Resveratrol ameliorates metabolic disorders and muscle wasting in streptozotocin-induced diabetic rats

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Chen KH, Cheng ML, Jing YH, Chiu DT, Shiao MS, Chen JK. Resveratrol ameliorates metabolic disorders and muscle wasting in streptozotocin-induced diabetic rats. Am J Physiol Endocrinol Metab 301: E853–E863, 2011. First published July 26, 2011; doi:10.1152/ajpendo.00048.2011.—Diabetes mellitus (DM) is characterized by dysregulated energy metabolism. Resveratrol (RSV) has been shown to ameliorate hyperglycemia and hyperlipidemia in diabetic animals. However, its overall in vivo effects on energy metabolism and the underlying mechanism require further investigation. In the present study, electrospray ionization-tandem mass spectrometry was employed to characterize the urine and plasma metabolomes of control, streptozotocin-induced DM and RSV-treated DM rats. Using principal component analysis (PCA) and heat map analysis, we discovered significant differences among control and experimental groups. RSV treatment significantly reduced the metabolic abnormalities in DM rats. Compared with the age-matched control rats, the level of carnitine was lower, and the levels of acetyl/carnitine and butyrylcarnitine were higher in the urine and plasma of DM rats. RSV treatment ameliorated the deranged carnitine metabolism in DM rats. In addition, RSV treatment attenuated the diabetic ketoacidosis and muscle protein degradation, as evidenced from the attenuation of elevated urinary methyl-histidine and plasma branched-chain amino acids levels in DM rats. The beneficial effects of RSV in DM rats were correlated with activation of hepatic AMP-activated protein kinase and SIRT1 expression, increase of hepatic and muscular mitochondrial biogenesis and inhibition of muscle NF-κB activities. We concluded that RSV possesses multiple beneficial metabolic effects in insulin-deficient DM rats, particularly in improving energy metabolism and reducing protein wasting.

diabetes; metabolomics; AMP-activated kinase; carnitine

Diabetes mellitus (DM) is characterized by dysregulated energy metabolism, and its incidence is growing to pandemic levels worldwide (28a). DM eventually leads to vascular diseases of the coronary arteries and cerebrovascular system, renal failure, blindness, neurological complications, and premature death (21, 29, 41). Current oral hypoglycemic agents, such as sulphonylureas, biguanides, and peroxisome proliferator-activated receptor-γ agonists induce one or more adverse effects, including hypoglycemia at high doses, liver toxicity, lactic acidosis, edema, and aggravated heart failure.

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Resveratrol (RSV; 3,5,4′-trihydroxy stilbene), a natural anti-oxidant found in plants and red wines, has anti-inflammatory, antiatherosclerotic, and anticancer properties (5). RSV has also been shown to reduce the synthesis of lipids in rat liver, interfere with arachidonate metabolism (33), inhibit platelet activation and aggregation (6), suppress TNF-induced activation of nuclear transcription factors (NF-κB) (25), inhibit the generation of reactive oxygen species (ROS) by human polymorphonuclear leukocytes (36), and exert cardioprotective effects against ischemia-reperfusion injuries in rat hearts (17). RSV has been reported to have significant hypoglycemic and hypolipidemic effects in streptozotocin (STZ)-induced DM rats (39). RSV improves the activity of the key enzymes for carbohydrate metabolism in diabetic rats (32). In addition, RSV and caloric restriction are similarly effective in delaying aging and mediating amelioration of type 2 DM and obesity in mice (4, 22, 38). These beneficial effects correlate with increased insulin sensitivity, AMPK and SIRT1 activities, and mitochondrial genesis. AMPK is a family of serine protein kinases that are regulated by alteration of the cellular energy state (14). Recently, AMPK has been shown to play an essential role in the metabolic effects of RSV (40). The aforementioned studies suggest that RSV may have potential for the treatment of DM-induced metabolic disorders. However, its overall in vivo effects on energy metabolism and its underlying mechanism require further study.

Metabolomics is a systemic approach for studying in vivo metabolic profiles and can provide information on environmental impact, disease states, drug effects, and gene function (13, 43). The major tools of the metabolomics approach include 1H-NMR spectroscopy, GC-MS, and liquid chromatography-mass spectrometry (LC-MS). Much of the original work in the metabolomics analysis of diabetes was performed using NMR spectroscopy. Zhang et al. (48) have reported the disturbances in glucose metabolism, the tricarboxylic acid (TCA) cycle and choline metabolism in STZ-induced DM rats. Additionally, Huo et al. (19) reported increased trimethylamine-N-oxide, 3-hydroxybutyrate and decreased glucose, N-acetyl glycoprotein, lipoprotein, lactate, acetoacetate, and unsaturated fatty acids in serum from metformin-treated patients compared with untreated ones. Electrospray ionization (ESI) tandem mass spectrometry (MS/MS), operated in combination with an initial liquid or gas-phase chromatographic purification, has been used in a number of metabolomic studies. ESI-MS/MS is highly sensitive and linear and is a sufficiently precise method
for the quantitative determination of disturbed levels of amino acids and acylcarnitines in mouse blood (34). The method also allows for large-scale screening when speed and cost-effectiveness are required.

This study employed HPLC-MS as a key tool in the analysis of the urine and plasma metabolomes of control, STZ-induced DM (DM) and RSV-treated STZ-induced DM (RSV-DM) rats and to elucidate the overall metabolic effects of RSV in diabetes.

MATERIALS AND METHODS

Reagents and chemicals. All aqueous solutions were prepared using deionized water (with a resistance of 18.2 MΩ·cm) from Millipore MilliQ system (Millipore, Billerica, MA). LC-MS solvents, including acetonitrile (ACN), methanol, and water (CHROMASOLV grade) were purchased from Fluka (Steinheim, Germany). Metabolite standards, chemical standards for structural validation of metabolites, RSV, and extra pure-grade ammonium formate (98–100%) were purchased from Sigma (St. Louis, MO). Primary antibodies to AMPKα1 (Thr172) and phospho-AMPKα1 were purchased from Cell Signaling Technology (Danvers, MA). The primary antibody to NF-κB was obtained from Millipore (Temecula, CA).

Animals and animal study. Male Sprague-Dawley (SD) rats, 8 to 10 wk old, were obtained from the National Laboratory Animal Center, Taipei, Taiwan. Animals were kept in an animal house at 22 ± 2°C and 55 ± 10% relative humidity and maintained under a 12:12-h light-dark cycle. Water and normal rodent chow (Sani-Chips; Montville, NJ) were provided ad libitum. Experimental protocols were approved by the Institutional Animal Care and Use Committee, Chang Gung University, and basic standards of laboratory animal care were followed.

Diabetes was induced with STZ (65 mg/kg body wt), and the confirmation of DM induction was performed as previously described (39). DM animals were randomly divided into two groups (n = 7 each) and were orally intubated with or without RSV at 0.75 mg/kg body wt, three times a day. RSV treatment continued for 8 wk after successful DM induction. The dose of RSV was chosen on the basis of our previous studies (20, 39). Age- and body-weight-matched rats were used as controls.

Urine and plasma samples were collected from control, DM, and RSV-DM rats at 8 wk after DM induction. Rats were transferred to metabolic cages 24 h before urine collection. Urine was collected for 5 min. The clear supernatants were pooled and dried in a nitrogen evaporator and stored at −20°C. Samples were resuspended in 100 µl of plasma. The mixture was sonicated and centrifuged at 10,000 × g for 25 min. The supernatant was collected into a separate tube, and the pellets were reextracted once with ACN. The residual pellets were reextracted with an equivalent volume of 50% aqueous methanol. The aqueous methanolic supernatant and two ACN supernatants were pooled and dried in a nitrogen evaporator and stored at −80°C. Samples were then resuspended in 100 µl of 95:5 water/ACN and centrifuged at 14,000 g for 5 min. The clear supernatant was collected for LC-MS analysis (44a).

Prior to analysis, urine samples were diluted with distilled water to a creatinine content of 20 µg/ml. Samples were centrifuged at 14,000 g for 5 min, and the supernatants were collected for LC-MS analysis.

Liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) analysis. Liquid chromatographic separation was achieved on a 100 mm × 2.1 mm Acquity 1.7-µm C18 column (Waters Corp, Milford, MA) by using an HPLC system (1200 rapid resolution system; Agilent Technologies, Santa Clara, CA). The column was maintained at 40°C with a flow rate of 0.25 ml/min. Samples were eluted from the LC column with linear gradients of solvent A (2 mM ammonium formate in water) and solvent B (100% ACN): 0–1 min: 0% B; 1–9.6 min: 0–98% B; 9.6–15 min: 98% B; and 15–18 min 0% B.

Mass spectrometry was performed on an Agilent Q-TOFMS (6510 Q-TOFMS; Agilent Technologies, Santa Clara, CA) operated in electrospray positive-ion (ESI+) and electrospray negative-ion (ESI−) modes. The scan range was from 50 to 1,000 m/z. The capillary and skimmer voltages were set at 4,000 and 65 V, respectively; the liquid nebulizer was set to 30 psig, and the nitrogen drying gas was set at a flow rate of 15 l/min; drying gas temperature was maintained at 350°C. To maintain a constant mass accuracy, purine ([M+H]+ = 121.05873), hexakis (1H,1H,3H-tetrafluoropropyl)phosphazene ([M+H]+ = 922.009798), or uric acid ([M−H]− = 119.036320) were used as internal reference ions. Data were collected in the profile mode by using data acquisition software (Agilent MassHunter Workstation).

Data processing. Information regarding the metabolites of interest was extracted from the raw data by using the molecular feature extraction algorithm (MassHunter, Agilent). In addition to time-aligned ion features, the algorithm gave monoisotopic neutral mass, retention time, and ion abundances. GeneSpring-MS was used to analyze and visualize the pattern of MassHunter data matrices. Principal component analysis (PCA) was used for clustering analysis and correlation analysis. The relative concentrations of metabolites were compared by ANOVA with Tukey HSD correction. The same software was also used for multivariate data analysis and representation. Accurate masses of features that were significantly different between control and test groups were searched against the METLIN, HMDB, and KEGG databases. Compound prediction was performed using Metabolite Database and Molecular Formula Generation software (Agilent Technologies).

Metabolites identification. For structural identification, we used identical chromatographic conditions that were used in the profiling experiment for the metabolite standards. Mass spectrometry using an Agilent 6510 Q-TOFMS was performed under the following conditions: gas temperature, 350°C; flow rate of drying gas, 10 l/min; nebulizer pressure, 30 psig; capillary voltage, 4,000 V; fragmentor voltage, 175 V; skimmer voltage, 65 V. MS and MS/MS spectra were both collected at 1.0 spectrum per second, with a medium isolation window of ~4 m/z. For MS/MS, the collision energy was set from 5 to 35 V. Several metabolites were further confirmed by an ion mobility mass spectrometer (SYNAPTO HDMS, Waters) under similar chromatographic conditions.

RT-PCR. RT-PCR was performed using total RNA isolated from the liver and gastrocnemius muscle of the rats. We used an ABI-Prism 7000 with SYBR Green 1 as a double-stranded DNA-specific dye, according to the manufacturer’s instructions (PE-Applied Biosystems, Cheshire, Great Britain). GAPDH mRNA expression served as an internal control. Primers (SIRT1: sense, 5′-GGA GGT TGC AGG AAT CCA AA-3′ and anti-sense, 5′-GGC AAG ATG CTG TTG-3′; IL-1β: sense, 5′-CAC CTC TCA AGC AGA GCA CAG-3′ and anti-sense, 5′-GGT TTC CAT GGT GAA GTA AAC-3′; IL-6: sense, 5′-TCC TAC CCC AAC TCT CCA TGC TC-3′ and anti-sense, 5′-TTG GAT GGT CCT GGT CCT TAG CC-3′) were constructed to be compatible with a single RT-PCR thermal profile (95°C for 10 min, 40 cycles of 95°C for 30 s and 60°C for 1 min). SIRT1 mRNA expression in liver, and IL-1β, IL-6 mRNA expression
in muscle relative to the GAPDH mRNA expression was recorded. The magnitude (in folds) of change in the gene expression was determined for DM, RSV-DM, and control animals.

Quantification of liver and muscle mitochondrial DNA. Relative amount of mitochondrial DNA (mtDNA) was determined by quantitative RT-PCR. The GAPDH DNA was simultaneously measured as an internal control for nuclear DNA. The ratio of mtDNA to nuclear DNA (GAPDH PCR) was performed using primers (mtDNA-specific PCR; forward, 5'-CCA GGA TGC CCT TTA GT-3' and reverse, 5'-AGA TTA CGG CTC CTG CTC A-3') and GAPDH PCR: forward, 5'-CTG CCA AGT ATG ATG ACA TCA AGA A-3' and reverse, 5'-AGC CCA GGA TGC CCT TTA GT-3') constructed to be compatible with a single RT-PCR thermal profile (95°C for 10 min, 40 cycles of 95°C for 30 s, and 60°C for 1 min).

Western blot analysis. Cytosolic and nuclear proteins were prepared using Pierce Nuclear and Cytoplasmic Extraction Reagent (NE-PER). Liver cytosolic proteins (40 µg) were separated on 10% SDS-PAGE and transferred onto a PVDF membrane, which was probed with anti-AMPKα1 (1:2,000) and anti-phospho-AMPKα1 (1:500) antibodies. Nuclear proteins of the gastrocnemius muscle (60 µg) were blotted with anti-NF-κB (1:1,000) and anti-β-actin (1:20,000) antibodies. Membranes were then blotted with horseradish peroxidase-conjugated secondary antibody (1:5,000–1:20,000), and the immunoreactive protein bands were visualized by enhanced chemiluminescence (Immobilon Western, Millipore).

Statistical analysis. All the data are expressed as means ± SE. One-way ANOVA was conducted for multiple-group comparisons and an LSD post hoc test was used to evaluate the significance of differences between group means. In all of the analyses, P ≤ 0.05 was considered significant.

RESULTS

Demographic characteristics. Plasma glucose levels of control, DM, and RSV-DM rats were 126.8 ± 5.3, 525.2 ± 24.5, and 450.0 ± 23.7 mg/dl, respectively. RSV-DM rats had plasma glucose levels that were 14.3% lower than those of the DM rats (P < 0.05) and plasma triglyceride levels that were 71.7% lower than those of the DM group (P < 0.01). The fasting plasma insulin of DM group was 32.7 ± 1.9 pmol/l and it was markedly lower than those of control rats (349.2 ± 23.9 pmol/l). The insulin levels of the DM rats were not significantly changed after the administration of RSV (33.7 ± 6.3 pmol/l). Although the amount of food consumption was similar, the mean body weight of the RSV-DM group was 30% higher than that of the DM group (Table 1). Urinary glucose level was high in DM rats but was toward normal in RSV-DM rats (Fig. 1A).

Urinary metabolite profiles. LC-MS-based analysis, coupled with univariate and multivariate statistical analysis, was used to generate the urine metabolomes that were compared in a global manner in control, DM, and RSV-DM groups. Typical base peak intensity chromatograms were obtained by scanning in ESI+ and ESI− modes (Fig. 1, A and B).

A total of 4,280 and 1,740 features were obtained by ESI+ and ESI− modes, respectively. A feature was defined as a unique m/z chromatographic retention time data for which a peak was present in at least one sample with intensity determined. Features present in all replicates within an experimental group were selected, and filtered mass lists were generated.

The filtered mass lists containing 2,068 ESI+ features and 796 ESI− features were subjected to one-way ANOVA. Accordingly, 383 ESI+ and 192 ESI− features showed significant difference (P ≤ 0.05) between experimental groups. Representative points of urine specimens from each experimental group were mapped in space defined by principal component 1 and principal component 2 orthogonal. The ESI+ and ESI− mode of the PCA spectra, corresponding to features within the filtered mass list that pass statistical filter were analyzed. The score plots exhibited clusters that corresponded to control, DM, and RSV-DM groups. The results indicated that the three groups were well distinguished (Fig. 1, C and D). The PCA showed an effective separation among groups.

When we restricted the analysis to those features that were only significantly different between DM and RSV-DM rats (with an average intensity difference >1.5-fold), 174 ESI+ and 153 ESI− features remained in the set for further analysis. The sets of features are illustrated in Fig. 1, E and F by using a heat map. The intensities of features (levels of metabolites) were conspicuous higher in DM rats than those in control and RSV-DM rats. The results suggested that the levels of many metabolites are significantly increased in the urine of diabetic rats and that RSV treatment reduces these metabolic disturbances.

Metabolites that had significantly different levels in the three groups were searched against the METLIN, HMDB, and KEGG databases. The mass accuracy of TOFMS was set at 5 parts per million (ppm) for all metabolites. Metabolites that were conspicuously high or low among groups are listed in Table 2. Twelve metabolites were identified on the basis of their chromatographic retention times and MS- and MS/MS information from the urine metabolite standards. The signal intensities of the targeted metabolites in the control, DM and RSV-DM groups are shown in Fig. 2.

In diabetic urine, the levels of acylcarboxylates, β-hydroxy acids, and amino acids metabolites were elevated. The urinary level of acetyl carnitine was 23.2-fold higher and the urinary level of butyrylcarnitine was 23.9-fold higher in DM rats than in control rats. However, the urinary levels of acetylcarnitine and butyrylcarnitine were lower in RSV-DM rats than in DM rats (Fig. 2, B and C). Urinary ketone bodies, namely 3-keto-butyrurate and 3-hydroxybutyrate, were also elevated in DM rats, but were significantly lower in RSV-DM rats (Fig. 2, F and J). Similar changes in urinary lysine and histidine metabolites, such as trimethyllysine, piperoc acid, and methylimidazole acetaldehyde, were also observed (Fig. 2, D, E, and H). In

<table>
<thead>
<tr>
<th>Table 1. Demographic characteristics and blood biochemical parameters of the control, DM, and RSV-DM rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Body weight, g</td>
</tr>
<tr>
<td>Food consumption, g/day</td>
</tr>
<tr>
<td>Water consumption, g/day</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
</tr>
<tr>
<td>Plasma triglycerides, mg/dl</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
</tr>
<tr>
<td>Urine output, ml, 24 h</td>
</tr>
</tbody>
</table>

Diabetes mellitus (DM) rats were fed without or with resveratrol (RSV) (0.75 mg/kg, three times per day) for 8 wk. *P < 0.05; †P < 0.01 between RSV-DM and DM rats; ‡P < 0.05 between DM and control rats.
The beneficial effects of RSV on the energy metabolism were shown as parts per million. The fold change reflects the averaged increase or decrease in abundance among control, DM, and RSV-DM rats.

### Table 2. Selected urine metabolites with significant differences in abundance between DM and RSV-DM rats

<table>
<thead>
<tr>
<th>Compound Name (Potential Candidates)</th>
<th>Mass (m/z)</th>
<th>Retention Time</th>
<th>Molecular Formula</th>
<th>Ionization Mode</th>
<th>Mass Error, ppm DM/Control</th>
<th>DM/Control</th>
<th>RSV-DM/DM</th>
<th>HMBDB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Glucose</td>
<td>180.0630</td>
<td>1.01</td>
<td>C₉H₁₄O₆</td>
<td>Positive</td>
<td>2.12</td>
<td>&gt;100</td>
<td>0.72</td>
<td>HMBDB00122</td>
</tr>
<tr>
<td>2 l-Lactic acid</td>
<td>90.0315</td>
<td>1.13</td>
<td>C₆H₁₂O₃</td>
<td>Negative</td>
<td>2.12</td>
<td>&gt;100</td>
<td>0.89</td>
<td>HMBDB00190</td>
</tr>
<tr>
<td>3 3-Ketobutyrate</td>
<td>102.0322</td>
<td>0.98</td>
<td>C₆H₁₀O₃</td>
<td>Negative</td>
<td>5.01</td>
<td>50</td>
<td>0.08</td>
<td>HMBDB00060</td>
</tr>
<tr>
<td>4 3-Hydroxybutyrate</td>
<td>104.0481</td>
<td>1.15</td>
<td>C₆H₁₀O₃</td>
<td>Negative</td>
<td>7.30</td>
<td>&gt;100</td>
<td>&lt;0.01</td>
<td>HMBDB00357</td>
</tr>
<tr>
<td>5 Picrolic acid</td>
<td>129.0783</td>
<td>1.56</td>
<td>C₇H₁₄NO₂</td>
<td>Positive</td>
<td>5.32</td>
<td>76.92</td>
<td>0.052</td>
<td>HMBDB00070</td>
</tr>
<tr>
<td>6 Methylimidazolone acetaldehyde</td>
<td>124.0635</td>
<td>6.27</td>
<td>C₆H₁₀N₂O₂</td>
<td>Positive</td>
<td>1.28</td>
<td>40.8</td>
<td>0.91</td>
<td>HMBDB04181</td>
</tr>
<tr>
<td>7 N₆,N₆, Trimeethyl-L-lysine</td>
<td>188.1514</td>
<td>1.08</td>
<td>C₆H₁₆N₂O₂</td>
<td>Positive</td>
<td>5.76</td>
<td>8.9</td>
<td>0.81</td>
<td>HMBDB01325</td>
</tr>
<tr>
<td>8 l-Carnitine</td>
<td>161.1047</td>
<td>1.12</td>
<td>C₆H₁₂NO₂</td>
<td>Positive</td>
<td>3.02</td>
<td>&lt;0.01</td>
<td>&gt;100</td>
<td>HMBDB00062</td>
</tr>
<tr>
<td>9 1-Methyl-histidine</td>
<td>169.0846</td>
<td>1.73</td>
<td>C₇H₁₁N₂O₃</td>
<td>Positive</td>
<td>3.16</td>
<td>10.87</td>
<td>0.67</td>
<td>HMBDB00001</td>
</tr>
<tr>
<td>10 3-Methyl-histidine</td>
<td>169.0849</td>
<td>1.29</td>
<td>C₇H₁₁N₂O₃</td>
<td>Positive</td>
<td>1.35</td>
<td>18.18</td>
<td>0.074</td>
<td>HMBDB00479</td>
</tr>
<tr>
<td>11 Citrate</td>
<td>192.0266</td>
<td>0.95</td>
<td>C₆H₁₀O₄</td>
<td>Negative</td>
<td>2.15</td>
<td>3.125</td>
<td>0.35</td>
<td>HMBDB00094</td>
</tr>
<tr>
<td>12 t-Acetylcarnitine</td>
<td>203.1169</td>
<td>1.44</td>
<td>C₇H₁₄NO₄</td>
<td>Positive</td>
<td>5.63</td>
<td>8.33</td>
<td>0.25</td>
<td>HMBDB00201</td>
</tr>
<tr>
<td>13 Prolylhydroxyproline</td>
<td>228.1120</td>
<td>5.97</td>
<td>C₈H₁₆N₂O₄</td>
<td>Positive</td>
<td>4.34</td>
<td>&gt;100</td>
<td>&lt;0.01</td>
<td>HMBDB00695</td>
</tr>
<tr>
<td>14 Butyrylcarnitine</td>
<td>231.1467</td>
<td>6.25</td>
<td>C₈H₂₁NO₄</td>
<td>Positive</td>
<td>1.58</td>
<td>27.03</td>
<td>0.488</td>
<td>HMBDB02013</td>
</tr>
<tr>
<td>15 Isobutyryl-L-carnitine</td>
<td>246.0869</td>
<td>1.70</td>
<td>C₇H₁₂N₂O₆</td>
<td>Negative</td>
<td>6.94</td>
<td>&gt;100</td>
<td>&lt;0.01</td>
<td>HMBDB00497</td>
</tr>
<tr>
<td>16 L-alpha-Aspartyl-L-hydroxyproline</td>
<td>274.1781</td>
<td>7.61</td>
<td>C₁₀H₂₆O₅</td>
<td>Negative</td>
<td>0.34</td>
<td>&gt;100</td>
<td>&lt;0.01</td>
<td>HMBDB00594</td>
</tr>
<tr>
<td>17 Tyramine glucuronide</td>
<td>313.1158</td>
<td>1.67</td>
<td>C₁₀H₂₆O₅</td>
<td>Positive</td>
<td>1.07</td>
<td>&gt;100</td>
<td>0.43</td>
<td>HMBDB10328</td>
</tr>
<tr>
<td>18 Dopamine glucuronide</td>
<td>329.1120</td>
<td>1.87</td>
<td>C₁₀H₂₆O₅</td>
<td>Positive</td>
<td>2.97</td>
<td>&gt;100</td>
<td>0.123</td>
<td>HMBDB10329</td>
</tr>
<tr>
<td>19 7-Ketodeoxocholic acid 3,7-Dihydroxy-12-oxo-cholanolic acid</td>
<td>406.2723</td>
<td>8.87</td>
<td>C₂₃H₃₈O₅</td>
<td>Negative</td>
<td>0.98</td>
<td>&gt;100</td>
<td>0.180</td>
<td>HMBDB00391</td>
</tr>
<tr>
<td>20 1β,3α,12α-Trihydroxy-5β-cholanolic acid</td>
<td>408.2870</td>
<td>8.45</td>
<td>C₂₃H₄₀O₃</td>
<td>Negative</td>
<td>1.42</td>
<td>&gt;100</td>
<td>0.183</td>
<td>HMBDB00326</td>
</tr>
</tbody>
</table>

Twenty metabolites were identified from the features based on the mass accuracy and retention time. The difference between observed and calculated m/z is shown as parts per million. The fold change reflects the averaged increase or decrease in abundance among control, DM, and RSV-DM rats.

In contrast, the urine carnitine level was 44% lower in DM rats than in controls, and RSV-DM urine carnitine level was twice as high as the DM urine carnitine level (Fig. 2A).

**Plasma metabolites.** The levels of several plasma metabolites were correlated with their levels in urine. Plasma levels of carnitine, acetylcarnitine, and butyrylcarnitine were altered in a similar manner as that in the urine of DM and RSV-DM rats (Fig. 3). In addition, plasma levels of glucose, 3-hydroxybutyrate, and branched-chain amino acids, such as leucine and isoleucine in DM rats, were higher than those in the control rats. In RSV-treated DM rats, plasma levels of these metabolites were significantly lower than in DM rats (Fig. 3).

**AMPK and SIRT1 expression and mitochondrial content in liver.** The beneficial effects of RSV on the energy metabolism in DM rats prompted us to examine the possible effect of RSV on the activities of AMPK, SIRT1 expression, and mitochondrial content in liver. Phospho-AMPK (p-AMPK) expression was depressed in DM rats compared with the levels in control rats (Figs. 4, A and B). P-AMPK level in RSV-DM rats was near normal. Liver SIRT1 mRNA levels were also examined. Fig. 4C shows that SIRT1 mRNA expression was significantly lower in the DM group than in the control. SIRT1 mRNA was closer to normal in the RSV-DM group than in the DM group. Further, as shown in Fig. 4D, the relative mtDNA content in liver was significantly elevated in the RSV-DM group than in the DM group. In addition, the relative mtDNA content in the gastrocnemius muscle was also significantly increased in RSV-DM group compared with that of DM group (Fig. 4E). The results suggested that RSV stimulates mitochondrial biogenesis in the liver and muscle of rats.

**Nucleic NF-kB, IL-1β, and IL-6 mRNA expression in gastrocnemius muscle.** Urinary levels of methyl-histidine and plasma branched-chain amino acids, such as leucine and isoleucine, were elevated in the DM group, indicating increased muscle protein wasting. Gastrocnemius muscle weight was
lower in DM rats (2.54 ± 0.73 g) than in control rats (5.91 ± 1.19 g), and RSV-DM rats (3.57 ± 0.91 g). In addition, as aggravated inflammation was an important contributor to muscle wasting in DM rats, nuclear NF-κB expression and downstream IL-1β, IL-6 mRNA expression were examined. As shown in Fig. 5, A and B, NF-κB expression was elevated in the DM group than in the control group. Fig. 5, C and D show that IL-1β and IL-6 mRNA expression was also significantly
increased in DM rats than in controls. RSV administration reverted the elevated mRNAs back toward normal.

DISCUSSION

The STZ-induced diabetic animal model is commonly used for studying type 1 DM. In cases of insulin deficiency, insufficient glucose utilization leads to an increased β-oxidation of long-chain fatty acids. In diabetic mice, the increased β-oxidation of fatty acids in muscle is correlated to increased expression of fatty acid oxidation enzymes (47). Excessive acyl-CoA may overwhelm TCA cycle machinery leading to acyl-CoA overload in the mitochondria. Under the circumstances, carnitine also functions as an acyl group acceptor and facilitates mitochondrial export of excessive carbons in the form of acylcarnitines. Previous studies have shown that plasma carnitine level is reduced in diabetic subjects (24, 44), and conditions of sustained metabolic stress, such as diabetes, aging, or obesity increase the carnitine requirement (30). Recently, it has been shown that plasma acetylcarnitine level is elevated in diabetic patients, indicating incomplete fatty acid β-oxidation and altered TCA cycle activity (1a). In the present study, we found that the plasma and urine levels of carnitine in DM rats were low, while the levels acetylcarnitine and butyrylcarnitine were high compared with those of control rats. The observations suggest that in DM rats, the facility for fatty acid oxidation was overloaded, and the TCA cycle activity was impaired. This study showed that treatment of DM rats with RSV effectively ameliorated the changes to the carnitine system associated with DM. In RSV-DM rats, plasma and urine carnitine levels were high and acylcarnitines (acetylcarnitine and butyrylcarnitine levels) were low compared with those of DM rats. The findings strongly suggest that RSV ameliorated the overloaded and disturbed fatty acid β-oxidation and improved the mitochondrial energy metabolism in STZ-induced DM rats.

Fig. 3. Box-and-whisker plot of the plasma metabolites with significant differences among control, DM, and RSV-DM rats. The integrated intensities of carnitine (A), acetylcarnitine (B), butyrylcarnitine (C), glucose (D), leucine (E), isoleucine (F) and 3-hydroxybutyrate (G) are as shown. The bottom and top of the boxes indicate the 25th and 75th percentiles, and the whiskers indicate the 5th and 95th percentiles. The P values were calculated by ANOVA with Tukey HSD correction. ° denotes the outlier. #Significant difference between DM and RSV-DM rats (P < 0.05).
With the improved glucose utilization in RSV-treated DM rats, the β-oxidation of fatty acids as an energy source can be reduced. This reduction in fatty acid oxidation affects carnitine levels. However, some authors have reported that plasma carnitine level is not related to glycemic control; that is, insulin treatments may not increase plasma carnitine levels or reduce acylcarnitines levels in diabetic patients (24, 44). In this study, the altered levels of plasma and urine carnitine and acylcarnitines were consistently observed across individuals in the DM and RSV-DM groups, strongly indicating that RSV treatment improved fatty acid metabolism in DM rats. Moreover, RSV has been shown to improve energy expenditure through
promotion of mitochondrial biogenesis and antioxidant activity (4). Diabetes and the accompanying hyperglycemia may induce oxidative stress by increasing ROS production or impairing antioxidant defenses (10, 26). Mitochondria are the principal source of cellular ROS production, and impairment of mitochondrial function has been reported in diabetes (7, 35). In mice fed a high-caloric diet, RSV treatment improves mitochondrial biogenesis and function in the liver (4) and skeletal muscle (22). In this study, the relative mtDNA content in liver and muscle was higher in RSV-DM rats than in DM rats. It is, therefore, possible that the improved energy metabolism in RSV-DM rats is due, at least in part, to an increased mitochondrial biogenesis in the major metabolic organs, such as liver and muscle. As more acyl-CoA is oxidized, less acyl carnitine will be synthesized in the mitochondria and released into plasma and urine. Our finding also suggests that the impaired TCA cycle activity in diabetes can be improved by RSV. This conclusion is further supported by the elevated citrate level observed in the urine of DM rats and the lower citrate levels observed in the urine of RSV-DM rats. Elevated citrate level in the urine is indicative of a reduced TCA cycle activity (49).

Type 1 DM is also characterized by elevated plasma and urine levels of ketone bodies, acetoacetate, and hydroxybutyrate. Elevated plasma ketone bodies may cause serious pathophysiological complications, such as diabetic ketoacidosis (31). Furthermore, increased ketone bodies have been reported to disturb fatty acid metabolism in the mitochondria of diabetic rats (15). In the present study, we showed that the levels of urinary 3-ketobutyrate and 3-hydroxybutyrate in DM rats were higher than those in controls but were not as elevated in RSV-DM rats. In the DM group, the level of plasma 3-hydroxybutyrate was higher than that in the control group but was reduced in the RSV-DM group. The reduction of diabetic ketoacidosis by RSV treatment could be attributed to its hypoglycemic and AMPK activation effects. Western blot analysis showed that the p-AMPK level was lower in the liver of DM rats than in controls and was closer to normal in RSV-DM rats. AMPK is an important kinase regulating the activity of the carnitine system, TCA cycle activity, and mitochondrial biogenesis. In addition, through the enhancement of hepatic AMPK activity, ketogenesis and hypertriglyceridemia were achieved at a relatively low dose and was not dose dependent (23, 28). Thus, RSV and insulin are similar in their protein-sparing effect was observed (8). In the present study, we showed, for the first time, that RSV attenuated skeletal muscle degradation in DM rats.

In the present study, DM rats treated with low-dose RSV (0.75 mg/kg, three times per day) had nearly normal plasma leucine and isoleucine, and urine methyl-histidine levels. The hypoglycemic effect of RSV was not optimal at this dose, suggesting a pleiotropic effect of low-dose RSV on the energy metabolism of diabetic rats. In human DM patients, the maximum inhibitory effect of insulin on protein degradation was achieved at a relatively low dose and was not dose dependent (23, 28). Thus, RSV and insulin are similar in their protein-sparing effects in diabetic individuals.

In summary, this metabolomic study showed that RSV treatment attenuated the diabetes-induced disturbances of the carnitine system, TCA cycle activity, and mitochondrial biogenesis. In addition, through the enhancement of hepatic AMPK activity, ketogenesis and hypertriglyceridemia were

Skeletal muscle atrophy, especially the gastrocnemius muscle in diabetes, has been linked to NF-κB upregulation and NF-κB-mediated chronic inflammation (9, 18, 27, 46). This study showed that NF-κB activation in gastrocnemius muscle of DM rats was reversed by RSV. Additionally, the increased expression of the inflammatory cytokines, namely IL-1β and IL-6, in gastrocnemius muscle of DM rats and was reversed by RSV.

It is particularly interesting that diabetes-related muscle wasting can be partially reversed by RSV. RSV has been shown to inhibit protein degradation and attenuate atrophy of skeletal muscle fibers in vitro (8, 37, 45). In C2C12 myotubes, RSV inhibited proteolysis inducing factor-induced IκBα and nuclear binding of NF-κB (37, 45). Although RSV has been consistently shown to ameliorate protein degradation in vitro, it is still debatable whether these effects also occur in vivo. RSV supplementation (1 mg·kg⁻¹·day⁻¹) to MAC 16 tumor-bearing rats has been shown to significantly attenuate body weight loss and muscle protein degradation via down-regulation of NF-κB activity (45). In contrast, in rats bearing Yoshida AH-130 ascites hepatoma or Lewis lung carcinoma, no such muscle protein-sparing effect was observed (8). In the present study, we showed, for the first time, that RSV attenuated skeletal muscle degradation in DM rats.

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Uncontrolled DM is associated with increased nitrogen excretion and accelerated protein degradation. Elevated plasma levels of branched-chain amino acids (leucine, isoleucine, and valine) and aromatic amino acids, have been observed in diabetes (3). In patients with type 1 DM, the circulating levels of branched-chain amino acids, such as leucine and isoleucine, are also elevated, suggesting an increased protein catabolism (11). Recently, the levels of these branched-chain and aromatic amino acids (tyrosine and phenylalanine) have been demonstrated to exhibit a significant association with the future development of diabetes (42). Similarly, we found circulating leucine and isoleucine were significantly elevated in DM compared with that of the control rats, and again, RSV treatment reverted these disturbances back to nearly normal.

Fig. 6. A proposed scheme for the effects of RSV on energy metabolism and the action sites of its antidiabetic effects.
short RSV ameliorates metabolic disorders in DM rats

REDUCED. The effects of RSV on the energy metabolism in DM rats on the basis of our metabolomic data are presented in Fig. 6. This study also demonstrated that RSV attenuated muscle protein wasting in DM rats, at least in part, through downregulation of the NF-κB activity.

REFERENCES


