Nutritional and Pharmacological Strategies to Modulate White Adipose Tissue Physiology in the Management of Insulin Resistance and Type 2 Diabetes

by

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White adipose tissue (WAT) is a crucial tissue implicated in metabolic health due to its roles in lipid handling and inflammation. In obesity, WAT fatty acid uptake and release is deregulated and released cytokines are primarily pro-inflammatory. Peroxisome proliferator-activated receptor-γ (PPARγ) and transforming growth factor-β (TGF-β) and its downstream Smad- and mothers against decapentaplegic (MAD)-related proteins (SMAD) cascade have been implicated in WAT physiology and whole-body carbohydrate and lipid homeostasis. However, the relationship between TGF-β/SMAD, PPARγ and WAT physiology remains to be elucidated.

Rosiglitazone (ROSI), a PPARγ agonist, and resveratrol (RSV) are used to prevent and treat metabolic disorders and type 2 diabetes. However, the use of ROSI in clinical settings is limited due to its adverse side effects. In addition, it remains unclear whether RSV induces metabolic benefits in humans. To date, the roles of RSV, alone or with ROSI, on WAT lipid handling and inflammation have yet to be examined. This thesis sought to investigate the effects of RSV and ROSI, individually and in combination, in rodent WAT in vivo and ex vivo and in human WAT in vitro.
In the first study of this thesis, chronic RSV supplementation improved glucose homeostasis in diabetic rodents, which was associated with depot-specific stimulation of WAT glyceroneogenesis, mitochondrial biogenesis and adiponectin secretion. In the second study of this thesis, ROSI-induced adipogenesis in Zucker diabetic fatty rats was associated with depot-specific dephosphorylation and deactivation of SMAD2 and SMAD3 along with increased protein content of inhibitory SMAD7 and SMAD ubiquitination regulatory factor 2 (SMURF2). Finally, the concomitant treatment of cultured WAT from morbidly obese patients with RSV and ROSI additively increased the expression of the glyceroneogenic genes PDK4 and PEPCK.

Taken together, this thesis highlights that RSV and ROSI, individually and in combination, favourably modulate lipid handling in WAT from rodents and humans in a depot-specific manner. In addition, it suggests that PPARγ agonism inhibits TGF-β/SMAD signalling, which is associated with WAT adipogenesis. Finally, these data support the concept of a combined pharmacological and nutraceutical approach to prevent and treat metabolic pathologies, which may improve the tolerability of current anti-diabetic therapies.
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<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>ATGL</td>
<td>adipose triglyceride lipase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>C/EBP</td>
<td>ccaat-enhancer-binding protein</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CGI-58</td>
<td>comparative gene identification-58</td>
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<td>Co-SMAD</td>
<td>common mediator SMAD</td>
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<td>COX4</td>
<td>cytochrome c oxidase complex IV</td>
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<td>DGAT</td>
<td>diacylglycerol acyltransferase</td>
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<tr>
<td>DsbA-L</td>
<td>disulfide-bond A oxidoreductase-like protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>eWAT</td>
<td>epididymal white adipose tissue</td>
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<tr>
<td>FFA</td>
<td>free fatty acids</td>
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<td>G3P</td>
<td>glycerol-3-phosphate</td>
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<tr>
<td>GDF</td>
<td>growth differentiation factors</td>
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<tr>
<td>GIP</td>
<td>glucose-dependent insulinotropic polypeptide</td>
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<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
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<td>Full Form</td>
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<tr>
<td>GNG</td>
<td>glyceroneogenesis</td>
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<td>GTT</td>
<td>glucose tolerance test</td>
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<td>HbA&lt;sub&gt;1C&lt;/sub&gt;</td>
<td>glycosylated hemoglobin</td>
</tr>
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<td>HF</td>
<td>high fat</td>
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<td>HOMA-IR</td>
<td>homeostatic model assessment – insulin resistance</td>
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<td>I-SMAD</td>
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<td>monoglyceride lipase</td>
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<tr>
<td>NS</td>
<td>non significant</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
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<td>phosphate buffered saline</td>
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<td>pyruvate dehydrogenase</td>
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<tr>
<td>PDK4</td>
<td>pyruvate dehydrogenase kinase 4</td>
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<td>protein kinase A</td>
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<td>PPARγ</td>
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<td>R-SMAD</td>
<td>receptor-associated SMAD</td>
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<td>ROSI</td>
<td>rosiglitazone</td>
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<td>rpWAT</td>
<td>retroperitoneal white adipose tissue</td>
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<tr>
<td>RSV</td>
<td>resveratrol</td>
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<tr>
<td>SARA</td>
<td>SMAD anchor for receptor activation</td>
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<td>scAT</td>
<td>subcutaneous white adipose tissue</td>
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<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<tr>
<td>SMAD</td>
<td>Sma- and MAD (mothers against decapentaplegic)-related proteins</td>
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<td>SMAD ubiquitination regulatory factor</td>
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<tr>
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<td>type 2 diabetes mellitus</td>
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<td>transforming growth factor-β</td>
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<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<td>uncoupling protein-1</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>ZDF</td>
<td>Zucker Diabetic Fatty</td>
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Chapter 1: Review of Literature

1.1 Adipose tissue physiology in health and disease

White adipose tissue (WAT) is no longer considered a passive storage tissue for excess energy, but is now recognized as having important metabolic, endocrine and inflammatory properties [75]. Consequently, WAT is now closely linked to the etiology of obesity, insulin resistance and type 2 diabetes mellitus (T2DM) [75]. In obesity and insulin resistance, WAT is characterized by adipocyte hypertrophy, pro-inflammatory macrophage infiltration, inadequate blood flow, increased free fatty acid release and altered cytokine secretion profile. More specifically, regulation of lipolysis [84], glyceroneogenesis (GNG) [219] and inflammation [194] in WAT from obese patients are compromised, which contribute to increased circulatory fatty acids, ectopic fat deposition and ultimately, insulin resistance [126].

1.1.1 Lipid handling in adipose tissue in health and disease

a. Lipolysis: regulation and role in metabolic disorders

Lipolysis is the process of hydrolyzing triglycerides (TG) to free fatty acids (FFA) and glycerol by lipase enzymes in times of caloric needs such as fasting and exercise. The resulting FFA are then transported to other tissues, such as skeletal muscle, and oxidized to provide energy. Lipolysis occurs through the sequential actions of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) (reviewed in [46]). Physiological conditions such as exercise [42] and
fasting [95] induce lipolysis through a β-adrenergic signalling cascade.

Catecholamines, such as epinephrine and norepinephrine, bind to G-coupled receptors to activate adenylyl cyclase and produce cyclic AMP (cAMP). cAMP binds and activates protein kinase A (PKA), resulting in the phosphorylation and activation of HSL. PKA also phosphorylates perilipin-1, which releases comparative gene identification-58 (CGI-58) and allows its stimulatory action on ATGL (reviewed in [25]). Conversely, insulin is a negative regulator of lipolysis, due to its actions on phosphodiesterase 3B to degrade cAMP and on protein phosphatase-1 to dephosphorylate HSL (reviewed in [25]).

In metabolic disorders, the sensitivity of adipocytes to the lipolytic actions of catecholamines and to the anti-lipolytic action of insulin is considerably compromised. Consequently, basal lipolysis is increased in obesity while catecholamine-stimulated lipolysis is reduced. In normal weight subjects with a family history of obesity, norepinephrine-stimulated lipolysis and activation of HSL were reduced by 50% compared to controls [84]. Accordingly, a weight-loss program in obese women increased lipolytic norepinephrine sensitivity by five-fold and reduced basal lipolysis by half in subcutaneous adipose tissue (scAT) explants [163]. Moreover, aerobic exercise training even without weight loss also increased the sensitivity of adipocytes to catecholamine and insulin signals and normalized basal and stimulated lipolysis in obese men [42]. Importantly, the deregulation of lipolysis in WAT from obese patients and the elevated basal FFA release contribute to excess circulatory FFA and ectopic fat deposition, two hallmarks of metabolic disorders.
b. Fatty acid reesterification: regulation and role in metabolic disorders

Lipolysis involves the sequential hydrolysis of three FFA units from one glycerol backbone molecule, but not all hydrolyzed FFAs reach the circulation. In fact, up to 50% of these fatty acids are recycled back into TG within the adipocytes, in a process called fatty acid reesterification [208]. Fatty acid reesterification is dependent upon the provision of glycerol-3-phosphate (G3P), which can come from three different sources (Figure 1.1). First, intracellular glycerol or glycerol taken up from the circulation can be directly phosphorylated by glycerol kinase [122]. Second, glucose can be converted into G3P through the glycolytic pathway and the action of glycerol phosphate dehydrogenase [57, 121]. Finally, non-carbohydrate molecules, such as pyruvate, lactate or amino acids can be converted into G3P in a process called glyceroneogenesis [162]. In adipose tissue, glyceroneogenesis is the main source of G3P, due to the limited expression of glycerol kinase [122] and the limited glucose uptake under fasting conditions.
Figure 1.1 Sources of glycerol-3-phosphate in adipocytes for fatty acid reesterification. G3P can be provided through 1- pyruvate, lactate or amino acids via glyceroneogenesis; 2- direct glycerol phosphorylation via glycerol kinase; 3- glucose via glycolysis. In adipocytes under fasting conditions, glyceroneogenesis is the main source of G3P. FA: fatty acids; G3P: glycerol-3-phosphate; G6P: glucose-6-phosphate; GPDH: glycerol phosphate dehydrogenase; GNG: glyceroneogenesis; OA: oxaloacetate; PEP: phosphoenolpyruvate; TG: triglycerides. Adapted from Festuccia et al, 2003 [57].

GNG is controlled by the concerted actions of pyruvate dehydrogenase kinase 4 (PDK4) and cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) [23]. In fact, overexpression of PEPCK specifically in adipose tissue increased the conversion of $^{14}$C-pyruvate to glycerol and fatty acid reesterification in mice [60]. Consequently, transgenic mice fed a standard chow diet were obese, showed elevated body fat and larger adipocyte size, but retained insulin sensitivity and glucose tolerance [60], suggesting that PEPCK is important in whole-body lipid homeostasis. Similarly,
treatment of adipocytes with leelamine, a PDK4 inhibitor, or with PDK4 siRNA, led to the reduction of pyruvate incorporation into triglycerides [23], illustrating how PDK4 is essential for the regulation of GNG. The role of PDK4 in the glyceroneogenic cascade is attributed to its inhibitory action on pyruvate dehydrogenase complex (PDC), preferentially directing pyruvate flux away from acetyl-coA and towards oxaloacetate and glycerol-3-phosphate [23] (Figure 1.2).

**Figure 1.2: Regulation of glyceroneogenesis in white adipocytes.** PDK4 and PEPCK-C activation and/or increased gene expression shuttles pyruvate towards oxaloacetate and the generation of glycerol-3-phosphate. FFA: free fatty acids; G3P: glycerol-3-phosphate; OA: oxaloacetate; PC: pyruvate carboxylase; PDC: pyruvate dehydrogenase complex; PDK4: pyruvate dehydrogenase kinase 4; PEPCK-C: cytosolic phosphoenolpyruvate carboxykinase. Adapted from Cadoudal et al, 2008 [23].

Both PDK4 and PEPCK mRNA expression are controlled by the transcription factor peroxisome proliferator-activated receptor γ (PPARγ). Mice with a disrupted PPARγ
binding site in the PCK1 gene promoter (the gene that controls PEPCK expression) showed reduced PEPCK mRNA in WAT, higher circulating FFA, obesity and insulin resistance on glucose tolerance tests and euglycemic-hyperinsulinemic clamps [129]. Furthermore, the addition of pyruvate in the incubation medium of epididymal WAT (eWAT) explants from wild-type mice reduced FFA release, but this effect was nullified in eWAT from PEPCK knockdown mice [140]. Increasing pyruvate availability in the medium provides additional substrate for GNG and hence promotes fatty acid reesterification. In knockdown animals, PEPCK activity is compromised, so the ability to convert pyruvate to glycerol-3-phosphate and sequester fatty acids declines [140]. Moreover, rosiglitazone (ROSI), a PPARγ ligand and activator, increases both PDK4 [23] and PEPCK [56, 193] mRNA expression. Treatment with ROSI decreased the ratio of FFA:glycerol in rats, an indirect measurement of fatty acid reesterification, and increased $^{14}$C-pyruvate incorporation into lipids in a PEPCK-dependent manner in adipocytes [193]. Finally, in vivo and ex vivo ROSI treatment of scAT from patients with T2DM directly increased PEPCK mRNA transcription [22]. Taken together, these data show that the transcription of PDK4 and PEPCK is dependent upon the action of PPARγ.

Fatty acid reesterification is essential to maintain lipid homeostasis as it sequesters fatty acids safely into adipose tissue thus reducing circulatory FFA and detrimental ectopic fat deposition [193, 218]. However, in obesity, fatty acid reesterification and PEPCK activity are compromised and precipitate the development of metabolic disturbances [122, 219]. For instance, in women, PEPCK-C activity and glyceroneogenesis are inversely correlated with body mass index (BMI) [122] and
fatty acid reesterification is decreased with abdominal obesity [219], which could increase lipotoxicity. Consequently, finding ways to maintain PEPCK and PDK4 activity and GNG in obesity may improve metabolic health.

1.1.2 Mitochondrial biogenesis in WAT: regulation and role in metabolic disorders

Mitochondrial content and function in skeletal muscle have been associated with the pathophysiology of metabolic disorders [133] but the causal relationship between mitochondria dysfunction and insulin resistance has been debated [86]. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is an important transcriptional coactivator of mitochondria in skeletal muscle [151]. More recently, the importance of mitochondrial biogenesis and PGC-1α in WAT has been examined in the etiology of metabolic diseases. In WAT, mitochondria are crucial components for effective thermogenesis and fatty acid oxidation [152]. Accordingly, PGC-1α mRNA and protein levels in omental and subcutaneous fat depots are negatively correlated with insulin resistance in humans [79, 169]. In addition, the recognition that mitochondria-rich brown adipose tissue [141] and brown adipocytes within WAT [173] are associated with improved glucose tolerance further supports the role of WAT mitochondria for optimal metabolic health. Furthermore, stimuli known to enhance metabolic health, such as exercise [186], calorie restriction [27] and PPARγ agonism [166], also stimulate mitochondrial biogenesis in WAT. Finally, adipose-specific deletion of PGC-1α in mice fed a high-fat diet was characterized by a greater
insulin resistance, glucose intolerance and hyperlipidemia than in WT mice fed the same diet [103].

Obesity is a common confounding factor in the investigation of insulin resistance as they are often – but not always – present together. In humans, there is evidence that mitochondrial function is negatively regulated with insulin resistance, independent from obesity. In adipose tissue from morbidly obese patients without T2DM, genes controlling β-oxidation and the citric acid cycle were higher in the insulin-sensitive compared to the insulin-resistant patients [153]. Furthermore, PGC-1α expression was decreased in omental fat from morbidly obese men and women with insulin resistance, but not in the insulin sensitive subgroup [214]. Overall, rodent and human data strongly support a role of PGC-1α and WAT mitochondrial biogenesis in maintaining whole-body metabolic health, independent of obesity.

1.1.3 Relationship between FFA reesterification, lipolysis and mitochondrial function

Lipolysis, fatty acid reesterification and mitochondrial biogenesis are three processes that are closely associated with one another in WAT. First, absolute rates of lipolysis parallel that of glyceroneogenesis [21], suggesting that similar stimuli could increase both lipolysis and GNG. In fact, epinephrine, a lipolytic signal, also increased the expression of PDK4 mRNA in rat eWAT [205]. In addition, the futile cycling of fatty acid during reesterification requires considerable cellular energy [47]. Accordingly, Gauthier and colleagues [67] reported that AMP-activated protein kinase (AMPK) is
activated in response to the acylation of fatty acids and the resulting increase in AMP:ATP ratio. The authors suggested that AMPK activation in response to lipolysis (and hence GNG) occurs to limit cellular energy depletion. Importantly, AMPK is a major regulator of mitochondrial biogenesis [66, 93] and is necessary for the regulation of PGC-1α in eWAT from rodents [204]. While this remains speculative, the activation of AMPK as a result of fatty acid acylation and AMPK’s actions on PGC-1α expression in WAT may provide a physiological link between the energy-costly process of fatty acid reesterification and cellular mitochondrial content.

1.1.4 Inflammation in WAT: regulation and role in metabolic disorders

In addition to lipid homeostasis, WAT plays a key role in the regulation of whole-body inflammation and cytokine secretion. In WAT, immune cells, such as macrophages and lymphocytes, interact closely with adipocytes to modulate local and systemic inflammatory status through the secretion of adipose-derived cytokines, or adipokines, which have paracrine and endocrine functions. While the family of adipokines is large and still expanding, this review will focus on three major molecules: the pro-inflammatory interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) and the anti-inflammatory adiponectin.

In obesity, adipose tissue expansion and adipocyte hypertrophy are associated with pro-inflammatory macrophage infiltration and altered adipokine secretion: while IL-6 and TNF-α release is increased, adiponectin secretion is decreased, thus contributing
to low-grade inflammation, a major characteristic of obesity and insulin resistance [194] (Figure 1.3).

**Figure 1.3:** Inflammatory molecule secretion and remodelling processes in white adipose tissue in lean and obese conditions. FFA: free fatty acids; IL-6: interleukin-6; TNF-α: tumour necrosis factor-α. Dotted lines indicate downregulated secretion; solid lines indicate upregulated secretion.

Adiponectin is an anti-inflammatory adipokine that is secreted from the adipocytes [172] and is inversely correlated with obesity [9] and insulin resistance [87]. Adiponectin exerts insulin-sensitizing effects on the liver and skeletal muscle, two key tissues in glucose homeostasis. Specifically, adiponectin suppresses hepatic glucose production [38] and enhances fatty acid oxidation in skeletal muscle [63]. Importantly, adiponectin is first regulated at the transcriptional level: the transcription factor PPARγ binds to a PPARγ-responsive element in the adiponectin promoter to stimulate its mRNA expression [92]. Secondly, there is also evidence that adiponectin is regulated at the post-transcriptional level. For instance, in 3T3-L1 adipocytes, troglitazone [154] and pioglitazone [160] increase the secretion of adiponectin without modulating its gene expression. This transcription-independent effect is
attributed to the endoplasmic reticulum protein Ero1-Lα that is responsive to PPARγ agonism and whose role is to form the disulfide bonds that are required for the multimerization of adiponectin [154]. Finally, adiponectin synthesis is closely linked to mitochondrial function in adipocytes. Indeed, mitochondrial biogenesis induced by rosiglitazone in db/db mice or through over-expression of nuclear respiratory factor-1 in cultured adipocytes both increased adiponectin mRNA and protein content [105]. Finally, other anti-diabetic agents, such as metformin [226] and sulfonylureas [107], also stimulate adiponectin secretion and expression. Taken together, these data suggest that adiponectin is positively regulated via PPARγ agonism and/or mitochondrial biogenesis at the transcriptional and post-transcriptional levels and that it has a clinically-relevant effect on insulin resistance.

Obesity is also associated with an increased secretion of pro-inflammatory adipokines, such as IL-6 and TNF-α. Interestingly, some studies reported that macrophage infiltration and inflammation precede the onset of insulin resistance in rodents [210, 212], suggesting a possible causal association between adipose tissue inflammation and metabolic disease. Pro-inflammatory macrophage infiltration within WAT stimulates IL-6 and TNF-α release [182], two molecules that act in a paracrine manner to further modulate inflammation and alter lipid homeostasis. Indeed, IL-6 [52] and TNF-α [224] act directly on the adipocytes to decrease adiponectin secretion. Furthermore, TNF-α participates in the release of saturated fatty acids from the adipocyte [182], which contributes to lipid imbalance in obesity and insulin resistance. Moreover, IL-6 treatment ex vivo blunts the mRNA expression and protein content of PEPCK and PDK4 in rodent eWAT and thus decreases fatty acid reesterification.
In addition, treatment of 3T3-L1 adipocytes with IL-6 increased glycerol release, suggesting a lipolytic action of IL-6 [146], but this effect was not replicated in rodent eWAT [202] or with infusion of recombinant IL-6 in humans [146]. Overall, obesity-related macrophages infiltration and the concomitant inflammatory processes contribute to the etiology of insulin resistance. Both IL-6 and TNF-α participate in positive feedback loops that contribute to sustained inflammation and increased FFA release from the adipose tissue.

1.1.5 TGF-β/SMAD signalling in adipose tissue in health and disease

The transforming growth factor-β (TGF-β) family signalling cascade is versatile but complex, involving up to 60 different ligands, dozens of receptors and several distinct intracellular molecules. Specificity of this pathway is achieved through cell-membrane receptor heterodimerization, intracellular molecular interactions with coactivators and corepressors and cross talk with parallel pathways (reviewed in [53]). TGF-β1, -β2 and -β3 are the best-known ligands of this superfamily and their associated intracellular signalling cascade is well characterized. TGF-β ligands bind to a heterodimer receptor complex comprised of TGF-β receptor type I and II, which have serine-threonine kinase activity [10]. Upon receptor binding, the traditional Smad- and mothers against decapentaplegic (MAD)-related protein (SMAD) 3 pathway becomes activated: phosphorylated SMAD3 proteins interact with SMAD4 in order to translocate to the nucleus and act as a transcription factor to modulate gene expression [91, 134].
a. Anti-adipogenic effects of TGF-β and SMAD3 in vitro

Recent data suggest that TGF-β signalling plays a role in the etiology of obesity and insulin resistance [189, 215]. TGF-β is a pro-inflammatory cytokine that is positively correlated with BMI and fat mass, and inversely associated with VO₂ max in humans [123, 215]. In vitro, the actions of TGF-β are anti-adipogenic. The inclusion of TGF-β into the differentiation media of 3T3-L1 adipocytes prevented their commitment to the adipocyte lineage [89], but this inhibitory effect was restricted to the early stages of adipocyte differentiation [179]. Similarly, the overexpression of TGF-β1 significantly reduced the size of all WAT fat pads in transgenic mice [37]. This anti-adipogenic effect is attributed to SMAD3 activity. As expected, treatment of mouse embryonic fibroblasts (MEF) with TGF-β significantly inhibited adipogenesis, but this effect was abolished in MEF from SMAD3⁻/⁻ animals [196]. Furthermore, in vitro, SMAD3 directly interacts with and inhibits CCAAT-enhancer-binding proteins (C/EBP) β and δ [35], two transcription factors that play a key role in adipocyte differentiation. While SMAD3 only weakly interacts with PPARγ, SMAD3 inhibitory effect on C/EBPs repressed PPARγ promoter transcription [35]. Overall, these data suggest that a SMAD3/CEBP/PPARγ pathway may be involved in the anti-adipogenic effects of TGF-β.

b. In vivo silencing of TGF-β/SMAD3 protects against diet-induced insulin resistance

Recently, two groups have shown that SMAD3⁻/⁻ mice are resistant to high-fat diet-induced obesity and insulin resistance [189, 215]. SMAD3⁻/⁻ mice exhibit decreased
adiposity, along with increased whole-body glucose uptake and insulin sensitivity. This phenotype was attributed to the modulation of WAT metabolism. In fact, total fat pad weight and adipocyte size were both decreased in knockout mice [215]. In addition, mitochondrial content and function were improved in WAT of SMAD3−/− mice as shown by elevated presence of brown adipocytes, increased oxygen consumption and expression of PGC-1α and uncoupling protein-1 (UCP-1) [215]. Conversely, the gene expression of PPARγ, involved in fatty acid storage, was decreased [189]. Interestingly, the treatment of high-fat fed mice with TGF-β neutralizing antibodies replicated the SMAD3−/− phenotype [189].

It is important to note that while in vitro data suggested an anti-adipogenic role of TGF-β and SMAD3, in vivo studies conversely demonstrated that the repression of SMAD3 and TGF-β decreased obesity. Moreover, the fact that TGF-β has anti-adipogenic effects in vitro but is positively correlated with adiposity in humans highlights another discrepancy. Clearly, the modulation of adipogenesis, PPARγ and adipose tissue metabolism by TGF-β and SMAD3 requires further investigation to reconcile data from cell lines and rodent models.

c. Involvement of R-SMADs, I-SMADs and Co-SMADs in the TGF-β/SMAD pathway

While most of the research has focused on the seminal protein SMAD3, the TGF-β signalling pathway includes numerous other intracellular modulators. In the traditional cascade, receptor-activated SMADs (R-SMADs or SMAD1, 2, 3, 5, and 8)
are phosphorylated upon ligand binding, interact with common mediator SMAD (Co-SMAD), SMAD4, to translocate into the nucleus, bind to SMAD binding elements and modulate gene expression. Modulation and regulation of this intracellular signalling pathway is achieved at numerous levels. First, different ligands activate specific parts of this pathway. For instance, TGF-β binding leads to the activation of SMAD2 and 3, while other members of the TGF-β superfamily, such as bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), phosphorylate SMAD1, 5, and 8 [32, 73]. In addition, these pathways can be negatively and selectively regulated by inhibitory SMADs (I-SMADs or SMAD6 and 7). While SMAD6 inhibits SMAD1, 5, and 8, SMAD7 acts specifically on SMAD2 and 3 [131, 217]. Both I-SMADs act via the recruitment of SMAD ubiquitination regulatory factor (SMURF) proteins that target activated SMADs for proteasomal degradation [99, 217]. Overall, this signalling cascade is elegantly regulated at several levels and many different proteins, in addition to SMAD3, should be considered when examining the role of TGF-β/SMAD in obesity and insulin resistance (Figure 1.4).
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**Figure 1.4: TGF-β and GDF/BMP signalling cascades.** Ligands from the TGF-β family bind specifically to TGF-β type 1 and 2 receptors to phosphorylate and activate SMAD2/3. Ligands from the GDF and BMP family bind to BMP receptors to phosphorylate and activate SMAD1/5/8. R-SMADs physically interact with Co-SMAD, SMAD4, in order to translocate to the nucleus and activate the transcription of target genes. The SMAD signalling cascade can be modulated by the actions of I-SMADs (SMAD6/7), SMURF2, SARA, JNK, and ERK. BMP: bone morphogenetic proteins; ERK: extracellular signal-regulated kinase; GDF: growth differentiation factor; JNK: c-Jun N-terminal kinases; P: phosphate group; SARA: SMAD anchor for receptor activation; TGF-β: transforming growth factor-β.

d. Regulation of TGF-β/SMAD pathway by PPARγ agonism

The modulation of the SMAD pathway by PPARγ remains elusive but several reports highlight that PPARγ agonism can inhibit TGF-β/SMAD signalling. In human Tenon’s fibroblasts, rosiglitazone (ROSI), a PPARγ agonist, effectively reversed TFG-β-induced SMAD2/3 phosphorylation [51]. In human aortic smooth muscle cells, PPARγ agonism through a prostaglandin ligand prevented TGF-β-induced
injury through a SMAD3 mechanism [64]. Downregulation of the TGF-β/SMAD pathway by PPARγ agonists was also reported in mesangial cells [76], epithelial cells [80], human lung fibroblasts [111], human peritoneal mesothelial cells [145], lung cancer cells [161] and renal interstitial fibroblasts [209].

Interestingly, the reciprocal regulation of TGF-β on PPARγ has also been reported. For instance, in human aortic smooth muscle cells, TGF-β inhibited PPARγ gene expression, an action attributed to the repressive action of SMAD3/4 on the PPARγ promoter [65]. In isolated pulmonary artery smooth muscle cells, hypoxia downregulated PPARγ expression, but the phenotype was normalized by TGF-β antagonism [72]. Overall, there is ample evidence that PPARγ agonism represses signalling of the TGF-β/SMAD pathway, but this evidence is restricted to cell line models. It is currently unknown 1- whether PPARγ agonism with ROSI would modulate TGF-β signalling in adipocytes and/or adipose tissue in vitro and in vivo and 2- whether this would be associated with metabolic benefits.

1.2 Approaches to prevent and treat metabolic pathologies

1.2.1 Resveratrol

Resveratrol (RSV; 3,5,4′-trihydroxy-trans-stilbene) is a polyphenolic compound found in plants and most commonly in the skin of grapes and in red wine [77]. From a biological standpoint, RSV is synthesized in plants in response to bacterial or fungal infection. Consequently, its concentration in plants varies considerably based on the growing conditions, presence of infection and temperature [124, 175].
The interest in RSV started in 1997, when the first in vitro study suggested that it had anti-carcinogenic effects [94]. Later on, another key study by Howitz and colleagues [88] suggested that RSV could act as a calorie restriction mimetic to extend yeast lifespan by 70% through the activation of SIRT1. While the results of the Howitz study [88] are still debated [96, 142], especially with regards to the direct activation of SIRT1 by RSV, this report sparked interest in possible use of RSV for metabolic benefits. Since then, several reports showed metabolic benefits of RSV, at least in cell culture and in animal models.

a. The effects of resveratrol on whole-body and skeletal muscle metabolism

RSV supplementation in rodents has produced exciting data with regards to metabolic health and survival. In high-fat fed rodents, supplementation with RSV prevented diet-induced obesity and insulin resistance, which was accompanied by enhanced mitochondrial content and function [113] and reduced diacglycerol and ceramide content in skeletal muscle [198]. RSV also decreased mortality by 31% in mice fed a high-calorie diet [12] and normalized plasma lipids in rodents [45, 164]. However, one study reported that RSV did not stimulate mitochondrial biogenesis in soleus or triceps in rodents [85]. Importantly, metabolic advantages of RSV are restricted to rodents that are prone to metabolic disorders through diet-induced obesity [12, 113], genetic manipulations [45, 164] or streptozotocin treatment [181]. In fact, the supplementation of RSV to healthy rodents does not translate into metabolic advantages [12, 85, 113, 164]. Finally, the daily dose of RSV provided to rodents in the aforementioned studies ranged widely from 10 mg/kg body weight [164] to 400
mg/kg body weight [113], making it challenging to conclude about possible benefits and mechanisms of action.

The whole-body effects of RSV are attributed, at least in part, to modulations of skeletal muscle metabolism. In skeletal muscle, RSV enhances mitochondrial oxidative capacity, enzyme activity and the mRNA expression of PGC-1α and PGC-1β, two essential mitochondrial transcriptional co-activators [12, 113, 150]. The activation of PGC-1α requires its deacetylation and, accordingly, RSV was reported to deacetylate PGC-1α [12, 113]. Both SIRT1 [150] and AMPK [85, 198] are necessary for RSV-induced PGC-1α deacetylation and activation, but the question remains whether SIRT1 and AMPK act in series or in a parallel manner and which of these molecules acts upstream of the cascade. Interestingly, RSV dosage may provide a solution for the conflicting reports. Price and colleagues [143] reported that low doses of RSV (25-30 mg/kg body weight daily) activated AMPK in a SIRT1-dependent manner, while high doses of RSV (215-230 mg/kg body weight daily) activated AMPK in a SIRT1-independent fashion. In support of the concept that AMPK can act upstream of SIRT1 with high doses of RSV, AMPK was shown to increase NAD⁺ levels and activate SIRT1 [24]. In addition, strong evidence suggested that high doses of RSV inhibit phosphodiesterases, which activate AMPK through Ca²⁺, upstream of SIRT1 and PGC-1α [143]. Taken together, these data suggest that skeletal muscle is a key tissue involved in the beneficial effects of RSV at the whole-body level, but the exact molecular mechanisms of action, involving SIRT1, AMPK and PGC-1α remain elusive and may be dose-dependent.
b. The effects of resveratrol on lipid handling in WAT

While RSV actions have been primarily investigated in skeletal muscle, a growing body of literature has demonstrated that RSV can act directly on adipose tissue to modulate lipid homeostasis and inflammation. *In vitro*, data suggest that RSV acts as an anti-adipogenic agent. In fact, in differentiated 3T3-L1 adipocytes [148, 187] and in adipose tissue explants [119], RSV inhibited triglyceride accumulation and potentiated FFA release in catecholamine-treated, but not in basal, conditions. However, RSV-potentiated lipolysis was abolished in eWAT explants from ATGL knockout mice, but not from HSL knockout animals [119]. Similarly, ATGL protein content was increased 16-fold with RSV treatment, while HSL remained unchanged [119], suggesting that RSV-potentiated lipolysis is regulated by ATGL. Moreover, RSV potentiated the formation of cAMP by epinephrine, but cAMP elevations did not fully explain the lipolytic actions of RSV [187]. In summary, RSV treatment *in vitro* potentiates catecholamines-stimulated lipolysis via ATGL in a cAMP-dependent and -independent fashion, which may contribute to an anti-adipogenic effect of RSV.

However, *in vivo*, the anti-adipogenic actions of resveratrol are more variable. In rodents, some studies [5, 33, 102, 198], but not all [4, 190], reported a decrease in fat pad weight and/or body fat with RSV treatment without differences in food intake. In humans, the data are controversial: most investigations do not show changes in adipose tissue weight with RSV [106, 149, 192]. In fact, Timmers and colleagues [192] reported that FFA and glycerol release were decreased – not increased – after supplementation with RSV. Taken together, these data suggest that the anti-adipogenic effect of RSV *in vitro* may not translate to *in vivo* models and especially
to humans. Aside from the inherent differences in the models used, the in vitro reports mostly investigated the effects of RSV on lipolysis and triglyceride synthesis, which are only two of the processes that regulate the size and lipid content of adipose tissue, and have not considered other key components of lipid homeostasis, such as glyceroneogenesis and fatty acid influx.

c. The effects of resveratrol on inflammatory processes in WAT

RSV has also been associated with anti-inflammatory properties in adipose tissue. In 3T3-L1 adipocytes, RSV pre-treatment for 6 h normalized TNF-α-induced increases in plasminogen activator inhibitor-1 (PAI-1) and IL-6 and decreases in adiponectin [2]. In the same model, RSV also decreased liposaccharide (LPS)-induced inflammation by normalizing IL-6 and TNF-α mRNA expression and protein content [98]. A similar anti-inflammatory effect was reported in human primary adipocytes: RSV reversed TNF-α-induced inflammation as evidenced by lower IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) concentrations in media [36]. In human scAT explants, treatment with IL-1β triggered a proinflammatory response (increases in IL-6, MCP-1, PAI-1 and decreases in adiponectin mRNA expression), which was partly prevented by concomitant RSV treatment [139]. This anti-inflammatory effect was replicated in primary human adipocytes [139], suggesting that human adipocytes are a direct target of RSV for its anti-inflammatory actions.

Adiponectin is one of the most studied adipokines with regards to its modulation by RSV. In animal studies, most reports [3, 45, 164], but not all [12, 198] showed that
RSV increases adiponectin secretion and/or mRNA expression. In human visceral adipocytes [39] and in human adipose tissue explants [139], adiponectin mRNA was increased with RSV. In addition to its effect on adiponectin transcription, RSV also improves the post-translational processing of adiponectin via upregulation of disulphide bond A oxidoreductase-like protein (DsbA-L) [206], a protein that stimulates the formation of disulfide bonds and promotes adiponectin multimerization in the endoplasmic reticulum. Overall, based on cell culture, tissue culture and animal models, RSV has anti-inflammatory effects, which can be explained, at least in part, by direct action on adipocytes.

d. The metabolic and anti-inflammatory effects of resveratrol in human clinical trials

There are only a few clinical studies that examined the effects of RSV supplementation on metabolic markers in humans and the potential benefits remain equivocal (Table 1.1). In 11 healthy obese men, 150 mg RSV daily for 30 days mildly improved fasting blood glucose, insulin and triglycerides [192] and decreased adipocyte size [106], but did not modulate glucagon-like peptide-1 (GLP-1) or glucose-dependent insulino tropic polypeptide (GIP) [104]. In other clinical trials, RSV had no effect on insulin sensitivity (measured by a euglycemic-hyperinsulinemic clamp), blood pressure, plasma biochemistry or ectopic lipid deposition in obese men [149] and postmenopausal women [222]. The effects of RSV may be more important in patients whose metabolism is compromised at baseline. Indeed, a small dose of 5 mg RSV twice daily for 4 weeks improved insulin sensitivity as assessed by homeostatic model assessment of insulin resistance (HOMA-IR) in patients with type
2 diabetes [19]. However, as a biomarker, HOMA-IR is less specific and less sensitive than glucose infusion rate during a euglycemic-hyperinsulinemic clamp, so it is still unknown whether RSV would improve insulin sensitivity in a diseased population as assessed by gold standard procedures.

RSV also only has a mild effect – if any – on inflammatory markers in humans. In healthy volunteers, supplementation with *Polygonum cuspidatum* extract containing 40 mg RSV for 6 weeks decreased plasma IL-6, TNF-α and C-reactive protein [68]. In patients with hypertension and T2DM, RSV decreased plasma IL-6 and mRNA expression of inflammatory markers in peripheral blood mononuclear cells. However, this anti-inflammatory effect was not replicated in several other randomized clinical trials [19, 138, 149, 192, 222]. Overall, literature examining the metabolic and anti-inflammatory effects of chronic resveratrol supplementation in humans is in its infancy. Several questions remain with regards to the type of population most likely to benefit from RSV interventions (if any), the appropriate dose, which biomarkers are more sensitive and whether the effects could be clinically significant. In addition, the mechanisms of action of RSV in human skeletal muscle, WAT and other tissues remain to be elucidated.
Table 1.1. Review of human clinical trials investigating the effects of chronic RSV supplementation on metabolic and/or inflammatory markers

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subject population</th>
<th>Intervention</th>
<th>RSV dose</th>
<th>Main findings</th>
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| [19]      | 19 males with T2DM | Double-blind, placebo-controlled, 4 wk randomization | 2 x 5 mg daily | RSV: ↓ HOMA-IR  
RSV: ↑ pAKT:AKT in platelets |
| [192]     | 11 healthy obese men | Double-blind, placebo-controlled, crossover, 30 d randomization | 150 mg daily | RSV: ↓ sleeping & resting MR  
RSV: ↑ AMPK, SIRT1, PGC-1α in SM  
RSV: ↓ plasma glucose, TG, insulin, TNFα  
RSV: ↓ PP lipolysis & glycerol release |
| [40]      | 10 elderly (7 F) subjects with IGT | Open-label, 4 wk supplementation | 1, 1.5 or 2 g daily | No change in fasting glucose  
RSV: ↓ PP peak glucose & AUC |
| [222]     | 29 non-obese, postmenopausal women with NGT | Double-blind, placebo-controlled, 12 wk randomization | 75 mg daily | No change in body composition, resting MR, plasma lipids or inflammatory markers  
No change in GIR on EH clamp  
No change in AMPK, SIRT1, PGC-1α in SM & AT |
| [104]     | 10 healthy obese men | Double-blind, placebo-controlled, crossover, 30 d randomization | 150 mg daily | No change on fasting or PP concentrations of GIP or GLP-1  
RSV: ↓ PP glucagon |
| [106]     | 11 healthy obese men | Double-blind, placebo-controlled, crossover, 30 d randomization | 150 mg daily | RSV: ↓ adipocyte size in scAT |
| [149]     | 24 healthy obese men | Double-blind, placebo-controlled, 4 wk randomization | 3 x 500 mg daily | No change on insulin sensitivity or EGP  
No change on lipid oxidation, ectopic fat deposition or inflammatory markers |
### Limitations of resveratrol as a nutraceutical agent

One of the main limitations of using RSV in humans resides in its poor bioavailability. While the absorption of RSV in the gastro-intestinal tract is rapid (within 30 min) and extensive (> 70%), RSV is also metabolized very quickly by the intestine and liver [200]. Consequently, only 2% of RSV products in plasma are intact RSV, while the remaining compounds are metabolites [70, 200]. In a human clinical trial investigating the bioavailability of RSV in healthy volunteers, supplementation of 5 g RSV for 29 days elevated the maximal plasma RSV concentration to 5 µmol/L [144]. In the study by Timmers and colleagues [192], plasma RSV concentration after treatment (150 mg RSV daily; 30 days) only reached 0.8 µmol/L. Notably, cell culture studies investigating the effects of RSV have used doses of 50-100 µmol/L, which is 10-100 fold higher than what is reported in human plasma after chronic RSV supplementation [144, 192]. Consequently, the differences between the doses used in...
clinical and pre-clinical studies constitute a major source of discrepancy between these models.

Furthermore, the doses of RSV given in both clinical and pre-clinical studies far exceed what is achievable through a regular diet. In fact, red wines, the most common dietary source of RSV, contain 2-12 mg RSV/L [13]. RSV doses administered to animals range from 10-400 mg RSV/kg body weight, which would translate to 60-2,400 L of wine daily. Consequently, it is not surprising that epidemiological studies examining the link between dietary RSV intake and health outcomes did not find any significant effect on all-cause mortality and inflammatory biomarkers [174]. Based on these data, RSV may well be better suited in a nutraceutical form than as a part of the diet. Interestingly, different forms of RSV [7] or mode of delivery [170] have the potential to increase the bioavailability and the effectiveness of RSV in humans.

1.2.2 Rosiglitazone

Rosiglitazone is a member of the thiazolidinedione (TZD) family, a class of drugs with anti-diabetic properties that acts primarily as a PPARγ agonist. PPARγ is a transcription factor that is widely expressed in WAT, is necessary for adipocyte differentiation and adipogenesis [168] and is essential to maintain whole-body glucose and lipid homeostasis [83]. In patients with T2DM, ROSI significantly improves glycemic and lipemic control: it decreases glycosylated haemoglobin (HbA1c) and fasting glucose, as well as plasma lipids such as FFA, total cholesterol and LDL-cholesterol [130, 159]. These whole-body effects are attributed to ROSI’s
PPARγ agonism in adipose tissue. In fact, in mice lacking WAT, treatment with ROSI or pioglitazone, another TZD, did not improve glycemic control [28]. In addition, PPARγ gain-of-function in adipocytes, but not in adipose tissue macrophages, is sufficient to replicate the whole-body insulin-sensitizing effects of ROSI in rodents [184]. This shows that the effectiveness of systemic PPARγ agonism may be attributed to its actions on WAT and adipocytes. Specifically, mitochondrial biogenesis, adipokine secretion and fatty acid handling in WAT are three physiological processes that are modulated by ROSI treatment and that can explain, at least in part, its insulin sensitizing effects.

a. The effects of rosiglitazone on lipid handling in WAT

In rodents and humans, ROSI treatment is characterized by increased adiposity [56, 132], which is attributed to PPARγ-dependent increases in fatty acid reesterification and lipid uptake. More specifically, ROSI is a strong activator of PEPCK [22, 69], PDK4 [23] and glycerol kinase [122]. The concerted actions of PEPCK and PDK4 contribute to the ROSI-induced glyceroneogenesis [23] while glycerol kinase activity provides another pathway to form glycerol-3-phosphate and enhance fatty acid reesterification. In addition, ROSI treatment stimulates triglyceride clearance from the circulation through increased lipoprotein lipase and fatty acid binding protein-4 activity in WAT [116]. Conversely, ROSI increases basal and norepinephrine-stimulated lipolysis from rat WAT explants [56], which is not surprising as lipolytic and glyceroneogenic rates are typically increased concomitantly [21]. However, the lipolytic actions of ROSI are largely compensated by a greater increase in fatty acid
reesterification and lipid uptake, which ultimately results in increased adiposity but lower ectopic fat deposition and lipotoxicity.

b. The effects of rosiglitazone on mitochondrial biogenesis and WAT remodelling

ROSI treatment results in mitochondrial biogenesis in adipose tissue, which is necessary for other ROSI-induced benefits such as fatty acid synthesis, adipogenesis and adipose tissue remodelling. In fact, the initiation of adipogenesis and mitochondrial biogenesis is temporally synchronized in adipocytes [43]. Conversely, inhibiting mitochondrial function prevents fat accumulation [97], a process that is correlated with PPARγ mRNA expression. The association between mitochondrial biogenesis and adipogenesis is perhaps not surprising given that 1- the transcription factors and coactivators PPARγ, CREB, ERRα and PGC-1α are involved in both processes [90, 180]; 2- adipogenesis is an energy-costly process as illustrated by the decrease in ATP during adipocyte differentiation [211] and 3- mitochondria provide essential intermediates, such as G3P, for triglyceride synthesis [23]. Overall, these data suggest that mitochondrial biogenesis is necessary for energy provision during adipogenesis and fatty acid synthesis.

In adipocytes [211], in rodent WAT [34, 166] and in human adipose tissue [17], ROSI increases respiration, as well as protein content and mRNA expression of mitochondrial markers. Furthermore, adipose tissue mitochondrial content and function are compromised in patients with T2DM but normalized with ROSI treatment [78]. Notably, mitochondrial biogenesis is associated with the development
of newer and smaller adipocytes and healthy adipose tissue remodelling [211]. Accordingly, ROSI-induced adiposity is characterized by healthy adipocyte hyperplasia as opposed to detrimental hypertrophy [184, 211]. In fact, ROSI promotes apoptosis of hypertrophied adipocytes while triggering the appearance of new, smaller adipocytes [44]. Taken together, hallmark features of ROSI include the induction of mitochondrial biogenesis, which allows adipocyte differentiation and healthy adipose tissue remodelling [211], while supporting the energy requirements of lipid accretion [67].

c. The effects of rosiglitazone on adipokine secretion and inflammation in WAT

In addition to its anti-lipemic effects, ROSI has important anti-inflammatory actions. ROSI treatment robustly increases adiponectin protein content and secretion in rodents [105, 195] and humans [130, 191]. ROSI specifically enhances the concentration of high molecular weight isomers [195], the adiponectin form that is most strongly correlated with insulin sensitivity [81]. As discussed above, adiponectin transcription is regulated by PPARγ [92] and is dependent upon mitochondrial function [105]. As ROSI is a PPARγ ligand [120] and stimulates mitochondrial biogenesis [166], it provides at least two different mechanisms by which ROSI may enhance adiponectin transcription and secretion. In addition, ROSI’s actions are much less potent in adiponectin knockout models [110, 135], suggesting that adiponectin is essential for ROSI’s actions. Therefore, adiponectin may explain, at least in part, how ROSI’s effects on WAT are translated into whole-body glycemic and lipemic control.
Overall, ROSI treatment acts directly on adipose tissue to promote beneficial remodelling and to increase the uptake and sequestering of fatty acids while indirectly enhancing fatty acid oxidation in peripheral tissues through adiponectin secretion. These coordinated actions lead to the normalization of blood lipids, insulin sensitization and an anti-inflammatory phenotype.

d. The effects of rosiglitazone in WAT are depot-specific

Importantly, the effects of ROSI on adipose tissue are depot-specific. TZD treatment is not only associated with increased adiposity, but also triggers a redistribution of fat away from visceral stores and towards subcutaneous depots [132, 178]. As subcutaneous adipose tissue is generally regarded as safer than visceral depots [109], ROSI’s depot-specific effects may largely contribute to its anti-diabetic actions. The preferential distribution of fat in subcutaneous stores is attributed to increases in TG-derived FFA uptake and in TG esterification via diacylglycerol acyltransferase-1 (DGAT1) specifically in scAT [54]. In addition, in the postprandial state, TG-derived lipid uptake and lipoprotein lipase (LPL) activity were robustly increased by PPARγ agonism in scAT, while being only moderately activated in visceral stores [114]. Conversely, PPARγ agonists act specifically in viscAT to increase fatty acid oxidation and thermogenesis, while basal lipolysis, fatty acid reesterification and mitochondrial biogenesis are increased to similar extents by PPARγ agonists in both depots [115]. Clearly, PPARγ agonism acts differentially depending on the location of the adipose tissue depots. While these depot-specific effects are well described in rodents, there are several limitations to translate this knowledge for humans. First, it
may be oversimplifying the differentiation between scAT and viscAT. For instance, viscAT refers to all depots located around internal organs, such as retroperitoneal, mesenteric and gonadal adipose tissue. There is evidence that, while they are all considered visceral depots, they may react differently to PPARγ agonism and other stimuli [114]. Second, there exist major differences between the anatomy and physiology of adipose tissue in humans and rodents. For instance, epididymal adipose tissue only exists in rodents and human AT is less responsive to the anti-lipolytic actions of insulin [26]. Therefore, there remain important questions to be answered with regards to depot-specific adipose tissue metabolism in humans with ROSI and other stimuli.

e. Limitations of rosiglitazone as an anti-diabetic pharmacological agent

Despite its efficacy on whole-body homeostatic control, ROSI treatment is seldom used in clinical settings due to its severe adverse effects. First, as described above, ROSI is adipogenic and, as such, is associated with significant weight gain [15]. Moreover, a meta-analysis [136] reported elevated risks of myocardial infarction (odds ratio [OR]: 1.43) and of cardiovascular mortality (OR: 1.64) in patients treated with ROSI. In addition, ROSI treatment was also associated with higher risk of fractures in men and women (OR: 1.39) [11]. Given that the risks associated with ROSI may exceed its benefits, limiting its use in clinical settings seems justified. However, ROSI remains an interesting tool to study adipose tissue physiology in the context of insulin resistance. Furthermore, distinct members of the TZD family behave differently in terms of adverse effects [130, 197], which suggests that finding
alternative ways to activate PPARγ and/or combining PPARγ agonists at lower concentrations with other agents may mimic the beneficial effects of ROSI while limiting the rates and severity of adverse effects.

1.3 Conclusions

Adipose tissue is an active organ that possesses inflammatory, endocrine and metabolic properties and that profoundly influences whole-body metabolism in health and disease. In WAT, the combined impacts of fatty acid uptake, lipolysis and fatty acid reesterification regulate whole-body lipid handling and play a crucial role in downregulating fat deposition and lipotoxicity in peripheral tissues. In addition, adipocytes and immune cells contained in WAT secrete adipokines whose inflammatory profile is linked to the size of the adipocytes and the extent of macrophage infiltration. Finally, adipose tissue mitochondrial biogenesis is recognized as an essential process to maintain metabolic health and is necessary for intact inflammatory and lipid handling functions in WAT.

Nutritional and pharmacological strategies to promote mitochondrial biogenesis, glyceroneogenesis and adiponectin secretion in adipose tissue have shown promise to prevent and treat insulin resistance and T2DM. Resveratrol, a polyphenolic compound found in grapes and wine, has anti-adipogenic effects in cell line models, improves metabolic markers at the whole-body, skeletal muscle and adipose tissue levels in rodents, but has yielded controversial data in humans. On the other hand, rosiglitazone, a PPARγ agonist from the thiazolidinedione family, is a potent anti-
diabetic agent that acts specifically on WAT, but has limited clinical use due to prevalent and severe adverse effects. Finally, recent data highlighted the importance of the TGF-β/SMAD pathway for adipose tissue and metabolic health. This ubiquitous pathway is regulated by PPARγ agonism in cell line models, but this relationship has not been examined in adipose tissue.

The investigation of resveratrol- and rosiglitazone-mediated modulations in rodent and human adipose tissue physiology would improve our understanding of adipose tissue in metabolic pathologies and would elucidate the effectiveness and mechanisms of actions of nutritional and pharmacological approaches to manage insulin resistance and T2DM.
Chapter 2: Aims of the Thesis

Obesity and insulin resistance are closely associated with the deregulation of white adipose tissue metabolism. Lipolysis and glyceroneogenesis are two crucial processes to maintain lipid homeostasis, while adipokine secretion controls whole-body inflammatory status and peripheral fuel use. In humans with metabolic pathologies, basal lipolysis is increased while catecholamine-stimulated lipolysis and glyceroneogenesis are reduced [84, 219]. This altered management of lipids within white adipose tissue contributes to excess circulatory fatty acids, ectopic fat deposition and lipotoxicity in peripheral tissues. Furthermore, in obese adipose tissue, interactions between adipocytes and immune cells lead to a pro-inflammatory adipokine profile that is characterized by an elevated secretion of IL-6 and TNF-α but reduced level of adiponectin. Nutraceutical and pharmacological strategies to normalize these processes may be promising in the management of insulin resistance and obesity.

Recent data have implicated the TGF-β/SMAD pathway in the etiology of insulin resistance and obesity and suggested that its signalling in white adipose tissue may be crucial for this effect [189, 215]. The PPARγ agonist rosiglitazone is an anti-diabetic agent that acts in a depot-specific fashion in white adipose tissue to promote healthy remodelling and fatty acid sequestering [54, 115]. It is currently unknown whether PPARγ agonism could modulate the TGF-β/SMAD signalling cascade. Given the importance of PPARγ in the regulation of adipogenesis and lipid homeostasis, it is crucial to determine whether rosiglitazone may alter TGF-β/SMAD signalling in
distinct adipose tissue depots. Furthermore, given some of the adverse effects associated with rosiglitazone treatment, it is important to determine whether less toxic agents could mimic its beneficial effects while limiting the risks of adverse effects [136].

Resveratrol is a nutraceutical compound with anti-diabetic and anti-inflammatory properties. To date, reports that examined the role of resveratrol on metabolic markers have focused on rodent models and on skeletal muscle metabolism [12, 113]. Moreover, the few investigations of RSV efficacy in humans have yielded inconsistent results [19, 104, 149, 192, 222]. Importantly, the understanding of the role of resveratrol on key homeostatic processes, such as lipolysis, glyceroneogenesis and adipokine secretion in rodent and human white adipose tissue is lacking. Based on its PPARγ activating effects [155], resveratrol may constitute a good candidate as a rosiglitazone mimetic.

The primary objective of this thesis was to investigate the individual and combined modulatory effects of resveratrol and rosiglitazone on white adipose tissue physiology in rodent models and human adipose tissue. To this end, three different studies were conducted:

The specific objectives of study one were:

1- to examine the effects of dietary resveratrol supplementation on glyceroneogenesis, mitochondrial biogenesis and adipokines secretion in white adipose tissue from Zucker Diabetic Fatty (ZDF) rats;
2- to assess whether resveratrol treatment could have direct modulatory effects on white adipose tissue using an *ex vivo* approach.

The specific objectives of study two were:

1- to characterize the ability of rosiglitazone to modulate the TGF-β/SMAD pathway in white adipose tissue from diabetic rodents *in vivo*;
2- to compare rosiglitazone’s modulatory actions on TGF-β/SMAD signalling between subcutaneous and visceral white adipose tissue depots.

The specific objective of study three was:

1- to investigate the individual and combined impacts of resveratrol and rosiglitazone on glyceroneogenic and inflammatory gene expression in cultured subcutaneous and visceral adipose tissue from morbidly obese subjects

The overall hypotheses of this thesis were that:

1- chronic dietary resveratrol supplementation would improve whole-body glucose tolerance and insulin sensitivity in diabetic rats, which would be associated with improved glyceroneogenesis, mitochondrial biogenesis and adiponectin secretion in white adipose tissue;
2- in ZDF rats, long-term treatment with rosiglitazone would improve whole-body glucose homeostasis, stimulate adipogenesis and would concurrently inhibit signalling from the TGF-β/SMAD pathway in white adipose tissue;
3- combined treatment of cultured white adipose tissue from morbidly obese patients with resveratrol and rosiglitazone would show additive effects to
increase glyceroneogenic gene expression and decrease inflammatory gene expression.
Chapter 3: Resveratrol supplementation improves white adipose tissue function in a depot-specific manner in Zucker Diabetic Fatty (ZDF) rats

As published with minor revisions:

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3.1 Abstract

Resveratrol (RSV) is a polyphenolic compound suggested to have anti-diabetic properties. Surprisingly, little is known regarding the effects of RSV supplementation on adipose tissue metabolism *in vivo*. The purpose of this study was to assess the effects of RSV on mitochondrial content and respiration, glyceroneogenesis (GNG), and adiponectin secretion in adipose tissue from Zucker Diabetic Fatty (ZDF) rats. Five-week old ZDF rats were fed a chow diet with (ZDF RSV) or without (ZDF chow) RSV (200 mg/kg body weight) for 6 weeks. Changes in adipose tissue metabolism were assessed in subcutaneous (scAT) and intra-abdominal (retroperitoneal [rpWAT], epididymal [eWAT]) adipose tissue depots. ZDF RSV rats showed lower fasting glucose and higher circulating adiponectin, as well as lower glucose area under the curve during i.p. glucose and insulin tolerance tests than ZDF chow. $^{14}$C-pyruvate incorporation into triglycerides and adiponectin secretion were higher in scAT from ZDF RSV rats, concurrent with increases in adipose tissue
triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and the phosphorylation of pyruvate dehydrogenase-E1α (PDH) (ser293) protein content in this depot. Moreover, uncoupled mitochondrial respiration and complex I and II-supported respiration were increased in both scAT and rpWAT, which correlated with increases in cytochrome C oxidase subunit IV (COX4) protein content. *In vitro* treatment of scAT with RSV (50 µmol/L; 24h) induced pyruvate dehydrogenase kinase 4 (PDK4) and PPARγ coactivator-1α (PGC-1α) mRNA expression. Collectively, these data demonstrate that RSV can induce adipose tissue mitochondrial biogenesis in parallel with increases in GNG and adiponectin secretion.

### 3.2 Introduction

Resveratrol (RSV; 3,5,4'-trihydroxy-trans-stilbene) is a polyphenolic compound found in plants, especially in the skins of red grapes. Its supplementation to rodents fed a high-fat diet improves insulin sensitivity [113] and life span [12]. In humans, small beneficial metabolic effects of RSV have been reported in obese subjects [192] and patients with type 2 diabetes (T2DM) [19], but not in individuals with normal glucose tolerance [222]. While the efficacy of RSV in humans remains controversial, the beneficial effects of RSV in rodents have been mostly attributed to enhanced mitochondrial biogenesis [113, 143] and activation of PGC-1α [113, 150, 198] in skeletal muscle.

While most investigations focus on the effects of RSV on skeletal muscle, an emerging body of evidence suggests that adipose tissue may also be responsive to RSV treatment. In addition to the anti-inflammatory effects of RSV on adipose tissue
[206], RSV has been shown to have anti-adipogenic effects [31, 112] and to increase lipolysis in adipocytes in vitro [119, 148, 187]. Lipolysis is a key process in the provision of free fatty acids (FFA) and occurs through the hydrolysis of fatty acids from the glycerol backbone through sequential reactions by adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL) (reviewed in [46]). Furthermore, phosphorylation of perilipin is necessary to allow access of lipases to the lipid droplet [46]. RSV-mediated lipolysis is associated with increases in ATGL protein content [119] and cAMP [187], an upstream activator of HSL via the activation of protein kinase A (PKA).

Importantly, up to 35% of FFA liberated through lipolysis is recycled back into TG within the adipocyte [208], and this is dependent upon the provision of glycerol-3-phosphate (G3P) through non-glucose precursors, such as pyruvate, in a process called glyceroneogenesis (GNG) [23]. GNG is regulated through the concerted actions of pyruvate dehydrogenase kinase 4 (PDK4) and phosphoenolpyruvate carboxykinase (PEPCK). In mice, the knockdown of pck1, the gene coding for PEPCK, results in whole-body insulin resistance and increased FFA and glycerol release during a euglycemic-hyperinsulinemic clamp [129]. Conversely, over-expression of PEPCK in adipose tissue led to obesity without insulin resistance [60]. Absolute rates of fatty acid reesterification mirror that of lipolysis [21] and the reesterification of fatty acids is one of the largest consumers of ATP in fat cells [165]. In fact, work from Ruderman’s group [67] has demonstrated that the activation of the energy-sensing enzyme 5’AMP activated protein kinase (AMPK) by β-adrenergic agonists occurs secondary to increases in fatty acid reesterification. AMPK is a
reputed mediator of mitochondrial biogenesis [66, 93] and thus RSV-mediated increases in ATGL and lipolysis may lead to increases in fatty acid reesterification, the activation of AMPK, and the induction of mitochondrial biogenesis in adipose tissue. In support of this premise, adipose tissue-specific over-expression of ATGL in mice resulted in increased lipolysis without elevations in plasma FFA, TG, or ectopic lipid deposition, along with increases in whole body TG cycling and adipose tissue mitochondrial enzyme gene expression [1]. To the best of our knowledge, it is currently unknown whether RSV can induce mitochondrial biogenesis and increase GNG in adipose tissue in vivo.

Adiponectin is an anti-inflammatory adipokine that stimulates FA oxidation in skeletal muscle and liver, and is associated with improved insulin sensitivity [105, 220]. RSV treatment increased adiponectin mRNA in human adipocytes isolated from visceral depots [39], and elevated adiponectin protein content in 3T3-L1 adipocytes [206] and in rodent epididymal and retroperitoneal adipose tissue [164]. Interestingly, mitochondrial biogenesis has been shown to be essential for adiponectin synthesis and secretion in adipocytes [105] and thus, it seems plausible that RSV-mediated increases in mitochondrial biogenesis could be associated with increases in adiponectin secretion. Conversely, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are pro-inflammatory adipokines that are negatively regulated by RSV in vitro [2, 98], but the effects of RSV on these cytokines in vivo remain to be elucidated.

The purpose of the current study was to examine the effects of dietary RSV supplementation on mitochondrial biogenesis, GNG, and adipokines secretion in white adipose tissue (WAT) from Zucker Diabetic Fatty (ZDF) rats, a rodent model
of T2DM. In addition, as RSV is known to improve skeletal muscle metabolism and whole-body glucose homeostasis [12, 113], it was important to determine whether RSV may have direct effects on adipose tissue. To address this question, we used an acute ex vivo approach to assess gene expression in adipose tissue explants from untreated ZDF rats. We hypothesized that RSV supplementation in ZDF rats would induce mitochondrial biogenesis, which would be associated with enhanced GNG, increased adiponectin secretion, and the prevention of insulin resistance. Furthermore, these effects could be mediated, at least in part, by direct actions of RSV onto adipose tissue.

3.3 Materials and Methods

3.3.1 Animals

Four-week old male ZDF rats (Charles River, St. Constant, QC, Canada) weighing 100g were individually housed in wire-bottom cages, in a temperature-controlled room with a reverse 12:12 h light-dark cycle, and were provided with food and water ad libitum. The 12 h light cycle started at 9pm, and experiments were performed between 9am and 4pm. After a 10-day acclimatization period, ZDF rats were fed either a standard powdered chow diet (Purina 5008 diet; Purina, St.Louis, MO, USA) (ZDF chow) or a chow diet supplemented with RSV (Cayman Chemical, Ann Arbor, MI, USA) ~200 mg/kg body weight (BW) (ZDF RSV) for 6 weeks. RSV was mixed directly into the powdered diet on a weekly basis, based on predicted body weight and food intake for that week. ZDF rats are characterized by the development of insulin resistance by 6 weeks of age and T2DM by 12 weeks, therefore we attempted
to delay the onset T2DM by treating with RSV between 5 and 11 weeks of age [185]. We acknowledge that the ZDF model is a genetic model of T2DM and that the dose of RSV used in the present study is likely not attainable through diet alone. However, we used this approach to study the development of T2DM. Moreover, while the RSV dose is large, it is similar to many other rodent-based reports in the literature [113, 150, 198]. Food intake was recorded 3 times weekly while BW was assessed weekly. All protocols followed Canadian Council on Animal Care guidelines and were approved by the Animal Care Committee at the University of Guelph.

3.3.2 Tolerance tests
Intraperitoneal glucose (ipGTT) and insulin (ipITT) tolerance tests were performed before (5-week old) and after (11-week old) the feeding intervention. After measurement of fasting (12 h overnight) blood glucose (Freestyle lite, Abbott Laboratories, St-Laurent, QC, Canada) through the tail vein, 1 g/kg BW glucose (BioShop, Burlington, ON, Canada) was administered via i.p. injection. Blood glucose was assessed at 15, 30, 45, 60, 90, and 120 min. For ipITTs, fed animals were administered 5 U/kg BW insulin i.p. (Humulin, Eli Lilly, Toronto, ON, Canada) and blood glucose was determined at 0, 5, 10, 15, 30, and 45 min through the tail vein.

3.3.3 Terminal procedures
After the 6-week feeding protocol, fasted (12 h overnight) animals were weighed and anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL, USA). Adipose tissue (AT) from the inguinal subcutaneous (scAT), epididymal (eWAT),
and retroperitoneal (rpWAT) depots were harvested. For each depot, 100 mg was used for GNG determination, 25 mg was used for respiration experiments, 50 mg was fixed in formalin, while the rest of the tissue was immediately frozen in liquid nitrogen and stored at -80°C for later determination of protein content and gene expression. Blood was collected in polypropylene tubes through cardiac puncture, allowed to clot at room temperature, centrifuged, and the supernatant was collected and stored at -20°C for further analysis of triglycerides (colorimetric assay: Sigma-Aldrich, Oakville, ON, Canada), free fatty acids (colorimetric assay: Wako Diagnostics, Richmond, VA, USA), total adiponectin (ELISA: Linco Research, St.Charles, MO, USA), insulin (ELISA: Millipore, St.Charles, MO, USA), IL-6 (ELISA: BioLegend, San Diego, CA, USA) and TNF-α (ELISA: BioLegend) through commercially-available kits.

a. *Ex vivo* incubation procedures

100 mg of adipose tissue from each of scAT, eWAT, and rpWAT was minced and placed in a plastic vial containing 3 mL oxygenated Krebs Ringer buffer (KRB: 118 mM NaCl, 4.8 mM KCl, 1.25 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 25 mM NaHCO$_3$; pH 7.4) containing 5 mM glucose and 2.5% FFA-free bovine serum albumin (BSA; MP Biomedicals, Santa Ana, CA, USA) and incubated at 37°C in a shaking water bath (60 rpm) for 2h. At the end of the incubation, media was collected in polypropylene tubes and stored at -20°C for later determination of total adiponectin, IL-6, and TNF-α using commercially available kits (see providers above).
b. $^{14}$C-pyruvate incorporation into triglycerides

Quantification of the incorporation of $^{14}$C-pyruvate into triglycerides is a measure of GNG within adipose tissue [115]. Adipose tissue (100 mg) from each of scAT, eWAT and rpWAT was minced and placed in a plastic vial containing 2 mL oxygenated KRB containing 5 mM pyruvate and 2% FFA-free BSA, and 1 µCi of $^{14}$C-labeled pyruvate (PerkinElmer, Woodbridge, ON, Canada). Tissue was incubated at 37°C in a shaking water bath (60 rpm) for 1 h. At the end of the incubation, the reaction was stopped by adding 250 µL of 5N sulfuric acid (Fisher Scientific, Ottawa, ON, Canada) into the vials and incubating them on ice for 1-2 min. AT explants were thoroughly rinsed with water and transferred to new tubes for lipid extraction with 5 mL of a 2:1 solution of chloroform:methanol (both from Fisher Scientific). One mL of phosphate buffer saline (PBS: 0.137 M NaCl, 2.68 mM KCl, 1.47 mM KH$_2$PO$_4$, 8.03 mM Na$_2$HPO$_4$; pH 7.4) was added, and the tubes were vortexed and centrifuged before removal of the upper aqueous phase. This washing step was repeated twice. The chloroform:methanol solution was allowed to evaporate overnight in a fume hood, after which two 500 µL aliquots were transferred to scintillation vials, 3 mL of scintillation fluid (MP Biomedicals) was added, and $^{14}$C-radiation was determined over 5 min in a β-counter (Beckman-Coulter, LS6500 Scintillation Counter, Mississauga, ON, Canada).

c. Respiration

Adipose tissue respiration was determined as previously described [108], with minor modifications [203]. Briefly, adipose tissue was excised, immediately placed in
polypropylene tubes containing 1 mL of MiR05 buffer (0.5 mM EGTA, 3 mM MgCl₂•6H₂O, 60 mM K-lactobionate, 10 mM KH₂PO₄, 20 mM HEPES, 20 mM taurine, 110 mM sucrose, 1g/L FFA-free BSA; pH 7.1) and minced with scissors. Adipose tissue was then transferred to 1 mL of fresh MiR05 buffer and left on ice for 10 min. Thereafter, tissue was blotted and 25 mg used to determine rates of oxygen consumption by high-resolution respirometry (Oroboros Oxygraph-2 k, Innsbruck, Austria). Digitonin or saponin are used to permeabilize cell membranes while leaving mitochondrial membranes intact due to their specificity to solubilize cholesterol, which exists in much higher concentrations on the plasma membrane. This is a common procedure to permit exogenously-added substrates free access for diffusion to the mitochondria. However, we found mincing without permeabilization to be sufficiently effective in disrupting the cell membrane without compromising mitochondrial intactness. Specifically, robust respiration was detected in minced adipose tissue and this was not increased with the addition of either digitonin or saponin following standard permeabilizing procedures. This has also been reported previously [108]. Therefore, all experiments were performed in the absence of either chemical. Respiration experiments were performed at 37°C in room air saturated MiR05 buffer. The sequential respiration protocol consisted of determining state IV (leak) respiration in the presence of 10 mM glutamate + 2 mM malate, state III respiration in the presence 5 mM ADP (Complex I), and 10 mM succinate (Complex I and II). The subsequent titration of Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) yielded maximal uncoupled respiration. Rates of oxygen consumption are expressed per mg wet weight.
d. Histochemistry

ScAT was fixed in 10% neutral buffered formalin (VWR, Mississauga, ON, Canada) dehydrated in xylene (Fisher Scientific) and embedded in paraffin. We chose to further analyze only scAT as this depot responded most robustly to RSV supplementation based on respiration, adiponectin, and $^{14}$C-incorporation measures. Sections were taken at 5 µm and mounted on charged 1.2 mm Superfrost slides (Fisher Scientific). Sections were stained using modified Harris hematoxylin and eosin stock with phloxine (all from Fisher Scientific). Sections were imaged using an Olympus FSX 100 light microscope and images were acquired in Cell Sense software (Olympus, Tokyo, Japan). For each image, 100 cells were randomly sampled and cross-sectional area was measured via ImageJ software (National Institute of Mental Health, Bethesda, MD).

e. Adipose tissue explant experiments

Adipose tissue explant incubation is a well-characterized technique that is used regularly to determine changes in adipose tissue metabolism and gene expression [186, 202, 205]. To assess the direct effects of RSV on gene expression, scAT and rpWAT from untreated 12-week old ZDF rats was minced, placed in a Petri dish with Media 199 (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 2.5% FFA-free BSA, and incubated at 37°C with 5% CO$_2$ for 1 h. Adipose tissue was then treated with RSV 50 µmol/L [113] or vehicle (dimethyl sulfoxide) for 24 h. At the end of the incubation period, adipose tissue was rinsed in ice-cold PBS, strained, frozen in liquid nitrogen, and stored at −80°C for further gene expression analysis.
We did not include epididymal adipose tissue in these experiments, as this depot did not respond to RSV supplementation in vivo.

f. Protein content and cAMP determination

Adipose tissue samples were homogenized in 2 volumes of ice-cold cell lysis buffer (Invitrogen, Burlington, ON, Canada) supplemented with protease inhibitor cocktail (as per manufacturer’s instructions, Sigma-Aldrich, Oakville, ON, Canada) and 0.05% phenylmethylsulfonyl fluoride (BioShop, Burlington, ON, Canada) in a homogenizer (FastPrep®-24, MP Biomedicals). Homogenized samples were centrifuged for 10 min at 1500 x g at 4°C. Lipids were carefully removed and the protein-containing infranatant was collected for determination of protein concentration using a bicinchoninic acid (BCA) assay [177] (ThermoScientific, Rockford, IL, USA). Western blotting was performed as previously described by our laboratory [186, 202, 205]. Membranes were incubated in primary antibodies diluted in TBST/5% non-fat dry milk (COX4, MitoSciences, Eugene, OR, USA; α-tubulin, perilipin A: Abcam, Toronto, ON, Canada; ATGL, p-HSL[ser660]: Cell Signaling, Danvers, MA, USA) or TBST/5% bovine serum albumin (p-PDH [ser293], UCP-1: Calbiochem, Mississauga, ON, Canada; PDH, MitoSciences; PEPCK, Cayman Chemicals, Ann Arbor, MI, USA; HSL, p-HSL[ser563], AMPK, p-AMPK[Thr172]: Cell Signaling; DGAT1, GPAT: Abcam; DGAT2: Imgenex, San Diego, CA, USA) overnight at 4°C. Subsequently, membranes were washed in TBST and incubated in appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West
Grove, PA, USA) diluted in TBST/1% non-fat dry milk for 1 h at room temperature. Signal was detected through enhanced chemiluminescence (ThermoScientific) and quantified by densiometry (Fluorchem HD2, ProteinSimple, Santa Clara, CA, USA). Intracellular cAMP content of scAT and rpWAT was determined by ELISA according to the manufacturer’s instructions (Enzo Life Sciences, Brockville, ON, Canada).

g. Real-time quantitative PCR (qPCR)
RNA was isolated from adipose tissue using an RNeasy lipid kit (Qiagen, Toronto, ON, Canada) according to the manufacturer’s directions. Total RNA (1 µg) was used to synthesize complementary DNA (cDNA) using SuperScript II Reverse Transcriptase, random primers and dNTP (all from Invitrogen, Burlington, ON, Canada). Gene expression was quantified in duplicate using 1 µL cDNA template by qPCR on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the appropriate gene expression assay (Applied Biosystems) as per the manufacturer’s recommendations. Relative differences in gene expression between groups were determined using the 2^{-ΔΔCt} method [125]. The amplification efficiencies of the gene of interest and the housekeeping gene were equivalent. The genes analyzed through qPCR include PDK4, PCK1, PGC-1α, PGC-1β, PPARγ, RIP140, and β-actin. Results were normalized to the mRNA expression of β-actin, which did not change with treatment (β-actin CT values for in vivo experiments; ZDF chow: 26.35 ± 0.25; ZDF RSV: 26.33 ± 0.34. β-actin CT values for explant experiments; vehicle-treated: 17.45 ± 0.05 [scAT], 18.31 ± 0.13 [rpWAT]; RSV-treated: 17.63 ±
0.12 [scAT], 18.64 ± 0.31 [rpWAT]).

3.3.4 Statistical analysis

Data are presented as mean ± SEM. Comparisons of blood metabolites, oxygen consumption, GNG, and adiponectin secretion between ZDF chow and ZDF RSV were made using a 2-tailed, unpaired student t-test for each depot (GraphPad Prism 5, La Jolla, CA, USA). Given that our in vivo data suggested in which direction to expect changes, the remaining comparisons (protein content, gene expression, and cell cross-sectional area) were analyzed using a 1-tailed, unpaired student t-test. Statistical significance was accepted at α≤0.05.

3.4 Results

3.4.1 Whole-body parameters

Body weight, body weight gain, and average daily food intake were not different between ZDF chow and ZDF RSV rats throughout the 6-week feeding protocol (Table 3.1). At sacrifice, fasting blood glucose was lower in RSV-treated animals (p=0.007), but other blood metabolites were comparable between groups (Table 3.1). Post-intervention, ZDF RSV rats showed a significantly lower glucose area under the curve (AUC) to ipGTT (p=0.03) and ipITT (p=0.0004) challenges compared to chow-fed animals (Figure 3.1).
Table 3.1 Body and organ weights and fasting blood parameters at sacrifice for ZDF chow (n=11) and ZDF RSV (n=13) animals.

<table>
<thead>
<tr>
<th></th>
<th>ZDF chow</th>
<th>ZDF RSV</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>381 ± 7</td>
<td>383 ± 5</td>
</tr>
<tr>
<td>Average daily food intake (g)</td>
<td>28.7 ± 1.0</td>
<td>27.3 ± 0.9</td>
</tr>
<tr>
<td>scAT (inguinal) weight (g)</td>
<td>21.1 ± 1.5</td>
<td>22.0 ± 1.3</td>
</tr>
<tr>
<td>eWAT weight (g)</td>
<td>7.2 ± 0.4</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>rpWAT weight (g)</td>
<td>5.2 ± 0.2</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>14.2 ± 0.6</td>
<td>13.1 ± 0.5</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>9.3 ± 1.6</td>
<td>5.1 ± 0.2*</td>
</tr>
<tr>
<td>Fasting insulin (pmol/L)</td>
<td>723 ± 145</td>
<td>1101 ± 208</td>
</tr>
<tr>
<td>Fasting FFA (mmol/L)</td>
<td>1.36 ± 0.16</td>
<td>1.40 ± 0.14</td>
</tr>
<tr>
<td>Fasting glycerol (mmol/L)</td>
<td>0.45 ± 0.03</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>FFA:glycerol ratio</td>
<td>3.0 ± 0.3</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>Fasting TG (mmol/L)</td>
<td>4.25 ± 0.43</td>
<td>3.90 ± 0.54</td>
</tr>
</tbody>
</table>

Values are mean ±SEM. *: p<0.05 compared to ZDF chow.

3.4.2 Glyceroneogenesis

RSV treatment resulted in increased incorporation of $^{14}$C-pyruvate into triglycerides in scAT (p=0.03) but not in intra-abdominal depots (eWAT: p=0.21; rpWAT: p=0.35) (Figure 3.2A). In scAT, phosphorylation of pyruvate dehydrogenase (PDH) at serine 293, a marker of PDK4 activity [183], was elevated in ZDF RSV rats (p=0.01), while total PDH (p=0.25) and PEPCK (p=0.25) protein content did not change with RSV treatment (Figure 3.2B). Consistent with the functional data, phosphorylation of PDH (ser293) remained unchanged in eWAT (p=0.21) and rpWAT (p=0.28) from RSV-treated animals (data not shown).
Figure 3.1 RSV treatment improves glucose tolerance and insulin sensitivity. Intraperitoneal GTT (A; n=10 for ZDF chow, n=14 for ZDF RSV) and ITT (B; n=8) and total AUC (inserts) for ZDF chow (☐) and ZDF RSV (■). Values are mean ± SEM. *: p<0.05 compared to ZDF chow.
Figure 3.2 RSV stimulates GNG in scAT in ZDF rats. Assessment of GNG through $^{14}$C-pyruvate incorporation into triglycerides in scAT, eWAT and rpWAT (A; n=8) and quantification of protein content (B) of PEPCK (n=10), PDH (n=7), and phosphorylation of PDH at serine 293 (n=7) in scAT for ZDF chow (☐) and ZDF RSV (■). Open bars represent ZDF chow while closed bars represent ZDF RSV animals. Values are mean ± SEM. *: p<0.05 compared to ZDF chow.
3.4.3 Mitochondrial content and function

In scAT, RSV did not alter State IV respiration (p=0.28), however there was a trend for increased ADP-stimulated respiration through complex I (40%; p=0.09) (Figure 3.3A). In addition, state III respiration supported by simultaneous electron entry through complex I and II increased by 23% (p=0.02), as did maximal uncoupled respiration by 29% (p=0.01) in scAT from ZDF RSV rats (Figure 3.3A). Similarly, in rpWAT, RSV enhanced complex I and II-supported respiration by 45% (p=0.009), uncoupled respiration by 37% (p=0.03), and there was a trend for increased complex I-supported respiration (33%; p=0.13) (Figure 3.3C). RSV did not increase oxygen consumption in eWAT (Figure 3.3B). Consistent with these respiration data, COX4 protein content, a marker of mitochondrial content, was increased in scAT (p=0.005) and rpWAT (p=0.02) of RSV-supplemented animals, but not in eWAT (p=0.38) (Figure 3.3D). We were unable to reliably detect Complex I and II protein content using commercially available antibodies. Uncoupling protein-1 (UCP-1) was unchanged in scAT and rpWAT in response to RSV supplementation (data not shown). In every depot, oxygen consumption normalized to COX4 protein content was similar between groups (data not shown).
Figure 3.3 RSV increases state III and uncoupled respiration in scAT and rpWAT of ZDF rats. Maximal oxygen consumption in scAT (A), eWAT (B) and rpWAT (C) supported by glutamate and malate (state IV), glutamate, malate, and ADP (state III [complex I]), glutamate, malate, ADP, and succinate (state III [complex I & 2]), and with the addition of FCCP (uncoupled respiration) in ZDF chow (☐) and ZDF RSV (■) (n=8). D) COX4 protein content in scAT and rpWAT in ZDF chow (☐) and ZDF RSV (■) (ZDF chow: n=11; ZDF RSV: n=13). Open bars represent ZDF chow while closed bars represent ZDF RSV animals. Values are mean ± SEM. *: p<0.05 compared to ZDF chow.
3.4.5 Adipokines

Fasting plasma adiponectin levels were increased by 43% in the ZDF RSV group compared to control (p=0.0005) (Figure 3.4A). Similarly, \textit{ex vivo} incubation of adipose tissue showed that adiponectin release from scAT (p=0.02), but not from eWAT (p=0.98) or rpWAT (p=0.49), was elevated in RSV-supplemented animals (Figure 3.4B). The release of IL-6 and TNF-\(\alpha\) were not altered with RSV supplementation in any depot (Table 3.2).

<table>
<thead>
<tr>
<th></th>
<th>ZDF chow</th>
<th>ZDF RSV</th>
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<tbody>
<tr>
<td>scAT IL-6 (pg/mL per mg tissue)</td>
<td>5.28 ± 0.80</td>
<td>4.38 ± 0.60</td>
</tr>
<tr>
<td>scAT TNF-(\alpha) ((\mu)g/mL per mg tissue)</td>
<td>261 ± 44</td>
<td>400 ± 106</td>
</tr>
<tr>
<td>eWAT IL-6 (pg/mL per mg tissue)</td>
<td>6.75 ± 0.65</td>
<td>6.65 ± 1.06</td>
</tr>
<tr>
<td>eWAT TNF-(\alpha) ((\mu)g/mL per mg tissue)</td>
<td>476 ± 134</td>
<td>324 ± 49</td>
</tr>
<tr>
<td>rpWAT IL-6 (pg/mL per mg tissue)</td>
<td>6.04 ± 1.22</td>
<td>6.30 ± 1.79</td>
</tr>
<tr>
<td>rpWAT TNF-(\alpha) ((\mu)g/mL per mg tissue)</td>
<td>433 ± 79</td>
<td>489 ± 81</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. There were no differences in IL-6 and TNF-\(\alpha\) release between the 2 groups, in any of the depots.
Figure 3.4 RSV stimulates adiponectin secretion from scAT and increases plasma adiponectin concentration in ZDF rats. Fasting plasma adiponectin (A; n=8) and adiponectin release ex vivo from scAT, eWAT, and rpWAT (B; n=8) for ZDF chow (☐) and ZDF RSV (■). Open bars represent ZDF chow while closed bars represent ZDF RSV animals. Values are mean ± SEM. *: p<0.05 compared to ZDF chow.

3.4.6 Lipolytic enzymes and cAMP content, cell cross-sectional area and gene expression in scAT

As our functional evidence strongly suggested that RSV exerts it effects specifically onto scAT, we sought to determine whether RSV could modulate protein content and mRNA expression in this depot. RSV supplementation increased total ATGL (p=0.05) and HSL (p=0.04) protein content by 18% and 24%, respectively, but did
not increase phosphorylation of HSL at serine 563 (p=0.21) or 660 (p=0.13) (Figure 3.5A). In addition, in scAT, RSV did not change the protein content of perilipin (p=0.68), or enzymes involved in TG synthesis such as DGAT1 (p=0.87), DGAT2 (p=0.33), and GPAT (p=0.64) (Figure 3.5A). The protein content (ZDF chow: 1.00 ± 0.11; ZDF RSV: 0.95 ± 0.18; p=0.83) and phosphorylation (ZDF chow: 1.00 ± 0.19; ZDF RSV: 0.98 ± 0.15; p=0.93) of AMPK were not altered. There were no differences between groups for the mRNA expression of PGC-1α (p=0.34), PGC-1β (p=0.28), PPARγ (p=0.26) or RIP140 (p=0.43), a repressor of mitochondrial biogenesis (Seth, 2007), in scAT (Figure 3.5C).

A recent paper reported that the anti-aging effects of RSV in rodents were mediated through the inhibition of phosphodiesterase 4 (PDE4), which resulted in elevated cAMP levels in C2C12 myotubes [143]. Consequently, we examined whether cAMP levels were elevated in adipose tissue from ZDF RSV rats. Intracellular cAMP concentration in scAT was similar in both groups (p=0.50) (Figure 5B). Finally, there were no differences for cell cross-sectional area between the groups (p=0.40) (Figure 3.5D-E).
Figure 3.5 RSV increases lipolytic protein content in scAT from ZDF rats but does not modulate cAMP levels, adipocyte size or transcription factors mRNA expression. A) Protein content in scAT for ATGL, HSL, phosphorylation of HSL at serine 563 and 660 (n=13), and DGAT1, DGAT2, GPAT, perilipin, and α-tubulin (n=7) for ZDF chow (☐) and ZDF RSV (■). Values are mean ± SEM. *:p<0.05 compared to ZDF chow. B) cAMP content of scAT for ZDF chow (☐) and ZDF RSV (■) animals (n=13). Values are mean ± SEM. C) mRNA expression in scAT for PGC-1α, PGC-1β, PPARγ, and RIP140 for ZDF chow (☐) and ZDF RSV (■) animals (n=8). Open bars represent ZDF chow while closed bars represent ZDF RSV animals. Values are mean ± SEM. D) Superimposed histogram of scAT cell cross-sectional area for ZDF chow (☐) and ZDF RSV (■) (n=5). E) Representative images for hematoxilin and eosin staining of scAT for ZDF chow and ZDF RSV.
3.4.7 Adipose tissue organ culture experiment

ScAT and rpWAT from ZDF rats was treated with RSV (50 µM) [113] to assess the
direct effects of RSV on gene expression. After 24 h, treatment with RSV increased
PDK4 (scAT: p=0.03; rpWAT: p=0.03) and PGC-1α (scAT: p=0.04; rpWAT:
p=0.02) mRNA compared to vehicle-treated samples (Figure 3.6). PPARγ (scAT:
p=0.37; rpWAT: p=0.26) remained unchanged with RSV treatment in both depots
(Figure 3.6B).

Figure 3.6 RSV acts directly on scAT and rpWAT from ZDF rats to increase
glyceroneogenic and mitochondrial biogenesis gene expression ex vivo. mRNA
expression of PDK4, PEPCK, PPARγ, and PGC-1α in scAT (A) and rpWAT (B)
explants treated with (■) or without (□) resveratrol (50 µM; 24 h) (n=12). Open bars
represent ZDF chow while closed bars represent ZDF RSV animals. Values are mean ± SEM. *:p<0.05 compared to control condition.
3.5 Discussion

Mitochondrial biogenesis in adipose tissue is essential to maintain metabolic homeostasis at both the tissue and whole-body levels. For instance, exercise [186, 213] or treatment with PPARγ agonists [34, 211] induces mitochondrial biogenesis in WAT. Moreover, recent work from Spigelman’s group [103] showed that mice with an adipose tissue-specific deletion of PGC-1α, a transcriptional co-activator and master regulator of mitochondrial biogenesis, became more insulin resistant than controls when fed a high-fat diet. Considering the severe adverse effects of PPARγ agonists such as rosiglitazone [136] and the limited long-term efficacy of exercise programs at the population level [221], it is important to find other avenues to improve adipose tissue metabolic health. In this investigation, we demonstrated that RSV treatment of ZDF rats increased oxygen consumption in scAT and rpWAT ex vivo, when supported by entry of electrons through complex I and II. We also found that maximal uncoupled respiration and COX4 protein content were increased in a depot-specific manner with RSV (Figure 3.3). Moreover, when respiration was normalized to COX4 protein content, values were similar between groups (data not shown), providing evidence that improvements in oxygen consumptions are driven by increases in mitochondrial content, and not due to enhancement in mitochondrial function per se. These data are consistent with previous reports of RSV-induced mitochondrial biogenesis in skeletal muscle [113] and liver [71], and expands this observation to adipose tissue.

Mitochondrial function is essential for adiponectin synthesis in adipocytes [105], and improvements in mitochondrial biogenesis are associated with increases in
adiponectin synthesis and release. In the present study, we reported an increase in circulating adiponectin following RSV treatment in vivo, which is consistent with enhanced adiponectin release from subcutaneous depots ex vivo (Figure 3.4).

Adiponectin expression is controlled by PPARγ [105] and undergoes extensive post-translational modifications before it is released into the circulation. Previous reports showed that RSV acts on DsbA-L protein (disulphide bond-A oxidoreductase-like protein) to promote adiponectin multimerization in 3T3-L1 adipocytes [206]. RSV treatment was also shown to increase adiponectin content in adipose tissue from obese Zucker rats [164], and to increase adiponectin gene expression in human adipocytes [39]. Here, we suggest that the main depot responsible for improvement in circulating adiponectin is scAT, which is also the depot that was most responsive to RSV with regards to mitochondrial biogenesis. The lack of effect on rpWAT despite RSV-induced mitochondrial biogenesis may be due to depot-specific regulation of adiponectin transcription and/or post-translational modifications.

Adiponectin is one of the principal adipokines linking adipose tissue to skeletal muscle and liver metabolism. For instance, the insulin-sensitizing effects of TZDs are attenuated in adiponectin knockout mice [135]. Therefore, increases in circulating adiponectin levels provide a possible mechanism to support the participation of adipose tissue in the improvements of whole-body glucose homeostasis following RSV supplementation. Indeed, in addition to lower glucose AUC during GTT and ITT, our group also showed muscle-specific insulin sensitizing effects with RSV [176]. The question remains whether adiponectin is required to observe the beneficial effects of RSV at the whole-body or skeletal muscle level.
Another important finding of this study is that 6-week RSV supplementation in ZDF rats increased $^{14}$C-pyruvate incorporation into TG in scAT, in parallel with increases in the phosphorylation of PDH at serine 293. PDH is inhibited by PDK4-induced phosphorylation at serine 293, which results in the shuttling of pyruvate away from acetyl-coA and towards oxaloacetate and the glyceroneogenic pathway [23]. The upregulation of GNG in scAT would be expected to result in a greater sequestration of fatty acids in adipose tissue, a lowering of plasma lipid levels, and concomitant improvements in insulin action. However, one of the caveats of our study is that increases in GNG did not result in lower circulating FFA or TG. This may be due to the genetic background of our model and/or severity of insulin resistance. Alternatively, the glyceroneogenic effect limited to scAT, and not in other depots, may not have been sufficient to lower circulating lipid levels.

Our *in vivo* data demonstrated that dietary RSV supplementation increases mitochondrial respiration, adiponectin secretion, and GNG. However, as RSV also improved whole-body glucose homeostasis, it was important to determine if the effects on adipose tissue were direct or secondary to alterations in glycaemia. We reported an induction of both PGC-1$\alpha$ and PDK4 mRNA following acute *ex vivo* treatment of scAT from untreated ZDF rats with RSV. These findings support that RSV can directly induce the expression of genes involved in mitochondrial biogenesis and GNG, suggesting that our observations may be attributed, at least in part, to direct effects of RSV on adipose tissue.

While our data showed that RSV induces mitochondrial biogenesis in adipose tissue, the mechanisms that mediate this effect are not clear. Importantly, FA reesterification
is dependent upon GNG and mirrors rates of lipolysis [21]. Interestingly, we found that RSV increased both GNG and the protein content of ATGL and HSL, two key enzymes involved in the control of lipolysis, suggesting FA reesterification would be increased in response to RSV. As FA reesterification is an energetically demanding process linked to the activation of AMPK [67], a reputed regulator of mitochondrial biogenesis, RSV-induced mitochondrial biogenesis may be the result of increases in GNG. Admittedly, this is speculative and requires further investigation. Notably, we did not observe a RSV-mediated increase in AMPK activation in vivo in the current study. This situation may not be surprising as a potential increase in the content and/or phosphorylation of AMPK is likely transient and timing may be an important parameter to consider. In addition, animals were killed after a 12 h-fast, which would trigger AMPK phosphorylation and could have, therefore, masked any potential treatment effect. Furthermore, while our data support a relationship between GNG and mitochondrial biogenesis in scAT, mitochondrial biogenesis was independent from GNG in rpWAT. While the reason for this discrepancy is still unknown, it would suggest that, at least in rpWAT, increases in mitochondrial function/content are not sufficient to enhance GNG, and would indicate that other factors, such as substrate provision and/or increased activity of glyceroneogenic enzymes such as PEPCK and/or PDK4, might be necessary.

3.6 Perspectives and Significance

In conclusion, the present study highlights that RSV supplementation in ZDF rats prevents the apparition of whole-body insulin resistance and glucose intolerance at 11
weeks of age, and is associated with increased mitochondrial content in scAT. This process may be triggered by the energetic demands of the lipolytic and glyceroneogenic pathways and the resulting activation of PGC-1α. Indeed, RSV-fed rodents showed increased mitochondrial respiration, enhanced GNG, and elevated adiponectin secretion in a depot-specific manner. Interestingly, the changes observed in adipose tissue metabolism following RSV supplementation are similar to the well-characterized effects of TZDs. While TZDs are effective in improving glucose homeostasis, they are associated with a wide range of negative side effects such as an increased risk of heart attack, osteoporosis and bladder cancer [136, 171]. In this light, our findings suggest that RSV could be used as a nutraceutical/nutritional adjunct that would allow for lower doses of TZDs to be prescribed. Clearly, further work is needed in order to examine the interactions between RSV and insulin-sensitizing medications.
Chapter 4: Novel effects of rosiglitazone on SMAD2 and SMAD3 signalling in white adipose tissue of diabetic rats

As published with minor revisions:


4.1 Abstract

Objective: To assess the effects of the PPARγ agonist rosiglitazone (ROSI) on the transforming growth factor (TGF)-β/SMAD signalling pathway in white adipose tissue (WAT) of diabetic rats.

Design and Methods: Six-week old, male ZDF rats were fed a chow diet with (ZDF ROSI) or without (ZDF chow) ROSI (100 mg/kg diet) for 6 weeks. Subcutaneous (scAT) and retroperitoneal (rpWAT) adipose tissue were excised to quantify protein content/phosphorylation.

Results: ZDF ROSI animals showed enhanced glucose tolerance and mitochondrial protein content in both depots. The protein content of enzymes involved in fatty acid handling was increased in scAT of ZDF ROSI animals. ZDF ROSI exhibited decreased phosphorylation of SMAD2 and SMAD3 exclusively in scAT, along with increases in inhibitory SMAD7 and the E3 ubiquitin ligase SMURF2. In contrast,
ROSI increased the protein content of SMAD4, TGF-β receptor I and II, and SARA in scAT.

Conclusions: For the first time, we demonstrate that ROSI inhibits SMAD2 and SMAD3 signalling in a depot-specific manner in diabetic rats. In scAT, ROSI reduced SMAD2 and SMAD3 phosphorylation, likely through the inhibitory actions of SMAD7 and SMURF2. Induction of proximal components of the SMAD pathway may constitute a feedback mechanism to counteract ROSI-induced lipid synthesis in scAT.

4.2 Introduction

Recent data suggest that TGF-β signalling plays a role in the etiology of obesity and insulin resistance [188, 189, 215]. TGF-β is a pro-inflammatory cytokine that is positively correlated with BMI and fat mass, and inversely associated with VO$_2$ max in humans [6, 123, 215]. The binding of TGF-β superfamily ligands to TGF-β type I and II receptors results in the activation of the traditional SMAD3 signalling cascade, in which phosphorylated SMAD proteins translocate to the nucleus and act as transcription factors to modulate gene expression [91, 134]. Recently, two groups have shown that SMAD3$^{-/-}$ mice are resistant to high-fat diet-induced obesity and insulin resistance [189, 215]. SMAD3$^{-/-}$ mice exhibit decreased adiposity, along with increased whole-body glucose uptake and insulin sensitivity. This phenotype is attributed to modulation of white adipose tissue (WAT) metabolism. In fact, WAT of SMAD3$^{-/-}$ mice show increased presence of brown
adipocytes and mitochondria [215] and decreased expression of genes involved in fatty acid storage (e.g. PPARγ) [189]. The treatment of high-fat fed mice with TGF-β neutralizing antibodies replicates the SMAD3⁻/⁻ phenotype [189]. While most of the research in this area has focused on the seminal protein SMAD3, the TGF-β signalling pathway includes numerous other proteins. In the traditional cascade, receptor-activated SMADs (R-SMADs or SMAD1, 2, 3, 5, and 8) are phosphorylated upon ligand binding, interact with SMAD4 to translocate into the nucleus, bind to SMAD binding elements, and modulate gene expression. However, the specificity and versatility of this pathway is dependent upon complex interactions between ligands, receptors, SMAD proteins, transcription factors, co-activators, co-repressors, and cross talk with parallel pathways (reviewed in [53]). First, different ligands activate specific parts of this pathway. For instance, TGF-β binding leads to the activation of SMAD2 and SMAD3, while other members of the TGF-β superfamily, such as bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), trigger the phosphorylation of SMAD1, 5, and 8 [32, 73]. In addition, these pathways can be negatively and selectively regulated by inhibitory SMADs (I-SMADs or SMAD6 and 7). While SMAD6 inhibits SMAD1, 5, and 8, SMAD7 acts specifically on SMAD2 and 3 [131, 217]. Both I-SMADs act via the recruitment of SMURF ubiquitin ligase proteins that target phosphorylated SMADs for proteasomal degradation [99, 217]. Furthermore, the traditional TGF-β/SMAD3 pathway is also modulated by other cytoplasmic kinases, such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), which inhibit and activate SMAD3, respectively (reviewed in [53], [48]). Consequently, this seemingly simple signalling
cascade is regulated at many different levels and several proteins, in addition to SMAD3, should be considered when examining the role of TGF-β/SMAD in obesity and insulin resistance.

Thiazolidinediones (TZD) are anti-diabetic agents acting through PPARγ agonism in WAT [28]. Treatment with PPARγ agonists, such as rosiglitazone (ROSI), in vivo [115, 216] and in vitro [147], leads to increased mitochondrial biogenesis, enhanced presence of brown adipocytes, an anti-inflammatory profile, and increases in whole-body insulin sensitivity. These changes are reminiscent of SMAD3 ablation in mice. Interestingly, some reports have linked PPARγ agonism to TGF-β activity. In vitro, PPARγ agonists decreased TGF-β signalling in human Tenon’s fibroblasts [51], in peritoneal mesothelial cells [145], and in adrenocarcinoma pancreatic and lung cell lines [161]. Collectively, these results suggest that ROSI treatment may attenuate SMAD signalling in white adipose tissue in vivo, though this possibility has not yet been explored. Within this context, the primary aim of this paper was to examine the in vivo effects of ROSI, a TZD, on the TGF-β/SMAD pathway in WAT from diabetic rats. We hypothesized that ROSI would inhibit SMAD3 signalling in WAT in conjunction with increases in mitochondrial proteins and enzymes involved in lipid synthesis.
4.3 Materials and Methods

4.3.1 Animals

Four-week old (~100g) male ZDF rats (Charles River, St. Constant, QC, Canada) were housed individually in wire-bottom cages, in a temperature-controlled room on a reverse (12:12) light-dark cycle. The experiments started at 9am, which coincided with the start of the dark cycle. After a 10-day acclimatization period, ZDF rats were fed *ad libitum* either a powdered chow diet (Purina 5008 diet; Purina, St.Louis, MO, USA) (ZDF chow) or the same diet supplemented with ROSI (Cayman Chemical, Ann Arbor, MI, USA) at a dose of 100 mg/kg diet (ZDF ROSI) for 6 weeks. This ROSI dose was chosen based on previous reports [54, 55, 116], as well as pilot studies performed in our laboratory. Food intake was tracked every other day, while body weight (BW) was recorded weekly. ZDF rats are characterized by the development of type 2 diabetes mellitus (T2DM) by 12 weeks [185]. Therefore, we chose to examine ZDF rats between 5 and 11 weeks of age, a timing that allowed us to focus on T2DM prevention. In addition, male Wistar rats (Charles River) (~200g) housed 2 per cage, in a 12:12-h light-dark cycle, were provided either chow (PMI Nutrition International, Shoreview, MN) or high-fat diet (60% kcals from fat, Harlan Laboratories, Madison, WI) *ad libitum*. Following 6 weeks of feeding, Wistar rats were anaesthetized with sodium pentobarbital, and scAT and rpWAT were removed, washed, and immediately frozen in liquid nitrogen. All protocols followed Canadian Council on Animal Care guidelines and were approved by the Animal Care Committee at the University of Guelph.
4.3.2 Glucose tolerance tests

Intraperitoneal glucose tolerance tests (ipGTT) were performed when ZDF rats were 11-week old. Blood glucose was assessed (Freestyle lite, Abbott Laboratories, St-Laurent, QC, Canada) through tail vein sampling at fasting, and at 15, 30, 45, 60, 90, and 120 min after i.p. injection of 1g/kg BW glucose (BioShop, Burlington, ON, Canada).

4.3.3 Terminal procedures

After the 6-week intervention, fasted (12 h) animals were weighed and anesthetised with isoflurane (Abbott Laboratories, North Chicago, IL, USA). Inguinal subcutaneous (scAT) and retroperitoneal (rpWAT) adipose tissue was weighed, frozen in liquid nitrogen, and stored at -80°C for later analysis. Blood was obtained through cardiac puncture, allowed to clot at room temperature and centrifuged (3000 x g; 10 min). Serum was stored at -20°C and later analyzed for triglycerides, glycerol (both colorimetric assays: Sigma-Aldrich, Oakville, ON, Canada), free fatty acids (colorimetric assay: Wako Diagnostics, Richmond, VA, USA), and insulin (ELISA: Millipore, St.Charles, MO, USA). Homeostatic model assessment of insulin resistance (HOMA-IR) was measured as described previously (fasting glucose x fasting insulin/22.5) [128].

4.3.4 Western blotting

Adipose tissue samples were homogenized (FastPrep®-24, MP Biomedicals, Santa Ana, CA, USA) in two volumes of ice-cold cell lysis buffer (Invitrogen, Burlington,
ON, Canada) supplemented with protease inhibitor cocktail (as per manufacturer’s instructions; Sigma-Aldrich, Oakville, ON, Canada) and 0.05% phenylmethylsulfonyl fluoride (BioShop, Burlington, ON, Canada). Homogenized samples were centrifuged for 10 min at 1500 x g at 4°C. Lipids were removed, the infranatant was collected, and protein concentration was determined with a bicinchoninic acid (BCA) assay [177] (ThermoScientific, Rockford, IL, USA). Western blotting was performed as previously described by our laboratory [14, 186, 202, 205]. Briefly, membranes were incubated in primary antibodies diluted in TBST/5% non-fat dry milk (COX4 [CAT#:MS407], CORE1 [CAT#: MS303]: MitoSciences, Eugene, OR, USA; β-actin [CAT#: ab8227]: Abcam, Toronto, ON, Canada; ATGL [CAT#:2138], p-SMAD2 [ser465/467] [CAT#:3101]: Cell Signaling, Danvers, MA, USA) or TBST/5% bovine serum albumin (PEPCK [CAT#:10004943]: Cayman Chemicals, Ann Arbour, MI, USA; ERK1/2 [CAT#:4695], p-ERK1/2 [thr202/tyr204] [CAT#:9101], HSL [CAT#:4107], SMAD2 [CAT#:5339], SMAD3 [CAT#: 9523], SMAD5 [CAT#:9517], p-SMAD1 [ser463/465] /5[ser463/465] /8[ser426/428] [CAT#: 9511], p-SMAD3 [CAT#: 9520], JNK [CAT#:9252], p-JNK [thr183/tyr185] [CAT#:4671], TGF-β Receptor I [CAT#:3712], TGF-β Receptor II [CAT#:11888]: Cell Signaling; SMAD1 [CAT#:sc-81378], SMAD8 [CAT#:sc-11393]: Santa Cruz Biotechnology, Dallas, TX, USA; SMAD4 [CAT#:ABE21]: Millipore, Billerica, MA, USA; SMAD6 [CAT#:PA1-410216], SMAD7 [CAT#:PA1-41506]: ThermoScientific; SMURF2 [CAT#:ab53316], Cytochrome C [CAT#:ab110325], DGAT1 [CAT#:ab54037], GPAT [CAT#:ab69990], α-tubulin [CAT#:ab7291]: Abcam; SARA [CAT#:GTX63430]: Genetex, Irvine, CA, USA; DGAT2 [CAT#:IMG-30279]:
Imgenex, San Diego, CA, USA; CD36 was generously provided by Dr. Tandon [127] overnight at 4°C. Following TBST washes, membranes were incubated at room temperature with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) diluted in TBST/1% non-fat dry milk for 1h. Enhanced chemiluminescence (ThermoScientific) was used to detect signals, which were then quantified by densiometry (Fluorchem HD2, ProteinSimple, Santa Clara, CA, USA).

4.3.5 Ex vivo incubation

One hundred (100) mg of scAT was minced, incubated in a plastic vial with 3 mL of oxygenated Krebs Ringer buffer (118 mM NaCl, 4.8 mM KCl, 1.25 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃; pH 7.4) supplemented with 5 mM glucose and 2.5% FFA-free bovine serum albumin (MP Biomedicals), and placed in a shaking water bath (60 rpm, 37°C, 2 h). After 2h, media was stored at -20°C for later measurement of active TGF-β1 via ELISA (Biolegend, San Diego, CA, USA).

4.3.6 Histochemistry

ScAT was fixed in 10% neutral buffered formalin (VWR, Mississauga, ON, Canada), dehydrated in xylene (Fisher Scientific), and embedded in paraffin. Five µm sections were mounted on 1.2 mm Superfrost slides, stained with modified Harris hematoxilyn and eosin stock with phloxine (all from Fisher Scientific), and imaged (Olympus FSX 100 light microscope, Olympus, Tokyo, Japan). One hundred cells were randomly sampled in each image to determine cross-sectional area (ImageJ software, National
Institute of Mental Health, Bethesda, MD, USA).

4.3.7 Statistical analysis

Data are presented as mean ± SEM. Total area under the curve for ipGTT was calculated using the trapezoid method (GraphPad Prism 5, La Jolla, CA, USA). Comparisons of blood metabolites, ipGTT AUC, cytokines, and protein content between ZDF chow and ZDF ROSI were made using 2-tailed, unpaired student t-tests for each depot (GraphPad Prism 5). Comparisons for ipGTT were made using a 2-way ANOVA testing for time and diet. Statistical significance was accepted at α≤0.05.

4.4 Results

4.4.1 ROSI improves whole-body metabolism in ZDF rats

As an initial step in characterizing the effects of ROSI treatment on SMAD signalling in white adipose tissue from ZDF rats, we first confirmed that ROSI prevented the development of overt diabetes in our model. ZDF ROSI animals showed lower fasting glucose, insulin, free fatty acids, and triglycerides (p<0.05) (Table 4.1). Furthermore, ROSI-treated animals showed a lower glucose response to an ipGTT (p<0.05) (Figure 4.1) and lower insulin resistance as assessed by HOMA-IR (p<0.05) (Table 4.1). There were no differences in food intake between groups (Table 4.1). Compared to ZDF chow animals, ROSI-treated rats were heavier, had more scAT, but less rpWAT (p<0.05). In addition, liver weight was significantly reduced in ROSI-treated animals (p<0.05) (Table 4.1).
Table 4.1 Weight, food intake, and fasting biochemistry data for ZDF chow and ZDF ROSI (n=8).

<table>
<thead>
<tr>
<th></th>
<th>ZDF chow</th>
<th>ZDF ROSI</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>386 ± 9</td>
<td>429 ± 12*</td>
</tr>
<tr>
<td>Average daily food intake (g)</td>
<td>31.5 ± 0.7</td>
<td>29.7 ± 0.9</td>
</tr>
<tr>
<td>scAT (inguinal) weight (g)</td>
<td>10.7 ± 1.0</td>
<td>14.9 ± 1.0*</td>
</tr>
<tr>
<td>rpWAT weight (g)</td>
<td>2.5 ± 0.1</td>
<td>1.9 ± 0.3*</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>14.7 ± 0.7</td>
<td>9.7 ± 0.5*</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>11.0 ± 1.8</td>
<td>4.8 ± 0.2*</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>1086 ± 140</td>
<td>512 ± 68*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>68.8 ± 16.6</td>
<td>16.0 ± 2.5*</td>
</tr>
<tr>
<td>FFA (mmol/L)</td>
<td>1.55 ± 0.16</td>
<td>0.94 ± 0.11*</td>
</tr>
<tr>
<td>Glycerol (mmol/L)</td>
<td>0.47 ± 0.04</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>4.32 ± 0.58</td>
<td>0.75 ± 0.04*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. * p<0.05 vs. ZDF chow. HOMA-IR: homeostatic model assessment of insulin resistance; scAT: inguinal subcutaneous adipose tissue. rpWAT: retroperitoneal white adipose tissue.
Figure 4.1 ROSI treatment improves glucose tolerance in ZDF rats. Intraperitoneal GTT (n=8) and total AUC (insert) for ZDF chow (□) and ZDF ROSI (■). Values are mean ± SEM. *: p<0.05 compared to ZDF chow.

4.4.2 ROSI induces markers of mitochondrial biogenesis, lipolysis, glyceroneogenesis and triglyceride synthesis in scAT

To gain insight into how potential alterations in SMAD signalling could be associated with adipose tissue metabolism, we examined the effects of ROSI treatment on the content of mitochondrial, lipolytic, glyceroneogenic, and lipogenic proteins. The improvement in glucose homeostasis associated with ROSI was accompanied by increases in the content of mitochondrial proteins cytochrome c oxidase complex IV (COX4), CORE1 and cytochrome c (p<0.05) in both scAT and rpWAT (Figure 4.2). ROSI treatment increased the protein content of adipose tissue triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and phosphoenolpyruvate carboxykinase (PEPCK) in scAT (Figure 4.3A). However, only ATGL was increased in response to
ROSI treatment in rpWAT (Figure 4.3B). ROSI increased the protein content of DGAT1, GPAT, and FAT/CD36, but not of DGAT2 (Figure 4.3A) in scAT. In contrast, ROSI did not modulate DGAT1, GPAT, or FAT/CD36, but DGAT2 was decreased, in rpWAT (Figure 4.3B). Finally, adipocyte cross-sectional area was reduced in both scAT and rpWAT of ZDF ROSI animals compared to their chow-fed counterparts (Figures 4.3C-D).
Figure 4.3 Protein content of lipolytic, glyceroneogenic and lipogenic proteins is increased in scAT of ZDF rats, but not in rpWAT, in response to rosiglitazone treatment. Protein content and representative blots of ATGL, HSL, PEPCK, DGAT1, DGAT2, GPAT, and FAT/CD36 in scAT (A) and rpWAT (B) for ZDF chow (□) and ZDF ROSI (■) (n=6-8). Superimposed histograms of cell cross-sectional area and representative images for hematoxilyn and eosin staining of scAT (C) and rpWAT (D) for ZDF chow (□) and ZDF ROSI (■) (n=3-5). Scale bars represent 50 µm. Values are mean ± SEM. *:p<0.05 compared to ZDF chow.
4.4.3 ROSI inhibits the phosphorylation of SMAD2 and 3 in scAT, but does not modulate SMAD1, 5 and 8

In scAT, ROSI treatment increased the total content of SMAD3, but decreased its phosphorylation (p<0.05) (Figure 4.4A). However, ROSI did not modulate SMAD3 content or its phosphorylation in rpWAT (Figure 4.4B). SMAD2 phosphorylation was also decreased in response to ROSI in scAT (p<0.05) (Figure 4.4A). Akin to SMAD3, there was no effect of ROSI on SMAD2 content or phosphorylation in rpWAT (Figure 4.4B). Due to the depot-specific effects of ROSI, the rest of the outcomes were evaluated only in scAT. In addition to SMAD2 and SMAD3, which are associated with TGF-β family ligands, R-SMADs include SMAD 1, 5, and 8, which are activated through ligands of the GDF and BMP families. In scAT, the total content and phosphorylation status of SMAD1, SMAD5, and SMAD8 did not respond to ROSI treatment (Figure 4.5).
Figure 4.4 TGF-β-associated R-SMADs are inhibited in scAT from ZDF rats, but not in rpWAT, in response to rosiglitazone treatment. Protein content and representative blots of SMAD3, p-SMAD3 (ser423/425), SMAD2, and p-SMAD2 (ser465/467) in scAT (A) and rpWAT (B) for ZDF chow (☐) and ZDF ROSI (■) (n=7). Values are mean ± SEM. *:p<0.05 compared to ZDF chow.
Figure 4.5 BMP- and GDF-associated R-SMADs are not modulated in scAT from ZDF rats in response to rosiglitazone treatment. Protein content and representative blots of SMAD1, SMAD5, SMAD8, and p-SMAD1/5/8 in scAT for ZDF chow (☐) and ZDF ROSI (■) (n=7). Values are mean ± SEM. *:p<0.05 compared to ZDF chow.

4.4.4 ROSI increases the content of inhibitory SMAD7 and SMURF2 in scAT

As SMAD2 and SMAD3 phosphorylation was reduced in scAT, we next examined the potential cellular mediators of this effect. Receptor-activated SMADs can be negatively regulated by inhibitory SMAD6, SMAD7, and SMURF2. In accordance with our R-SMAD data, ROSI increased the content of SMAD7 (p<0.05) and SMURF2 in scAT (p<0.05), but did not modulate SMAD6 (Figure 4.6A). Finally, ROSI treatment in ZDF rats did not modulate the total content or phosphorylation of ERK1/2 or JNK (Figure 4.6B).
In order to examine the relationship between alterations in systemic glucose homeostasis and the modulation of SMAD signalling in adipose tissue, we measured SMAD3 content/phosphorylation and SMAD7 content in adipose tissue from chow...
and high-fat fed rats. Despite increases in body weight, fat pad mass and fasting glucose (as previously reported in [62]), there were no differences in SMAD3 phosphorylation and the content of SMAD3 and SMAD7 in scAT and rpWAT between groups (Appendix A). When analyzing these same variables in lean and obese ZDF rats (please note the obese ZDF data is the same presented in this manuscript), there were no differences between groups (Appendix B), indicating that differences in glucose homeostasis alone are not sufficient to alter SMAD signalling.

### 4.4.5 ROSI increases TGF-β receptors and SMAD4 protein in scAT

While our data suggest that ROSI inhibits SMAD signalling, some elements of the pathway were robustly increased in scAT in response to TZD treatment. First, TGF-β receptor I and II were significantly increased in ZDF ROSI compared to ZDF chow animals (p<0.05) (Figure 4.7A). Furthermore, SMAD Anchor for Receptor Activation (SARA), an adapter protein that facilitates the interaction between TGF-β receptors and R-SMADs, was also increased in scAT following ROSI treatment (p<0.05) (Figure 4.7A). Similarly, in scAT, ROSI triggered a 6-fold increase in SMAD4 (p<0.05) (Figure 4.7B), a Co-SMAD whose main role is to allow translocation of R-SMADs into the nucleus.

Finally, as we reported decreased activation of SMAD2 and SMAD3 and since these proteins are associated with the TGF-β ligand family, we sought to determine whether ROSI treatment could decrease TGF-β1 secretion. Active TGF-β1 secretion by scAT was 37% lower in the ZDF ROSI group compared to ZDF chow, but failed to reach
significance (p=0.3) (Figure 4.7C). TGF-β1 was undetectable in plasma of both ZDF chow and ZDF ROSI animals.

**Figure 4.7** Proximal parts of the TGF-β pathway, but not TGF-β1 secretion, are increased in scAT of ZDF rats in response to rosiglitazone treatment. Protein content and representative blots of TGF-β Receptor I, TGF-β Receptor II, and SARA (A) and SMAD4 (B) in scAT for ZDF chow (□) and ZDF ROSI (■) (A) (n=7). TGF-β secretion from scAT for ZDF chow (□) and ZDF ROSI (■) (C) (n=7). Values are mean ± SEM. *:p<0.05 compared to ZDF chow.
4.5 Discussion

The present work demonstrates that ROSI-mediated improvements in glucose homeostasis in ZDF rats are accompanied by a depot-selective inhibition of SMAD2 and SMAD3 phosphorylation and that these changes are associated with increases in the protein content of SMAD7 and SMURF2. To our knowledge, this is the first study reporting a modulation of SMAD2 and SMAD3 following a pharmacological intervention and provides additional support for interventions targeting this signalling cascade in the treatment of insulin resistance.

As expected, ROSI treatment for 6 weeks prevented whole-body insulin resistance, and it led to the normalization of blood lipid biochemistry in ZDF rats. The anti-diabetic effects of ROSI are attributed to enhanced mitochondrial content as well as augmented lipolysis, glyceroneogenesis, and triglyceride synthesis in WAT [56, 115, 159]. Accordingly, in our model and in other reports [56], ROSI increased mitochondrial biogenesis in both subcutaneous and abdominal fat depots, whereas the content of enzymes involved in fatty acid uptake and synthesis was only increased in scAT.

The TGF-β/SMAD pathway is a complex signalling cascade in which two main branches have been identified. On one hand, SMAD2 and SMAD3 are phosphorylated and activated upon TGF-β ligand binding to the TGF-β receptor I and II heterodimeric complex, while activation of SMAD1, 5, and 8 are dependent upon BMP and/or GDF ligands [32, 53, 73]. In the present study, we show, for the first time, that ROSI specifically decreases SMAD2 and SMAD3 phosphorylation in scAT, but does not modulate the BMP/GDF arm of the pathway. In contrast to SMAD3−/−
mice, SMAD2−/− mice are non-viable [137], which illustrates that SMAD2 and SMAD3 may serve different functions in embryogenesis and development. It is still unknown whether SMAD2 and SMAD3 have differential actions on adipose tissue metabolism in the context of obesity and diabetes, but, as both were dephosphorylated in response to ROSI treatment, our findings would suggest that SMAD2 and SMAD3 in white adipose tissue are reduced by ROSI in vivo.

TGF-β1, a ligand that activates SMAD2 and SMAD3, has been correlated with BMI in obese humans [6] and its release from scAT has been positively correlated with women’s body fat content [50]. In our model, the active form of TGF-β1 in the plasma was below the detectable level of the assay in both groups (data not shown). However, in scAT explant experiments, TGF-β1 release was lower by 37% (NS) in the ZDF ROSI group compared to ZDF chow, despite increased adiposity of ZDF ROSI animals. In contrast, TGF-β receptor type I and II were significantly increased by 42% and 134%, respectively. Taken together, our results demonstrate that downregulation at the ligand/receptor level is likely not responsible for the intracellular inhibition of SMAD2 and SMAD3. Admittedly, TGF-β1 constitutes only one of several ligands able to activate this cascade, and other ligands may be implicated in this regulation.

The TGF-β/SMAD pathway is negatively regulated by inhibitory SMAD6 and SMAD7. While SMAD6 primarily inhibits SMAD1, 5, and 8, SMAD7 targets SMAD2 and SMAD3 [217]. In the current study, SMAD6 did not respond to ROSI treatment, which is consistent with the lack of effect on SMAD1, 5, and 8. However, SMAD7 and SMURF2 were increased in ZDF ROSI. As there were no differences in
the content or phosphorylation of ERK and JNK, kinases that also regulate SMAD3 phosphorylation (reviewed in [53], [48]), this suggests that, in our model, increases in SMAD7 and SMURF2 could be causally linked to the ROSI-mediated reductions in SMAD2 and SMAD3 phosphorylation.

In regards to the regulation of mitochondrial enzymes, both ROSI treatment [115] and SMAD3 knockout [215] result in enhanced mitochondrial content in WAT. This phenotypic similarity suggests that ROSI could act, at least in part, through the inhibition of SMAD2 and SMAD3, via the intracellular actions of SMAD7 and SMURF2, to induce mitochondrial enzyme expression in scAT. However, mitochondrial proteins were increased in rpWAT in the absence of any detectable reductions in SMAD signalling, providing evidence that decreases in SMAD signalling is not required for ROSI-mediated increases in adipose tissue mitochondrial biogenesis, at least in rpWAT.

In addition to mitochondrial biogenesis, ROSI also triggers adipogenesis and triglyceride synthesis. In this study, ROSI-induced triglyceride synthesis was restricted to scAT. In scAT, fat pad weight and the protein content of PEPCK, DGAT1, GPAT, and CD36 were increased in parallel with reductions in SMAD2 and SMAD3 phosphorylation in response to the 6-week ROSI treatment. In contrast, in rpWAT, ROSI did not trigger lipid synthesis nor did it modulate SMAD signalling. This association between lipid synthesis and SMAD proteins is consistent with recent work demonstrating that TGF-β attenuates PPARγ mRNA expression and adipogenesis in a SMAD3-dependent manner in mouse embryonic fibroblasts [196]. While changes in SMAD2 and SMAD3 are associated with triglyceride synthesis,
they do not correlate with reduction in adipocytes’ cross-sectional area, which was observed in both scAT and rpWAT. Interestingly, these findings contrast with the observed phenotype in SMAD3−/− mice, which show decreased adiposity and triglyceride synthesis [189, 215]. This may not be surprising, as SMAD3−/− mice have a lifelong, whole-body deletion of SMAD3, while SMAD3 is downregulated for a much shorter duration with ROSI treatment. The fact that SMAD2 and SMAD3 phosphorylation, fat pad weight, and lipid synthesis enzymes were not increased in rpWAT from ZDF rats treated with ROSI suggest that decreases in SMAD2 and SMAD3 phosphorylation could be required for ROSI-mediated increases in enzymes involved in lipid synthesis.

In addition to the inhibition of SMAD2 and SMAD3 phosphorylation, we observed a robust induction of proximal components of the TGF-β/SMAD pathway. TGF-β receptors type I and II, and their adapter protein SARA, were increased in scAT of ZDF ROSI animals. Furthermore, we reported a 6-fold induction of SMAD4 in scAT in response to ROSI. SMAD4 is a Co-SMAD implicated in nuclear translocation and has been shown to repress PPARγ transcription and hence inhibit adipogenesis [35]. The strong induction of proximal parts of the TGF-β/SMAD pathway may be surprising in the context of decreased SMAD2 and SMAD3 phosphorylation and the increases in scAT mass and enzymes involved in lipid uptake and synthesis. However, based on the negative role of TGF-β and SMAD4 on adipogenesis, we hypothesize that this upregulation may constitute a counter-regulatory mechanism in response to the robust ROSI-induced lipid synthesis.
In conclusion, the present study provides novel evidence demonstrating that PPARγ agonism leads to the inhibition of SMAD signalling in WAT in vivo. The attenuation of SMAD3 phosphorylation would not appear to be secondary to alterations in glucose homeostasis, though we cannot rule out an indirect effect of ROSI on SMAD signalling in white adipose tissue in vivo. Our findings constitute the first report showing that inhibition of SMAD signalling cascade is possible through pharmacological treatments and it supports the use of SMAD2 and SMAD3 antagonism as a potential treatment for insulin resistance.
Chapter 5: Additive effects of resveratrol and rosiglitazone on glyceroneogenic enzymes in human adipose tissue from morbidly obese subjects

Submitted to *Int J Obesity*:

Beaudoin MS, Gaudio N, Reed K, Foute-Nelong J, Mutch DM, Wright DC

5.1 Abstract

Resveratrol (RSV) and rosiglitazone (ROSI) increase glyceroneogenesis and decrease inflammation in rodent adipose tissue. The purpose of this study was to evaluate the individual and combined roles of RSV and ROSI on glyceroneogenic and inflammatory gene expression in human adipose tissue from obese individuals undergoing bariatric surgery. Subcutaneous (scAT) and visceral (viscAT) adipose tissue was treated for 24h with vehicle, RSV 50µM, ROSI 0.5µM, RSV+ROSI, or epinephrine 10µM. ROSI increased pyruvate dehydrogenase 4 (PDK4) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA in both depots. Furthermore, RSV+ROSI showed additive effects on PDK4 and PEPCK mRNA in viscAT, and the induction of these genes occurred independently of lipolysis. RSV+ROSI also decreased IL-6 gene expression in viscAT, but did not affect adiponectin mRNA expression or protein secretion. These genes were not affected in scAT. Therefore, in human viscAT, RSV+ROSI additively stimulate glyceroneogenic gene expression in a lipolysis-independent manner and decrease markers of inflammation. These data
show that RSV can potentiate the effects of ROSI in vitro in humans, and support the concept of combination therapy using pharmaceutical and nutraceutical agents to improve treatment efficacy while possibly reducing ROSI’s adverse effects.

5.2 Introduction

Rosiglitazone (ROSI) is an anti-diabetic agent that acts as a ligand to peroxisome proliferator-activated receptor γ (PPARγ) and induces adipogenesis [115]. ROSI’s adipogenic effect can be attributed, at least in part, to fatty acid reesterification; a process that is dependent upon the generation of glycerol-3-phosphate through glyceroneogenesis. Phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate dehydrogenase kinase 4 (PDK4) are two key regulators of glyceroneogenesis [23]. Despite its metabolic benefits, the clinical use of ROSI is limited due to severe adverse effects [167]. Thus, the identification of alternate approaches to mimic the beneficial metabolic outcomes of ROSI is warranted.

Recent work from our laboratory showed that the polyphenol compound resveratrol (RSV), when fed to Zucker Diabetic Fatty rats, increases adipose tissue mitochondrial content and respiration, glyceroneogenesis and the secretion of the anti-inflammatory adipokine adiponectin [14]. These effects are comparable to what has been reported with ROSI treatment. Moreover, recent data showed that RSV and ROSI shared similar PPARγ deacetylation actions in vitro [155]. Collectively, these findings suggest that RSV treatment may mimic the beneficial effects of ROSI. Examining the combined effects of ROSI and RSV is an important first step to determine if RSV could lower the required dose of ROSI and thus mitigate its adverse effects.
Consequently, the purpose of this study was to evaluate the independent and combined effects of ROSI and RSV on the expression of genes involved in lipid handling and inflammation in cultured adipose tissue from morbidly obese subjects. We hypothesized that RSV+ROSI would have additive effects on increasing the mRNA expression of glyceroneogenic enzymes and improving the inflammatory state in both subcutaneous (scAT) and visceral (viscAT) adipose tissue.

5.3 Materials and Methods

5.3.1 Subject recruitment

This study included morbidly obese men and women who were recruited from the Guelph General Hospital (GGH; Ontario, Canada). All participants consumed a standardized OPTIFAST 900 powdered diet for a minimum of two weeks prior to surgery. Participants were fasted overnight before undergoing laparoscopic bariatric surgery. Baseline measurements of weight, height, body mass index (BMI) and glycosylated haemoglobin were made 2-4 weeks before the surgery following standard clinical protocols. All subjects provided written consent and the study was approved by the Ethics Board at the University of Guelph and GGH.

5.3.2 Adipose tissue organ culture (ATOC)

ATOC is an established technique to assess the modulations in adipose tissue physiology and gene expression [61]. ScAT and viscAT was obtained before surgery and was immediately carried to the laboratory in oxygenated media 199 (M199;
Sigma-Aldrich, Oakville, ON, Canada). After rinsing with sterile PBS, 400 mg of adipose tissue was minced and placed into petri dishes with M199 supplemented with 1% antibiotic/antimycotic, insulin (350 pmol/L) and dexamethasone (2.5 nmol/L), and cultured at 37°C with 5% CO₂. After an overnight incubation, explants were transferred into fresh M199 supplemented with 2.5% FFA-free bovine serum albumin (MP Biomedicals, Santa Ana, CA, USA) and treated for 24 h with vehicle (dimethyl sulfoxide; Sigma-Aldrich), resveratrol 50 µM (Cayman Chemicals, Ann Arbor, MI, USA), rosiglitazone 0.5 µM (Cayman Chemicals), resveratrol 50 µM + rosiglitazone 0.5 µM or epinephrine 10 µM (Sigma-Aldrich). Doses of RSV and ROSI were based on previous reports [148, 155] to provide an effective dose of RSV and a submaximal dose of ROSI. After treatment, tissue was drained, rinsed, frozen in liquid nitrogen and stored at -80°C. Media was stored at -20°C until further analysis.

5.3.3 Real-Time PCR (qPCR)

RNA extraction, complementary DNA synthesis and mRNA quantification were performed as described previously [14]. Relative differences in gene expression were determined using the $2^{-\Delta\Delta CT}$ method [125]. The amplification efficiency of the gene of interest and the housekeeping gene were equivalent. Results were normalized to the mRNA expression of 18S ribosomal RNA, which did not change with treatment.
5.3.4 Lipolysis and adiponectin

Colorimetric assays were used to quantify FFA (Wako Diagnostics, Richmond, VA, USA) and glycerol (Sigma-Aldrich) in media. Adiponectin in media was quantified using an ELISA (Millipore, Billerica, MA, USA). Raw values were normalized to tissue’s wet weight.

5.3.5 Statistical analysis

A repeated-measure analysis of variance was used to compare gene expression, adiponectin, FFA and glycerol in each depot (SigmaPlot, Systat Software, San Jose, CA, USA). When appropriate, post-hoc analysis was conducted using a Tukey test. Data are presented as mean ± SEM and an $\alpha=0.05$ was considered statistically significant.

5.4 Results and Discussion

Subject characteristics are presented in Table 5.1. As hypothesized, the mRNA expression of the glyceroneogenic enzymes PDK4 and PEPCK was increased by the treatments. In scAT, the 20% increase in PDK4 expression with RSV was not significant; however, ROSI ($p=0.08$) and RSV+ROSI ($p<0.05$) both showed a 2.6-fold increase compared to control (Fig. 5.1A). PEPCK was increased by ROSI (3.4 fold; $p<0.05$) and by RSV+ROSI (4.2 fold; $p<0.05$), but only 22% by RSV (NS).
Table 5.1. Subjects’ characteristics

<table>
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<tr>
<th></th>
<th>Whole population</th>
<th>scAT</th>
<th>viscAT</th>
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<tbody>
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<td>28 (3/25)</td>
<td>9 (1/8)</td>
<td>20 (3/17)</td>
</tr>
<tr>
<td>Age (yr)</td>
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<td>42.3 ± 2.0</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
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<td>46.1 ± 2.1</td>
<td>46.2 ± 2.1</td>
</tr>
<tr>
<td>HbA$_{1c}$ (%)</td>
<td>0.067 ± 0.003</td>
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Data are mean ± SEM. There were no differences between groups in any of the parameters. BMI: body mass index. HbA$_{1c}$: glycosylated haemoglobin.

In viscAT, the actions of RSV+ROSI on PDK4 and PEPCK were additive. PDK4 mRNA was increased 42% by RSV (NS), 81% by ROSI (p<0.05) and 180% by RSV+ROSI (p<0.05) (Fig. 5.1B). Notably, with a perfectly additive effect, we would anticipate RSV+ROSI to increase PDK4 by ~120%. While this is admittedly speculative, this 180% increase suggests synergistic effects of the two compounds on PDK4. On the other hand, PEPCK was increased 2.4, 4.5 and 7.3 fold by RSV, ROSI and RSV+ROSI, respectively (all p<0.05) (Fig. 5.1B). The increase seen with RSV+ROSI (7.3 fold) is very similar to the theoretical additive effect of 6.9 fold. Based on a report that adipose-specific deletion of PGC-1α in mice decreased PDK4 mRNA expression [103], we examined PGC-1α gene expression, but it was unchanged with treatment (Fig. 5.1A-B).

Absolute rates of glyceroneogenesis parallel lipolysis [21], so PEPCK and PDK4 could be increased as a consequence of lipolysis [201]. In the present study, only epinephrine (the positive control) increased FFA or glycerol release in both depots (Fig. 5.1E-F). The lack of induction of basal lipolysis by RSV is not surprising as
most reports of RSV-induced lipolysis examined it in the stimulated state [119, 148]. Consequently, the actions of RSV and ROSI on PDK4 and PEPCK expression are independent from changes in lipolysis, which suggests a direct action on the induction of these genes.

Aside from glyceroneogenesis, glycerol-3-phosphate, which is essential for fatty acid reesterification, can also be generated via glycerol kinase [122]. In adipose tissue, this process is considered less prevalent than glyceroneogenesis [122]. In the current study, none of the treatments modulated the gene expression of glycerol kinase or of the glycerol channel aquaporin 7 in viscAT (Appendix C). Overall, in human cultured adipose tissue, RSV induced glyceroneogenesis without changes in lipolysis, which may explain why the anti-adipogenic effects of RSV reported in cell models [119, 148] failed to translate to human studies [106].

Both ROSI [105] and RSV [14] have powerful anti-inflammatory actions. In scAT and viscAT, none of the treatments modulated the expression of TNF-α or adiponectin (Fig. 5.1C-D). However, IL-6 mRNA in scAT was reduced 45% and 32% with RSV and RSV+ROSI, respectively. While this failed to reach statistical significance due to inter-individual variability, it is an effect that deserves further attention. There was a similar trend in viscAT: both RSV (NS) and RSV+ROSI (p<0.05) reduced IL-6 mRNA (Fig. 5.1C-D). Furthermore, there was no effect of treatment on adiponectin secretion in the media of either depot (Appendix D). Admittedly, the current protocol was not designed to induce inflammation; therefore possibly limiting the likelihood of observing anti-inflammatory effects.
Interestingly, most studies reported that RSV decreased PPARγ mRNA and/or protein expression in adipocytes [30, 148]. This is not consistent with the present data and with previous reports from our laboratory [14] and others [58, 98]. Here, we report the induction of PEPCK with RSV, a target of PPARγ, suggesting that RSV activates PPARγ. Of note, activation of PPARγ promotes its own proteasomal degradation [82]. Consequently, the decrease in PPARγ content reported in numerous studies [30, 148] cannot be interpreted as PPARγ inhibition. Accordingly, ROSI and RSV both deacetylate and activate PPARγ in cell lines [155], suggesting that these two compounds could be acting, at least in part, via similar mechanisms.

In summary, we are the first to demonstrate that RSV and ROSI show lipolysis-independent and additive effects on glyceroneogenic enzymes in cultured visceral adipose tissue from morbidly obese patients. As RSV and ROSI share at least some common mechanisms [155], these additive effects suggest that RSV could potentiate the effects of ROSI. While speculative, RSV-induced PPARγ deacetylation could result in a conformational change of PPARγ, which could 1-promote TZD ligand binding affinity or 2-promote TZD-induced PPARγ phosphorylation and further activation [155].

Finally, this study design is admittedly limited by several factors, including the large variability inherent to human studies. However, it examined, for the first time, the effects of RSV and ROSI in two distinct adipose tissue depots from morbidly obese individuals and showed novel additive effects of RSV+ROSI in viscAT. These findings are particularly intriguing, as it would suggest that combining nutraceutical
and pharmacological approaches might mitigate the harmful effects of anti-diabetic drugs by reducing the required doses.
Figure 5.1 Resveratrol + rosiglitazone additively increase glyceroneogenic and decrease inflammatory gene expression in cultured visceral adipose tissue from morbidly obese patients, independent of lipolysis. A-D: mRNA expression of glyceroneogenic & mitochondrial genes (A) and inflammatory genes (C) in subcutaneous cultured adipose tissue (n=7-9). mRNA expression of glyceroneogenic & mitochondrial genes (B) and inflammatory genes (D) in visceral cultured adipose tissue (n=17-20). Data are expressed relative to subject’s own control condition. *: p<0.05 vs. control, #: p<0.05 vs. RSV, $: p<0.05 vs. ROSI. E-F: Free fatty acid and glycerol concentrations in media of subcutaneous (E) or visceral (F) cultured human adipose tissue. Data are normalized to tissue’s wet weight and expressed relative to subject’s own control. $: p<0.05 vs. control, RSV, ROSI, and RSV+ROSI conditions, n=25-28. Adipose tissue was treated with resveratrol 50 µM (open bars), rosiglitazone 0.5 µM (gray bars), resveratrol 50 µM + rosiglitazone 0.5 µM (black bars), or epinephrine 10 µM (dashed bars) for 24 h. mRNA expression is not shown for the epinephrine treatment.
Chapter 6: Integrative Discussion

The overall objective of this thesis was to examine the individual and combined effects of resveratrol and rosiglitazone on white adipose tissue physiology in rodent and human models in a context of obesity and insulin resistance. Given the limited long-term success of exercise and weight loss programs at the population level [221], nutraceutical and pharmacological strategies to promote metabolic health may provide valuable tools to manage the burden of the metabolic complications of obesity. Prior to this thesis, RSV was recognized as a calorie-restriction mimetic in yeast [88] and rodents [12] and its whole-body effects were mostly attributed to the modulation of skeletal muscle metabolism [12, 113]. Currently, the investigations of RSV’s actions on adipose tissue physiology have been restricted to cell-culture models, limited to a few biomarkers and have not taken into account the inherent differences between adipose tissue depots.

On the other hand, ROSI’s PPARγ ligand actions in adipose tissue are well characterized but its severe adverse effects limit its use in clinical settings [136]. Nonetheless, PPARγ agonism remains an essential signal for adipogenesis and lipogenesis in adipose tissue. However, the effects of PPARγ on the TGF-β/SMAD pathway, which has recently been implicated in the development of obesity and insulin resistance [189, 215], have never been studied. Finally, in the light of RSV’s and ROSI’s similar mechanisms of actions [155], it was important to elucidate whether a combined treatment of RSV+ROSI would have additive or synergistic actions on lipid handling and inflammation in human adipose tissue.
The overall hypotheses of this thesis were that:

1- chronic dietary resveratrol supplementation would improve whole-body glucose tolerance and insulin sensitivity in diabetic rats, which would be associated with improved glyceroneogenesis, mitochondrial biogenesis and adiponectin secretion in white adipose tissue;

2- in ZDF rats, long-term treatment with rosiglitazone would improve whole-body glucose homeostasis, stimulate adipogenesis and would concurrently inhibit signalling from the TGF-β/SMAD pathway in white adipose tissue;

3- combined treatment of cultured white adipose tissue from morbidly obese patients with resveratrol and rosiglitazone would show additive effects to increase glyceroneogenic gene expression and decrease inflammatory gene expression.

### 6.1 Summary of results

This thesis first reports that RSV supplementation improves whole-body glucose homeostasis in a rodent model of T2DM and that can be attributed, at least in part, to direct modulatory effects on adipose tissue metabolism. Consistent with the hypothesis, RSV supplementation in ZDF rats improved insulin sensitivity and glucose tolerance while stimulating glyceroneogenesis, mitochondrial biogenesis and adiponectin secretion in a depot-specific manner. Markers of mitochondrial content and function were increased in both scAT and rpWAT while glyceroneogenesis,
lipolytic proteins and adiponectin secretion *ex vivo* were stimulated exclusively in scAT.

Given that silencing TGF-β and/or SMAD3 *in vivo* result in loss of adiposity [189, 215] but that, paradoxically, *in vitro* treatment with TGF-β inhibits adipocyte differentiation and lipid accretion [37, 89, 179, 196], this thesis sought to determine how ROSI, a known adipogenic stimulus, would modulate TGF-β/SMAD signalling in rodent adipose tissue *in vivo*. In accordance with the operating hypothesis, ROSI inhibited TGF-β ligand family signalling concurrently with increases in adipogenesis and lipogenesis specifically in scAT. This conclusion was based on decreased phosphorylation of TGF-β-associated R-SMADs (SMAD2 and SMAD3), paralleled with increased protein content of I-SMAD (SMAD7) and ubiquitin ligase SMURF2. Conversely, the protein content of proximal parts of the pathway, such as TGF-β receptor type 1 and 2, the receptor adapter protein SARA and SMAD4, was robustly upregulated. This suggests that adipogenesis and inhibition of TGF-β signalling are associated in scAT and that upregulation of proximal parts of the pathway constitutes an attempt to maintain cellular homeostasis in response to the massive adipogenic stimulus from PPARγ agonism.

Finally, given the direct effects of RSV and ROSI in rodent WAT, their individual and combined impacts were examined in cultured human adipose tissue from morbidly obese subjects. In accordance with the hypothesis, there was an additive, lipolysis-independent, effect of RSV and ROSI on glyceroneogenic gene expression in viscAT. Similarly, in viscAT, RSV and RSV+ROSI decreased IL-6 gene
expression, suggesting an anti-inflammatory effect of both RSV alone and the combined treatment.

Overall, this thesis shows a direct and depot-specific modulatory effect of RSV on lipid handling in WAT. In addition, ROSI-induced adipogenesis occurred in parallel with TGF-β/SMAD inhibition specifically in scAT. Finally, the combined treatment of RSV+ROSI showed additive beneficial effects on glyceroneogenesis in vitro in WAT from morbidly obese individuals. Taken together, this thesis provides support for the combined use of nutraceutical and pharmacological compounds to treat and prevent metabolic pathologies.

6.2 Is resveratrol a promising nutraceutical strategy to prevent and treat insulin resistance?

Based on a large body of literature in animal models, RSV constitutes a promising nutraceutical compound to alleviate the metabolic complications of obesity. Its supplementation in rodents improves whole-body insulin sensitivity, endurance performance, skeletal muscle mitochondrial biogenesis and survival [12, 113, 143, 150, 176, 198]. Furthermore, the present thesis showed that at least part of the metabolic effects of RSV occurs through direct modulations of WAT physiology. In fact, RSV stimulated GNG, mitochondrial biogenesis and adiponectin secretion, which are implicated in the prevention of lipotoxicity in other tissues. Additional investigations also reported RSV-induced benefits in other metabolic tissues, such as the liver [71], the kidneys [101] and the heart [158]. Taken together, these data show
that RSV acts in a variety of tissues and exert pleiotropic beneficial effects at the molecular and tissue level in rodents.

However, the current data in humans are less convincing: little to no benefits are reported with supplementation with RSV in healthy and diseased individuals [19, 104, 106, 149, 192, 222]. One of the main limitations of RSV in humans resides in its poor bioavailability. The gastro-intestinal tract and the liver extensively and rapidly metabolize RSV, resulting in only a mild elevation of plasma RSV and limited accumulation in tissues [8, 200]. Currently, researchers are exploring avenues to extend RSV’s half-life through the development of synthetic RSV derivatives or routes of administration that would bypass the gastro-intestinal tract and first-pass metabolism [7, 199].

It is plausible that RSV’s actions in the gastro-intestinal tract modulate at least some of its whole-body effects. In fact, mice supplemented with RSV showed an increase in intestinal active GLP-1 secretion, which also promoted insulin secretion [41], but this effect was not replicated in humans [104]. Furthermore, in the first study of this thesis [14], fasting insulin was upregulated, albeit not significantly, in rodents supplemented with RSV. Interestingly, the beneficial effects of RSV supplementation were abolished in GLP-1 receptor knockout animals but intensified with concomitant treatment with dipeptidyl peptidase-4 (DPP-4) inhibitor sitagliptin [41]. This suggests that GLP-1 may be necessary for RSV benefits, but this hypothesis requires further testing.

In addition, increasing attention is currently given to the role of the gut microbiota in health and disease. While this field of research is still at its infancy, RSV
supplementation normalized the profile of caecal bacterial composition in high-fat fed mice [41] and in a model of induced colitis [117]. The modulation of gut microbiota is also present with red wine consumption [157], one of the main dietary sources of RSV. Furthermore, RSV-induced modulations of the gut microbiota were correlated with RSV metabolic benefits at the whole-body level [156]. Overall, these data suggest that modulations of the gastro-intestinal tract metabolism, through gut microbiota and/or incretin hormone stimulation, may constitute an additional plausible mechanism of action for RSV.

Another potential avenue to reconcile RSV’s limited bioavailability with its metabolic benefits is to examine the actions of RSV’s metabolites (Appendix E). In addition to the gastro-intestinal tract, RSV metabolites accumulate more strongly in liver, with intermediate levels detected in WAT and the lowest quantities reported in skeletal muscle [8]. Recent in vitro data suggest that RSV metabolites have bioactive effects that are similar to intact RSV on adipose tissue inflammation and lipid handling. In 3T3-L1 adipocytes, RSV metabolites trans-resveratrol-3-0-glucuronide, trans-resveratrol-4’-O-glucuronide and trans-resveratrol-3-O-sulphate, three of the most common RSV metabolites in rodents and humans [223], favourably modulated the mRNA expression of the adipokines apelin, visfatin and leptin [49]. Furthermore, treatment of adipocytes with RSV’s glucuronide metabolites reduced triglyceride accumulation and increased SIRT1 mRNA expression [118]. Interestingly, some aspects of the metabolism of RSV are dependent upon the nature of the individual’s gut microbiota and show a strong inter-individual variability [16, 74]. Taken together, the formation and accumulation of RSV metabolites may explain why RSV has
beneficial effects despite its limited bioavailability. Individual variations in gut microbiota and RSV metabolites formation may account for the lack of consistent data with RSV supplementation in humans.

In conclusion, RSV remains a promising avenue to prevent and treat metabolic disorders based on comprehensive rodent data and preliminary human clinical trials. Future research should focus on effective dosing in humans, with an emphasis on RSV metabolites and/or synthetic molecules to increase bioavailability. In addition, individual responsiveness to RSV supplementation should be examined, based on clinical characteristics and/or the nature of the gut microbiota.

6.3 Is combining RSV + ROSI a promising strategy to prevent and treat insulin resistance?

One of the overall objectives of this thesis was to determine whether RSV might be used in conjunction with other anti-diabetic drugs, such as ROSI, to prevent and treat metabolic pathologies and alleviate some of the adverse effects of pharmacological treatment. Overall, this thesis suggests that RSV and ROSI, individually and in combination, modulate lipid handling and inflammation in WAT from rodents and humans in vivo and in vitro (Table 6.1). RSV and ROSI both increased PDK4 and PEPCK mRNA expression in human and rodents, which was associated with an increase in $^{14}$C-pyruvate incorporation into triglycerides in rodent WAT (Appendix F). This increase in glyceroneogenic markers and fatty acid reesterification may explain why the lipolytic effects of RSV in vitro [119, 148, 187] do not consistently translate
to \textit{in vivo} models. In fact, resveratrol supplementation in obese men decreased, rather than increased, the size of subcutaneous adipocytes \cite{106}. Stimulating glyceroneogenesis and fatty acid reesterification may constitute a valuable tool to safely sequester fatty acids within WAT and prevent ectopic fat deposition and lipotoxicity in other tissues.

Table 6.1 Mechanisms by which resveratrol and rosiglitazone, individually and in combination, modulate lipid handling and inflammation in rodent and human white adipose tissue.

<table>
<thead>
<tr>
<th></th>
<th>Resveratrol</th>
<th>Rosiglitazone</th>
<th>Resveratrol + Rosiglitazone\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceroneogenesis</td>
<td>+</td>
<td>++</td>
<td>+++ (PEPCK/PDK4 gene expression)</td>
</tr>
<tr>
<td>Mitochondrial biogenesis</td>
<td>+</td>
<td>+</td>
<td>No change (PGC-1\textalpha gene expression)</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>+ (secretion)</td>
<td>+\textsuperscript{b}</td>
<td>No change (secretion &amp; gene expression)</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>No change (secretion)</td>
<td>No change</td>
<td>- (gene expression)</td>
</tr>
<tr>
<td>SMAD2/3 phosphorylation</td>
<td>NA</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>Inhibitory SMAD7 and SMURF2 protein content</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>TGF-\beta receptors, SARA, SMAD4 protein content</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The combined effects of resveratrol+rosiglitazone were only examined \textit{in vitro} in this thesis, while the individual effects were investigated \textit{in vivo} and \textit{in vitro}. \textsuperscript{b}Not investigated directly in this thesis, but supported by other reports \cite{105}. NA: not investigated in the present thesis. PDK4: pyruvate dehydrogenase kinase 4; PECK-C: cytosolic phosphoenolpyruvate carboxykinase; PG\textC-1\textalpha: peroxisome-proliferated activator receptor-\gamma coactivator-1\alpha; SARA: SMAD anchor for receptor activation; TGF-\beta: transforming growth factor-\beta.
This thesis also reports inhibitory effects of RSV and ROSI on IL-6 gene transcription, an adipokine that is involved in the etiology of insulin resistance. In addition, the present thesis documented RSV-induced secretory effects of the anti-inflammatory adipokine adiponectin and others reported a robust stimulatory effect of ROSI on adiponectin [105]. However, this thesis fails to show RSV- or ROSI-mediated effects on adiponectin gene expression in cultured human adipose tissue, suggesting that this effect may occur at the post-transcriptional level, is species-specific, or is abolished in adipose tissue from morbidly obese participants. Overall, a large body of literature documents the anti-inflammatory actions of RSV and ROSI, independently. Those data, in addition to the inhibitory effect of RSV+ROSI on IL-6, suggest that the combination of RSV and ROSI may be helpful in alleviating WAT inflammation present in metabolic disorders.

In addition, this thesis documents, for the first time, that ROSI-induced adipogenesis is associated with downregulation of the TGF-β/SMAD signalling pathway in scAT. The illustration that TGF-β and SMAD3 are involved in whole-body glucose homeostasis [189, 215] provides novel targets for nutraceutical and pharmacological interventions in the treatment and prevention of insulin resistance. Interestingly, preliminary data suggest that RSV can also inhibit the TGF-β/SMAD signalling cascade in kidneys from diabetic rats [29], in squamous cell carcinoma [100] and in A549 lung cancer cells [207]. Given the inhibitory effects of ROSI on TGF-β/SMAD signalling in adipose tissue described in this thesis, it would be important to elucidate the combined effects of ROSI and RSV on this pathway. Potential additive effects of
RSV+ROSI on TGF-β/SMAD signalling in WAT may provide additional support for their combined use in the management of metabolic pathologies.

Finally, while the data on lipid handling and inflammation support the combined use of RSV and ROSI, these data are admittedly preliminary as they were generated in tissue culture and animal models. Therefore, further investigations in clinical populations, using additional markers, are warranted to solidify the possibility of combining nutraceutical and pharmacological approaches. In addition, the safety of such treatment, especially with regards to ROSI-induced cardiac and osteoporotic complications, should be given close attention.

6.4 Adipose tissue responds in a depot-specific manner to RSV and ROSI treatments

In this thesis, depot-specific effects of RSV and ROSI, individually and in combination, were consistently reported. In study one, RSV-induced glyceroneogenesis and adiponectin secretion were both described specifically in scAT. In study two, ROSI increased adipogenic markers in conjunction with downregulation of TGF-β signalling exclusively in scAT. These depot-specific effects are consistent with other reports that described that PPARγ agonism stimulates lipid uptake, lipogenesis and fatty acid reesterification to a larger extent in rodent scAT compared to viscAT [54, 115]. However, this thesis is the first to report a preferential action of RSV on scAT, similar to what has been described with ROSI treatment. These
findings are significant as scAT is generally viewed as the safer depot to store fatty acid [109] and may contribute to the anti-diabetic activity of both ROSI and RSV.

Interestingly, study three documented more robust actions of RSV and ROSI in viscAT, which contrasts with the two previous studies. This apparent discrepancy could be explained by several factors. First, study designs were considerably different between study one and two and study three. Indeed, species differences between rats (studies one & two) and humans (study three) may explain these data. Second, and perhaps more importantly, depot specificity was stronger in in vivo (studies one & two) compared to in vitro (study three) designs. In fact, the effective dose with in vivo supplementation would have been much lower than that provided in an adipose tissue culture model. Consequently, it is possible that higher doses may override depot-specific sensitivity to RSV and ROSI and thus provide similar responses between scAT and viscAT. Moreover, depot specificity may be conferred by signals from other tissues, such as the myokines irisin [18], IL-6 [203] and myostatin [225] or from the innervation from the nervous system [20]. By isolating WAT in culture models, all of these factors would have been removed and thus depot specificity may have been lost. Finally, it cannot be ruled out that the small sample size in study three may have prevented an accurate description of the true effects of RSV and ROSI in scAT.

In fact, viscAT analysis included 17-20 subjects, while the analysis for scAT only included 7-9 participants. The sample size for scAT was limited due to technical difficulties in isolating RNA from this specific depot. Given the inherent inter-subject variability in human studies, it is not surprising that the most robust effects of RSV and ROSI were reported in viscAT compared to scAT. Importantly, the same trends
were seen in both depots, suggesting that scAT may be as responsive to treatment as viscAT but that the sample size limited the strength of these conclusions.

Overall, there is strong evidence from this thesis and other reports [54, 115] that different adipose tissue depots respond uniquely to stimuli such as RSV and ROSI. While the exact molecular modulators of depot-specificity remain to be elucidated, these effects are more evident in *in vivo* study designs, suggesting that factors outside of adipose tissue *per se* are involved in this process.

### 6.5 Concluding remarks

In summary, the present thesis highlights that RSV and ROSI, individually and in combination, modulate lipid handling and inflammatory processes in a depot-specific manner in white adipose tissue from rodents and humans, which contributes to improvements in whole-body insulin resistance and glucose intolerance. Specifically, RSV and ROSI stimulate glyceroneogenesis and fatty acid reesterification, in parallel with increases in mitochondrial biogenesis and adiponectin secretion. Finally, this thesis also describes that ROSI’s adipogenic and healthy WAT remodelling effects may be modulated, at least in part, through the inhibition of the TGF-β/SMAD signalling cascade in scAT, which provides a novel target pathway for obesity and metabolic pathologies.

Overall, this thesis provides support, from *in vivo* rodent models and *in vitro* human models, for the combined use of ROSI and RSV to promote glucose homeostasis in metabolic disorders and highlights a direct role of WAT in modulating RSV and
ROSI’s anti-diabetic effects. Further studies should involve clinical populations and examine whether combined pharmacological and nutraceutical treatments could be a viable alternative to increase treatment efficacy while decreasing its adverse side effects.
References


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Miyazaki Y and DeFronzo RA. Rosiglitazone and pioglitazone similarly improve insulin sensitivity and secretion, glucose tolerance and


Appendix A

Protein content of SMAD proteins in male Wistar rat adipose tissue in response to high-fat (HF) feeding. Protein content and representative blots of SMAD3, p-SMAD3 (ser423/425), and SMAD7 in scAT (A) and rpWAT (B) for chow-fed (□) and HF-fed (■) (n=5). Values are mean ± SEM. There were no differences between groups.
Appendix B

Protein content of SMAD proteins in adipose tissue of lean and obese ZDF rats fed a chow diet. Protein content and representative blots of SMAD3, p-SMAD3 (ser423/425), and SMAD7 in scAT (A) and rpWAT (B) for lean (☐) and ZDF (■) rats (n=7-8). Values are mean ± SEM. There were no differences between groups.
Appendix C

mRNA expression of glycerol kinase and aquaporin 7 in visceral adipose tissue from morbidly obese patients treated with vehicle, RSV, ROSI or RSV+ROSI (n=18-19).

<table>
<thead>
<tr>
<th></th>
<th>RSV</th>
<th>ROSI</th>
<th>RSV+ROSI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycerol kinase</strong></td>
<td>1.09 ± 0.14</td>
<td>1.25 ± 0.13</td>
<td>0.95 ± 0.11</td>
</tr>
<tr>
<td>(fold increase above control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Aquaporin 7</strong></td>
<td>1.13 ± 0.12</td>
<td>1.18 ± 0.15</td>
<td>1.22 ± 0.21</td>
</tr>
<tr>
<td>(fold increase above control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. There were no differences between groups.
### Appendix D

Adiponectin secretion in media from cultured scAT and viscAT from morbidly obese patients treated with vehicle, RSV, ROSI or RSV+ROSI (n=9-10).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>RSV (fold increase above control)</th>
<th>ROSI (fold increase above control)</th>
<th>RSV+ROSI (fold increase above control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scAT</td>
<td>$1.14 \pm 0.11$</td>
<td>$1.22 \pm 0.07$</td>
<td>$1.04 \pm 0.08$</td>
</tr>
<tr>
<td>viscAT</td>
<td>$1.02 \pm 0.04$</td>
<td>$1.02 \pm 0.04$</td>
<td>$0.95 \pm 0.05$</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. Data are normalized to tissue’s wet weight and expressed relative to their own control. There were no differences between groups.
Appendix E

Quantification of resveratrol metabolites (nmol/g tissue) in scAT from rodents fed 60 mg trans-resveratrol/kg body weight daily for 6 weeks. Adapted from Andres-Lacueva and colleagues (2012) [8].

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-resveratrol</td>
<td>nq</td>
</tr>
<tr>
<td>trans-resveratrol-3-O-glucuronide</td>
<td>1.08 ± 0.29</td>
</tr>
<tr>
<td>trans-resveratrol-4’-O-sulfate</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>trans-resveratrol-3-O-sulfate</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>cis-resveratrol-3-O-sulfate</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>trans-resveratrol-3-4’-disulfate</td>
<td>nq</td>
</tr>
<tr>
<td>Dihydropresveratrole³</td>
<td>nq</td>
</tr>
<tr>
<td>Dihydropresveratrol glucuronide³</td>
<td>nq</td>
</tr>
<tr>
<td>Dihydropresveratrol sulfate³</td>
<td>1.02 ± 0.37</td>
</tr>
</tbody>
</table>

³: indicates resveratrol metabolites from microbial origins. nq: indicates that the metabolite was non-quantifiable.
Appendix F

Assessment of glyceroneogenesis through $^{14}$C-pyruvate incorporation into triglycerides (nmol/L per g tissue) in subcutaneous, epididymal and retroperitoneal adipose tissue from ZDF rats treated with or without rosiglitazone for 6 weeks (n=7-8).

<table>
<thead>
<tr>
<th>Adipose tissue depot</th>
<th>ZDF chow</th>
<th>ZDF ROSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>scAT</td>
<td>28.61 ± 4.66</td>
<td>71.34 ± 9.96*</td>
</tr>
<tr>
<td>eWAT</td>
<td>47.76 ± 4.71</td>
<td>63.14 ± 5.78*</td>
</tr>
<tr>
<td>rpWAT</td>
<td>41.71 ± 3.14</td>
<td>59.62 ± 3.21*</td>
</tr>
</tbody>
</table>

* p<0.05 vs. ZDF chow. Data are presented as mean ± SEM. Data from the ZDF chow group is the same as published in Beaudoin and colleagues (2013) [14].