Coumestrol Induces Mitochondrial Biogenesis by Activating Sirt1 in Cultured Skeletal Muscle Cells

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ABSTRACT: The mitochondrion is a central organelle in cellular energy homeostasis; thus, reduced mitochondrial activity has been associated with aging and metabolic disorders. This paper provides biological evidence that coumestrol, which is a natural isoflavone, activates mitochondrial biogenesis. In cultured myocytes, coumestrol activated the silent information regulator two ortholog 1 (Sirt1) through the elevation of the intracellular NAD+/NADH ratio. Coumestrol also increased the mitochondrial contents and induced the expression of key proteins in the mitochondrial electron transfer chain in cultured myocytes. A Sirt1 inhibitor and Sirt1-targeting siRNAs abolished the effect of coumestrol on mitochondrial biogenesis. Similar to an increase in mitochondrial content, coumestrol improved myocyte function with increased ATP concentration. Taken together, the data suggest that coumestrol is a novel inducer of mitochondrial biogenesis through the activation of Sirt1.

KEYWORDS: coumestrol, mitochondrial biogenesis, Sirt1, myocyte

INTRODUCTION

The mitochondrion produces most of the cellular ATP via aerobic respiration and regulates various cellular processes, including signaling pathways, growth, differentiation, cell cycle, and apoptosis.1 Because the maintenance of mitochondrial functions is critical for maintaining energy homeostasis, mitochondrial biogenesis, which maintains cellular mitochondrial content, has been considered a critical biomarker to assess cellular longevity and functionality. Mitochondrial biogenesis is regulated by multiple factors, one of which is a nuclear-encoded transcription factor, nuclear respiratory factor 1 (NRF1), that induces the expression of transcription factor A mitochondria (Tfam) and other mitochondria-related genes in nucleus DNA.2,3 Tfam is a key mitochondrial transcription factor, which is responsible for the regulation of mitochondrial DNA (mtDNA)-encoded genes and the replication of mtDNA. Therefore, mitochondrial biogenesis is regarded as a complex process that is coordinated by nuclear (by NRF1) and mitochondrial (by tfam) genes.1

Although the molecular mechanism of mitochondrial biogenesis is not fully understood, it has been reported that peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC1α) has been reported as a positive regulator that directly interacts with NRF1 to form a transcription complex.1 PGC1α forms a transcription complex with NRF1 to increase the transcriptional activity of NRF1. In addition, PGC1α also regulates glucose and lipid homeostasis by interaction with various transcription factors, such as peroxisome proliferator-activated receptor γ, retinoid X receptor, and cAMP response element-binding protein.1,4–6

Silent information regulator two ortholog 1 (Sirt1), which is an NAD+-dependent histone deacetylase, is a mammalian homologue of the silent mating-type information regulation 2 (Sir2) gene inSaccharomyces cerevisiae, which has been studied as a “longevity gene”; thus, Sir2 overexpression results in a lifespan extension in small-animal models, such as budding yeasts, nematodes, and fruit flies.7–9 Calorie restriction has been known to exhibit a positive effect on lifespan extension,10 whereas the deletion of Sir2 diminished the effect on lifespan extension.11 These results imply that Sir2 activity mediates the effect of calorie restriction on longevity. Therefore, Sir2 is regarded as a central molecule in the network of energy metabolism and lifespan. In mammals, Sirt1 has been reported to regulate energy metabolism and response to calorie restriction.12 For these reasons, small molecules that target Sirt1 are being developed to overcome metabolic disorders, including obesity, insulin resistance, and type 2 diabetes.

In addition to the roles in longevity, Sirt1 has been proposed to be one of the most important positive regulators for regulating cellular mitochondrial number.13 Upon activation, Sirt1 facilitates the deacetylation and activation of PGC1α.13 As above-mentioned, activated PGC1α can trigger mitochondrial biogenesis through interaction with NRF1. Thus, it is also feasible that mitochondrial biogenesis can be achieved by the activation of Sirt1.

Coumestrol, which is a daidzein-derived coumestan, is a natural organic compound that is found in some plants, including alfalfa, legumes, Brussels sprouts, spinach, clover, and soybeans.14 Various biological activities of coumestrol have been reported, including antioxidant activity,15 the inhibition of osteoclast differentiation,16 the inhibition of adipocyte differentiation, and reduced lipid accumulation.17 As a phytoestrogen, coumestrol also exerts an estrogenic effect, with strong binding affinities for estrogen receptors α and β.14,18 and the positive effects of coumestrol on bone metabolism and uterine...
growth are well-studied in ovariectomized animals.\textsuperscript{19,20} Finally, coumestrol attenuated tumorigenesis\textsuperscript{21,22} However, the molecular target and mechanism of action of coumestrol have not been clearly understood.

Recently, it has been proposed that flavonoids and their derivatives induce mitochondrial biogenesis through the activation or expression of Sirt1 in proximal renal tubular cells.\textsuperscript{23} Therefore, here we investigated whether coumestrol has activation or expression of Sirt1 in proximal renal tubular derivatives induce mitochondrial biogenesis through the activity of coumestrol with resveratrol, which is a compound that is known as a Sirt1 activator. Sirt1 activity was determined, and coumestrol-induced mitochondrial biogenesis was assessed by mitochondrial content and by the expression of mitochondria-related genes in differentiated myocytes. The effect of Sirt1 activation on the coumestrol-mediated induction of mitochondrial biogenesis was confirmed by utilizing a Sirt1 inhibitor and Sirt1-specific siRNAs. The effect of coumestrol-mediated mitochondrial biogenesis was confirmed by the measurement of cellular ATP concentrations.

\section*{Materials and Methods}

\textbf{Reagents.} Coumestrol and nicotinamide were purchased from Sigma (St. Louis, MO, USA). The antibodies for NADH dehydrogenase subunit 1 (SDHA), ubiquinol cytochrome c reductase core protein 2 (UQRC2C), cytochrome oxidase subunit 1 (COX1), ATP synthase mitochondrial F1 complex subunit 1 (ATP5a), PGC1, and NRF1, as well as scrambled and Sirt1 siRNAs, were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The \(\beta\)-actin antibody was purchased from Cell Signaling Technology (Danvers, MA, USA), and horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, and anti-goat secondary antibodies were purchased from Bio-Rad (Hercules, CA, USA).

Cell Culture. C2C12 mouse myocytes were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco’s modified Eagle medium (DMEM, Lonza), which was supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA). All media were supplemented with 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma), and C2C12 cells were grown at 37°C in a humidified atmosphere, which contained 5% CO\(_2\). For differentiation, confluent C2C12 cells were maintained with DMEM, which was supplemented with 2% horse serum (HyClone) for 4 days. During differentiation, the medium was changed daily.

\textbf{Sirt1 Activity Assay.} Sirt1 activity was examined using a SIRT1 Fluorimetric Drug Discovery Kit (Enzo Life Sciences, Farmingdale, NY, USA) with minor modifications, as described previously.\textsuperscript{24} Briefly, total protein (25 \(\mu\)g) was dissolved in the assay buffer and incubated in the presence of NAD\(^+\) (100 \(\mu\)M) at 37°C for 30 min. The acetylated substrate (Fluor-de-Lys-Sirt1 substrate) was then added, and the mixture was incubated at 37°C for 45 min. After incubation, deacetylated substrates were detected on a TECAN M200 PRO fluorometric plate reader (Tecan, Männedorf, Switzerland).

\textbf{Measurement of Cellular NAD\(^+\)/NADH Ratio.} The NAD\(^+\)/NADH ratio was determined using an NAD/NADH assay kit (Biovision, Milpitas, CA, USA), following the manufacturer’s protocol. Briefly, cells were lysed with extraction buffer (400 \(\mu\)L) by the freeze-thaw method. Extracts were vortexed for 10 s and centrifuged at 18000 rcf for 5 min. The supernatants were then equally divided into two microcentrifuge tubes. Half of the samples were incubated at 60°C for 30 min to disrupt NAD\(^+\) and remain NADH only. Unheated samples (NAD\(\overline{D}\); NAD\(^+\) plus NADH) and heated (NAD\(^{D}\) only) samples were incubated with a NAD cycling enzyme, which converts NAD\(^+\) to NADH at room temperature for 5 min. Then, the mixtures were mixed with the NADH developing agent and incubated at room temperature for 1 h. NADH levels were determined by measuring the absorbance at 450 nm using a TECAN M200 PRO fluorometric plate reader. NAD\(^+\) levels were calculated by subtracting NADH from NADH.

\textbf{Measurement of Mitochondrial Content.} Mitochondrial content was measured using the MitoTracker Green FM (Invitrogen, Carlsbad, CA, USA) reagent. Briefly, cells were rinsed with PBS and incubated with MitoTracker Green FM (50 nmol) for 30 min. Fluorescence was measured using a TECAN M200 PRO fluorometric plate reader to determine the density of mitochondria.

\textbf{Western Blotting.} An equal amount (60 \(\mu\)g) of protein per sample was separated on NaPAGe Novex Tris–acetate gels (Invitrogen) and transferred to membranes using an iBlot dry transfer device (Invitrogen). Membranes were blocked with 5% skim milk (Sigma) in Tris-buffered saline (Bio-Rad), which was supplemented with 0.1% Tween-20 (Sigma) (TBST) at room temperature for 30 min. After blocking, the membranes were incubated with primary antibodies, which were dissolved in TBST/5% bovine serum albumin (BSA, Sigma) with gentle shaking at 4°C overnight. After three washings with TBST, the blots were hybridized with HRP-conjugated secondary antibodies in TBST/5% BSA at room temperature for 2 h and washed three times with TBST. After washing, the membranes were incubated with enhanced chemiluminescence reagents (Santa Cruz Biotechnology) and analyzed with an LAS-3000 imaging system (Fujifilm, Tokyo, Japan). Relative expression levels of proteins were measured using Multi gauge software (Fujifilm).

\textbf{siRNA Transfection.} For the knockdown of Sirt1, differentiated C2C12 myocytes were transfected with scrambled or mouse Sirt1 siRNA using the Lipofectamine 2000 reagent (Invitrogen). After transfection, cells were incubated in Opti-MEM (Invitrogen) medium for 4 h. After transfection, the medium was replaced with fresh DMEM, which contained 10% FBS.

\textbf{Measurement of ATP.} ATP content was determined using an ATP determination kit (Invitrogen). Briefly, extracts were mixed with a reaction solution, which contained firefly luciferase and \(\beta\)-luciferin. ATP content was measured using a Wallac 1420 Victor\(\textsuperscript{\textregistered}\) multilabel counter microplate reader (PerkinElmer, Waltham, MA, USA) and normalized with the protein concentration of each sample.

\textbf{Glucose Uptake Assay.} Glucose uptake was assessed by using a glucose uptake cell-based assay kit (Cayman Chemical Co., Ann Arbor, MI, USA), following the manufacturer’s protocol with minor modifications. Briefly, cells in a 96-well black, clear-bottom plate were washed with PBS three times and the medium was changed into glucose-free DMEM (Lonza) containing 150 \(\mu\)g/mL 2-NBDG (fluorescent-labeled deoxyglucose analogue). Cells were incubated for a further 1 h, and the plate was centrifuged at 400 rcf for 5 min. After centrifugation, supernatant was discarded and cell-based assay buffer (200 \(\mu\)L) was added to each well. The plate was centrifuged at 400 rcf for 5 min, the supernatant was discarded, and cell-based assay buffer (100 \(\mu\)L) was added one more time. The amount of 2-NBDG taken up by the cells was immediately measured at the wavelength of 485/535 nm (excitation/emission) using a TECAN M200 PRO fluorometric plate reader.

\textbf{Statistical Analyses.} The results are representative of the data from three or more independent experiments. All data points represent the average of triplicate samples. Error bars represent the standard deviation (SD). The \(p\) values, which were calculated from a one-way ANOVA followed by Dunnett’s procedure, that were <0.01 were interpreted as statistically significant.

\section*{Results}

\textbf{Coumestrol Activates Sirt1.} Coumestrol significantly and dose-dependently increased Sirt1 activity in differentiated C2C12 myocytes (Figure 1A). The effect of coumestrol (CMS; 1 and 10 \(\mu\)M) on Sirt1 activity was comparable with that of resveratrol (Res; 50 \(\mu\)M), which indicated that coumestrol is a potent inducer of Sirt1 activity. Sirt1 activity is directly regulated by the intracellular NAD\(^+\)/NADH ratio; thus, we measured the intracellular NAD\(^+\)/NADH ratio in cells that were stimulated with coumestrol. Corresponding with Sirt1 activity, coumestrol caused a dose-dependent induction of the intracellular NAD\(^+\)/NADH ratio, which demonstrated that
Coumestrol activates Sirt1, at least in part, through the elevation of the intracellular NAD+/NADH ratio (Figure 1B).

**Figure 1.** Coumestrol activates Sirt1. (A) Sirt1 activity assay. Differentiated C2C12 cells were treated with DMSO, resveratrol (Res; 50 μM), nicotinamide (NAM; 1 mM), or coumestrol (CMS; 1 or 10 μM, respectively) for 24 h, and Sirt1 activity assay was assessed as described under Materials and Methods. Relative Sirt1 activity is shown as a bar graph. AFU, arbitrary fluorescence unit. (B) Intracellular NAD+/NADH ratio. C2C12 myocytes were treated with Res (25 μM) or CMS (0.1, 1, 5, and 10 μM, respectively) for 12 h. Cells were rinsed with PBS, and intracellular NAD+/NADH ratio was measured. Values that do not share the same letter are significantly different \((p < 0.01)\).

### Coumestrol Induces Mitochondrial Biogenesis

We demonstrated that coumestrol activates Sirt1 (Figure 1), which induces mitochondrial biogenesis.\(^{13}\) To determine whether coumestrol promotes mitochondrial biogenesis similar to other Sirt1 activators,\(^{25,26}\) we quantified the mitochondrial density in differentiated C2C12 myocytes that were treated with coumestrol. Coumestrol increased the mitochondrial content in a dose-dependent manner (Figure 2A). Moreover, the protein expression of mitochondrial electron transfer chain (ETC) components, such as NDUFA9, SDHA, UQCRC2, COX1, and ATP5a, and transcriptional regulators that are responsible for mitochondrial biogenesis, such as PGC1 and NRF1, were augmented with coumestrol stimulation (Figure 2B,C). Corresponding with protein expression, the mRNA expression of mitochondria-related genes was also up-regulated by coumestrol (Supporting Information Figure S1), which suggested that coumestrol can induce mitochondrial biogenesis through the transcriptional regulation of the expression of mitochondrial biogenesis-related genes.

**Figure 2.** Coumestrol induces mitochondrial biogenesis. C2C12 myotubes were treated with resveratrol (Res; 25 μM) or coumestrol (CMS; 0.1, 1, 5, and 10 μM, respectively) for 24 h. (A) Mitochondrial density was measured using the Mitotracker Green FM reagent. Relative mitochondrial density is shown as a bar graph. (B) After rinsing with PBS, cells were harvested, and proteins were extracted and blotted with antibodies against specific proteins (NDUFA9, SDHA, UQCRC2, COX1, ATP5a, PGC1, and NRF1). β-Actin was used as a loading control. (C) Protein expression level of each protein shown in (B). Each number under the bar graph indicates the lane number in (B). Values that do not share the same letter are significantly different \((p < 0.01)\).
3B). These data suggest that Sirt1 mediates the mitochondriogenic effect of coumestrol in myocytes.

**Knockdown of Sirt1 Lessens the Effect of Coumestrol on Mitochondrial Biogenesis.** The nicotinamide treatment indicated that the inhibition of Sirt1 activity abolishes the effect of coumestrol on mitochondrial biogenesis (Figure 3). To confirm the Sirt1-dependent effect of coumestrol, the expression of Sirt1 was knocked down with Sirt1-specific siRNAs (Supporting Information Figure S2). Similar to the nicotinamide pretreatment, knockdown of the Sirt1 expression also abolished the effect of coumestrol on mitochondrial biogenesis (Figure 4A) and mitochondrial ETC protein

**Figure 3.** Nicotinamide prevents Sirt1-mediated mitochondrial biogenesis. C2C12 cells were pretreated with DMSO (control) or nicotinamide (NAM; 1 mM) for 2 h. Then, cells were treated with resveratrol (Res; 25 μM) or coumestrol (CMS; 10 μM) for an additional 24 h. (A) After incubation, mitochondrial density was determined. (B) After washing with PBS, cells were harvested, and proteins were blotted with specific antibodies. β-Actin was used as a loading control. (C) Densitometry of Western blots shown in (B). Values that do not share the same letter are significantly different (p < 0.01).

**Figure 4.** Sirt1 siRNA blunts the effect of coumestrol on mitochondrial biogenesis. C2C12 cells were transfected with scrambled or Sirt1 siRNA. After transfection, cells were treated with resveratrol (Res; 25 μM) or coumestrol (CMS; 10 μM) for 24 h. (A) Mitochondrial density was determined. (B) Proteins were blotted with antibodies that targeted mitochondrial proteins and their regulators. β-Actin was used as a loading control. The expression of each protein was normalized and shown as bar graphs (C). Values that do not share the same letter are significantly different (p < 0.01).
expression (Figure 4B). Similar results were obtained with resveratrol. Taken together, these results demonstrate that both resveratrol and coumestrol induce mitochondrial biogenesis in a Sirt1-dependent manner.

**Coumestrol Increases ATP Content in a Sirt1-Dependent Manner and Augments Glucose Uptake.** To confirm whether coumestrol contributes to ATP production through the induction of mitochondrial content, we measured cellular ATP concentrations in cells that were stimulated with coumestrol. In agreement with increased mitochondrial content (Figure 2), coumestrol induced cellular ATP concentrations in C2C12 myocytes (Figure 5A); however, the blockade of Sirt1 activity with nicotinamide prevented coumestrol-mediated ATP production (Figure 5B). Collectively, these data indicate that coumestrol induces mitochondrial biogenesis and, consequently, increases cellular ATP level through the activation of Sirt1.

Glucose is the primary energy source for ATP production. Increased ATP content in coumestrol-treated myocytes (Figure 5) indicates that coumestrol might be associated with glucose uptake. To elucidate whether coumestrol enhances glucose uptake in skeletal muscle, we assessed the glucose uptake assay. As shown in Figure 6, coumestrol slightly but significantly increased glucose uptake, compared to control. These data suggest that coumestrol is able to enhance energy metabolism.

**DISCUSSION**

As “power plants” of cells and regulators of diverse cellular activities, mitochondria play an important role in cellular energy balance and survival. Not surprisingly, mitochondrial dysfunction is associated with several cellular dysfunctions and diseases, including metabolic disorders. For example, mitochondrial dysfunction has been reported to be closely associated with β-cell failure. Additionally, impaired mitochondrial bioenergetic capacity is observed in the skeletal muscle of type 2 diabetes patients. In fat and muscle tissues from obese rodents, mitochondrial density, mitochondrial function, and ATP content are down-regulated. Accordingly, it is likely that mitochondrial dysfunction can be observed in various organs or in tissues of metabolic syndromes. Therefore, maintaining mitochondrial function by inducing mitochondrial biogenesis is required to sustain a healthy life.

Here, we demonstrated that coumestrol is able to induce Sirt1 activity and mitochondrial biogenesis. By promoting mitochondrial content, coumestrol increased cellular ATP levels and glucose uptake, implying that coumestrol could improve energy metabolism. Interestingly, the expression and activity of Sirt1 gradually decline with aging, and mitochondrial content and function are also age-dependently decreased. Considering the mitochongriogenic effect of Sirt1, it is feasible that decreased Sirt1 activity could be a causal factor of blunted mitochondrial biogenesis, which thereby diminishes mitochondrial density and causes aging-related dysfunctions in aged cells. As for other Sirt1 activators, we demonstrated that coumestrol also increases mitochondrial content through the activation of Sirt1 in muscle cells. Therefore, it is feasible that coumestrol has the potential to attenuate aging-related mitochondrial dysfunction.

The benefit of Sirt1 activation is not limited to mitochondrial rejuvenation. For example, Sirt1 modulates glucose homeostasis by regulating PGC1α and forkhead box O1 activity in the liver. The loss of function of Sirt1 in the pancreas is associated with decreased insulin secretion, and Sirt1 is activated during glucose-stimulated insulin secretion. These data imply that Sirt1 may be involved in the regulation of glucose homeostasis. Further, Sirt1 is also able to control lipid metabolism. In fact, Sirt1 deacetylases and deactivates sterol regulatory element binding protein 1c (SREBP1c), which is a master transcriptional regulator of lipogenesis-related genes. Conversely, Sirt1 enhances lipid catabolism through the activation of AMP-activated protein kinase (AMPK), which is a master regulator of energy metabolism. Additionally, Sirt1 is implicated in the expression and secretion of adiponectin.

Figure 6. Coumestrol augments glucose uptake in differentiated myocytes. Differentiated C2C12 myocytes were treated with DMSO (for control), resveratrol (Res; 50 μM), or coumestrol (CMS; 0.1, 1, 5, and 10 μM, respectively) for 24 h. Cellular glucose uptake was measured as described under Materials and Methods and shown as a bar graph. Values that do not share the same letter are significantly different (p < 0.01).
SREBP1c and fatty acid synthase) in adipocytes and hepatocytes, whereas coumestrol increased fatty acid oxidation-related genes in myocytes (e.g., acyl-CoA oxidase and carnitine-palmitoyl transferase 1) (Supporting Information Figure S3), indicating that coumestrol could also modulate lipid metabolism to ameliorate metabolic disorders.

Although Sirt1 is beneficial to the regulation of energy metabolism, the effect of Sirt1 in cancer development is controversial. The ectopic expression of Sirt1 attenuated colon cancer progression in mice,44 and an allelic loss or a mutation of Sirt1 is present in various tumor cells.45 In contrast, Sirt1 mediates the deacetylation of metabolism and on the correlation between AMPK and Sirt1.46 Further, a specific Sirt1 activator has been reported to promote tumor cell migration and invasion in mice.45 As an oncogene, Sirt1 activation could lead to tumorigenesis. However, coumestrol displayed anti-cancer activities by suppressing some tumor development with the modulation of estrogen receptor β and casine kinase II activities.41,22 Thus, the collective effects of coumestrol appear beneficial without affecting cancer risk. The molecular mechanism of the coumestrol-mediated inhibition of tumor progression requires further study.

As regulators of energy metabolism and mitochondrial biogenesis, it has been proposed that Sirt1 and AMPK activate each other. AMPK has been reported to increase NAD+ to activate Sirt1.46 In contrast, Sirt1 mediates the deacetylation of liver kinase B1,47 which is an upstream regulator of AMPK. On the basis of these results, it is likely that Sirt1 and AMPK might assemble a positive feedback loop to enhance energy catabolism. According to the previous data, it might be possible that coumestrol could activate AMPK, which is a regulator of Sirt1. Although it remains unclear which one, Sirt1 or AMPK, is the upstream regulator of the other, we observed that coumestrol increased the phosphorylation level of the AMPKα catalytic subunit (Supporting Information Figure S4). Similar to Sirt1, AMPK is also reported to regulate mitochondrial biogenesis and catabolism.38 On the basis of these data, it is likely that AMPK activation may boost the favorable effect of coumestrol on mitochondrial biogenesis and energy metabolism. Further studies, including in vivo experiments, will reveal the effect of coumestrol on energy metabolism and on the correlation between AMPK and Sirt1.

In conclusion, we demonstrated that coumestrol is an effective inducer of mitochondrial biogenesis. Coumestrol caused a small elevation in the intracellular NAD+/NADH ratio, but a major induction of Sirt1 activation, which suggests that other factors in addition to NAD+/NADH ratios are involved in this effect. The coumestrol-mediated mitochondrial biogenesis was abolished by the inhibition of Sirt1 activity. Increased mitochondrial content induced by coumestrol caused an elevation of cellular ATP levels and an increase of glucose uptake. Collectively, our data suggest that coumestrol is an effective Sirt1 activator and enhancer of mitochondrial number and function.

## ASSOCIATED CONTENT

### Supporting Information

Figures S1–S4. Figure S1. mRNA expression of mitochondria-related genes upon coumestrol treatment. C2C12 cells were treated with Res (50 μM) or CMS (0.1, 1, and 10 μM, respectively) for 24 h. After incubation, cells were rinsed with PBS. RNAs were isolated using the TRizol reagent (Invitrogen), and cDNAs were synthesized using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). The relative mRNA level of mitochondria-related genes was measured by qPCR (Rotor-Gene 3000, Corbett Research) and normalized to GAPDH. Values that do not share the same letter are significantly different (p < 0.01). Figure S2. Reduced Sirt1 mRNA expression by siRNA transfection. C2C12 myocytes were transfected with scrambled or Sirt1 siRNA, as described in Figure 4. After transfection, cells were rinsed with PBS. RNAs were isolated, and cDNAs were synthesized using the TRizol reagent and a RevertAid First Strand cDNA Synthesis Kit as described in Figure S1. The relative mRNA expression of Sirt1 was determined by qPCR and normalized to GAPDH. Values that do not share the same letter are significantly different (p < 0.01). Figure S3. Coumestrol represses lipogenic gene expression and enhances fatty acid oxidation-related genes. For adipocyte differentiation, confluent 3T3-L1 cells were incubated with DMEM supplemented with 10% FBS, 0.39 μg/mL dexamethasone, 115 μg/mL isobutylmethylxanthine, and 10 μg/mL insulin (DMI) for 48 h. After DMI treatment, the medium was changed into DMEM supplemented with 10% FBS and 10 μg/mL of insulin every 2 days for 2 weeks to complete adipocyte differentiation. HepG2 human hepatoma cells were grown in DMEM supplemented with 10% FBS. All cells were grown at 37 °C in a humidified atmosphere, which contained 5% CO2, and all media were supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. (A) Differentiated 3T3-L1 adipocytes, (B) HepG2 hepatoma cells, and (C) differentiated C2C12 myocytes were treated with Res (50 μM) or CMS (0.1, 1, and 10 μM, respectively) for 24 h. After incubation, cells were rinsed with PBS. RNAs were isolated and cDNAs were synthesized using the TRizol reagent and a RevertAid First Strand cDNA Synthesis Kit, as described in Figure S1. The relative mRNA expression of indicated genes was determined by qPCR and normalized to GAPDH. Values that do not share the same letter are significantly different (p < 0.01). Figure S4. Coumestrol activates AMPK and increases glucose uptake. (A) C2C12 myotubes were treated with Res (50 μM) or CMS (0.1, 1, and 10 μM, respectively) for 2 h. After incubation, cells were rinsed with PBS, and proteins were extracted. Proteins were blotted with antibodies that target AMPK and p-AMPK. β-Actin was used as a loading control. (B) p-AMPK/AMPK ratio is shown as a bar graph. (C) Differentiated C2C12 myocytes were starved with low-glucose DMEM supplemented with 0.1% BSA overnight. After starvation, cells were treated with DMSO (for control), Res (50 μM), or CMS (0.1, 1, and 10 μM, respectively) for 2 h. Cellular glucose uptake was measured as described under Materials and Methods and shown as a bar graph. Values that do not share the same letter are significantly different (p < 0.01). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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■ ABBREVIATIONS USED
Sirt1, silent information regulator two ortholog 1; NRF1, nuclear respiratory factor 1; Tfam, transcription factor A mitochondria; mtDNA, mitochondrial DNA; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; Sir2, silent mating-type information regulation 2; NDUF9A9, NADH dehydrogenase 1α subcomplex 9; SDHA, succinate dehydrogenase complex subunit A; UQCRCC2, ubiquinol cytochrome c reductase core protein 2; COX1, cytochrome oxidase subunit 1; ATP5a, ATP synthase mitochondria F1 complex α subunit 1; HRP, horseradish peroxidase; DMEM, Dulbecco’s modified Eagle medium; FBS, fetal bovine serum; ETC, electron transfer chain; SREBP1c, sterol regulatory element binding protein 1c; AMPK, AMP-activated protein kinase

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