Improving Glucose Metabolism With Resveratrol in a Swine Model of Metabolic Syndrome Through Alteration of Signaling Pathways in the Liver and Skeletal Muscle

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Hypothesis: We hypothesized that supplemental resveratrol would affect glucose metabolism in the skeletal muscle and liver to improve blood glucose control.

Design: Case-control study.

Setting: Hospital laboratory.

Subjects: Yorkshire miniswine.

Intervention: The swine developed metabolic syndrome by consuming a high-calorie, high-fat/cholesterol diet for 11 weeks. Pigs were fed either a normal diet (control) (n=7), a hypercholesterolemic diet (HCC) (n=7), or a hypercholesterolemic diet with supplemental resveratrol (100 mg/kg/d) (HCRV) (n=7). Animals underwent dextrose challenge prior to euthanasia and tissue collection.

Main Outcome Measures: Measurements of glucose and insulin levels, skeletal muscle and liver protein expression, and liver function test results.

Results: The HCC group had significantly increased blood glucose levels at 30 minutes as compared with the control and HCRV groups. The HCC group demonstrated increased fasting serum insulin levels and levels of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase. Oil red O staining demonstrated increased lipid deposition in the livers of the HCC animals. Immunoblotting in the liver showed increased levels of mammalian target of rapamycin, insulin receptor substrate 1, and phosphorylated AKT in the HCRV group. Immunoblotting in skeletal muscle tissue demonstrated increased glucose transporter type 4 (Glut 4), peroxisome proliferating activation receptor γ coactivator 1α, peroxisome proliferator-activated receptor α, peroxisome proliferator-activated receptor γ, and phosphorylated AKT at threonine 308 expression as well as decreased retinol binding protein 4 in the HCRV group. Immunofluorescence staining for Glut 4 in the skeletal muscle demonstrated increased Glut 4 staining in the HCRV group compared with the HCC or control groups.

Conclusion: Supplemental resveratrol positively influences glucose metabolism pathways in the liver and skeletal muscle and leads to improved glucose control in a swine model of metabolic syndrome.

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cose metabolism, contributing to the development of T2DM.8
Caloric restriction often leads to weight loss and a modification of the adverse effects of obesity including reversal of insulin resistance/prediabetes.9 In the long-term, however, especially in obese individuals, the rate of maintained weight control is very low.10,11
Resveratrol (trans-3,4′,5-trihydroxystilbene), a polyphenol extract from plants, including red grapes, is thought to be the “heart-healthy” component of red wine.12 Resveratrol has been shown to biochemically simulate caloric restriction and to have a number of beneficial effects, including antioxidant and anticancer as well as antidiabetic properties.13,14 Small-animal studies have shown that resveratrol can restore insulin sensitivity and signaling in the skeletal muscle and liver as well as facilitate transport of cholesterol from peripheral tissue to the liver, ameliorating some of the dysfunctional lipid metabolism of T2DM.15,16
To date, there have been few studies performed in humans or clinically relevant large-animal models on the effects of resveratrol in metabolic syndrome. The aim of this article is to demonstrate the potential efficacy of resveratrol in a porcine model of metabolic syndrome. We hypothesize that supplemental resveratrol will increase sensitivity to insulin, normalize blood glucose levels, reduce weight gain, and prevent pathologic changes associated with metabolic syndrome through alterations in liver and skeletal muscle metabolic signaling.

METHODS

ANIMAL MODEL
All experiments were approved by the hospital institutional animal care and use committee. Animals were cared for in compliance with the Harvard Medical Area Institutional Animal Care and Use Committee and in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals.17

Yorkshire miniswine (Parsons Research, Amherst, Massachusetts) were fed 1 of 3 diets throughout the 11-week experiment. The first group was given 500 g of a hypercholesterolemic diet daily (HCC) (n=7) providing 2248 kcal/d composed of 4% cholesterol, 17.2% coconut oil, 2.3% corn oil, 1.5% sodium cholate, and 75% regular chow. A second group was fed the same hypercholesterolemic diet supplemented with 100 mg/kg/d of resveratrol (ChromaDex, Irvine, California) orally (HCRV) (n=7) on the third group of swine was fed regular chow (control) (n=7, 1824 kcal/d) and served as the control. Animals were observed during feeding to ensure complete consumption of food and supplement.

After 11 weeks, swine were anesthetized, the heart was exposed, and physiologic measurements were taken, followed by euthanasia. Heart, liver, and skeletal muscle samples were collected. Tissue samples were rapidly frozen in liquid nitrogen (molecular studies) or placed in formalin, 10% (immunohistochemistry studies).

INTRAVENOUS GLUCOSE TOLERANCE TEST

Intravenous glucose tolerance testing was performed prior to euthanasia. A fasting baseline blood glucose level was measured and then dextrose, 0.5 g/kg, was infused. Blood glucose levels were measured 30 minutes postinfusion.

LIVER FUNCTION AND SERUM INSULIN TESTS

Serum liver chemistry and fasting insulin level analyses were performed with a Beckman DXC 800 Chemistry Analyzer (Beckman Coulter, Brea, California).

WESTERN BLOT ANALYSIS

Whole-cell tissue sections were isolated from the homogenized tissue samples with radioimmunoprecipitation assay buffer (Boston BioProducts, Worcester, Massachusetts). Sixty micrograms of total protein were fractionated by 4% to 20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (Invitrogen, San Diego, California) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, Massachusetts). Membranes were incubated with specific antibodies. Expression levels of a protein that increases the expression of molecules important to metabolic homeostasis, peroxisome proliferator-activated receptor γ (PPAR-γ) (Cayman Chemical, Ann Arbor, Michigan), was assessed. Expression of retinol binding protein 4 (RBP4) (Abnova, Taipei City, Taiwan), a protein shown to inhibit proper insulin signaling, as well as glucose transporter type 4 (Glu4), a protein that transports glucose across the cytoplasmic membrane of skeletal muscle, were assessed. Expression of phosphorylated AKT (phospho-AKT) at threonine 308 (T308) and serine 473 (S473), involved in stimulating translocation of Glu4 to the cell membrane, were assessed. The level of insulin receptor substrate 1 (IRS-1), which when stimulated by insulin activates a signaling cascade through phospho-AKT resulting in improved glucose transport into the cell, was determined. Expression of PPAR-α, which increases insulin sensitivity, and mammalian target of rapamycin (mTOR) (Cell Signaling Technology, Danvers, Massachusetts), which helps activate phospho-AKT to diminish the amount of glucose released from the liver, were assessed. The membranes were incubated for 1 hour in diluted appropriate secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania). Immune complexes were visualized with the enhanced chemiluminescence detection system (Amersham, Piscataway, New Jersey). Bands were quantified by densitometry digital images taken of the membranes (Syngene, Frederick, Maryland). Ponceau staining was used to ensure equal protein loading.

IMMUNOHISTOCHEMICAL ANALYSIS

Frozen tissue sections (12 µm in thickness) were equilibrated at room temperature and then fixed for 20 minutes in formalin at ~20°C. Sections were blocked with bovine serum albumin, 1%, in phosphate-buffered saline for 1 hour at room temperature, labeled with anti-Glut 4 antibody (Epitomics, Burlingame, California), and incubated overnight at 4°C. Sections were incubated with a 1:100 dilution of secondary antibody (DyLight 488-conjugated antirabbit; Jackson ImmunoResearch) in the dark for 45 minutes, then mounted in Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame). Images were obtained with a Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan) at ×100 and ×200 magnification.

HEMATOXYLIN-EOSIN STAINING AND OIL RED O STAINING IN THE LIVER

Hematoxylin staining and eosin counterstaining as well as oil red O staining for lipids counterstained with hematoxylin were performed and on frozen liver sections in a standard fashion by the Pathology and Histology core facility at Rhode Island Hospital.
Table. Weight, Liver Function, and Blood Glucose Clinical Data Obtained From Resveratrol-Treated and Untreated Swine

<table>
<thead>
<tr>
<th>Data</th>
<th>Control Group</th>
<th>HCC Group</th>
<th>HCRV Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prediet weight, kg</td>
<td>18.14 (1.65)</td>
<td>18.61 (0.796)</td>
<td>17.23 (0.7150)</td>
<td>.60</td>
</tr>
<tr>
<td>Postdiet weight, kg</td>
<td>29.66 (1.541)</td>
<td>38.46 (1.024)</td>
<td>31.02 (0.673)</td>
<td>&lt;.001b</td>
</tr>
<tr>
<td>Core body temperature, °C</td>
<td>37.44 (0.302)</td>
<td>37.83 (0.333)</td>
<td>37.60 (0.351)</td>
<td>.69</td>
</tr>
<tr>
<td>Baseline glucose level, mg/dL</td>
<td>32.33 (6.835)</td>
<td>59.00 (6.156)</td>
<td>52.80 (5.276)</td>
<td>.03b</td>
</tr>
<tr>
<td>30-min blood glucose level, mg/dL</td>
<td>119.7 (7.740)</td>
<td>171.0 (5.682)</td>
<td>132.4 (14.11)</td>
<td>&lt;.001b</td>
</tr>
<tr>
<td>Serum insulin level, ng/mL</td>
<td>0.046 (0.002)</td>
<td>0.099 (0.01)</td>
<td>0.053 (0.009)</td>
<td>.002b</td>
</tr>
<tr>
<td>Liver function levels, mg/dL</td>
<td>19.92 (1.270)</td>
<td>29.70 (1.2210)</td>
<td>25.10 (1.337)</td>
<td>&lt;.001b</td>
</tr>
<tr>
<td>AST</td>
<td>42.86 (1.895)</td>
<td>57.17 (4.929)</td>
<td>41.00 (4.017)</td>
<td>.01b</td>
</tr>
<tr>
<td>ALT</td>
<td>113.3 (4.497)</td>
<td>146.8 (10.72)</td>
<td>116.2 (10.68)</td>
<td>.03b</td>
</tr>
<tr>
<td>ALP</td>
<td>4.300 (0.111)</td>
<td>4.500 (0.123)</td>
<td>4.383 (0.172)</td>
<td>.57</td>
</tr>
<tr>
<td>Total protein</td>
<td>1.189 (0.026)</td>
<td>1.333 (0.042)</td>
<td>1.200 (0.045)</td>
<td>.03b</td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hypercholesterolemic diet; HCRV, hypercholesterolemic diet with supplemental resveratrol.

SI conversion factor: To convert glucose to nanomoles per liter, multiply by 0.0555.

Clinical parameters, including weight, blood glucose levels, and liver function data, are shown from the HCC group, HCRV group, and control animals.

Significant.

DATA ANALYSIS

All results are expressed as mean (SEM). Western blots were analyzed using National Institutes of Health ImageJ 1.40g software (National Institutes of Health, Bethesda, Maryland). Comparisons between groups were analyzed by 1-way analysis of variance with a Newman-Keuls multiple comparison post hoc test. A probability value of less than .05 was considered significant.

RESULTS

ANIMAL MODEL

All animals lived through the entire procedure. The swine had similar body weights at baseline. Immediately prior to euthanasia, the animals in the HCC group had significantly increased body mass compared with controls (P < .001), while the HCRV animals had body weights similar to the control group. Core body temperature at euthanasia was similar among all 3 groups (Table).

INTRAVENOUS GLUCOSE CHALLENGE TESTING

At baseline, all groups were within normal ranges of blood glucose level, despite elevation of both the HCC and HCRV groups (T308) over the control group (P < .05 for both). Thirty minutes after dextrose infusion, the HCC group had significantly higher blood glucose levels than both the HCRV and control groups (P < .001), with no significant difference between the HCRV and control groups (mean [SEM], 171.0 [5.68] mg/dL in the HCC group, 119.7 [7.74] mg/dL in the control group, and 132.4 [14.11] mg/dL in the HCRV group [to convert glucose to nanomoles per liter, multiply by 0.0555]) (Table).

SKELETAL MUSCLE PROTEIN EXPRESSION

Expression of Glut 4, peroxisome proliferating activation receptor γ coactivator 1α (PGC-1α), and phospho-AKT (T308), proteins involved in glucose metabolism, were all significantly elevated in the HCRV group compared with the HCC and control groups (P < .001, P < .001, and P = .03, respectively). Peroxisome proliferator-activated receptor α and PPAR-γ were more highly expressed in the control group compared with the HCC group (P = .04 and P = .02, respectively). Retinol binding protein 4, a protein implicated in glucose intolerance, was expressed most highly in the HCC group (P = .01) (Figure 1).

LIVER PROTEIN EXPRESSION

The ratio of phospho-AKT (T308) to total AKT was significantly higher in the HCRV group compared with the HCC group (P = .008). Mammalian target of rapamycin expression was elevated in the HCC group over the control group and the HCRV group over the HCC group, but neither difference was significant (P > .05 in both cases). Insulin receptor substrate 1 expression in the HCRV group was significantly higher than both the control group and the HCC group (P < .05 for both) (Figure 2).

LIVER FUNCTION TEST MEASUREMENTS

Markers of liver function (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, and albumin) were similar between the control and HCRV groups. The HCC group had higher levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and albumin compared with both the control and HCRV groups (Table).

FASTING SERUM INSULIN

Fasting insulin levels were measured at the culmination of the experiment, prior to euthanasia, and we found that HCC animals had significantly higher insulin levels compared with the control and HCRV groups, which were statistically similar (Table).
IMMUNOHISTOCHEMICAL ANALYSIS OF SKELETAL MUSCLE PROTEINS

The HCC group demonstrated the least intense staining for membrane-bound Glut 4 of the 3 groups. The control group was similar in that there were small clusters of positively stained cells, but there were more of these clusters in the control group compared with the HCC group. In contrast, almost all HCRV skeletal muscle cells stained positively for membrane-bound Glut 4 (Figure 3).

LIVER TISSUE HISTOLOGIC ANALYSIS

There was no morphological difference between the 3 groups when the liver tissue was stained with hematoxylin-eosin and examined for any evidence of inflammatory cell infiltrate, oxidative stress, steatosis, or cirrhosis (data not shown).
OIL RED O STAINING

The control group demonstrated very limited lipid deposition in the liver. The HCRV group showed increased amounts of lipid, and the HCC group showed the highest level of hepatic lipid deposition (Figure 4).

COMMENT

WEIGHT CONTROL

Weight control is a major component in the treatment of T2DM as well as the other comorbidities associated with metabolic syndrome. A possible mechanism for resveratrol-induced weight control is through the activation of SIRT1. Mimicking the pathway activated by caloric restriction, resveratrol is thought to interact with SIRT1 to activate PGC-1α, which increases the use of cellular energy stores in brown adipose tissue and skeletal muscle. Peroxisome proliferating activation receptor γ coactivator 1α activation leads to uncoupling of adenosine triphosphate production from the electron transport chain across the membrane of the mitochondria, ultimately burning calories by thermogenesis. Mice and rats have large stores of brown adipose tissue as juveniles and adults whereas pigs and humans have almost

Figure 3. Skeletal muscle immunofluorescence staining for glucose transporter 4 (Glut 4) (original magnification ×100). Green staining represents Glut 4 (second column) and blue staining is for all nuclei (third column). A, Composite image of B and C of control animal. D, Composite image of E and F of hypercholesterolemic diet (HCC) animal. G, Composite image of H and I of hypercholesterolemic diet with supplemental resveratrol (HCRV) animal. The HCRV animal shows higher levels of membrane Glut 4 staining than the control and HCC animals.
none as adults and rely on skeletal muscle for thermogenesis. Resveratrol has been found to induce the conversion of nonoxidative fast-twitch skeletal muscle into oxidative endurance muscles that are capable of providing thermogenesis.\cite{14,19} Although thermogenesis in mice and rats often raises body temperature, we did not observe this in the pig model, likely because of an increased body mass to surface area ratio, which allows for more efficient dissipation of heat.

**BLOOD GLUCOSE CONTROL**

In response to the intravenous glucose tolerance testing, only the HCC group developed blood glucose levels higher than the threshold of glucose intolerance (160 mg/dL). This effect of the high-cholesterol diet was prevented by resveratrol supplementation. Serum insulin levels were also significantly reduced in the HCRV group. A hallmark of T2DM is an increase in circulating insulin level as compensation for peripheral insulin resistance. Decreased fasting serum insulin concentration in the HCRV group suggests an absence of insulin resistance.

**SKELETAL MUSCLE GLUCOSE METABOLISM**

Retinol binding protein 4 is a peptide that is produced in adipose tissue and signals other tissues.\cite{20} One of the many links between obesity and insulin resistance, RBP4 inhibits insulin signaling in skeletal muscle by impairing downstream signaling of phosphoinositide 3-kinase. A 2006 study by Graham et al\cite{21} showed a linear correlation between a patient’s body mass index and levels of RBP4 and an inverse relationship with insulin sensitivity. Similarly, we found significantly increased levels of RBP4, increased body weight, and impaired glucose tolerance in the HCC group compared with the control group. Resveratrol treatment maintained RBP4 levels in the HCRV group to that of the control group, further supporting the antidiabetic properties of the supplement.

Skeletal muscle clears 80% of circulating glucose from the blood primarily via Glut 4. Regulation of Glut 4 expression is not entirely understood, but skeletal muscle Glut 4 levels are a good indicator of whole-body insulin sensitivity.\cite{22} Several proteins have been shown to upregulate the expression of Glut 4, including insulin\cite{23} and PGC-1α.\cite{22} Peroxisome proliferating activation receptor γ coactivator 1α, a SIRT1-activated protein, is involved in mitochondrial metabolism and oxidation of energy stores and has been linked to increased insulin sensitivity.\cite{22} Our findings that PGC-1α and Glut 4 levels are increased in resveratrol-treated animals are consistent with previous studies that demonstrated upregulation of PGC-1α and Glut 4 expression by resveratrol.\cite{14,19} Proper insulin signaling is required for the translocation of Glut 4 from intracellular vesicles to the cell membrane, where it becomes active.\cite{24,25} This is accomplished mainly through phosphorylation of AKT at T308,\cite{26} which we found to be significantly increased in the HCRV group compared with the control and HCC groups. Predictably, we also saw a striking increase in the amount of membrane-bound Glut 4 in the HCRV group compared with both the control and HCC groups, suggesting improved translocation of Glut 4 to the cell membrane in these animals and increased ability to absorb circulating glucose.

We also investigated PPAR-α and PPAR-γ, nuclear receptors that regulate the gene expression of important metabolic proteins such as IRS-2. Peroxisome proliferator-activated receptor α has been shown to reduce levels of...
Thus, PPAR-α mediates lipid deposition in peripheral tissue that contributes to insulin resistance; which also increases insulin sensitivity. Peroxisome proliferator-activated receptor (PPAR) expression of proteins important to metabolic homeostasis, such as IRS-2, increases insulin sensitivity. Peroxisome proliferator-activated receptor (PPAR) expression of Glut 4 and activates PPAR-α.

Insulin resistance reduces the expression of these molecules. Peroxisome proliferator-activated receptor (PPAR) decreases levels of PPAR-α in the HCC group, which was attenuated by resveratrol treatment in the HCRV group. It has been suggested that resveratrol reduces lipid peroxidation, protecting the liver against oxidative damage.

Insulin resistance leads to hepatocellular fat deposition and liver damage, including nonalcoholic steatohepatitis and cirrhosis. However, in our study, there were no differences in hepatic histology between the groups on hematoxylin-eosin staining. A possible explanation is that the course of the study was too short for hepatic cellular damage to achieve visible levels. A 2006 study in mice conducted over 110 weeks described significant fatty liver pathology, which was completely abrogated by resveratrol treatment. On the other hand, there were differences in lipid deposition between groups on oil red O staining. Resveratrol treatment appeared to reduce hepatic lipid accumulation to normcholesterolemic control levels, likely contributing further to the protective effects of resveratrol.

HEPATOCELLULAR EFFECTS OF RESVERATROL

Free fatty acids, which are increased by a high-fat diet, are known to be directly toxic to hepatocytes, causing mitochondrial dysfunction and membrane damage. As expected, we observed an elevation in liver transaminase levels in the HCC group, which was attenuated by resveratrol treatment in the HCRV group. It has been suggested that resveratrol reduces lipid peroxidation, protecting the liver against oxidative damage.

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HEPATIC PROTEIN EXPRESSION

Resveratrol influenced a number of pathways regulating glucose metabolism in the liver. Insulin receptor substrate 1 (IRS-1) is a key regulator of insulin signaling. When stimulated by insulin, activates phosphoinositide 3-kinase (PI3K), which then activates AKT by phosphorylation at threonine 308 (phosphorylated AKT (pAKT)). Resveratrol also increases the expression of mammalian target of rapamycin (mTOR), which activates AKT by phosphorylation at serine 473. Activation of AKT enhances glycosynthetic activity by inhibiting its inhibitor, glycogen synthase kinase 3 (GSK3). Increased glycogen synthesis activity increases glycogen production, facilitating increased movement of glucose into the liver from the circulation and reducing blood glucose levels. AKT activation also signals for forkhead box O1 (FOXO1) to be transported out of the nucleus. This prevents transcription of proteins needed for gluconeogenesis and thus reduces the level of gluconeogenesis, which diminishes the amount of glucose released from the liver, also reducing the blood glucose level.
phosphorylation of forkhead box O1, a nuclear transcription factor, and glycogen synthase kinase 3. Following phosphorylation, forkhead box O1 moves out of the nucleus and suppresses the expression of certain proteins that promote gluconeogenesis.32 Phosphorylation of glycogen synthase kinase 3 removes its constitutive inactivation of glycogen synthase. Thus, through inhibition of glycogen synthase kinase 3, phospho-AKT enhances synthesis of glycogen and reduces hyperglycemia.33 Through these 2 pathways, IRS-1, mTOR, and phospho-AKT enhance insulin sensitivity and reduce the circulating blood glucose level. We found that all 3 of these targets were more highly expressed in the HRVC group than the HCC group, suggesting a mechanism by which resveratrol prevents insulin resistance in the liver.

LIMITATIONS OF THE STUDY

This study was performed in a porcine model, which, while it more closely approximates human metabolic response than do smaller-animal models, still represents a different species. Additionally, the relatively short time frame of the study may have limited the long-term effects of the high-cholesterol diet. Furthermore, we investigated only 1 dose of resveratrol, which has been shown to have varying effects at different doses.34

CONCLUSIONS

In a porcine model of metabolic syndrome, we found that resveratrol counteracts the obesity, weight gain, and insulin resistance induced by a high-calorie, high-fat diet. Resveratrol accomplishes this by influencing several insulin-dependent metabolic pathways in skeletal muscle and the liver. We believe that resveratrol is a supplement that has the potential to be a novel pharmacological agent for the prevention of diet-induced insulin resistance, T2DM, and metabolic syndrome.

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Author Contributions: Mr Burgess and Dr Robich had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Robich and Sellke. Acquisition of data: Burgess and Chu. Analysis and interpretation of data: Burgess, Robich, Bianchi, and Sellke. Drafting of the manuscript: Burgess and Sellke. Critical revision of the manuscript for important intellectual content: Burgess, Robich, Chu, Bianchi, and Sellke. Statistical analysis: Burgess, Robich, and Chu. Obtained funding: Sellke. Administrative, technical, and material support: Chu, Bianchi, and Sellke. Study supervision: Robich, Chu, Bianchi, and Sellke.

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REFERENCES


