Resveratrol action in the duodenum and the regulation of insulin sensitivity

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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General Abstract

Oral resveratrol improves insulin sensitivity in obese or diabetic rodents, but the underlying mechanisms remain elusive. Resveratrol’s effects have been attributed, in part, to the activation of SIRT1 and/or AMPK in various tissues, although the low bioavailability of resveratrol raises questions about whether these effects are direct. We here show that intraduodenal resveratrol infusion activates duodenal SIRT1 and AMPK to enhance insulin sensitivity to lower glucose production (GP) in high-fat diet (HFD)-induced insulin resistance, while duodenal SIRT1 knockdown in chow fed-rats induces hepatic insulin resistance. The glucoregulatory role of preabsorptive resveratrol is dependent on a gut-brain neuronal network to remotely restore hypothalamic insulin sensitivity and GP regulation. Lastly, intraduodenal resveratrol infusion lowers GP and plasma glucose levels in two complementary obese and type 2 diabetic animal models. These studies highlight the therapeutic relevance of duodenal resveratrol and SIRT1/AMPK activation in improving insulin sensitivity and glucose control in obesity and diabetes.
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<tr>
<td>ABC transporter</td>
<td>ATP binding cassette transporter</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl CoA carboxylase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate activate protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistant protein</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DJB</td>
<td>Duodenal jejunal bypass</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DVC</td>
<td>Dorsal vagal complex</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic peptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon like peptide-1</td>
</tr>
<tr>
<td>GP</td>
<td>Glucose production</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment of insulin resistance</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Jak-2</td>
<td>Janus kinase 2</td>
</tr>
<tr>
<td>LCFA-CoA</td>
<td>Long chain fatty acyl-coenzyme A</td>
</tr>
<tr>
<td>LCFAs</td>
<td>Long chain fatty acids</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
</tr>
<tr>
<td>MBH</td>
<td>Mediobasal hypothalamus</td>
</tr>
<tr>
<td>MRP2</td>
<td>Multidrug resistant-associated protein 2</td>
</tr>
<tr>
<td>MRP3</td>
<td>Multidrug resistant-associated protein 3</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>Ob-Rb</td>
<td>Long form leptin receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>Ra</td>
<td>Rate of appearance</td>
</tr>
<tr>
<td>Rd</td>
<td>Rate of disappearance</td>
</tr>
<tr>
<td>RYGB</td>
<td>Roux-en-Y gastric bypass</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SGLT</td>
<td>Sodium-glucose transporter</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin1</td>
</tr>
<tr>
<td>SLR</td>
<td>Soluble leptin receptor</td>
</tr>
<tr>
<td>SRIF</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>VSG</td>
<td>Vertical sleeve gastrectomy</td>
</tr>
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<td>ZDF rats</td>
<td>Zucker diabetic fatty rats</td>
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Chapter 1
Introduction

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1.1 Obesity and type 2 diabetes

Obesity has become an increasing concern worldwide, as the World Health Organization estimates that as of 2008, more than 1.4 billion adults were overweight (BMI $\geq$ 25), and of those, approximately 500 million were considered obese (BMI $\geq$ 30)$^1$. The health consequences of obesity are extensive and include increased risk for cardiovascular diseases, some cancers, and importantly, type 2 diabetes. Indeed, nearly 80% of those affected with type 2 diabetes are considered overweight or obese$^2$, which can be partly attributed to overconsumption of a high calorie diet and interactions with the environment in individuals with genetic predispositions to the disease$^3$. Obesity itself is associated with disruptions in energy homeostasis, where excess in adipose stores can lead to insulin resistance and an inability to effectively control glucose homeostasis. This is manifested by the inability of the liver to suppress glucose production, and by the inability of skeletal muscle and adipose tissue to stimulate glucose uptake in response to insulin, which can lead to hyperglycemia. While the majority of obese individuals do not
develop hyperglycemia as the pancreas can compensate for the loss of insulin sensitivity by an increase in β-cell mass, a failure of this adaptation results in hyperglycemia and the development of type 2 diabetes.

It is estimated that more than 346 million individuals are affected by diabetes worldwide and approximately 9 million Canadians alone are type 2 diabetic. Type 2 diabetes is characterized by fasting hyperglycemia, where plasma glucose levels exceed 7.0 mM in the fasted state. Chronic hyperglycemia damages endothelial cells of blood vessels, and leads to both micro- and macrovasculature damage such as nephropathy, neuropathy, and retinopathy. Therapeutic approaches to treat diabetes and to lower its associated economic burden are focused on regulating glucose levels and hepatic glucose production. As such, the most prescribed antidiabetic drug, metformin, suppresses glucose production to normalize glycemia in diabetic individuals. However, the side effects of metformin treatment are wide, which has led to the development and success of another class of oral hypoglycemic agents, aimed at increasing the actions of gut-derived incretins (GLP-1 and GIP). While the physiological and pathophysiological mechanisms of glucose production regulation still remain largely unknown, these promising treatment options highlight the importance of the gastrointestinal tract in regulating glucose homeostasis. Thus, it is of interest to develop novel therapeutic approaches to improve glucose control and insulin action in both obesity and diabetes, focusing on the gut as the site for these interventions.
1.2 Gut nutrient sensing and the regulation of metabolic homeostasis

The gut has been demonstrated to be an important mediator of metabolic homeostasis. As one of the first sites to come into contract with ingested nutrients after a meal, the small intestine is positioned as a primary line of defense against nutrient excess by controlling both food intake and glucose control under normal conditions. In obesity and diabetes, nutrient-sensing mechanisms aimed at maintaining metabolic homeostasis fail, leading to energy and glucose dysregulation\textsuperscript{9,10}.

As nutrients enter the most proximal section of the small intestine, the duodenum, after a meal, nutrient sensing mechanisms are triggered in order to maintain metabolic homeostasis by eliciting appropriate physiological responses to adapt to the influx of nutrients. Studies in both rodents and humans demonstrate the ability of lipid accumulation and administration of long chain fatty acids (LCFAs) with 12 or more carbons in the duodenum to suppress food intake\textsuperscript{11-20}. The satiation effect caused by these upper intestinal lipids requires a gut-brain neuronal network, as co-infusion of lipids with tetracaine, an anesthetic, and subdiaphragmatic vagotomy, abolish their effects on energy intake\textsuperscript{11}.

In addition to regulating energy homeostasis, intraduodenal lipids also suppress hepatic glucose production during the pancreatic (basal insulin) euglycemic clamp. After an intraduodenal administration of the lipid emulsion Intralipid, triglycerides are hydrolyzed by lipases to release LCFAs for absorption into enterocytes\textsuperscript{21}. LCFAs are subsequently esterified into LCFA-CoA by acyl-coenzyme A synthetase, a step necessary for lipids to lower glucose production, as co-infusion of intraduodenal lipids with triascin C, an acyl-coenzyme A synthetase inhibitor, abolished their effect to regulate glucose production\textsuperscript{21}. Furthermore, upper intestinal lipids trigger a neuronal gut-brain-liver axis to regulate glucose control. When co-infused with the
anesthetic tetracaine, Intralipid had no effects, indicating the need of neurotransmission activation within the duodenum for lipids to lower glucose production\textsuperscript{21}. Specifically, the vagal afferents innervating the gut mediate this effect, as lipids did not lower glucose production in rats with vagal deafferentation surgery\textsuperscript{21}. The vagal afferent neurons innervating the intestine are known to terminate in the nucleus of the solitary tract (NTS), and N-methyl-D-aspartate (NMDA) receptor activation in this region is necessary for the glucose lowering effect of duodenal lipids, as blockade of NMDA receptors using MK-801 abolished the ability of duodenal lipids to lower glucose production. Moreover, infusion of Intralipid into rats that had received hepatic vagotomy (a surgical technique that interrupts the vagal innervation between the brain and liver) also abolished the ability of lipids to lower glucose production\textsuperscript{21}.

Interestingly, while duodenal nutrient sensing and the subsequent activation of this gut-brain-liver axis lowers glucose production in healthy chow-fed rats, it fails to do so in rats fed a high fat diet for 3 days\textsuperscript{21}.

Although it was previously believed that the absorption of nutrients only occurred in the proximal section of the small intestine\textsuperscript{22}, studies demonstrate that nutrients reach the distal small intestine after a meal in both rodents\textsuperscript{23-25} and humans\textsuperscript{26,27}, suggesting a potential role for the distal gut in mediating glucose homeostasis. Indeed, the second portion of the small intestine, the jejunum, has been illustrated to sense both glucose\textsuperscript{28} and lipids\textsuperscript{28-32} to lower food intake, although direct administration of proteins into the jejunum had no effects on feeding\textsuperscript{28}. Jejunal sensing also has a glucoregulatory role, as the jejunum senses both glucose and lipids to lower glucose production during a pancreatic (basal insulin) euglycemic clamp\textsuperscript{33}. Uptake into enterocytes and metabolism is necessary for each, respectively, to lower glucose production and similar to duodenal sensing, a neuronal gut-brain-liver network also mediates the effect of jejunal nutrients to affect glucose control\textsuperscript{33}. In order to test the effects of jejunal nutrients
sensing in a more physiological setting, a fasting refeeding experiment was performed to induce nutrient sensing mechanisms after a meal. During the refeeding, nutrient sensing was inhibited by blocking both the uptake of glucose into cells by infusing a SGLT inhibitor as well as the metabolism of lipids by infusing triasin C\textsuperscript{33}. Interestingly, blockade of either nutrients had no effects on glucose homeostasis, suggesting that these sensing mechanisms may only play a role in glucose control in settings where nutrient flow is disrupted\textsuperscript{33}. Such conditions occur during bariatric surgery in obese and type 2 diabetic patients, when sections of the upper intestinal tract are removed and/or bypassed, leading to enhanced nutrient flux to the distal small intestine.

While bariatric surgery is widely used as a weight loss tool in morbidly obese patients, this form of surgery has recently been termed “metabolic surgery”, as patients with type 2 diabetes often undergo a rapid remission of their diabetes\textsuperscript{34}. This normalization in glycemia occurs independent of weight loss in the majority of cases\textsuperscript{8,35-37}. There are two components to bariatric surgeries, which lead to normalization of glucose levels in diabetic individuals: 1) a restrictive component, where the stomach size is reduced, and 2) a malabsorptive component, where the anatomy of the intestinal tract is changed. Both Roux–en-Y gastric bypass (RYGB -includes both a restrictive and malabsorptive component) and vertical sleeve gastrectomy (VSG - includes restricting 80% of the stomach) have been shown to improve glycemia in rodents and humans independent of weight loss\textsuperscript{38,39}. To better understand the involvement of the malabsorptive component of bariatric surgery in the normalization of glycemia, duodenal jejunal bypass (DJB) surgery was developed. DJB surgery does not have a restrictive component, but instead involves bypassing the duodenum and proximal jejunum. Thus, nutrients flow directly from the stomach into the jejunum, bypassing the duodenum entirely. In a rodent model of type 2 diabetes, DJB surgery has been demonstrated to have a rapid antidiabetic effect independent of changes in body weight\textsuperscript{40}. Similar results were found in non-obese or mild obese type 2
diabetic humans, who had weight-independent improvements in glycemia after DJB surgery\textsuperscript{41-44}. These studies underline the importance of intestinal rearrangement in the improvements of glycemia, suggesting a potential role for jejunal nutrient sensing mechanisms in mediating the glucose lowering effect of DJB surgery.

To begin to dissect the role of jejunal nutrient sensing in mediating the antidiabetic effects of DJB, this surgical technique was performed in the diabetes-prone Biobreeding rats and streptozotocin (STZ)-induced uncontrolled diabetic rats\textsuperscript{33}. These are two rodent models of insulin deficient diabetes that are induced by a genetic autoimmune response and by the destruction of the pancreatic β-cells by a toxic injection of STZ, respectively. In both of these models, rapid reductions in glycemia independent of weight loss were seen 2 days after the surgery compared to sham-operated rats\textsuperscript{33}. This glucose lowering effect induced by DJB was mediated by jejunal nutrient sensing since during a fasting-refeeding experiment, blocking nutrient sensing in the jejunum reversed the beneficial glucose-lowering effects of DJB\textsuperscript{33}.

As DJB surgery was effective to improve glycemia in two insulin-deficient rodent models, this suggests that insulin action does not mediate the beneficial effects of this bariatric surgery. Moreover, in obese type 2 diabetic rodents, DJB surgery also led to reductions in glucose production independent of insulin action\textsuperscript{45}. These studies highlight an insulin-independent mechanism of DJB surgery to rapidly improve glycemia. Although insulin action has traditionally been thought to be a primary mediator of glycemia by acting on the liver, adipose tissue, muscle, and most recently, the brain\textsuperscript{46}, it may not be the sole mediator of glucose homeostasis, as the hormone leptin is able to lower plasma glucose levels in uncontrolled diabetic rodents, independent of insulin action\textsuperscript{47-49}. These findings highlight the possibility that
while insulin may regulate glucose in some settings, other intestinal mechanisms may play a role in the antidiabetic effect of DJB surgery.

As discussed above, the adipocyte derived hormone leptin has implications in energy homeostasis and glucose regulation. The glucoregulatory actions of leptin have specifically been studied in the brain. Hypothalamic leptin is demonstrated to bind to the long-form leptin receptor (Ob-Rb) to activate downstream signaling molecules, including PI3K via phosphorylation of the insulin receptor substrate\(^{50}\), and STAT3 by activation of Jak-2\(^{51,52}\), to improve insulin sensitivity\(^{50}\) and lower glucose production\(^{51}\). Importantly, subcutaneous leptin improves glycemia independent of insulin action\(^{47-49}\), an effect most likely mediated by its central effects, as i.c.v. leptin lowers glucose production and plasma glucose levels in STZ-induced insulin deficient uncontrolled diabetic rodents\(^{53,54}\). These studies emphasize the ability of leptin to affect glycemic control independent of insulin.

Interestingly but often disregarded, gastric leptin is released upon nutrient ingestion, but its actions in the gastrointestinal tract beyond food intake regulation remain to be elucidated\(^{55}\). As opposed to adipocyte-derived leptin, gastric leptin is released from the stomach’s fundic region, primarily from pepsinogen secreting gastric chief cells in an exocrine fashion\(^{56,57}\). It is hypothesized that to withstand the proteolytic environment of the stomach and to avoid degradation, leptin binds to the soluble leptin receptor (SLR), with which it is secreted\(^{58}\). This enables leptin to reach the intestine in its intact form. While the contribution of the SLR in enabling leptin to avoid degradation is debated, leptin has been detected in duodenal juices, highlighting its ability to reach the duodenum intact\(^{55}\). Leptin in the intestine activates the Ob-Rb, which is expressed both on enterocytes as well as on vagal afferents innervating the small intestine\(^{59-63}\).
Ob-Rb activation in the intestine by leptin has various functions, including maintaining the intestinal environment in both mice and humans, and protecting against infection by activating downstream STAT3. Furthermore, intestinal leptin modulates short-term satiation, mediates both intestinal lipid and carbohydrate absorption and leads to increased neuronal activity in the NTS. Interestingly, intestinal leptin has been demonstrated to suppress the recruitment of sodium glucose transporters to the intestinal apical membrane in order to inhibit glucose absorption, demonstrating its ability to mediate glucose control. Indeed, leptin’s well-established glucoregulatory actions in the hypothalamus and its insulin independent actions to regulate glycemia suggest a potential role for intestinal leptin to regulate glucose homeostasis and to mediate the antidiabetic effects of DJB surgery.

Intestinal leptin action was tested in normal rodents during a pancreatic (basal insulin) euglycemic clamp. While duodenal leptin action had no effects on glucose kinetics, jejunal leptin suppressed glucose production. The intestinal Ob-Rb was required for this effect, as blocking its activity with a chemical inhibitor abolished the ability of leptin to suppress glucose production, and jejunal leptin had no effects in rodents lacking the leptin receptor (Koletsky fa/k fa/k rats and db/db mice). Jejunal leptin was found to activate jejunal PI3K, but not STAT3, as well as to trigger a neuronal network to suppress glucose production. Thus, it was found that jejunal leptin triggers an Ob-Rb → PI3K neuronal mediated signaling pathway to affect glucose homeostasis.

Further, jejunal leptin was tested in two diseased models, as recent studies highlight leptin as an antidiabetic therapy. Leptin action in the jejunum remained intact in rats fed a 3 day high fat diet, which is known to both cause hepatic insulin resistance leading to an inability to lower
hepatic glucose production, as well as to disrupt lipid sensing in the duodenum, as described above. Additionally, leptin’s antidiabetic actions were tested in uncontrolled STZ-induced diabetic rats. In this insulin-deficient model, jejunal leptin (similar to jejunal nutrient sensing) was successful in suppressing glucose production and plasma glucose levels. These findings highlight a potential role for gastric leptin in mediating the antidiabetic actions of DJB surgery.

Thus, DJB surgery was performed in uncontrolled STZ-induced diabetic rats and a fasting-refeeding experiment was performed to stimulate the physiological release of leptin from the stomach. While the glucose-lowering effect of DJB remained intact in rats receiving a jejunal saline infusion during the fasting refeeding protocol, blocking jejunal leptin signaling during this experimental protocol disrupted the glucose control observed. However, blocking leptin signaling did not completely abolish the ability of DJB to restore glucose homeostasis, illustrating that other factors influencing glucose control could be at play. It is possible that jejunal nutrient-sensing mechanisms and jejunal leptin signaling converge to lead to the antidiabetic effects of DJB surgery.

The existence of a gut-brain-liver axis involving both nutrients and leptin to regulate glucose homeostasis after bariatric surgery suggests the importance of the gastrointestinal tract in glucose control. Indeed, the activation of intestinal-sensing mechanisms renders the gut a suitable physiological and surgical target for the treatment of diabetes. It remains to be determined whether the gut is also a pharmacological target. Targeting the small intestine with drug therapies could potentially mimic the anti-diabetic effects of bariatric surgery to lower blood glucose levels in diseased states.
1.3 Resveratrol

As discussed above, nutrient and hormonal sensing in the small intestine have been studied extensively in both the regulation of food intake and glucose homeostasis, highlighting the physiological, pathological, and therapeutic relevance of intestinal sensing mechanisms to regulate glucose kinetics in normal and diseased states. However, the pharmacological nature of intestinal-sensing mechanisms remains unclear.

Traditionally, pharmacological targets used to treat diabetes and metabolic disorders have been described as being “islet-centered” – focusing primarily on insulin secretagogues aimed to act on the liver, muscle, and adipose tissue as primary targets\textsuperscript{46}. However, mounting evidence highlights the importance of the gastrointestinal tract in mediating glucose control to potentially contribute to the initial dysregulation of metabolic homeostasis in diabetes\textsuperscript{10}. As discussed previously, one of the most successful treatments of type 2 diabetes is bariatric surgery, where the gut is anatomically remodeled leading to a rapid remission of diabetes independent of changes in weight loss. While the exact mechanisms leading to the normalization of hyperglycemia after this metabolic surgery remain to be elucidated, they are likely gut-initiated events that trigger and restore insulin-dependent and neuronal-mediated insulin-independent mechanisms. These findings not only highlight the importance of the gastrointestinal tract in glucose control, but also underscore the potential of targeting these mechanisms directly in the gut via pharmacological agents without performing invasive bariatric surgery. Thus, it is of importance to dissect the pharmacological role of intestinal sensing in diabetes and obesity.

Laboratories consistently describe the insulin-sensitizing actions of the anti-diabetic agent resveratrol in diabetic and obese rodents\textsuperscript{71-73}. However, the glucoregulatory mechanisms of resveratrol remain elusive. While resveratrol’s actions on peripheral tissues is limited by its low
bioavailability (reviewed in 74), the intestine may be a potential site of action as there is a high enrichment of resveratrol in the intestinal mucosa after oral administration75,76. Thus, in this thesis, we have chosen to test the ability of the gastrointestinal tract to mediate the antidiabetic actions of resveratrol.

Resveratrol (trans-3,5,4’-trihydroxystilbene) is a polyphenolic compound belonging to the stilbene class77. As a phytoalexin, resveratrol is synthesized by plants under conditions of stress, such as due to chemical stressors, ultraviolet radiation, and during microbial infections78. Thus, it is found in a variety of foods and food products including peanuts and grapes, and consequently, red wine. Resveratrol action has been described in various pharmacological settings both in vitro and in vivo, having many health properties including providing anticancer79, cardioprotective80, and antioxidant81 benefits, although there are few clinical trials assessing its efficacy in humans. Importantly, resveratrol has also been demonstrated to exert antidiabetic effects.

Resveratrol has been described as a caloric restriction mimetic, where caloric restriction is defined as reduced caloric intake by approximately 30-40% without malnutrition82. The beneficial actions of restriction of food intake are well documented, which include the mitigation of many late-onset diseases as well as the extension of lifespan82,83. Moreover, caloric restriction also exerts favourable metabolic actions including decreases in plasma glucose levels and improvements in insulin action82,83.

Similar to caloric restriction, resveratrol was shown to improve the health and lifespan of yeast and vertebrates in 2003 and 2004, respectively84,85. In 2006, two studies implicated resveratrol in improving metabolism. The Sinclair group demonstrated that chronic oral administration of
resveratrol in mice fed a 60% HFD for 6 months not only improved the lifespan of these mice, but shifted their physiology to that of standard diet-fed mice\textsuperscript{71}. This included the improvements of various plasma biomarkers involved the prediction of the onset of type 2 diabetes, including decreased fed and fasted plasma glucose, insulin, and IGF-1 levels\textsuperscript{71} as well as significantly improving their oral glucose tolerance. Moreover, Lagouge et al. showed similar findings in high fat-fed mice treated with resveratrol administered orally in the diet for 15 weeks. When these mice were subjected to the hyperinsulinememic euglycemic clamp, which is the gold standard for measuring insulin sensitivity, mice with resveratrol treatment had a significantly increased glucose infusion rate needed to maintain euglycemia when compared to high fat-fed mice without resveratrol supplementation, indicating improved insulin sensitivity\textsuperscript{86}.

Since these studies, chronic oral resveratrol administration has been shown to improve insulin sensitivity in various rodent models of insulin resistance. Sun et al. demonstrated that resveratrol treatment in high fat fed mice significantly improved insulin response during an insulin tolerance test, improved glucose tolerance, and attenuated plasma insulin levels compared to high fat fed mice without treatment\textsuperscript{87}. They confirmed these results \textit{in vitro} as resveratrol treatment of C2C12 myotubes and rat adipocytes enhanced insulin stimulated glucose uptake under normal conditions and insulin resistant conditions, increasing the phosphorylation and subsequent activation of various insulin signaling molecules in both states\textsuperscript{87}. These findings are further supported by three studies in which rats and mice were fed a HFD to induce insulin resistance and were supplemented with resveratrol, leading to improved insulin sensitivity as measured by the homeostatic model assessment of insulin resistance (HOMA-IR) and improved glucose tolerance compared to controls\textsuperscript{88-90}. HOMA-IR is an index of insulin resistance, calculated using fasting plasma insulin and glucose levels. While the HOMA index is a convenient method for measuring insulin resistance and its results correlate well with the
hyperinsulinemic euglycemic clamp, the clamp technique remains the gold standard in measuring insulin sensitivity. Shang et al. performed the hyperinsulinemic euglycemic clamp in rats fed a HFD that were supplemented orally with resveratrol for 16 weeks. Using this method, they found the need for a significantly increased glucose infusion rate needed to maintain euglycemia during the clamp in the resveratrol treated rats compared with saline treated control rats, indicating improved insulin sensitivity and further confirming the above groups’ results.

In addition to being tested in high-fat fed models of insulin resistance, two studies administered resveratrol in rats supplemented with fructose to mimic Western eating habits. In the first study, high-fructose fed rats were given oral resveratrol daily for 8 weeks, which led to improvements in glucose tolerance as well as plasma insulin levels. While improvements in glucose homeostasis were seen, decreases in food intake and body weight were also recorded; meaning the improvements in insulin action could be due to weight loss instead of direct resveratrol action. In the second study, rats were fed a high cholesterol diet and supplemented with fructose in drinking water for 15 weeks to induce insulin resistance, including hyperinsulinemia and impaired glucose tolerance with normal body weight gain. The importance of the rats not developing obesity lies in the ability to discern the effects of resveratrol on insulin action itself, and not because of resveratrol’s action to lower body weight gain which could itself affect insulin sensitivity. Resveratrol supplemented by oral gavage daily shifted the metabolic parameters of diet-induced insulin resistant rats to that of rats fed regular chow. When subjected to the hyperinsulinemic euglycemic clamp, resveratrol treated rats has significantly improved insulin sensitivity compared to controls, which was due to improved insulin-stimulated glucose uptake into muscle but not adipose tissue.
Lastly, resveratrol has also been demonstrated to improve insulin action in models of type 2 diabetes. Rats treated with nicotinamide and STZ to induce experimental type 2 diabetes and fed regular chow were administered oral resveratrol daily for 30 days. Resveratrol treated rats displayed normalized glucose tolerance and significantly decreased plasma insulin levels. Further, two studies assessed the effects of resveratrol in two different genetic models of diabetes induced by lack of leptin action. The first are leptin-deficient ob/ob mice that display profound glucose intolerance and insulin resistance. These were treated with resveratrol orally for 4 weeks, resulting in significant anti-hyperglycemic activity, and leading to improved glucose tolerance and decreased insulin levels. The second model used was the ZDF rat, which has a loss of function of the leptin receptor and is therefore insulin resistant and hyperglycemic. ZDF rats were administered resveratrol daily for 8 weeks. Resveratrol treatment led to normoglycemia and decreased insulin levels in these rats, as well as improved insulin sensitivity as measured by HOMA-IR.

Taken together, the above studies demonstrate the significant insulin sensitizing actions of chronic resveratrol treatment in various rodent models of insulin resistance. While treatment durations vary widely between studies, as do doses used, these studies highlight the antidiabetic effects of resveratrol administered orally.

The success of oral resveratrol administration to improve glucose homeostasis and insulin sensitivity in rodent models of metabolic diseases has lead to various clinical trials being conducted to test the efficacy of resveratrol supplementation in humans. In 2011, the first clinical study administered resveratrol to obese (non-diabetic) men. In addition to decreasing hepatic lipid content and circulating glucose levels, resveratrol supplementation also improved insulin sensitivity as measured by HOMA-IR. While the early success of this trial was
promising, another trial studying resveratrol’s effect in obese men with mild insulin resistance showed no improvements in metabolic parameters, including insulin sensitivity, as measured both by a hyperinsulinemic euglycemic clamp and HOMA-IR\textsuperscript{98}. Importantly, these clinical trials did not assess the glucose-lowering effect of resveratrol in individuals with type 2 diabetes or more severe glucose intolerance. In two trials where resveratrol was administered to overweight and normal weight type 2 diabetic patients, resveratrol led to decreased plasma glucose levels and increased whole body insulin sensitivity, and lowered glycosylated hemoglobin levels\textsuperscript{99,100}. Moreover, four weeks of resveratrol supplementation in obese humans with impaired glucose tolerance decreased plasma glucose levels and improved insulin sensitivity as assessed by the MATSUDA index of insulin resistance\textsuperscript{101}. These studies together demonstrate that a minimal level of metabolic abnormality may have to be present in order for resveratrol to have beneficial effects. This is further demonstrated as 12 weeks of resveratrol supplementation had no effects on insulin sensitivity as assessed by the hyperinsulinemic euglycemic clamp in non-obese women with normal glucose tolerance\textsuperscript{102}.

The different trial lengths, resveratrol doses, and, importantly, severity of the metabolic diseases studied may explain the discrepancies between these clinical trials. Indeed, studies conducted in normal rodents show that resveratrol has no beneficial effects on metabolism\textsuperscript{103-107} supporting the above clinical trials. Nonetheless, resveratrol’s beneficial effects on glucose metabolism and insulin sensitivity have been demonstrated in diseased rodent models and humans with various metabolic disorders, but the mechanism of action of resveratrol responsible for its metabolic actions remains unclear.

While resveratrol is demonstrated to be efficacious \textit{in vivo} after oral administration in both rodents and humans, resveratrol has been criticized as an effective antidiabetic pharmacological
agent due to its low bioavailability and fast metabolism. Indeed, although absorption of resveratrol in humans is approximately 75%, extensive metabolism in both the small intestinal mucosa and the liver results in a low bioavailability of less than 1%\textsuperscript{108-112}. Consumption of more than 1g/day of resveratrol in humans has been demonstrated to cause mild gastrointestinal side effects, preventing the use of higher doses of resveratrol to compensate for its low ability to reach known target tissues\textsuperscript{112,113}.

After oral administration, the majority of resveratrol enters enterocytes via transepithelial diffusion without the involvement of carrier proteins\textsuperscript{114}. The uptake of resveratrol via the intestinal apical membrane was demonstrated to be high and rapid in Caco-2 cells\textsuperscript{115}. Once inside enterocytes, resveratrol is metabolized extensively to glucuronide and sulfate conjugates by UDP-glucuronosyltransferases and sulfotransferases. The major metabolites formed are resveratrol-3-O-sulfate, resveratrol-4’-O-glucuronide and resveratrol-3-O-glucuronide\textsuperscript{109}. After intestinal metabolism, metabolites have two fates: 1) they are released into the portal circulation and 2) they undergo efflux from enterocytes back into the intestinal lumen\textsuperscript{116}. It is thought that metabolites that are released into the portal circulation leave the enterocyte via MRP3, a subset of ABC transporters responsible for oral drug absorption, and undergo subsequent metabolism in the liver. These are then released into the systemic circulation or can undergo enterohepatic transport in the bile to be recycled back to the intestine\textsuperscript{110}. Conversely, MRP2 and BCRP transporters on the apical membrane can release metabolites present in enterocytes directly back into the intestinal lumen\textsuperscript{116}, a process known as phase III metabolism, described as efflux of already metabolized molecules\textsuperscript{116}. These two processes together contribute to the low bioavailability of oral resveratrol.
However, despite the low concentration of unmetabolized resveratrol reaching the systemic circulation, resveratrol has been shown to reach target tissues. In a study where healthy mice were administered $^{14}$C-resveratrol, plasma levels remained low throughout the experiment, but three hours after administration, radioactivity was found in various organs, including the liver, lungs and the brain$^{117}$. This radioactivity was found to be low levels of intact resveratrol as well as a relatively high concentration its sulfate and glucuronide conjugates$^{117}$. These findings are supported by a study that treated isolated rat jejunum and ileum small intestinal segments with resveratrol for 90 minutes. After perfusion, only small amounts of intact resveratrol were found on the basolateral segment of enterocytes, while high concentrations of metabolites were detected$^{118}$.

Thus, the ability of resveratrol to exert beneficial actions on insulin sensitivity may be due to resveratrol metabolites retaining biological activity$^{119}$ or as Patel et al. have recently demonstrated, resveratrol can be generated from its sulfate conjugates in tissues, and formation via this route could result in sustained exposure to the intact parent compound to exert beneficial actions$^{120}$.

Nonetheless, the bioavailability of resveratrol is low and the ability of metabolites to exert beneficial actions remains unclear. After intragastric administration in rats, resveratrol is almost undetectable in tissues other than the small intestine after 60 minutes$^{75}$ and mice that receive intragastric resveratrol had an approximate 40-fold increase in resveratrol concentration in the intestinal mucosa compared to serum levels$^{76}$. In fact, resveratrol stays present for as long as 24 hours in the intestinal mucosa of mice after oral administration.
Thus, while only a small amount of resveratrol reaches the circulation, there is a high enrichment of resveratrol in the intestine following oral administration. These findings, along with the fact that the intestine mediates glucose control, as described above, highlight the possibility of the intestine being a target for resveratrol action.
1.4 Mechanism of action of resveratrol - SIRT1 and AMPK

The mechanism of resveratrol action as an effective antidiabetic agent is unclear. Indeed, although resveratrol’s bioavailability is low, it remains efficacious in improving glucose homeostasis and insulin action. As stated above, resveratrol has been described as a caloric restriction mimetic. Various molecular signaling pathways, including activation of SIR2 proteins and AMPK, mediate the beneficial effects of caloric restriction. Both sirtuins and AMPK have also been implicated in resveratrol’s actions to improve metabolic parameters.

SIR2 proteins have been implicated in caloric restriction’s effects on increasing lifespan in *Drosophila* and *C. elegans*\(^{84,85}\). The sirtuin proteins are the mammalian homologs of SIR2 and are a class of NAD\(^+\) dependent deacetylases involved in regulating proteins involved in metabolism. While there are seven sirtuin proteins, SIRT1 has been implicated in both caloric restriction and resveratrol’s effects. SIRT1 is a nuclear protein expressed widely, which has been linked to the regulation of lipid and glucose homeostasis and insulin action\(^{121}\). The metabolic actions of SIRT1 have been studied extensively. Defects in SIRT1 signaling have been demonstrated to result in metabolic dysfunction, while activation of SIRT1 via genetic or pharmacological means protects against insulin resistance, type 2 diabetes, and diet induced metabolic disorders.

SIRT1 has been demonstrated to be necessary for survival, as inbred mice with whole body deletion of SIRT1 do not survive\(^{122}\). Moreover, outbred global SIRT1 knockout mice are viable, but display developmental abnormalities, including metabolic problems\(^{123}\). Partial global SIRT1 knockdown in mice leads to hepatic dysfunctions, such as steatosis\(^{124}\) and inefficient liver mitochondria\(^{123}\). In contrast to these findings, Bordone et al. demonstrated that SIRT1 knock out mice displayed improve glucose tolerance and metabolic homeostasis compared to wildtypes\(^{125}\),
but this may be due to the fact that these mice are significantly smaller and have decreased fat mass compared to wildtype mice\textsuperscript{123}. Adult inducible whole body SIRT1 deletion in mice resulted in no significant differences between knockout and wildtype mice under normal conditions, although when challenged with a high fat diet, weight gain and insulin resistance was more prominent in knockout mice\textsuperscript{126}.

Conversely, whole body SIRT1 overexpression has been demonstrated to provide metabolic benefits. The Guarente group generated SIRT1 transgenic mice that displayed overexpression of SIRT1 in adipose tissue and the brain, but did not show increased SIRT1 levels in liver or muscle. These mice displayed an improved metabolic phenotype, including reduced fasting plasma glucose and insulin levels, as well as improved glucose tolerance\textsuperscript{127}. Further confirming these results, while mild SIRT1 overexpression in all tissues of mice did not improve glucose tolerance per se, it prevented the adverse effects of obesity on glucose metabolism and improved hepatic insulin sensitivity as measured by a hyperinsulinemic euglycemic clamp\textsuperscript{128}. Indeed, in another study, moderate overexpression of SIRT1 resulted in improved glucose tolerance and protection from hepatic steatosis when mice were chronically exposed to a high fat diet\textsuperscript{129}.

To further dissect the role of SIRT1 on metabolism, several groups have knocked out or overexpressed SIRT1 in specific metabolic tissues. Rodgers and Puigserver found that mice with a liver-specific SIRT1 knockdown displayed improved insulin sensitivity, although this improvement was only observed under fasting conditions\textsuperscript{130}, while other groups demonstrated an increased susceptibility to hepatic steatosis and chronic hyperglycemia with hepatic SIRT1 knockdown\textsuperscript{131,132}. Moreover, hepatic SIRT1 overexpression resulted in varied outcomes, including increased hepatic glucose production by one group\textsuperscript{130} and attenuation of hepatic glucose production and insulin resistance in ob/ob diabetic mice by another group\textsuperscript{131}.
Unlike the varied effects of hepatic SIRT1 deletion and gain of function, both adipose tissue and central deletion of SIRT1 result in higher susceptibility to metabolic dysfunction\textsuperscript{133-136}, while SIRT1 overexpression in adipose tissue results in positive outcomes on metabolism\textsuperscript{137}. SIRT1 knockdown in a macrophage cell line resulted in increased inflammatory gene expression and activated inflammatory pathways, and these results were mirrored in \textit{in vivo} studies where SIRT1 activation improved glucose tolerance and insulin sensitivity in parallel to reducing tissue inflammatory markers and macrophage pro-inflammatory state in adipose tissue\textsuperscript{138}. Altogether, these studies indicate that SIRT1 deletion usually results in metabolic dysfunction while overexpression improves glucose homeostasis.

As such, SIRT1 has been a target for pharmacological agents in order to improve glucose control. Resveratrol has been demonstrated to activate SIRT1 to mediate its glucoregulatory actions. In 2003, the Sinclair group first discovered that resveratrol could activate SIRT1 directly in budding yeast\textsuperscript{84}, and in 2004, reproduced these results in \textit{C. elegans} and \textit{Drosophila}\textsuperscript{85}. These studies were followed by a 2006 study where mice were administered with resveratrol and concurrently placed on a high calorie diet for 6 months. Resveratrol treatment led to improved glucose homeostasis (as discussed in section 2.3), which was accompanied by increased SIRT1 activity in the liver of resveratrol-treated mice\textsuperscript{71}. Further, Lagouge et al. also found increased SIRT1 activity in muscle and brown adipose tissue of high fat fed mice treated chronically with resveratrol compared to high fat fed control, in parallel with overall improved whole body insulin sensitivity\textsuperscript{86}. Similar findings have been demonstrated in humans treated with resveratrol as well. A 30-day supplementation of resveratrol in obese insulin resistant men that resulted in an overall improvement of insulin sensitivity as assessed by HOMA-IR resulted in increased SIRT1 expression and SIRT1 activity in muscle\textsuperscript{97}. 

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Resveratrol has also been implicated in activating central SIRT1 to regulate glucose homeostasis. A chronic intracerebroventricular (i.c.v.) infusion of resveratrol in diet-induced obese and diabetic mice normalized hyperglycemia and improved hyperinsulinemia by activating brain SIRT1 and suppressing NF-kB, without affecting hepatic SIRT1 levels. These results suggest a potential role for central SIRT1 in regulating resveratrol’s actions by suppressing central diet-induced inflammation. Additionally, when infused into the hypothalamus specifically, acute resveratrol resulted in suppressed glucose production as assessed by the pancreatic (basal insulin) euglycemic clamp technique. This effect also required SIRT1 activation as co-infusion of resveratrol with the SIRT1 specific inhibitor EX527 resulted in lack of suppression of glucose production. Moreover, resveratrol and subsequent SIRT1 activation required a neuronal brain-liver network to exert effects on glycemic control in these two studies.

In addition to activating SIRT1, resveratrol has also been demonstrated to activate the serine/threonine kinase AMPK. AMPK is a conserved energy sensor responsible for maintaining energy homeostasis by phosphorylating downstream effectors in order to regulate metabolic pathways. It is activated in settings of low energy, specifically by increased levels of AMP:ATP and ADP:ATP. AMPK agonists are shown to protect against metabolic syndrome, where metformin, a potent AMPK activator, is the most widely prescribed antidiabetic agents used to treat type 2 diabetes in humans. Indeed, metformin treatment lowers plasma glucose levels in both rodents and humans by inhibiting hepatic glucose production. The liver is thought to be the main site of action of metformin treatment, thus highlighting hepatic AMPK activation in mediating glucose control. Overexpression of AMPK in the liver has previously been demonstrated to control hyperglycemia in a murine model of diabetes. Moreover,
decreases in LKB1, the kinase responsible for phosphorylating and activating AMPK, results in inactive hepatic AMPK leading to increased fasting blood glucose\(^{147}\). Whole-body AMPK deficiency leads to hyperglycemia, glucose intolerance and insulin resistance in mice\(^{149}\) and muscle AMPK ablation exacerbates HFD-induced insulin resistance in mice\(^{150}\). Altogether, these studies demonstrate the importance of AMPK in glucose regulation.

Resveratrol also activates AMPK to have insulin-sensitizing actions. Shang et al. cultured HepG2 hepatocytes and treated them with 50 uM of resveratrol, leading to a 3.7 fold increase in the phosphorylation, and thus activation, of AMPK. Furthermore, after acute administration of resveratrol in rats fed a HFD, AMPK phosphorylation in the liver increased by 164\% \(^{91}\). Chronic resveratrol treatment in rats fed a HFD also activated hepatic AMPK\(^{91}\). Baur et al. presented similar results in high calorie fed mice, where levels of phosphorylated AMPK increased significantly after chronic resveratrol treatment, along with increased phosphorylation and inactivation of downstream indicators of AMPK activity, ACC and fatty acid synthase, two enzyme involved in fatty acid synthesis\(^{71}\). Increased phosphorylation and activation of both AMPK and ACC were also seen in the liver and visceral adipose tissue of ZDF rats after resveratrol treatment\(^{96}\). Moreover, resveratrol activated AMPK in muscle cells to stimulate glucose uptake and insulin signaling\(^{151}\).

In order to determine if AMPK is necessary for resveratrol’s glucose lowering and insulin sensitizing effects, Um et al. treated wildtype and AMPK\(\alpha_1\) and AMPK\(\alpha_2\) knockout mice on a HFD with resveratrol. These knockout mice were deficient in AMPK’s catalytic subunit, which has two isoforms (\(\alpha_1\) and \(\alpha_2\)), expressed in different tissues\(^{90}\). After treatment with resveratrol, impaired glucose tolerance and insulin resistance caused by HFD improved significantly in wildtype mice, whereas only moderate improvements were seen in both AMPK\(\alpha_1\) and AMPK\(\alpha_2\).
knockout mice. These results suggest that AMPK is at least partly responsible for resveratrol’s actions. In contrast, Knight et al. assessed AMPK activation after central administration of resveratrol in normal rats. Activation of AMPK was found with AICAR infusion, an AMPK activator, but not with resveratrol infusion.

While rodent studies have shown that resveratrol is capable of activating AMPK, which may be necessary for it to have beneficial health effects on glucose and insulin homeostasis, Timmers et al. demonstrated that resveratrol treatment in obese men for 30 days resulted in an increased level of phosphorylated, and thus activated, muscle AMPK by 1.22 fold compared to controls. Although this is only a small increase in activation, there is evidence that resveratrol does exert its effect through AMPK in both rodents and humans.

Convincing evidence highlights the necessity of both SIRT1 and AMPK in mediating resveratrol’s action, but the exact signaling pathway activated by resveratrol remains debated. AMPK activation has been demonstrated to be upstream of SIRT1. Upregulated AMPK activity leads to increased NAD+ levels, thereby activating SIRT1, and more recently, resveratrol was shown to directly activate AMPK via phosphodiesterases, also leading to increased SIRT1 activity due to higher NAD+ levels. In contrast to these studies, SIRT1 has also been down to be upstream of AMPK in resveratrol’s action as SIRT1 activation by resveratrol has been shown to deacetylate LKB1, leading to subsequent AMPK activation. Moreover, mice with a whole body inducible SIRT1 knockout did not display increased mitochondrial biogenesis, AMPK activation, or decreased NAD+ levels in skeletal muscle compared to controls, further supporting the idea that SIRT1 lies upstream of AMPK. It is important to note that in this same study, a higher dose of resveratrol activated AMPK independently of SIRT1. Nonetheless,
both SIRT1 and AMPK are expressed in the intestine\textsuperscript{156-159}, and could therefore mediate the insulin sensitizing effects of resveratrol.

In addition to triggering SIRT1 and/or AMPK, resveratrol has the potential of mediating glucose homeostasis through the release of intestinal gut peptides. Indeed, local hormonal signaling at the level of the gut has been implicated in the ability of intestinal sensing to mediate glucose control. CCK is released from enteroendocrine I cells, GIP is released from K cells, and GLP-1 and GLP-2 are released from L cells along the length of the gastrointestinal tract, which all act on their receptors centrally, or locally in the intestine on vagal afferents or in proximity to enteroendocrine cells to exert effects on energy and/or glucose homeostasis.

CCK is released mainly in response to lipid ingestion\textsuperscript{160} from I cells of the proximal small intestine to regulate both food intake and glycemia. The release of CCK requires intestinal mucosa PKC\textdelta activation\textsuperscript{161,162}, where CCK can subsequently bind and activate its receptor located on vagal afferents\textsuperscript{163}. This leads to activation of PKA, which in turn is sufficient to trigger a neuronal relay to the brain and liver via the hepatic vagus nerve to lower hepatic glucose production\textsuperscript{164}. Interestingly, after only 3 days of high fat feeding, duodenal CCK resistance occurs at the level of the CCK receptor, but direct PKA activation in this state remains able to lower glucose production\textsuperscript{164,165}.

While lipids stimulate CCK release, GLP-1 is secreted in response to both glucose and fatty acids. The potent glucoregulatory effect of GLP-1 was first demonstrated almost three decades ago, and its incretin effect has since been well-established\textsuperscript{166}. GLP-1 acts on its receptor located on pancreatic \( \beta \) cells, which results in insulin secretion via both PKA dependent and independent pathways. However, due to the rapid degradation of GLP-1 by DPP-IV upon its
release, it is thought that its glucoregulatory actions may be mediated by a neuronal gut-brain-periphery network. Indeed, GLP-1 activates the vagal afferents terminating at the level of the NTS to control glucose flux. The vagus nerve innervating the intestine is of particular importance for this effect as surgically removing the hepatic branch of the vagus did not abolish the glucoregulatory effects of endogenous GLP-1. Therapeutic treatments aimed at increasing GLP-1 action in the intestine to trigger a gut-brain-periphery axis have been developed for the treatment of type 2 diabetes. In fact, sitagliptin administration, which diminished DPP-IV activity to increase intestinal GLP-1 content led to an enhancement of glycemic control and plasma insulin levels via activation of the GLP-1 receptor on the vagus nerve.

In addition to activating SIRT1 and AMPK, it is plausible that resveratrol could improve insulin sensitivity by causing the release of intestinal gut peptide to trigger a neuronal axis to regulate glycemia. A recent paper has demonstrated that an oral dose of resveratrol causes the release of GLP-1 from intestinal enteroendocrine L cells in mice, leading to increased intestinal content of active GLP-1. These results were accompanied by improvements in glucose homeostasis and insulin action, which were negated in GLP-1 receptor knock out mice. While the involvement of SIRT1 or AMPK was not assessed in this study, AMPK activation has been linked to GLP-1 release and signaling. Two AMPK activators, metformin and AICAR, both stimulate GLP-1 release from murine and rat intestinal L cells, as well as in normal mice in vivo. Thus intestinal release of CCK or GLP-1, and subsequent binding to their receptor located on vagal afferents could mediate the antidiabetic effect of intestinal resveratrol. Moreover, GLP-1 has been demonstrated to lead to the activation of PKA, and direct PKA activation in the duodenum bypasses duodenal sensing resistance caused by high fat feeding to lower glucose production in a state of HFD-induced insulin resistance. Thus, it is possible that resveratrol could activate intestinal SIRT1 and/or AMPK to improve insulin action and glucose
homeostasis through the release of gut hormones and subsequent activation of PKA on the vagal nerves innervating the intestine.

Altogether, the above studies highlight the importance of intestinal mediated signals in controlling glycemia and insulin action. Although the physiological, therapeutic, and surgical relevance of intestinal sensing mechanisms have been uncovered, the potential of the intestine in mediating pharmacological actions in obesity and diabetes remains unknown. With the study outlined in this thesis, we aim, for the first time to our knowledge, to uncover the intestine as a site of resveratrol action to improve insulin action in insulin resistant states.
Chapter 2
General Hypothesis and Aims

Intestinal sensing plays an important role in mediating energy and glucose homeostasis. The small intestine senses both nutrients and gut peptides to regulate glucose production in healthy and diseased states, and these mechanisms have been demonstrated to be physiological, pathological, and surgical in nature. However, the pharmacological nature of intestinal-sensing mechanisms in the context of obesity and diabetes remains unclear.

The overall aim of this thesis is to evaluate the pharmacological relevance of intestinal sensing mechanisms to regulate glucose homeostasis and insulin action in multiple disease models, including insulin resistance, obesity, and type 2 diabetes. More specifically, this thesis focuses on the role of duodenal resveratrol in remotely improving hepatic insulin sensitivity and the downstream mechanisms required to do so.

It has been previously shown that oral resveratrol improves insulin sensitivity by activating SIRT1 and/or AMPK, both of which are expressed in the intestine. Due to the high intestinal enrichment of resveratrol after oral administration, we hypothesize that duodenal resveratrol triggers duodenal SIRT1 and/or AMPK to improve hepatic insulin sensitivity.

Moreover, an independent brain-liver neuronal network has been implicated in hypothalamic resveratrol’s ability to lower glucose production in diabetic and obese rodents, and hypothalamic insulin resistance disrupts hepatic glucose production in diabetes and obesity. Thus, we hypothesize that duodenal resveratrol triggers a neuronal network and...
subsequently remotely improves hypothalamic insulin sensitivity to lower hepatic glucose production.

**General Hypothesis:**
Duodenal resveratrol triggers duodenal SIRT1 and/or AMPK and a subsequent neuronal network to remotely improve hypothalamic insulin sensitivity in insulin resistance.

**Aims:**

**Aim 1:** To determine whether preabsorptive duodenal resveratrol can improve insulin sensitivity.

**Aim 2:** To examine the downstream molecular mechanisms required for duodenal resveratrol to improve insulin sensitivity.

**Aim 3:** To investigate whether a neuronal network is required for duodenal resveratrol to improve insulin sensitivity.

**Aim 4:** To determine whether duodenal resveratrol can remotely improve mediobasal hypothalamic (MBH) insulin sensitivity.

**Aim 5:** To assess whether duodenal resveratrol can lower glucose production and plasma glucose levels in obese insulin resistant rodents and in an early onset type 2 diabetic rodent model.
Figure 2.1. Schematic representation of the working hypothesis.
Preabsorptive resveratrol activates duodenal SIRT1 and/or AMPK and triggers a neuronal network to remotely improve hypothalamic insulin sensitivity and lower glucose production and plasma glucose levels in insulin resistance and type 2 diabetes.
Chapter 3  
Materials and Methods

3.1 Animals

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University Health Network. Eight week-old male Sprague-Dawley rats (280-300g) were obtained from Charles River Laboratories (Montreal, QC, Canada) and maintained on a 12-hour light-dark cycle, with access to chow (Table 3.1) and water *ad libitum*.

3.2 Animal Models

3.2.1 Three day HFD-induced insulin resistant model

Rats were placed on a lard-oil enriched diet (TestDiet #571R, Purina Mills, IN, USA) (Table 3.1) one day after duodenal and vascular cannulations for three days before undergoing either the pancreatic (basal insulin) euglycemic clamp experiment or the hyperinsulinemic euglycemic clamp experiment. Rats were restricted to 103 kcal the night prior to the experiment. Rats that overeat this diet for three days develop hypothalamic\(^{177}\) and hepatic\(^{70}\) insulin resistance, but have normal peripheral insulin sensitivity\(^{70}\). Rats that did not overeat were excluded from the study.

3.2.2 Four week HFD-induced obese/insulin resistant model

Rats were placed on lard-oil enriched HFD (described above, Table 3.1) for 28 days. Duodenal and vascular cannulations were performed on day 24 post placement on HFD and the hyperinsulinemic euglycemic clamp experiment was performed on day 28 after recovery from surgical procedures. Rats were restricted to 103 kcal the night prior to the experiment.
3.2.3 Nicotinamide-STZ HFD-induced diabetic model

Rats were treated with a single injection of nicotinamide (170 mg/kg i.p.) followed 15 minutes later by a single injection of STZ (65 mg/kg) to induce mild hyperglycemia without compensatory hyperinsulinemia (e.g. normal insulin levels)\textsuperscript{178}. Four to five days later, rats underwent duodenal and vascular cannulations and were placed on lard-oil enriched HFD (described above, Table 3.1) to induce insulin resistance. Five to six days after surgery, rats underwent the basal [3-\textsuperscript{3}H]glucose infusion protocol. Rats were restricted to 103 kcal the night prior to the experiment.

3.3 Surgical Procedures

Rats were anesthetized using a cocktail of ketamine (60-90 mg/kg) (Ketalean; Bimeda-MTC, Cambridge, Ontario) and xylazine (8-10 mg/kg) (Rompun; Bayer) administered i.p. before performing all surgical procedures. Prior to surgery, the abdominal and neck areas were shaved and cleaned with 70\% ethanol and 10\% povidone-iodine (Betadine solution, ON, Canada). Rats were monitored after surgery to ensure recovery by measuring food intake and body weight gain for four to six days.

3.3.1 Duodenal catheterization

Duodenal catheterization was performed 4 days prior to the clamp experiment. A laparotomic incision was made on the ventral midline and through the abdominal muscle wall to expose the gastrointestinal tract within the peritoneal cavity. The duodenum was identified as being distal to the pyloric sphincter and was isolated. A 21-gauge needle was used to make a small hole on the ventral aspect of the duodenum, approximately 1.5 cm distal to the pyloric sphincter, in a region with the least vascularization to minimize bleeding. A 18 cm catheter (0.04 in ID, 0.085 in OD; Sil-Tec, Technical Products, USA) with a 0.2 cm extension of small silicone tubing
(0.025 in ID, 0.037 in OD; Sil-Tec, Technical Products, USA) was inserted into this hole and anchored to the outer serosal surface of the duodenum with 3M adhesive (Vetbond) and a 0.5 cm$^2$ piece of Marlex mesh sewn to the surface with 6-0 silk suture. To ensure the cannula was placed in the duodenal lumen and no leakage occurred, the cannula was flushed with saline. The proximal portion of the catheter exited the abdominal cavity through the laparotomic incision and the abdominal wall was sutured closed (4-0 silk suture). A 2 cm incision was made in the skin at the back of the neck and the catheter was tunneled subcutaneously to exit the incision. This 2 cm incision was then closed with 4-0 silk sutures and the catheter was closed with a metal pin. The catheter was flushed daily with 0.1 mL of saline to ensure patency on the day of the experiment.

### 3.3.2 Vascular surgery

Indwelling catheters were made with polyethylene tubing (PE50, Clay Adams, Boston, MA) with cuff extensions (15mm internal diameter of 0.02 inches) of Silastic tubing (Dow Corning, Midland, MI). An approximate one-inch incision was made near the midline of the neck. After blunt dissecting through skin, fat and connective tissue, the right internal jugular vein and left carotid artery were isolated. The exposed vessels were ligated at the cranial end using 4-0 silk thread and another thread was loosely tied at the caudal end of the vessels. The two ligatures were pulled taught to restrict flow of blood. A small incision was made into the vessel walls and indwelling catheters filled with heparinized saline were inserted past the overlap of the tubing with the cuff extension. The catheters were secured by tightening the loose ligature around the vessel. Blood withdrawal and infusion were tested from the catheters. The catheters were then tunneled subcutaneously with a 16 gauge needle to the back of the neck, filled with 10% heparinized saline to maintain patency, and closed with a metal pin until the day of the experiment.
3.3.3 Duodenal lentivirus injection

A subgroup of rats receiving duodenal virus injection underwent the procedure at the same time as duodenal and intravenous (i.v.) catheterization surgeries and another subset of rats received the duodenal virus injection 2 weeks prior to the clamp experiments to determine the effects of long-term knock down of duodenal SIRT1 on insulin sensitivity. The virus injection was performed as previously described\(^{162}\). A laparotomic incision was made on the ventral midline and through the abdominal muscle wall to expose the gastrointestinal tract within the peritoneal cavity. The duodenum was identified as being distal to the pyloric sphincter. A 5 cm region of the duodenum starting 1 cm distal to the pyloric sphincter was isolated and ligated using 4-0 silk thread in order to restrict inflow or outflow of duodenal juices from this region. Using a 23 gauge needle a hole was made on the ventral aspect of the duodenum in a region with the least vascularization to minimize bleeding, and 0.5 mL of saline was flushed directly into the duodenal lumen. The ligated portion of the duodenum was then massaged and its contents were gently flushed out. Either the lentivirus expressing mismatch or SIRT1 shRNA (0.2 mL, 1.0 x 10\(^6\) IFU) (Santa Cruz, CA, USA) was injected into the duodenal lumen using a 23-gauge needle. The injection site was closed using 6-0 silk sutures and the intestines were covered with saline-soaked gauze, and the virus was left for 20 minutes to optimize infection. After 20 minutes, the silk suture was opened a duodenal catheter was inserted in the site of the virus injection as described above. The abdominal wall was closed using 4-0 silk sutures.

3.3.4 Stereotaxic surgery

A subgroup of rats underwent stereotaxic surgery 6 days prior to duodenal and vascular surgeries to implant a bilateral cannula into the MBH, in accordance with the atlas of the rat
brain. The rats were fixed onto a stereotaxic machine (David Kopf Instruments, Tunjunga, CA) with ear bars and a nose piece set to +5.00mm. A 26-gauge stainless steel double guide cannula was used for implantation into the MBH. The cannula was secured to the skull using instant adhesive (Loctite) and dental cement, and a bilateral dummy cannula (C235; Plastics One Inc.) was inserted into the guide cannula to prevent clogging. The stereotaxic coordinates for insertion of the bilateral guide cannula were 3.1 mm posterior to bregma and 9.6 mm below the skull surface at the midsagittal suture.

3.4 Hyperinsulinemic-euglycemic clamp and pancreatic (basal insulin) euglycemic clamp procedures

The night prior to the clamp, rats were restricted to 57 kcal of regular chow or 103 kcal of HFD. The clamp procedure was 200 minutes in duration, performed in unrestrained rats in vivo. At the onset of the experiment (t = 0 min), a primed i.v. infusion of $[^{3}\text{H}]$ glucose (Perkin Elmer; 40 $\mu$Ci bolus; 0.4 $\mu$Ci/ min) was started and continued throughout the experiment (t = 200 min) in order to measure glucose kinetics using the tracer-dilution methodology. A subset of rats received MBH infusions, where saline and insulin infusions were started at t = 0 min and continued throughout the clamp (t = 200 min) and the insulin receptor antagonist S961 was pre-infused for an hour prior to the start of the clamp and infused, or co-infused with insulin, for the remainder of the experiment (t = 200 min). Blood samples were collected in heparinized tubes at 10 minute intervals and subjected to centrifugation at 6000 rpm to separate the plasma, and plasma glucose was measured as described below (3.12.1 Plasma Glucose) to obtain basal glucose readings (t = 60-90 min). At t = 90 min until the end of the experiment (t = 200), the clamp was started, where a continuous i.v. infusion of somatostatin (3 $\mu$g/kg/min) was initiated
and maintained to inhibit endogenous insulin and glucagon secretion and an insulin infusion was simultaneously administered at a dose of 3.6 mU/kg/min for hyperinsulinemic clamps or replaced back to basal levels at a dose of 1.2 mU/kg/min during the pancreatic (basal-insulin) clamp. Additionally, a variable 25% glucose infusion was initiated at \( t = 90 \) minutes and adjusted every 10 minutes to maintain euglycemia (from \( t = 120 \) to \( t = 200 \) min). At \( t = 150 \) minutes, the intraduodenal treatment (0.01 ml/min) was started and continued throughout the experiment (\( t = 200 \) min). Plasma samples were obtained every 10 minutes to determine the specific activity of \([3^{-3}H]\)glucose and measure insulin levels. At the end of the experiments, rats were anesthetized and tissue samples were collected, immediately flash frozen, and stored at -80 °C until use. The Harvard Apparatus PHD 2000 infusion pumps (MA, USA) were used for all infusions during the clamp.

3.5 Basal \([3^{-3}H]\)glucose infusion protocol (non-clamp conditions)

The night prior to the infusion studies, rats were restricted to 103 kcal of HFD. These studies were performed in diabetic model described above (Section 3.2.3). The infusion experiment was 140 min in duration, and performed in unrestrained rats \textit{in vivo}. A primed continuous infusion of \([3^{-3}H]\)glucose was started at \( t = 0 \) min and continued until \( t = 140 \) min. A duodenal infusion was started at \( t = 90 \) min and continued throughout the experiment. Plasma samples were collected every 10 minutes from \( t = 60 \) min to \( t = 90 \) min and from \( t = 100 \) min and \( t = 140 \) min to determine plasma glucose levels and \([3^{-3}H]\)glucose specific activity. At the end of the experiments, rats were anesthetized and tissue samples were collected and immediately flash frozen. Tissues were stored at -80 C until use.
3.6 Treatments

The following treatments were infused into the lumen of the duodenum during the in vivo experiments at a rate of 0.01 ml/min for 50 minutes: 1) saline, 2) DMSO (0.88%, Sigma-Aldrich, St. Louis, MO, USA), 3) resveratrol (60 ng/min (26.3 µM total); Sigma-Aldrich;), 4) EX527 (12 µM) Sigma-Aldrich), 5) tetracaine (0.01 mg/min, Sigma-Aldrich), 6) compound C (100 µM, Millipore), SRT1720 (16 µM, Selleck Biochem). The resveratrol dose was selected based on the following rationale: Previous studies administering resveratrol into the MBH at 0.3 ng/min (200 µM) for 360 min lowers GP\(^72\). In order to address the preabsorptive effect of resveratrol, the gut infusion protocol was limited to 50 min duration; therefore we first selected a dose 100 fold higher than the brain (30 ng/min) but saw minimal effects on GP regulation (glucose production ~7 mg kg\(^{-1}\) min\(^{-1}\), n=2). We then increased resveratrol to 60 ng/min and discovered a consistent lowering effect of GP as reported in Fig. 4.1. The EX527 dose was selected based on the same study described above\(^72\), and was adjusted by the same factor as the resveratrol dose.

The following treatments were infused into the MBH during the pancreatic (basal-insulin) euglycemic clamp at a rate of 0.006 µL/min: 1) saline, 2) insulin (total of 1.26 mU)\(^179\) (Sigma-Aldrich), 3) S961 (1.3 mg/ml) (gift from Dr. Schäffer, Novo Nordisk).

3.7 Tissues collection and preparation for western blotting

The duodenal mucosa was separated from the duodenal smooth muscle after removal from anesthetized rats in petri dishes filled with 0.9% saline on ice with a spatula immediately after termination of the experiments. The separated layers were transferred to separate eppendorf tubes and stored at -80°C until use. The liver was freeze clamped using steel tongs pre-cooled in liquid nitrogen. The tissues were lysed on ice with a handheld homogenizer in a buffer
containing 50 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 % (w/v) Nonidet P40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 µM Dithiotritolo (DTT) and protease inhibitor cocktail (Roche). After homogenization, the tissues were spun at 12000 rpm for 15 minutes at 4°C. The protein concentration of homogenized tissues was determined using the Pierce 660 nm protein assay (Thermo Scientific).

### 3.8 Protein Assay

The Thermo Scientific Pierce 660nm Protein Assay (Thermo Scientific, IL, USA) was used to measure the protein concentration of different tissue samples with BSA used as a standard. This is a colorimetric assay based on the binding of a dye-metal complex to protein under acidic conditions, which causes a shift in the dye’s maximum absorption, measured at 630nm. The tissue samples were aliquoted for the protein assay, thawed, vortexed and kept on ice. The samples were diluted 1:20 with sterile water. 10 µl of the BSA standards were transferred to a 96 microwell plate in duplicate to prepare a curve ranging from 0 to 2 mg/ml and10 µl of the diluted tissue samples were added to the plate in triplicate. 150 µl of the Thermo Scientific Pierce 660nm Protein Assay Reagent was added to each well and allowed to change color. After 5 minutes, the plate was transferred to a spectrophotometer and the absorbance was read at 630nm. Through interpolation, the protein concentrations of the tissue samples were determined.

### 3.9 Western blotting

20 ug of tissues lysates (prepared as described above) were subjected to electrophoresis on 10% acrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated for 1 hour with blocking buffer (either TBS-T containing 5% (w/v) BSA or 5% skim milk). The
membranes were then incubated with the indicated primary antibodies (SIRT1; 1:500 (Upstate Biotechnology, MA, USA), B actin; 1:30,000 (Sigma-Aldrich, MO, USA) diluted in the blocking buffer for 16 hours at 4 °C. The membranes were washed 3 times with TBS-T and incubated with the appropriate secondary HRP-conjugated antibodies (diluted 1:4,000 for SIRT1 and 1:30,000 for β actin in 5% skim milk) at room temperature for 1 hour. Finally, the membranes were washed in TBS-T 5 times for 5 minutes, and the signal was detected using enhanced chemiluminescence reagent (Pierce, IL USA). Immunoblots were developed using a film automatic processor (SRX-101; Konica Minolta Medical), and films were scanned with the GS-800 Calibrated Densitometer (BioRad). Protein levels were quantified by densitometry with the Quantity One 1-D Analysis Software (BioRad) and normalized to β actin levels.

3.10 Cell culture

HEK 293 cells were treated with the same doses of resveratrol (Sigma-Aldrich) and/or EX527 (Sigma-Aldrich) as the clamp studies for 24 hours. Cells were then lysed in a buffer containing: 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (w/v) Nonidet P40, 1mM sodium orthovanadate, 50mM sodium fluoride, 5mM sodium pyrophosphate, 0.27M sucrose, and 1 mM Dithiotritolo (DTT) and the protein concentration was determined using the Pierce 660 nm protein assay (Thermo Scientific). Five hundred micrograms of protein was used in the SIRT1 activity assay.

3.11 SIRT1 activity assay

SIRT1 activity was measured using the Abcam SIRT1 Activity Assay Kit (Fluorometric) (Abcam, MA, USA). SIRT1 was immunoprecipitated from 500 ug of cell lysates with 8 ug of SIRT1 antibody (Upstate Biotechnology, N, USA). The antibody was incubated for 2 hours on a
rotating wheel at 4 °C, then 25 ul of 25% protein A/G beads (Santa Cruz, CA, USA) were added, and the lysates were incubated for an additional hour at 4 °C on a rotating wheel. The beads were washed 3 times with 750 uL of lysis buffer (see above). The beads were then re-suspended in assay reagents provided by the kit according to the manufacturer’s instructions. The reaction was stopped after 30 minutes, and fluorescence was measured at Ex/Em = 350-380 nm/440-460 nm.

3.12 Biochemical analysis

3.12.1 Plasma Glucose

The measurements of plasma glucose concentrations were conducted by the glucose oxidase methods using a GM9 Analox Glucose Analyzer (Analox Instruments, Lunenbertg, MA). Blood samples were collected into heparinized tubes and centrifuged at 6000 rpm to separate the plasma. Upon calibration of the analyzer with a provided standard, a 10 µl D-glucose containing plasma sample was pipetted into the reaction well containing a solution with glucose oxidase and oxygen. The following reaction occurs after injection of a sample:

\[ \beta-D-\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} D-\text{gluconic acid} + H_2O_2 \]

The rate of oxygen consumption is proportional to the amount of glucose in the plasma sample. A polarographic sensor measures the rate of oxygen consumption to determine the plasma glucose concentration. More specifically, the partial pressure of oxygen in the sample is measured as Clark-type amperometric oxygen electrodes are immersed in the sample and a potential is applied between them that reduces dissolved oxygen at the working electrode. Results are obtained within 20 seconds of inserting the sample into the apparatus.
3.12.2 Plasma Glucose Tracer Specific Activity

50 µl of plasma was used to determine the specific activity of [3-\(^3\)H] in the plasma. The samples were first deproteinized by the addition of 100 µl of Ba(OH)\(_2\) and ZnSO\(_4\) followed by vortexting and centrifugation at 13200 rpm for 5 minutes at 4°C. The supernatant of each sample was transferred to scintillation vials and evaporated to dryness to remove tritiated water (since tritium on the C-3 position of glucose is lost to water during glycolysis). Thus, radioactivity would be represented from the [3-\(^3\)H] glucose in the plasma only. Scintillation fluid (Bio-Safe Scintillation Cocktail, Research Products International Corp., Mount Prospect, IL, USA) was added to the dried sample to amplify the radioactive signal and counted in a LS6500 Multipurpose Scintillation Counter (Beckman, USA).

3.12.3 Plasma Insulin

A radioimmunoassay (RIA) was used to determine plasma insulin concentrations using a rat insulin kit (100% specificity) from Linco research (St. Charles, MO). The antigen-antibody binding principle is used in the RIA. Briefly, the amount of insulin present in the plasma sample is in competition for binding to antibodies raised against insulin (guinea pig anti-rat insulin antibody) with a labeled tracer antigen (\(^{125}\)I labeled insulin). Thus the amount of radiolabeled \(^{125}\)I-labeled insulin that binds is in reverse proportion to the known standards and the amount of insulin in the plasma sample. Separation of the \(^{125}\)I-labeled insulin and unbound fractions is conducted through the use of a double antibody solid phase.

Specifically, a 2-day protocol as per the supplier’s instructions was used. First, the generation of standard curve is constructed with the use of 50 µl of standards with a range of known concentrations. Then 50 µl of the plasma samples was pipetted into appropriate tubes and the addition of 50 µl of \(^{125}\)I-labeled insulin and 50 µl of the rat insulin antibody is added to
both the standards and samples, and were vortexed. 1.0 ml of precipitating reagent is added after overnight incubation at 4°C followed by vortexing and incubation at 4°C for 20 minutes. To pellet the bound insulin, the samples were then centrifuged. A gamma counter (Perkin Elmer 1470) is used to count the radioactivity of the pellet. The radioactivity counts (B) for the standards and samples are expressed as a percentage of the mean counts of total binding reference tubes (B₀):

\[
\% \text{ total binding} = \% \frac{B}{B_0} = \frac{\text{Standard or sample}}{B_0} \times 100\%
\]

A standard curve is constructed by plotting the \( \% \frac{B}{B_0} \) for each standard against the known concentration. Through interpolation, the concentration of the insulin samples was determined.

### 3.13 Calculations

During the euglycemic clamp and non-clamp experiments, a radioactive [3–³H] glucose tracer was infused at a constant rate to allow for equilibration of the tracer glucose with the glucose in the body. After equilibration, using the steady state formula, glucose production and uptake can be determined. That is, in the steady state basal condition, the rate of glucose uptake (Rd) is equal to the rate of glucose appearance (Ra) or rate of endogenous glucose appearance. Thus, using the steady state formula, the Ra and Rd can be determined by the following equation:

\[
Ra = Rd = \frac{\text{Constant tracer infusion rate (\( \mu \text{Ci/min} \))}}{\text{Specific activity (\( \mu \text{Ci/mg} \))}}
\]

During the euglycemic clamp where an exogenous glucose infusion is given to maintain euglycemia, glucose production is calculated by subtracting the exogenous glucose infusion rate from the Rd:
3.14 Statistical Analysis

Unpaired Student’s t-test was performed in statistical analyses of two groups. Where comparisons were made across more than two groups, ANOVA was performed; if significant, this was followed by Tukey’s post hoc test, which enabled comparisons of all treatment groups. A probability of $P < 0.05$ was accepted as significant. The statistical software program Prism (GraphPad Software Inc., CA, USA) was used for statistical calculations. Data are presented as means ± s.e.m. For the clamp experiments, the time period of 60–90 min was averaged for the basal condition, and the time period of 180–200 min was averaged for the clamp condition. For non-clamp experiments, the time period 60-90 min was averaged for the basal condition, and the time period from 140-150 min was averaged for treatment conditions.
**Table 3.1** Diet content of the regular chow and the lard-oil enriched high fat diet.

<table>
<thead>
<tr>
<th>Calories provided</th>
<th>Chow diet</th>
<th>High fat diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates (%)</td>
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<td>44</td>
</tr>
<tr>
<td>Protein (%)</td>
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<td>22</td>
</tr>
<tr>
<td>Fat (%)</td>
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<td>34</td>
</tr>
<tr>
<td>Saturated</td>
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<td>14.3</td>
</tr>
<tr>
<td>Monounsaturated</td>
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<td>14.8</td>
</tr>
<tr>
<td>Polyunsaturated</td>
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<td>4.8</td>
</tr>
<tr>
<td>Total metabolizable energy provided (kcal/g)</td>
<td>3.1</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Chapter 4
Results

SIRT1 is expressed in the duodenal mucosa

We first confirmed the presence of SIRT1 in the duodenum\textsuperscript{156} by western blot analysis, using liver samples of fed vs. 24 h fasted rats (i.e., fasting induces hepatic SIRT1 protein levels\textsuperscript{180}) as positive controls (Fig. 4.1a). Interestingly, we additionally discovered that SIRT1 was primarily expressed in the duodenal mucosa, with relatively low levels in the duodenal smooth muscle of rodents (Fig. 4.1a). Thus, subsequent SIRT1 protein analysis was localized to the intestinal mucosa.

Intraduodenal resveratrol improves insulin sensitivity in the preabsorptive state

In order to begin addressing the insulin-sensitizing actions of intraduodenal resveratrol, we infused resveratrol directly into the duodenal lumen in 3 d hyperphagic HFD (Table 2.1)-fed rats using the hyperinsulinemic-euglycemic clamps (Fig. 1b; plasma insulin levels were elevated to >2 fold, Table 4.1). We first established that during an intraduodenal saline infusion, the exogenous glucose infusion rate required to maintain euglycemia was significantly reduced in HFD-fed rats compared to chow-fed rats (Fig. 4.1c), due to the inability of circulating hyperinsulinemia to suppress glucose production (GP) (Fig. 4.1d,e) and not on the inability to simulate glucose uptake (Fig. 4.1f). These findings are consistent with previous studies\textsuperscript{26} indicating that 3 d of HFD feeding selectively impairs circulating hyperinsulinemia to lower GP.
Importantly, when resveratrol (60 ng/min) was infused into the duodenal lumen for only 50 min, the glucose infusion rate (Fig. 4.1c) and GP suppression (Fig. 4.1d,e) were normalized to levels of chow-fed rats, with comparable plasma insulin and glucose levels (Table 4.1). Furthermore, resveratrol infused i.v. at a dose and duration equal to that of intraduodenal resveratrol failed to affect insulin action (Fig. 4.1c-f), ensuring that our observed insulin-sensitizing effect was due to local duodenal resveratrol action. These findings collectively indicate that preabsorptive intraduodenal resveratrol infusion normalizes insulin sensitivity to lower glucose production.

**Intraduodenal resveratrol normalizes SIRT1 expression in HFD-fed rats**

To begin dissecting the role of duodenal SIRT1 in intraduodenal resveratrol’s actions, we first determined the effects of a 50 min infusion of resveratrol on duodenal SIRT1 expression. We discovered that HFD feeding decreased SIRT1 expression in duodenal mucosa tissues obtained immediately following saline infusion-clamp studies, while a 50 min intraduodenal resveratrol infusion was sufficient to normalize SIRT1 protein levels to that of chow-fed rats (Fig. 4.2a). These findings indicate that intraduodenal resveratrol infusion normalizes duodenal SIRT1 levels in HFD-fed rats.

**SIRT1 knockdown is restricted to the duodenum, and duodenal SIRT1 knockdown in chow fed-rats induces hepatic insulin resistance**
To examine the physiological contribution of duodenal SIRT1 on insulin sensitivity, we performed a chronic 2-week SIRT1 knockdown, restricted to the duodenum, in chow-fed rats \textit{in vivo}. We developed a genetic approach to knock down SIRT1 protein levels in the duodenum \textit{in vivo} (Fig. 4.3a). Two weeks before the infusion-clamp experiments, a lentivirus expressing either mismatch (LV-mismatch) or SIRT1 shRNA (LV-SIRT1 shRNA) was injected into the duodenal lumen, and left for 20 minutes to optimize local infection, as described\textsuperscript{162}. Western blot analysis validated the knockdown, as SIRT1 protein levels were decreased in the duodenal mucosa of LV-SIRT1 shRNA vs. LV-mismatch injected rats (Fig. 4.4a). Importantly, SIRT1 protein levels did not differ in the jejunal mucosa (Fig. 4.4b), ileal mucosa (Fig. 4.4c), or liver (Fig. 4.4d) of LV-SIRT1 shRNA and LV-mismatch injected rats, indicating that SIRT1 knockdown was specific to the duodenum. Interestingly, duodenal SIRT1 knockdown in chow-fed rats resulted in reduced hepatic insulin sensitivity as assessed by the hyperinsulinemic euglycemic clamp (Fig. 4.4e-g) when compared to rats with intact duodenal SIRT1 expression, with no changes in glucose uptake (Fig. 4.2h). These findings highlight the importance of duodenal SIRT1 in regulating glucose control.

**Duodenal SIRT1 activity is required for intraduodenal resveratrol to improve insulin sensitivity, and direct duodenal SIRT1 activation improves insulin sensitivity**

We then investigated whether an increase in duodenal SIRT1 levels is necessary for the insulin-sensitizing effect of resveratrol. Using the same molecular approach described above (Fig. 4.3a), we knocked down SIRT1 protein levels three days before the infusion-clamp experiments. While duodenal LV-mismatch-injected HFD-fed rats responded to intraduodenal resveratrol
infusion identically to non-injected HFD-fed rats during the infusion-clamp studies (Fig. 4.5a-d), intraduodenal resveratrol infusion failed to increase the glucose infusion rate and lower GP in LV-SIRT1 shRNA injected HFD-fed rats (Fig. 4.5a-c) at comparable plasma insulin and glucose levels (Table 4.1). Thus, increased duodenal SIRT1 expression is necessary for the insulin-sensitizing effect of preabsorptive resveratrol in HFD-fed rats.

To alternatively assess the involvement of duodenal SIRT1, we co-infused resveratrol with the SIRT1-specific inhibitor EX527 (Fig. 4.3a). Co-infusion of EX527 with resveratrol into the duodenum abolished the increase in glucose infusion rate (Fig. 4.6a) and suppression of glucose uptake (Fig. 4.6b,c) induced by resveratrol, with no changes in glucose uptake or plasma insulin and glucose levels (Fig. 4.6d, Table 4.1) in HFD-fed rats, while infusion of EX527 alone in both chow and HFD-fed rats had no effects on glucose metabolism (Fig. 4.6a-d). To ensure that EX527 specifically attenuated the ability of resveratrol to increase SIRT1 activity, HEK293 cells were treated with the same resveratrol and EX527 doses used in vivo and a SIRT1 activity assay was performed. In these cells, resveratrol increased SIRT1 activity, whereas in the presence of resveratrol and EX527, SIRT1 was not activated (Fig. 4.6e). Taken together, these in vivo and in vitro chemical loss-of-function approaches suggest that duodenal SIRT1 activation is necessary for the insulin-sensitizing effect of preabsorptive resveratrol.

Since SIRT1 action is required for duodenal resveratrol to improve insulin sensitivity, we investigated whether direct SIRT1 activation in the duodenum has insulin-sensitizing effects. We infused the SIRT1 activator SRT1720, which has previously been demonstrated to mediate glucose kinetics when given orally, directly in the duodenum of 3d HF-fed rats during the hyperinsulinemic euglycemic clamp (Fig 4.3a). Interestingly, intraduodenal SRT1720 increased
the glucose infusion rate needed to maintain euglycemia (Fig. 4.6a) and decreased GP (Fig 4.6b,c), with no changes in glucose uptake, (Fig. 4.6d), indicating improved insulin sensitivity. Hence, these data support the hypothesis that resveratrol requires duodenal SIRT1 to improve insulin sensitivity, and that SIRT1 action in the duodenum is important in the regulation of glucose control and insulin action.

Intraduodenal resveratrol triggers an AMPK - SIRT1 pathway to improve insulin sensitivity

After identifying duodenal SIRT1 as an important target of resveratrol to improve insulin sensitivity, we wished to identify further molecular mechanisms to explain how resveratrol and downstream SIRT1 activation affect glucose kinetics. As AMPK has previously been implicated in resveratrol’s actions to improve insulin sensitivity, and AMPK is expressed in the duodenum\textsuperscript{157,159}, we investigated the necessity of duodenal AMPK in intraduodenal resveratrol’s insulin sensitizing effect. We co-infused the AMPK inhibitor compound C with resveratrol during the infusion-clamp experiment. Interestingly, compound C abolished the ability of duodenal resveratrol to increase the glucose infusion rate (Fig. 4.7a) and to lower GP (Fig. 4.7b,c) in HFD-fed rats, with no changes in glucose uptake (Fig. 4.7d). An infusion of compound C alone in chow-fed and HFD-fed rats had no effects on the glucose infusion rate, GP or glucose uptake (Fig. 4.7a-d) during the infusion-clamp experiments.

Because AMPK action has been implicated to be both upstream\textsuperscript{152,153} and downstream\textsuperscript{155} of SIRT1 in resveratrol action in various tissues, we sought to investigate the interfunctional relationship of SIRT1 and AMPK in mediating glucose control in the duodenum. To do so, we
activated duodenal SIRT1 with the activator SRT1720 and blocked AMPK with the inhibitor compound C in the duodenum in 3 d HFD-fed rats during the hyperinsulinemic euglycemic clamp. When intraduodenal SRT1720 (at the same dose that improved insulin sensitivity as described above) was co-infused with compound C, compound C did not affect the ability of SIRT1 activation to increase the glucose infusion rate (Fig. 4.7a) and suppress glucose production (Fig. 4.7b,c), with no changes in glucose uptake (Fig. 4.7d). These data indicate that activation of AMPK is not required for duodenal SIRT1 action to improve insulin sensitivity, and thus AMPK may lie upstream of SIRT1. In line with this, western blot analysis of total AMPK protein in HEK293 cells treated with DMSO, resveratrol, or resveratrol and the SIRT1 inhibitor EX527 (the same cells that were used to assess SIRT1 activity revealed increased AMPK expression in cells treated with resveratrol compared to DMSO-treated cells, while treatment of resveratrol with EX527 did not abolish this increase in total AMPK protein (Fig. 4.7e). Taken together, the above data indicate that intraduodenal resveratrol triggers a potential AMPK - SIRT1 signaling cascade to improve insulin sensitivity.

Intraduodenal resveratrol activates a neuronal network to improve insulin sensitivity

To assess whether preabsorptive resveratrol triggers the nerve terminals innervating the intestine to improve insulin sensitivity to suppress GP (Fig. 4.8a), we infused the anesthetic tetracaine into the duodenum. Co-infusion of resveratrol with tetracaine abolished the ability of resveratrol to increase the glucose infusion rate (Fig. 4.9a) and decrease GP (Fig. 4.9b,c) at similar plasma insulin and glucose levels during the hyperinsulinemic-euglycemic clamps (Table 4.1), while tetracaine alone had no effects (Fig. 4.9a-d) and no differences in glucose uptake were detected.
(Fig. 4.9d). This set of findings indicates duodenal resveratrol requires a neuronal innervation for its insulin-sensitizing action.

Intraduodenal resveratrol remotely improves mediobasal hypothalamic insulin sensitivity

Thus, given that a gut-brain neuronal axis is subsequently activated by preabsorptive resveratrol, coupled with the facts that (i) hyperinsulinemic-euglycemic clamps partly suppress GP through a hypothalamic insulin receptor-mediated signaling pathway\textsuperscript{182}, and (ii) hypothalamic insulin resistance disrupts GP regulation and glucose homeostasis in HFD feeding\textsuperscript{46}, we reasoned that the insulin-sensitizing effect of preabsorptive duodenal resveratrol may occur via a gut-brain neuronal axis to remotely reverse hypothalamic insulin resistance (Fig. 4.8a).

To test this hypothesis, we performed pancreatic-euglycemic clamps in chow and HFD-fed rats while plasma insulin levels were maintained at basal levels (Fig. 4.10a, Table 4.1). While mediobasal hypothalamic (MBH) saline in chow-fed rats has no effect on GP\textsuperscript{182}, in the presence of duodenal saline infusion, we confirmed that direct insulin infusion into the MBH increased the glucose infusion rate and decreased GP in chow-fed but not in 3 d HFD-fed rats (Fig. 4.10b-d). Importantly, while intraduodenal resveratrol infusion for 50 min had no effects on the glucose infusion rate or GP in HFD-fed rats infused with MBH saline during the basal insulin clamp, duodenal resveratrol restored the effect of MBH insulin infusion to increase the glucose infusion rate and suppress GP (Fig. 4.10b-d). To assess whether the selective improvement of hypothalamic insulin action in GP regulation is specific to insulin receptor-mediated events, we co-infused MBH insulin with the insulin receptor antagonist S961 (at a established dose that negates the ability brain insulin action to lower GP\textsuperscript{179}) in the presence of a duodenal resveratrol
infusion. S961 co-administration with MBH insulin and with duodenal resveratrol abolished the increase in glucose infusion rate and the decrease in GP in HFD-fed rats, with no changes in glucose uptake (Fig. 4.10d-d) while a MBH S961 infusion alone had no effects on glucose metabolism (Fig. 4.10b-d). Taken together, our findings indicate that duodenal resveratrol triggers a gut-brain neuronal network to remotely reverse hypothalamic insulin resistance in HFD-fed rodents and thus restores the ability of hypothalamic insulin signaling to effectively lower GP.

Intraduodenal resveratrol improves insulin sensitivity in long-term high-fat fed rodents and lowers glucose production and plasma glucose levels in a type 2 diabetic rodent model

To further assess the therapeutic relevance of duodenal resveratrol action, we finally tested intraduodenal resveratrol’s effects in two chronic diseased rodent models. Firstly, we tested the effects of intraduodenal resveratrol on insulin sensitivity in obese rats fed a HFD (Table 3.1) for 28 days during a hyperinsulinemic euglycemic clamp. We established in rats infused with intraduodenal saline that this obese model displays both hepatic and peripgceral insulin resistance, as there was an inability of circulating hyperinsulinemia to suppress hepatic glucose production as well as stimulate peripheral glucose uptake compared with rats fed regular chow for 28 days (Fig. 11a-c). Interestingly, 28 d HF-fed rats infused with intraduodenal resveratrol had improved insulin sensitivity as the glucose infusion rate needed to maintain euglycemia was
significantly higher than saline-infused rats (Fig 11a), which was due to a suppression of hepatic glucose production as opposed to changes in glucose uptake (Fig. 11b-d).

Secondly, we tested the ability of resveratrol to affect glucose kinetics in an unclamped setting in a type 2 diabetic rodent model with mild hyperglycemia. Rats were injected with nicotinamide (NA) and streptozotocin (STZ) and placed on a HFD for 5-6 d to induce hyperglycemia and insulin resistance but to prevent insulin-deficiency as described\textsuperscript{30} (Fig. 4.12a). Consistent with previous findings\textsuperscript{178}, GP and plasma glucose levels were significantly higher in NA-STZ/HFD treated rats than HFD-fed and/or regular chow-fed rats (Fig 4.12b). Importantly, in a non-clamped condition, intraduodenal resveratrol vs. saline infusion for 50 min was sufficient to lower GP (Fig. 4.12c) and plasma glucose levels (Fig. 4.12d) in the presence of comparable basal plasma insulin levels (Resveratrol: 0.9 +/- 0.1 ng/ml vs. Saline: 1.3 +/- 0.2 ng/ml).

Taken together with the data described above, these overall findings collectively suggest that resveratrol action in the duodenum is sufficient to improve insulin sensitivity and lower GP and plasma glucose levels in obesity and diabetes.
Figure 4.1. Intraduodenal resveratrol infusion improves insulin sensitivity in HFD-fed rodents. 

**a.** SIRT1 protein expression in the duodenal mucosa or smooth muscle, using liver samples from 24h fasted or fed rats as a positive control. 

**b.** Experimental procedure and hyperinsulinemic euglycemic clamp protocol. 

**c.** The glucose infusion rate during the clamp in chow fed rats with intraduodenal saline or DMSO and HFD-fed rats with intraduodenal saline or resveratrol, or i.v. resveratrol. 

**d-e.** The rate of endogenous glucose production and suppression of glucose production expressed as the percentage reduction from basal during the clamp. 

**f.** The rate of glucose uptake during the clamp. (*p < 0.05 vs. other groups, †p < 0.05 versus chow + saline). Values are shown as mean ± SEM. n = 6-10 rats per group.
Figure 4.2. Intraduodenal resveratrol normalizes SIRT1 protein levels in HFD-fed rodents. a. Duodenal mucosa SIRT1 protein expression normalized to β actin in regular chow-fed rats with intraduodenal saline, HFD-fed rats with intraduodenal saline, and HFD-fed rats with 50 min intraduodenal resveratrol.
Figure 4.3. Schematic representation of the working hypothesis. a. Intraduodenal resveratrol activates duodenal SIRT1 to improve insulin sensitivity, and direct SIRT1 action mediates hepatic insulin sensitivity.
Figure 4.4. SIRT1 knockdown is restricted to the duodenum, and duodenal SIRT1 knockdown in chow fed-rats induces hepatic insulin resistance. **a.** Duodenal SIRT1 protein expression normalized to β actin is decreased in SIRT1 shRNA injected rats versus mismatch-injected rats. **b-d.** SIRT1 protein levels normalized to β actin do not differ in the jejunum, ileum, or liver of mismatch or SIRT1-injected rats. **e.** The glucose infusion rate during the hyperinsulinemic euglycemic clamp in rats with a 2-week lentiviral-mediated SIRT1 knockdown restricted to the duodenum, versus mismatch controls. **f-g.** The rate of endogenous glucose production and suppression of glucose production expressed as the percentage reduction from basal during the clamp. **h.** The rate of glucose uptake during the clamp. (*p < 0.05 vs. other groups). Values are shown as mean ± SEM. n = 5-6 rats per group.
Figure 4.5. Duodenal SIRT1 is required for intraduodenal resveratrol to improve insulin sensitivity. **a.** The glucose infusion rate during the hyperinsulinemic euglycemic clamp in duodenal mismatch or SIRT1-knockdown rats with an intraduodenal saline or resveratrol infusion. **b-c.** The rate of endogenous glucose production and suppression of glucose production expressed as the percentage reduction from basal during the clamp. **d.** The rate of glucose uptake during the clamp. (*p < 0.05 vs. other groups). Values are shown as mean ± SEM. n = 5-6 rats per group.
Figure 4.6. Duodenal SIRT1 activity is required for intraduodenal resveratrol to improve insulin sensitivity and direct duodenal SIRT1 activation improves insulin sensitivity. 

a. The glucose infusion rate during a hyperinsulinemic clamp in rats with an intraduodenal infusion of the SIRT1 specific inhibitor EX527 alone in chow or HFD-fed rats, or in combination with resveratrol, and of the SIRT1 specific activator SRT1720 in HFD-fed rats. 

b-c. The rate of endogenous glucose production and suppression of glucose production expressed as the percentage reduction from basal during the clamp. 

d. The rate of glucose uptake during the clamp. 

e. SIRT1 activity measured by fluorometric activity assay in HEK293 cells, reaction stopped after 30 min. (*p < 0.05, **p < 0.01 vs. other groups). Values are shown as mean ± SEM. n = 5-10 rats per group.
Figure 4.7. Duodenal AMPK activity is required for resveratrol to improve insulin sensitivity, but is not necessary for direct SIRT1 action to improve insulin sensitivity. 

a. The glucose infusion rate during the hyperinsulinemic euglycemic clamp in rats with an intraduodenal infusion of the AMPK inhibitor compound C alone in chow fed or HFD-fed rats, or in combination with resveratrol or the SIRT1 specific activator SRT1720 in HFD-fed rats. 

b-c. The rate of endogenous glucose production and suppression of glucose production expressed as the percentage reduction from basal during the clamp. 

d. The rate of glucose uptake during the clamp. 

e. Total AMPK protein expression normalized to β actin in HEK 293 cells treated with DMSO, resveratrol, or resveratrol and EX527 for 24 hours. (*p < 0.05 vs. other groups). Values are shown as mean ± SEM. n = 5-10 rats per group.
Figure 4.8. Schematic representation of the working hypothesis. a, Intraduodenal resveratrol triggers a neuronal network and remotely improves hypothalamic insulin sensitivity.
Figure 4.9. Intraduodenal resveratrol triggers a neuronal network to improve hypothalamic insulin sensitivity. a. The glucose infusion rate during the hyperinsulinemic euglycemic clamp in rats with intraduodenal administration of tetracaine alone in chow and HFD-fed rats, or in combination with resveratrol in HFD-fed rats. b-c. The rate of endogenous glucose production and suppression of glucose production expressed as the percentage reduction from basal during the clamp. d. The rate of glucose uptake during the clamp. (*p < 0.05 vs. other groups). Values are shown as mean ± SEM. n = 5-10 rats per group.
Figure 4.10. Intraduodenal resveratrol remotely improves hypothalamic insulin sensitivity. 
a. Experimental procedure and pancreatic (basal-insulin) euglycemic clamp protocol. b. The glucose infusion rate during the pancreatic (basal insulin) euglycemic clamp with MBH insulin and intraduodenal saline in regular chow fed rats, and MBH insulin, saline, S961, or insulin + S961 and intraduodenal saline or resveratrol in HFD-fed rats. c–d. The rate of endogenous glucose production and suppression of glucose production expressed as the percentage reduction from basal during the clamp. e. The rate of glucose uptake during the clamp. (*p < 0.05 vs. other groups). Values are shown as mean ± SEM. n  = 5-6 rats per group.
Figure 4.11 Intraduodenal resveratrol improves insulin sensitivity in obese insulin resistant rodents. 

a. The glucose infusion rate during the hyperinsulinemic euglycemic clamp in rats with intraduodenal administration of saline or resveratrol in 28 day chow-fed or 28d HFD-fed obese rats

b. The rate of endogenous glucose production and suppression of glucose production expressed as the percentage reduction from basal during the clamp.

c. The rate of glucose uptake during the clamp. (*p < 0.05 vs. other groups). Values are shown as mean ± SEM. n = 5-6 rats per group.
Figure 4.12. Intraduodenal resveratrol lowers glucose production and plasma glucose levels in a rodent model of type 2 diabetes. a. Experimental procedure and non-clamp experiment protocol. b. The rate of GP and plasma glucose levels in the basal state of chow-fed, 3d HFD-fed, and NA-STZ/HFD induced hyperglycemic rats. c,b. The rate of GP and GP % suppression in NA-STZ/HFD induced hyperglycemic rats with intraduodenal saline or resveratrol. d. Plasma glucose levels and plasma glucose percent suppression from basal in NA-STZ/HFD induced hyperglycemic rats with intraduodenal saline or resveratrol. (*p < 0.05 **p < 0.01 versus other groups, †p < 0.05 versus chow). Values are shown as mean ± SEM. n = 5 rats per group.
Table 4.1. Plasma insulin and glucose concentrations of the groups receiving duodenal and/or MBH infusions during the basal and clamp conditions.

<table>
<thead>
<tr>
<th></th>
<th>Chow + saline (N = 7)</th>
<th>HFD + saline (N = 10)</th>
<th>HFD + resveratrol (N = 6)</th>
<th>HFD + SIRT1 shRNA + resveratrol (N = 6)</th>
<th>HFD + resveratrol + EX527 (N = 5)</th>
<th>HFD + resveratrol + tetracaine (N = 5)</th>
<th>HFD + MBH insulin + duodenal resveratrol (basal clamp) (N = 6)</th>
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<tbody>
<tr>
<td><strong>Basal</strong></td>
<td></td>
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<tr>
<td>Insulin (ng/mL)</td>
<td>0.9 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>0.9 ± 0.3</td>
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<tr>
<td>Glucose (mM)</td>
<td>6.7 ± 0.1</td>
<td>7.6 ± 0.4</td>
<td>6.9 ± 0.3</td>
<td>7.7 ± 0.1</td>
<td>6.9 ± 0.2</td>
<td>7.2 ± 0.3</td>
<td>6.9 ± 0.3</td>
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<tr>
<td><strong>Clamp</strong></td>
<td></td>
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<tr>
<td>Insulin (ng/mL)</td>
<td>2.4 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>2.0 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>6.8 ± 0.4</td>
<td>7.6 ± 0.3</td>
<td>7.3 ± 0.4</td>
<td>7.7 ± 0.1</td>
<td>7.5 ± 0.2</td>
<td>7.4 ± 0.3</td>
<td>6.5 ± 0.4</td>
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Chapter 5
Discussion

In this study, we reveal the duodenum as a pharmacological target for antidiabetic agents. The duodenum is capable of sensing resveratrol to activate an AMPK - SIRT1 signaling pathway and a subsequent neuronal network to normalize insulin sensitivity in rodents with diet-induced hepatic insulin resistance. Importantly, duodenal resveratrol remotely improves hypothalamic insulin sensitivity to lower hepatic glucose production, with no effects on glucose uptake. We also demonstrate the importance of duodenal SIRT1 in mediating hepatic insulin sensitivity.

While the clinical relevance of resveratrol remains debated, encouraging data exists supporting resveratrol’s insulin-sensitizing effects in obese and diabetic individuals. Although some clinical studies point to the inability of resveratrol to improve glucose homeostasis in humans, various disease states and severities may play a role in the different effects observed with resveratrol treatment. Indeed, resveratrol does not appear to improve glucose metabolism in individuals without pre-existing metabolic abnormalities, such as non-obese women with normal glucose tolerance, and mixed results have been reported in obese men with only mild insulin resistance, who are in relatively good health. In direct contrast, chronic resveratrol generally improves glycemic control and insulin sensitivity in glucose intolerant and/or diabetic individuals. Nonetheless, studies denoting the antidiabetic action of resveratrol and SIRT1 activation in various rodent models are extensive, highlighting the importance of SIRT1 activation in improving metabolic parameters. While chronic resveratrol treatment activates hepatic and muscle SIRT1, it is still debated...
whether oral resveratrol intake can reach adequate concentrations in target tissues to produce its observed beneficial effect via direct mechanisms, due to its short initial half-life and high metabolism\(^8^3\). In light of this, our current findings indicate that acute resveratrol administration into the duodenum potently acts to improve circulating hyperinsulinemia to suppress GP in HFD-fed rodents with insulin resistance, and to lower GP and improve glucose homeostasis in NA-STZ/HFD induced type 2 diabetic rodents. These findings collectively suggest accumulation of resveratrol in the duodenum in a preabsorptive state\(^1^2^0\) could recapitulate its chronic, whole body effects, offering a potential resolution to the paradox of resveratrol’s efficacy despite poor bioavailability.

We first demonstrated the importance of duodenal SIRT1 in mediating insulin sensitivity. In rats fed regular chow, duodenal SIRT1 knockdown led to a decreased ability to lower hepatic glucose production under insulin-stimulated conditions with no effects on peripheral glucose uptake. Further, we saw that rats fed a HFD for three days had significantly decreased SIRT1 expression in the duodenum, which could contribute to the hepatic insulin resistance of these HFD-fed rats. SIRT1 activation in these rats achieved both by intraduodenal resveratrol and SRT1720 was also sufficient to restore insulin sensitivity, further highlighting the importance of duodenal SIRT1 in mediating insulin action.

To ensure that the insulin-sensitizing effect of intraduodenal resveratrol is localized to the duodenum and is exerted in the preabsorptive state, we mimicked a condition of 100% leakage of intraduodenal-infused resveratrol into the circulation, and found that i.v. resveratrol did not alter insulin sensitivity. Second, with molecular and chemical inhibition of duodenal mucosal SIRT1 expression and activity, the insulin-sensitizing effect of intraduodenal resveratrol infusion was abolished independent of changes in hepatic SIRT1 expression. Third, a direct
blockade of duodenal neuronal innervation negated the insulin-sensitizing effect of duodenal resveratrol, all suggesting preabsorptive resveratrol activates duodenal SIRT1 to trigger a gut-brain neuronal axis to improve insulin sensitivity to suppress GP. These findings collectively raise two important questions: (i) What are the downstream effectors of duodenal SIRT1? (ii) What site(s) of insulin resistance did preabsorptive resveratrol reverse to restore GP control in HFD-fed rodents?

AMPK has been demonstrated to be required for many of resveratrol’s effects. While SIRT1 can activate AMPK via deacetylation of liver kinase B1, AMPK can reciprocally activate SIRT1 by increasing the abundance of its cosubstrate, NAD$^{+}$. It is debated whether SIRT1 lies upstream or downstream of AMPK in the effects of resveratrol, as one study indicates that AMPK is activated via inhibition of phosphodiesterases and subsequently enhances SIRT1 activity by increasing NAD$^{+}$ levels while another reports that resveratrol activates AMPK via SIRT1 in the muscle and subsequently improves mitochondrial function. Nonetheless, as AMPK is expressed in the intestine, we investigated its involvement in duodenal resveratrol’s actions. We found that blockade of duodenal AMPK negated the glucose-lowering effect of resveratrol while direct SIRT1 activation did not require AMPK to suppress glucose production, suggesting that AMPK may lie upstream of SIRT1. Future studies are warranted to confirm this pathway.

If hypothalamic insulin resistance is one of the primary defects in obesity that dysregulates GP and glucose homeostasis, it is possible that a resveratrol-duodenal AMPK-SIRT1 activated signaling axis could remotely improve hypothalamic insulin sensitivity to restore glucose homeostasis. To directly address this hypothesis, we performed the pancreatic (basal insulin)-euglycemic clamps, and as opposed to the hyperinsulinemic-euglycemic clamps (where the
whole body, including both the brain and the liver, are stimulated by insulin), we selectively increased MBH insulin levels. We discovered that in the face of HFD-induced hypothalamic insulin resistance, duodenal resveratrol selectively and fully restored the ability of hypothalamic insulin action to lower GP. Importantly, this restoration was negated when the hypothalamic insulin receptor was blocked, suggesting that preabsorptive resveratrol specifically enhances hypothalamic insulin receptor-mediated signaling events to regain control of GP in HFD-fed rats. However, the underlying signaling events that lead to the reversal of hypothalamic insulin resistance remain to be identified. It is noteworthy that although intestinal neuronal innervation (i.e., tetracaine experiment) is fully responsible for the effect of duodenal resveratrol, future studies are warranted to elucidate the contribution of reversing hypothalamic insulin resistance in the whole-body insulin sensitizing effect of preabsorptive resveratrol. Nonetheless, one should not underestimate the beneficial contribution that preabsorptive resveratrol has on hypothalamic insulin action since it fully reverses hypothalamic insulin resistance in GP regulation and illustrates, for the first time to our knowledge, the ability of duodenal-sensing mechanisms to remotely improve hypothalamic insulin sensitivity. We demonstrate that this ability of resveratrol is dependent upon duodenal SIRT1 and AMPK activity and SIRT1 action itself mediates insulin action, and one potential therapeutic approach would therefore be to selectively target duodenal SIRT1 and/or AMPK. As opposed to treatments aimed at directly altering the CNS, remotely targeting the insulin-signaling pathway in the brain via intestinal SIRT1 and/or AMPK would minimize direct CNS drug-related side effects while potentially achieving a similar impact on hypothalamic insulin sensitivity.

In summary, we unveil the duodenum as a pharmacological site of resveratrol action to improve insulin sensitivity and lower GP in HF-fed/diabetic rodents through a neuronal network, and identify duodenal SIRT1 and AMPK as potential targets for novel antidiabetic agents.
Chapter 6
Limitations

While our findings provide advancement in the understanding of resveratrol action in the duodenum to regulate insulin sensitivity, our findings are not without limitations.

1. While we show the involvement of AMPK and SIRT1 in resveratrol’s ability to mediate insulin sensitivity, we have only used a chemical approach to block AMPK activity. We could also infect the duodenum with an adenovirus expressing the dominant negative of AMPK in order to suppress its activity, and infuse resveratrol during a hyperinsulinemic euglycemic clamp. Further, while we show that direct SIRT1 activation does not require AMPK to improve insulin sensitivity, we have not fully defined the intermolecular relationship between AMPK and SIRT1. We could block SIRT1 activity using either the lentiviral or molecular approach described previously, and activate AMPK (eg. AICAR infusion), to determine whether AMPK indeed does lie upstream of SIRT1.

2. Another limitation to our study is that the hyperinsulinemic clamp technique measures insulin sensitivity independent of changes in circulating glucoregulatory hormones, which does not address the physiological relevance of our study. Thus, we performed non-clamp experiments, in which we only infused tritiated glucose to measure glucose kinetics during an intraduodenal infusion of resveratrol, in a setting where circulating hormones can change at will. We were able to measure glucose production and plasma glucose levels during this experiment, but we did not measure insulin sensitivity per se.
Thus, we could additionally perform an insulin tolerance test to determine the effects of intraduodenal resveratrol on insulin sensitivity in a non-clamp setting.

3. Lastly, we have demonstrated that intraduodenal resveratrol remotely improves hypothalamic insulin sensitivity. In the HFD-induced insulin resistance model that we use, rats develop both central$^{177}$ and hepatic insulin resistance$^{70}$. Thus, resveratrol could also remotely improve insulin sensitivity at extra-hypothalamic sites. A previous study has demonstrated that 3 days of HF-feeding also causes insulin resistance at the level of the DVC in the brainstem$^{179}$. Moreover, insulin signaling at the level of the liver itself is disrupted upon HF-feeding$^{185}$. Thus, in addition to restoring insulin sensitivity in the hypothalamus, intraduodenal resveratrol could also remotely improve DVC and/or hepatic insulin resistance via a gut-brain or a gut-brain-liver neuronal network, respectively. To test this, we could perform a hyperinsulinemic euglycemic clamp with an intraduodenal resveratrol infusion, while blocking insulin signaling in the hypothalamus. If an increase in glucose infusion rate and a suppression of glucose production are seen during this experiment, this indicates that duodenal resveratrol also mediates insulin sensitivity at a site other than the hypothalamus.
In this thesis, we examined the potential of intestinal sensing to mediate the pharmacological effect of the insulin-sensitizer resveratrol. Specifically, we demonstrated that in HFD-induced hepatic insulin resistant rats, preabsorptive intraduodenal resveratrol improves insulin sensitivity. This effect is mediated by duodenal SIRT1, and we further demonstrate the ability of duodenal SIRT1 itself to affect insulin sensitivity. Moreover, we discovered that resveratrol requires AMPK activation to regulate insulin action, and that AMPK may be located upstream of SIRT1 in a potential resveratrol $\rightarrow$ AMPK $\rightarrow$ SIRT1 signaling pathway. Moreover, duodenal resveratrol triggers a neuronal network and remotely improves insulin sensitivity specifically in the hypothalamus to lower hepatic glucose production. We finally demonstrate that resveratrol is effective in improving insulin sensitivity in both obese and type 2 diabetic rodents.

Future studies are necessary to further elucidate the downstream signaling mechanisms involved in duodenal resveratrol’s effects, and to dissect the potential of the intestine in mediating the anti-diabetic effects of classic therapeutic treatments for diabetes and obesity.
We have successfully demonstrated that duodenal resveratrol activates both SIRT1 and AMPK to improve insulin sensitivity, although the interrelationship between AMPK and SIRT1 remains to be assessed in resveratrol’s effects, as discussed in the Chapter 6. Moreover, the downstream signaling pathway remains to be elucidated. Since AMPK can activate PKA\textsuperscript{186} and duodenal PKA activation bypasses intestinal sensing resistance in high fat feeding, PKA may lie downstream of AMPK/SIRT1 activation in mediating resveratrol’s effects. The involvement of PKA in resveratrol’s effects could suggest the potential of gut peptides mediating its actions on glucose homeostasis. We have previously shown that duodenal lipid sensing triggers the release of CCK from duodenal I cells, which necessitates the CCK receptor and downstream PKA activation to lower glucose production\textsuperscript{10}. In addition to CCK, the duodenum also comprises enteroendocrine L cells responsible for the release of the incretin GLP-1 as well as other peptides. Importantly, GLP-1 has previously been demonstrated to activate PKA\textsuperscript{174,175}. Chronic resveratrol treatment has been shown to increase intestinal GLP-1 secretion, and fails to improve glucose homeostasis and insulin action in GLP-1 receptor knockout mice\textsuperscript{89}. Thus, a potential AMPK $\rightarrow$ SIRT1 $\rightarrow$ GLP-1/GLP-1 receptor $\rightarrow$ PKA pathway could mediate the insulin sensitizing effects of resveratrol in insulin resistance. Performing studies to elucidate the mediators of duodenal resveratrol would uncover potential therapeutic targets in the intestine for treatment of insulin resistance in obesity and type 2 diabetes.

Moreover, the discovery of a novel glucoregulatory pathway mediating the insulin sensitizing effects of oral resveratrol treatment gives credence to the idea that other classical therapeutic
treatments could similarly improve central and/or peripheral glucose homeostasis by remotely targeting the gastrointestinal tract. Indeed, recent evidence suggests that metformin, the most widely prescribed type 2 diabetic drug, interacts with the gastrointestinal tract to alter glucose homeostasis despite the classical view of a direct hepatic effect on lowering gluconeogenesis. For example, intraduodenal administration of metformin leads to the greatest drop of plasma glucose levels compared to intraportal and i.v. administration\textsuperscript{187} highlighting the possibility of a gut-mediated glucoregulatory effect. Furthermore, both gut microbiota\textsuperscript{188} and bile acid homeostasis\textsuperscript{189} are altered during metformin treatment. Thus, it is plausible that similar to resveratrol acting via the gastrointestinal tract and a subsequent neuronal network, oral metformin treatment can remotely alter glucose homeostasis via a gut-brain-liver axis. Although the precise mechanisms mediating this gut-brain signaling pathway remain unknown, the fact that duodenal lipids lower hepatic glucose production via a gut-brain-liver axis involving the gut peptide CCK, coupled with the fact that metformin can alter the release of another gut peptide, GLP-1, leads to the possibility that metformin could promote gut peptide release and activation of their respective receptors on local sensory nerve afferents to signal via a gut-brain-liver axis to lower Gp.

Similar to resveratrol activating intestinal AMPK as described previously, metformin treatment of excised intestines increases phosphorylation of AMPK\textsuperscript{157}. As we demonstrate that resveratrol’s improvement of peripheral insulin sensitivity is SIRT1 and AMPK dependent, it is possible that metformin signals through a similar AMPK-SIRT1 pathway. Interestingly, despite the overlap in the signaling pathways of these two treatments, such that both drugs potentially activate both intestinal SIRT1 and AMPK, resveratrol and metformin could target divergent glucoregulatory mechanisms. Thus, while resveratrol enhances peripheral insulin sensitivity to lower GP, metformin could activate a unique and separate gut-brain-liver neuronal pathway that
additionally lowers GP, similar to what has been shown with lipids. Therefore, this thesis lays the groundwork for the potential development of specific gut targeted treatments that could simultaneously activate intestinal energy sensor proteins, SIRT1 and AMPK, to lower GP via distinct independent neuronal pathways, thus overcoming commonly observed therapeutic developed resistances, by additively or synergistically lowering GP production to improve glycemia.


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