Resveratrol supplementation improves white adipose tissue function in a depot-specific manner in Zucker diabetic fatty rats

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Beaudoin M, Snook LA, Arkell AM, Simpson JA, Holloway GP, Wright DC. Resveratrol supplementation improves white adipose tissue function in a depot-specific manner in Zucker diabetic fatty rats. Am J Physiol Regul Integr Comp Physiol 305: R542–R551, 2013. First published July 3, 2013; doi:10.1152/ajpregu.00200.2013.—Resveratrol (RSV) is a polyphenolic compound suggested to have anti-diabetic properties. Surprisingly, little is known regarding the effects of RSV supplementation on adipose tissue (AT) metabolism in vivo. The purpose of this study was to assess the effects of RSV on mitochondrial content and respiration, glyceroneogenesis (GNG), and adiponectin secretion in adipose tissue from Zucker diabetic fatty (ZDF) rats. Five-week-old ZDF rats were fed a chow diet with (ZDF RSV) or without (ZDF chow) RSV (200 mg/kg body wt) for 6 wk. Changes in adipose tissue metabolism were assessed in subcutaneous (scAT) and intra-abdominal [retroperitoneal (rpWAT), epididymal (eWAT)] adipose tissue depots. ZDF RSV rats showed lower fasting glucose and higher circulating adiponectin, as well as lower glucose area under the curve during intraperitoneal glucose and insulin tolerance tests than ZDF chow. [14C]pyruvate incorporation into triglycerides and adiponectin secretion were higher in scAT from ZDF RSV rats, concurrent with increases in adipose tissue triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL) (12). Furthermore, phosphorylation of perilipin is necessary to allow access of lipases to the lipid droplet (12). RSV-mediated lipolysis is associated with increases in ATGL protein content (25) and cAMP (41), an upstream activator of HSL via the activation of protein kinase A (PKA).

Importantly, up to 35% of FFA liberated through lipolysis is recycled back into triglyceride (TG) within the adipocyte (48), and this is dependent on the provision of glycerol-3-phosphate (G3P) through nonglucose precursors, such as pyruvate, in a process called glyceroneogenesis (GNG) (7). GNG is regulated through the concerted actions of pyruvate dehydrogenase kinase 4 (PDH) (Ser293) protein content in this depot. Moreover, uncoupled mitochondrial respiration and complex I and II-supported respiration were increased in both scAT and rpWAT, which correlated with increases in cytochrome c oxidase subunit IV (COX4) protein content. In vitro treatment of scAT with RSV (50 μmol/l; 24 h) induced pyruvate dehydrogenase kinase 4 (PDH4) and peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) mRNA expression. Collectively, these data demonstrate that RSV can induce adipose tissue mitochondrial biogenesis in parallel with increases in GNG and adiponectin secretion.

mitochondrial biogenesis; glyceroneogenesis; glucose homeostasis; adipose tissue; rat

RESVERATROL (RSV; 3,5,4′-trihydroxy-trans-stilbene) is a polyphenolic compound found in plants, especially in the skins of red grapes. Its supplementation to rodents fed a high-fat diet improves insulin sensitivity (23) and life span (4). In humans, small beneficial metabolic effects of RSV have been reported in obese subjects (42) and patients with Type 2 diabetes (T2DM) (5) but not in individuals with normal glucose tolerance (54). While the efficacy of RSV in humans remains controversial, the beneficial effects of RSV in rodents have been mostly attributed to enhanced mitochondrial biogenesis (23, 30) and activation of proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) (23, 32, 43) in skeletal muscle.

While most investigations focus on the effects of RSV on skeletal muscle, an emerging body of evidence suggests that adipose tissue may also be responsive to RSV treatment. In addition to the anti-inflammatory effects of RSV on adipose tissue (47), RSV has been shown to have anti-adipogenic effects (3, 9, 22) and to increase lipolysis in adipocytes in vitro (25, 31, 41). Lipolysis is a key process in the provision of free fatty acids (FFA) and occurs through the hydrolysis of fatty acids from the glycerol backbone through sequential reactions by adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL) (12). Furthermore, phosphorylation of perilipin is necessary to allow access of lipases to the lipid droplet (12). RSV-mediated lipolysis is associated with increases in ATGL protein content (25) and cAMP (41), an upstream activator of HSL via the activation of protein kinase A (PKA).

Importantly, up to 35% of FFA liberated through lipolysis is recycled back into triglyceride (TG) within the adipocyte (48), and this is dependent on the provision of glycerol-3-phosphate (G3P) through nonglucose precursors, such as pyruvate, in a process called glyceroneogenesis (GNG) (7). GNG is regulated through the concerted actions of pyruvate dehydrogenase kinase 4 (PDH4) and phosphoenolpyruvate carboxykinase (PEPCK). In mice, the knockdown of pck1, the gene coding for PEPCK, results in whole body insulin resistance and increased FFA and glycerol release during a euglycemic-hyperinsulinemic clamp (27). Conversely, overexpression of PEPCK in adipose tissue led to obesity without insulin resistance (13). Absolute rates of fatty acid (FA) reesterification mirror that of lipolysis (4), and the reesterification of FAs is one of the largest consumers of ATP in fat cells (34). In fact, work from Ruderman’s group (15) has demonstrated that the activation of the energy-sensing enzyme 5′-AMP-activated protein kinase (AMPK) by β-adrenergic agonists occurs secondary to increases in FA reesterification. AMPK is a reputed mediator of mitochondrial biogenesis (14, 17) and thus RSV-mediated increases in ATGL and lipolysis may lead to increases in FA reesterification, the activation of AMPK, and the induction of mitochondrial biogenesis in adipose tissue. In support of this premise, adipose tissue-specific overexpression of ATGL in mice resulted in increased lipolysis without elevations in plasma FFA, TG, or ectopic lipid deposition, along with increases in whole body TG cycling and adipose tissue mitochondrial enzyme gene expression (1). To the best of our knowledge, it is currently unknown whether RSV can induce mitochondrial biogenesis and increase GNG in adipose tissue in vivo.

Adiponectin is an anti-inflammatory adipokine that stimulates FA oxidation in skeletal muscle and liver and is associated with improved insulin sensitivity (20, 52). RSV treatment increased adiponectin mRNA in human adipocytes isolated...
from visceral depots (11) and elevated adiponectin protein content in 3T3-L1 adipocytes (47) and in rodent epidymal and retroperitoneal adipose tissue (33). Interestingly, mitochondrial biogenesis has been shown to be essential for adiponectin synthesis and secretion in adipocytes (20) and thus, it seems plausible that RSV-mediated increases in mitochondrial biogenesis could be associated with increases in adiponectin secretion. Conversely, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are pro-inflammatory adipokines that are negatively regulated by RSV in vitro (2, 18), but the effects of RSV on these cytokines in vivo remain to be elucidated.

The purpose of the current study was to examine the effects of dietary RSV supplementation on mitochondrial biogenesis, GNG, and adipokines secretion in white adipose tissue (WAT) from Zucker diabetic fatty (ZDF) rats, a rodent model of T2DM. In addition, as RSV is known to improve skeletal muscle metabolism and whole body glucose homeostasis (4, 23), it was important to determine whether RSV may have direct effects on adipose tissue. To address this question, we used an acute ex vivo approach to assess gene expression in adipose tissue explants from untreated ZDF rats. We hypothesized that RSV supplementation in ZDF rats would induce mitochondrial biogenesis, which would be associated with enhanced GNG, increased adiponectin secretion, and the prevention of insulin resistance. Furthermore, these effects could be mediated, at least in part, by direct actions of RSV onto adipose tissue.

METHODS

Animals. Four-week-old male ZDF rats (Charles River, St. Constant, QC, Canada) weighing ~100 g were individually housed in wire-bottom cages, in a temperature-controlled room with a reverse 12:12 h light-dark cycle, and were provided with food and water ad libitum. The 12-h light cycle started at 9 PM, and experiments were performed between 9 AM and 4 PM. After a 10-day acclimatization period, ZDF rats were fed either a standard powdered chow diet (Purina 5008 diet; Purina, St. Louis, MO) or a chow diet supplemented with RSV (Cayman Chemical, Ann Arbor, MI) ~200 mg/kg body wt (BW) (ZDF RSV) for 6 wk. RSV was mixed directly into the powered diet on a weekly basis, based on predicted body weight and food intake for that week. ZDF rats are characterized by the development of insulin resistance by 6 wk of age and T2DM by 12 wk, therefore, we attempted to delay the onset T2DM by treating with RSV between 5 and 11 wk of age (39). We acknowledge that the ZDF model is a genetic model of T2DM and that the dose of RSV used in this study is higher than that typically used in rodent models. However, this study was designed to determine whether RSV can impair mitochondrial biogenesis, GNG, and adiponectin secretion. Retinoic acid (RA) is known to induce mitochondrial biogenesis, which may be relevant to the present study, but the effects of RA on GNG and adiponectin secretion remain to be elucidated. Therefore, we chose to use a genetic model of T2DM to study the effects of RSV on mitochondrial biogenesis, GNG, and adiponectin secretion in vivo.

Adipose tissue respiration was determined as previously described (21), with minor modifications (46). Briefly, adipose tissue was excised, immediately placed in polypropylene tubes containing 2 ml oxygenated Krebs-Ringer buffer (KRB; 118 mM NaCl, 4.8 mM KCl, 1.25 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3; pH 7.4) containing 5 mM glucose and 2.5% FFA-free bovine serum albumin (BSA; MP Biomedicals, Santa Ana, CA) and incubated at 37°C in a shaking water bath (60 rpm) for 2 h. At the end of the incubation, medium was collected in polypropylene tubes and stored at −20°C for later determination of protein content and gene expression. Blood was collected in polypropylene tubes through cardiac puncture, allowed to clot at room temperature, and centrifuged, and the supernatant was collected and stored at −80°C for further analysis of triglycerides (colorimetric assay: Sigma-Aldrich, Oakville, ON, Canada), FFAs (colorimetric assay: Wako Diagnostics, Richmond, VA), total adiponectin (ELISA: Linco Research, St. Charles, MO), insulin (ELISA: Millipore, St. Charles, MO), IL-6 (ELISA: BioLegend, San Diego, CA), and TNF-α (ELISA: BioLegend) through commercially available kits.

Ex vivo incubation procedures. Adipose tissue (100 mg) from each of scAT, eWAT, and rpWAT was minced and placed in a plastic vial containing 3 ml oxygenated Krebs-Ringer buffer (KRB; 118 mM NaCl, 4.8 mM KCl, 1.25 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3; pH 7.4) containing 5 mM glucose and 2.5% FFA-free bovine serum albumin (BSA; MP Biomedicals, Santa Ana, CA) and incubated at 37°C in a shaking water bath (60 rpm) for 1 h. At the end of the incubation, medium was collected in polypropylene tubes and stored at −20°C for later determination of total adiponectin, IL-6, and TNF-α using commercially available kits (see providers above).

14C-[14C]pyruvate incorporation into triglycerides. Quantification of the incorporation of [14C]pyruvate into triglycerides is a measure of GNG within adipose tissue (24). Adipose tissue (100 mg) from each of scAT, eWAT, and rpWAT was minced and placed in a plastic vial containing 2 ml oxygenated KRB containing 5 mM pyruvate and 2% FFA-free BSA, and 1 μCl of [14C]pyruvate (PerkinElmer, Waltham, MA) was added to all vials. Tissue was incubated at 37°C in a shaking water bath (60 rpm) for 1 h. At the end of the incubation, the reaction was stopped by adding 250 μl of 5 N sulfuric acid (Fisher Scientific, Ottawa, ON, Canada) into the vials and incubating them on ice for 30 min. Adipose tissue explants were thoroughly rinsed with water and methanol, transferred to new tubes for lipid extraction with 5 ml of a 2:1 solution of chloroform:methanol (both from Fisher Scientific). One milliliter of chloroform:methanol (both from Fisher Scientific) was added, and [14C] radiation was measured. Chloroform:methanol solution was allowed to evaporate overnight in a fume hood, and the remaining lipid extract was acetylated with 10 μl of acetic anhydride and 10 μl of pyridine. The acetylated lipid extracts were then used for quantification of triglycerides.

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

Respiration. Adipose tissue respiration was determined as previously described (21), with minor modifications (46). Briefly, adipose tissue was excised, immediately placed in polypropylene tubes containing 1 ml of MiR05 buffer (0.5 mM EGTA, 3 mM MgCl2-6H2O, 60 mM K-lactobionate, 10 mM KH2PO4, 20 mM HEPES, 20 mM taurine, 110 mM sucrose, 1 μl FFA-free BSA; pH 7.1), and minced with scissors. Adipose tissue was then transferred to 1 ml of fresh MiR05 buffer and left on ice for 10 min. Thereafter, tissue was blotted and 25 mg used to determine rates of oxygen consumption by high-resolution respirometry (Oroboros Oxygraph-2 k, Innsbruck, Austria). Digitonin or saponin are used to permeabilize cell membranes while leaving mitochondrial membranes intact due to their specificity to solubilize cholesterol, which exists in much higher concentrations on the plasma membrane. This is a common procedure to permit exogenously added substrates free access for diffusion to the mitochondria. However, we found mincing without permeabilization...
at 37°C with 5% CO2 for 1 h. Adipose tissue was then placed in a Petri dish with medium 199 (Sigma-Aldrich, Oakville, ON, Canada) in a homogenizer (FastPrep-24, MP Biomedicals). Homogenized samples were centrifuged for 10 min at 1,500 g at 4°C. Lipids were carefully removed, and the protein-containing infranatant was collected for determination of protein concentration using a bicinchoninic acid assay (37) (ThermoScientific, Rockford, IL). Western blotting was performed as previously described by our laboratory (40, 44, 45).

Membranes were incubated in primary antibodies diluted in TBST/5% nonfat dry milk (COX4, MitoSciences, Eugene, OR; α-tubulin, perilipin A: Abcam, Toronto, ON, Canada; ATGL, p-HSL[Ser660]: Cell Signaling, Danvers, MA) or TBST/5% bovine serum albumin [p-PDH (Ser293), uncoupling protein-1 (UCP-1): Calbiochem, Missisauga, ON, Canada; PDH, MitoSciences; PEPC, Camyan Chemicals, Ann Arbor, MI; HSL, p-HSL[Ser563], AMPK, p-AMPK-[Thr172]: Cell Signaling; diglyceride acyltransferase (DGAT1), glycerolphosphate acyltransferase (GPAT): Abcam; DGAT2: Immunex, San Diego, CA) overnight at 4°C. Subsequently, membranes were washed in TBST and incubated in appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) diluted in TBST/1% nonfat dry milk for 1 h at room temperature. Signal was detected through enhanced chemiluminescence (ThermoScientific) and quantified by densitometry (Fluorchem HD2, ProteinSimple, Santa Clara, CA). Intracellular cAMP content of scAT and rpWAT was determined by ELISA according to the manufacturer’s instructions (Enzo Life Sciences, Brockville, ON, Canada).

Table 1. Body and organ weights and fasting blood parameters at euthanasia for ZDF chow and ZDF RSV animals

<table>
<thead>
<tr>
<th></th>
<th>ZDF Chow (n = 11)</th>
<th>ZDF RSV (n = 13)</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>381 ± 7</td>
<td>383 ± 5</td>
</tr>
<tr>
<td>Average daily food</td>
<td>28.7 ± 1.0</td>
<td>27.3 ± 0.9</td>
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<tr>
<td>intake, g</td>
<td></td>
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<tr>
<td>scAT (inguinal) weight, g</td>
<td>21.1 ± 1.5</td>
<td>22.0 ± 1.3</td>
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<tr>
<td>eWAT weight, g</td>
<td>7.2 ± 0.4</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>rpWAT weight, g</td>
<td>5.2 ± 0.2</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>14.2 ± 0.6</td>
<td>13.1 ± 0.5</td>
</tr>
<tr>
<td>Fasting glucose, mmol/l</td>
<td>9.3 ± 1.6</td>
<td>5.1 ± 0.2*</td>
</tr>
<tr>
<td>Fasting insulin, pmol/l</td>
<td>723 ± 145</td>
<td>1101 ± 208</td>
</tr>
<tr>
<td>Fasting FFa, pmol/l</td>
<td>1.36 ± 0.16</td>
<td>1.40 ± 0.14</td>
</tr>
<tr>
<td>Fasting glycerol, mmol/l</td>
<td>0.45 ± 0.03</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>FA:glycerol ratio</td>
<td>3.0 ± 0.3</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>Fasting TG, mmol/l</td>
<td>4.25 ± 0.43</td>
<td>3.90 ± 0.54</td>
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Values are means ± SE; n, number of animals; ZDF, Zucker diabetic fatty rats; RSV, resveratrol; scAT, subcutaneous adipose tissue; eWAT, epididymal adipose tissue; rpWAT, retroperitoneal adipose tissue; FFA, free fatty acid; TG, triglycerides. *P < 0.05 compared with ZDF chow.

To be sufficiently effective in disrupting the cell membrane without compromising mitochondrial intactness, Specifically, robust respiration was detected in minced adipose tissue and this was not increased with the addition of either digitonin or saponin following standard permeabilizing procedures. This has also been reported previously (21). Therefore, all experiments were performed in the absence of either chemical. Respiration experiments were performed at 37°C in room air-saturated MiR05 buffer. The sequential respiration protocol consisted of determining state IV (leak) respiration in the presence of 10 mM glutamate + 2 mM malate, state III respiration in the presence of 5 mM ADP (complex I), and 10 mM succinate (complex I and II). The subsequent titration of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) yielded maximal uncoupled respiration. Rates of oxygen consumption are expressed per milligram wet weight.

Histochemistry. scAT was fixed in 10% neutral buffered Formalin (VWR, Mississauga, ON, Canada) dehydrated in xylene (Fisher Scientific) and embedded in paraffin. We chose to further analyze only scAT as this depot responded most robustly to RSV supplementation (23) or vehicle (dimethyl sulfoxide) for 24 h. At the end of the incubation period, adipose tissue was rinsed in ice-cold PBS, strained, frozen in liquid nitrogen, and stored at −80°C for further gene expression analysis. We did not include epididymal adipose tissue in these experiments, because this depot did not respond to RSV supplementation in vivo.

Adipose tissue explant experiments. Adipose tissue explant incubation is a well-characterized technique that is used regularly to determine changes in adipose tissue metabolism and gene expression (40, 44, 45). To assess the direct effects of RSV on gene expression, scAT and rpWAT from untreated 12-wk-old ZDF rats were minced, placed in a Petri dish with medium 199 (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 2.5% FFA-free BSA, and incubated at 37°C with 5% CO2 for 1 h. Adipose tissue was then treated with RSV 50 μmol/L (23) or vehicle (dimethyl sulfoxide) for 24 h. At the end of the incubation period, adipose tissue was rinsed in ice-cold PBS, strained, frozen in liquid nitrogen, and stored at −80°C for further gene expression analysis. We did not include epididymal adipose tissue in these experiments, because this depot did not respond to RSV supplementation in vivo.

Protein content and cAMP determination. Adipose tissue samples were homogenized in 2 volumes of ice-cold cell lysis buffer (Invitrogen, Burlington, ON, Canada) supplemented with protease inhibitor cocktail (as per manufacturer’s instructions, Sigma-Aldrich) and 0.05% phenylmethanesulfonyl fluoride (BioShop, Burlington, ON, Canada) in a homogenizer (FastPrep-24, MP Biomedicals). Homogenized samples were centrifuged for 10 min at 1,500 g at 4°C. Lipids were carefully removed, and the protein-containing infranatant was collected for determination of protein concentration using a bicinchoninic acid assay (37) (ThermoScientific, Rockford, IL). Western blotting was performed as previously described by our laboratory (40, 44, 45). Membranes were incubated in primary antibodies diluted in TBST/5% nonfat dry milk (COX4, MitoSciences, Eugene, OR; α-tubulin, perilipin A: Abcam, Toronto, ON, Canada; ATGL, p-HSL[Ser660]: Cell Signaling, Danvers, MA) or TBST/5% bovine serum albumin [p-PDH (Ser293), uncoupling protein-1 (UCP-1): Calbiochem, Mississauga, ON, Canada; PDH, MitoSciences; PEPC, Camyan Chemicals, Ann Arbor, MI; HSL, p-HSL[Ser563], AMPK, p-AMPK-[Thr172]: Cell Signaling; diglyceride acyltransferase (DGAT1), glycerolphosphate acyltransferase (GPAT): Abcam; DGAT2: Immunex, San Diego, CA) overnight at 4°C. Subsequently, membranes were washed in TBST and incubated in appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) diluted in TBST/1% nonfat dry milk for 1 h at room temperature. Signal was detected through enhanced chemiluminescence (ThermoScientific) and quantified by densitometry (Fluorchem HD2, ProteinSimple, Santa Clara, CA). Intracellular cAMP content of scAT and rpWAT was determined by ELISA according to the manufacturer’s instructions (Enzo Life Sciences, Brockville, ON, Canada).
Real-time quantitative PCR. RNA was isolated from adipose tissue using an RNeasy lipid kit (Qiagen, Toronto, ON, Canada) according to the manufacturer’s directions. Total RNA (1 μg) was used to synthesize complementary DNA (cDNA) using SuperScript II Reverse Transcriptase, random primers, and dNTP (all from Invitrogen, Burlington, ON, Canada). Gene expression was quantified in duplicate using 1 μl cDNA template by quantitative PCR (qPCR) on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the appropriate gene expression assay (Applied Biosystems) as per the manufacturer’s recommendations. Relative differences in gene expression between groups were determined using the 2^−ΔΔCT method (26). The amplification efficiencies of the gene of interest and the normalized to the mRNA expression of β-actin. Results were normalized to the mRNA expression of β-actin, which did not change with treatment (β-actin cycle threshold (CT) values for in vivo experiments; ZDF chow: 26.35 ± 0.25; ZDF RSV: 26.33 ± 0.34. β-actin CT values for explant experiments; vehicle treated: 17.45 ± 0.05 (scAT), 18.31 ± 0.13 (rpWAT); RSV treated: 17.63 ± 0.12 (scAT), 18.64 ± 0.31 (rpWAT)).

Statistical analysis. Data are presented as means ± SE. Comparisons of blood metabolites, oxygen consumption, GNG, and adipokine secretion between ZDF chow and ZDF RSV were made using a two-tailed, unpaired Student’s t-test for each depot (GraphPad Prism 5, La Jolla, CA). Given that our in vivo data suggested in which direction to expect changes, the remaining comparisons (protein content, gene expression, and cell cross-sectional area) were analyzed using a one-tailed, unpaired Student’s t-test. Statistical significance was accepted at α ≤ 0.05.

RESULTS

Whole body parameters. Body weight, body weight gain, and average daily food intake were not different between ZDF chow and ZDF RSV rats throughout the 6-wk feeding protocol (Table 1). At euthanasia, fasting blood glucose was lower in RSV-treated animals (P = 0.007), but other blood metabolites were comparable between groups (Table 1). Postintervention, ZDF RSV rats showed a significantly lower glucose area under the curve (AUC) to ipGTT (P = 0.03) and ipITT (P = 0.0004) challenges compared with chow-fed animals (Fig. 1).

Glyceroneogenesis. RSV treatment resulted in increased incorporation of [14C]pyruvate into triglycerides in scAT (P = 0.03) but not in intra-abdominal depots (eWAT: P = 0.21; rpWAT: P = 0.35) (Fig. 2A). In scAT, phosphorylation of pyruvate dehydrogenase (PDH) at Ser 293, a marker of PDK4 activity (38), was elevated in ZDF RSV rats (P = 0.01), whereas total PDH (P = 0.25) and PEPCK (P = 0.25) protein content did not change with RSV treatment (Fig. 2B). Consistent with the functional data, phosphorylation of PDH (Ser293) remained unchanged in eWAT (P = 0.21) and rpWAT (P = 0.28) from RSV-treated animals (data not shown).

Mitochondrial content and function. In scAT, RSV did not alter state IV respiration (P = 0.28); however, there was a trend for increased ADP-stimulated respiration through complex I (40%; P = 0.09) (Fig. 3A). In addition, state III respiration supported by simultaneous electron entry through complex I and II increased by 23% (P = 0.02), as did maximal uncoupled respiration by 29% (P = 0.01) in scAT from ZDF RSV rats (Fig. 3A). Similarly, in rpWAT, RSV enhanced complex I- and II-supported respiration by 45% (P = 0.009) and uncoupled respiration by 37% (P = 0.03), and there was a trend for increased complex I-supported respiration (33%; P = 0.13) (Fig. 3B). RSV did not increase oxygen consumption in eWAT (Fig. 3B). Consistent with these respiration data, COX4 protein content, a marker of mitochondrial content, was increased in scAT (P = 0.005) and rpWAT (P = 0.02) of RSV-supplemented animals but not in eWAT (P = 0.38) (Fig. 3D). We were unable to reliably detect complex I and II protein content using commercially available antibodies. UCP-1 was unchanged in scAT and rpWAT in response to RSV supplementation (data not shown). In every depot, oxygen consumption normalized to COX4 protein content was similar between groups (data not shown).

Adipokines. Fasting plasma adiponectin levels were increased by 43% in the ZDF RSV group compared with control (P = 0.0005) (Fig. 4A). Similarly, ex vivo incubation of adipose tissue...
showed that adiponectin release from scAT ($P = 0.02$), but not from eWAT ($P = 0.98$) or rpWAT ($P = 0.49$), was elevated in RSV-supplemented animals (Fig. 4B). The release of IL-6 and TNF-α were not altered with RSV supplementation in any depot (Table 2).

Lipolytic enzymes and cAMP content, cell cross-sectional area, and gene expression in scAT. As our functional evidence strongly suggested that RSV exerts it effects specifically onto scAT, we sought to determine whether RSV could modulate protein content and mRNA expression in this depot. RSV supplementation increased total ATGL ($P = 0.05$) and HSL ($P = 0.04$) protein content by 18% and 24%, respectively, but did not increase phosphorylation of HSL at serine 563 ($P = 0.21$) or 660 ($P = 0.13$) (Fig. 5A). In addition, in scAT, RSV did not change the protein content of perilipin ($P = 0.68$) or enzymes involved in TG synthesis such as DGAT1 ($P = 0.87$), DGAT2 ($P = 0.33$), and GPAT ($P = 0.64$) (Fig. 5A). The protein content (ZDF chow: 1.00 ± 0.11; ZDF RSV: 0.95 ± 0.18; $P = 0.83$) and phosphorylation (ZDF chow: 1.00 ± 0.19; ZDF RSV: 0.98 ± 0.15; $P = 0.93$) of AMPK were not altered. There were no differences between groups for the mRNA expression of PGC-1α ($P = 0.34$), PGC-1β ($P = 0.28$), PPARγ ($P = 0.26$), or RIP140 ($P = 0.43$), a repressor of mitochondrial biogenesis (36), in scAT (Fig. 5C).

A recent paper reported that the anti-aging effects of RSV in rodents were mediated through the inhibition of phosphodiesterase 4 (PDE4), which resulted in elevated cAMP levels in C2C12 myotubes (30). Consequently, we examined whether cAMP levels were elevated in adipose tissue from ZDF RSV

Fig. 3. Maximal oxygen consumption in scAT (A), eWAT (B), and rpWAT (C) supported by glutamate and malate (state IV), glutamate, malate, and ADP [state III (complex I)], glutamate, malate, ADP, and succinate [state III (complex I and 2)], and with the addition of carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone (FCCP) (uncoupled respiration) in ZDF chow (●) and ZDF RSV (■) ($n = 8$).

D: COX4 protein content in scAT and rpWAT in ZDF chow (●) and ZDF RSV (■) (ZDF chow: $n = 11$; ZDF RSV: $n = 13$). Open bars represent ZDF chow, whereas closed bars represent ZDF RSV animals. Values are means ± SE. *$P < 0.05$ compared with ZDF chow.

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rats. Intracellular cAMP concentration in scAT was similar in both groups (P = 0.50) (Fig. 5B). Finally, there were no differences for cell cross-sectional area between the groups (P = 0.40) (Fig. 5, D and E).

Adipose tissue organ culture experiment. ScAT and rpWAT from ZDF rats were treated with RSV (50 μM) (23) to assess the direct effects of RSV on gene expression. After 24 h, treatment with RSV increased PDK4 (scAT: P = 0.03; rpWAT: P = 0.03) and PGC-1α (scAT: P = 0.04; rpWAT: P = 0.02) mRNA compared with vehicle-treated samples (Fig. 6). PPARγ (scAT: P = 0.37; rpWAT: P = 0.26) remained unchanged with RSV treatment in both depots (Fig. 6B).

**DISCUSSION**

Mitochondrial biogenesis in adipose tissue is essential to maintain metabolic homeostasis at both the tissue and whole body levels. For instance, exercise (40, 50) or treatment with PPARγ agonists (10, 49) induces mitochondrial biogenesis in WAT. Moreover, recent work from Spigelman’s group (19) showed that mice with an adipose tissue-specific deletion of PGC-1α, a transcriptional coactivator and master regulator of mitochondrial biogenesis, become more insulin resistant than controls when fed a high-fat diet. Considering the severe adverse effects of PPARγ agonists such as rosiglitazone (29) and the limited long-term efficacy of exercise programs at the population level (53), it is important to find other avenues to improve adipose tissue metabolic health. In this investigation, we demonstrated that RSV treatment of ZDF rats increased oxygen consumption in scAT and rpWAT ex vivo, when supported by entry of electrons through complex I and II. We also found that maximal uncoupled respiration and COX4 protein content were increased in a depot-specific manner with RSV (Fig. 3). Moreover, when respiration was normalized to COX4 protein content, values were similar between groups (data not shown), providing evidence that improvements in oxygen consumptions are driven by increases in mitochondrial content and not due to enhancement in mitochondrial function per se. These data are consistent with previous reports of RSV-induced mitochondrial biogenesis in skeletal muscle (23) and liver (16) and expands this observation to adipose tissue.

Mitochondrial function is essential for adiponectin synthesis in adipocytes (20), and improvements in mitochondrial biogenesis are associated with increases in adiponectin synthesis and release. In the present study, we reported an increase in circulating adiponectin following RSV treatment in vivo, which is consistent with enhanced adiponectin release from subcutaneous depots ex vivo (Fig. 4). Adiponectin expression is controlled by PPARγ (20) and undergoes extensive post-translational modifications before it is released into the circulation. Previous reports showed that RSV acts on DsbA-L protein (disulfide bond-A oxidoreductase-like protein) to promote adiponectin multimerization in 3T3-L1 adipocytes (47). RSV treatment was also shown to increase adiponectin content in adipose tissue from obese Zucker rats (33) and to increase adiponectin gene expression in human adipocytes (11). Here, we suggest that the main depot responsible for improvement in circulating adiponectin is scAT, which is also the depot that was most responsive to RSV with regards to mitochondrial biogenesis. The lack of effect on rpWAT despite RSV-induced mitochondrial biogenesis may be due to depot-specific regulation of adiponectin transcription and/or posttranslational modifications.

Adiponectin is one of the principal adipokines linking adipose tissue to skeletal muscle and liver metabolism. For instance, the insulin-sensitizing effects of TZDs are attenuated in adiponectin knockout mice (28). Therefore, increases in circulating adiponectin levels provide a possible mechanism to support the participation of adipose tissue in the improvements of whole body glucose homeostasis following RSV supple-

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**Table 2. Release of IL-6 and TNF-α from scAT, eWAT, and rpWAT in ZDF rats fed a chow diet with or without RSV (n = 8) during a 2-h ex vivo incubation**

<table>
<thead>
<tr>
<th>Condition</th>
<th>scAT</th>
<th>eWAT</th>
<th>rpWAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZDF Chow</td>
<td>5.28 ± 0.80</td>
<td>261 ± 44</td>
<td>476 ± 134</td>
</tr>
<tr>
<td>ZDF RSV</td>
<td>4.38 ± 0.60</td>
<td>400 ± 106</td>
<td>324 ± 49</td>
</tr>
<tr>
<td>IL-6, pg·ml⁻¹·mg tissue⁻¹</td>
<td>6.75 ± 0.65</td>
<td>6.65 ± 1.06</td>
<td>6.04 ± 1.22</td>
</tr>
<tr>
<td>TNF-α, µg·ml⁻¹·mg tissue⁻¹</td>
<td>476 ± 134</td>
<td>324 ± 49</td>
<td>433 ± 79</td>
</tr>
</tbody>
</table>

Values are means ± SE. There were no differences in interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) release between the two groups, in any of the depots.
mentation. Indeed, in addition to lower glucose AUC during GTT and ITT, our group also showed muscle-specific insulin-sensitizing effects with RSV (Smith BK, Perry CGR, Herbst EAF, Ritchie IR, Beaudoin MS, Smith JC, Neufer PD, Wright DC, Holloway GP; unpublished observations). The question remains whether adiponectin is required to observe the beneficial effects of RSV at the whole body or skeletal muscle level.

Another important finding of this study is that 6-wk RSV supplementation in ZDF rats increased [14C]pyruvate incorporation into TG in scAT, in parallel with increases in the phosphorylation of PDH at serine 293. PDH is inhibited by PDK4-induced phosphorylation at serine 293, which results in the shuttling of pyruvate away from acetyl-coA and toward oxaloacetae and the glyceroneogenic pathway (7). The up-regulation of GNG in scAT would be expected to result in a greater sequestering of fatty acids in adipose tissue, a lowering of plasma lipid levels, and concomitant improvements in insulin action. However, one of the caveats of our study is that

Fig. 5. A: protein content in scAT for adipose tissue triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and phosphorylation of HSL at serine 563 and 660 (n = 13), and DGAT1, DGAT2, GPAT, perilipin, and α-tubulin (n = 7) for ZDF chow (○) and ZDF RSV (●) animals (n = 13). Values are means ± SE. *P < 0.05 compared with ZDF chow. B: cAMP content of scAT for ZDF chow (○) and ZDF RSV (●) animals (n = 13). Values are means ± SE. C: mRNA expression in scAT for proliferator-activated receptor (PPAR)γ coactivator-1α (PGC-1α), PGC-1β, PPARγ, and RIP140 for ZDF chow (○) and ZDF RSV (●) animals (n = 8). Open bars represent ZDF chow, whereas closed bars represent ZDF RSV animals. Values are means ± SE. D: superimposed histogram of scAT cell cross-sectional area for ZDF chow (○) and ZDF RSV (●) (n = 5). E: representative images for hematoxolyn and eosin staining of scAT for ZDF chow and ZDF RSV.
increases in GNG did not result in lower circulating FFA or TG. This may be due to the genetic background of our model and/or severity of insulin resistance. Alternatively, the glyceroneogenic effect limited to scAT, and not in other depots, may not have been sufficient to lower circulating lipid levels.

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

While our data showed that RSV induces mitochondrial biogenesis in adipose tissue, the mechanisms that mediate this effect are not clear. Importantly, FA reesterification is dependent on GNG and mirrors rates of lipolysis (6). Interestingly, we found that RSV increased both GNG and the protein content of ATGL and HSL, two key enzymes involved in the control of lipolysis, suggesting FA reesterification would be increased in response to RSV. As FA reesterification is an energetically demanding process linked to the activation of AMPK (15), a reputed regulator of mitochondrial biogenesis, RSV-induced mitochondrial biogenesis may be the result of increases in GNG. Admittedly, this is speculative and requires further investigation. Notably, we did not observe a RSV-mediated increase in AMPK activation in vivo in the current study. This situation may not be surprising as a potential increase in the content and/or phosphorylation of AMPK is likely transient and timing may be an important parameter to consider. In addition, animals were killed after a 12 h-fast, which would trigger AMPK phosphorylation and could have, therefore, masked any potential treatment effect. Furthermore, while our data support a relationship between GNG and mitochondrial biogenesis in scAT, mitochondrial biogenesis was independent from GNG in rpWAT. While the reason for this discrepancy is still unknown, it would suggest that, at least in rpWAT, increases in mitochondrial function/content are not sufficient to enhance GNG and would indicate that other factors, such as substrate provision and/or increased activity of glyceroneogenic enzymes such as PEPCK and/or PDK4, might be necessary.

**Perspectives and Significance**

In conclusion, the present study highlights that RSV supplementation in ZDF rats prevents the apparition of whole body insulin resistance and glucose intolerance at 11 wk of age and is associated with increased mitochondrial content in scAT. This process may be triggered by the energetic demands of the lipolytic and glyceroneogenic pathways and the resulting activation of PGC-1α. Indeed, RSV-fed rodents showed increased mitochondrial respiration, enhanced GNG, and elevated adiponectin secretion in a depot-specific manner. Interestingly, the changes observed in adipose tissue metabolism following RSV supplementation are similar to the well-characterized effects of TZDs. While TZDs are effective in improving glucose homeostasis, they are associated with a wide range of negative side effects such as an increased risk of heart attack, osteoporosis, and bladder cancer (29, 35). In this light, our findings suggest that RSV could be used as a nutraceutical/nutritional adjunct that would allow for lower doses of TZDs to be prescribed. Clearly, further work is needed to examine the interactions between RSV and insulin-sensitizing medications.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

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