Resveratrol induces brown-like adipocyte formation in white fat through activation of AMP-activated protein kinase (AMPK) α1

ARTICLE *in* INTERNATIONAL JOURNAL OF OBESITY (2005) · MARCH 2015

Impact Factor: 5.39 · DOI: 10.1038/ijo.2015.23 · Source: PubMed

10 AUTHORS, INCLUDING:

Qiyuan Yang
Washington State University
6 PUBLICATIONS **18** CITATIONS
SEE PROFILE

Min Du
Washington State University
178 PUBLICATIONS **3,086** CITATIONS
SEE PROFILE

International Journal of Obesity (2015) **39,** 967–976

www.nature.com/ijo

ORIGINAL ARTICLE

Resveratrol induces brown-like adipocyte formation in white fat through activation of AMP-activated protein kinase (AMPK) α1

S Wang[1,2], X Liang[2], Q Yang[2], X Fu[2], CJ Rogers[2], M Zhu[3], BD Rodgers[2], Q Jiang[1], MV Dodson[2] and M Du[2]

OBJECTIVE: Development of brown-like/beige adipocytes in white adipose tissue (WAT) helps to reduce obesity. Thus we investigated the effects of resveratrol, a dietary polyphenol capable of preventing obesity and related complications in humans and animal models, on brown-like adipocyte formation in inguinal WAT (iWAT).
METHODS: CD1 female mice (5-month old) were fed a high-fat diet with/without 0.1% resveratrol. In addition, primary stromal vascular cells separated from iWAT were subjected to resveratrol treatment. Markers of brown-like (beige) adipogenesis were measured and the involvement of AMP-activated protein kinase (AMPK) α1 was assessed using conditional knockout.
RESULTS: Resveratrol significantly increased mRNA and/or protein expression of brown adipocyte markers, including uncoupling protein 1 (UCP1), PR domain-containing 16, cell death-inducing DFFA-like effector A, elongation of very long-chain fatty acids protein 3, peroxisome proliferator-activated receptor-γ coactivator 1α, cytochrome *c* and pyruvate dehydrogenase, in differentiated iWAT stromal vascular cells (SVCs), suggesting that resveratrol induced brown-like adipocyte formation *in vitro*. Concomitantly, resveratrol markedly enhanced AMPKα1 phosphorylation and differentiated SVC oxygen consumption. Such changes were absent in cells lacking AMPKα1, showing that AMPKα1 is a critical mediator of resveratrol action. Resveratrol also induced beige adipogenesis *in vivo* along with the appearance of multiocular adipocytes, increased UCP1 expression and enhanced fatty acid oxidation.
CONCLUSIONS: Resveratrol induces brown-like adipocyte formation in iWAT via AMPKα1 activation and suggest that its beneficial antiobesity effects may be partly due to the browning of WAT and, as a consequence, increased oxygen consumption.

International Journal of Obesity (2015) **39,** 967–976; doi:10.1038/ijo.2015.23

INTRODUCTION

Mammals have two morphologically and functionally distinct types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), both of which are involved in energy homeostasis. WAT mainly stores energy in the form of lipids (triglycerides) in unilocular white adipocytes and secretes a number of adipokines and other factors, such as leptin, adiponectin, tumor necrosis factor α and interleukin-6, to regulate energy metabolism and immune function.[1,2] Excessive WAT accumulation that occurs in obesity is a major risk factor for developing insulin resistance, type 2 diabetes mellitus and cardiovascular diseases.[3,4] In contrast, BAT specializes in dissipating energy as heat due to its high mitochondrial content and expression of uncoupling protein 1 (UCP1).[5,6] However, the amount of BAT found in adults is typically quite low.[7] Recently, brown-like adipocytes were discovered in WAT, so called beige adipocytes.[8,9] Similar to brown adipocytes, beige adipocytes express UCP1 to dissipate energy. Thus stimulating the development of beige adipocytes in WAT, so called 'browning', might reduce adverse effects of WAT and could help to improve metabolic health.[10–12]

There are many transcriptional regulators, including PR domain-containing 16 (PRDM16), peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC1α), CCAAT/enhancer-binding protein α and PPARγ, as well as various secreted mediators, such as bone morphogenetic protein 7, Irisin, fibroblast growth factor 21, atrial and brain natriuretic peptides, that can induce the formation of brown-like adipocytes.[6,10,11,13,14] Meanwhile, certain pharmacological and nutritional agents are also involved in promoting WAT browning[15,16] by activating transcription factors or related regulatory signaling pathways.[17] As a nutritional or dietary supplement, resveratrol, a natural polyphenol present in the skin of grapes and other plants, has remarkable beneficial effects on energy metabolism and related disorders in mammals.[18,19] It has been reported that resveratrol protects against high-fat-diet-induced obesity in mice[20,21] and elicits beneficial effects on obese persons.[22,23] Resveratrol also inhibits adipogenesis[24–26] and enhances fat mobilization.[27–29] Resveratrol increased UCP1 expression in 3T3-L1 cells[25] and enhanced the mitochondrial DNA content and UCP1 expression in primary mouse embryonic fibroblast (MEF)-derived adipocytes.[30] To date, studies regarding resveratrol in adipose tissue mainly focus on the white adipogenesis and lipid metabolism, and the effects of resveratrol on the formation of brown-like or beige adipocytes remains sparsely studied. To our knowledge, there is no report about mechanisms in which resveratrol induces the formation of brown-like adipocytes.

[1]College of Animal Science, ALLTECH-SCAU Animal Nutrition Control Research Alliance, South China Agricultural University, Guangzhou, China; [2]Department of Animal Sciences, Washington Center for Muscle Biology, Washington State University, Pullman, WA, USA and [3]School of Food Sciences, Washington State University, Pullman, WA, USA. Correspondence: Dr M Du, Department of Animal Sciences, Washington Center for Muscle Biology, Washington State University, 100 Dairy Road, Pullman, WA 99164, USA. E-mail: min.du@wsu.edu
Received 29 July 2014; revised 25 November 2014; accepted 2 December 2014; accepted article preview online 12 March 2015; advance online publication, 31 March 2015

The effects of resveratrol on metabolic health are due, at least in part, to its ability to activate the AMP-activated protein kinase (AMPK), a master regulator of energy metabolism.[15,19] AMPK consists of one α-catalytic subunit and two regulatory subunits, β and γ. The catalytic subunit of AMPK has two isoforms, α1 and α2, which have different tissue expression patterns. In adipose tissue, the α1 catalytic subunit is the predominant isoform expressed,[31,32] while the α2 isoform is highly expressed in muscle and liver but at a low level in adipose tissue.[33,34] The measurement of AMPK activity indicates that the α1 isoform accounts for the majority of the total activity of this kinase in adipose tissue.[32,35] Although there are a number of studies on the role of AMPK in adipose tissue metabolism,[21,36,37] it is unclear whether AMPK is involved in the browning of white adipocytes.

In the present study, we sought to elucidate the role of resveratrol in brown-like adipocyte formation in WAT and to explore the mechanism underlying this process. Our data show that resveratrol induces browning of white fat, a process mediated by AMPKα1.

MATERIALS AND METHODS

Animals

Twelve adult CD1 female mice (5-month old) were randomly divided into two groups: a control group, which was fed a high-fat diet (HFD; 45% energy from fat, D12451, Research Diet, New Brunswick, NJ, USA), and a resveratrol (Resv) group, which was fed a HFD containing 0.1% (w/w) resveratrol. Mice were housed in environmentally controlled rooms on a 12-h light–dark cycle with free access to food and water. Before and after the treatment, we measured the basal metabolic rate (oxygen consumption (VO_2), CO_2 production (VCO_2) and respiratory exchange ratio (RER)) of mice during the day (quiescent phase) using a CLAMS (Columbus Instruments, Columbus, OH, USA) indirect open circuit calorimetry system. We deprived the mice of food for 4 h prior to measurement and continuously measured for 3 h (with water provided), taking a measurement every 30 s.[38,39] We used the lowest 10 consecutive measures (5 min) as the estimate of basal metabolic rate.

Body weight and food intake were measured weekly. At the end of 4 weeks of treatment, mice were killed by carbon dioxide anesthesia. Inguinal WAT (iWAT) was rapidly isolated and weighed. One side of the adipose tissues were frozen in liquid nitrogen and stored at − 80 °C until further analyses. A middle portion of the other side was fixed in 4% paraformaldehyde for sectioning and staining. Another portion of the other side was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium for tissue oxygen consumption measurement. Wild-type and $Rosa^{Cre}$/$AMPK\alpha1^{flox/flox}$ C57BL/6 mice (Jackson Lab, Bar Harbor, ME, USA) were housed in environmentally controlled rooms on a 12-h light–dark cycle with free access to food and water. All animal experiments and care procedures were performed according to protocols preapproved by the Institutional Animal Care and Use Committees (IACUC) at Washington State University.

Antibodies and chemicals

Antibodies against AMPKα (no. 2532), phospho-AMPKα at Thr172 (no. 2535), pyruvate dehydrogenase (PDH) (no. 2784), cytochrome *c* (Cyto C; no. 4280), β-tubulin (no. 2146) and goat anti-rat antibody Alexa Fluor 488 (no. 4416) were purchased from Cell Signaling (Danvers, MA, USA). Anti-PRDM16 polyclonal antibody (no. ABD130) was purchased from Millipore (Billerica, MA, USA). Anti-UCP1 polyclonal antibody (no. sc28766) was bought from Santa Cruz Biotechnology (Dallas, TX, USA). Goat anti-rabbit IRDye 800CW (no. 926–32211) and goat anti-rabbit IRDye 680RD (no. 926-68070) secondary antibodies for western blotting were purchased from LI-COR (Lincoln, NE, USA). Fluoro-Gel II with 4,6-diamidino-2-phenylindole (DAPI) (no. 17985–50) was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Insulin, dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine, Triiodothyronine (T3), Oil-Red O and compound C were purchased from Sigma (St Louis, MO, USA). Collagenase D and dispase II were purchased from Roche Diagnostics (Indianapolis, IN, USA). DMEM/F12 and fetal bovine serum were purchased from Life Technologies (Grand Island, NY, USA).

Stromal vascular cell (SVC) isolation and *in vitro* differentiation

SVCs were isolated from iWAT as previously described.[40] The medium was changed every other day.[41] To induce brown adipogenic differentiation of SVCs, confluent SVCs were cultured in DMEM/F12 containing 10% fetal bovine serum, 1% penicillin–streptomycin solution with 5 μg ml^{-1} insulin, 1 nM T_3, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 0.125 mM indomethacin for 2 days. The cells were then switched to DMEM/F12 supplemented with 10% fetal bovine serum and 5 μg ml^{-1} insulin for 5 more days, and the medium was changed every other day.[42] For SVCs from iWAT of weaning $Rosa26^{Cre}$/$AMPK\alpha1^{flox/flox}$ mice, confluent SVCs were treated with 250 nM 4-hydroxytamoxifen (4-OHT) for 2 days to delete AMPKα1 before being induced to undergo brown adipogenic differentiation.[43]

In vitro O_2 consumption assay

In vitro O_2 consumption measurement was performed with Thermo Scientific Orion 3-Star Dissolved Oxygen meter and probe (Thermo Electron Corporation, Madison, WI, USA).[44] Equal numbers of iWAT SVCs were seeded and treated with vehicle (control) or 10 μM resveratrol (Resv) to induce differentiation. On day 7, the differentiated SVCs were changed to fresh DMEM/12 for 30 min. The dissolved oxygen in the medium were measured at the start and end of incubation. For the tissues, a thin slice (50 mg) of iWAT from control and resveratrol-fed mice were cultured in medium for 1 h, and dissolved oxygen was measured before and after incubation. O_2 consumption of differentiated SVCs or iWAT were calculated as the rate of decrease in dissolved oxygen.[45]

Oil-Red O staining

Differentiated cells were subjected to Oil-Red O staining as previously described.[46]

Immunostaining of cells and tissue sections

Immunofluorescence staining of cells was conducted as previously described.[43] Fluorescence was examined and images were acquired using an EVOS fl fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA). As for iWAT, paraffin-embedded iWAT sections (5-μm thick) were either stained with hematoxylin and eosin (H&E)[47] or used for UCP1 immunohistochemical (IHC) staining.[48] Adipocyte diameters were analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Real-time quantitative PCR

Total RNA was extracted from differentiated iWAT SVCs using TRIzol reagent (Sigma) according to the manufacturer's protocol, and cDNA was synthesized from 0.5 μg of total RNA using a reverse transcription kit (Bio-Rad, Hercules, CA, USA). Real-time quantitative PCR was carried out in the final 10-μl volume of the amplification mixture containing 2× Qprecise Green Master Mix (EarthOx, LLC, San Francisco, CA, USA), primers, and cDNA using a CFX RT-PCR detection system (Bio-Rad). Delta cycle threshold (CT) was used to calculate the differences between the target CT value and the control (18 S) for each sample: ΔCT = CT (target) − CT (control). The relative expression level was calculated using $2^{-\Delta CT}$. The following cycle parameters were used: 40 two-step cycles of 95 °C for 15 s and 58 °C for 60 s.[46] Primer sequences (with their respective PCR fragment lengths) are shown in Table 1.

Western blotting analysis

Western blot was conducted as previously described.[46] Immunoreactive proteins in the membrane were scanned and analyzed by Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE, USA). Band density was normalized according to the β-tubulin content.

Statistical analysis

The *in vitro* data were generated from three independent experiments and three parallels were used in each experiment. The *in vivo* data were obtained from one experiment, with six mice in each treatment. Data are presented as means ± s.e.m. Statistical analysis was performed using Sigmaplot 12.5 (Systat Software, Inc., San Jose, CA, USA). Differences between means were determined using Student's *t*-test or one-way analysis of variance followed by Duncan's multiple test when appropriate and a confidence level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Resveratrol exerts dose-dependent effects on brown adipogenic differentiation of iWAT SVCs

First, we investigated the effects of resveratrol on brown adipogenic differentiation of iWAT SVCs. The result of Oil-Red O staining demonstrated that the higher concentrations (20 or 40 μM) of resveratrol significantly ($P < 0.001$) inhibited lipid accumulation in the differentiated iWAT SVCs after 7-day brown adipogenic differentiation (Figures 1a and b) and suppressed the expression of adipogenic markers PPARγ and aP2 (Figures 1c and d). Similar inhibitory effects of resveratrol on white adipogenesis were observed previously.[24,25] On the other hand, at the concentrations ⩽10 μM, resveratrol had no effect on lipid accumulation.

Resveratrol promotes formation of brown-like adipocytes in differentiated iWAT SVCs

Although low concentrations of resveratrol had no effect on lipid accumulation during brown adipogenic differentiation of iWAT SVCs, we further determined whether resveratrol stimulated the generation of brown-like adipocytes by analyzing the mRNA expression of brown adipocyte-specific genes. As shown in Figure 2a, resveratrol increased the mRNA level of PRDM16 (3.6-fold versus control, $P < 0.05$), a key transcription factor regulating brown adipogenesis. UCP1 expression, which is specific to brown adipocytes and does not occur in white adipocytes, was also markedly elevated (2.2-fold versus control, $P < 0.05$). In addition, the expression of cell death-inducing DFFA-like effector A (Cidea), a gene predominantly expressed in brown adipocytes, and elongation of very long-chain fatty acids protein 3 (Elovl3), a very long chain fatty acid elongase that is expressed in brown but not in white fat, increased 3.4-fold ($P < 0.01$) and 1.8-fold ($P < 0.05$), respectively, in the resveratrol group. Moreover, resveratrol increased the mRNA expression of PGC1α (3.3-fold, $P < 0.01$), the master regulator of mitochondrial biogenesis and oxidative phosphorylation. Finally, resveratrol promoted the mRNA expression of beige adipocyte selective markers, such as CD137 (1.8-fold, $P < 0.05$), Tbx1 (1.9-fold, $P < 0.01$) and TMEM26 (2.1-fold, $P < 0.01$).

Immunostaining results showed that the expression of UCP1 in the resveratrol-treated group was higher than that of the control

Table 1. Primer sequences used for real-time quantitative PCR

Gene	*Forward (5′ → 3′)*	*Reverse (3′ → 5′)*	*Amplicon size (bp)*	*Gene access number*
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151	NR_046233.2
Cidea	ATCACAACTGGCCTGGTTACG	TACTACCCGGTGTCCATTTCT	136	NM_007702.2
CD137	GTCGACCCTGGACGAACTGCTCT	CCTCTGGAGTCACAGAAATGGTGGTA	132	NM_001077509.1
Elovl3	GATGGTTCTGGGCACCATCTT	CGTTGTTGTGTGGCATCCTT	73	XM_006526624.1
PGC1α	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC	161	XM_006503779.1
PRDM16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG	87	NM_001291029.1
Tbx1	TGAAGAAGAACCCGAAGGTGG	ACTTGGAACGTGGGGAACATT	133	XM_006536887.1
TMEM26	GAAACCAGTATTGCAGCACCCAAT	AATATTAGCAGGAGTGTTTGGTGGA	205	NM_177794.3
UCP1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG	123	NM_009463.3

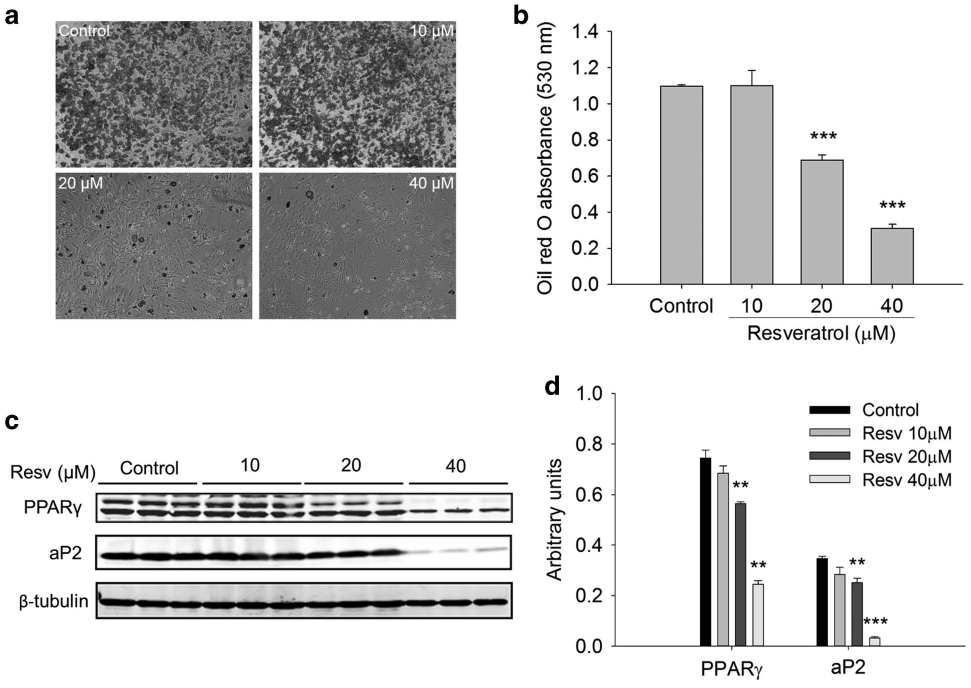

Figure 1. Effects of resveratrol on the lipid accumulation and the expression of adipogenic marker genes in differentiated iWAT SVCs. (**a**) Oil-Red O staining was conducted in the differentiated iWAT SVCs after 7-day brown adipogenic differentiation. Microscopic pictures were taken on day 7 with × 100 magnification. (**b**) The stained Oil-Red O was extracted with isopropanol. The absorbance of the extracted Oil-Red O was spectrophotometrically determined at 530 nm to measure triglyceride accumulation. (**c**, **d**) Western blotting analysis of adipogenic marker genes (PPARγ and aP2) in the differentiated iWAT SVCs after 7-day brown adipogenic differentiation, and β-tubulin was used as a loading control (**c**). Mean ± s.e.m. of immunoblotting bands of PPARγ and aP2 (**d**). The intensities of the bands were expressed as arbitrary units. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control. A full color version of this figure is available at the *International Journal of Obesity* journal online.

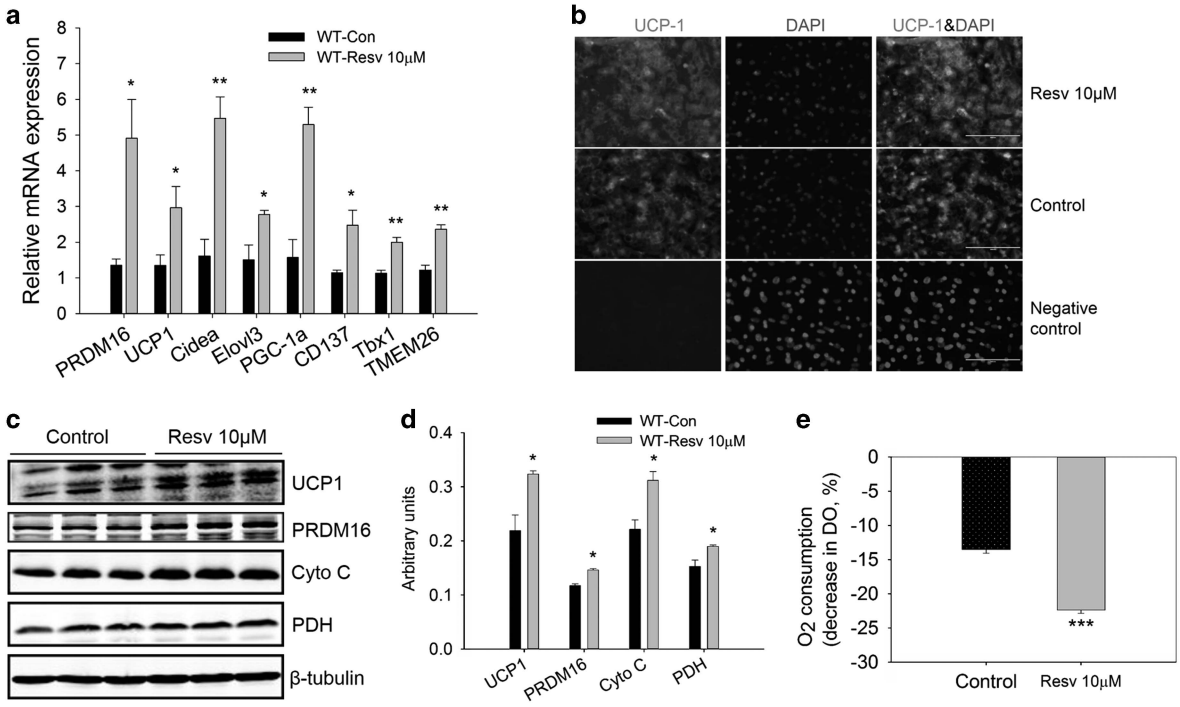

Figure 2. Resveratrol promoted formation of brown-like adipocytes in the differentiated iWAT SVCs from wild-type mice after 7-day adipogenic differentiation. (**a**) Relative mRNA levels of brown adipocyte selective genes (PRDM16, UCP1, Cidea, Elovl3 and PGC-1a) and beige adipocyte selective genes (CD137, Tbx1 and TMEM26). (**b**) UCP1 immunofluorescence staining for UCP1 in the differentiated iWAT SVCs. Nuclei were stained with DAPI (scale bar, 100 μm). (**c**, **d**) Western blotting analysis of brown adipocyte selective genes (UCP1, PRDM16, Cyto C and PDH) in the differentiated iWAT SVCs, and β-tubulin was used as a loading control (**c**); mean ± s.e.m. of immunoblotting bands of UCP1, PRDM16, Cyto C and PDH (**d**). The intensities of the bands were expressed as arbitrary units. (**e**) Basal O_2 consumption of differentiated iWAT SVCs from control and resveratrol-treated groups. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control. A full color version of this figure is available at the *International Journal of Obesity* journal online.

group (Figure 2b), and this was confirmed by western blotting as the UCP1 protein level in resveratrol-treated cells was 1.5-fold higher than that of the control cells ($P < 0.05$). Consistent with mRNA expression, the protein level of PRDM16 was also markedly increased in the resveratrol-treated group (1.2-fold versus control, $P < 0.05$). Moreover, the protein levels of Cyto C (1.4-fold versus control, $P < 0.05$) and PDH (1.2-fold versus control, $P < 0.05$), which represent the mitochondrial content, were also elevated by resveratrol (Figures 2c and d). The mRNA and protein expression data together provided evidence that resveratrol promoted the formation of brown-like adipocytes of iWAT SVCs.

Browning of WAT is expected to similarly increase cellular respiration. To investigate whether resveratrol elevated cellular respiration, O_2 consumption of differentiated iWAT SVCs was measured after 7 days of treatment. Consistent with increased browning, the basal oxygen consumption in the resveratrol group was 1.6-fold higher than that of the control cells ($P < 0.001$; Figure 2e).

Resveratrol stimulates the phosphorylation of AMPKα in SVCs

In order to determine whether AMPKα was involved in the resveratrol-mediated browning effects, we examined the effects of resveratrol on the phosphorylation of AMPKα (p-AMPKα). As shown in Figure 3, resveratrol increased the phosphorylation of AMPKα in differentiated wild-type iWAT SVCs (1.3-fold versus control, $P < 0.05$), with no effect on total AMPKα (t-AMPKα). In addition, the ratio pAMPKα/t-AMPKα was elevated (1.2-fold versus control, $P < 0.01$) in the resveratrol-treated group. Furthermore, the protein level of Sirt1 was also higher (1.5-fold versus control, $P < 0.05$) due to resveratrol treatment. When the confluent iWAT SVCs were treated with 4-OHT to knockout AMPKα1 acutely before brown adipogenic differentiation, the expression levels of p-AMPKα, t-AMPKα and Sirt1 in differentiated iWAT SVCs were much lower than seen in wild-type cells. Moreover, we found that resveratrol had no effect on the protein levels of p-AMPKα, t-AMPKα or Sirt1 in AMPKα1 knockout SVCs.

AMPK inhibition or AMPKα1 deletion eliminate the browning effects of resveratrol on mouse iWAT SVCs

AMPK inhibitor Compound C was used to examine the effects of AMPK inhibition on the resveratrol-mediated browning effects on mouse iWA SVCs. We found that Compound C (1 μM) did inhibit the activation of AMPK (p-AMPK/t-AMPK) (Figures 4a and b). While Compound C had no effects on the expression of UCP1, PRDM16, Cyto C and PDH. However, Compound C inhibited the promotional effects of resveratrol on the expression of these genes (Figures 4a and b).

We also tested whether acute Ampkα1 deletion affected the browning effects of resveratrol on iWAT SVCs. To this end, iWAT SVCs isolated from weaning $Rosa^{cre}/Ampk\alpha1^{flox/flox}$ mice that ubiquitously express a tamoxifen-inducible Cre recombinase were treated with 4-OHT to induce AMPKα1 knockout acutely. In the absence of AMPKα1, resveratrol had no effects on the mRNA expression of PRDM16, UCP1, Cidea, Elovls and PGC1a (Figure 4c). Consistently, after deletion of AMPKα1, the protein levels of UCP1, PRDM16, Cyto C and PDH in the resveratrol-treated group did not differ from those in the control group (Figures 4e and f). Furthermore, after knocking out AMPKα1, the basal oxygen consumption of differentiated iWAT SVCs was not affected by resveratrol treatment (Figure 4d). These results suggested that AMPKα1 has a major role in mediating the browning effect of resveratrol on iWAT SVCs.

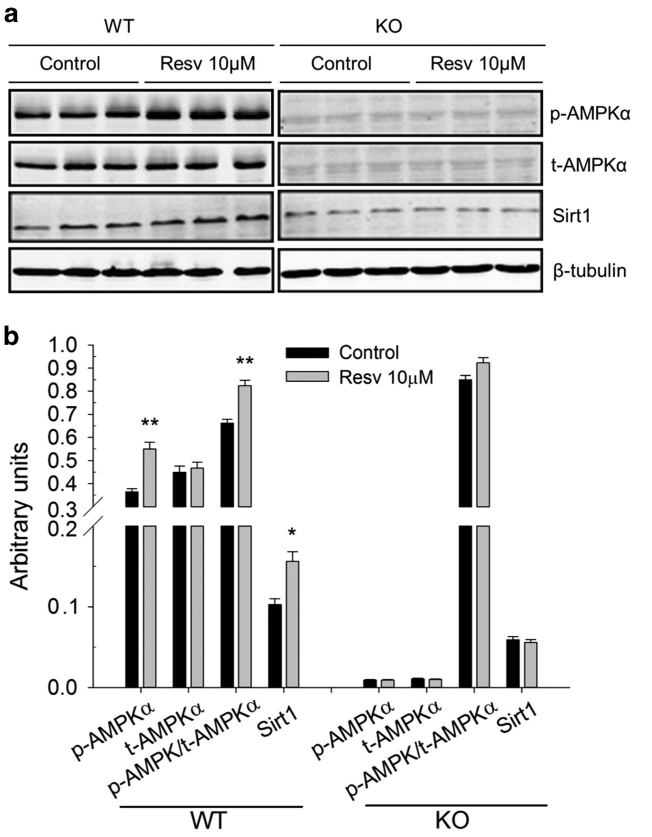

Figure 3. Effects of resveratrol on the phosphorylation of AMPKα and Sirt1 in wild-type and AMPKα1 knockout iWAT SVCs. (**a**) Western blotting analysis of phospho-AMPKα (p-AMPKα), t-AMPKα (t-AMPKα) and Sirt1 in the differentiated iWAT SVCs of wild type (left part) and AMPKα1 deletion (right part). β-Tubulin was used as a loading control. (**b**) Mean ± s.e.m. of immunoblotting bands of p-AMPKα, t-AMPKα, p-AMPKα/t-AMPKα and Sirt1 in wild-type and AMPKα1 knockout cells. The intensities of the bands were expressed as arbitrary units. *$P < 0.05$ and **$P < 0.01$ versus control.

Resveratrol reduces body weight and iWAT index and stimulates browning of iWAT

To further analyze the browning effects of resveratrol on iWAT *in vivo*, 5-month-old CD1 mice were treated HFD or HFD containing 0.1% resveratrol for 4 weeks. Although there was no apparent difference in food intake (Figure 5a), the body weight gain in the resveratrol-supplemented group was lower than that of the control group (2.99 ± 0.91 versus 5.91 ± 0.90 g of control, $P < 0.05$; Figure 5b). In addition, resveratrol markedly reduced the body iWAT index (iWAT mass/body weight) (24.15 ± 2.56 versus 34.57 ± 0.76 of control, $P < 0.05$; Figure 5c), with no effect on blood glucose level. H&E staining results revealed that the average adipocyte diameter in the resveratrol-supplemented group was much smaller than that of the control group (33.29 ± 1.90 versus 81.07 ± 1.94 μm of control, $P < 0.001$; Figure 5d). Inspection of the distribution of cell sizes indicated that diameter of most adipocytes (about 70%) in the control group was in the range of 61–100 μm. In contrast, diameter of most adipocytes (about 90%) in the resveratrol-supplemented group was smaller than 60 μm (Figure 5e). Moreover, iWAT from resveratrol-treated mice showed the appearance of multiocular adipocytes within white fat, a characteristic of brown adipocytes (Figure 5d), suggesting that resveratrol induced brown-like remodeling (browning) of iWAT. Subsequently, IHC staining of UCP1 indicated an enhanced UCP1 staining in resveratrol-treated mice (Figure 5d). In agreement, the UCP1 protein content in the resveratrol group was 1.5-fold higher than that of the control group ($P < 0.05$). Furthermore, resveratrol supplement resulted in increased protein contents of PRDM16 (1.8-fold versus control, $P < 0.01$) and Cyto C (1.3-fold versus control, $P < 0.05$), which was accompanied by the elevated expression of p-AMPKα (1.5-fold versus control, $P < 0.01$; Figures 5f and g), suggesting that AMPKα was involved in the resveratrol-induced browning of iWAT. We also found that resveratrol supplement had no effect on the protein expression levels of PPARγ and aP2.

We also analyzed the serum profiles (Table 2). Although there was no difference in non-fasting glucose, the insulin level was lower in resveratrol-treated mice ($P < 0.05$). In addition, the triglyceride concentration was also reduced in resveratrol-supplemented compared with control mice ($P < 0.05$).

Resveratrol promotes lipid oxidation in iWAT

To explore why resveratrol feeding reduced the body weight gain and iWAT mass under HFD, we measured the oxygen consumption of mice. Resveratrol treatment significantly increased the oxygen consumption (VO_2) of mice (2359 ± 43 versus 2103 ± 61 ml kg^{-1} h^{-1} of control, $P < 0.01$; Figure 6a). There was no difference in CO_2 production (VCO_2) between the control and resveratrol group before and after treatment (Figure 6b). As a result, resveratrol decreased the RER (VCO_2/VO_2) (0.731 ± 0.017 versus 0.791 ± 0.038 of control, $P < 0.01$; Figure 6c), suggesting that there was a shift to primarily utilize fatty acids for oxidation in the resveratrol group. This was consistent with the reduced serum triglyceride concentration in the resveratrol group (Table 2). Furthermore, resveratrol exerted the tendency to increase average heat production (0.727 ± 0.024 versus 0.671 ± 0.010 kCal h^{-1} of control, $P = 0.065$; Figure 6d). Moreover, resveratrol increased the basal oxygen consumption (2.1-fold versus control, $P < 0.01$; Figure 6e) of iWAT *in vitro*.

DISCUSSION

In this paper, we investigated the effects of resveratrol on the formation of brown-like adipocytes and the mechanism underlying this process. Our results demonstrated that resveratrol induces the browning of mouse iWAT by promoting the expression of brown adipocyte selective genes through the activation of AMPKα1. It has been reported that resveratrol reduces adiposity,[19] via inhibiting white adipogenesis[24] and stimulating the lipolysis.[27,28] To date, however, no study assessed resveratrol's effects on the brown adipogenesis or the formation of brown-like adipocytes. Furthermore, the concentrations used in previous *in vitro* studies of white adipogenesis[24,25] are much higher than the plasma concentration.[20,49] These concentrations can stimulated apoptosis[25,50] and might be less relevant to the physiological effects of resveratrol. In the present study, we found that high concentrations (20 or 40 μM) of resveratrol inhibited lipid accumulation during the brown adipogenic differentiation of iWAT SVCs. And these results agreed with previous reports that high concentration (50 μM) of resveratrol inhibits the adipogenic differentiation of 3T3-L1[24,25] and Simpson–Golabi–Behmel syndrome preadipocytes.[51] However, at the lower concentrations (⩽10 μM), which is closer to the plasma concentration,[20,49] resveratrol did not affect lipid content in induced brown adipocytes. It has been reported that the plasma resveratrol concentration is 1.56 ± 0.28 μM in rat fed a HFD containing 4 g resveratrol per kg diet.[49] While in mice fed HFD containing 0.4% resveratrol, the highest plasma resveratrol concentration is about 0.5 μM.[20] Thus relative low resveratrol concentration (10 μM) was selected to investigate its role in the formation of brown-like adipocytes during the brown adipogenic differentiation of iWAT SVCs.

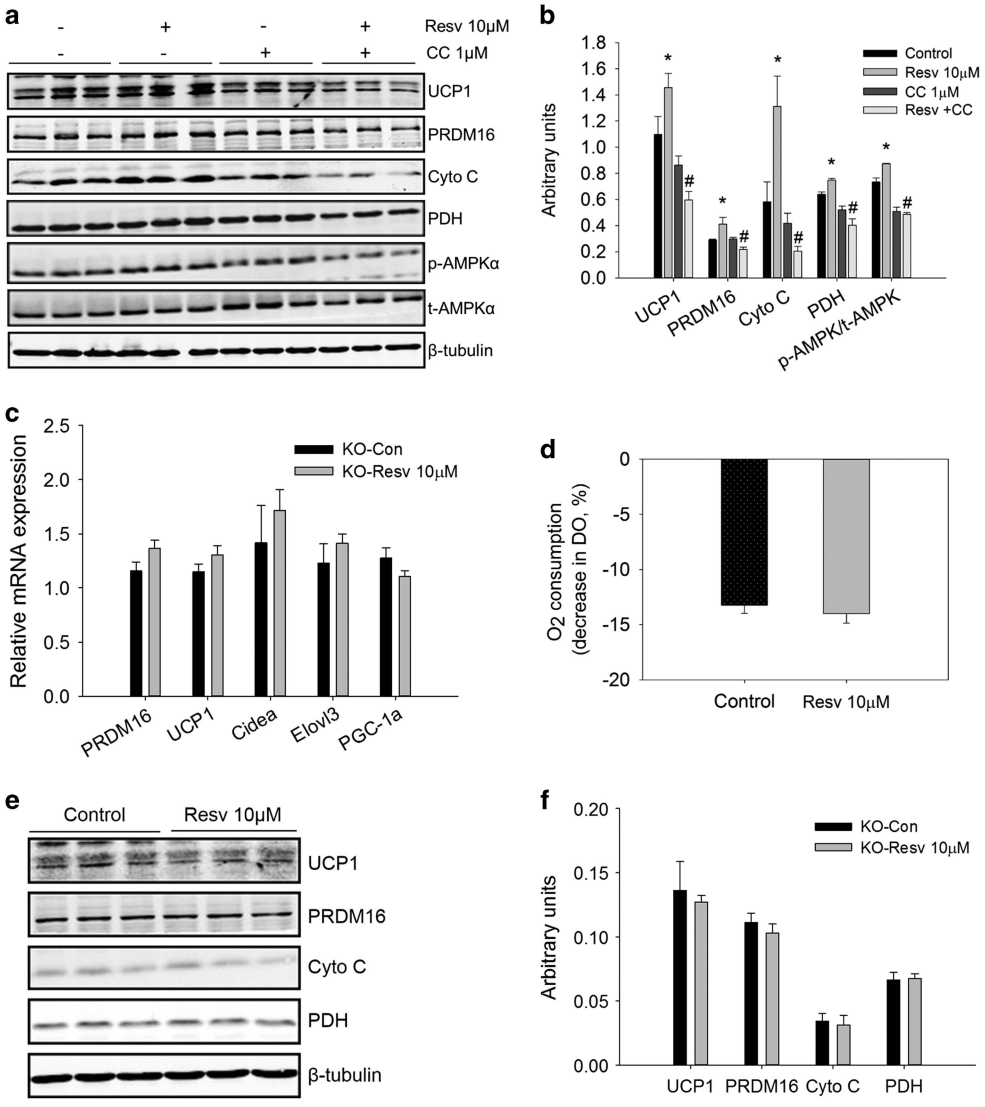

Figure 4. AMPK inhibition or AMPKα1 deletion eliminated the browning effects of resveratrol on mouse differentiated iWAT SVCs. (**a**) Effects of AMPK inhibitor Compound C (CC) in the protein contents of UCP1, PRDM16, Cyto C, PDH, phospho-AMPKα (p-AMPKα) and t-AMPKα (t-AMPKα) in the differentiated iWAT SVCs after 7-day brown adipogenic differentiation. β-Tubulin was used as a loading control. (**b**) Mean ± s.e.m. of immunoblotting bands of UCP1, PRDM16, Cyto C, PDH and p-AMPKα/t-AMPKα. The intensities of the bands were expressed as arbitrary units. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus Resv 10 μM. (**c**) Relative mRNA levels of brown adipocyte selective genes (PRDM16, UCP1, Cidea, Elovl3 and PGC1α) in the differentiated SVCs after 7-day differentiation with classical brown adipogenic induction cocktails. SVCs from iWAT of weaning $Rosa26^{Cre}/AMPK\alpha1^{flox/flox}$ mice were treated with 4-OHT to delete AMPKα1 before being induced to undergo brown adipogenic differentiation. (**d**) Basal O_2 consumption of differentiated AMPKα1 knockout iWAT SVCs from control and resveratrol-treated groups. (**e**, **f**) Western blotting analysis of brown adipocyte selective genes (UCP1, PRDM16, Cyto C, and PDH) in the differentiated SVCs after 7-day brown adipogenic differentiation, and β-tubulin was used as a loading control (**e**). Mean ± s.e.m. of immunoblotting bands of UCP1, PRDM16, Cyto C and PDH (**f**). The intensities of the bands were expressed as arbitrary units.

Our results showed that resveratrol boosts UCP1 mRNA expression in differentiated iWAT SVCs, which is consistent with the reports in maturing 3T3-L1 preadipocytes[25] and primary MEF-derived adipocytes.[30] In addition, the mRNA expression of other brown adipocyte selective genes such as PRDM16, Cidea, Elovl3, and PGC1α as well as the protein levels of UCP1, Cyto C and PDH were also markedly elevated by resveratrol treatment. Moreover, the expression levels of beige adipocyte selective markers such as CD137, Tbx1 and TMEM26 in the resveratrol-treated group were much higher than those of the control group. These data strongly support the notion that resveratrol promotes the formation of brown-like adipocytes in differentiated mouse iWAT SVCs.

In vivo studies were conducted to further address the biological effects of resveratrol on the formation of brown-like adipocytes in WAT. We found that resveratrol significantly decreased the body weight gain compared with the control group when challenged with an obesogenic diet. The reduced body weight gain in resveratrol-treated mice might be due to lower body fat accumulation. Our findings confirmed the body fat-lowering effects of resveratrol, which have been reported in both animals[21,29,52] and humans.[22] It has been reported that thermogenesis is involved in the body fat-lowering effects of resveratrol.[53,54] However, in these previous studies, their primary focuses were on the BAT and/or skeletal muscle but not on WAT. In our study, we found that resveratrol resulted in decreased adipocyte size in WAT, which is in agreement with a recent report in humans.[23] More importantly, we observed brown-like adipocytes, with an appearance of multiocular lipid droplets, in iWAT, which has not been observed before. The presence of brown-like adipocytes was further confirmed by UCP1 IHC staining.

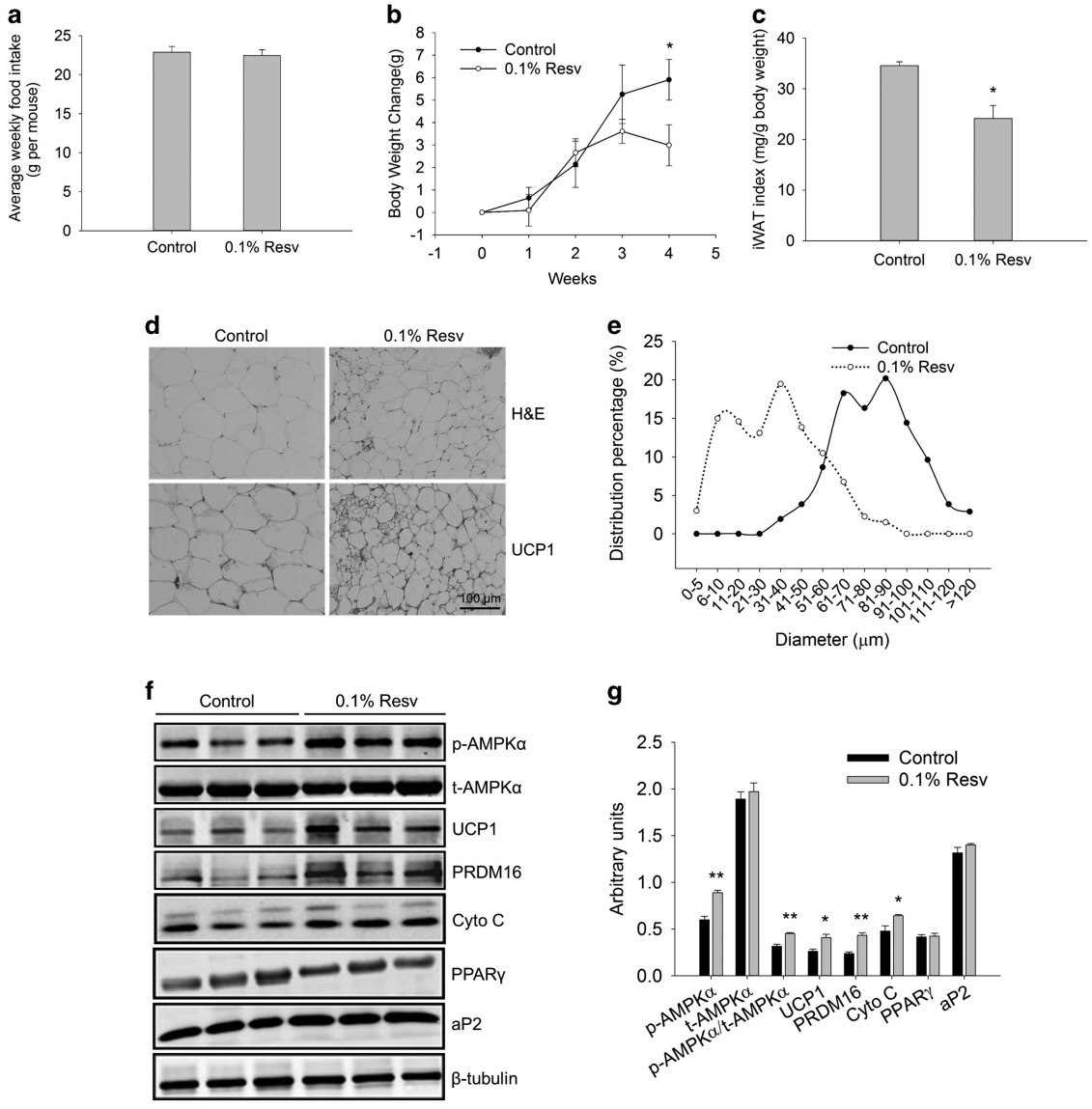

Figure 5. Resveratrol induced brown-like adipocytes in iWAT. (**a**) Weekly food intake were measured in the control ($n=6$) and 0.1% Resv ($n=6$) groups. (**b**) Body weight changes were compared between the control and 0.1% Resv groups during 4 weeks. (**c**) iWAT index was compared between the control and 0.1% Resv groups. (**d**) Representative images of H&E and UCP1 IHC staining in sections of iWAT of control and 0.1% Resv-treated mice. All images were obtained at ×400 magnification. (**e**) Distribution percentage of adipocyte diameters from control and 0.1% Resv-treated mice. Data analysis from the H&E staining sections. (**f**, **g**) Western blotting analyses of p-AMPKα, t-AMPKα, UCP-1, PRDM 16, Cyto C and adipogenic marker genes (PPARγ and aP2) were performed in iWAT of control and resveratrol-treated mice, and β-tubulin was used as a loading control (**f**). Mean ± s.e.m of immunoblotting bands of p-AMPKα, t-AMPKα, p-AMPKα/t-AMPKα, UCP-1, PRDM16, Cyto C, PPARγ and aP2 (**g**). The intensities of the bands were expressed as arbitrary units.*$P<0.05$, **$P<0.01$ versus control. A full color version of this figure is available at the *International Journal of Obesity* journal online.

Moreover, the UCP1 protein content was also elevated in the resveratrol group, accompanied with the elevated expression of PRDM16 and Cyto C, two markers of brown adipogenesis.[55,56] These findings strongly suggested the browning effects of resveratrol on iWAT.

Increased browning of iWAT could lead to increased energy expenditure and oxygen consumption. It has been reported that resveratrol improves mitochondrial oxidation function in BAT and skeletal muscle,[20] but whether resveratrol elicits similar effects in iWAT has not been evaluated. Our findings indicate that resveratrol increased oxygen consumption (VO_2) and decreased RER (CO_2 production/O_2 uptake) in mice, which is highly consistent with our *in vitro* data. RER is commonly used to determine the relative contribution of carbohydrate and lipids to overall energy expenditure. A high RER indicates that carbohydrates are being predominantly catabolized, whereas a low RER suggests lipid oxidation.[57] Thus the decreased RER in resveratrol-treated mice suggests that a higher ratio of lipids were being oxidized. We also found that resveratrol had the tendency to increase ($P=0.065$) the average heat production. It should be noted that the increased oxygen consumption (VO_2), heat production and lipid oxidation might be partially due to the activation of BAT by resveratrol.[53] Meanwhile, we also found that oxygen consumption of tissue (iWAT) and cells (differentiated iWAT SVF) in the resveratrol-treated group was higher than that of the control group. Thus a lower iWAT adipocyte size in the HFD-fed mice supplemented with resveratrol might be due to the increase of lipolysis and subsequent elevated fat oxidation and

heat production with increased oxygen consumption. Moreover, the expression of genes related to mitochondrial fatty acid oxidation such as PGC1α, PDH, Cyto C was elevated in the resveratrol group. These data were consistent with the enhanced fatty acid oxidation observed in 3T3-L1 and MEF-derived adipocytes following resveratrol treatment.[30] Together, our data suggested that the antiobesity effects of resveratrol at least partially resulted from the enhanced fat oxidation in iWAT.

It has been reported that resveratrol may exert its effects on metabolic health in part through the activation AMPK.[49,58] To investigate whether AMPK was involved in the resveratrol-mediated browning of iWAT, we first analyzed the activation of AMPKα (the ratio of AMPKα/t-AMPK) in the differentiated iWAT SVCs and found that the ratio of p-AMPKα/t-AMPKα was increased in the resveratrol-treated group. Meanwhile, AMPKα inhibition by Compound C, which could inhibit the activation of AMPKα, led to the complete elimination of the stimulating effects of resveratrol on the expression of markers of beige adipocytes, including UCP1, PRDM16, Cyto C and PDH. Furthermore, our *in vivo* study also revealed the increased AMPKα phosphorylation and p-AMPKα/t-AMPKα ratio in iWAT of resveratrol-treated mice, in agreement with the previous report.[21] These results suggested that AMPKα is involved in the browning effects of resveratrol on iWAT.

Because the predominant isoform of α catalytic subunit expressed in adipose tissue is α1,[31,32] we speculated that AMPKα1 but not AMPKα2 participated in resveratrol-induced browning effects. To verify our hypothesis, we acutely delete AMPKα1 by treating the confluent iWAT SVCs isolated from $Rosa26^{Cre}/Ampka1^{flox/flox}$ mice with 4-OHT and then induced brown adipogenic differentiation. As expected, only trace amount of p-AMPKα and t-AMPKα was detected in SVCs after the acute deletion of AMPKα1, showing that α1 isoform accounts for most of the total activity of this kinase in SVCs.[32,35] In the absence of AMPKα1, the effects of resveratrol on the expression of brown adipocyte selective genes were abolished, suggesting that AMPKα1 is the key mediator linking resveratrol to the browning of iWAT. Our study is consistent with a previous study showing that AMPKα1 knockout abolished the effect of resveratrol on metabolic rate in mice,[21] though no brown adipogenesis or browning of white adipocytes were examined. Here, building on that study, for the first time, we demonstrate that resveratrol improves metabolism at least partially through enhancing brown-like or beige adipogenesis in WAT, which is mediated by AMPKα1.

In a recent study, resveratrol was shown to induce thermogenesis by increasing Sirt1 expression.[53] And Sirt1 is required for the mitochondrial biogenesis induced by resveratrol.[58] Consistently, in our study, the Sirt1 content in SVCs was also activated due to resveratrol treatment, which was absent in AMPKα1 KO cells; in addition, AMPKα1 deficiency dramatically reduced Sirt1 content. These data suggest that AMPK and Sirt1 likely reinforce each other to induce the browning of iWAT. Indeed, AMPK and Sirt1 coordinate to regulate mitochondriogenesis.[59]

In conclusion, we provide evidence that resveratrol induces the formation of brown-like adipocytes in mouse iWAT by increasing the expression of genes specific to brown adipocytes and stimulating fatty acid oxidation, which appeared to be primarily mediated by AMPKα1. These data demonstrate, in addition to the inhibition of adipogenesis and stimulation of lipolysis, a novel browning role of resveratrol in WAT, which contributes to the beneficial effects of resveratrol in metabolism. Moreover, it extends our knowledge on dietary polyphenols and beige adipogenesis and provides new strategies for the prevention and treatment of obesity and related diseases.

Table 2. Serum profiles of Con and resveratrol-treated CD1 mice fed a high-fat diet

Treatments	*Control (Con)*	*Resveratrol (Resv)*	P-*value*
Insulin (ng ml^{-1})	1.48 ± 0.279	0.85 ± 0.136	< 0.05
Triglyceride (mg dl^{-1})	145.0 ± 28.2	87.4 ± 9.33	< 0.05
Glucose (mg dl^{-1})	188 ± 16.7	196 ± 8.2	NS

Mice were fed a high-energy diet with 45% energy from fat for 4 weeks and with/without 0.1% resveratrol. Mice were not fasted before collection of blood samples for analyses. $n = 6$.

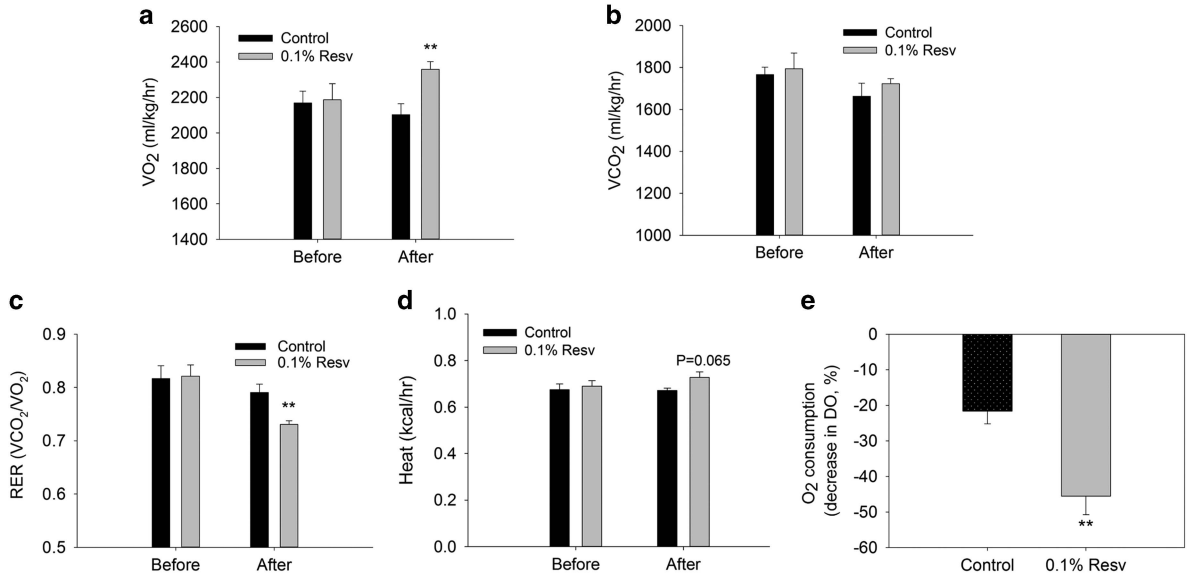

Figure 6. Resveratrol promoted the lipid oxidation of iWAT. (**a**) O_2 consumption of control and resveratrol-treated mice were recorded during a 3-h period. (**b**) CO_2 production of control and resveratrol-treated mice was recorded during a 3-h period. (**c**) RER of control and resveratrol-treated mice was recorded during a 3-h period. (**d**) Average heat production of control and resveratrol-treated mice during a 3-h period. (**e**) O_2 consumption of iWAT of control and resveratrol-treated mice was measured as the decrease in dissolved oxygen (DO). $^{**}P < 0.01$ versus control.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Special thanks to Joseph Maricelli for his help in the measurement of basal metabolic rate. This work was supported by grants from National Institutes of Health (R01HD067449), the National Natural Science Foundation of China (31372397), the Muscular Dystrophy Association (216602) and the National Science Foundation (1147275). This activity was also funded, in part, with an Emerging Research Issues Internal Competitive Grant from the Agricultural Research Center at Washington State University, College of Agricultural, Human, and Natural Resource Sciences.

REFERENCES

1 Vazquez-Vela ME, Torres N, Tovar AR. White adipose tissue as endocrine organ and its role in obesity. *Arch Med Res* 2008; **39**: 715–728.
2 Coelho M, Oliveira T, Fernandes R. Biochemistry of adipose tissue: an endocrine organ. *Arch Med Sci* 2013; **9**: 191–200.
3 Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature* 2006; **444**: 875–880.
4 Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* 2008; **9**: 367–377.
5 Saito M. Brown adipose tissue as a regulator of energy expenditure and body fat in humans. *Diabetes Metab J* 2013; **37**: 22–29.
6 Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. *Nat Med* 2013; **19**: 1252–1263.
7 Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T *et al.* Functional brown adipose tissue in healthy adults. *N Engl J Med* 2009; **360**: 1518–1525.
8 Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH *et al.* Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* 2012; **150**: 366–376.
9 Beranger GE, Karbiener M, Barquissau V, Pisani DF, Scheideler M, Langin D *et al.* In vitro brown and "brite"/"beige" adipogenesis: human cellular models and molecular aspects. *Biochim Biophys Acta* 2013; **1831**: 905–914.
10 Bartelt A, Heeren J. Adipose tissue browning and metabolic health. *Nat Rev Endocrinol* 2014; **10**: 24–36.
11 Peschechera A, Eckel J. "Browning" of adipose tissue--regulation and therapeutic perspectives. *Arch Physiol Biochem* 2013; **119**: 151–160.
12 Park A, Kim WK, Bae KH. Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells. *World J Stem Cells* 2014; **6**: 33–42.
13 Lo KA, Sun L. Turning WAT into BAT: a review on regulators controlling the browning of white adipocytes. *Biosci Rep* 2013; **33**: 711–719.
14 Villarroya F, Vidal-Puig A. Beyond the sympathetic tone: the new brown fat activators. *Cell Metab* 2013; **17**: 638–643.
15 Bonet ML, Oliver P, Palou A. Pharmacological and nutritional agents promoting browning of white adipose tissue. *Biochim Biophys Acta* 2013; **1831**: 969–985.
16 Carpene C, Gomez-Zorita S, Deleruyelle S, Carpene MA. Novel strategies for preventing diabetes and obesity complications with natural polyphenols. *Curr Med Chem* 2014; **22**: 150–164.
17 Wang S, Moustaid-Moussa N, Chen L, Mo H, Shastri A, Su R *et al.* Novel insights of dietary polyphenols and obesity. *J Nutr Biochem* 2014; **25**: 1–18.
18 Szkudelska K, Szkudelski T. Resveratrol, obesity and diabetes. *Eur J Pharmacol* 2010; **635**: 1–8.
19 Lam YY, Peterson CM, Ravussin E. Resveratrol vs. calorie restriction: data from rodents to humans. *Exp Gerontol* 2013; **48**: 1018–1024.
20 Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F *et al.* Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 2006; **127**: 1109–1122.
21 Um JH, Park SJ, Kang H, Yang S, Foretz M, McBurney MW *et al.* AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol. *Diabetes* 2010; **59**: 554–563.
22 Timmers S, Konings E, Bilet L, Houtkooper Riekelt H, van de Weijer T, Goossens Gijs H *et al.* Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. *Cell Metab* 2011; **14**: 612–622.
23 Konings E, Timmers S, Boekschoten MV, Goossens GH, Jocken JW, Afman LA *et al.* The effects of 30 days resveratrol supplementation on adipose tissue morphology and gene expression patterns in obese men. *Int J Obesity (Lond)* 2014; **38**: 470–473.
24 Mitterberger MC, Zwerschke W. Mechanisms of resveratrol-induced inhibition of clonal expansion and terminal adipogenic differentiation in 3T3-L1 preadipocytes. *J Gerontol A Biol Sci Med Sci* 2013; **68**: 1356–1376.
25 Rayalam S, Yang JY, Ambati S, Della-Fera MA, Baile CA. Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. *Phytother Res* 2008; **22**: 1367–1371.
26 Zhang XH, Huang B, Choi SK, Seo JS. Anti-obesity effect of resveratrol-amplified grape skin extracts on 3T3-L1 adipocytes differentiation. *Nutr Res Pract* 2012; **6**: 286–293.
27 Lasa A, Schweiger M, Kotzbeck P, Churruca I, Simon E, Zechner R *et al.* Resveratrol regulates lipolysis via adipose triglyceride lipase. *J Nutr Biochem* 2012; **23**: 379–384.
28 Gomez-Zorita S, Treguer K, Mercader J, Carpene C. Resveratrol directly affects in vitro lipolysis and glucose transport in human fat cells. *J Physiol Biochem* 2013; **69**: 585–593.
29 Baile CA, Yang JY, Rayalam S, Hartzell DL, Lai CY, Andersen C *et al.* Effect of resveratrol on fat mobilization. *Ann NY Acad Sci* 2011; **1215**: 40–47.
30 Mercader J, Palou A, Bonet ML. Resveratrol enhances fatty acid oxidation capacity and reduces resistin and Retinol-Binding Protein 4 expression in white adipocytes. *J Nutr Biochem* 2011; **22**: 828–834.
31 Daval M, Diot-Dupuy F, Bazin R, Hainault I, Viollet B, Vaulont S *et al.* Anti-lipolytic action of AMP-activated protein kinase in rodent adipocytes. *J Biol Chem* 2005; **280**: 25250–25257.
32 Lihn AS, Jessen N, Pedersen SB, Lund S, Richelsen B. AICAR stimulates adiponectin and inhibits cytokines in adipose tissue. *Biochem Biophys Res Commun* 2004; **316**: 853–858.
33 Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T *et al.* Mammalian AMP-activated protein kinase subfamily. *J Biol Chem* 1996; **271**: 611–614.
34 Gaidhu MP, Fediuc S, Ceddia RB. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside-induced AMP-activated protein kinase phosphorylation inhibits basal and insulin-stimulated glucose uptake, lipid synthesis, and fatty acid oxidation in isolated rat adipocytes. *J Biol Chem* 2006; **281**: 25956–25964.
35 Gaidhu MP, Ceddia RB. The role of adenosine monophosphate kinase in remodeling white adipose tissue metabolism. *Exerc Sport Sci Rev* 2011; **39**: 102–108.
36 Bijland S, Mancini SJ, Salt IP. Role of AMP-activated protein kinase in adipose tissue metabolism and inflammation. *Clin Sci (Lond)* 2013; **124**: 491–507.
37 Daval M, Foufelle F, Ferré P. Functions of AMP-activated protein kinase in adipose tissue. *J Physiol* 2006; **574**: 55–62.
38 Duarte LC, Vaanholt LM, Sinclair RE, Gamo Y, Speakman JR. Limits to sustained energy intake XII: is the poor relation between resting metabolic rate and reproductive performance because resting metabolism is not a repeatable trait? *J Exp Biol* 2010; **213**: 278–287.
39 Speakman JR. Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. *Front Physiol* 2013; **4**: 34.
40 Aune UL, Ruiz L, Kajimura S. Isolation and differentiation of stromal vascular cells to beige/brite cells. *J Vis Exp* 2013; **28**: 50191.
41 Wang S, Zhou G, Shu G, Wang L, Zhu X, Gao P *et al.* Glucose utilization, lipid metabolism and bmp-smad signaling pathway of porcine intramuscular preadipocytes compared with subcutaneous preadipocytes. *Cell Physiol Biochem* 2013; **31**: 981–996.
42 Xue R, Wan Y, Zhang S, Zhang Q, Ye H, Li Y. Role of bone morphogenetic protein 4 in the differentiation of brown fat-like adipocytes. *Am J Physiol Endocrinol Metab* 2014; **306**: E363–E372.
43 Fu X, Zhao JX, Zhu MJ, Foretz M, Viollet B, Dodson MV *et al.* AMP-activated protein kinase alpha1 but not alpha2 catalytic subunit potentiates myogenin expression and myogenesis. *Mol Cell Biol* 2013; **33**: 4517–4525.
44 Diepart C, Verrax J, Calderon PB, Feron O, Jordan BF, Gallez B. Comparison of methods for measuring oxygen consumption in tumor cells in vitro. *Anal Biochem* 2010; **396**: 250–256.
45 Zhao G, Zhang X, Xu X, Wolin MS, Hintze TH. Depressed modulation of oxygen consumption by endogenous nitric oxide in cardiac muscle from diabetic dogs. *Am J Physiol Heart Circ Physiol* 2000; **279**: H520–H527.
46 Yang QY, Liang JF, Rogers CJ, Zhao JX, Zhu MJ, Du M. Maternal obesity induces epigenetic modifications to facilitate zfp423 expression and enhance adipogenic differentiation in fetal mice. *Diabetes* 2013; **62**: 3727–3735.
47 Cardiff RD, Miller CH, Munn RJ. Manual hematoxylin and eosin staining of mouse tissue sections. *Cold Spring Harb Protoc* 2014; **2014**: 655–658.
48 Chi V, Chandy KG. Immunohistochemistry: paraffin sections using the Vectastain ABC kit from vector labs. *J Vis Exp* 2007; **8**: e308.
49 Higashida K, Kim SH, Jung SR, Asaka M, Holloszy JO, Han DH. Effects of resveratrol and SIRT1 on PGC-1alpha activity and mitochondrial biogenesis: a reevaluation. *PLoS Biol* 2013; **11**: e1001603.
50 Mader I, Wabitsch M, Debatin KM, Fischer-Posovszky P, Fulda S. Identification of a novel proapoptotic function of resveratrol in fat cells: SIRT1-independent sensitization to TRAIL-induced apoptosis. *FASEB J* 2010; **24**: 1997–2009.
51 Fischer-Posovszky P, Kukulus V, Tews D, Unterkircher T, Debatin KM, Fulda S *et al.* Resveratrol regulates human adipocyte number and function in a Sirt1-dependent manner. *Am J Clin Nutr* 2010; **92**: 5–15.

52 Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A *et al.* Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006; **444**: 337–342.
53 Alberdi G, Rodriguez VM, Miranda J, Macarulla MT, Churruca I, Portillo MP. Thermogenesis is involved in the body-fat lowering effects of resveratrol in rats. *Food Chem* 2013; **141**: 1530–1535.
54 Andrade JM, Frade AC, Guimaraes JB, Freitas KM, Lopes MT, Guimaraes AL *et al.* Resveratrol increases brown adipose tissue thermogenesis markers by increasing SIRT1 and energy expenditure and decreasing fat accumulation in adipose tissue of mice fed a standard diet. *Eur J Nutr* 2014; **53**: 1503–1510.
55 Kajimura S, Seale P, Kubota K, Lunsford E, Frangioni JV, Gygi SP *et al.* Initiation of myoblast to brown fat switch by a PRDM16-C/EBP-beta transcriptional complex. *Nature* 2009; **460**: 1154–1158.
56 Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S *et al.* PRDM16 controls a brown fat/skeletal muscle switch. *Nature* 2008; **454**: 961–967.
57 Ramos-Jimenez A, Hernandez-Torres RP, Torres-Duran PV, Romero-Gonzalez J, Mascher D, Posadas-Romero C *et al.* The respiratory exchange ratio is associated with fitness indicators both in trained and untrained men: a possible application for people with reduced exercise tolerance. *Clin Med Circ Respirat Pulm Med* 2008; **2**: 1–9.
58 Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ *et al.* SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. *Cell Metab* 2012; **15**: 675–690.
59 Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC *et al.* AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* 2009; **458**: 1056–1060.