International Journal of Sport Nutrition and Exercise Metabolism, 2014, 24, 2-13
http://dx.doi.org/10.1123/ijsnem.2013-0045

INTERNATIONAL JOURNAL OF
SPORT NUTRITION AND
EXERCISE METABOLISM
www.IJSNEM-Journal.com
ORIGINAL RESEARCH

Effects of Resveratrol in Patients With Type 2 Diabetes Mellitus on Skeletal Muscle SIRT1 Expression and Energy Expenditure

Kian Peng Goh, Hwei Yee Lee, Dawn Pingxi Lau, Wilma Supaat, Yiong Huak Chan, and Angela Fang Yung Koh

Objectives: The primary aims of the study were to examine the effect of resveratrol on skeletal muscle SIRT1 expression and energy expenditure in subjects with Type 2 diabetes mellitus (T2DM). ***Background***: Animal and in vivo studies indicate that resveratrol increases SIRT1 expression that stimulates PGC1α activity. Subsequent upregulation of AMPK and GLUT4 expression are associated with improved insulin sensitivity in peripheral tissues. ***Methods***: Ten subjects with T2DM were randomized in a double-blind fashion to receive 3g resveratrol or placebo daily for 12 weeks. Secondary outcomes include measures of AMPK, p-AMPK and GLUT4 expression levels, energy expenditure, physical activity levels, distribution of abdominal adipose tissue and skeletal muscle fiber type composition, body weight, HbA1c, plasma lipid subfraction, adiponectin levels, and insulin sensitivity. ***Results***: There was a significant increase in both SIRT1 expression (2.01 vs. 0.86 arbitrary units [AU], $p = .016$) and p-AMPK to AMPK expression ratio (2.04 vs. 0.79 AU, $p = .032$) in the resveratrol group compared with the placebo group. Although the percentage of absolute change (8.6 vs. –13.9%, $p = .033$) and percentage of predicted resting metabolic rate (RMR; 7.8 vs. –13.9%, $p = .013$) were increased following resveratrol, there was a significant reduction in average daily activity (–38 vs. 43.2%, $p = .028$) and step counts (–39.5 vs. 11.8%, $p = .047$) when compared with placebo. ***Conclusions***: In patients with T2DM, treatment with resveratrol regulates energy expenditure through increased skeletal muscle SIRT1 and AMPK expression. These findings indicate that resveratrol may have beneficial exercise-mimetic effects in patients with T2DM.

Keywords: AMPK, PGC1μ, GLUT4

Animal and in vivo studies suggest that resveratrol (trans-3,4',5-trihydroxystilbene), a phytoalexin enriched in red grape-skin and a constituent of red wine, is associated with improved insulin sensitivity and may have a role in treating Type 2 diabetes mellitus (T2DM). Its effects were postulated to be mediated through the activation of SIRT1, a nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase and a member of the Sir2 family (sirtuins) known to regulate lifespan in response to caloric restriction in several species (Baur & Sinclair, 2006; Baur et al., 2006).

An important target of SIRT1 is peroxisome proliferator–activated receptor coactivator-α (PGC1α), a major metabolic regulator which is also stimulated by AMP-activated protein kinase (AMPK).(Baur et al., 2006; Jager et al., 2007) Activated AMPK improves insulin sensitivity through glucose transporter type 4 (GLUT4) upregulation and increased mitochondrial biogenesis from the transformation of fast glycolytic skeletal fibers to slow oxidative ones (Fujii et al., 2006; Jager et al., 2007; Lagouge et al., 2006).

Two recent trials demonstrated that resveratrol improved insulin sensitivity in subjects with T2DM and obesity.(Brasnyo et al., 2011; Timmers et al., 2011) This trial was a proof-of-concept study primarily designed to examine for the first time in humans, the effect of 12 weeks of oral resveratrol on skeletal muscle SIRT1 expression and energy expenditure in 10 patients with T2DM. Additional important efficacy measures included the change in AMPK, phosphorylated-AMPK-Thr^{172} (p-AMPK) and GLUT4 expression levels, body weight, glycated hemoglobin (HbA1c), plasma lipid subfraction, adiponectin levels, insulin sensitivity, physical activity levels, distribution of abdominal adipose tissue and skeletal muscle fiber type composition.

Methods

The study was approved by the Health Sciences Authority and the National Healthcare Group Domain Specific

Goh and Koh are with the Division of Endocrinology and Lau the Clinical Research Unit, Khoo Teck Puat Hospital, Singapore. Lee and Supaat are with the Department of Pathology, Tan Tock Seng Hospital, Singapore. Chan is with the Biostatistics Unit, Yong Loo Lin School of Medicine, Singapore.

Institutional Review Board for Medical Research Ethics Committee (DSRB-C/08/351). Written informed consent was taken from all subjects. The study was registered with Clinicaltrials.gov (Clinical Trials Registration Number: NCT01677611).

Eligibility Criteria

Patients were recruited between July 2009 and May 2010 from a single hospital in Singapore. Eligible patients were Chinese males, aged between 40 and 69 years old, with T2DM with a HbA1c of 7.1–12.0% (54–108 mmol/mol IFCC) and who had been on a stable oral hypoglycemic regimen for the past 3 months. They must be willing to abstain from ingesting large quantities of resveratrol-containing foods including alcohol during the study period. Subjects who were insulin-dependent, on 3 or more oral hypoglycemic agents (OHA), or had renal or liver impairment were excluded.

Study Design

The study is a randomized, double-blind, parallel group trial consisting of a screening visit, a 2-week placebo run-in period, and a 12-week treatment period.

Protocol

Randomization was centrally performed during treatment assignment with a 2-digit reference code placed in sealed opaque envelopes maintained by the person responsible for the preparation of the intervention drug and placebo. Transresveratrol extract from *Polygonum Cuspidatum* (Mega Resveratrol, Danbury, USA) was used in the trial. After screening, all subjects underwent a 2-week run-in period with placebo. The placebo was not distinguishable by color, form, or taste from the active drug.

Following run-in, subjects were given a starting dose of 500 mg daily of either resveratrol or placebo and instructed to abstain from foods with high resveratrol content during the entire duration of the trial. The dose was increased by 500 mg per day every 3 days to a maximum dose of 3 g per day in three divided doses if there was no hypoglycemia. Compliance was determined by pill counting at the end of the trial period. Subjects in both arms were instructed to continue on their current OHA and not allowed to commence any new exercise regimen during the study period. Subjects who dropped out of the trial would not be replaced. All variables were measured at baseline and at the end of the treatment period.

Histological and Molecular Analysis

Skeletal muscle needle biopsy was performed on the dominant vastus lateralis using a modified Allendale needle as previously described (Dietrichson et al., 1987).

Tissue Preparation. Isopentane (2-methyl butane, Sigma) was placed in a small metal container in a liquid nitrogen bath. When it was ready for use, the isopentane became slightly viscous and formed a white laminate lining the inside of the metal container (temperature ~ minus 160 °C). The muscle biopsy samples was removed from the needle, dissected free of connective tissue and other nonmuscle tissue. They were then washed in ice-cold phosphate-buffered saline and dried on gauze to remove blood and serum before being vertically orientated and placed in a cryomold filled with O.C.T. embedding medium. Thereafter, they were slowly dipped into the isopentane for about 10 s. Once frozen, the samples were placed in the cryovial and deep frozen at –70 °C for fiber typing studies. Samples for protein extraction were immediately placed in liquid nitrogen for subsequent storage at –70 °C.

Skeletal Muscle Protein Extraction. Approximately 50 mg of frozen muscle for each sample were rinsed with cold PBS to remove excess blood and lysed in homogenization buffer (50mM Tris-HCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100 and 0.1% 2-mercaptoethanol). Protease inhibitor (Complete ETDA-Free Cocktail Tablets, Roche, Switzerland) and Phosphatase inhibitor (PhosSTOP Phosphatase inhibitor Cocktail tablets, Roche, Switzerland) were added before use, Samples were homogenized using a sharp surgical scissors for 2 min followed by TissueRuptor (QIAGEN, Germany) for 10 s. The samples were spun down at 13,000g at 4 °C for 10 min and the supernatant removed for protein determination. The protein concentration in the supernatant was determined using Bradford protein assay (Quick StartBradford Protein Assay, Biorad, USA).

Western Blotting. The following antibodies were used with the corresponding dilutions: rabbit polyclonal SIRT1 at 1:200 (H-300, SC-15404; Santa Cruz Biotechnology, USA), rabbit polyclonal AMPK-alpha at 1:500 (2532; Cell Signaling Technology, USA), rabbit monoclonal phospho-AMPK-alpha at 1:500 (Thr172, 2535; Cell Signaling Technology, USA), rabbit polyclonal GLUT4 at 1:20 (CBL243; Millipore, USA), rabbit polyclonal PGC1α at 1:100 (H-300, SC-13067; Santa Cruz Biotechnology, USA) and mouse monoclonal GAPDH at 1:5000 (ab8245; Abcam, UK). The specificity of all primary antibodies was previously established (Gurd et al., 2010; Kraniou et al., 2006; Sriwijitkamol et al., 2007).

Equal aliquots of 22.5 ug of skeletal muscle protein samples were solubilized in 3× Laemmli SDS sample buffer and was loaded and separated by SDS- polyacrylamide gel electrophoresis on a 10% acrylamide gel. The proteins were then transferred onto an Immobilon PVDF Membrane (Millipore Corporation, USA) at 100V for 1 hr. After the transfer, membranes were blocked for 1 hr in 10% milk in Tris-buffered saline + 0.1% Tween (TBST). Membranes were then incubated overnight at 4 °C with the appropriate primary antibody in TBST. The membrane was then washed three times in TBST for at least 5 min with gentle agitation at room temperature before incubation for 2 hr in 5% milk containing horseradish-peroxidase (HRP)-conjugated secondary antibody (Amersham ECL-HRP Linked Secondary Antibodies, GE

Healthcare, USA). Antibody binding was detected using enhanced chemiluminescence HRP substrate detection kit (Luminata Western HRP substrates, Millipore Corporation, USA). Pre and post treatment samples from any given subject were always run on the same gel. All values were normalized to their corresponding GAPDH loading control. At least two independent experiments as duplicates were performed for all samples. Images were captured using (ChemiDOC imager, Biorad, USA), and the band intensities were quantified using National Institutes of Health (NIH) Image J 1.45 software (Rasband, 1997–2011).

Muscle Fiber Composition. Serial transverse sections of the embedded muscle samples were subjected to the following stains: ATPase at pH 4.3 and 9.4, reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), succinic dehydrogenase (SDH), Gomori trichrome, Periodic acid-Schiff and Oil Red O. All sections were reviewed and ATPase at pH 4.3 (ATPase 4.3) was identified as the stain demonstrating the best fiber type differentiation. The number of dark (Type 1) fibers and light (Type 2) fibers were manually counted on each section stained with ATPase 4.3 and the corresponding percentages derived for each subject.

Energy Expenditure

Resting Metabolic Rate (RMR) RMR was measured by indirect calorimetry using a breath-by-breath metabolic gas analyzer via the facemask method (Quark b2, Cosmed, Rome, Italy), which has been validated in previous studies (Duffield et al., 2004). Measurements were performed using the facemask method with correction made for dead space, after an overnight fast and avoidance of smoking for 2 hr before testing. Before each measurement, the equipment was calibrated with gas mixtures of known oxygen and carbon dioxide contents according to the manufacturer's instructions. After a 20-min rest in the sitting position, RMR was measured for 15 min with the subjects were awake in the sitting position. The test was performed in a quiet room with an ambient temperature of 24 °C to 26 °C and subjects were instructed not to move their limbs during the test. Data were collected using a standardized software protocol with the first 5 min of measurements discarded and RMR calculated using standard validated equations from the manufacturer's software (Elia & Livesey, 1992). Validity of the test was checked by comparing the ventilation, respiratory frequency and resting heart rate with predicted values. The predicted RMR was calculated after adjustment for age, weight, and sex and measured RMR was expressed as both as an absolute value and a percentage of the predicted as previously described (Holmer et al., 2010).

Physical Activity All subjects wore an accelerometer (Model GT1M or GT3×, Actigraph, Florida), previously validated in large epidemiological studies, for 7 days (Copeland & Esliger, 2009; Troiano et al., 2008). Subjects were instructed to secure the unit at the upper thigh level upon waking up in the morning and to take it off only while bathing or swimming and before retiring to bed in the night. The accelerometers collected and reported physical activity in terms of activity counts, which were an accumulation of filtered acceleration measured during the set 10-s epochs and provided a quantitative measurement of activity over time. Changes in acceleration were measured 30 times per second. A valid hour was defined as <30 consecutive "zero" intensity counts and a valid day contained 10 valid hours/day. Nonvalid days were dropped from the analysis and subjects were not required to rewear the units unless there were <3 valid days. The accelerometers were initialized to collect activity and steps in 10-s epochs. The daily epoch data were computed in terms of total activity counts, peak activity counts (highest count for the day) and average activity counts (summed epoch values/number of epoch samples). This data were then used to derive the average for the total number of valid days. Similar computation was applied to step counts. Caloric output was calculated with the manufacturer's software using a combination of the work-energy theorem (kcals per minute = 0.0000191 × counts per minute x body mass in kg) and Freedson equation (kcals per min = 0.00094 × counts per minute + 0.1346 × body mass in kg - 7.37418). Metabolic equivalent of tasks (METs) was derived from the Crouter 2 regression model (Version 1.0.18, Actigraph, Florida; Crouter & Bassett, 2008). Activity was monitored over a range of 3–6 valid days with all subjects having at least 4 valid days and 85% of the samples having 5 valid days or more. The data were visually inspected to identify malfunctioning units and cleaned before analysis.

Abdominal Fat Measurement

Abdominal adipose tissue (AT) measurement was performed using a noncontrast magnetic resonance imaging (MRI) method on a multislice MRI scanner with one body array and spine coil (Model Symphony 1.5T or Avanto, Siemens, Munich, Germany). A 10 cm × 10 cm area on the anterior abdominal wall, between the xiphoid and umbilicus, was marked using 4 Vitamin E pills placed 5 cm from midline, with the subject supine on table with body array placed on top of abdomen. Subjects were instructed to hold their breath during scanning. Slice imaging was then performed with slice thickness fixed at 10 mm. The field of view was between 380 mm to 450 mm and the total number of slices was variable depending on the subject's size. For both in-phase and out-phase scan sequence, the time of repetition was between 100 ms to 153 ms. The time of excitation for out-phase scans was fixed at 2.4 ms. Postprocessing calculation was done by manually outlining the subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). The volume of the respective AT in each slice was calculated by multiplying the AT area by the slice thickness and the final AT volume was the summation of the volumes of all slices. The total AF volume was calculated as a sum of the volumes of SAT and VAT.

Biochemical Parameters

All biochemical measurements were performed at the hospital's central laboratory except for plasma adiponectin which was done at the metabolic research laboratory using a commercially available enzyme-linked immunosorbent assay (ELISA, Millipore). The Cobas C analyzer (Roche/Hitachi) was used to measure levels glycated hemoglobin (HbA1c) using a turbidimetric inhibition immunoassay (Roche Diagnostics) and fasting plasma low-density lipoprotein (LDL), triglyceride and high-density lipoprotein (HDL) cholesterol lipids using an enzymatic colorimetric assay (Roche Diagnostics). Fasting glucose and insulin were measured using the hexokinase method and electrochemiluminescence immunoassay respectively to derive the hepatic insulin resistance with the Homeostasis Model Assessment (HOMA) 2 calculator (Version 2.2.2, Diabetes Trials Unit, University of Oxford, UK). (Diabetes Trials Unit, University of Oxford, UK, 2004) Complete blood count, serum creatine kinase, electrolytes, creatinine and liver transaminases were monitored as part of the safety process.

Statistical Analysis

We estimated that a sample size of 5 patients per group (Total $N = 10$) was needed to provide 80% power to detect an effect size of 5% and 15% for the low and high SIRT1 expression groups respectively and a *SD* of 2.0 for each group (Lenth, 2012).

Data are presented as mean ± *SD* (*SD*). Normal distribution was verified with the Kolmogorov-Smirnov test. Differences between the two groups were assessed using Fischer's exact test for categorical variables, 2-sided student's *t* test for continuous variables and Mann-Whitney *U* test for nonnormally distributed variables.

All data were analyzed according to the intention-to-treat principle. Statistical significance was accepted when the P value was <0.05 on a 2-sided *t* test, assuming a common standard deviation (*SD*) of 1.1%. All analyses were performed with the use of SPSS software, version 20 (IBM).

Results

Study Patients

Of the 11 patients who were screened, 10 patients were enrolled and randomized with 5 in each group. All 10 patients completed the trial with all patients achieving at least 70% compliance by the end of the study. All patients were continued on their medication with no adjustments made by their primary doctor during the study period. The mean duration of T2DM was 9.5 ± 5.5 years. There were no significant differences among the groups with regard to the baseline characteristics (Table 1).

Safety

A total of four patients experienced adverse events. One patient in the resveratrol group with a history of fatty liver experienced an asymptomatic and mild elevation of alanine transaminase (ALT). Another patient in the resveratrol group developed diarrhea and mild hypoglycemia which resolved spontaneously. 1 patient from each group developed mild cellulitis at the biopsy site which resolved with oral antibiotics. The proportions of patients with adverse events did not differ significantly among the study groups ($p = .419$). There were no deaths in the study.

Outcome (Table 2)

Skeletal Muscle Protein Expression (Figure 1). SIRT1 expression was significantly increased at the end of intervention in the resveratrol group (2.01 ± 0.64 arbitrary units [AU]) compared with the placebo group (0.86 ± 0.38 AU; $p = .016$). The ratio of p-AMPK to AMPK

Table 1 Baseline Characteristics of Subjects

Characteristic	All Patients (*n* = 10)	Placebo (*n* = 5)	Resveratrol (*n* = 5)	*p*
Demographic				
age (yr)	56.3 ± 6.0	56.8 ± 5.3	55.8 ± 7.3	.75
duration of T2DM (yr)				
mean	9.5 ± 5.5	9.6 ± 6.3	9.4 ± 5.3	.75
current smoker (no.; %)	3 (30)	2 (20)	1 (10)	1.0
Clinical				
weight (kg)	77.6 ± 22.3	68.3 ± 13.7	87.0 ± 26.6	.08
body-mass index (kg/m^2)	26.9 ± 5.8	24.4 ± 3.6	29.4 ± 6.8	.25
oral hypoglycemic agents (no.; %)				
any metformin	9 (90)	5 (50)	4 (40)	1.0
any sulphonylurea	9 (90)	4 (40)	5 (50)	1.0
any glitazone	1 (10)	0 (0)	1 (10)	1.0

(continued)

Table 1 *(continued)*

Characteristic	All Patients (*n* = 10)	Placebo (*n* = 5)	Resveratrol (*n* = 5)	*p*
Skeletal muscle protein expression (arbritrary units)				
SIRT1	0.68 ± 0.32	0.86 ± 0.19	0.49 ± 0.33	.15
AMPK	1.01 ± 0.61	1.27 ± 0.79	0.75 ± 0.24	.15
p-AMPK	0.80±.0.54	0.83 ± 0.27	0.78 ± 0.77	.55
p-AMPK/AMPK	0.87 ± 0.68	0.75 ± 0.29	0.99 ± 0.96	1.00
GLUT4	2.11 ± 2.23	2.88 ± 3.02	1.35 ± 0.81	.15
PGC1α	0.61 ± 0.20	0.62 ± 0.11	0.60 ± 0.29	.88
Skeletal muscle fiber type				
Type 1 (%)	33.3 ± 9.2	35.5 ± 13.1	31.0 ± 3.7	1.00
Type 2 (%)	66.8 ± 9.2	64.5 ± 13.1	69.0 ± 3.7	1.00
Energy expenditure				
resting metabolic rate				
absolute (Kcal/day)	1731 ± 557	1634 ± 344	1828 ± 745	.60
percentage of predicted (%)	107.2 ± 16.0	112.0 ± 12.3	102.4 ± 19.1	.60
caloric output (Kcal/day)	638.8 ± 243.5	490.8 ± 137.2	786.8 ± 244.5	.08
Daily physical activity (counts)				
total activity	397118 ± 149214	355367 ± 91715	438870 ± 193200	.60
peak activity	2106 ± 1596	1913 ± 1116	2298 ± 2095	.92
average activity	77.3 ± 61.0	69.5 ± 57.0	85.1 ± 70.6	.75
total steps	7411 ± 2798	7286 ± 2060	7536 ± 3651	.90
peak steps	33.0 ± 28.4	37.2 ± 35.3	28.8 ± 23.0	.18
average steps	1.4 ± 1.0	1.4 ± 1.2	1.4 ± 0.9	.92
metabolic equivalent tasks	1.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	.08
Abdominal fat distribution (L)				
subcutaneous fat volume	2.2 ± 1.1	2.1 ± 1.3	2.3 ± 1.0	.47
visceral fat volume	2.3 ± 1.3	2.3 ± 1.7	2.4 ± 1.1	.47
Biochemical				
Glycated hemoglobin: Mean				
– % NGSP	8.9 ± 1.2	8.2 ± 0.7	9.5 ± 1.3	.10
– mmol/mol IFCC	74 ± 13.2	66 ± 7.7	80 ± 14.3	.10
fasting plasma glucose (mmol/L)	10.7 ± 2.4	9.5 ± 1.0	11.8 ± 2.9	.12
fasting plasma insulin (mU/L)	10.1 ± 5.8	7.6 ± 4.8	12.7 ± 6.0	.12
fasting plasma C-peptide (nmol/L)	0.66 ± 0.23	0.60 ± 0.23	0.76 ± 0.23	.35
HOMA (B) (%)	29.0 ± 16.7	26.4 ± 9.6	31.6 ± 22.8	.92
HOMA (S) (%)	86.8 ± 48.0	112.5 ± 50.5	61.1 ± 31.2	.08
HOMA (IR)	1.6 ± 1.0	1.1 ± 0.7	2.0 ± 1.0	.09
plasma cholesterol				
high-density lipoprotein (mmol/L)	1.30 ± 0.45	1.56 ± 0.53	1.05 ± 0.06	.12
low-density lipoprotein (mmol/L)	2.87 ± 0.72	2.93 ± 0.33	2.80 ± 1.03	.18
triglycerides (mmol/L)	1.59 ± 0.43	1.39 ± 0.44	1.79 ± 0.36	.08
adiponectin (µg/ml	7.6 ± 4.6	8.3 ± 5.2	6.8 ± 4.4	.60
serum creatinine (µmol/L)	79.6 ± 8.8	79.6 ± 8.8	79.6 ± 8.8	1.00
alanine transferase (U/L)	34.3 ± 18.6	27.2 ± 12.5	41.4 ± 22.2	.25

Note. Plus-minus values are mean ± *SD*. The BMI is the weight in kilograms divided by the square of the height in meters.

Table 2 Outcome and Changes from Baseline After 12 Weeks

Variable	Placebo (*n* = 5)	Resveratrol (*n* = 5)	*p*
Clinical			
weight (kg)	0.0 ± 1.0	0.6 ± 1.5	NS
body-mass index (kg/m^2)	0.0 ± 0.2	0.4 ± 0.6	NS
Skeletal muscle protein expression (arbitrary units)			
SIRT1	0.86 ± 0.38	2.01 ± 0.64	.016
AMPK	0.93±.50	1.07 ± 0.68	NS
p-AMPK	0.74 ± 0.41	2.37 ± 2.94	NS
p-AMPK/AMPK	0.79 ± 0.28	2.04 ± 1.29	.032
GLUT4	0.76 ± 0.29	1.61 ± 0.77	NS
PGC1α	1.03 ± 0.11	0.91 ± 0.24	NS
Skeletal muscle fiber type (%)			
Type 1	28.9 ± 34.7	17.2 ± 29.8	NS
Type 2	–7.8 ± 22.0	-8.7 ± 16.2	NS
Energy expenditure			
resting metabolic rate			
absolute (Kcal per day)	–236.2 ± 167.3	101.7 ± 129.6	.022
percentage of absolute (%)	–13.9 ± 9.4	8.6 ± 10.0	.033
percentage of predicted (%)	–13.9 ± 10.0	7.8 ± 7.9	.018
caloric output (%)	41.6 ± 103.6	–18.3 ± 24.6	NS
Daily physical activity (%)			
total activity counts	43.2 ± 105.2	–17.3 ± 25.7	NS
peak activity counts	8.9 ± 32.6	–6.2 ± 48.9	NS
average activity counts	43.2 ± 105.3	–38.0 ± 25.7	.028
total steps counts	8.6 ± 43.4	–13.5 ± 41.9	NS
peak steps counts	–10.1 ± 10.6	–17.8 ± 30.7	NS
average steps counts	11.8 ± 43.2	–39.5 ± 18.8	.047
metabolic equivalent tasks	21.7 ± 33.4	–5.7 ± 6.7	.043
Abdominal fat distribution (L)			
subcutaneous fat	0.0 ± 0.2	0.1 ± 0.2	NS
visceral fat	0.0 ± 13.9	0.1 ± 0.2	NS
Biochemical			
HbA1c—absolute change			
– % NGSP	0.1 ± 1.1	–0.8 ± 0.6	NS
– mmol/mol IFCC	1.1 ± 12.1	–8.8 ± 6.6	NS
fasting plasma glucose—mmol/L	–0.5 ±0.6	–0.3 ± 1.7	NS
fasting plasma insulin—mU/L	1.0 ± 1.1	0.6 ± 5.1	NS
fasting plasma C-peptide—nmol/L	–0.03 ± 0.10	–0.03 ± 0.20	NS
HOMA (B; %)	23.9 ± 18.0	21.8 ± 47.2	NS
HOMA (S; %)	–11.0 ± 16.0	–5.4 ± 41.9	NS
HOMA (IR; %)	13.6 ± 19.6	22.8 ± 54.9	NS
cholesterol			
high-density lipoprotein—mmol/L	0.05 ± 0.12	–0.06 ± 0.20	NS
low-density lipoprotein—mmol/L	–0.28 ± 0.39	0.44 ± 0.30	.016
triglycerides—mmol/L	–0.02 ± 0.49	0.37 ± 0.75	NS
adiponectin—µg/ml	0.2 ± 3.0	–0.4 ± 1.3	NS
serum creatinine—µmol/L	0.0 ± 8.8	0.0 ± 8.8	NS
alanine transferase—U/l	–2.6 ± 9.0	12.6 ± 23.5	NS

Note. Plus-minus values are mean ± *SD*. The BMI is the weight in kilograms divided by the square of the height in meters.

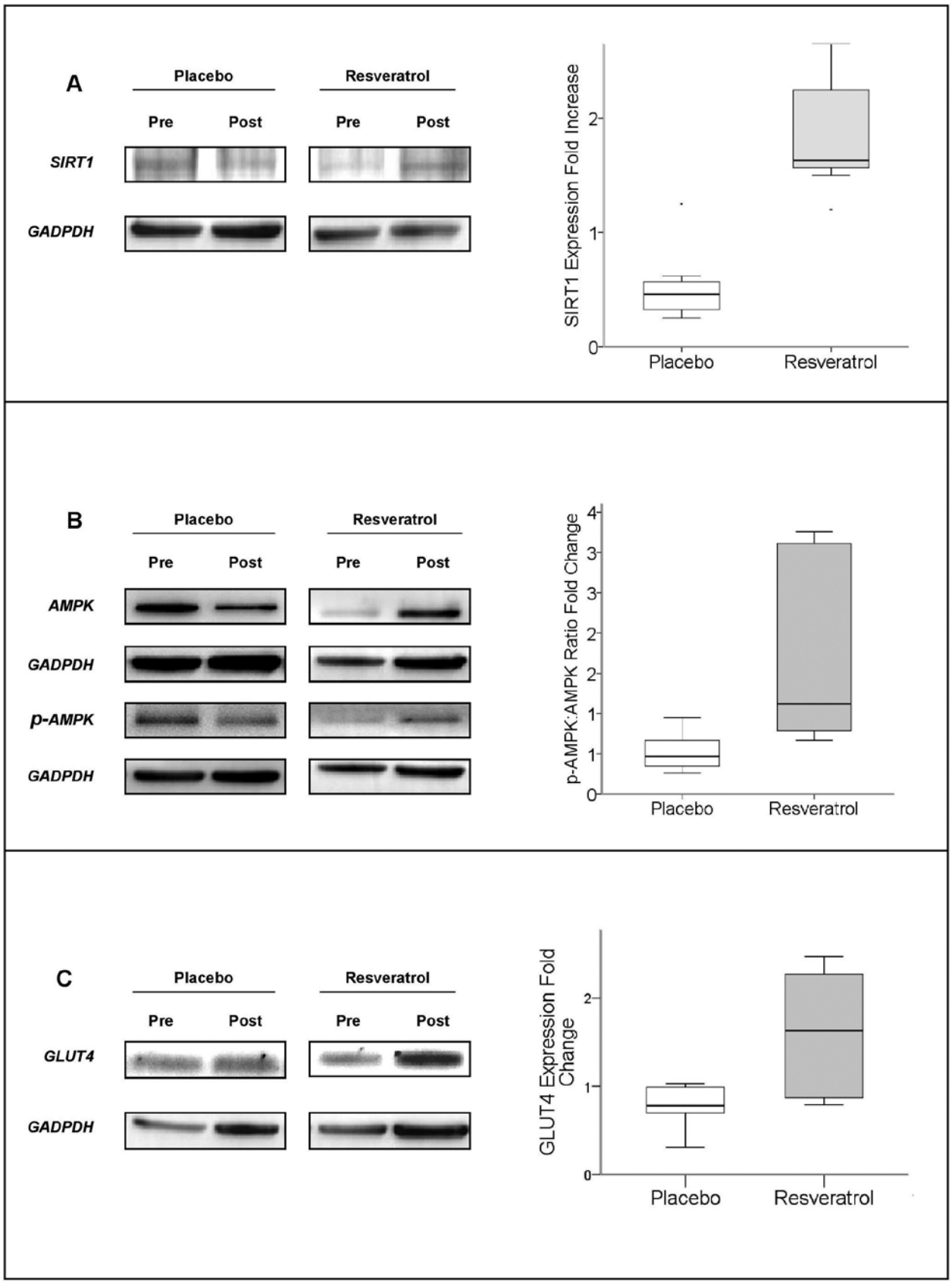

Figure 1 — Protein expression of SIRT1, AMPK and p-AMPK Expression in Skeletal Muscle Fibers. Representative Western blots and skeletal muscle protein expression levels of SIRT1 (Panel A), AMPK and p-AMPK (Panel B) and GLUT4 (Panel C) at baseline and after 12 weeks of intervention for the resveratrol and placebo groups are shown ($N = 5$ in each group).

protein expression level in the resveratrol group was also found to be significantly increased (2.04 ± 1.29 vs 0.79 ± 0.28 AU, $p = .032$). The same pattern was observed for the remaining proteins AMPK, p-AMPK and GLUT4, although they did not reach statistical significance.

Skeletal Muscle Fiber Typing. The mean total numbers of fibers analyzed per subject were 1129 and 1071 for the placebo and resveratrol groups respectively. Fiber typing was not performed for 2 subjects, 1 from each group, due to suboptimal sample preservation. Both groups had more Type 2 fibers at baseline. At the end of the study period, the proportion of type 1 fibers increased in both groups with a concomitant decrease in Type 2 fibers. Both pre- and poststudy differences were not significant.

Energy Expenditure (Figure 2). Due to a technical problem, poststudy indirect calorimetry could not be done for 2 subjects in the resveratrol group. Compared with the placebo group, subjects in the resveratrol group demonstrated a significant increase in RMR from baseline in terms of the absolute change (101.7 vs -236 kcal per day, $p = .022$), percentage of absolute change (8.6 vs -13.9%, $p = .033$) and the percentage of predicted RMR (7.8 vs -13.9%, $p = .013$). This change was also significant within subjects in both groups on General Linear Model analysis for repeated measures using multivariate analysis ($p = .025$).

Accelerometry data by study group are presented in Table 2. There were no differences in valid days of wear-time by study groups. The resveratrol group registered a decrease in average daily activity (-38 vs 43.2%, $p = .028$) and step counts (-39.5 vs 11.8%, $p = .047$) compared with the placebo group. There was a similar observation when the change in average daily metabolic equivalent of tasks (METs) was compared between the 2 groups (-5.7 vs 21.7%, $p = .047$).

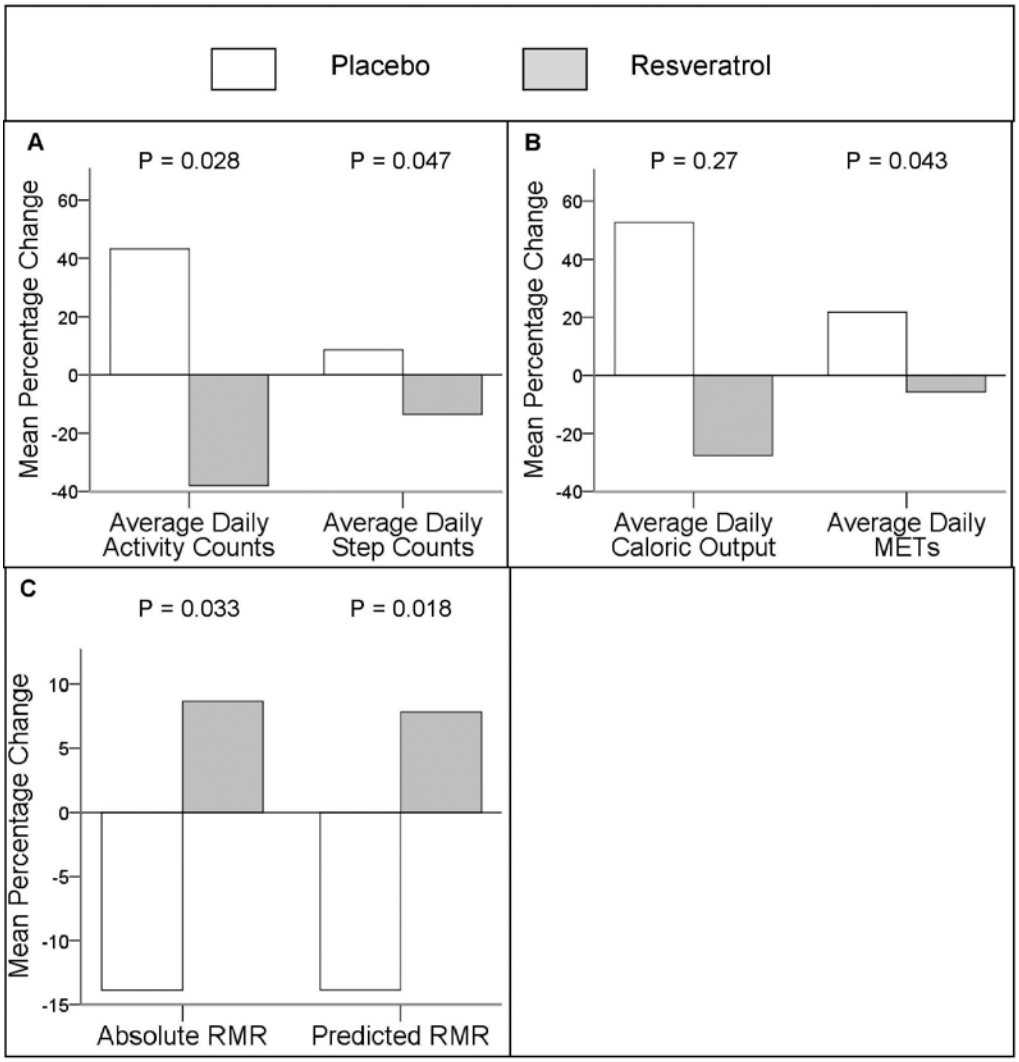

Figure 2 — Changes in measures of energy expenditure from baseline. Mean percentage changes after 3 months were plotted for average daily activity counts and step counts (Panel A), average daily caloric output and metabolic equivalent of tasks (METs; Panel B), and absolute and predicted resting metabolic rate (RMR; Panel C).

Glycemic Control and Lipid Profile (Figure 3). There was a nonsignificant trend toward a reduction in HbA1c with a mean absolute change from baseline of -0.8% (8.8 mmol/lmol IFCC) and 0.1% (1.1 mmol/lmol IFCC) in the resveratrol and placebo groups respectively ($p = .13$). LDL cholesterol increased by a mean of 17.0 mg per deciliter (0.44 mmol per liter) in the resveratrol group ($p = .016$).

Insulin Sensitivity, Body Weight and Abdominal Fat Distribution. There were no significant changes in weight, AT distribution and volume or HOMA-IR.

Discussion

To our knowledge, our study is the first to prospectively examine the effects of resveratrol on SIRT1 expression and energy expenditure in individuals with T2DM. The results showed that resveratrol supplementation was associated with a significant increase in skeletal muscle SIRT1 expression compared with placebo. Expression levels of GLUT4, AMPK, p-AMPK and ratio of p-AMPK to AMPK were correspondingly increased as well with the latter reaching statistical significance. These results are consistent with the action of AMPK on GLUT4 upregulation through the phosphorylation of its catalytic site Thr 172 (p-AMPK). (Hardie et al., 2006) Although no difference was detected in total PGC1a expression, this may be explained by the mechanism which SIRT1 uses to increase PGC1a stability and activity, which is through deacetylation and not via increased synthesis (Baur et al., 2006).

Our data also shows for the first time the effect of resveratrol on energy expenditure in humans. Obligatory expenditure as measured by the RMR was increased in the resveratrol group, although this was offset by a reduction in average daily METs and physical activity, which could explain the lack of observable differences in weight and BMI. This is consistent with the role of AMPK as a cellular energy deficit sensor. Once activated, AMPK switches on ATP-generating catabolic pathways, which increases RMR. However, in an effort to prevent energy wastage there is inhibition of nonessential ATP-consuming biosynthetic activities such as unnecessary physical activity (Hardie et al., 2006). Energy balance is thus maintained. Interestingly, clinical trials have demonstrated that chronic starvation is associated with a reduction in physical activity as well as nonexercise activity thermogenesis (NEAT), a substantial contributor to overall energy balance (Levine, 2004). Park et al. recently reported an indirect effect of resveratrol on SIRT1 via the inhibition of cAMP-degrading phosphodiesterases. This resulted in elevated cAMP levels which is also a consequence of nutrient deprivation (Park et al., 2012). While these studies support the effect of caloric restriction on SIRT1 activation as shown in many species, the exact mechanisms by how SIRT1 affects energy expenditure and its net effect are far from clear.

The decrease in RMR in the control group may be due to the restriction of strenuous physical exercise as part of the study protocol. If this explanation is correct, then the data in the intervention group suggests that resveratrol not only attenuates this effect but is also associated with an increased RMR without a corresponding increase in physical activity level. (Bostrom et al., 2012)

Our data also provides important evidence to support the concept of resveratrol as a potential glucose-lowering agent in T2DM, either directly through SIRT1 activation or via AMPK. While a nonsignificant trend toward a reduction in glycated hemoglobin was seen, it was nonetheless a trend in the right direction in the context of an

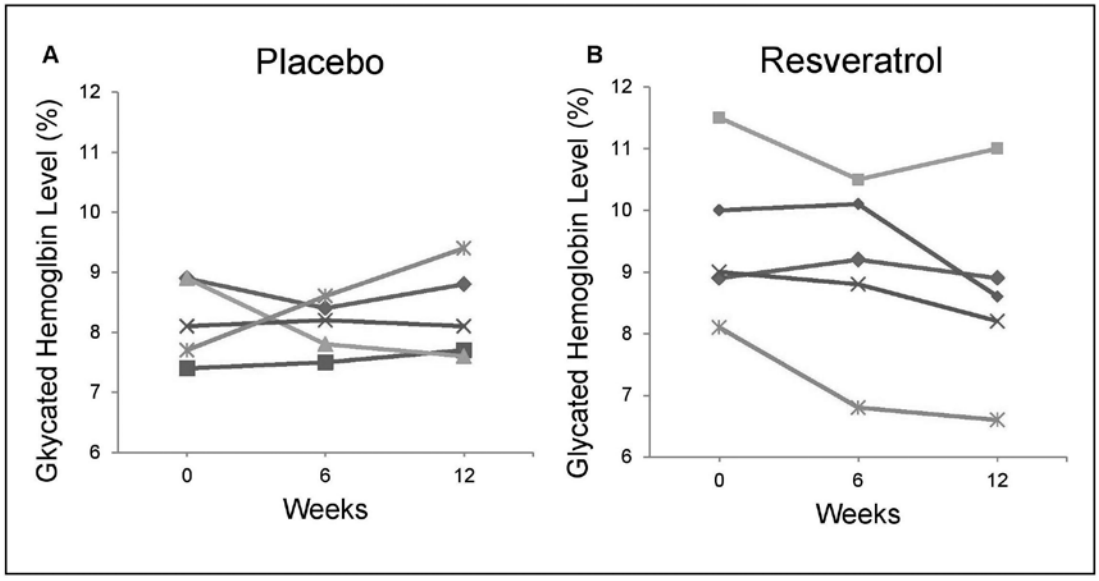

Figure 3 — Changes in HbA1c from baseline. Values for glycated hemoglobin were plotted at baseline, 6 and 12 weeks for placebo group (Panel A) and resveratrol group (Panel B; $p = .13$).

underpowered sample size. In fact, two commonly used OHA, biguanides and thiazolidinediones, have already been shown to activate AMPK (Hardie et al., 2006). There is also in vivo evidence that SIRT1 over-expression in pancreatic beta cells and down-regulation in monocytes are associated with increased glucose tolerance and insulin resistance respectively (Baur & Sinclair, 2006; de Kreutzenberg et al., 2010).

A recent study showed that resveratrol improved insulin sensitivity in subjects with T2DM, although SIRT1 expression level was not measured. Another study which measured SIRT1 level in obese subjects also showed an improvement in the HOMA index. However, the duration of resevratrol supplementation in both studies was only 4 weeks and the doses used were much lower compared with our protocol (Brasnyo et al., 2011; Timmers et al., 2011). Our study also provides for the first time in humans, information regarding the tolerability of oral resveratrol of 3g per day for up to 12 weeks. These safety data are of clinical importance as GlaxoSmithKline (GSK) recently terminated a Phase 2 trial involving SR501, a proprietary form of resveratrol, in patients with multiple myeloma as a result of limited efficacy and possible exacerbation of renal impairment (NCT00920556). Additional clinical trials on the efficacy of resveratrol in T2DM are currently underway.

Interestingly, LDL was increased in the resveratrol group. This was in contrast to the study by Yashiro which showed an upregulation of LDL receptor expression in hepatocytes (Yashiro et al., 2012) A possible explanation is the increased conversion of small dense LDL to larger less atherpogenic particles similar to what was observed with thiazolidinediones (Brackenridge et al., 2009; van Wijk et al., 2003). More studies are needed to further elucidate the effects of resveratrol on lipid metabolism.

Finally, there was no difference in skeletal fiber type proportion, even though AMPK and PGC1α are known to play a major role in mitochondrial biogenesis and fiber type transformation (Spangenburg & Booth, 2003). It is possible that our study period was too short to detect an observable difference and the results might have been different had the treatment duration been longer.

Overall, our results are provocative in several aspects. Firstly, they demonstrate the efficacy of resveratrol at the dose used to upregulate SIRT1 expression. Secondly, our observations provide evidence that skeletal muscle AMPK signaling can be pharmacologically upregulated in T2DM. This is significant as the skeletal muscle is a critical regulator of energy homeostasis and glucose metabolism by virtue of its large mass and oxidative capacity. In fact, T2DM patients have been shown to have defective oxidative capacity and mitochondrial function (Garvey et al., 1992; Mogensen et al., 2007). In this regard, resveratrol may partially function as an exercise-mimetic with SIRT1 as a new molecular target in the treatment of T2DM. Finally, our study may bridge the link between the mechanistic science of the SIRT1 signaling pathway and the phenotypic variables of T2DM, in particular energy expenditure and glycemic control.

Our study has limitations. Despite fulfilling the sample-size criteria for the primary efficacy variable in a proof-of-concept study, our sample was relatively small and larger studies will be required to confirm these findings as there was insufficient power to detect differences in the secondary end-points. Missing data from two subjects on fiber type composition and indirect calorimetry meant that no firm conclusion can be made regarding these secondary measures. Immunohistochemistry using antibodies to the various MHC isoforms was not used in skeletal fiber differentiation. Hence, any transformation between a fast (2X) and an intermediate fiber type (2A) would not have been detected. Although the duration of resveratrol used was the longest in any published trial to date, any outcome beyond this period, especially on SIRT1 activation is unclear. Finally, there is a possibility that resveratrol may exert pleiotropic effects beyond SIRT1 activation, which this study was not designed to examine.

In conclusion, the study results have shown for the first time that a 12-week supplementation of oral resveratrol is associated with increased skeletal muscle SIRT1 expression in subjects with T2DM. It is also the first time in which increased basal energy expenditure and decreased physical activity were demonstrated after oral resveratrol intake. These observations suggest that SIRT1 may be implicated in the derangements of energy metabolism that occur in T2DM. We hope our results will provide the impetus for a large-scale clinical trial to determine whether these signal transduction effects of resveratrol-induced SIRT1 activation can be translated into actual clinical benefit.

Acknowledgments

We thank the following for their valuable assistance with the study: Dr. Toy Wan Ching (Clinical Research Unit, Alexandra Health), Ms. Elsie Swee Lan Kok (Department of Pathology, Tan Tock Seng Hospital), and Drs. Subramaniam Tavintharan, Lim Su Chi, and Sum Chee Fang from the Diabetes Centre, Alexandra Health.

Declaration of Funding Source

This work was supported by the National Medical Research Council (Grant number: NMRC/NIG/0035/2008). The drug manufacturer had no role in the design of the study, in the accrual or analysis of the data, or in the preparation of the manuscript. All authors vouch for the accuracy and completeness of the reported data and an external data safety monitoring board monitored safety throughout the study period. The authors report no potential conflict of interests or relationship with any industry of interest relevant to this article.

References

Baur, J.A., Pearson, K.J., Price, N.L., Jamieson, H.A., Lerin, C., Kalra, A., . . . Sinclair, D.A. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. *Nature, 444*(7117), 337–342. PubMed doi:10.1038/nature05354

Baur, J.A., & Sinclair, D.A. (2006). Therapeutic potential of resveratrol: The in vivo evidence. *Nature Reviews. Drug Discovery, 5*(6), 493–506. PubMed doi:10.1038/nrd2060

Bostrom, P., Wu, J., Jedrychowski, M. P., Korde, A., Ye, L., Lo, J. C., . . . Spiegelman, B. M. (2012). A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature, 481*(7382), 463-468.

Brackenridge, A. L., Jackson, N., Jefferson, W., Stolinski, M., Shojaee-Moradie, F., Hovorka, R., . . . Russell-Jones, D. (2009). Effects of rosiglitazone and pioglitazone on lipoprotein metabolism in patients with Type 2 diabetes and normal lipids. *Diabetic Medicine: A Journal of the British Diabetic Association, 26*(5), 532-539.

Brasnyó, P., Molnár, G.A., Mohás, M., Markó, L., Laczy, B., Cseh, J., . . . Wittmann, I. (2011). Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the akt pathway in Type 2 diabetic patients. *The British Journal of Nutrition, 106*(3), 383–389. PubMed doi:10.1017/S0007114511000316

Copeland, J.L., & Esliger, D.W. (2009). Accelerometer assessment of physical activity in active, healthy older adults. *Journal of Aging and Physical Activity, 17*(1), 17–30. PubMed

Crouter, S.E., & Bassett, D.R., Jr. (2008). A new 2-regression model for the actical accelerometer. *British Journal of Sports Medicine, 42*(3), 217–224. PubMed doi:10.1136/bjsm.2006.033399

de Kreutzenberg, S.V., Ceolotto, G., Papparella, I., Bortoluzzi, A., Semplicini, A., Dalla Man, C., . . . Avogaro, A. (2010). Downregulation of the longevity-associated protein sirtuin 1 in insulin resistance and metabolic syndrome: Potential biochemical mechanisms. *Diabetes, 59*(4), 1006–1015. PubMed doi:10.2337/db09-1187

Diabetes Trials Unit, University of Oxford, UK. (2004). HOMA2 calculator version 2.2.2. Retrieved from http://www.dtu.ox.ac.uk/homacalculator/index.php

Dietrichson, P., Coakley, J., Smith, P.E., Griffiths, R.D., Helliwell, T.R., & Edwards, R.H. (1987). Conchotome and needle percutaneous biopsy of skeletal muscle. *Journal of Neurology, Neurosurgery, and Psychiatry, 50*(11), 1461–1467. PubMed doi:10.1136/jnnp.50.11.1461

Duffield, R., Dawson, B., Pinnington, H. C., & Wong, P. (2004). Accuracy and reliability of a cosmed K4b2 portable gas analysis system. *Journal of Science and Medicine in Sport / Sports Medicine Australia, 7*(1), 11-22.

Elia, M., & Livesey, G. (1992). Energy expenditure and fuel selection in biological systems: The theory and practice of calculations based on indirect calorimetry and tracer methods. *World Review of Nutrition and Dietetics, 70,* 68–131. PubMed

Fujii, N., Jessen, N., & Goodyear, L.J. (2006). AMP-activated protein kinase and the regulation of glucose transport. *American Journal of Physiology. Endocrinology and Metabolism, 291*(5), E867–E877. PubMed doi:10.1152/ajpendo.00207.2006

Garvey, W.T., Maianu, L., Hancock, J.A., Golichowski, A.M., & Baron, A. (1992). Gene expression of GLUT4 in skeletal muscle from insulin-resistant patients with obesity, IGT, GDM, and NIDDM. *Diabetes, 41*(4), 465–475. PubMed doi:10.2337/diab.41.4.465

Gurd, B.J., Perry, C.G., Heigenhauser, G.J., Spriet, L.L., & Bonen, A. (2010). High-intensity interval training increases SIRT1 activity in human skeletal muscle. *Applied Physiology, Nutrition, and Metabolism, 35*(3), 350–357.

Hardie, D.G., Hawley, S.A., & Scott, J.W. (2006). AMP-activated protein kinase–development of the energy sensor concept. *The Journal of Physiology, 574*(Pt 1), 7–15. PubMed doi:10.1113/jphysiol.2006.108944

Holmer, H., Pozarek, G., Wirfalt, E., Popovic, V., Ekman, B., Bjork, J., & Erfurth, E.M. (2010). Reduced energy expenditure and impaired feeding-related signals but not high energy intake reinforces hypothalamic obesity in adults with childhood onset craniopharyngioma. *The Journal of Clinical Endocrinology and Metabolism,*. PubMed

Jäger, S., Handschin, C., St-Pierre, J., & Spiegelman, B.M. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proceedings of the National Academy of Sciences of the United States of America, 104*(29), 12017–12022. PubMed doi:10.1073/pnas.0705070104

Kraniou, G. N., Cameron-Smith, D., & Hargreaves, M. (2006). Acute exercise and GLUT4 expression in human skeletal muscle: Influence of exercise intensity. *Journal of Applied Physiology (Bethesda, Md.: 1985), 101*(3), 934-937.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., . . . Auwerx, J. (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell, 127*(6), 1109–1122. PubMed doi:10.1016/j.cell.2006.11.013

Lenth, R. (2007). Java applets for power and sample size. Retrieved June 6, 2012, from http://homepage.stat.uiowa.edu/~rlenth/Power.

Levine, J.A. (2004). Nonexercise activity thermogenesis (NEAT): Environment and biology. *American Journal of Physiology. Endocrinology and Metabolism, 286*(5), E675–E685. PubMed doi:10.1152/ajpendo.00562.2003

Mogensen, M., Sahlin, K., Fernstrom, M., Glintborg, D., Vind, B.F., Beck-Nielsen, H., & Hojlund, K. (2007). Mitochondrial respiration is decreased in skeletal muscle of patients with Type 2 diabetes. *Diabetes, 56*(6), 1592–1599. PubMed doi:10.2337/db06-0981

Park, S. J., Ahmad, F., Philp, A., Baar, K., Williams, T., Luo, H., . . . Chung, J. H. (2012). Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. *Cell, 148*(3), 421-433.

Rasband, W. S. (1997–2011). ImageJ. *U. S. National Institutes of Health, Bethesda, USA,*

Spangenburg, E.E., & Booth, F.W. (2003). Molecular regulation of individual skeletal muscle fibre types. *Acta Physiologica Scandinavica, 178*(4), 413–424. PubMed doi:10.1046/j.1365-201X.2003.01158.x

Sriwijitkamol, A., Coletta, D.K., Wajcberg, E., Balbontin, G.B., Reyna, S.M., Barrientes, J., . . . Musi, N. (2007). Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with Type 2 diabetes: A time-course and dose-response study. *Diabetes, 56*(3), 836–848. PubMed doi:10.2337/db06-1119

Timmers, S., Konings, E., Bilet, L., Houtkooper, R.H., van de Weijer, T., Goossens, G.H., . . . Schrauwen, P. (2011). Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. *Cell Metabolism, 14*(5), 612–622. PubMed doi:10.1016/j.cmet.2011.10.002

Troiano, R.P., Berrigan, D., Dodd, K.W., Masse, L.C., Tilert, T., & McDowell, M. (2008). Physical activity in the united states measured by accelerometer. *Medicine and Science in Sports and Exercise, 40*(1), 181–188. PubMed doi:10.1249/mss.0b013e31815a51b3

van Wijk, J.P., de Koning, E.J., Martens, E.P., & Rabelink, T.J. (2003). Thiazolidinediones and blood lipids in Type 2 diabetes. *Arteriosclerosis, Thrombosis, and Vascular Biology, 23*(10), 1744–1749. PubMed doi:10.1161/01.ATV.0000090521.25968.4D

Yashiro, T., Nanmoku, M., Shimizu, M., Inoue, J., & Sato, R. (2012). Resveratrol increases the expression and activity of the low density lipoprotein receptor in hepatocytes by the proteolytic activation of the sterol regulatory element-binding proteins. *Atherosclerosis, 220*(2), 369–374. PubMed doi:10.1016/j.atherosclerosis.2011.11.006