}. This leads to attenuated insulin-stimulated IRS-1 tyrosine phosphorylation, PI3K activity and Akt phosphorylation\textsuperscript{38,40,49,51}.

Adding to the uncertainty of lipotoxicity induced insulin resistance, it has been suggested that fatty acid composition affects insulin signaling, with certain fatty acids inducing resistance and others playing a preventative role against resistance\textsuperscript{52}. Saturated fatty acids (SFAs) in particular are associated with the induction of insulin resistance\textsuperscript{52,53}. Specifically, SFAs in adipose tissue are implicated in the degradation of IRS1 as well as macrophage activation and
subsequent pro-inflammatory molecule release as is discussed in detail below\textsuperscript{53,54}. These effects may be partially mediated through the production of ceramides, as inhibition of ceramide production prevented lipid infusion induced insulin resistance in rodent models\textsuperscript{46,54,55}. Excessive accumulation of SFAs in hypertrophied adipocytes leads to an increase in SFA release into the circulation, resulting in the activation of inflammatory pathways and IRS1 degradation in liver and skeletal muscle, further promoting whole-body insulin resistance\textsuperscript{53}. It is likely that the various inhibitory effects of lipids listed above do not occur in isolation, but rather work in a coordinated manner in order to attenuate whole body insulin sensitivity.

\textbf{1.3.3 Inflammation}

Obesity-induced inflammation has been shown to occur in skeletal muscle\textsuperscript{56} and liver\textsuperscript{25,57}, however adipose tissue experiences a greater degree of inflammation\textsuperscript{8,26,56,58} and may induce insulin resistance in the aforementioned tissues through secretion of adipokines and pro-inflammatory molecules into the systemic circulation\textsuperscript{26,33,59}. Because of this, our examination of inflammation-mediated insulin resistance will focus on adipose tissue.

Increased adiposity is believed to play a role in the onset of inflammation through white adipose tissue (WAT) hypoxia\textsuperscript{58,60} and FFA accumulation\textsuperscript{47,61}. Hypoxia develops in hypertrophied adipose tissue as angiogenesis is unable to match the rapid increase in adipose tissue expansion\textsuperscript{60}. Studies have shown that hypoxia increases pro-inflammatory gene expression in adipocytes and macrophages\textsuperscript{58}, serum levels of inflammatory markers\textsuperscript{50}, and the infiltration of macrophages and cytotoxic T-cells into adipose tissue\textsuperscript{58,60}. Alternatively, FFAs may directly effect immune cell activation through interacting with toll-like receptors on the cell surface, although this has only been shown to occur at supraphysiological FFA concentrations\textsuperscript{47,62}. 
Classically activated, pro-inflammatory macrophages have previously been shown to express toll-like receptors, which activate the c-Jun NH2-terminal protein kinase (JNK) inflammatory pathway, leading to an increased expression of pro-inflammatory molecules such as tumor necrosis factor α (TNF-α)\textsuperscript{33,47}. Due to the close proximity of adipocytes and macrophages in adipose tissue, it is likely that released fatty acids from insulin resistant adipose tissue will activate macrophages, and subsequent release of TNF-α will in turn induce lipolysis in adipocytes, leading to a pathological feed-forward cycle that preserves inflammation and insulin resistance\textsuperscript{47,62}.

Obesity induced inflammation is characterized by the infiltration of adipose tissue with a variety of immune cells; including neutrophils\textsuperscript{63}, B cells\textsuperscript{64} and T cells\textsuperscript{64–66}. However, macrophages are the most robustly increased and are suggested to be key components of obesity-induced insulin resistance\textsuperscript{8,26,33,67}. Pro-inflammatory macrophages increase with obesity through two processes: (I) monocyte infiltration into adipose tissue\textsuperscript{8,33,47,66} and (II) switches in macrophage polarization, exhibited by a larger proportion of total macrophages displaying a pro-inflammatory phenotype\textsuperscript{26,47,67,68}.

Infiltrating macrophages in WAT release a variety of pro-inflammatory cytokines with paracrine and endocrine effects\textsuperscript{62}. Macrophages in obese WAT exhibit stimulation of JNK, extracellular signal-regulated kinase (ERK) and nuclear factor kappa B (NFκB) pro-inflammatory signaling\textsuperscript{47,62,69}. These pathways subsequently amplify WAT pro-inflammatory molecule expression and secretion in obesity, such as that of TNF-α\textsuperscript{47,62,69–71}. Increased TNF-α leads to a reduction in GLUT4 and WAT lipoprotein lipase expression\textsuperscript{71–73}, effectively reducing the availability of insulin stimulated glucose transporters and increasing circulating TAG molecules. These pro-inflammatory molecules also appear to have direct detrimental effects on
insulin signaling, as TNF-α has been found to inhibit insulin-stimulated IR autophosphorylation and IRS phosphorylation\textsuperscript{74–77}. Interestingly, a positive feedback system exists through which pro-inflammatory molecules propagate the inflammatory response by activating the pathways that produce them\textsuperscript{76}. Studies have shown a positive correlation between pro-inflammatory molecule expression and whole-body insulin sensitivity, as measured by increased plasma insulin, decreased glucose disposal and impaired inhibition of HGP\textsuperscript{70}. Moreover, inhibition of macrophage NFκB signaling promotes skeletal muscle and liver insulin sensitivity in high-fat fed mice, elucidating the impact of inflammation in whole-body insulin sensitivity\textsuperscript{69}.

1.3.4 Mitochondrial Dysfunction

Mitochondria are cellular organelles responsible for various metabolic processes geared towards ATP production, including oxidative phosphorylation and beta-oxidation\textsuperscript{78}. Importantly, insulin resistance is associated with mitochondrial dysfunction in both skeletal muscle and adipose tissue. Within skeletal muscle, mitochondrial content and size are reduced in obese and diabetic subjects when compared to lean individuals\textsuperscript{34,79}. Corresponding reductions in mitochondrial DNA and peroxisome proliferator activating receptor γ co-activator 1 alpha (PGC-1α) responsive oxidative phosphorylation genes have also been described\textsuperscript{9,80}. Functional consequences of these changes in skeletal muscle mitochondrial volume include reduced oxidative capacity and electron transport chain activity, an increased reliance on anaerobic metabolism and subsequent increases in lipid storage due to impaired beta oxidation\textsuperscript{9,34,81}. Therefore, mitochondrial dysfunction is believed to promote skeletal muscle lipotoxicity through incomplete lipid oxidation, further worsening insulin sensitivity\textsuperscript{82}. The oxidative capacity of skeletal muscle is in fact a superior predictor of insulin sensitivity over markers of lipotoxicity\textsuperscript{39}. However, changes in mitochondrial content and oxidative capacity may not play a causative role
in insulin resistance, potentially representing a secondary manifestation following the development of insulin resistance\textsuperscript{83}.

Similar to the changes observed in skeletal muscle, mitochondrial content is reduced in diabetic and insulin resistant mouse WAT and is inversely related to adiposity\textsuperscript{11,33,84}. PGC-1\textbeta and PGC-1\textalpha expression are also reduced in WAT, along with electron transport chain protein expression, mitochondrial DNA and oxidative capacity\textsuperscript{10,11,84}. The importance of PGC-1\textalpha in WAT was further explored using a tissue specific PGC-1\textalpha knockout model\textsuperscript{85}. Loss of PGC-1\textalpha worsened HFD-induced insulin resistance, which may be mediated through observed reductions in mitochondrial and thermogenic genes\textsuperscript{85}. HFD-induced mitochondrial dysfunction in WAT is suggested to be a result of increases in FFAs, as \textit{ex vivo} studies show similar reductions in mitochondrial protein expression following palmitate treatment\textsuperscript{10}. FFAs may overwhelm WAT mitochondrial oxidative capacity and result in accumulation of incompletely oxidized lipids, as has been shown in skeletal muscle. Studies in 3T3-L1 adipocytes using TNF\textalpha induced insulin resistance have shown that TNF\textalpha also induces mitochondrial dysfunction, with associated reductions in mitochondrial size, PGC-1\textalpha expression, membrane potential and intracellular adenosine triphosphate (ATP) production\textsuperscript{86}. Inhibition of NFkB inflammatory signaling has also been shown to improve insulin resistance induced mitochondrial dysfunction in 3T3-L1 adipocytes, through changes in mitochondrial fission and fusion and associated reductions in TNF\textalpha production. NFkB inhibition further restored insulin stimulated glucose uptake in insulin-resistant adipocytes\textsuperscript{87}. As such, inflammation likely plays a causative role in insulin resistance associated mitochondrial dysfunction in adipose tissue\textsuperscript{86,87}. However, reduced WAT mitochondrial content has been found to occur after impairments in glucose homeostasis have been established and therefore likely does not play a causative role in insulin resistance\textsuperscript{10}.  

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Regardless, a link between whole-body insulin resistance and mitochondrial dysfunction in the aforementioned tissues is well established, and mitochondrial biogenesis remains a potential target for anti-diabetic therapies.

1.4. Current Treatments

Given the increasing prevalence of diabetes\(^1\), it is important to explore potential treatment options in order to reestablish insulin sensitivity and glucose homeostasis. This necessity is made even more pressing given the understanding that insulin resistance is a prerequisite for the development of the metabolic syndrome and chronic cardiovascular complications leading to an increased risk of mortality\(^3\). Although exercise and dietary interventions have been shown to have beneficial effects on improving insulin sensitivity\(^88\text{-}90\), the inability of many diabetic individuals to commit to long-term lifestyle interventions illustrates the importance of developing pharmacological interventions\(^88\text{-}90\). Two commonly prescribed anti-diabetic drugs are thiazolidinediones (TZDs) and metformin (MET). Additionally, it has been determined that many nutraceuticals have insulin-sensitizing effects, with resveratrol (RSV) being the most highly studied.

1.4.1 Thiazolidinedione’s (TZDs)

The insulin sensitizing effects of TZDs are well established. TZDs significantly reduce plasma glucose and insulin concentrations in models of insulin resistance\(^11,91\text{-}93\). Moreover, TZDs increase whole-body insulin sensitivity and glucose disposal rates following treatment\(^91,92,94,95\). This is associated with improvements in insulin-induced effects on whole-body glucose and lipid metabolism which will be discussed below\(^94\).
TZDs are a family of selective peroxisome proliferator activating receptor gamma (PPARγ) agonists. PPARγ in turn is an important regulator of adipogenesis, lipid metabolism and mitochondrial genes. Therefore, it is not surprising that the insulin sensitizing effects of TZDs are mediated through these PPARγ targeted pathways. PPARγ is highly expressed in adipose tissue and, although TZDs promote insulin sensitivity at the liver and skeletal muscle, TZD activity appears to be localized to the adipose tissue. This tissue specificity is evidenced by the absence of TZD-induced insulin sensitizing effects in lipoatrophic mice, which lack adipose tissue.

Increased adipogenesis following TZD treatment results in increases in the number of adipocytes and body weight. The increase in adiposity appears to be localized to subcutaneous adipose tissue (scAT), which is significant given that visceral adiposity is associated with metabolic abnormalities. Lipid release from adipose tissue is reduced and lipid uptake is enhanced, while glyceroneogenesis (GNG), glycerol phosphorylation and TAG synthesis enzymes are upregulated in order to promote lipid deposition following TZD treatment. Specifically, the expression and activity of phosphoenolpyruvate carboxykinase (PEPCK) and glycerol kinase (GyK), key regulators of separate glycerol-3-phosphate producing pathways important in fatty acid re-esterification, are upregulated in WAT of human, rodent and cell culture models of insulin resistance following TZD treatment. Importantly, the expression of diacylglycerol acyltransferase-1 (DGAT1), which catalyzes the committed step in TAG synthesis, has also been shown to increase with TZD treatment. These changes effectively counteract the increased lipolysis associated with TZDs and reduces fatty acid secretion through promotion of FFA trapping in WAT. Increases in TAG synthesis regulating pathways in turn reduce local WAT inflammation by attenuating FFA-induced
increases in macrophage infiltration and inflammatory gene expression\textsuperscript{8,47,104}. Importantly, increased lipid storage capacity of WAT effectively reduces circulating lipids and ectopic lipid storage\textsuperscript{91–94,101}. Reduced ectopic lipid deposition may explain the whole-body insulin sensitizing effects of TZDs, given the role of lipotoxicity in insulin resistance.

TZD-induced increases in fat oxidation and energy consumption may also explain the reductions in circulating lipids and ectopic lipid storage\textsuperscript{97}. Obesity and insulin resistance are associated with reductions in WAT mitochondrial content and gene expression, resulting in a reduced capacity to oxidize fat and carbohydrates\textsuperscript{11,84,105–107}. Mitochondria are responsible for energy production to facilitate the aforementioned TAG synthesis and GNG processes. Treatment with TZDs has been shown to induce increases in WAT mitochondrial biogenesis and mitochondrial function, as evidenced by enhanced oxidative capacity, fat oxidation and non-shivering thermogenesis, effectively reversing the effects of diabetes and insulin resistance\textsuperscript{11,84,105–107}. Mitochondrial gene expression is positively correlated to plasma insulin and glucose levels, suggesting that mitochondrial biogenesis is essential to the insulin sensitizing effects of TZDs\textsuperscript{106}.

Mitochondria also appear to be important in the production of adiponectin, an adipocyte derived cytokine involved in tissue communication and regulation of whole-body metabolism and insulin sensitivity\textsuperscript{108}. Specifically, adiponectin has been shown to increase glucose uptake into skeletal muscle and improve insulin sensitivity through suppression of macrophage cytokine production and increased fatty acid oxidation\textsuperscript{95,104,109–112}. This has been supported by a high correlation between adiponectin secretion and the rate of glucose disposal and negative correlations with basal circulating insulin and TAG concentrations\textsuperscript{95,104,109–112}. Interestingly, circulating adiponectin is reduced in insulin resistant human and rodents and recovered following
associated increases in mitochondrial biogenesis with TZD treatment\textsuperscript{95,104,108,113}. The importance of adiponectin in mediating the insulin sensitizing effects of TZDs has been explored in adiponectin null mouse models\textsuperscript{114,115}. Reduced adiponectin secretion was associated with severe liver specific insulin resistance and exposure to a HFD resulted in the rapid induction of impaired glucose homeostasis\textsuperscript{115}. TZD induced improvements in glucose tolerance were in fact attenuated in adiponectin null mice, with associated reductions in liver and skeletal muscle AMPK activation\textsuperscript{115}. Higher doses of TZDs than required to elicit effects in control insulin resistant mice were found to be capable of eliciting insulin sensitizing effects in insulin resistant adiponectin null mice\textsuperscript{114}. However, mice lacking adiponectin did not experience decreases in HGP and improvements in insulin sensitivity were limited to increased skeletal muscle glucose uptake, while insulin resistant control mice exhibited improvements in both HGP attenuation and skeletal muscle glucose uptake\textsuperscript{114}. Therefore, it appears that TZDs are capable of improving insulin action through both adiponectin dependent and adiponectin independent mechanisms.

Although the insulin sensitizing effects of TZDs are promising, a multitude of adverse side effects have been identified. TZDs are associated with weight gain, hepatic toxicity and increased risk of congestive heart failure, myocardial infarction, osteoporosis and bladder cancer\textsuperscript{14,116–119}. Furthermore, there is an increase in the risk of mortality as a result of these complications\textsuperscript{14,116–119}.

\subsection*{1.4.2 Metformin (MET)}

MET is a commonly prescribed anti-diabetic drug that has been shown to improve glucose homeostasis\textsuperscript{120}. This is supported by improvements in glucose tolerance and reduced post-meal plasma glucose following metformin treatment\textsuperscript{120–124}. In addition, MET reduces
fasting and basal plasma TAG, glucose and insulin concentrations in models of diabetes and high-fat feeding\textsuperscript{12,124–127}. These effects are thought to occur through two primary mechanisms; improved peripheral glucose uptake\textsuperscript{121} and reduced HGP\textsuperscript{12,126}.

Given the improved rate of glucose disposal during glucose tolerance testing, it is not surprising that MET improves peripheral glucose uptake\textsuperscript{121,125,127}. As such, increases in skeletal muscle\textsuperscript{12,125}, cardiac\textsuperscript{128,129} and liver\textsuperscript{130} glucose clearance have all been found to occur following MET treatment. The fraction of glucose disposal accounted for by muscle has been found to increase\textsuperscript{12} and MET induced improvements in muscle glucose uptake provides an additive effect to insulin stimulation\textsuperscript{125}. MET counteracts insulin-induced skeletal muscle, cardiac and liver FFA incorporation into TAGs and prevents suppression of FFA oxidation\textsuperscript{125,128,131}, which may induce improvements in insulin sensitivity through reduced lipotoxicity. However, although significant, these lipid effects were minor and therefore are unlikely to be the primary mechanism through which MET increases glucose disposal. Research using cardiomyocytes shows that MET increases Akt phosphorylation and reduces the rate of GLUT4 endocytosis post-insulin stimulation\textsuperscript{129}. Increased IR tyrosine kinase activity in both muscle and liver treated with MET suggest that MET increases insulin action in these tissues\textsuperscript{120,130}. The metabolic fate of increased glucose taken into peripheral tissues remains controversial, as some studies show increased muscle glycogen content\textsuperscript{123,130} while others identify increased glucose oxidation\textsuperscript{121,122}.

Although many studies have shown improved peripheral glucose uptake and insulin sensitivity, the primary mechanism of MET action appears to be through reduced HGP\textsuperscript{12,122,126}. MET has been shown to reduce basal and glucagon-induced glucose output\textsuperscript{126}. This effect is likely the result of observed reductions in hepatic gluconeogenic transcription factor activity and subsequent suppression of PEPCK and glucose-6-phosphatase (G6Pase) expression, two key
regulatory enzymes of glucose production\textsuperscript{132}. Moreover, there appears to be a high correlation between plasma glucose concentration and hepatic glucose output before and after administration of MET\textsuperscript{12}, suggesting that the reduced HGP is essential to the glucose lowering effects of MET.

MET treatment results in the dose-dependent accumulation of AMP and an increase in the AMP:ATP ratio in the liver through direct inhibition of complex I of the electron transport chain and subsequent impairments in oxygen consumption\textsuperscript{126,133–135}. This reduced energy state may be essential for mediating the beneficial effects of MET on insulin resistance and plasma glucose concentration, as inhibition of the electron transport chain attenuates HGP and increases skeletal muscle glucose utilization in a similar manner to that observed with MET\textsuperscript{135}. AMP acts as an allosteric inhibitor or activator of various molecules, including adenylate cyclase (AC) and AMP-activated protein kinase (AMPK), respectively\textsuperscript{126,136}. AC is responsible for mediating the formation of cyclic-AMP from ATP and subsequent protein kinase A activation\textsuperscript{126}. MET effectively reduces glucagon-induced glucose output through AMP induced AC inhibition, as cyclic-AMP is a required second messenger in glucagon signalling\textsuperscript{126}. Additionally, AMPK is an energy responsive enzyme that is indirectly activated by MET treatment in liver, muscle and the heart\textsuperscript{125,129,131,136}. AMPK regulates glucose and lipid metabolism and increases in AMPK activity have been shown to inhibit HGP gene expression and stimulate FFA oxidation and glucose uptake\textsuperscript{123,132}. MET-induced inhibition of HGP is dependent on AMPK activation\textsuperscript{125}.

In contrast to TZDs, MET does not adversely effect cardiac function and is associated with weight loss and infrequent, minor adverse effects\textsuperscript{12,120,125,128,136}. These include gastrointestinal symptoms, such as diarrhea and nausea\textsuperscript{124,127,137}. Due to the low risk of MET treatment it has also been examined as a potential preventative therapy in individuals at risk of diabetes, resulting in reduced fasting plasma glucose and incidence of diabetes similar to life-
1.4.3 Combination Therapy: Thiazolidinedione’s & Metformin

Combinations of various pharmacological treatments have been widely examined\(^{138}\). As the primary defects in impaired glucose homeostasis are attenuated insulin-stimulated glucose uptake and increased HGP, utilizing individual therapies that target these pathways in combination will enhance the efficacy of treatment. Numerous studies have directly compared the effects of TZDs and MET *in vivo*, supporting improvements in whole-body insulin sensitivity and plasma glucose concentrations, with MET acting through reduced HGP and TZDs increasing muscle and WAT glucose uptake\(^{13,79,139–141}\). However, TZDs induced greater improvements in insulin stimulated glucose disposal\(^{13,79,139,140}\). When utilized in combination, fasting plasma glucose decreased to a greater extent than that of either therapy independently\(^{13}\). Importantly, combination therapy also increased the rate of glucose disposal and decreased post-prandial plasma glucose compared to when given separately\(^{13}\). However, the impact of combination therapy on TZD-induced adverse effects has not been thoroughly examined. Therefore, it is important to examine alternative molecules that may provide additive beneficial effects in combination with MET without increasing the risk of adverse effects.

1.5. Resveratrol (RSV)

Resveratrol (RSV) is a polyphenol found in many plant species, red grapes and red wine\(^{16,17}\). Within plants, RSV protects against fungal infections, functioning as a phytoalexin\(^{142}\). Recently, RSV has gained attention for proposed anti-aging and anti-diabetic effects\(^{143,144}\).
Prophylactic administration of RSV produces a protective effect against genetic and diet-induced insulin resistance. This effect has been supported with findings of improved whole-body insulin sensitivity, reduced plasma glucose and insulin levels and increased plasma adiponectin\textsuperscript{15,143,145}. Beneficial effects of RSV do not appear to be limited to one mechanism of action, providing diverse outcomes in various tissues that together promote whole-body insulin sensitivity. Although RSV-induced improvements in insulin sensitivity are partially mediated through enhanced mitochondrial biogenesis similar to the previously defined pharmacological interventions, prophylactic administration of RSV does not appear to be tissue specific, as it induces similar mitochondrial effects in skeletal muscle\textsuperscript{145}, liver\textsuperscript{143} and adipose tissue\textsuperscript{15,145,146}. Moreover, RSV treatment enhances whole body energy expenditure, in the absence of changes in respiratory exchange ratio and activity\textsuperscript{145,146}.

The mechanism of action through which RSV establishes metabolic changes remains controversial. The most commonly described mediator of RSV is activation of PGC-1\textalpha\textsuperscript{145,147}. However, the mechanism through which RSV stimulates PGC-1\textalpha is not clear, as studies have shown that either sirtuin 1 (SIRT1), a deacetylase that regulates PGC-1\textalpha acetylation, or AMPK may be responsible\textsuperscript{143,146–149}. Studies have shown that SIRT1 is directly activated by RSV, enhancing deacylation activity\textsuperscript{148,149} which increases PGC-1\textalpha activity in liver and skeletal muscle resulting in increased mitochondrial biogenesis\textsuperscript{143,145,147}. Moreover, SIRT1 knockdown was found to block RSV-induced expression of PGC-1\textalpha regulated mitochondrial genes in muscle\textsuperscript{145}. However, recent work has shown that measures of SIRT1 activation by RSV \textit{in vitro} are fraught with technical issues, as problems with activity assays have been identified\textsuperscript{148,149}, and \textit{in vivo} work suggests that SIRT1 may play a small role in comparison to AMPK in regulating insulin sensitizing effects of RSV\textsuperscript{147,150}. Alternatively, AMPK phosphorylation is increased by
RSV in liver, skeletal muscle and WAT\textsuperscript{143,147}. AMPK knockout and inhibitor studies have shown that RSV induced improvements in insulin sensitivity, metabolic rate and skeletal muscle mitochondrial biogenesis are AMPK dependent\textsuperscript{144,147}. Importantly, AMPK knockout prevents RSV-induced decreases in PGC-1\(\alpha\) acetylation and SIRT1 knockout cells conserve RSV-induced AMPK activation and subsequent PGC-1\(\alpha\) expression\textsuperscript{147}. Therefore, AMPK appears to regulate SIRT1 activity upstream, which is believed to prolong a state of increased PGC-1\(\alpha\) activity\textsuperscript{147}. However, recent work in PGC-1\(\alpha\) knockout mice shows that although PGC-1\(\alpha\) is necessary for skeletal muscle mitochondrial biogenesis following RSV treatment, improvements in energy expenditure and body composition are independent of PGC-1\(\alpha\)\textsuperscript{146}. Therefore, the exact mechanisms through which RSV acts to produce an improved metabolic state require further consideration.

Within skeletal muscle, RSV-induced mitochondrial biogenesis and content has been well described\textsuperscript{145–147}. Skeletal muscle mitochondrial respiration rates and oxidative capacity were enhanced in glycolytic fibers following treatment\textsuperscript{145,151}. These functional effects are stimulated by increased expression of genes involved in electron transport, oxidative phosphorylation and uncoupling\textsuperscript{145,146,151}. Increases in skeletal muscle basal glucose uptake have been described and found to be dependent on AMPK activation. Moreover, RSV augments insulin-stimulated glucose uptake through AMPK dependent increases in Akt signaling\textsuperscript{144,151}. However, the role of skeletal muscle in RSV-induced whole-body metabolic effects appears to be minor\textsuperscript{146}.

In the liver, RSV promotes mitochondrial biogenesis through deacetylation of PGC-1\(\alpha\) in the absence of changes in PGC-1\(\alpha\) expression. Deacetylation results in PGC-1\(\alpha\) activation, subsequent increases in mitochondrial content and changes in TCA cycle and glycolysis pathways\textsuperscript{143,145}. As such, RSV-induced mitochondrial biogenesis in the liver appears to be
dependent on SIRT1, rather than AMPK as was found following MET treatment. In addition to promoting mitochondrial biogenesis, RSV attenuates increases in liver weight and hepatic lipid droplet accumulation. This is largely explained through reduced expression of genes responsible for lipogenesis and FFA re-esterification in the liver, likely reducing obesity-induced lipotoxicity.

Adipose tissue plays an important role in RSVs beneficial effects on whole-body insulin sensitivity. Mitochondrial biogenesis in WAT is promoted through PGC-1α and subsequent increases in the expression of mitochondrial genes similar to TZDs, although the mechanisms of action of these molecules are functionally distinct. However, RSV effects are not limited to WAT, as PGC-1α and uncoupling protein-1 (UCP-1) gene expression increased in both WAT and brown adipose tissue, as did mitochondrial size and density of mitochondrial cristae. scAT and retroperitoneal white adipose tissue exhibit increased mitochondrial respiration, which is associated with increased protein content of cytochrome c oxidase subunit IV (COX IV). In parallel with increased mitochondrial biogenesis, RSV induces enhanced GNG and adiponectin secretion in WAT, again mirroring the effects of TZDs. Moreover, increased lipolytic rate and re-esterification was observed in scAT with RSV.

In keeping with the multifactorial causes of insulin resistance, it is of note that RSV prevents pharmacological (lipopolysaccharide) and diet-induced production of TNF-α, reducing inflammation-stimulated changes in adipokine expression and enhancing insulin action through IR, IRS1 and Akt phosphorylation. Although no differences in body weight were observed in most studies, RSV decreases body fat content through reductions in retroperitoneal, subcutaneous and epididymal adipose depot size. This may be partially explained through reductions in basal and insulin-stimulated lipogenesis from glucose in adipocytes.
Therefore, RSV effectively prevents the development of the three main contributors to insulin resistance through reduced lipotoxicity in liver, decreased inflammation in WAT and increased mitochondrial biogenesis in skeletal muscle, liver and WAT.

Despite the overwhelming evidence supporting protective anti-diabetic effects of RSV listed above, few studies have examined the treatment potential of RSV in animal models of established insulin resistance. Chemically (streptozotocin-nicotinamide) induced type 1 diabetic rats display reduced plasma glucose, reduced markers of inflammation including TNFα, and increased plasma insulin following RSV treatment\(^\text{155,157}\). These improvements are associated with increased hepatic glycogen content and changes in hepatic activity of carbohydrate metabolism regulating enzymes, including glycogen phosphorylase, glycogen synthase, pyruvate kinase, fructose-1,6-bisphosphatase, hexokinase and G6Pase\(^\text{157}\). Similarly, a genetic model of obese rats exhibited improvements in plasma glucose and insulin concentrations and whole-body insulin sensitivity following 4 weeks of treatment\(^\text{16}\). Plasma FFA and TAG concentrations were also reduced in parallel with reductions in hepatic lipid content\(^\text{16}\). RSV treatment decreases visceral fat content and TNFα expression and increases adiponectin expression\(^\text{16}\). Therefore, WAT appears to be a major contributor to the metabolic effects of RSV treatment, as was determined in prophylactic studies. Interestingly, although many prophylactic studies have examined the protective effects of RSV in animal models of diet-induced insulin resistance, to our knowledge no diet-induced insulin-resistant animal models have assessed the treatment potential of RSV.

Although beneficial, anti-diabetic effects of RSV have been supported in animal and in vitro models, human data is scarce and inconsistent\(^\text{158,159}\). Clinical trials have shown that RSV improves energy expenditure and resting metabolic rate in diabetic subjects, with no
corresponding increases in average daily activity\textsuperscript{160}. This was associated with increases in skeletal muscle SIRT1 and AMPK expression\textsuperscript{160}. Moreover, RSV has been found to reduce lipoprotein production and post-prandial glucagon responses in diabetic subjects\textsuperscript{161,162}. The insulin sensitizing effects of RSV are however controversial in humans, with many studies finding no significant improvements in insulin sensitivity, glucose production or oxidation, and energy expenditure in obese, non-diabetic subjects following RSV treatment\textsuperscript{158,161}. These finding were further supported by no observable changes in ectopic lipid deposition, plasma lipid concentrations or inflammation following treatment\textsuperscript{158,161}. Given the inconsistent findings in human studies, as well as between studies in obese subjects rather than diabetic, a meta-analysis set out to evaluate RSV insulin sensitizing effects by investigating the findings of eleven clinical trials comprising approximately 400 subjects\textsuperscript{159}. It was determined that RSV significantly reduced fasting glucose and insulin concentrations and improved insulin sensitivity in diabetic subjects, but had no significant effects on non-diabetic subjects, explaining the inconsistent results outlined above\textsuperscript{159}. Moreover, the administration of RSV to diabetic individuals concurrently taking metformin and other hypoglycemic agents was found to improve glycemic control compared to the hypoglycemic agents alone, supporting potential synergistic effects of RSV in improving insulin sensitivity in humans\textsuperscript{163}.

Although the beneficial effects and mechanisms of action of RSV are highly supported, studies examining the absorption and metabolism of RSV suggest that \textit{in vivo} bioavailability is low. RSV degradation is highly sensitive to light, pH and temperature. Transition to the less active cis-RSV isoform occurs rapidly upon exposure to light, while degradation is exponentially increased when exposed to an alkaline pH of 7.4\textsuperscript{164}. Moreover, temperature has no effect on RSV when maintained in acidic pH, although degradation in an alkaline environment is accelerated.
when temperature is raised over 25°C\textsuperscript{164}. Although the rate of oral RSV absorption is high, it is rapidly metabolized \textit{in vivo} into one of two metabolites, namely trans-resveratrol-3-sulfate (R3S) and trans-resveratrol-3-glucuronide (R3G), in the liver, intestines and lungs of humans and rodents prior to entering systemic circulation\textsuperscript{165–169}. Approximately 70% of absorbed RSV is metabolized, resulting in very low amounts of free RSV in plasma for tissue delivery\textsuperscript{166,170}. In fact, the majority of absorbed RSV is metabolized to R3G in the small intestine before entering the blood stream\textsuperscript{165}. These metabolites have increased solubility and, although they exhibit reduced cell permeability, the \textit{in vivo} effects of RSV regardless of low bioavailability suggests that R3G and R3S may in fact be the bioactive forms of RSV\textsuperscript{166,170}. Importantly, R3S possesses biological activity, while biological activity of R3G has yet to be reported\textsuperscript{168}. A recent study has compared the concentrations of these metabolites in liver, adipose tissue and skeletal muscle of rats and found that the greatest concentration of metabolites were found in liver, followed by adipose tissue and low amounts in skeletal muscle\textsuperscript{171}. The large concentration of metabolites in liver is not surprising, given the role of the liver in metabolizing RSV, while the intermediate concentrations in adipose tissue suggest potential targeting of adipose tissue by RSV. Given the low bioavailability of RSV, recent studies have focused on identifying means of increasing bioavailability of free RSV through structural modifications or modified delivery\textsuperscript{170,172,173}.

\textit{1.5.1 Combination Therapy: Potential of Resveratrol}

The low potency of RSV and correspondingly high doses required to elicit beneficial effects establish a need to develop means of increasing its metabolic effectiveness. Using RSV in conjunction with a pharmacological anti-diabetic therapy represents an interesting therapeutic approach, as RSV induces similar metabolic effects to TZDs in WAT and TZDs are successfully used with other medications\textsuperscript{15,138}. Unlike TZDs, which are known to increase weight gain and the
risk of cardiovascular mortality\textsuperscript{14,116–118}, RSV is well-tolerated in animals and produces beneficial effects in the absence of adverse side effects\textsuperscript{145}. RSV has in fact been found to produce cardioprotective effects such as decreased blood pressure and increased aortic mitochondrial biogenesis to pre-diabetic levels in diabetic mouse models\textsuperscript{16,155,174,175}.

MET is a well-tolerated pharmaceutical that produces anti-diabetic effects through targeting HGP and skeletal muscle insulin sensitivity, in the absence of WAT effects observed with RSV. Given that MET has separate mechanisms of action and is known to produce beneficial effects when provided in combination with TZDs\textsuperscript{138}, MET provides an ideal pharmaceutical to test in combination with RSV. One study to date has examined the therapeutic potential of RSV and MET in combination. However, hydroxymethylbutyrate (HMB), a leucine metabolite, was also used in this examination of RSV and MET combination, as it was previously shown to synergistically improve insulin sensitivity with RSV at concentrations that had no sensitizing effects independently. MET, RSV and HMB combination therapy was found to increase fat oxidation and both AMPK and SIRT1 activity in adipocyte and skeletal muscle cell cultures when compared to each compound in isolation\textsuperscript{176}. In a mouse model of genetically induced diabetes, combination therapy increased insulin sensitivity and decreased both visceral fat and liver weight, although no improvements were seen with each compound independently at the selected doses\textsuperscript{176}. Therefore, RSV could not only provide a synergistic therapeutic potential to MET, but also reduce the required dose necessary to promote anti-diabetic effects. However, this study failed to examine the mechanisms through which these therapies acted to increase insulin action and to our knowledge no studies have examined these effects in a more physiologically relevant model, such as diet-induced insulin resistance. As such, more research is required to validate these findings in models of diet-induced diabetes.
Table 1.1: Summary of the therapeutic potential of TZDs, MET and RSV on factors deemed to be important in the development of insulin resistance.

<table>
<thead>
<tr>
<th></th>
<th>Thiazolidinedione’s (TZDs)</th>
<th>Metformin (MET)</th>
<th>Resveratrol (RSV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Target</strong></td>
<td>WAT 88,93</td>
<td>Liver 12,114</td>
<td>WAT</td>
</tr>
<tr>
<td><strong>Body Weight</strong></td>
<td>▲ 94.96</td>
<td>▼ 12,112</td>
<td>▼ 15,138,139</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td>▲ 46,100</td>
<td>=</td>
<td>▼ 15,138,140,149</td>
</tr>
<tr>
<td><strong>Adiposity</strong></td>
<td>▲ (scAT) 94.96</td>
<td>=</td>
<td>▼ 15,138,140,149</td>
</tr>
<tr>
<td><strong>HGP</strong></td>
<td>▼ 105,106</td>
<td>▼ 12,114</td>
<td>▲ 15</td>
</tr>
<tr>
<td><strong>Plasma Adiponectin</strong></td>
<td>▲ 105,106</td>
<td>=</td>
<td>=</td>
</tr>
</tbody>
</table>

1.6. Conclusion & Future Directions

Insulin resistance is a multifactorial pathology characterized by lipotoxicity, inflammation and mitochondrial dysfunction. As insulin resistance is a prerequisite for many other pathological conditions, such as type 2 diabetes and the metabolic syndrome, it is important to explore potential therapeutic options. Although weight loss and life-style interventions are successful in reversing insulin resistance, it is often difficult for individuals to commit to prolonged exercise or dietary interventions. As such, many pharmacological interventions have been developed to improve insulin resistance. However, many current medications have varying adverse side effects, from MET-induced gastrointestinal discomfort to the increased risk of cardiovascular related mortality associated with TZDs. RSV, a natural polyphenol, provides an interesting therapeutic approach, as many studies have shown anti-diabetic effects. However, RSV has a very low bioavailability and as a result requires high daily intake to achieve the described effects. Moreover, more work is required to establish a treatment effect in animal models of diet-induced insulin resistance, as most work has focused on utilizing RSV in prevention through prophylactic administration.

Future work is required to identify means of reducing the adverse effects of current medications, or alternatively improving the effectiveness of natural compounds. Studies have been performed to examine RSV analogues in an attempt to increase biological activity, while
medications are commonly used in combination therapy to improve insulin sensitivity. Utilizing RSV in combination with MET could provide various synergistic effects. RSV represents an ideal adjunct for use in combination with MET, as RSV may target the same metabolic processes as TZDs, although through separate means, and would remove the associated adverse effects. Although RSV has low potency, using it alongside MET could reduce the required dose through targeting multiple contributors of insulin resistance, given the divergent mechanisms of these compounds.
Chapter 2: Aims of Thesis

The whole-body and tissue specific anti-diabetic effects of RSV and MET have been widely examined. MET primarily targets HGP and is known to produce synergistic effects with other anti-diabetic therapies, while RSV targets adipose tissue inflammation, mitochondrial biogenesis and adiponectin secretion as well as skeletal muscle mitochondrial biogenesis. However, RSV effects have been largely supported in preventative models, and support of therapeutic effects in treatment of established insulin resistance is lacking. Moreover, the potential for RSV and MET to produce synergistic beneficial effects on glucose homeostasis has yet to be evaluated.

Therefore, the purposes of this thesis were to:

1. To examine the treatment effects of RSV and MET, independently and in combination, on whole-body glucose homeostasis in a mouse model of diet-induced insulin resistance;
2. To examine the preventative effects of RSV and MET, independently and in combination, on the development of diet-induced insulin resistance;
3. To identify if changes in mitochondrial content, inflammation or HGP could explain any differences in insulin resistance following RSV and MET treatment.

It was hypothesized that:

1. RSV and MET will synergistically improve whole-body insulin and glucose tolerance in a model of established insulin resistance;
2. Prophylactic treatment with RSV and MET will both prevent the development of impairments in glucose homeostasis with a HFD and the combined effects will be greater than either treatment alone;
3. The treatment and prevention of impaired glucose homeostasis with RSV or MET will be associated with enhanced WAT and skeletal muscle insulin signaling, mitochondrial content, attenuated inflammation and reductions in enzymes involved in HGP. These effects will be greater with combined treatment than either compound independently.
Chapter 3: Resveratrol and Metformin Combination Therapy in Prevention and Treatment of Insulin Resistance

3.1 Methods

3.1.1 Materials

Trans-resveratrol (CAT# 70675) and metformin (CAT# 13118) were purchased from Cayman Chemicals (Ann Arbor, MI). Low-fat (LFD: 10% kcal from fat; CAT# D12450B) and high-fat (HFD: 60% kcal from fat; CAT# D12492) diets (Table 3.1) were purchased from Research Diets (New Brunswick, NJ). Insulin was from Eli Lilly and glucose from BioShop. Reagents, molecular weight marker and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad. Antibodies against and GAPDH (CAT# ab8245) was obtained from Abcam, PEPCK (CAT# 10004943) was from Cayman Chemicals and G6Pase (CAT# 25840) was from Santa Cruz. Total Akt (CAT# 4685), p-AKT threonine 308 (CAT# 9275), p-AKT serine 473 (CAT# 9271), total JNK (CAT# 9252), P-JNK (CAT# 4671), total ERK (CAT# 4695S), P-ERK (CAT# 9101S), total p38 (CAT# 9212S) and P-p38 (CAT# 9211S) antibodies were from Cell Signaling (Danvers, MA). Western Lightening Plus Enhanced Chemiluminescence substrate was purchased from Perkin Elmer (CAT# NEL105001EA). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). SuperScript II Reverse Transcriptase, random primers and dNTP were products from Invitrogen (Burlington, ON). RNeasy mini kits and Quiazo were from Quiagen. Taqman gene expression assays for mouse GAPDH (CAT# 4352932) and adiponectin (CAT# Mm00456425) were from Applied Biosystems.
Table 3.1: Macronutrient composition of the low and high fat diets used, measured as both a percentage of total grams and percentage of total kilocalories. Total kcal/g is also displayed.

<table>
<thead>
<tr>
<th></th>
<th>LFD</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent of total mass (g%)</td>
<td>Percent of total kcal (kcal%)</td>
</tr>
<tr>
<td>Protein</td>
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<tr>
<td>Carbohydrate</td>
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<tr>
<td>Fat</td>
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<td>10</td>
</tr>
<tr>
<td>Total kcal/g</td>
<td>3.85</td>
<td></td>
</tr>
</tbody>
</table>

3.1.2 Experimental Design

8 week old male C57BL/6 mice were purchased from Charles River (Wilmington, MA) and individually housed with a 8:00 to 20:00 12:12 hour light:dark cycle and *ad libitum* access to standard rodent chow and water. All protocols were approved by the University of Guelph Animal Care Committee and met the Canadian Council on Animal Care (CCAC) guidelines. Following 1 week of acclimatization, the standard rodent chow was replaced by either LFD (*n*=12; 10% kcal from fat) or HFD (*n*=40; 60% kcal from fat). Although a 60% kcal from fat HFD is not representative of the typical diet in obese humans, we are using this model to induce obesity and ensure impairments in glucose homeostasis are achieved. This HFD formulation has been suggested for use with the C57BL/6 mouse model and we have previously used this diet to induce insulin and glucose intolerance. Animal and food weights were monitored one and three days/week, respectively. The dietary intervention continued for 9 weeks, at which point glucose and insulin tolerance testing (IPGTT and IPITT) were performed, 48 hours apart, in order to verify differences in glucose homeostasis between LFD and HFD fed animals (Figure 3.1). The HFD animals were randomly assigned to 4 groups, HFD control, HFD - metformin (MET; 250 mg/kg/day), HFD - resveratrol (RSV; 100 mg/kg/day), and a combination of both (COM; MET: 250 mg/kg/day and RSV: 100 mg/kg/day). Animals were matched so body
weights and measures of glucose and insulin tolerance were similar between groups at the onset of treatment (Appendix A). Drug treatments were mixed into food weekly, with drug dilutions determined based on average daily food consumption and body weight within each group (Appendix B). Doses were selected based on pilot work comparing effects of various metformin and resveratrol doses on glucose and insulin tolerance (Appendix C and D). During the fifth week of drug treatment IPGTT and IPITT protocols were repeated, with 48 hours between tests.

In a separate study, 8 week old male C57BL/6 mice were assigned into the aforementioned groups (LFD, HFD, MET, RSV and COM; \(n=11\)/group) at the onset of the HFD intervention in order to examine the preventative effects of treatment during the development of insulin resistance (Figure 3.1). Animal and food weights were monitored one and three days/week, respectively. The dose of each drug was equal to that of the treatment study in order to allow comparisons to be made between the treatment and prevention studies. IPGTTs and IPITTs were performed in these mice during week thirteen of the dietary intervention (Figure 3.1).

![Figure 3.1: Time-line of the experimental design. The design of the treatment study is displayed above the line, while the prevention study is below the line. The initiation of the HFD and drug interventions are displayed, along with the times of GTT and ITT measures.](image-url)
3.1.3 Glucose and Insulin Tolerance Testing

Intraperitoneal (IP) glucose (IPGTT) and insulin (IPITT) tolerance testing were performed as measures of glucose homeostasis. The IPGTT assesses the capacity of animals to clear a glucose load. Mice were fasted for 6h prior to an IP injection of glucose (2 g/kg body weight) for the IPGTT. Blood glucose concentrations were determined via tail vein sampling at baseline and 15, 30, 45, 60, 90 and 120 minutes post-injection using a hand-held glucometer (Freestyle Lite, Abbott).

The IPITT measures the ability of insulin to reduce blood glucose concentrations and is a rough measure of whole body insulin action. Food was removed immediately prior to an IP injection of insulin (0.5 U/kg). Blood glucose concentrations were determined via tail vein sampling at baseline and at 15, 30, 45, 60, 90 minutes as described above. IPGTT and IPITT measures were performed 48 hours (2 days) apart, with ad libitum access to their respective diets maintained between tests. Changes in glucose over time were plotted per mouse, and the average total area under the curve (AUC) for each group was calculated using the trapezoid rule. As such, \( \Delta X \times (Y_1 + Y_2)/2 \) represents the AUC between two corresponding plots, with the \( \Delta X \) denoting the time difference between two plots and the \( Y \) values representing the mmol/L concentration of glucose at each plot. Therefore, AUC is presented in mmol/L*time. Baseline was set to \( Y=0 \).

For the treatment study, the relative difference in IPGTT and IPITT measures from HFD were calculated with treatment groups and MET and RSV measures were used to predict the effects of combined therapy. HFD was set to 100 in order to allow differences from treatment groups to be observed as a percent change. Observed COM and predicted combined effects on IPGTT and IPITT measures were then compared.
3.1.4 Metabolic Caging

Two days following the final IPGTT and IPITT (week 13 and week 12 in the treatment and prevention studies, respectively) mice were placed in a Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH) for a 24-hour period beginning at 7:00 am. The light:dark cycle was maintained as described above, and water and respective diets were provided ad libitum while in the CLAMS. Oxygen consumption (VO2), respiratory exchange ratio (RER), heat production and activity levels were determined. VO2 and activity are measured directly, while RER and heat production are calculated by the CLAMS. As such:

\[
RER = \frac{\text{Carbon Dioxide Production (VCO2)}}{\text{Oxygen Consumption (VO2)}}
\]

Heat production = \((3.815 + 1.232 \times RER) \times VO2\)

VCO2 used in calculation of RER is measured directly. The calculation for heat is based off a least squares linear fit model and VO2 must be in liters/hour to yield a heat value in kcal/hour. Measures were not analyzed from the first 6 hours of the light cycle, allowing time for the mice to acclimate to the metabolic caging. The light cycle data was averaged or summed (for activity) per animal over a 6 hour period, from 14:00 – 20:00, and dark cycle data was averaged or summed over a 12 hour period, from 20:00 – 7:00.

3.1.5 Terminal Procedures

Mice were anesthetized with sodium pentobarbital (5mg/100g body weight, IP injection) in week 14 in the treatment study and week 13 in the prevention study. Given our interest in insulin action and glucose homeostasis, terminal surgeries were performed in order to allow examination of tissue specific insulin action. Inguinal subcutaneous and epididymal adipose tissue depots as
well as triceps from the left side of mice were excised, weighed and flash frozen or fixed in formalin. Mice were then injected with insulin (1.25 U/kg) and 10 minutes post injection subcutaneous and epididymal adipose tissue and triceps from the right side of mice, as well as liver were excised, weighed and flash frozen. Intrathoracic blood was collected at this time. Tissues were stored at -80°C until further analysis.

3.1.6 Histological Analysis

Epididymal adipose tissue was fixed in 10% neutral-buffered formalin (VWR, Mississauga, ON, Canada), dehydrated in 70% ethanol (Fisher Scientific) and embedded in paraffin. Five micrometer sections were mounted on 1.2mm Superfrost slides, stained with Harris hematoxylin and eosin stock (Fisher Scientific) and imaged (Olympus FSX 100 light microscope, Olympus, Tokyo, Japan). Cells (>100/image) were sampled in each image (2 images/mouse) to determine cross-sectional area (>200 cells/mouse) (ImageJ software, National Institute of Mental Health, Bethesda, MD).

3.1.7 Western Blotting

Samples were homogenized in 3 (adipose tissue), 20 (skeletal muscle), or 30 (liver) volumes of NP40 Cell Lysis Buffer (Life Technologies; CAT# FNN0021) supplemented with Phenylmethylsulfonyl Fluoride and Protease Inhibitor Cocktail (Sigma; CAT# 78830, CAT# P2714) using a FastPrep-24 Tissue Homogenizer (MP Biomedicals). Homogenized samples were centrifuged at 4°C for 10 minutes at 1,500 X g and the supernatant collected from liver and skeletal muscle, while the infranatant was collected from adipose tissue. A Bicinchoninic Acid assay was performed to determine protein content of the homogenate. Equal amounts of protein were then electrophoretically separated on 10% SDS-PAGE gels and then transferred to
nitrocellulose membranes at 100V for 1 hour at 4°C. Membranes were then blocked for 1 hour at room temperature in 5% non-fat dry milk-TBST (tris buffered saline/0.1% tween 20). Membranes were then incubated in primary antibody diluted 1:1000 in 5% BSA (Bovine Serum Albumin)-TBST overnight with gentle agitation at 4°C. Following a 1-hour incubation at room temperature with appropriate donkey anti-rabbit or goat anti-mouse HRP-conjugated secondary antibodies (diluted in 1% non-fat dry milk-TBST), membranes were briefly washed and proteins visualized by Western Lightning Plus-ECL using a Flourochem HD2 imager (Cell Biosciences) and bands quantified using Alpha Innotech software (Santa Clara, CA). A housekeeping protein (GAPDH) was measured in each gel to ensure equal loading. Phosphorylated protein measures were made relative to total protein content of the protein of interest.

3.1.8 RT-PCR

Changes in mRNA expression of adiponectin were determined using real time qPCR as described in detail previously by our laboratory\textsuperscript{15}. RNA was isolated from inguinal subcutaneous and epididymal adipose tissue using the Qiagen RNeasy kit according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized from 1µg of total RNA using SuperScript II Reverse Transcriptase, random primers and dNTP. Real time PCR was carried out using the CFX Connect Real-Time PCR Detection System (Bio-Rad). Each well contained 1 µL of cDNA template, 8 µL of RNase free water, 1 µL gene expression assay and 10 µL of Taqman Fast Universal PCR Master Mix. Relative differences between groups were determined using the $2^{-\Delta\Delta CT}$ method\textsuperscript{178}. The amplification efficiencies of the gene of interest and the housekeeping gene were equivalent and the expression of GAPDH did not change between groups.
3.1.9 Statistical Analysis

A two-way repeated measures ANOVA (diet and time), followed by Tukey’s post-hoc analysis, was used to examine differences in LFD and HFD IPGTT and IPITT curves at individual time-points (0, 15, 30, 45, 60, 90 and 120 minutes) at 9 weeks of the treatment study. An unpaired one-tailed t-test was performed to measure differences between LFD and HFD IPGTT and IPITT AUC’s prior to drug-intervention in the treatment study. A one tailed t-test was chosen a priori based on the prevalence of literature demonstrating impaired glucose and insulin tolerance following long-term HFD\textsuperscript{26,32,177}.

One-way ANOVA with Tukey’s post-hoc analysis was used to determine differences in body and tissue weights, eWAT cell size, metabolic caging outcomes, liver Akt phosphorylation, inflammatory protein phosphorylation, protein content of HGP regulating enzymes and mitochondrial proteins and adiponectin expression. To examine differences in Akt phosphorylation between groups (LFD, HFD, MET, RSV and COM) and within groups (basil vs. insulin stimulated) in triceps, eWAT and scAT, a two-way repeated measures ANOVA with Tukey’s post-hoc analysis was used.

When data were not normally distributed, as measured with Shapiro-Wilk normality testing, log transformation was performed in order to achieve normal distribution. If normality was not achieved, a non-parametric Kruskal-Wallis test with Dunn’s multiple comparisons testing was performed on measures of one-way ANOVA. Although scAT Akt S473 phosphorylation in the prevention study was not normally distributed, the repeated measures two-way ANOVA with Tukey’s post-hoc was performed as described above. Statistical significance was set at $p<0.05$. 
3.2 Results

3.2.1 Treatment Study

a. Diet-Induced Changes in Whole-Body Parameters

Prior to treatment intervention, high-fat fed animals (HFD, MET, RSV and COM) weighed significantly more than LFD at 9 weeks of dietary intervention (Table 3.2). Importantly, no differences in body weight were observed between high-fat fed groups at this time. 9 weeks of high-fat feeding significantly reduced whole-body glucose and insulin tolerance, as evidenced by a higher glucose area under the curve (AUC) in the IPGTT and IPITT, respectively (Figure 3.2). Treatment interventions (MET, RSV and COM) were initiated at this time.

### Table 3.2: Body weight at onset of treatment and body and tissue weights at termination.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Liver Weight</th>
<th>eWAT Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Onset of Treatment</td>
<td>Post-treatment</td>
<td>Absolute (g)</td>
</tr>
<tr>
<td>LFD</td>
<td>34.7 ± 0.9</td>
<td>40.2 ± 0.8</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>HFD</td>
<td>41.2 ± 1.5</td>
<td>46.6 ± 1.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>MET</td>
<td>42.2 ± 0.7</td>
<td>46.4 ± 0.7</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>RSV</td>
<td>43.4 ± 1.1</td>
<td>47.3 ± 1.0</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>COM</td>
<td>43.7 ± 0.6</td>
<td>44.5 ± 0.6</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM for n=9-17/group. Significance (p<0.05) is denoted by letters: a different than LFD, b different than HFD, c different than MET, d different than RSV and e different than COM.
Figure 3.2: HFD impairs glucose and insulin tolerance. IPGTT (a), IPGTT AUC (b) (n=12 LFD, n=51 HFD), IPITT (c) and IPITT AUC (d) (n=12 LFD, n=47 HFD) for LFD and HFD C57BL/6 mice. Values are mean ± SEM. Significance (*) = p<0.05 compared to LFD.

b. Effects of MET, RSV and COM Therapy on Body and Tissue Weights

Five weeks of MET (231.28 ± 12.24 mg/kg/day), RSV (93.68 ± 3.51 mg/kg/day) or COM (MET 232.01 ± 17.12 mg/kg/day; RSV 92.77 ± 6.92 mg/kg/day) therapy had no effect on HFD-induced weight gain and high-fat fed groups remained significantly heavier than LFD at 14 weeks (Table 3.2). No differences in absolute liver weight were observed between groups, although relative liver weight in RSV treated mice was increased compared to HFD (Table 3.2). Absolute and relative eWAT weight was reduced in RSV compared to MET and COM. No differences were
observed between groups in adipocyte cell size in eWAT (Figure 3.3) or in food intake (Kcal/day) (data not shown).

Figure 3.3: No differences in adipocyte cell size were observed between groups (n=4/group). Values are mean ± SEM.

c. Whole-Body Glucose Homeostasis and Insulin Sensitivity

Following 13 weeks of high-fat feeding, HFD mice continued to display glucose intolerance, as glucose AUC was significantly higher in IPGTT compared to LFD mice (Figure 3.4b). Four weeks of treatment with MET or RSV did not improve HFD induced glucose intolerance, as IPGTT AUC’s of mice treated with each compound independently were not different from HFD mice (Figure 3.4b). However, COM treatment significantly improved glucose tolerance, as displayed by a lower IPGTT AUC compared to HFD (Figure 3.4b). The predicted change in glucose tolerance with combined treatment was calculated based on the observed changes of RSV and MET independently. The observed change in AUC for IPGTT, relative to HFD, with COM mirrored the predicted effects of COM (Figure 3.4c).
Similarly to the IPGTT findings, HFD mice remained insulin intolerant at 13 weeks, as IPITT glucose AUC was higher compared to LFD (Figure 3.4e). Although RSV failed to significantly improve insulin tolerance compared to HFD, RSV treated mice were not significantly impaired when compared to LFD IPITT glucose AUC measures (Figure 3.4e). MET and COM significantly improved insulin tolerance compared to HFD (Figure 3.4e). The predicted change in insulin tolerance with combined therapy was calculated, as described above, and the observed change in blood glucose AUC for IPITT, relative to HFD, with COM was similar to the predicted effects of COM (Figure 3.4c).

**Figure 3.4:** COM therapy improves HFD induced glucose and insulin intolerance. Predicted combined effects of MET and RSV mirror the observed effects of COM. IPGTT (a,b) and IPITT (d,e) measures, as well as percent change in IPGTT (c) and IPITT (f) from HFD, are presented as mean ± SEM (n=11-18/group). Significance (p<0.05) is denoted by letters: a different than LFD, b different than HFD, c different than MET, d different than RSV and e different than COM.
d. Whole-Body Energy Expenditure

No differences in oxygen consumption or RER were observed between groups during the light (Figure 3.5a and 3.5c) or dark cycles (Figure 3.5b and 3.5d). Moreover, heat production and total activity were similar between groups (Figure 3.5e-h).

**Figure 3.5:** Whole-body RER, oxygen consumption, heat and total activity are not affected by diet or treatment. Light and dark VO2 (a,b), RER (c,d), heat (e,f) and total activity (g,h) values are presented as mean ± SEM (n=2-4/group).
**e. Tissue Specific Insulin Action**

In order to evaluate tissue specific effects of MET, RSV and COM on insulin action, Akt phosphorylation at S473 and T308 was examined in liver, triceps, eWAT and scAT. Akt phosphorylation was measured following insulin injection in the liver, and before and after insulin injection in triceps, eWAT and scAT (Figure 3.6). Importantly, a maximal dose of insulin was used to examine tissue specific insulin action, and as such these measures are representative of maximal insulin action and are not to be interpreted as differences in insulin sensitivity at the tissue level. Therefore, HFD does not exhibit reduced insulin action in liver, triceps or scAT compared to LFD, although eWAT Akt phosphorylation at S473 and T308 was significantly reduced with HFD (Figure 3.7).

COM significantly increased liver Akt phosphorylation at S473 compared to LFD (Figure 3.7a). No differences in liver T308 phosphorylation were observed between groups (Figure 3.7b). Insulin significantly increased S473 and T308 AKT phosphorylation in all groups compared to basal (Figure 3.6 and Figure 3.7c-d). Insulin stimulated triceps S473 phosphorylation was significantly increased in COM compared to LFD, HFD and RSV. MET increased insulin-induced S473 phosphorylation compared to RSV (Figure 3.7c). No differences were observed in triceps T308 phosphorylation between groups (Figure 3.7d).

Insulin increased S473 and T308 AKT phosphorylation in eWAT in all groups compared to basal, with the exception of RSV which did not exhibit increased Akt phosphorylation at either site following insulin stimulation (Figure 3.6 and Figure 3.7e-f). Insulin-stimulated S473 phosphorylation was significantly reduced in HFD eWAT compared to LFD. RSV had no effect on HFD induced impairments in S473 AKT phosphorylation and remained significantly reduced compared to LFD. Interestingly, MET and COM Akt S473 phosphorylation was not impaired
compared to LFD (Figure 3.7e). HFD, RSV and COM exhibited significantly reduced insulin stimulated Akt T308 phosphorylation compared to LFD, while MET was not different from LFD or HFD. MET exhibited increased Akt T308 phosphorylation compared to RSV (Figure 3.7f).

Insulin increased scAT Akt phosphorylation on both sites, in all groups, compared to basal (Figure 3.6 and Figure 3.7g-h). Although there was no overall treatment effect for scAT S473 phosphorylation (p=0.0808), COM significantly increased insulin stimulated S473 phosphorylation compared to RSV (Figure 3.7g). COM exhibited significant increases in T308 phosphorylation with insulin stimulation compared to HFD and RSV (Figure 3.7h).

**Figure 3.6:** Representative blots for total and phosphorylated Akt (S473 and T308) in liver (a), triceps (b), eWAT (c) and scAT (d).
Figure 3.7: Differences in liver (a,b), triceps (c,d), eWAT (e,f) and scAT (g,h) Akt S473 and T308 phosphorylation at basal (☐) and following insulin stimulation (■). Values are mean ± SEM (n=7-11/group). Significance compared to basal within group (*) = p<0.05. Between groups significance (p<0.05) is denoted by letters: a different than LFD, b different than HFD, c different than MET, d different than RSV and e different than COM.
**f. Adiponectin Expression**

As adiponectin is known to have insulin sensitizing and glucose lowering effects\(^{109-111}\), we examined whether changes in adiponectin expression in eWAT and scAT were associated with COM induced changes in insulin action and glucose homeostasis. No diet or treatment intervention induced changes in adiponectin expression in eWAT or scAT were observed between groups (Figure 3.8a and 3.8b).

**Figure 3.8:** Adiponectin expression is unaffected by HFD or treatment in eWAT (a) and scAT (b). Values are mean ± SEM (n=6/group).
### 3.2.2 Prevention Study

#### a. Body and Tissue Weights

HFD mice weighed significantly more than LFD mice (Table 3.3). Mice prophylactically treated with MET (244.76 ± 16.30 mg/kg/day), RSV (95.14 ± 4.11 mg/kg/day) or COM (MET 240.81 ± 11.49 mg/kg/day; RSV 96.49 ± 4.58) for 13 weeks weighed significantly less than HFD, effectively preventing the high-fat diet induced increases in body weight (Table 3.3). MET significantly decreased absolute liver weight compared to HFD and relative liver weight compared to LFD (Table 3.3). No differences were observed in absolute eWAT weight, although relative eWAT weight was significantly increased in MET compared to LFD and RSV (Table 3.3). Food intake (Kcal/day) was not different between high-fat fed groups (data not shown).

#### Table 3.3: Body and tissue weights at termination.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Liver Weight</th>
<th>eWAT Weight</th>
</tr>
</thead>
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<td></td>
<td>Post-treatment</td>
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<td></td>
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<td>45.1 ± 1.2</td>
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<td>41.0 ± 1.4</td>
</tr>
<tr>
<td>MET</td>
<td>40.7 ± 0.9 b</td>
<td>1.5 ± 0.1 b</td>
<td>38.6 ± 1.5 a</td>
</tr>
<tr>
<td>RSV</td>
<td>40.8 ± 1.5 b</td>
<td>1.7 ± 0.1 b</td>
<td>41.2 ± 1.0</td>
</tr>
<tr>
<td>COM</td>
<td>39.7 ± 1.3 b</td>
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</table>

<table>
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<tr>
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<th>Absolute (g)</th>
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<th>Absolute (g)</th>
<th>Relative (mg/g BW)</th>
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<td>45.5 ± 1.5</td>
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</tr>
<tr>
<td>HFD</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MET</td>
<td>56.3 ± 2.7 a</td>
<td>45.1 ± 2.0 c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV</td>
<td>56.3 ± 2.7 a</td>
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</table>

Values are presented as mean ± SEM for n=8-11/group. Significance (p<0.05) is denoted by letters: a different than LFD, b different than HFD, c different than MET, d different than RSV and e different than COM.

#### b. Whole-Body Glucose Homeostasis and Insulin Sensitivity

At 12 weeks, HFD mice were significantly glucose and insulin intolerant compared to LFD, as measured through IPGTT AUC and IPITT AUC, respectively (Figure 3.9). MET, RSV and COM therapy prevented HFD induced glucose intolerance, with no significant differences between treatment groups and LFD (Figure 3.9a and 3.9b). Moreover, MET, RSV and COM all
prevented HFD induced insulin intolerance, and in fact displayed significantly higher insulin tolerance compared to HFD mice (Figure 3.9c and 3.9d).

**Figure 3.9:** MET, RSV and COM therapy prevent HFD induced glucose and insulin intolerance. IPGTT (a), IPGTT AUC (b), IPITT (c) and IPITT AUC (d) for LFD, HFD, MET, RSV and COM C57BL/6 mice (n=8-11/group). Values are mean ± SEM. Between groups significance (p<0.05) is denoted by letters: a different than LFD, b different than HFD, c different than MET, d different than RSV and e different than COM.

c. **Whole-Body Energy Expenditure**

Similar to the treatment study, no differences in oxygen consumption were observed between groups during the light or dark cycle (Figure 3.10a and 3.10b). However, MET displayed lower RER compared to LFD during the dark phase, indicating a greater reliance on fat for energy production (Figure 3.10c and 3.10d). Heat production and total activity was similar between all groups (Figure 3.10e and 3.10f).
Figure 3.10: MET reduces RER compared to LFD during the light and dark cycles (c,d). No changes in oxygen consumption (a,b), heat (e,f) or total activity (g,h) were observed between groups. Values are mean ± SEM (n=3-5/group). Between groups significance (p<0.05) is
denoted by letters: a different than LFD, b different than HFD, c different than MET, d different than RSV and e different than COM.

d. Tissue Specific Insulin Action

As described for the treatment study, tissue specific effects of MET, RSV and COM on insulin action were examined through measuring Akt phosphorylation in liver, triceps, eWAT and scAT (Figure 3.11). Liver Akt S473 phosphorylation was significantly increased by MET compared to LFD and HFD mice (Figure 3.12a). As was described in the treatment study, a maximal insulin dose was used to examine changes in Akt phosphorylation and these measures are representative of maximal insulin action, not insulin sensitivity, at the tissue level. Therefore, HFD does not exhibit reduced insulin action at any tissue compared to LFD, with the exception of reduced eWAT S473 Akt phosphorylation. No differences in liver T308 phosphorylation were observed between groups (Figure 3.12b).

Insulin significantly increased Akt S473 and T308 phosphorylation in triceps in all groups (Figure 3.11 and Figure 3.12c-d). Insulin stimulated Akt S473 phosphorylation in triceps was significantly increased in COM compared to all other groups. No differences were observed in triceps Akt T308 phosphorylation between groups (Figure 3.12d).

With the exception of the HFD group on S473, insulin significantly increased eWAT Akt phosphorylation in all groups (Figure 3.11 and Figure 3.12e-f). Insulin-stimulated S473 phosphorylation was significantly reduced in HFD eWAT compared to LFD. MET, RSV and COM therapy prevented HFD induced impairments in S473 phosphorylation, as all three treatment groups exhibited significantly higher insulin stimulated S473 phosphorylation compared to HFD (Figure 3.12e). Although there was no overall treatment effect for eWAT T308 phosphorylation (p=0.0505), MET and COM significantly increased insulin stimulated T308 phosphorylation compared to HFD (Figure 3.12f).
All groups exhibited significantly increased insulin stimulated scAT S473 and T308 phosphorylation compared to basal levels within each group (Figure 3.11 and Figure 3.12g-h). COM had significantly higher insulin stimulated S473 phosphorylation compared to LFD, HFD and RSV (Figure 3.12g). MET displayed significantly higher insulin stimulated T308 phosphorylation compared to HFD (Figure 3.12h). COM significantly increased insulin stimulated T308 phosphorylation compared to all groups (Figure 3.12h).

**Figure 3.11**: Representative blots for total and phosphorylated Akt (S473 and T308) in liver (a), triceps (b), eWAT (c) and scAT (d).
Figure 3.12: Differences in liver (a,b), triceps (c,d), eWAT (e,f) and scAT (g,h) Akt S473 and T308 phosphorylation at basal (□) and following insulin stimulation (■). Values are mean ± SEM (n=6-11/group). Significance compared to basal within group (*) = p<0.05. Between groups significance (p<0.05) is denoted by letters: a different than LFD, b different than HFD, c different than MET, d different than RSV and e different than COM.
e. Protein content of Enzymes Regulating HGP

MET is thought to act primarily through attenuation of HGP\textsuperscript{12,122,126}. We therefore examined the protein content of two key regulators of this process, glucose 6 phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK), in the liver. No differences in G6Pase protein content were observed between groups (Figure 3.13a and 13.3c). Surprisingly, PEPCK content increased in both MET and COM compared to LFD and HFD (Figure 3.13b-c). However, it is important to note that protein content is not a direct measure of HGP.

![Liver G6Pase and PEPCK expression](image)

Figure 3.13: Liver G6Pase (a,c) and PEPCK (b,c) expression are unaffected by HFD, while MET and COM exhibit increased PEPCK content (b,c). Values are mean ± SEM (n=6-11/group).

f. Regulation of Inflammatory Pathways

Inflammation has been established as a key contributor to the development of insulin resistance\textsuperscript{8,56,57}. We therefore sought to determine if the beneficial effects of MET, RSV and COM on whole-body glucose homeostasis could be attributed to reductions in pro-inflammatory signaling. HFD did not exhibit increased phosphorylation of JNK, Erk or p38 in scAT or eWAT, three proteins important in pro-inflammatory signaling (Figure 3.14). Moreover, RSV, MET and COM did not significantly reduce phosphorylation at any site, and MET in fact exhibited increased p38 phosphorylation compared to LFD in scAT.
Figure 3.14: Activation of key inflammation regulating pathways JNK, erk and p38 (a-b) is unchanged by HFD and not reduced following MET, RSV and COM treatment in scAT (a,c-e) and eWAT (b,f-h) (n=6-11/group).
Chapter 4: Discussion

This thesis presents a novel examination of the potential synergistic effects of RSV and MET in prevention and treatment of insulin resistance. The tissue specific effects of RSV, MET and COM treatment on insulin action were examined, as well as potential mechanisms of action. To our knowledge, this is the first study to examine the combined effects of RSV and MET on insulin action and glucose homeostasis. Moreover, as most research on the anti-diabetic effects of RSV has been performed in prophylactic models, and recent work in cell culture suggests that RSV is only effective when given prior to establishing insulin resistance, this study provides novel insight into the differential effects of RSV in prevention and treatment of insulin resistance in vivo.

4.1 Treatment Study

4.1.1 Whole-Body Glucose Homeostasis and Tissue Specific Insulin Action

The treatment study was performed in order to examine the synergistic effects of RSV and MET. The selected doses were chosen as they did not independently reduce glucose and insulin tolerance in preliminary experiments (Appendix A). RSV failed to significantly reduce HFD induced insulin resistance when administered to mice with established insulin resistance. In contrast, previous work has shown that 4-10 weeks of RSV treatment increases insulin sensitivity, independent of changes in body weight. Interestingly, insulin action was reduced in eWAT by RSV compared to LFD, exhibiting similar reductions to those observed with HFD. Perhaps more alarming, eWAT Akt phosphorylation was not significantly increased by insulin stimulation in RSV treated mice compared to basal levels, indicating pronounced insulin resistance in this tissue. This finding is supported by the identification of RSV induced reductions in insulin action in 3T3-L1 adipocytes.
Although MET induced improvements in insulin sensitivity are highly supported\textsuperscript{120,127,139,176}, we found that MET treatment in insulin resistant mice did not improve glucose tolerance compared to HFD, although a non-significant reduction in IPGTT AUC was observed. Moreover, MET remained significantly glucose intolerant compared to LFD. However, MET significantly increased insulin tolerance compared to HFD. The lack of a MET effect on liver insulin signaling was unexpected, as previous reports have identified increases in liver insulin signaling in diabetic rats and potentiated insulin responsiveness in hepatocytes with MET\textsuperscript{120,130}.

Perhaps the most interesting finding is that COM therapy significantly improved whole-body insulin sensitivity in the treatment study. This is a novel finding, as RSV and MET were incapable of improving glucose tolerance when administered independently, although MET improved insulin tolerance. Therefore, RSV and MET additively improved glucose tolerance. In fact, the observed COM improvements in glucose and insulin tolerance relative to HFD are nearly identical to the sum of RSV and MET improvements independently. These findings are in agreement with those of a previous study that found improved whole-body insulin sensitivity with a combination of MET, RSV and HMB\textsuperscript{176}. However, the RSV dose (12.5 mg/kg diet) was much lower to that used in our study, while HMB (2 g/kg diet) was given at a comparatively larger dose than both RSV and MET (0.25-0.75g/kg diet)\textsuperscript{176}. Therefore, the findings of the aforementioned study may be more representative of the combined effects of HMB and MET.

COM also induced superior tissue specific insulin signaling in scAT and triceps. Although MET is not known to effect WAT insulin action, long-term RSV is known to increase insulin action in this tissue\textsuperscript{146}. However, it appears likely that RSV and MET act synergistically to induce these improvements, as RSV and MET independently did not increase insulin signaling
in scAT. Alternatively, RSV has been shown to increase myotube insulin action and MET induces increases in insulin action in muscle\textsuperscript{120,125,144}, suggesting a potential for insulin sensitizing effects to occur with COM in triceps. To our knowledge, this is the first study to examine the combined effects of RSV and MET on tissue specific insulin action.

### 4.1.2 Association of Insulin Sensitivity with Body Weight

As obesity is a risk factor in the development of insulin resistance, it is not surprising that weight loss is associated with increased insulin sensitivity. RSV did not reduce body weight when given as a treatment for established insulin resistance, supporting the findings of others\textsuperscript{16,145}. MET therapy is commonly associated with reduced body weight, although this has only been established with long-term treatment\textsuperscript{12,88,120,139,181}. However, no differences in body weight were observed from HFD with treatment in our model. This is supported by short-term (2–4 weeks) MET treatment findings, as no differences in body weight or fat mass were observed\textsuperscript{127,176}. The effects of COM therapy on body weight were identical to those of RSV and MET, as no changes were observed in treatment compared to HFD. Therefore, increased glucose and insulin tolerance cannot be explained by weight loss in COM.

### 4.2 Prevention Study

#### 4.2.1 Whole-Body Glucose Homeostasis and Tissue Specific Insulin Action

We found that RSV prevented HFD induced glucose and insulin intolerance when administered prophylactically. These findings are similar to previous work from our lab, which has shown that RSV prevents glucose and insulin tolerance in Zucker Diabetic Fatty (ZDF) rats, a genetic model of insulin resistance\textsuperscript{15}. Moreover, others have observed increased whole-body insulin sensitivity with RSV in prophylactically treated rodents\textsuperscript{143,145,146}. RSV also significantly increased insulin action in eWAT compared to HFD. This was expected, as a similar 13 week
prevention model found that RSV induced increases in insulin signaling in WAT. Targeting of WAT insulin signaling by RSV is supported by bioavailability studies in rodents, which show greater concentrations of RSV and its metabolites, R3G and R3S, in WAT compared to skeletal muscle. Therefore, it is not surprising that we did not observe increased insulin action in skeletal muscle, as RSV concentrations have been shown to be below levels of detection in this tissue.

MET prevented HFD-induced glucose intolerance and significantly improved insulin tolerance compared to HFD. These findings are supported not only by previous treatment findings, but also by reported reductions in the incidence of type 2 diabetes following prophylactic administration of MET in at risk subjects. Previous reports have identified increases in liver insulin action in diabetic rats and potentiated insulin responsiveness in hepatocytes with MET. Our findings in the prevention study support this, as MET treatment increased liver insulin action compared to HFD. No differences in triceps insulin action were observed with MET, although MET may have improved glucose and insulin tolerance through an indirect mechanism to additively increase insulin action in skeletal muscle. Our novel findings of increased insulin signaling in eWAT and scAT identify WAT as a potential target for improved glucose homeostasis effects of MET.

The prevention effects of COM therapy on glucose and insulin tolerance are identical to those of RSV and MET independently, and therefore there does not appear to be an additive effect of RSV and MET in this model. However, it is plausible that reducing RSV and MET doses would increase the potential to detect synergistic or additive effects, although this would limit our ability to compare our prevention and treatment experiments. COM was found to increase eWAT insulin signaling compared to HFD, similar to the effects of MET and RSV.
However, COM has greater effects on tissue specific insulin action in scAT and triceps and therefore RSV and MET may synergistically increase tissue specific insulin action in these tissues. The synergistic effects of RSV and MET in triceps are expected, given that RSV has been shown to increase myotube insulin action and MET induces increases in insulin signaling in muscle\textsuperscript{120,125,144}.

4.2.2 Association of Insulin Sensitivity with Body Weight

The insulin sensitizing effects of RSV in the prevention study may be explained by our observed reductions in body weight, as weight loss is associated with increased insulin sensitivity. RSV has previously been shown to prevent HFD induced increases in body weight when given prophylactically\textsuperscript{145–147}. Similar to RSV, MET therapy reduced body weight compared to HFD in the prevention study. This is in agreement with previous findings that body weight is reduced with MET\textsuperscript{12,88,120,139,181}. The effects of COM therapy on body weight were identical to those of RSV and MET, as significant decreases compared to HFD were observed. Body weight is regulated by caloric intake and energy expenditure\textsuperscript{145}. However, reduced body weight with RSV, MET and COM cannot be explained by food intake or energy expenditure, as no differences were observed compared to HFD.

4.3 Differences in Responses to Treatment and Prevention

RSV produced opposing effects on glucose and insulin tolerance between treatment and prevention studies, with only the prevention study exhibiting improved insulin sensitivity. Although RSV is known to prevent HFD induced increases in body weight when given prophylactically\textsuperscript{145–147}, it does not appear to produce similar effects on body weight when given as a treatment for established insulin resistance\textsuperscript{16,145}. Therefore, it appears that although RSV may effectively prevent increases in body weight, it is not capable of reducing body weight.
RSV induced insulin sensitizing effects in the prevention study may be mediated through reduced body weight compared to HFD as obesity is associated with insulin resistance. Differences in the length of RSV administration between the treatment and prevention studies could explain the different responses to RSV, as the treatment persisted for 4 weeks and the prevention persisted for 12 weeks. However, this is not likely to be the case, as a previous study has examined the differences in response to RSV with 4 and 13 weeks of prophylactic administration, with only the 4 week treatment exhibiting significantly decreased GTT AUC\textsuperscript{146}. Importantly, the insulin sensitivity responses to various lengths of RSV administration has yet to be examined in a treatment model and thus the impact of treatment length cannot be conclusively negated as a factor mediating the differential responses between our models.

Conversely, MET increased insulin tolerance in both prevention and treatment, although glucose tolerance was only improved in prevention. Importantly, increased insulin tolerance by MET in both studies may be mediated through indirect mechanisms on insulin action, as MET has been shown to additively increase insulin-stimulated skeletal muscle glucose disposal without changing insulin signaling directly\textsuperscript{120,125}. Similar to RSV, MET had no effect on body weight with treatment, although body weight was significantly reduced in the prevention study compared to HFD. The length of the treatment period appears to be important in MET induced changes in body weight, as no differences are observed following short-term (2-4 weeks) MET administration\textsuperscript{127,176}. Therefore, different MET effects on body weight in the prevention and treatment studies are not surprising, as MET was administered for 12 weeks and 4 weeks, respectively.

As COM increased glucose and insulin tolerance in a similar manner in both studies, it is unlikely that improvements in glucose homeostasis can be solely attributed to weight loss, as the
treatment study did not exhibit weight loss. Therefore, other mechanisms are likely responsible. Increased insulin action in scAT and triceps may explain improved whole body insulin sensitivity with COM, as these effects were greater than RSV and MET independently in both studies. To our knowledge, this is the first set of studies to examine the effects of RSV and MET, independently and in combination, on insulin sensitivity in treatment and prevention in vivo.

4.4 Conclusion

In conclusion, this thesis presents novel insight into the combined effects of RSV and MET in prevention and treatment of insulin resistance. We have shown that RSV and MET are potent prophylactic treatments in preventing the development of insulin resistance, while treatment effects were not observed in our model. Perhaps the most important finding of this study is the improvement in whole-body glucose homeostasis and insulin sensitivity observed with COM in the treatment study. This is interesting, given that RSV and MET had no significant effects when provided at the same dose independently, although MET increased insulin tolerance compared to HFD.

Combination therapy is important to examine in diabetic subjects, as they frequently experience poor responses to monotherapy. Although controversial, a few studies have provided evidence supporting that RSV improves whole body insulin sensitivity in humans. Moreover, MET is known to independently improve whole body insulin resistance in humans and acts synergistically with other anti-diabetic therapies that induce similar effects to RSV. Therefore our findings may potentially extend to the human population. However, future work in human clinical trials is required using RSV and MET combination therapy.
References


Appendix A

Treatment study body weight, IPITT AUC and IPGTT AUC averages for high-fat fed groups prior to treatment. Animals were matched for body weight and glucose and insulin tolerance to ensure groups were similar at the onset of treatment.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>IPITT (AUC)</th>
<th>IPGTT (AUC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFD</td>
<td>41.18 ± 1.50</td>
<td>686.36 ± 40.17</td>
<td>1898.73 ± 173.67</td>
</tr>
<tr>
<td>MET</td>
<td>42.23 ± 0.72</td>
<td>695.18 ± 47.64</td>
<td>1838.98 ± 148.94</td>
</tr>
<tr>
<td>RSV</td>
<td>43.38 ± 1.10</td>
<td>688.88 ± 42.61</td>
<td>1952.18 ± 136.18</td>
</tr>
<tr>
<td>COM</td>
<td>43.65 ± 0.58</td>
<td>694.64 ± 36.38</td>
<td>1869.27 ± 105.09</td>
</tr>
</tbody>
</table>
Appendix B

Drug dilution calculation:

Dose (mg/kg/day) = drug consumed/day (mg) ÷ mouse weight (kg)

= [food consumed/day (g)*drug dilution in food (mg/g)] ÷ mouse weight (kg)

Desired dose (mg/kg/day), avg mouse weight (g) and avg food consumed/day (mg) are known.

Therefore,

Drug dilution in food (mg/g) = [dose (mg/kg/day)*mouse weight (kg)] ÷ food consumed/day (g)
Appendix C

MET doses for the treatment study were selected based on pilot work comparing effects of various doses on glucose and insulin tolerance. High (HFD-HM; 250mg/kg/day), medium (HFD-MM; 125mg/kg/day) and low (HFD-LM; 62.5mg/kg/day) doses of MET were compared to HFD and HFD-HM was chosen for the treatment study, as it improved both glucose and insulin tolerance although it was not significantly different from HFD.

**Metformin**

**a)** IPGTT

**b)** Glucose Tolerance

**c)** IPITT

**d)** Insulin Tolerance

IPGTT (a), IPGTT AUC (b), IPITT (c) and IPITT AUC (d) for LFD, HFD, HFD-HM, HFD-MM and HFD-LM C57BL/6 mice (n=6/group). Values are mean ± SEM. Between groups significance (p<0.05) is denoted by letters: a different than LFD, b different than HFD, c different than HFD-HM, d different than HFD-MM and e different than HFD-LM.
Appendix D

RSV doses for the treatment study were selected based on pilot work comparing effects of various doses on glucose and insulin tolerance. High (HFD-HR; 200mg/kg/day) and low (HFD-LR; 100mg/kg/day) doses of RSV were compared to HFD and HFD-LR was chosen for the treatment study, as it improved both glucose and insulin tolerance although it was not significantly different from HFD, while HFD-HR exhibited worsened glucose and insulin tolerance. The worsening with HFD-HR may not be surprising, as a previous study has suggested that larger doses of RSV may be associated with poor tolerance\textsuperscript{170}.

**Resveratrol**

IPGTT (a), IPGTT AUC (b), IPITT (c) and IPITT AUC (d) for LFD, HFD, HFD-HR and HFD-LR C57BL/6 mice (n=6/group). Values are mean ± SEM. Between groups significance (p<0.05) is denoted by letters: a different than LFD, b different than HFD, c different than HFD-HR and d different than HFD-LR.