ADIPOSE MITOCHONDRIAL BIOGENESIS IS SUPPRESSED IN DB/DB & HIGH-FAT DIET-FED MICE AND IMPROVED BY ROSIGLITAZONE

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Running title: Mitochondrial biogenesis in db/db & HFD mice

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ABSTRACT

Objective: The objective of this study was to further establish and confirm the relationship of adipose mitochondrial biogenesis in diabetes/obesity and the effects of rosiglitazone (RSG), a PPARγ agonist, by systematically analyzing mitochondrial gene expression and function in two mouse models of obesity and type II diabetes.

Research Design & Methods: Using microarray technology, adipose mitochondrial gene transcription was studied in db/db, high-fat diet-fed C57BL/6 (HFD), and respective control mice with or without RSG treatment. The findings were extended using mitochondrial staining, DNA quantification, and measurements of citrate synthase activity.

Results: In db/db and HFD mice, gene transcripts associated with mitochondrial ATP production, energy uncoupling, mitochondrial ribosomal proteins, outer and inner membrane translocases, and mitochondrial heat-shock proteins were decreased in abundance, compared to db/+ and standard-fat diet-fed control mice, respectively. RSG dose-dependently increased these transcripts in both db/db and HFD mice, and induced transcription of mitochondrial structural proteins and cellular antioxidant enzymes, responsible for removal of reactive oxygen species generated by increased mitochondrial activity. Transcription factors, including PGC1β, PGC1α, ERRα and PPARα, were suppressed in both models and induced by RSG. The effects of RSG on adipose mitochondrial genes were confirmed by quantitative RT-PCR, and further supported by mitochondrial staining, mitochondrial DNA quantification, and citrate synthase activity.

Conclusions: Adipose mitochondrial biogenesis was overwhelmingly suppressed in both mouse models of diabetes/obesity and globally induced by RSG. These findings suggest an important role of adipose mitochondria in diabetes/obesity, and the potential for new treatment approaches targeting adipose mitochondria.
PPARγ agonists, including rosiglitazone (RSG), are effective drugs for the treatment of type II diabetes mellitus (T2DM). It is well established that RSG induces adipogenesis, and causes body-wide lipid repartitioning by increasing adipose triglyceride content, thereby lowering free fatty acids, glycerol, triglycerides and glucose in the circulation, which is associated with increased insulin sensitivity of the liver, muscle and other organs. Critical to this process, adipose tissue, which highly expresses PPARγ, serves not only as a lipid storage depot, but also as an endocrine organ producing adipokines that regulate the activity of other tissues (reviewed in (1,2)).

There has been growing interest in exploring the involvement of adipose mitochondria in the regulation of whole-body energy homeostasis (3-9). Recent studies suggest that diabetes/obesity is accompanied by a decrease in the expression of adipose mitochondrial genes in ob/ob mice (8) and impaired adipose mitochondria in db/db mice (9), and that the compromised mitochondrial conditions in ob/ob and db/db mice were reversible by RSG (8,9). Mitochondrial dysfunction in adipose tissue, like that in skeletal muscle (10,11), is linked to diabetes/obesity in humans (3).

Mitochondria produce >95% of cellular ATP, required by adipocytes for triglyceride synthesis and adipokine synthesis and secretion, in addition to other general cellular processes. Mitochondria are also indispensable in non-shivering thermogenesis, which takes place in brown adipose tissue (BAT) in small mammals and relies on energy uncoupling via short-circuiting proton gradient across the mitochondrial inner membrane (12,13). The relationship of adipose mitochondrial gene expression in diabetes/obesity has not been studied systematically in db/db or high-fat diet-fed (HFD) mice, two widely used models of T2DM and obesity. While both models are characterized by high blood glucose and insulin levels, dyslipidemia, and obesity, different mechanisms underlie the disease. Db/db mice carry a spontaneous mutation in the leptin receptor (14), and suffer severe glucose intolerance, hyperglycemia and hyperinsulinemia. In contrast, HFD mice have milder glucose intolerance and other metabolic symptoms, reversible with a low-fat diet (15).

In this study, transcriptional profiling was conducted using microarray technology, and focused on adipose mitochondrial gene expression in db/db and HFD mice and the effects of RSG treatment. The expression of genes regulating mitochondrial ATP production, energy uncoupling, mitochondrial structure, mitochondrial protein translation, transportation and folding were examined, as well as the expression of transcription factors regulating mitochondrial biogenesis. The results indicate both mouse models exhibit compromised mitochondrial biogenesis in adipose tissue, which is improved dose-dependently by RSG. The transcriptional evidence was confirmed by quantitative real-time RT-PCR (QRT-PCR), mitochondrial staining, mitochondrial DNA content, and examination of citrate synthase activity. The findings support a new understanding of PPARγ actions in diabetes/obesity treatment.

**RESEARCH DESIGN AND METHODS**

**ANIMALS AND DIETS**

Male, four-week-old C57BL/6J and C57BL/Ks db/db and db/+ mice were obtained from the Jackson Laboratory (Bar Harbor, MA) and housed in a
temperature- and humidity-controlled environment with a 12-h light/dark cycle. Db/db and db/+ mice were fed a standard chow (TestDiet, Richmond, IN; Purina 5001) throughout the experiments. For the HFD model, 5-week-old C57BL/6J mice were placed on either a high-fat diet (58% kcal from fat; Research Diets Inc., New Brunswick, NJ; D12331), or a standard-fat diet (11% kcal; D12329) with coconut oil as the main fat source, matching that in the high-fat diet (15). All animals were allowed free access to food and water. Care and use of the animals were in accordance with protocols approved by the GlaxoSmithKline Institutional Animal Care and Use Committee.

**RSG ADMINISTRATION**

RSG maleate (BRL49653C) was obtained from GlaxoSmithKline, Harlow, UK. For the HFD model, RSG treatment began after 8 weeks on either the high-fat or standard-fat diet. For the db/db model, treatment began in db/db and db/+ mice at 8 weeks of age. In both models, mice received RSG (1, 3, or 10 mg/kg) or vehicle (0.5% methylcellulose) by oral gavage twice daily for 14 days. Blood samples were taken on Day 0 and 14 after 6-hr fasting. Ten mice were used in each treatment group in each model.

**PLASMA MEASUREMENTS**

Plasma glucose and insulin was measured using an automated clinical chemistry analyzer (Olympus AU400; Olympus America Inc., Melville, NY) and an ELISA kit (ALPCO Diagnostics, Inc., Windham, NH), respectively. HbA1c levels were measured from whole blood using the Variant II (BioRad; Hercules, CA).

**AFFYMETRIX MICROARRAY**

All adipose tissues used in the microarray experiment were inguinal subcutaneous adipose tissue. RNA isolation, DNase I treatment, reverse transcription, and cRNA synthesis, labelling, and hybridization with Genechips® Mouse Genome 430A Array (Affymetrix Microarray Suite, Santa Clara, CA) were conducted following the standard Affymetrix protocol (Supplemental Materials). Total RNA (5 µg) from single animals was used for each chip. The RNA samples were randomized before cRNA synthesis and re-randomized before hybridization to avoid batch effects. Array intensity data were captured by Genechip Computer Operating System using the algorithm, MAS 5.0 (Affymetrix).

**IMMUNOHISTOCHEMISTRY & MORPHOMETRIC ANALYSIS**

Inguinal adipose tissue fixed in 10% neutral buffered formalin was paraffin-embedded and sectioned (5-µm in thickness). After deparaffinization and antigen retrieval (16), the sections were stained with goat anti-cytochrome c (Santa Cruz, Santa Cruz, CA; 1:500 dilution), goat anti-Hspd1 (Santa Cruz; 1:200), or rabbit anti-UCP1 (Abcam, Cambridge, UK; 1:2000) polyclonal antibodies (Supplemental Materials).

Quantification of cytochrome c-positive area was performed by two blinded pathologists using eCognition (Definiens AG, Germany), following manufacturer’s instruction. Details of image acquisition and morphometric analysis were provided in Supplemental Materials. Cytochrome c-positive area was calculated as cytochrome c-positive area divided by the total area of examined images, and expressed as µm²/mm². UCP1-positive multilocular cells on acquired images
were identified and counted by blinded pathologists, and expressed as number of cells per 0.705 mm².

**QUANTIFICATION OF RNA AND MITOCHONDRIAL DNA BY QUANTITATIVE REAL-TIME PCR**

Transcriptomics results from the microarray analysis were confirmed using representative genes by QRT-PCR (Supplemental Materials). Relative amounts of nuclear and mitochondrial DNA were determined by quantitative real-time PCR using GAPDH and cytochrome b, respectively (17) (Supplemental Materials). The ratio of mitochondrial DNA to nuclear DNA reflects the tissue concentration of mitochondria per cell.

**CITRATE SYNTHASE ACTIVITY IN ADIPOSE TISSUE**

Citrate synthase activity was measured similarly to Bogacka et al (3) using a Sigma kit (Supplemental Materials). Protein concentrations in the lysate were determined using a Micro BCA Protein Assay kit (Pierce Technology, Holmdel, NJ). The citrate synthase activity was expressed as µmol · min⁻¹ · g protein⁻¹.

**STATISTICAL ANALYSES**

Plasma measurements are presented as mean±standard error (SE), and were analyzed using two-way repeated measures ANOVA with a post-hoc analysis using the Student Newman Keuls multiple comparison test. p <0.05 was considered statistically significant.

Microarray analysis was based on 10 chips per treatment group (1 chip for each mouse), 5 groups in each mouse model. Probe sets with intensity values ≥22.1, which represented the 95 percentile of the built-in Affymetrix negative control probe sets for all the chips, were considered present. Probe set intensity values were log10-transformed and analyzed using both ANOVA and the false discovery rate (FDR) (18). p <0.01 from ANOVA was used to select genes with a significant change, because in our analyses this criterion was found to be more stringent than that using an FDR q <0.1 (10% FDR rate), an acceptable false positive rate in microarray literature (e.g., (19)). In addition, a 1.2-fold cut-off for median fold changes, previously used by others (e.g., (20)) and consistently generating reproducible results in our microarray experiments (Hansen et al, personal communications), was used for further gene selection.

Cytochrome c-positive areas, UCP1-positive cell numbers, relative mitochondrial DNA content and citrate synthase activity are presented as mean±SE. Statistical analyses of the differences between vehicle-treated and rosiglitazone-treated db/db mice, and between vehicle-treated db/+ and vehicle-treated db/db mice were conducted using one-way ANOVA followed by Dunnett test using vehicle-treated db/db mice as the control, and Student’s t-test, respectively. p <0.05 was considered statistically significant.

**RESULTS**

**PLASMA MEASUREMENTS IN DB/DB & HFD MICE**

Db/db mice had significantly higher glucose, insulin and HbA1c levels than db/+ mice (Table 1). Fourteen days of RSG treatment significantly decreased glucose and insulin levels at all 3 doses. HbA1c levels were reduced with significant changes occurring with the 3 and 10 mg/kg of RSG. RSG significantly
increased body weight and inguinal adipose weight (data not shown).

HFD mice had significantly higher glucose, insulin and HbA1c levels than standard-fat diet-fed mice (SFD) (Table 2), but the levels were generally lower than those in db/db mice. In HFD mice, only the highest dose of RSG (10 mg/kg) significantly decreased glucose after 14 days of treatment, but all RSG doses significantly decreased insulin levels. There were no significant reductions in HbA1c with RSG treatment in the HFD mice. Body weight and inguinal adipose weight were significantly increased in mice treated with 3 and 10 mg/kg RSG (data not shown).

**TRANSCRIPTION OF ADIPOSE MITOCHONDRIAL PROTEINS REGULATING ATP PRODUCTION WAS SUPPRESSED IN DB/DB & HFD MICE, AND INDUCED BY RSG.**

Mitochondria host enzymes necessary for the tricarboxylic acid (TCA) cycle, fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) pathways, all of which contribute to ATP production. As listed in Supplemental Table 1, of the 22,626 probe sets (each identifying one gene transcript) on Affymetrix mouse chip 430A, 248 represent genes encoding proteins localized to mitochondria and related to OXPHOS/TCA/FAO, based on Affymetrix chip annotation and confirmed by Entrez Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene).

Of the 248 probe sets, 241 (97%) were called “present” in the inguinal adipose tissue, composed of BAT and white adipose tissue (WAT) (21). **Fig. 1** shows the expression of the majority of the OXPHOS/TCA/FAO genes in the tissue was suppressed in db/db and HFD mice, compared to those in db/+ and SFD mice, respectively. In a dose-dependent manner, RSG induced the expression of most of the OXPHOS/TCA/FAO genes in both db/db and HFD mice. At 10 mg/kg, RSG increased the expression of the majority of the genes to a level equivalent to or higher than that in db/+ and SFD mice. This suggests RSG is capable of correcting the compromised adipose mitochondrial gene expression in db/db and HFD mice.

Among the regulated genes were those encoding key subunits of enzymes regulating the control points (22) in the TCA cycle, namely, pyruvate dehydrogenase, isocitrate dehydrogenase 3, and α-glutarate dehydrogenase, and those encoding electron transporters in OXPHOS, including cytochrome c and cytochrome c1. The rate of FAO is controlled by carnitinepalmitoyl transferase I (CPT1) (22). CPT1b, the major CPT1 isoform in BAT (23), was significantly decreased in HFD mice (to 29% of that in SFD), and induced by RSG at 10 mg/kg dose by 6.2- and 4.9-fold in db/db and HFD mice respectively.

In addition to enzymes/proteins directly involved in OXPHOS/TCA/FAO, those responsible for the transport of metabolites and intermediates for ATP production and FAO across mitochondrial membranes, including voltage-dependent anion channels, ATP/ADP shuttles, carnitine/acyl carnitine shuttles, were suppressed in the adipose tissue from db/db and HFD mice, and were dose-dependently induced by RSG (**Fig. 1**).

**TRANSCRIPTION OF UNCOUPLING PROTEINS (UCPS) WAS SUPPRESSED IN DB/DB AND HFD MICE, AND INDUCED BY RSG.**

To date, three UCP isoforms have been identified in mammalian cells (24), UCP1, UCP2, and UCP3, which share >57% homology (25). In mice, UCP1 is
specifically expressed in mitochondria-rich BAT, and plays a key role in non-shivering thermogenesis (25,26). Ectopic expression of UCP1 in WAT mitigates obesity induced by genetic or dietary factors (25,26). UCP3 is predominantly expressed in skeletal muscle, heart and BAT, and has been shown to facilitate FAO (27), whereas UCP2 is localized to the pancreas, immune system, WAT, and the brain, and has been linked to insulin secretion and modulation of inflammatory response (24,25).

UCP1 was detected in the inguinal adipose tissue of db/+ mice and SFD mice, reaffirming the presence of BAT in the tissue (21). Interestingly, UCP1 expression in the tissue from SFD mice was ~10-fold higher than that in db/+ mice (Supplemental Table 1), indicating the tissue from SFD mice had a greater proportion of the BAT-like component. UCP1 expression in the tissue was lower in db/db than in db/+ mice, and was even more dramatically decreased in HFD compared to SFD mice (Table 3). RSG treatment remarkably increased UCP1 expression (by 37 and 34 fold respectively) in both db/db and HFD mice.

Interestingly, the expression of Cidea, a modulator of UCP1 and another marker of BAT-like tissue (5,28,29), was decreased in db/db and HFD mice, compared to db/+ and SFD mice respectively, and was increased by RSG in both models (Table 3). UCP3 levels also decreased in db/db and HFD, compared to db/+ and SFD mice respectively, and were increased by RSG (Table 3). UCP2 levels were not affected by diabetes/obesity or RSG treatment.

**RSG INDUCED TRANSCRIPTION OF MITOCHONDRIAL STRUCTURAL PROTEINS.**

To investigate whether the observed changes were associated with changed mitochondrial mass, the regulation of mitochondrial structural proteins not directly participating in metabolic activities was examined. These proteins include mitofillin, localized to mitochondrial inner membrane and regulating morphology of mitochondrial cristae (30); optic atrophy 1 (OPA1), anchored to mitochondrial inner membrane and localized to the intermembrane space, and implicated in maintenance of mitochondrial genome and network (31); Surf1, localized to the inner membrane and involved in assembly of cytochrome c oxidase complex (32); and BCS1L, a human homologue of yeast BCS1 localized to the inner membrane and involved in assembly of complex III of mitochondrial respiratory chain (33). Fig. 2A shows that the transcription of these proteins was induced by RSG in db/db mice, and so was that of mitofillin and OPA1 in HFD mice.

**RSG INDUCED TRANSCRIPTION OF PROTEINS MEDIATING MITOCHONDRIAL GENE TRANSLATION, PROTEIN TRANSPORT AND FOLDING.**

To further investigate the roles of diabetes/obesity and RSG on mitochondrial mass/biogenesis, transcription of genes directly participating in mitochondrial replication was examined. Fig. 2B shows 28% and 26% of transcripts representing mitochondrial ribosomal proteins were down-regulated in db/db and HFD mice, respectively, while the rest of the mitochondrial ribosomal protein
transcripts, with a few exceptions, were unchanged. Similarly, 27% transcripts encoding translocases that transport cytosolic proteins across mitochondria outer and inner membrane (TOMMs & TIMMs, respectively) were suppressed in both db/db and HFD mice. Furthermore, 42% transcripts for mitochondrial heat-shock proteins/chaperones (HSPs), responsible for proper protein folding and transport, were down-regulated, while the rest were unchanged, in db/db mice.

At 10 mg/kg, RSG treatment induced the transcription of 76% and 60% of mitochondrial ribosomal proteins, and 80% and 70% of TIMMs & TOMMs, in db/db and HFD mice, respectively. The RSG effect was dose-dependent, and the dose-dependency was more evident in db/db than in HFD mice. In addition, RSG (10 mg/kg) induced in db/db and HFD mice 71% and 58% of the transcripts, respectively, for mitochondrial HSPs, including mitochondrial HSP70 (Hspa9a) and HSP60 (Hspd1), while had no overall effect on cytosolic HSPs (such as Hspa4 and Tcp1, which belong to the cytosolic HSP70 and HSP60 family, respectively), or ER chaperones (e.g., calnexin, calreticulin, and GRP94), indicating the induction by RSG was selective for mitochondrial proteins.

**REGULATION OF TRANSCRIPTION FACTORS INVOLVED IN MITOCHONDRIAL BIOGENESIS**

Mitochondrial biogenesis involves the integration of multiple transcriptional pathways controlling both nuclear and mitochondrial gene expression. PPARγ co-activators (PGC) 1α and 1β have been implicated as key regulators of mitochondrial biogenesis in adipose tissue (3,6,34-37) and muscle (11,36-40). Table 3 shows PGC1β transcription was suppressed in the inguinal fat in both db/db and HFD mice, and was induced by RSG in both models. PGC1α transcription in C57BL/6 mice was not influenced by diet, i.e., there was no difference between HFD and SDF mice; however, its transcription was suppressed in db/db compared to db/+ mice. RSG elicited expression of PGC1α in HFD (p <0.01) and db/db (p =0.06) mice.

The expression of nuclear receptors ERRα and PPARα was suppressed in db/db and HFD mice, and induced by RSG. RIP140, a recently identified transcription co-repressor negatively regulating mitochondrial biogenesis and metabolic functions in adipocytes (6,28), was induced in HFD but not in db/db mice. Surprisingly, RIP140 expression was induced by RSG in db/db mice and not affected in HFD mice. This indicates RSG-induced mitochondrial biogenesis might not involve suppression of RIP140.

**TRANSCRIPTION OF CELLULAR ANTIOXIDANT ENZYMES WERE CONCORDANTLY REGULATED WITH RSG-INDUCED MITOCHONDRIAL BIOGENESIS**

Increased mitochondrial metabolic activity is associated with an increased chance of leaking O₂⁻, leading to formation of other reactive oxygen species (ROS), followed by oxidative stress, cell injury, and eventually cell death. Fig. 3 shows the transcription of antioxidant enzymes catalase (Cat), superoxide dismutase 1 (Sod1), superoxide dismutase 2 (Sod2), glutathione peroxidase 1 (Gpx1) and methionine sulfoxide reductase A (Msra) was generally increased by RSG. Meanwhile, RSG did not affect the transcription of mitochondrial proteins favoring apoptosis, such as Bax and Bad, or cytosolic regulators of apoptosis, such as caspases, in db/db and HFD mice.
VALIDATION OF MICROARRAY FINDINGS & MITOCHONDRIAL BIOGENESIS

The transcription of 18 genes representing control point enzymes, structural proteins, or those involved in mitochondrial replication, or antioxidant enzymes, were examined by QRT-PCR using inguinal adipose RNA samples from the db/db model. The results, generally consistent with the microarray data (Supplemental Table 2A), supported the notion that mitochondrial transcription was down-regulated in diabetes/obesity and induced by RSG. The microarray findings were also confirmed at the protein level by immunohistochemistry and histomorphometric analysis of a few representative mitochondrial markers, i.e., cytochrome C, Hspd1, and UCP1 (see below).

To test whether diabetes/obesity and RSG also affected mitochondrial gene expression in epididymal adipose tissue, predominantly WAT (21), the transcription of the 18 representative genes was examined by QRT-PCR. As expected, Cidea was not detected, and UCP1 expression was very low in epididymal adipose tissue. UCP1 was surprisingly induced under the diabetes/obesity condition, but was dramatically induced with RSG treatment. The regulation of the other genes was generally consistent with that in inguinal adipose tissue (Supplemental Table 2B).

Staining of inguinal adipose tissue showed that adipocyte size in db/db mice was generally larger than that in db/+ mice (Fig. 4A), and decreased with RSG treatment (41). Cytochrome c, a mitochondrial marker, was significantly decreased in the db/db mice. RSG increased cytochrome c staining in virtually all adipocytes, indicating the conversion of mitochondria-poor to mitochondria-rich adipocytes. There was also increased presence of smaller, multilocular cells enriched with cytochrome c (Fig. 4A, arrows). The increase of total cytochrome c staining was RSG-dose dependent (Fig. 4B), consistent with the microarray results. Similar observations were made with immunostaining of Hspd1, another mitochondrial marker (Fig. 4C). UCP1 staining was limited to the multilocular cells, confirming their brown adipocyte nature (Fig. 4D). RSG dose-dependently increased brown adipocyte numbers in inguinal adipose tissue (Fig. 4F).

Mitochondrial DNA content in inguinal adipose tissue from db/db mice was slightly decreased compared to db/+ mice, but this did not reach statistical significance. However, mitochondrial DNA content in the tissue was increased by RSG dose-dependently, and at 3 and 10 mg/kg doses, was significantly greater than vehicle-treated mice (Fig. 5A).

Citrate synthase, localized exclusively to the mitochondrial matrix, catalyzes the conversion of acetyl-CoA to citrate, the initial step in the TCA cycle. Citrate synthase activity has been used as a reference to determine changed mitochondrial function associated with mitochondrial biogenesis in adipose (3) and muscle (42). The activity in inguinal adipose tissue from db/db mice was significantly lower than that from db/+ mice (Fig. 5B). RSG dose-dependently increased the activity, reaching a plateau at 3 and 10 mg/kg.

DISCUSSION

The effects of diabetes/obesity and insulin sensitizers, such as rosiglitazone, on adipose mitochondrial biogenesis are only beginning to be understood and have not been systematically investigated in either db/db or HFD mice. The current study comprehensively evaluated mitochondrial nuclear DNA-encoded
proteins involved in important metabolic functional pathways, including the control-point enzymes of each individual pathway. In addition, the regulation of transcription factors, mitochondrial structural proteins, and the machinery for mitochondrial replication were evaluated. In both mouse models, adipose mitochondrial gene transcription was overwhelmingly decreased in the disease states, and RSG dose-dependently increased the expression of many of the mitochondrial genes. The current results are consistent with recent observations in several in vivo models (3,8,9). The results on gene expression were extended by mitochondrial staining, quantification of mitochondrial DNA and citrate synthase activity, and further support a link between adipose mitochondria and the pathogenesis of diabetes/obesity.

The exact mechanisms by which adipose mitochondrial dysfunction and RSG-induced mitochondrial biogenesis contribute to whole-body energy homeostasis are unclear. Impaired mitochondria may lead to a lack of ATP (9), thereby attenuating triglyceride synthesis from fatty acids and carbohydrates in adipocytes, leading to a reduced adipose capacity for energy storage. Indeed, the insulin-sensitizing action of PPARγ agonists in muscle and liver is believed to be due to promoting triglyceride synthesis in adipose tissue, and thus, partitioning of circulating lipids away from muscle and the liver (1). A decrease in ATP may also impair the synthesis and secretion of adipokines that serve as endocrine signals. In fact, low serum levels of adiponectin, the most abundant secretory protein from adipocytes (43) and an adipokine that improves insulin sensitivity in other organs including the liver and muscle (44), is associated with diabetes/obesity in vivo. PPARγ activation increased circulating adiponectin levels in others’ (reviewed by (1,44)) and the current study (data not shown).

Adipose mitochondria also regulate whole-body energy metabolism via dissipating energy and generating heat in BAT. The inguinal adipose tissue consists of both BAT and WAT (21). The expression of BAT markers UCP1, Cidea, and CPT1b, were coordinately suppressed in the disease states, and coordinately induced by RSG treatment in both models (Table 3). This indicates the plasticity of the adipose tissue—diabetes/obesity induces the conversion of adipose from a semi-BAT phenotype to a more classical WAT phenotype, and RSG reverses the process by promoting the increase of mitochondria in pre-existing WAT (Fig. 4A, Supplemental Table 2B) and the expansion of the BAT component (Fig. 4D & 4E). This supports the long-standing interest (e.g., refs (5,26)) in converting WAT to BAT and transforming adipose tissue into fat-oxidizing machinery as a therapeutic approach for obesity and/or diabetes.

In the current study, RSG significantly increased mitochondrial activity. Inherent to increased mitochondrial activity is the potential of increased ROS generation. Interestingly, RSG induced the transcription of antioxidant enzymes, responsible for removal of excess ROS. This effect may prevent potential cell injury resulting from RSG-induced mitochondrial metabolic activity. The dual RSG actions on mitochondria and antioxidant enzymes could work through the up-regulation of one or both of the PGC1 proteins (Table 3) that have been shown to regulate both mitochondrial biogenesis and the antioxidant machinery (13,45). A recent study also showed up-regulation of antioxidant enzymes per se may be beneficial for improving insulin resistance (46).
In addition to genes involved in mitochondrial biology, a number of inflammation genes were coordinately regulated, but in an opposite direction to the mitochondrial genes, by diabetes/obesity and RSG treatment (data not shown), which is consistent with previous reports (47-49). Therefore, anti-inflammatory properties of PPARγ agonists represent yet another potential mechanism that may be involved in their efficacy in T2DM. The present study cannot discriminate whether mitochondrial effect is responsible for inflammation changes, or vice versa. In addition, the study cannot rule out that RSG-induced improvements in mitochondrial function are an indirect consequence of improved glycemia; however, separate studies indicated metformin, which decreased glucose levels in db/db mice, did not cause similar changes in adipose mitochondrial gene expression (Supplemental Table 3). Furthermore, a similar effect on mitochondrial genes was observed with RSG in murine 3T3 L1, C3H10T1/2, and human primary adipocyte cultures that were free of inflammatory cell types and exposed to a constant concentration of glucose in the medium (Rong et al, unpublished data, also see refs (34,35)). Taken together, this suggests that RSG is likely having direct actions on adipose mitochondrial function.

In conclusion, in this study transcriptional profiling combined with phenotypic, molecular and functional measurements indicates a deficiency in adipose mitochondrial function under diabetes/obesity conditions, and RSG treatment improves mitochondrial function. The results suggest a new area for the investigation of RSG action, that of promoting mitochondrial activity and health in metabolic tissues. These findings may bring new insights on the mechanisms of RSG action on diabetes/obesity treatment, and new treatment approaches targeting adipose mitochondria.

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**TABLES**

Table 1. Glucose, HbA1c & insulin levels in the db/db model.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Day</th>
<th>db/+</th>
<th>Vehicle</th>
<th>db/db</th>
<th>Vehicle</th>
<th>RSG-1</th>
<th>RSG-3</th>
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<tr>
<td></td>
<td></td>
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<td>Mean</td>
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*p <0.05 vs. db/+; †p <0.05 vs. Day 0.

Table 2. Glucose, HbA1c & insulin levels in the HFD model.

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<th>Measurement</th>
<th>Day</th>
<th>11% Diet (SFD)</th>
<th>Vehicle</th>
<th>Vehicle</th>
<th>RSG-1</th>
<th>RSG-3</th>
<th>RSG-10</th>
<th>58% Diet (HFD)</th>
<th>Vehicle</th>
<th>Vehicle</th>
<th>RSG-1</th>
<th>RSG-3</th>
<th>RSG-10</th>
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<td>Mean</td>
<td>SE</td>
<td>Mean</td>
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<td>4.16</td>
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<td>2.41*</td>
<td>0.21</td>
<td>1.78†</td>
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<td>2.03†</td>
<td>0.23</td>
<td>1.51†</td>
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*p <0.05 vs. SFD; †p <0.05 vs Day 0.
Table 3. Transcriptional regulation of UCPs, Cidea, and transcription factors.
Median fold-changes of probe set intensities were calculated between the treatment groups (n = 10 mice/treatment group) indicated in column titles. Positive and negative values represent increase and decrease in fold changes (p <0.01, unless otherwise specified), respectively.

<table>
<thead>
<tr>
<th>Gene</th>
<th>db/db vs. db/+</th>
<th>RSG 10 mg/kg vs. Veh in db/db</th>
<th>HFD vs. SFD</th>
<th>RSG 10 mg/kg vs. Veh in HFD</th>
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<td>Ucp2</td>
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<td>NC</td>
<td>NC</td>
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<td>RIP140*</td>
<td>NC</td>
<td>1.2</td>
<td>1.4</td>
<td>NC</td>
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</table>

*Two probe sets existed for UCP3 and RIP140 on the chip, respectively. †p =0.06. NC, no change.
Figure Legends

Figure 1. Transcriptional regulation of genes involved in mitochondrial ATP production. Shown is a heatmap of median fold-changes of probe set intensities between the treatment groups indicated in column titles (n = 10 mice/group). Each row represents one probe set. Only fold-changes significantly different (p < 0.01) were shown in colors (red or green). NV stands for Normal (db/+ or SDF) mice treated with Vehicle. DV, DT1, DT3, and DT10 stand for Diseased (db/db or HFD) mice treated with Vehicle, 1, 3, and 10 mg/kg of RSG, respectively.

Figure 2. Transcriptional regulation of mitochondrial structural proteins (A) and mitochondrial ribosomal proteins, TIMMs & TOMMs, and heat shock proteins (B). Shown is a heatmap of median fold-changes of probe set intensities between the treatment groups indicated in column titles (n = 10 mice/group). Each row represents one probe set. Only fold-changes significantly different (p < 0.01) were shown in colors (red or green). NV stands for Normal (db/+ or SDF) mice treated with Vehicle. DV, DT1, DT3, and DT10 stand for Diseased (db/db or HFD) mice treated with Vehicle, 1, 3, and 10 mg/kg of RSG, respectively.

Figure 3. Transcriptional regulation of antioxidant enzymes. Shown is a heatmap of median fold-changes of probe set intensities between the treatment groups indicated in column titles (n = 10 mice/group). Each row represents one probe set. Only fold-changes significantly different (p < 0.01) were shown in colors (red or green). NV stands for Normal (db/+ or SDF mice) treated with Vehicle. DV, DT1, DT3, and DT10 stand for Diseased (db/db or HFD mice) treated with Vehicle, 1, 3, and 10 mg/kg of RSG, respectively.

Figure 4. Mitochondria and brown adipocyte content. Inguinal adipose tissues from db/+ or db/db mice with the indicated treatments (n = 10/group) were stained with mitochondrial markers. A, C, & D. Representative pictures from cytochrome c (A), Hspd1 (C), or UCP1 (D) staining. Arrows indicate cytochrome c-enriched brown adipocytes in A. Magnification = 200x; when present, scale bar = 50 µm. B. Morphometric analysis of cytochrome c-stained areas. F. Averaged UCP1-positive, brown adipocyte numbers in the acquired images (total area = 0.705 mm²). All numerical data were expressed as mean±SE. #p < 0.05 vs. db/db Veh.

Figure 5. Mitochondrial DNA content (A) & citrate synthase activity (B). Relative mitochondrial DNA content and citrate synthase activity were determined in inguinal adipose tissues from db/+ or db/db mice with the indicated treatments (n = 10/group). Data were expressed as mean±SE. #p < 0.05 vs. db/db Veh.
Figure 3

<table>
<thead>
<tr>
<th></th>
<th>db/db model</th>
<th>HFD model</th>
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<tr>
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Fold change:

- <0.33
- 1.0
- ≥3
Figure 5A

Relative Mitochondrial DNA

Figure 5B

Specific Activity (umol/min/g protein)