Resveratrol Improves Mitochondrial Function and Protects against Metabolic Disease by Activating SIRT1 and PGC-1α

Marie Lagouge,[8,1] Carmen Argmann,[8,1] Zachary Gerhart-Hines,[2] Hamid Meziane,[3] Carles Lerin,[2] Frederic Daussin,[4] Nadia Messadeq,[3] Jill Milne,[5] Philip Lambert,[5] Peter Elliott,[5] Bernard Geny,[4] Markku Laakso,[6] Pere Puigserver,[2] and Johan Auwerx[1,3,7,*]

[1] Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS / INSERM / ULP, 67404 Illkirch, France
[2] Department of Cell Biology, John Hopkins University School of Medicine, Baltimore, MD 21205, USA
[3] Institut Clinique de la Souris, BP10142, 67404, Illkirch, France
[4] Department of Respiratory, Cardiocirculatory and Exercise Physiology, Hôpitaux Universitaires, 67000 Strasbourg, France
[5] Sirtris Pharmaceutical, Cambridge, MA 02139, USA
[6] Department of Medicine, University of Kuopio, 70211 Kuopio, Finland
[7] IGBMC-ICS, 67404 Illkirch, France
[8] These authors contributed equally to this work.
*Contact: auwerx@igbmc.u-strasbg.fr
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SUMMARY

Diminished mitochondrial oxidative phosphorylation and aerobic capacity are associated with reduced longevity. We tested whether resveratrol (RSV), which is known to extend lifespan, impacts mitochondrial function and metabolic homeostasis. Treatment of mice with RSV significantly increased their aerobic capacity, as evidenced by their increased running time and consumption of oxygen in muscle fibers. RSV's effects were associated with an induction of genes for oxidative phosphorylation and mitochondrial biogenesis and were largely explained by an RSV-mediated decrease in PGC-1α acetylation and an increase in PGC-1α activity. This mechanism is consistent with RSV being a known activator of the protein deacetylase, SIRT1, and by the lack of effect of RSV in SIRT1$^{-/-}$ MEFs. Importantly, RSV treatment protected mice against diet-induced-obesity and insulin resistance. These pharmacological effects of RSV combined with the association of three *Sirt1* SNPs and energy homeostasis in Finnish subjects implicates SIRT1 as a key regulator of energy and metabolic homeostasis.

INTRODUCTION

Mitochondria are the principal energy sources of the cell that convert nutrients into energy through cellular respiration (Wallace, 2005). Compromised mitochondrial function has been linked to numerous diseases, including those of the metabolic and cardiovascular systems (Petersen et al., 2003). The genetic basis of such a tight link in the rat was illustrated by the cosegregation of cardiovascular and metabolic risk factors with low aerobic capacity and reduced muscle expression of genes required for mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) (Wisloff et al., 2005). In humans, insulin resistance in the skeletal muscle has been associated with a lower ratio of oxidative type 1 to type 2 glycolytic type muscle fibers, decreased mitochondrial oxidative capacity and ATP synthesis, and, finally, decreased expression of genes that control mitochondrial activity (Mootha et al., 2003, 2004; Patti et al., 2003; Petersen et al., 2003). One gene whose decreased expression is consistently implicated in the human or animal diabetic muscle is the peroxisome proliferator-activated receptor γ coactivator, PGC-1α (Mootha et al., 2004; Patti et al., 2003; Sparks et al., 2005). PGC-1α is a coactivator with pleiotropic functions (Knutti and Kralli, 2001; Lin et al., 2005). Most importantly, PGC-1α controls mitochondrial biogenesis and function, which in the muscle can contribute to fiber-type switching (Lin et al., 2002a) and, in the brown adipose tissue (BAT), to adaptive thermogenesis (Puigserver et al., 1998).

Recently SIRT1 has been shown to function together with PGC-1α to promote adaptation to caloric restriction (CR) by regulating the genetic programs for gluconeogenesis and glycolysis in the liver (Rodgers et al., 2005). SIRT1 is one of seven mammalian homologs of Sir2 that catalyzes NAD$^+$-dependent protein deacetylation, yielding nicotinamide and *O*-acetyl-ADP-ribose (Blander and Guarente, 2004). Originally described as a factor regulating longevity, apoptosis and DNA repair (Blander and Guarente, 2004; Sinclair, 2005), SIRT1 also facilitates the conversion of changes in the nutritional status, which it senses via NAD$^+$ levels, into modulation of cellular metabolism (Brunet et al., 2004; Lin et al., 2002b; Picard et al., 2004;

Rodgers et al., 2005). SIRT1 physically interacts with and deacetylates PGC-1α at multiple lysine sites, consequently increasing PGC-1α activity leading to the induction of liver gluconeogenic gene transcription (Rodgers et al., 2005). Given the role of SIRT1 as a mediator of CR and longevity and the central role for reactive oxygen species (ROS), mainly produced as a consequence of mitochondrial functioning in promoting aging, it is plausible that PGC-1α and SIRT1 functions converge in tissues beyond the liver that have a high level of mitochondrial activity, such as the muscle and BAT. Since such a convergence could potentially impact on metabolic diseases, we addressed our hypothesis not in the context of CR but under conditions of caloric excess using the specific SIRT1 activator, resveratrol (RSV) (Borra et al., 2005; Howitz et al., 2003).

RSV is a natural polyphenolic compound mainly found in the skin of grapes and is well known for its phytoestrogenic and antioxidant properties (Baur and Sinclair, 2006). It has been shown to significantly increase SIRT1 activity through an allosteric interaction, resulting in the increase of SIRT1 affinity for both NAD$^+$ and the acetylated substrate (Howitz et al., 2003). These findings are consistent with the fact that in various species, RSV treatment mimics Sir2-dependent lifespan extension during CR (Howitz et al., 2003; Lin et al., 2000; Rogina and Helfand, 2004).

In this study we tested whether RSV, through increasing SIRT1 activity, could modulate PGC-1α functions in vivo and ultimately impact on the regulation of energy homeostasis. Our data reveal that RSV potently induces mitochondrial activity, through activating PGC-1α, as evidenced by the increase in oxidative type-muscle fibers, enhanced resistance to muscle fatigue, and increased tolerance to cold, all PGC-1α-dependent effects. Importantly, these effects, induced by RSV, rendered the animals resistant to diet-induced obesity and insulin resistance. In support of the importance of SIRT1 in the control of energy homeostasis, we also report a significant association between three single-nucleotide polymorphisms (SNPs) in the human *Sirt1* gene and energy homeostasis, extending the impact of our animal studies to human pathophysiology.

RESULTS

### Metabolic Consequence of RSV in Diet-Induced Obesity

The metabolic effect of RSV was initially evaluated in a cohort of male C57Bl/6J mice that were given a dose of 200 or 400 mg/kg/day (mpk) of RSV administered in either a chow diet or high fat (HF) diet for 15 weeks. With this protocol, the plasma level of RSV was dose-related and ranged from 10–120 ng/ml. Under chow-fed conditions, RSV-treated mice tended to gain less weight as compared to controls (Figure 1A). However, this effect became significant when the animals were challenged with an HF diet, such that RSV-treated, HF-fed mice weighed almost the same as the chow-fed mice (Figure 1B). This de-

creased body mass was accounted for by a decrease in fat as illustrated by dual X-ray absorptiometry (Figure 1B) and was also reflected in the mass of the different white fat pads (Figure 1C). Morphological analysis of epididymal white adipose tissue (WAT) sections by hematoxylin and eosin (HE) staining also showed smaller adipocytes upon RSV treatement (Figure S1). These beneficial effects of RSV on body weight and fat mass were not due to decreased food intake, as the amount of kcal of food consumed per mouse over a 24 hr period was unchanged (Figure 1D). RSV, at the dose given, did not induce hepatic toxicity, since the serum levels of alanine aminotransferase and aspartate aminotransferase (data not shown) were unchanged, as was the liver histo-morphology (Figure S1). In addition, stool composition, coat maintenance, and water intake (data not shown) were unaffected, indicating that overall, RSV was well tolerated by the animals. Finally, fecal lipid kcal content was minimally affected by RSV treatment, and greater than 98% of all dietary-derived lipid was absorbed in both groups (data not shown).

The critical parameters contributing to body-weight maintenance include caloric intake and energy homeostasis (Lowell and Spiegelman, 2000). As caloric intake is unaffected by RSV (Figure 1D), we assessed the effect of this compound on energy expenditure (EE) by indirect calorimetry. Basal EE, as measured by oxygen (O_2) consumption, was significantly increased in HF-fed mice treated with RSV (Figure 1E), but their respiratory quotient (RQ) was not changed (data not shown). To assess the effect of RSV on the capacity for adaptive thermogenesis, we performed a cold test. RSV enhanced this capacity, since it maintained the body temperature higher as compared to that of nontreated animals (Figure 1F). In the mouse, the major contributor to the production of heat is the BAT, and morphometric analysis of the BAT mitochondria, by electron microscopy, revealed clearly larger mitochondrial structures attributed to an increased presence of cristae in RSV-treated mice as compared to that of HF-fed animals (Figure 2A). This amplification of the mitochondria was reflected both in the quantification of mitochondrial size (Figure 2A, right panel) and mitochondrial DNA content (mtDNA, Figure 2D). Consistent with enhanced mitochondrial activity, a marked decrease in the lipid-droplet size was also noted.

RSV Increases the Aerobic Capacity of the Muscle

In the adult human, little BAT is present, and it is mainly the skeletal muscle that possesses the mitochondrial capacity for EE. The changes in the muscle mitochondrial morphology, however, paralleled those observed in the BAT of RSV-treated mice (Figure 2B). Whereas the oxidative fibers of the gastrocnemius were unaffected, the nonoxidative fibers in RSV-treated mice had larger and denser mitochondria aggregated between adjacent myofibrils. Mitochondrial expansion was evidenced by increased mitochondrial size (Figure 2B, right panel) and mtDNA content (Figure 2D). Histological sections of muscle stained for the presence of the mitochondrial enzyme, succinate
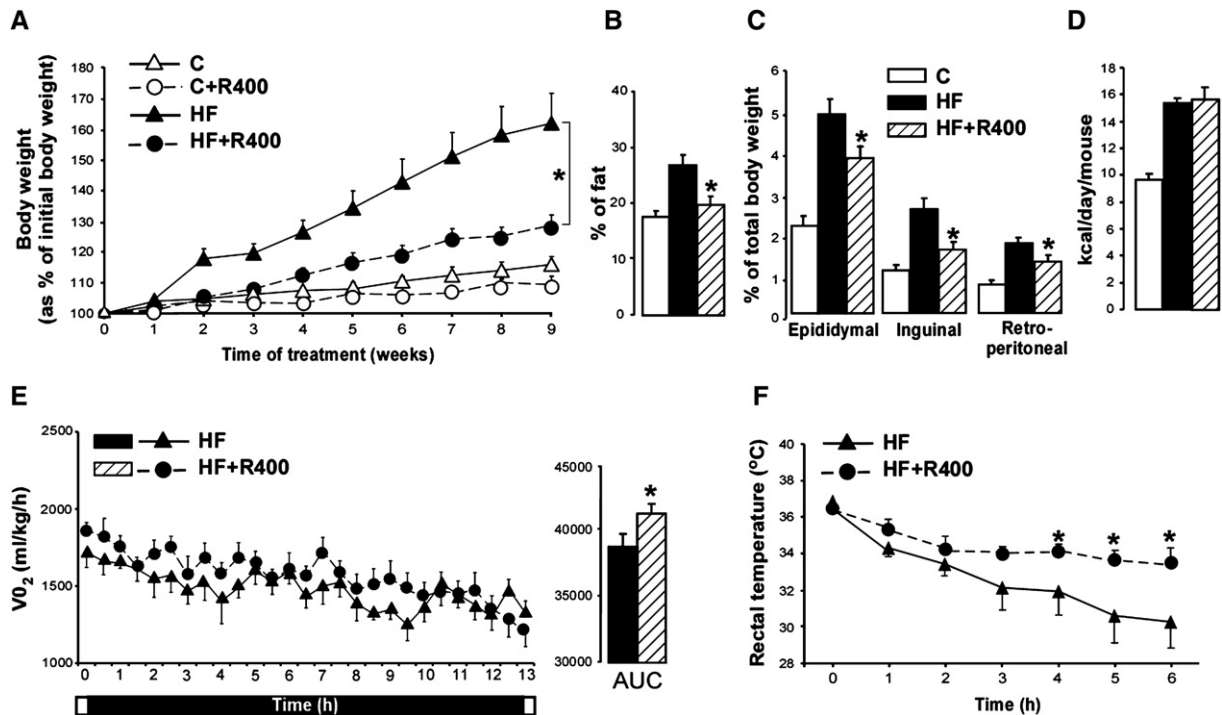
Figure 1. RSV Prevents Diet-Induced Obesity

C57Bl/6J mice were fed a chow diet (C) or high-fat diet (HF) alone or supplemented with RSV (400 mpk, R400) for 15 weeks.

(A) Evolution of body weight gain expressed as percentage of initial body weight.

(B) Body fat content expressed as percentage of total body mass as analyzed by DEXA.

(C) Weight of the WAT depots, expressed as percentage of total body weight.

(D) Average food intake expressed as kcal/mouse/day.

(E) EE as measured by changes in VO_2 consumption in indirect calorimetry during 13 hr (time 0 is 7:00 p.m.). The mean areas under the curves (AUC) are shown in the right side graph (n = 7).

(F) The evolution of the body temperature during a cold test (4°C for 6 hr). * = $P < 0.05$ and n = 10 animals/group unless stated otherwise. Values represent means ± SEM.

dehydrogenase (SDH, Figure 2C), and the increase in citrate synthase activity in muscle homogenates, furthermore indicates that RSV enhanced mitochondrial enzymatic activity (Figure 3A). Finally, in the isolated nonoxidative muscle fibers of RSV-treated mice, there was a significantly higher maximum VO_2 rate, indeed suggesting an increased oxidative capacity (Figure 3B). The combination of the increased mitochondria size and density, mtDNA content, SDH, and citrate synthase activities and oxidative capacity is highly suggestive that RSV increases the ratio of oxidative to nonoxidative type-muscle fibers.

Following the hypothesis that RSV induces a fiber-type switch and knowing that oxidative type 1 fibers are associated with an increased resistance to muscle fatigue (Booth et al., 2002), we evaluated the effect of RSV administration in an endurance test. In HF-fed animals treated with RSV, the distance run to exhaustion was twice that of the HF-fed controls (Figure 3C). To account for the potentially confounding significant weight difference between RSV-treated and nontreated HF-fed mice, we redid the test using RSV-treated and nontreated chow-fed mice, which did not significantly differ in body weight (Figure 1A). The

RSV-treated mice, however, still outran the control chow-fed mice by nearly double the distance (Figure 3C). Thus, RSV treatment significantly increases the animal's resistance to muscle fatigue, consistent with increased mitochondrial activity and the transformation of muscle toward a slow type phenotype.

No Behavioral Defects, but Improved Motor Function in RSV-Treated Mice

Since it was reported that SIRT1 is required for increased physical activity in response to CR (Chen et al., 2005), we carefully investigated whether the RSV-mediated increase in resistance to muscle fatigue was a result of a behavioral response or was truly a metabolic consequence. We initially examined the effect of RSV on spontaneous activity in mice by assessing their circadian activity. No significant difference was observed between chow- and HF-fed mice (data not shown). However, in RSV-treated HF-fed mice, there was a significant decrease in ambulatory locomotor activity as well as a tendency to decrease the number of rears (Figure 4A). These observations indicated that the effect of RSV on EE and weight gain could not be explained
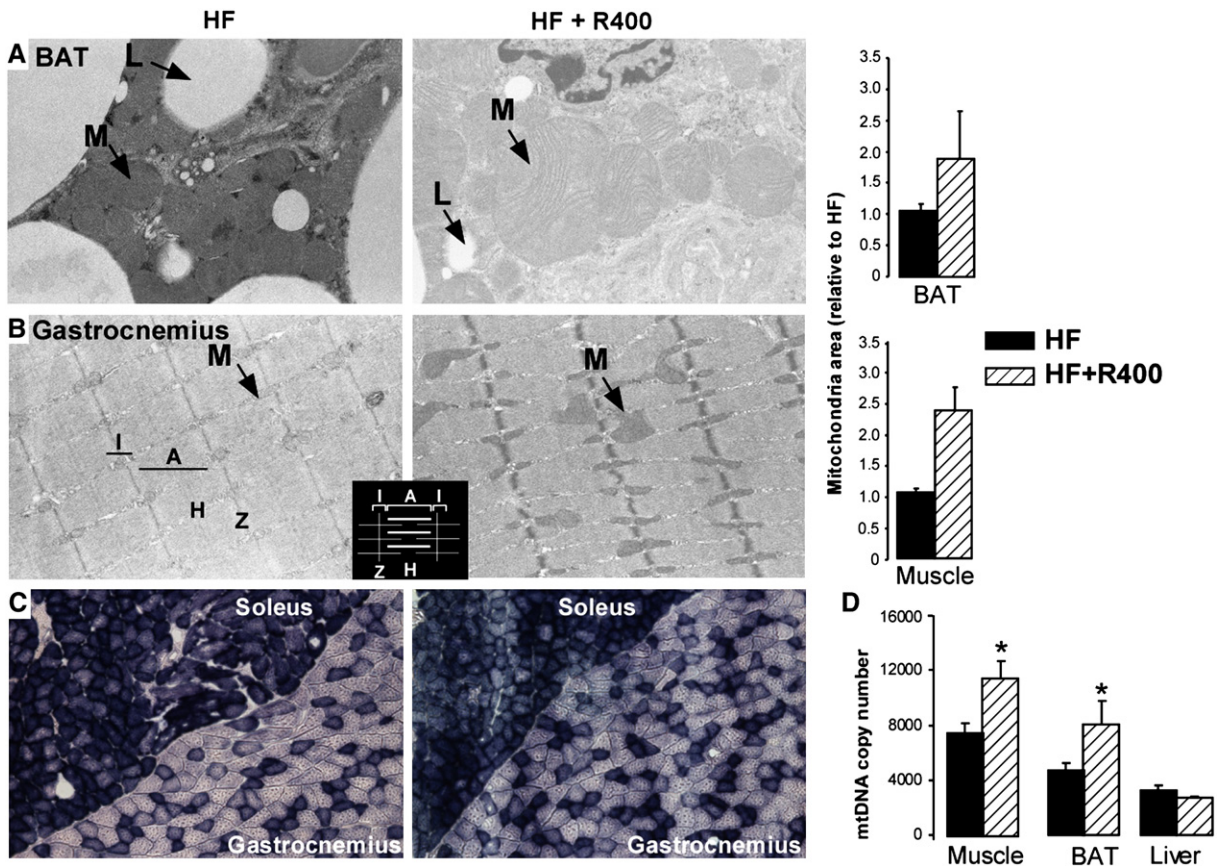
Figure 2. RSV Increases Mitochondrial Activity in the BAT and Muscle

(A and B) Transmission electronic microscopy (magnification of 20,000) image and corresponding quantification of mitochondria size in BAT (A) and nonoxidative fibers of gastrocnemius muscle (B) from RSV-treated (HF+R400) and nontreated HF-fed animals. Arrows indicate the position of mitochondria (M) and lipid droplets (L), and the inset shows the schematic organization of muscle fiber anatomy. Quantification was performed on 2 animals/group and is expressed relative to HF controls.

(C) SDH staining of gastrocnemius and soleus muscle from RSV-treated and nontreated HF-fed animals.

(D) mtDNA copy number of gastrocnemius muscle, BAT, and liver from RSV-treated and nontreated HF-fed mice (n = 4 animals/group). Values represent means ± SEM.

by an increase in spontaneous activity. In fact, the reduced level of activity in RSV-treated mice is in line with the decrease in resting heart rate (Figure 4B). Cardiodetrimental effects are, however, not suspected due to the lack of significant effect of RSV on blood pressure (Figure 4C), various echocardiography parameters (data not shown), PGC-1 activity (see below), and cardiac gene expression (Figure S3).

To discount the potential of central nervous system (CNS)-mediated behavioral effects and to determine the effect of RSV treatment on other motor abilities, we evaluated anxiety and sensorimotor function. No significant effects were observed between RSV-treated and nontreated HF-fed mice on anxiety, as evaluated by open field (Figure 4D), light/dark box (Figure 4E), and elevated-plus-maze tests (Figure 4F). The absence of a difference between RSV-treated and nontreated HF-fed mice in pain sensitivity, as measured in the hot-plate test (data not shown), also discounted the possibility that fatigue re-

sistance might be due to altered pain sensitivity. Interestingly, as compared to nontreated HF-fed mice, the RSV-treated mice displayed increased muscle strength (Figure 4G) and markedly improved motor coordination and traction force as revealed in the rotarod (Figure 4H) and string tests (Figure 4I). These tests support the data obtained in the exercise test and suggest that RSV may improve neuromuscular function.

RSV Reprograms Muscle Gene Expression

To make the molecular connection between RSV treatment and the apparent myofiber remodeling, we profiled the expression of ±40,000 genes by microarray analysis. As the coordination of muscle plasticity is a complex event, composed of many small but cumulatively significant changes, we used a gene-set enrichment analysis (GSEA) to look for coordinate expression within treated samples of a priori-defined groups of genes (Mootha et al., 2003; Subramanian et al., 2005). Genes were ranked
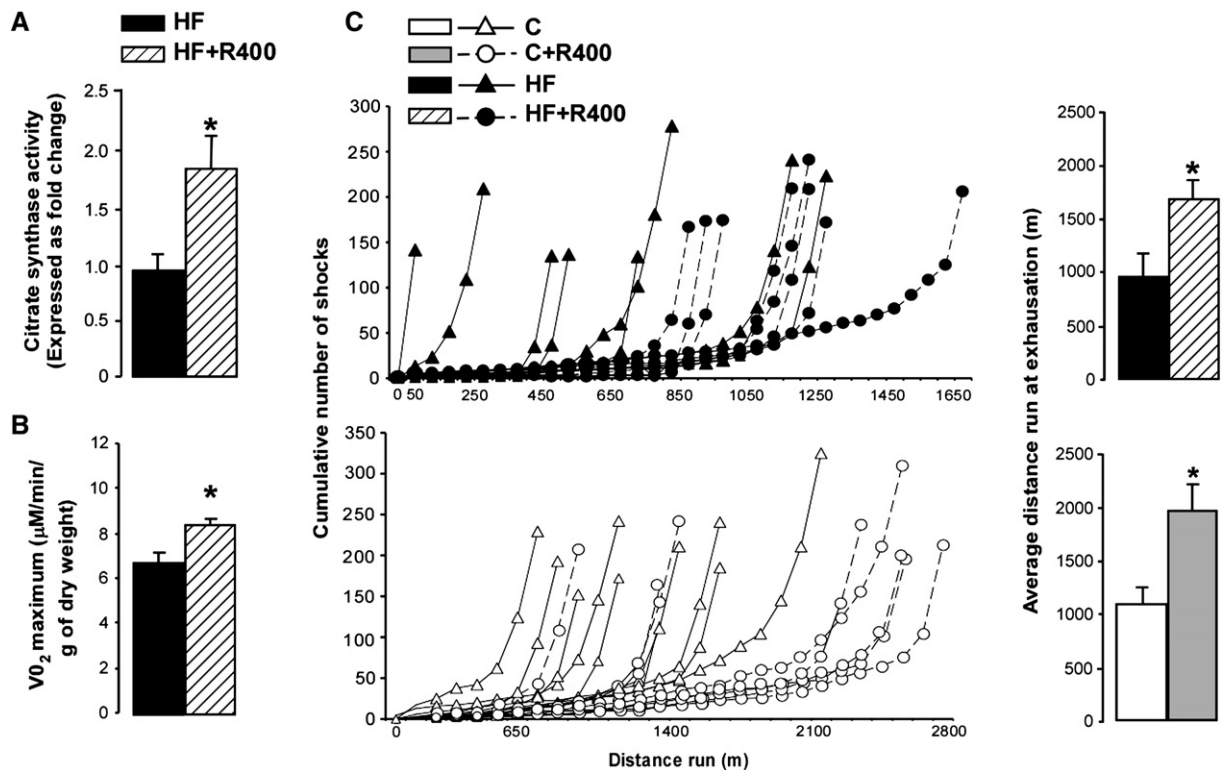
Figure 3. Enhanced Oxidative Capacity and Endurance in RSV-Treated Mice

(A) Activity of the citrate synthase, as measured in homogenates of gastrocnemius fibers isolated from RSV-treated (HF + R400) and nontreated HF-fed mice. N = 3 animals/group, and values are expressed relative to control.

(B) Maximum VO$_2$ consumption in isolated gastrocnemius fibers measured ex vivo. N = 5 animals/group.

(C) The effect of RSV on endurance, as measured by an exercise test. Individual animal performances (graphs on the left) as well as the average distance run until exhaustion (graphs on the right) are presented for animals treated with HF or HF + R400 (top) or chow diet (C) or chow diet and RSV at 400 mpk (C + R400) (bottom). N = ∼8 animals/group. * = P < 0.05. Values represent means ± SEM.

according to their correlation to RSV treatment, and then the position of each gene-set member was identified, and a maximum enrichment score (MES) for each gene set was calculated. Amongst the top 30 gene sets, which were significantly enriched in RSV-treated mice, were ribosomal mRNA processing, striated muscle contraction, electron transport chain, OXPHOS, and ATP synthesis (Table S1). Three representative GSEA-scoring plots and their corresponding heat maps are shown (Figure 5A). Of particular note is their increase in gene expression under RSV treatment. Individual genes in the enriched pathways were related to muscle contraction (e.g., troponins) as well as enhanced oxidative metabolic status, including components of the respiratory apparatus (e.g., NDUFB8), oxidative enzymes (e.g., CoxVa), and ATPases (e.g., ATP5G3). These could provide a slow but stable, long-lasting supply of ATP, which would explain the increased muscle endurance associated with RSV. In addition, sets of genes supporting organelle biogenesis such as those encoding RNA-processing enzymes and ribosomal subunits were also enriched (Figures 5 and S2). Thus, this global molecular fingerprint of RSV identified coordinated changes in the expression of groups of genes functionally involved

in mitochondrial biogenesis and function underpinning the enhanced oxidative capacity of the muscle.

To further evaluate the hypothesis that mitochondrial activity was affected by RSV treatment, we measured the expression of PGC-1α and several of its targets by Q-RT-PCR in gastrocnemius muscle. PGC-1α mRNA was significantly induced upon RSV treatment, which also translated into an increase in PGC-1α protein (Figures 5C and 5D). We also noted an increase in PGC-1β, which has several overlapping functions with that of PGC-1α in inducing genes related to OXPHOS (Lin et al., 2002c). The estrogen-related receptor α (ERRα), which mediates many of the downstream effects of activated PGC-1α on mitochondrial function and is itself a target of PGC-1α (Huss et al., 2002; Schreiber et al., 2003; Schreiber et al., 2004; Tcherepanova et al., 2000), was markedly increased by RSV, as was the ERRα/PGC-1 target, nuclear respiratory factor-1 (NRF-1) (Mootha et al., 2004; Patti et al., 2003). Mitochondrial transcription factor A (Tfam), a nuclear encoded mitochondrial transcription factor that is indispensable for the expression of key mitochondrial-encoded genes (Larsson et al., 1998) and a target of NRF-1, was also increased. In addition to the transcription
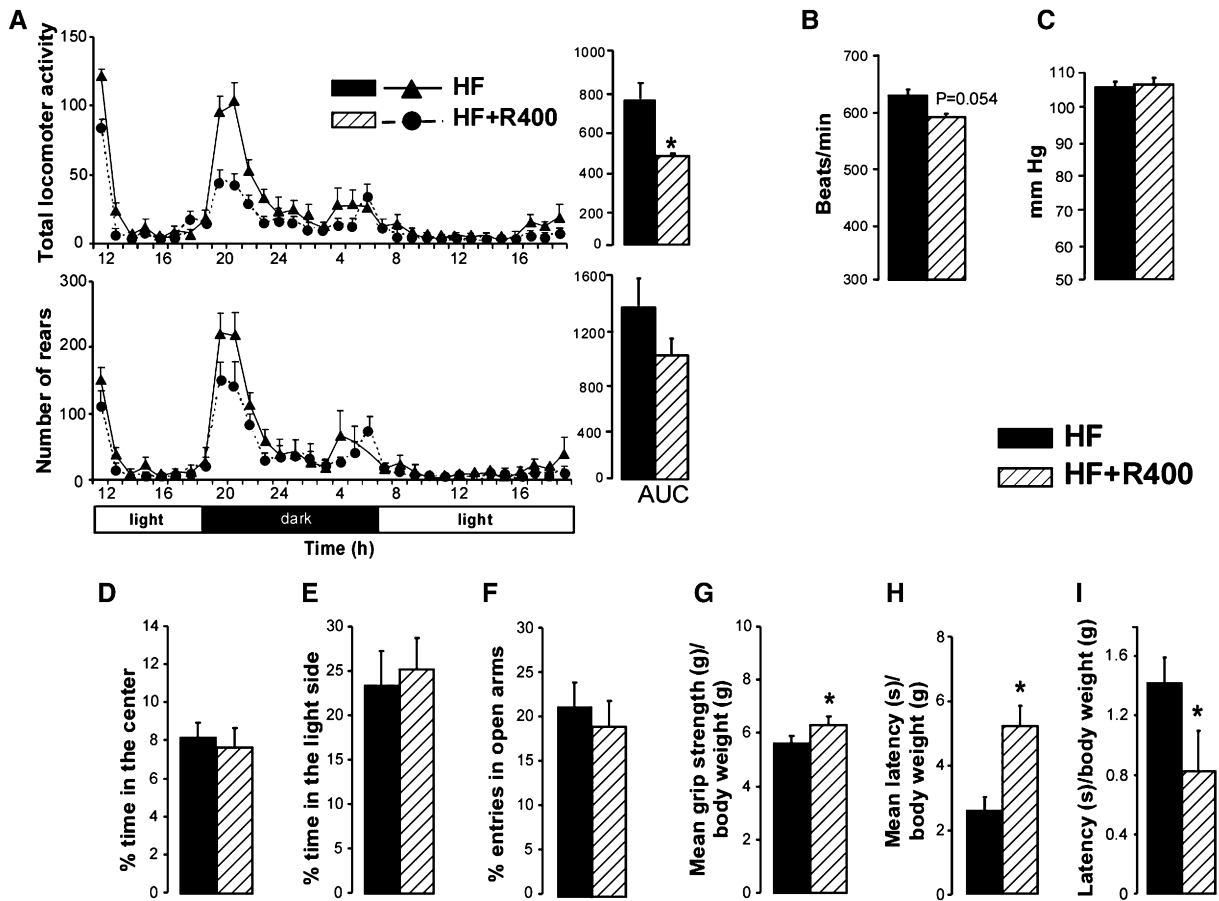
Figure 4. The Increase in Endurance and EE by RSV Is Not Explained by Increased Spontaneous Locomotor Activity or Altered Behavior

C57Bl/6J mice were fed an HF diet or HF diet and RSV 400 mpk (HF + R400). N = 8–10 animals/group.

(A) Circadian activity, measured as the total ambulatory locomotor activity (top graph) and the number of rears (bottom graph). The mean AUC are shown on the right.

(B and C) Heart rate as beats/min (B) and blood pressure in mm Hg (C).

(D–I) Behavior tests to evaluate mouse anxiety, including open field (D), light/dark box (E) and elevated-plus-maze (F), and sensorimotor function, including grip strength (G), rotarod (H), and string test (I). * = P < 0.05. Values represent means ± SEM.

factors, an array of additional downstream targets of PGC-1α (Lin et al., 2005), including genes involved in fatty-acid oxidation (medium chain acyl-CoA dehydrogenase, MCAD), uncoupling, and protection against ROS (uncoupling protein 3, UCP-3), and fiber-type markers (myoglobin and troponin 1) were induced by RSV.

As predicted from the electron microscopy and the cold test results, we noted a significant increase in gene expression in pathways related to energy homeostasis (Figure S3A) in BAT. PGC-1α, peroxisome proliferator-activated receptor α (PPARα), and UCP-1 mRNA levels were all induced by RSV. Like ERRα, PPARα induces genes that facilitate β-oxidation of fatty acids (Schoonjans et al., 1997), and UCP-1 is largely responsible for the uncoupling of respiration from ATP synthesis resulting in the production of heat in the BAT (Ricquier, 2005). Interestingly, however, mitochondrial changes were not evident in the heart, as reflected by a lack of changes in gene expression of

PGC-1α and related target genes (Figure S3B), which corroborates the insignificant effects on heart physiology. We also surveyed the liver and found no changes in expression of gluconeogenic genes but a tendency for increased expression in genes related to OXPHOS (Figure S3B).

RSV Induces PGC-1α Activity through SIRT1

In spite of the RSV-mediated induction in PGC-1α mRNA and protein expression (Figure 5D), PGC-1α can also be regulated at the posttranslational level, as modifications, such as acetylation, significantly impact on its activity (Rodgers et al., 2005). Therefore, we compared PGC-1α acetylation in gastrocnemius muscle, BAT, and heart between mice that were fed an HF diet in the presence or absence of RSV (Figure 6A). In gastrocnemius muscle and BAT, we observed that the ratio of acetylated nuclear PGC-1α to total nuclear PGC-1α protein was significantly decreased in RSV-treated mice, suggesting that PGC-1α
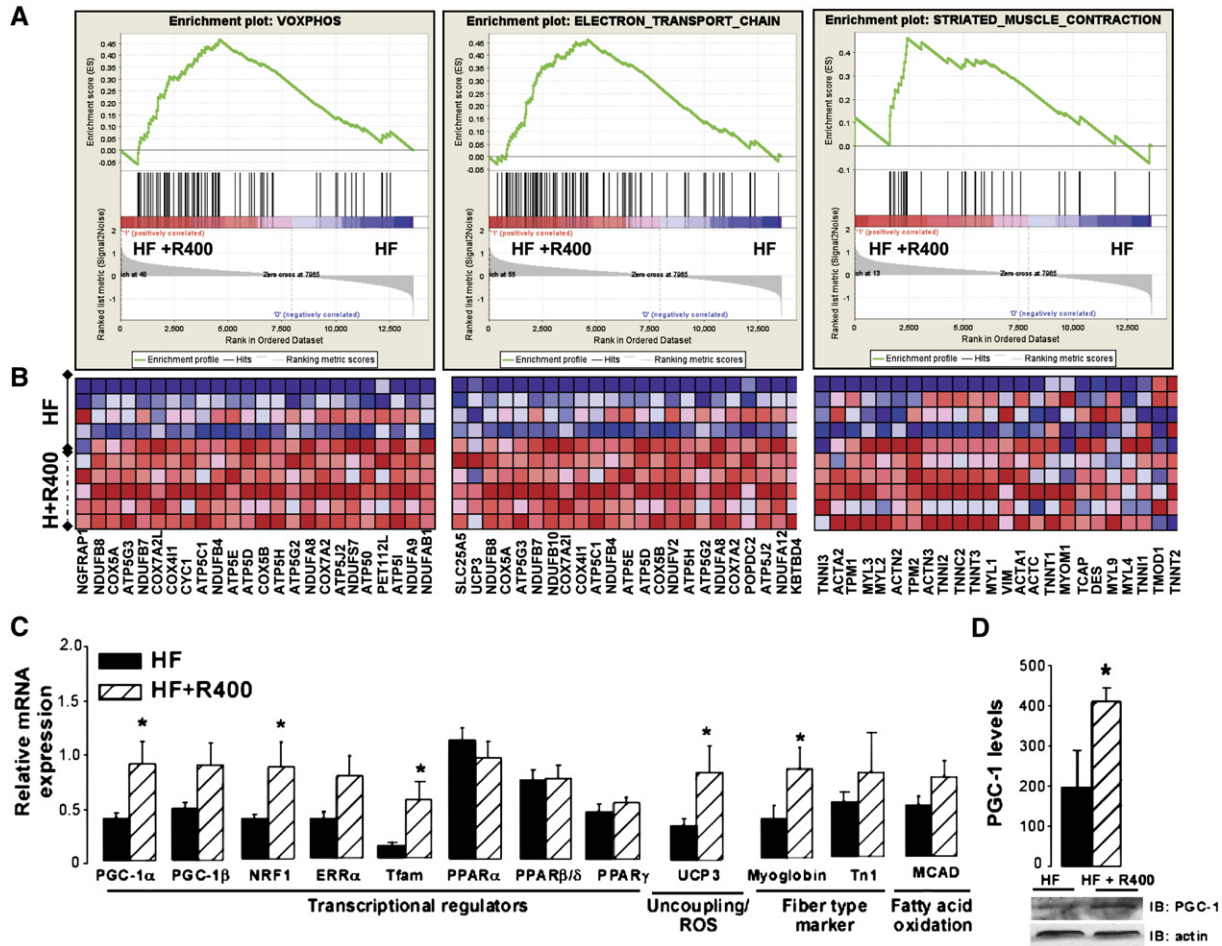
Figure 5. The Gene-Expression Profile of Skeletal Muscle from RSV-Treated Mice Is Enriched in Pathways Related to Mitochondrial Biogenesis and Function

(A) Gene-set enrichment analysis (GSEA) of gene-expression profile in gastrocnemius muscles isolated from HF-fed male C57Bl/6J mice treated with or without RSV (400 mpk, HF + R400). N = 5 animals/group. Three plots are shown where the FDR was <10% for both oxidative phosphorylation pathway and electron transport chain and <33% for striated muscle contraction. The top part of each plot shows the progression of the running enrichment score and the maximum peak therein. The middle part shows the genes in the gene set as "hits" against the ranked list of genes. The lower part shows the histogram for the ranked list of all genes in the expression data set. See Experimental Procedures and Results for more detailed description.

(B) The corresponding heat maps show the expression values for the top subset of genes of each pathway, which contributes most to the enrichment score in the five mice of each group. Results are transformed into colors, where red indicates a high and blue a low expression value.

(C) Relative mRNA expression levels of key genes related to mitochondrial function as measured by Q-RT-PCR in the gastrocnemius muscle of HF or HF + R400 mice. N=5-6 animals/group. Data represent mRNA levels relative to cyclophilin and are given as means ± SEM.
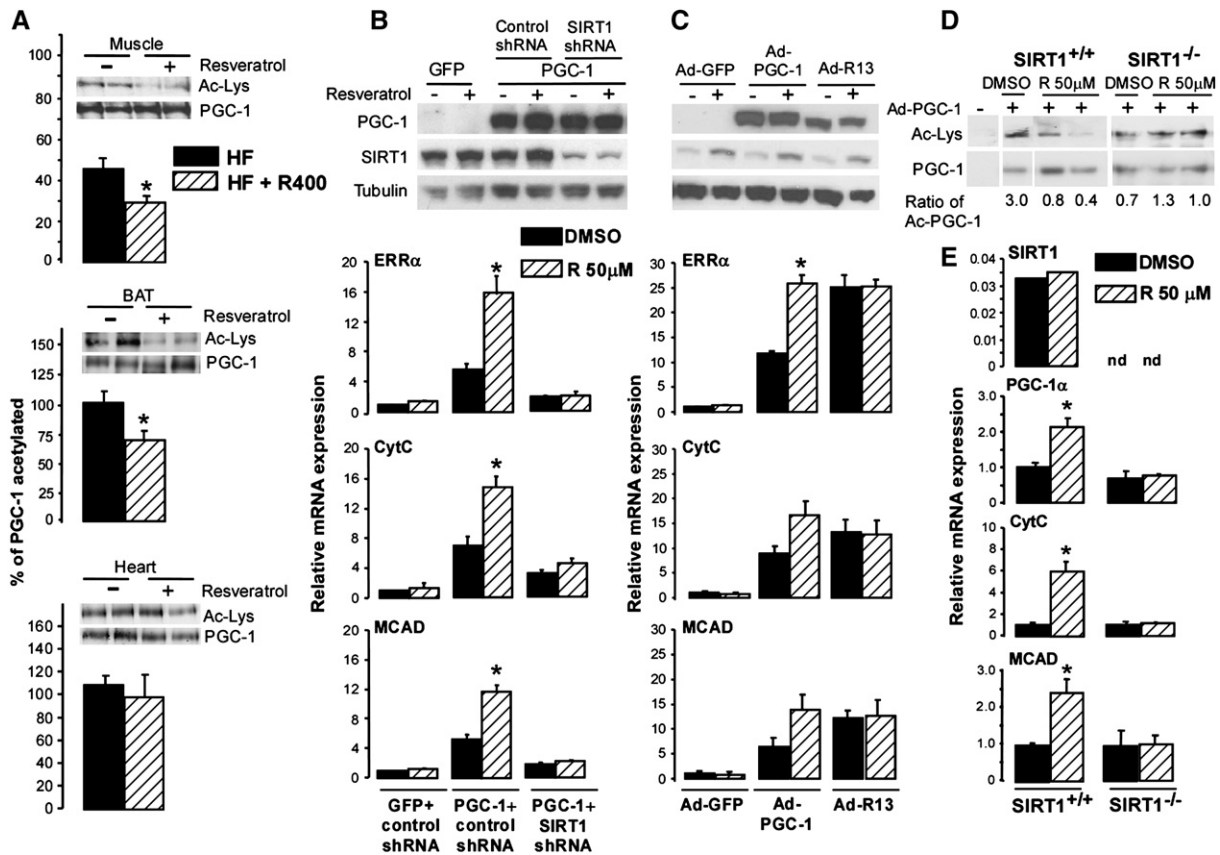
(D) Quantification and representative western blot of PGC-1 protein in nuclear extracts from the gastrocnemius muscle of RSV treated and nontreated HF fed animals. Values represent arbitrary density units ± SEM (n = 3 animals/group), and actin serves as loading control. * = P < 0.05.

activity was also increased (Figure 6A). In contrast, no effect on PGC-1α acetylation was observed in heart of the RSV-treated HF mice (Figure 6A), indicating a certain tissue specificity in RSV's effects.

To test whether RSV's effects on mitochondrial function are mediated by SIRT1 and PGC-1α, we coinfected C2C12 myotubes with an adenovirus expressing PGC-1α, together with either a specific short hairpin RNA (shRNA) directed against SIRT1 or a control shRNA. This strategy effectively reduced endogenous SIRT1 levels while still maintaining high PGC-1α expression (Figure 6B,

top panel). Importantly the knockdown of the SIRT1 protein largely blocked the RSV-induced increase in MCAD, cytochrome C (CytC), and ERRα expression, (Figure 6B, bottom panels). This experiment also demonstrated the dependence of RSV's effect on PGC-1α, since no significant increases in mRNA expression of CytC, MCAD, or ERRα were observed in RSV-treated C2C12 cells in the absence of PGC-1α (Figure 6B).

To further consolidate our hypothesis that SIRT1 deacetylates and hence activates PGC-1α, we compared the efficacy of RSV to induce gene expression in cells infected

**Figure 6. Muscle PGC-1α Is a Molecular Target of RSV In Vivo**

(A) Representative western blots and quantification showing the relative amount of acetylated versus total PGC-1 protein, for gastrocnemius muscle, BAT, and heart. PGC-1 was immunoprecipitated (IP) from nuclear extracts and then immunoblotted with either an antiacetylated lysine antibody to determine the extent of PGC-1 acetylation (Ac-Lys) or a PGC-1 antibody to determine the total amount of PGC-1. N > 3 animals/group.

(B and C) C2C12 myotubes were coinfected with an adenovirus expressing either PGC-1α (Ad-PGC-1) or GFP and a SIRT1 shRNA or a control shRNA or a PGC-1α acetylation mutant (Ad-R13). Following 24 hr incubation with DMSO or RSV (R, 50 μM), cells were harvested for protein and RNA extraction. A representative western blot showing the protein expression levels of PGC-1 (wild-type or acetylation mutant) and SIRT1 in these cells is shown. Tubulin was used as a loading control. The mRNA expression levels of ERRα, CytC, and MCAD were determined by Q-RT-PCR. Values represent the mRNA levels relative to the housekeeping gene 36B4, * = P < 0.05.

(D) SIRT1$^{-/-}$ and SIRT$^{+/+}$ MEFs were infected with Ad-PGC-1 and treated with DMSO or RSV (R 50 μM) for 24 hr. PGC-1 was immunoprecipitated from lysates with a flag M2 antibody. Acetylated PGC-1 (Ac-Lys) was revealed by an antiacetylated-lysine antibody and total PGC-1 levels by an HA antibody. A representative western blot is shown and the quantification of the ratio of acetylated to nonacetylated PGC-1, n = 2 animals/group.

(E) Gene-expression levels are as measured in SIRT1$^{-/-}$ and $^{+/+}$MEFs following a 24 hr incubation with either DMSO or RSV (R, 50 μM). mRNA levels are relative to the 36B4 gene, * = P < 0.05 (n = 3). n.d. = not detected. Values represent means ± SEM.

with an adenovirus that either encoded the wild-type (WT) PGC-1α or the R13-PGC-1α protein in which 13 of the potential lysine acetylation sites were mutated into arginine (Rodgers et al., 2005) (Figure 6C). Since the capacity for acetylation is impaired for the R13-PGC-1α protein, it was no surprise that expression of the R13-PGC-1α mutant alone induced the PGC-1α target genes, ERRα, CytC, and MCAD, to a higher level as compared to WT PGC-1α. Importantly, addition of RSV failed to further induce the expression level of these PGC-1α target genes in the R13-PGC-1α infected C2C12 cells (Figure 6C), which is in sharp contrast to cells infected with WT PGC-1α and consistent with the dependence of RSV on

SIRT1-mediated deacetylation of PGC-1α to activate PGC-1α transcriptional programs.

Finally, we sought in vivo support for the dependency of RSV effects on SIRT1 by using mouse embryonic fibroblasts (MEFs) isolated from SIRT1$^{+/+}$ and SIRT$^{-/-}$ mice (Chua et al., 2005). In contrast to WT MEFs, in SIRT1$^{-/-}$ MEFs, RSV did not decrease PGC-1 acetylation (Figure 6D), and there was no significant effect of RSV on expression of CytC, MCAD, and PGC-1α (Figure 6E), results entirely consistent with the crucial role of SIRT1 in mediating RSV's activity. The demonstration that RSV treatment results in deacetylation of PGC-1α and modulation of the expression of PGC-1α target genes in the muscle and
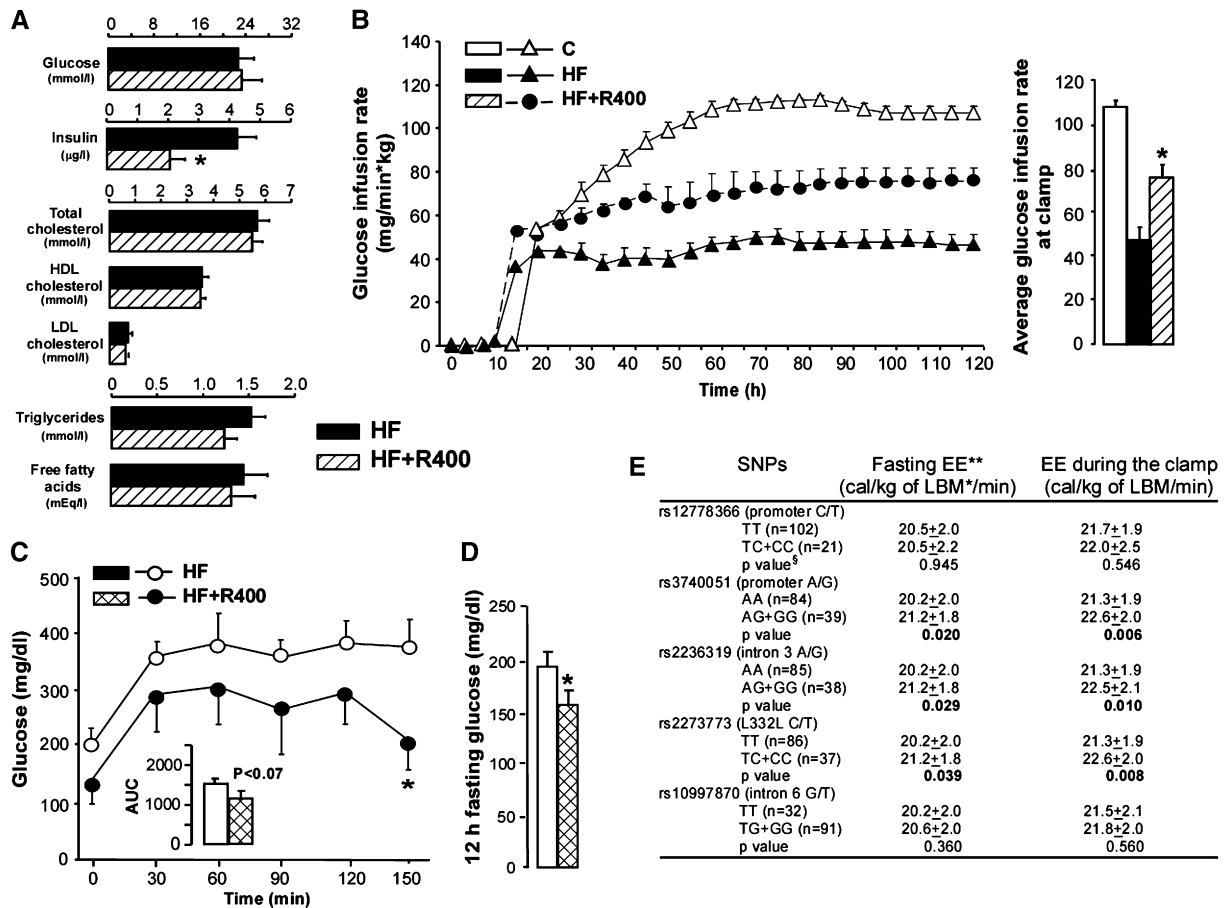
Figure 7. RSV Increases Insulin Sensitivity and the Association of the Sirt1 gene with Energy Expenditure in Humans

(A) Average glucose, insulin, and lipid levels in HF-fed C57Bl/6J mice treated with or without RSV (HF + R400) for 16 weeks. N = 8–10 animals/group.

(B) Evolution of the glucose infusion rate (GIR) during the hyperinsulinemic euglycemic clamp on C57Bl/6J mice treated with chow (C) or HF diet or HF + R400. N = 4 animals/group. The average GIR at clamp is shown in the bar graph.

(C and D) Eight week-old male KKAy mice were treated with HF diet or HF diet plus RSV at a dose of 400 mpk (HF+R400) for 8 weeks. N = 5 animals/group. OGTT was performed, and the AUCs are shown in the inset bar graph (C). Fasting (12 hr) and nonfasting plasma glucose in KKAy mice treated with HF or HF + R400 (D). Values represent means ± SEM. * = P < 0.05.

(E) The association of SNPs of the *Sirt1* gene with EE as measured in normal weight offspring of probands with type 2 diabetes (n = 123). *LBM, lean body mass, ** EE, energy expenditure mean ± SD, p values adjusted for age, gender, and familial relationship.

BAT (Figure 6A), in combination with the absence of an effect of RSV on gene expression, when SIRT1 expression is reduced or eliminated (Figures 6B and 6E) and/or when the acetylation sites specifically targeted by SIRT1 were mutated in PGC-1α (Figure 6C), is highly suggestive that RSV relies to a large extent on SIRT1 activation and PGC-1 deacetylation to achieve its effects on PGC-1α-dependent gene expression in vivo.

Improved Insulin Sensitivity in RSV-Treated Mice

Since genomic profiling of human diabetic muscle revealed a coordinated decrease in expression of genes related to OXPHOS (Mootha et al., 2003), we determined whether the effects of RSV on mitochondrial metabolism translated into changed insulin sensitivity. Although fasting glucose levels were not altered by RSV, fasting insulin levels were significantly reduced, suggesting an insulin sensitization (Figure 7A). We thus performed a hyperinsulinemic euglycemic clamp study in these mice. In line with the fact that diet-induced obesity decreases insulin sensitivity, a significant decrease in the glucose infusion rate (GIR) was observed in HF compared to chow-fed mice (Figure 7B). Importantly, however, the GIR in RSV-treated HF mice was significantly higher as compared to HF control animals, indicating that RSV improves insulin sensitivity in a diet-induced obesity model (Figure 7B). No major impact on blood lipid levels was observed after RSV (Figure 7A). We also assessed the effects of RSV in a genetic mouse model of diabesity, the KKAy mouse. KKAy mice were treated with an HF diet without or with RSV (at 400 mpk for 10 weeks). Although in this model RSV did not significantly affect weight gain, glucose tolerance,

as assessed by an oral glucose tolerance test (OGTT, 2 g glucose/kg), was significantly improved by RSV (Figure 7C). This was paralleled by a significant decrease in fasting glucose levels, suggesting that RSV possesses intrinsic antidiabetic effects that are independent of its effects on body weight (Figure 7D).

Genetic Variation in the Human *Sirt1* Gene Is Associated with EE

To determine whether common alleles in *Sirt1* might contribute to heritable phenotypic variation in EE in humans, we investigated the effects of five genetic variants in the *Sirt1* gene on EE as profiled in a cohort of healthy, normal-weight (body mass index < 26.0 kg/m^2), nondiabetic offspring of type 2 diabetic patients (Ferrannini et al., 1988). Three out of five SNPs tested (i.e., rs3740051 [promoter A/G], rs2236319 [intron 3 A/G], and rs 2273773 [L332L C/T]) were significantly associated with whole body EE as evaluated either during fasting or during a hyperinsulinemic clamp (Figure 7E). These data indicate that in humans, *Sirt1* genetic polymorphisms covary with the degree of EE, which provides an independent genetic argument that bolsters the direct involvement of SIRT1 in modulating EE that we uncovered by manipulating its activity pharmacologically with RSV in mice.

DISCUSSION

Our data demonstrate that the SIRT1 activator, RSV, induces PGC-1α activity by facilitating SIRT1-mediated deacetylation. The effects of RSV on PGC-1α target gene expression were dependent on the presence of the WT PGC-1α protein and were lost in cases where the acetylation sites in PGC-1α that are targeted by SIRT1 were mutated or when SIRT1 expression was disrupted in either C2C12 myotubes by RNAi or in MEFs isolated from SIRT1-deficient mice. The effects of RSV were seen in both muscle and BAT and resulted in an increase in mitochondrial function, which translated into an increase in EE, improved aerobic capacity, and enhanced sensorimotor function. Importantly, mice on an HF diet were consequently protected from the development of obesity and remained insulin sensitive when they were treated with RSV. Our observations therefore extend the function of the SIRT1-PGC-1α axis beyond control of liver gluconeogenesis (Rodgers et al., 2005) to adaptive thermogenesis in the BAT and muscle function. Although most of our conclusions are based on cellular studies and pharmacological interventions in mice, the novel association between genetic variations in the *Sirt1* gene and energy homeostasis in man reveals a significant place for our work in the context of human pathophysiology.

In the BAT, RSV treatment induced striking mitochondrial morphological changes and also increased UCP-1 expression levels and thus poised the mitochondria for uncoupling of respiration (Puigserver et al., 1998). This effect is consistent with the observed increase in cold tolerance and goes a long way in explaining their increase in EE and resistance to weight gain. Surprisingly, though, we did not observe similar changes in mitochondrial biogenesis by RSV in the heart, despite the coexpression of PGC-1α and SIRT1 (Figure S3). PGC-1α expression, PGC-1α acetylation, and heart function were not altered by RSV. As cardiac-specific PGC-1α overexpression in mice ultimately results in cardiomyopathy and death (Lehman et al., 2000), the absence of an effect of RSV on mitochondrial biogenesis in the heart is interesting. Although in the liver, changes in expression of genes related to OXPHOS showed a tendency to increase, other known PGC-1 target genes related to gluconeogenesis were unaffected by RSV. Therefore, we suspect that cell-specific associations between PGC-1α and other transcription factors or cofactors may exist to modulate tissue-specific transcriptional consequences of RSV.

A striking feature of the myofiber is its ability to transform and remodel in response to environmental demands (Booth et al., 2002). The most notable is exercise training, which transforms the metabolic status of the myofiber to one of increased oxidative metabolism and switches the fiber from one of a fast twitch type 2 to a slow twitch type 1 (Booth et al., 2002). In this study, RSV treatment of HF-fed mice induced a similar myofiber remodeling but in the absence of increased physical activity. The myofibers from RSV-treated mice were enriched in mitochondria, exhibited enhanced oxidative capacity, and displayed a higher resistance to fatigue because of the concerted activation of a genetic program geared for aerobic metabolism. Although we were unable to prove that these progressive changes in oxidative capacity by RSV lasted long enough to induce a complete type 1 fiber transformation, we did see advanced improvement in motor function, which is a component of the integrated physiological response required to improve exercise performance. Comparable changes in muscle fiber types have been recapitulated in genetically engineered mouse models that trigger calcium regulatory pathways (Wu et al., 2002), mimic PPARβ/δ activation (Wang et al., 2004), or enhance PGC-1α activity (Lin et al., 2002a). The fact that RSV induces a muscle fiber type switch in the absence of genetic engineering underscores its powerful pharmacological activities. RSV could hence be viewed as a performance-enhancing drug, which, in contrast to other pharmacological mediators, such as anabolic steroids, improves performance by changing myofiber specificity rather than by increasing muscle mass.

Different cells and tissues have distinct sensitivities and requirements of mitochondrial function. Neurons appear particularly vulnerable to mitochondrial dysfunction, as testified by the many neurodegenerative diseases, including Alzheimer's and Huntington's diseases, which have been associated with abnormal mitochondrial activity and dynamics (Chan, 2006). Interestingly, we noted a significant improvement in motor coordination and traction force, as well as enhanced aerobic performance, in RSV-treated mice, suggesting a potential beneficial neuronal effect of RSV. In the brain, PGC-1α deficiency in mice leads

to certain behavioral abnormalities, including profound hyperactivity with neurodegeneration, reminiscent of Huntington's disease (Leone et al., 2005; Lin et al., 2004). Interestingly, we noted a significant decrease in spontaneous locomotor activity in RSV-treated mice, which is converse to the hyperactive phenotype of the PGC-1$\alpha^{-/-}$ mice, thus pointing once more to a potential connection between mitochondrial activation and PGC-1α, this time in the CNS. In fitting with these potential neuroprotective effects has been the recent observations that RSV rescued neuronal dysfunction induced by the polyglutamine tracts in the Huntington protein in *C. elegans* (Parker et al., 2005) and significantly delayed the age-dependent decay of locomotor activity and cognitive performances in the short-lived vertebrate, *N. furzeri* (Valenzano et al., 2006). A potential connection with SIRT1 becomes apparent in the remarkable protection against axonal degeneration afforded by SIRT1 activation in the Wallerian degeneration slow mice, an effect that can be reproduced in vitro on dorsal root ganglion cultures by RSV (Araki et al., 2004).

Mitochondrial function can impact on whole-body metabolism. This is most evident in the muscle, a metabolically flexible tissue that switches between carbohydrate and lipid as substrates in order to meet the energy demands (Kelly and Scarpulla, 2004). Indeed, impaired mitochondrial function that directs fatty acids toward storage, as opposed to oxidation, may contribute considerably to intramyocellular lipid accumulation, which has been linked to insulin resistance in obesity and type 2 diabetes in humans (Patti et al., 2003; Petersen et al., 2004; Virkamaki et al., 2001). In line with this, RSV significantly improved both muscle oxidative capacity and sensitivity to insulin in HF-fed mice. Although the RQ, reflective of whole-body substrates use, was unchanged under RSV treatment, gene-expression analysis in the gastrocnemius supported an increase in fatty-acid oxidation since MCAD (Figure 5C) expression was increased and glucose utilization reduced as PDK4 levels were increased (data not shown) (Kim et al., 2006). Complimenting the effects on tissues metabolizing fat, such as muscle and BAT, was the effect of RSV on storage tissues such as WAT, where it reduced both fat pad mass and adipocyte size. Consistent with such wide-spread effects of RSV on fat and muscle was the previous work showing increased fat mobilization by genetically manipulating SIRT1 activity (Picard et al., 2004), as was the capacity of SIRT1 to modulate muscle-cell differentiation (Fulco et al., 2003).

Admittedly, RSV is reported to have pleiotropic properties, including the activation of signaling pathways involving AMPK, thyroid hormone, and estrogen (Baur and Sinclair, 2006; Baur et al., 2006). However, in the muscle-microarray analysis, we did not observe enrichment of gene expression in pathways related, for example, to estrogen or thyroid signaling. Together, with our data demonstrating that the muscle gene-expression changes are critically dependent on the presence of SIRT1, our data confirm the fact that SIRT1 is the main target of RSV's metabolic actions (Howitz et al., 2003). At this point, we cannot

determine whether PGC-1α is the only target of RSV-activated SIRT1. However, evidence supporting the importance of PGC-1α in mediating effects of RSV on mitochondrial gene expression in muscle cells is the fact that RSV's effects were not observed unless the wild-type PGC-1α protein was overexpressed and that these effects are lost in cases where the acetylation sites, targeted by SIRT1, were mutated in PGC-1α. Furthermore, the effects of RSV in the muscle and BAT recapitulate those observed by stimulating PGC-1α activity and are hence consistent with the convergence between SIRT1 and PGC-1α activation described in the hepatocyte (Rodgers et al., 2005). Despite this, we cannot exclude unequivocally that PGC-1α is the sole target of SIRT1, as SIRT1 interacts with and deacetylates other substrates (Blander and Guarente, 2004), including potential regulators of metabolism and mitochondrial function such as FOXO1 (Brunet et al., 2004; Motta et al., 2004) and p53 (Matoba et al., 2006). Finally, it is possible that the consequence of RSV activation of SIRT1 is different in other tissues, since unlike the stimulation of PGC-1α activity seen here in muscle and previously reported in liver (Rodgers et al., 2005), in the PC12 adrenal cell line, PGC-1α activity was inhibited by SIRT1 (Nemoto et al., 2005).

Since mitochondria are recognized organelles for aerobic production of high-energy phosphates and bear a central role in cellular metabolism, especially in tissues with high metabolic intensity, it is not surprising that their dysfunction has been associated with cardiovascular, metabolic, and neurodegenerative diseases. Our studies genetically and pharmacologically associate SIRT1 with PGC-1α and EE and warrant the further evaluation of SIRT1 activators as a strategy to prevent and/or treat these common disorders. This could be particularly appealing in the metabolic arena, where physical activity and dietary restriction, the cornerstones of clinical management of the metabolic syndrome, are known to enhance mitochondrial activity. It is tempting to speculate that the basis of the French paradox and the beneficial effect of RSV on life span could be attributed in part to the prevention of chronic cardiovascular, metabolic, and neurodegenerative diseases, important determinants of mortality in the industrialized world. This claim is supported by data in a concurrent study, which demonstrated that long-term RSV administration extended life spans of mice (Baur et al., 2006).

EXPERIMENTAL PROCEDURES

In Vivo Analysis

Four to eight week male C57Bl/6J mice from Charles River (L'Arbresle, France) and 8 week male KKAy mice from Clea (Tokyo, Japan) were housed in specific pathogen-free conditions with a 12 hr light-dark cycle and had free access to water and food. RSV (Orchid, Chennai, India) was mixed with either powdered chow (DO4, UAR, France) or HF diet (D12327, Research diet, New Brunswick, USA) at a concentration of 4 g/kg of food to provide a 400 mpk dose, and pellets were then reconstituted. Control groups received pellets without drug. Body weight and caloric intake were monitored throughout the experiments.

The protocols used to assess behavioral, cardiac, and metabolic phenotypes included the following: body composition by DEXA; EE by indirect calorimetry (13 hr, food and water) and 4°C cold test (6 hr); circadian activity by metabolic cage monitoring (32 hr); anxiety by open field, elevated-plus-maze, and light/dark test; locomotor function by rotarod, string and grip strength test; blood pressure and heart rate by tail-cuff system; cardiac anatomy and systolic and diastolic function by echocardiography; glucose sensitivity by an oral glucose tolerance test (16 hr fasted, 2 g glucose/kg mouse), and hyperinsulinemic euglycemic clamp (4 hr fast, 18 mU insulin/kg/min, clamped at 5.5 mmol/L for 60 min); and endurance test by variable speed belt treadmill and incremental speed protocol (range from 18 cm/s to 40 cm/s on habituated 2 hr fasted mice). These tests were performed as outlined in the standard operating procedures (SOP) linked to the EMPReSS website http://empress.har.mrc.ac.uk and as described in the Supplemental Experimental Procedures.

Ex Vivo Analysis

O_2 consumption was measured in glycolytic fibers isolated from gastrocnemius muscle, using the technique as described (N'Guessan et al., 2004). See Supplemental Experimental Procedures for a brief description.

Histological and Biochemical Analysis

Histological analysis, including HE and SDH staining and electron microscopy (EM), were performed as outlined on the EMPReSS website (http://empress.har.mrc.ac.uk) and described in the Supplemental Experimental Procedures. Mitochondria in EM images were quantified using Image J version 1.36b.

Citrate synthase activity in gastrocnemius muscle extracts was determined spectrophotometrically (Ceddia et al., 2000). Fecal lipids, including triglyceride and cholesterol content, were measured enzymatically, using commercially available kits and manufacturers protocol (WAKO, Richmond, VA), following a Folch extraction. Blood plasma was analyzed for insulin by ELISA (Crystal Chem, Downers Grove, IL), glucose by glucose oxidase kit (Sigma, Lyon, France) and free fatty acids, triglycerides, HDL, LDL, AST, ALT, and total cholesterol using enzymatic assays (Boehringer-Mannheim, Mannheim, Germany) on an Olympus automated analyzer.

In Vitro Analysis

SIRT1$^{-/-}$ and $^{+/+}$ MEFs (Chua et al., 2005) and the C2C12 mouse myoblast cell line were maintained in culture as described previously (Rodgers et al., 2005). Following C2C12 myotube differentiation, cells were infected with adenovirus expressing either Flag-HA-PGC-1α, Flag-HA-R13, or Sirt1 shRNA (Rodgers et al., 2005). MEFs and C2C12 were treated for 24 hr with 50 μM RSV or DMSO.

DNA, RNA, and Protein Analysis

Total DNA was extracted as described in the Supplemental Experimental Procedures, and quantitative (Q) PCR was performed using mitochondrial DNA and genomic DNA-specific primers.

RNA was extracted using the TRIzol® reagent (Invitrogen, Carlsbad, CA). Q-RT-PCR was performed as described (Watanabe et al., 2004). Primer details are listed online (Table S2). Affymetrix mouse 430_2 microarray analysis was performed according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Data were analyzed by Affymetrix MAS 5.0 software and GSEA (http://www.broad.mit.edu/gsea) (Mootha et al., 2003; Subramanian et al., 2005).

Nuclear protein fractions from gastrocnemius muscle were prepared as described online. Protein extracts were separated by SDS-PAGE and immunoblotted using antibodies against PGC-1 (H-300, Santa Cruz, CA), SIRT1 (anti-Sir2, Upstate, Norcross, GA), tubulin (Upstate) and actin (Santa Cruz, CA). PGC-1 acetylation was analyzed by immunoprecipitation of PGC-1 from nuclear lysates (50 μg) with anti-PGC-1 antibody (1 μg) followed by western blot using an acetyl-lysine antibody (Cell Signaling, Danvers, MA) (Rodgers et al., 2005).

Clinical Genetic Study

The collection of subjects and the study protocol have been published (Salmenniemi et al., 2004), and a brief summary is available online. The study protocol was approved by the Ethics Committee of the University of Kuopio, and all subjects gave an informed consent. The mean age and BMI of the subjects was 34 years and 23 kg/m^2, respectively. All subjects underwent an OGTT. Indirect calorimetry was performed in the fasting state and during hyperinsulinemia (40 mU/m^2/min insulin infusion for 120 min) as described (Salmenniemi et al., 2004). The rates of EE were calculated according to Ferrannini et al. (1988). Selection of the SNPs of Sirt1 was based on linkage disequilibrium and haplotype block analysis of the HapMap project data (http://www.hapmap.org; Public Release #20/Phase II, January 24, 2006; population: Utah residents with ancestry from northern and western Europe).

Statistics

Statistical analyses were performed with the Student's t test for independent samples (nonparametric), and data are expressed as means ± SEM unless specified otherwise. P value > 0.05 was considered as statistically significant.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/127/6/1109/DC1/.

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