ORIGINAL

Bergenin promotes mitochondrial biogenesis via the AMPK/SIRT1 axis in hepatocytes

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Abstract : Aging and obesity trigger liver mitochondrial decline, impairing liver function and energy metabolism. Effective hepatic mitochondrial biogenesis helps maintain and restore hepatocyte function. The effects of bergenin, a polyphenol with various pharmacological effects, on hepatic mitochondrial biogenesis remain unclear. Therefore, we aimed to determine its effects on mitochondrial biogenesis in hepatocytes. We measured mitochondrial content in human HepG2 hepatocytes using MitoTracker Green FM ; intracellular ATP content using an ATP assay kit ; and mitochondrial DNA (mtDNA) using the ratio of mtDNA to nuclear DNA by qPCR. Protein levels were analyzed using immunoblotting. Nuclear translocation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) was assessed by immunofluorescence staining and immunoblotting. In human HepG2 hepatocytes, bergenin increased mitochondrial content, elevated mitochondrial DNA and constituent proteins, and enhanced intracellular ATP levels and PGC-1α nuclear translocation, possibly promoting mitochondrial biosynthesis. SIRT1 expression was induced in bergenin-treated cells and may be responsible for bergenin-inducible mitochondrial biogenesis, which was abolished by the SIRT1 inhibitor EX-527. Furthermore, bergenin activated AMP-activated protein kinase (AMPK). Compound C, an AMPK inhibitor, abolished bergenin-induced SIRT1 expression and mitochondrial biogenesis. Overall, bergenin activates hepatic mitochondrial biogenesis through the AMPK/SIRT1 axis, which could help to prevent and ameliorate serious aging- and obesity-related liver diseases. J. Med. Invest. 72 : 66-75, February, 2025

Keywords **:** *mitochondria, bergenin, biogenesis, hepatocytes*

INTRODUCTION

Mitochondria are organelles that play an important role in producing more than 80% of intracellular ATP through oxidative phosphorylation and are particularly abundant in cells derived from tissues and organs that control motor and metabolic functions, such as the heart, liver, and skeletal muscle (1). In addition to ATP production, mitochondria are responsible for various important functions, such as regulating apoptosis and intracellular Ca^{2+} concentration. Mitochondrial quality control is maintained by the regulatory mechanisms of mitochondrial biogenesis and clearance (2).

Mitochondrial biogenesis proceeds not through de novo generation but through the growth and division of pre-existing mitochondria in a multistep process involving fusion and fission events. Mitochondria have a circular genome, mitochondrial DNA (mtDNA), which encodes several proteins, including all the essential subunits of the electron transport system, while the remaining mitochondrial proteins are encoded by nuclear DNA (nDNA) located in the nucleus (1). Therefore, coordinated expression of mtDNA and nDNA genes is required for mitochondrial biogenesis and is considered integratively regulated by the transcription coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (3). PGC-1α is activated and translocated to the nucleus via deacetylation by the NAD^+-dependent deacetylase sirtuin 1 (SIRT1) and activates the expression of mitochondria-associated proteins through nuclear respiratory factor 1 (NRF-1) (4). Furthermore, NRF-1 and PGC-1α couple to induce the expression of mitochondrial transcription factor A, which promotes mtDNA protection and transcription (5). AMP-activated protein kinase (AMPK) is a nutrient and energy sensor that maintains cellular energy homeostasis and regulates mitochondrial biogenesis (4). In gain-of-function studies using mice overexpressing a mutant form of the AMPK γ3 subunit, mitochondrial biogenesis was increased in skeletal muscle (6).

Numerous reports have suggested that mitochondrial dysfunction is involved in major phenotypes associated with aging (4). Increased mtDNA mutations and a decline in respiratory chain capacity have been observed in aged human tissues and organs (7). In addition, there is evidence that the activity of several key regulators responsible for mitochondrial function and number declines with aging, further impairing mitochondrial function. Sahin *et al*. generated mice genetically engineered to gradually lose telomeres and showed a link between the nuclear and mitochondrial aging processes (8). In these mice, mitochondrial dysfunction was also observed in addition to aging features due to p53-mediated processes in the nucleus. They also showed that the mitochondrial dysfunction was due to decreased activity of PGC-1α and PGC-1β. Thus, evidence suggests that a decline in PGC-1 activity contributes to mitochondrial aging in quiescent organs, such as the heart, brain, and liver.

Research has shown that obesity can lead to mitochondrial dysfunction (9). A decline in respiratory chain function has been

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observed in obesity, along with increased oxidative stress and apoptosis. It is suggested that the decline in the content and biogenesis of mitochondria is associated with impaired mitochondrial function in obesity (9). A significant decrease in the content and biosynthesis of mitochondria has been observed in obese humans (10). Holmström *et al*. showed that mitochondrial respiratory capacity and protein expression of PGC-1α decreased in the livers of obese diabetic mice (11). Another report showed that overexpression of PGC-1α increased mitochondrial content and function and decreased triglyceride accumulation and secretion in liver cells (12). Thus, mitochondrial biosynthesis is regarded as a therapeutic or preventive target leading to the restoration of mitochondrial function and the amelioration of symptoms of obesity (13).

In our preliminary experiment with 47 food ingredients, we found that bergenin could potentially be the most potent accelerator of mitochondrial biogenesis (Supplementary Fig. 1). Bergenin is a *C*-glucoside of 4-*O*-methyl gallic acid that is not hydrolyzed by glucosidases and has potent anti-inflammatory and antioxidant activities (Fig. 1). Bergenin has many known pharmacological properties, including antihepatotoxic, antiulcerogenic, antiviral, antifungal, hepatoprotective, antiarrhythmic, neuroprotective, anti-inflammatory, immune enhancement, and wound-repair activities (14). Diverse health effects of bergenin have been reported, but whether it promotes mitochondrial biogenesis has not been reported.

Herein, we aimed to determine whether bergenin enhances mitochondrial biogenesis in human hepatocytes. We believe that our findings can help to prevent and ameliorate liver diseases caused by aging and obesity.

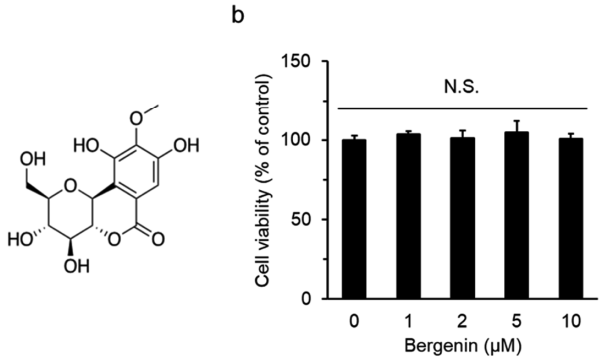

Figure 1. Evaluation of bergenin cytotoxicity against HepG2 hepatocytes
(a) Structure of bergenin. (b) HepG2 hepatocytes were cultured with or without bergenin (0–10 μM) for 24 h. After incubation, we performed Neutral Red assays. All values are presented as mean ± standard error ($n = 3$). N.S. : not significant (analysis of variance, Dunnett's multiple comparison test).

MATERIALS AND METHODS

Reagents

Bergenin was purchased from Tokyo Chemical Industry (Tokyo, Japan). EX-527 and compound C were obtained from Cayman Chemical Co. (MI, USA). All other chemicals used were of the highest commercially available grade.

Cell cultures

The human hepatocyte cell line HepG2 (RIKEN Cell Bank, Tsukuba, Japan) was cultured in Dulbecco's modified Eagle's medium (DMEM ; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Invitrogen, MA, USA), 100 units/mL streptomycin, and 100 μg/mL penicillin at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every alternate day until the culture reached approximately 50% confluence, after which it was changed daily. The cells were passaged when they reached 80-90% confluence. For each test, the culture medium was replaced with serum-free DMEM. After 12 h of incubation, cells were cultured in the presence or absence of bergenin, EX-527, and compound C.

Neutral Red assay

The cells were exposed to 150 μg/ml Neutral Red solution for 2 h at 37°C in a humidified atmosphere with 5% CO_2. After incubation, cells were washed twice with phosphate-buffered saline (PBS) and lysed using a cell extraction solution (EtOH/water/acetic acid, 50/49/1, v/v/v). The absorbance of the samples was measured at 540 nm using a Sunrise Remote microplate reader (Tecan Austria GmbH).

Assessments of mitochondrial content

Hepatocytes were stained by incubation with 100 nM MitoTracker Green FM (Invitrogen) in serum-free DMEM for 30-45 min at 37°C in a humidified atmosphere with 5% CO_2. After incubation, cells were washed twice with PBS, and cell images were obtained using a BIOREVO all-in-one fluorescence microscope (BZ-9000, Keyence, IL, USA) with excitation at 485 nm and emission at 520 nm. Fluorescence intensity was measured using an MTP-900 fluorescent microplate reader (Corona Electric, Ibaraki, Japan) under the same fluorescence wavelength settings used for microscopic observation.

ATP assay

Intracellular ATP levels were measured using a "Cell" ATP assay reagent Ver.2 kit (Toyo B-net, Tokyo, Japan) according to the manufacturer's instructions. Cells were exposed to ATP assay reagent dissolved in serum-free DMEM for 10 min in an MTP-900 fluorescent microplate reader (Corona Electric, Ibaraki, Japan) set at 23°C, and luminescence was measured.

Extraction and quantification of mtDNA

Cells were washed twice with ice-cold PBS and incubated with a DNA extraction buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, 100 mM ethylenediamine-*N, N, N', N'*-tetraacetic acid (EDTA), and 1% sodium dodecyl sulfate (SDS) for 1 h at 37°C in a humidified atmosphere with 5% CO_2. The upper aqueous layer was transferred to a fresh tube, RNase (1 mg/ml) was added, and the tube was incubated for 1 h at 37°C. After incubation, proteinase K (>700 units/ml) was added to the resulting samples and further incubated for 1 h at 50°C, followed by the addition of NaCl to a final concentration of 0.5 M. After the addition of an equal amount of isopropanol, the mixture was gently stirred for 10 min at 24°C. After centrifugation at 16,000 ×g for 10 min at 4°C, the supernatant was discarded. The pellet was washed with ice-cold 70% EtOH and dried using a centrifugal evaporator. The DNA sample was suspended in TE buffer and stored at −20°C until use.

For the quantification of mtDNA, DNA samples were reacted with THUNDERBIRD SYBR quantitative polymerase chain reaction (qPCR) Mix (Toyobo, Osaka, Japan) and one set of mtDNA- or nuclear DNA-specific primers targeting *succinate-ubiquinone oxidoreductase* (*SQR*) or *18S rRNA*, respectively, using a Thermal Cycler Dice Real Time System II (Takara Bio, Shiga, Japan). The primers used were as follows : human *SQR*, 5′-CAAACCTACGCCAAAATCCA-3′ and 5′-GAAATGAATGAGCCTACAGA-3′ ; human *18S rRNA*, 5′-TTTGCGAGTACTCAACACCAACATC-3′ and 5′-GAGCATATCTTCGGCCCACAC-3′. PCR was performed for

41 cycles, consisting of the first cycle for the initial denaturation (for 1 min at 95°C) and the subsequent 40 cycles for 2-step PCR (for 15 s at 95°C and 1 min at 60°C). All primers were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The ratio of mtDNA to nuclear DNA in each sample was calculated as an estimate of the mtDNA number.

Immunoblotting

HepG2 hepatocytes were lysed with a lysis buffer containing 150 mM NaCl, 50 mM Tris HCl (pH 7.0), 1 mM EDTA, 1% NP-40, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, a phosphatase inhibitor cocktail (Nacalai Tesque), and a protease inhibitor cocktail (Nacalai Tesque). Proteins in each lysate were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes using a semi-dry blotting apparatus (ATTO, Tokyo, Japan). After the transfer, the membranes were blocked with PBS with 0.1% Tween-20 (PBST) containing 20% Blocking One (Nacalai Tesque) for 1 h to reduce nonspecific binding. The membranes were then incubated with primary antibodies overnight at 4°C, washed, and incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit/mouse IgG ; Cell Signaling Technology, MA, USA) for 1 h. Proteins were detected using a chemiluminescent reagent (EZ West Lumi One, ATTO) and LAS-4000 chemiluminescence imager (Fujifilm, Tokyo, Japan). The following primary antibodies were used : anti-p-AMPKα (p-Thr172 ; 1 : 3,000, Cell Signaling Technology), anti-β-actin (1 : 20,000, Santa Cruz Biotechnology, CA, USA), anti-cytochrome c oxidase subunit 4 (COX IV, 1 : 3,000, Santa Cruz Biotechnology), anti-NADH dehydrogenase 1 alpha subcomplex subunit 3 (NDUFA3, 1 : 2,000, Santa Cruz Biotechnology), anti-PGC-1α (1 : 3,000, Santa Cruz Biotechnology), anti-succinate dehydrogenase complex iron sulfur subunit B (SDHB, 1 : 2,000, Santa Cruz Biotechnology), anti-ubiquinol-cytochrome c reductase core protein 2 (UQCRC2, 1 : 8,000, Santa Cruz Biotechnology), anti-AMPK (1 : 3,000, Cusabio Technology, TX, USA), anti-SIRT1 (1 : 5,000, Cusabio Technology), and anti-cytochrome c oxidase subunit 1 (MTCO1, 1 : 3,000, ABGENT, CA, USA). The quantification of band intensity was performed using the ImageJ software (National Institutes of Health, MD, USA).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed three times with PBS, and permeabilized using 0.1% Triton X-100 in PBS for 30 min. After three PBS washes, the cells were blocked with PBST containing 20% Blocking One (Nacalai Tesque), washed with PBST, and incubated with anti-PGC-1α antibody (1 : 500) diluted in Signal Enhancer HIKARI Solution A (Nacalai Tesque) overnight at 4°C. Next, the cells were washed three times with PBST and incubated with Cy3-labeled anti-rabbit IgG antibody (1 : 500) diluted in Signal Enhancer HIKARI Solution B (Nacalai Tesque) for 1 h. The cells were washed three times with PBST and mounted with Fluoro-KEEPER Antifade Reagent Non-Hardening Type (Nacalai Tesque) with 4'-6-diamidino-2-phenylindole (DAPI) for 30 min. The fluorescence signal of the cells was imaged using a BIOREVO all-in-one fluorescence microscope (BZ-9000 ; Keyence, IL, USA).

Nuclear/cytosolic fractionation

Cells were washed twice with ice-cold PBS and scraped from the culture plates after being soaked with a lysis buffer for the cytosol extraction containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail. After centrifugation at 1,000 ×g for 5 min at 4°C, the supernatant was stored as the cytosolic fraction at −20°C. The residual pellets were suspended with a lysis buffer containing 0.4 M NaCl, 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% NP-40, 1 mM DTT, 10% glycerol, and protease inhibitor cocktail for nuclear extraction and then homogenized with an ultrasonic generator (SONICSTAR 85, As ONE, Osaka, Japan). After centrifugation at 16,000 ×g for 10 min at 4°C, the supernatant was stored as a nuclear fraction at −20°C until use. The protein concentration of each fraction was calculated using a BCA Protein Assay Reagent Kit (Nacalai Tesque).

Statistical analyses

The results are presented as mean ± standard error of the mean of at least three independent experiments. Statistical analyses were carried out using software Statcel3 (OMS, Tokyo, Japan). One-way analysis of variance was conducted followed by the Dunnett's multiple comparison or Tukey–Kramer test to evaluate differences among means. Statistical significance was set at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

Bergenin enhances mitochondrial biogenesis in HepG2 hepatocytes

As shown in the results of a preliminary study for mitochondrial biogenesis enhancing the activity of various food ingredients (Supplementary Figure 1), bergenin could be the most potent accelerator of mitochondrial biogenesis in human HepG2 hepatocytes. First, the Neutral Red assay was performed to evaluate the cytotoxicity of bergenin in human HepG2 hepatocytes. HepG2 hepatocytes were exposed to bergenin (0-10 μM) for 24 h. Bergenin showed no significant cytotoxicity at concentrations of up to 10 μM (Fig. 1b).

Mitochondria in bergenin-treated HepG2 hepatocytes (0-2 μM) were fluorescently stained with MitoTracker Green FM to verify whether bergenin promoted mitochondrial biogenesis. The fluorescence intensity increased in a concentration-dependent manner (Fig. 2a). In addition, quantification using a fluorescent microplate reader showed that bergenin significantly increased mitochondrial content (Fig. 2b). We examined whether bergenin increases intracellular ATP levels in HepG2 hepatocytes. In the ATP assay, intracellular ATP content in HepG2 hepatocytes was significantly increased by bergenin treatment (Fig. 2c). To evaluate changes in mtDNA content during bergenin treatment, we used qPCR to analyze the mtDNA/nDNA ratio in the genomic DNA extracted from the cells. mtDNA content increased in a dose-dependent manner after bergenin treatment (Fig. 2d). These results suggest that bergenin promotes mitochondrial biogenesis in HepG2 hepatocytes.

Bergenin increases protein levels of mitochondrial constituents and induces PGC-1α nuclear translocation in HepG2 hepatocytes

To confirm the increase in mitochondrial contents due to bergenin, we examined the protein expression levels of mitochondrial constituents, such as NDUFA3, SDHB, UQCRC2, MTCO1, and COX IV. Levels of all five proteins significantly increased after bergenin treatment (Fig. 3a-e).

The regulation of mitochondrial biogenesis is governed by a transcriptional network centered on PGC-1α (3). We focused on whether bergenin enhances the nuclear translocation of PGC-1α. Immunofluorescence staining using anti-PGC-1α antibody revealed that PGC-1α translocated into the nucleus 15 h after bergenin treatment (Fig. 4a). To confirm that PGC-1α nuclear translocation was induced by bergenin, we analyzed the nuclear fraction from bergenin-treated cells by immunoblotting. Bergenin treatment increased PGC-1α protein levels in the nuclear

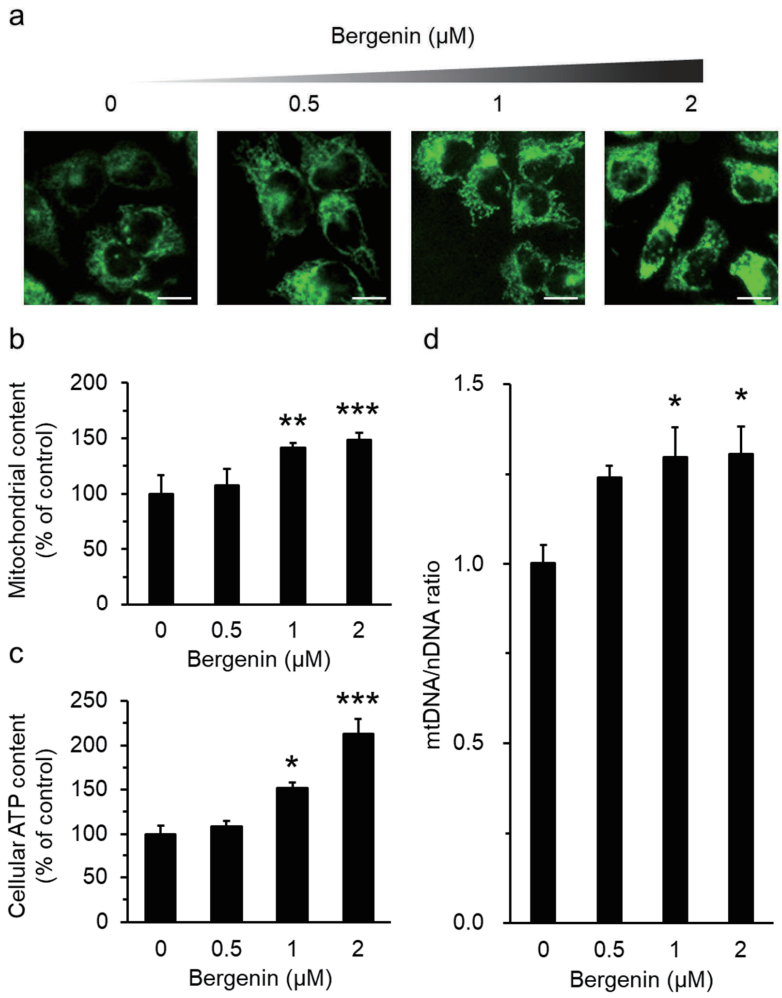

Figure 2. Bergenin promotes mitochondrial biogenesis in HepG2 hepatocytes
HepG2 hepatocytes were treated with 0–2 μM bergenin for 24 h. (a) Cells were stained with MitoTracker Green FM, and cell images were obtained using fluorescence microscopy. Scale bar, 10 μm. (b) Fluorescence intensity was measured using a fluorescence microplate reader with excitation at 485 nm and emission at 520 nm. (c) Intracellular ATP content was measured using an ATP assay kit. (d) After extracting genomic DNA from the cells, the mtDNA/nDNA ratio was analyzed using qPCR by targeting the *SQR* and *18S rRNA* genes as mtDNA and nDNA, respectively. The results are presented as the mean ± S.E. ($n = 3$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, comparison with the vehicle-treated control (analysis of variance, Dunnett's multiple comparison test)

fraction in a dose-dependent manner (Fig. 4b). PGC-1α protein expression in HepG2 hepatocytes was not affected by bergenin (data not shown). These results indicate that bergenin increases mitochondrial constituents and induces PGC-1α nuclear translocation.

SIRT1 plays a key role in bergenin-induced mitochondrial biosynthesis in HepG2 hepatocytes

SIRT1 positively regulates mitochondrial biogenesis by deacetylating and activating PGC-1α (4). We investigated protein levels of SIRT1 following bergenin treatment. SIRT1 protein levels were significantly increased in bergenin-treated HepG2 cells (Fig. 5a). To confirm the relationship between SIRT1 deacetylase activity and PGC-1α nuclear translocation, we used EX-527, a SIRT1 inhibitor, before bergenin treatment. We observed that EX-527 pretreatment abolished PGC-1α nuclear translocation (Fig. 5b). EX-527 also inhibited the increase in mitochondrial content following bergenin treatment (Fig. 5c). These data suggest that bergenin induces PGC-1α nuclear translocation and mitochondrial biogenesis via the increase in SIRT1 expression in hepatocytes.

Bergenin induces AMPK phosphorylation and leads to an increase in SIRT1 expression and mitochondrial biosynthesis in HepG2 hepatocytes

Several previous studies have shown that bergenin is a potent AMPK inducer (15). AMPK mediates the activation of SIRT1 (16-18). Thus, we speculated that bergenin enhances mitochondrial biosynthesis in HepG2 hepatocytes by activating AMPK. AMPK consists of a catalytic subunit (α) and two regulatory subunits (β and γ), which are activated by the phosphorylation of threonine 172 within the catalytic subunit. Thus, we evaluated phosphorylation levels of AMPK in bergenin-treated HepG2 cells. We found that bergenin significantly promoted AMPK phosphorylation (Fig. 6a), suggesting that bergenin induces AMPK-mediated activation of SIRT1. Moreover, to determine whether AMPK activation is associated with the increase in SIRT1 expression induced by bergenin, we pretreated HepG2 hepatocytes with an inhibitor of AMPK (compound C) before bergenin treatment. Compound C completely abolished

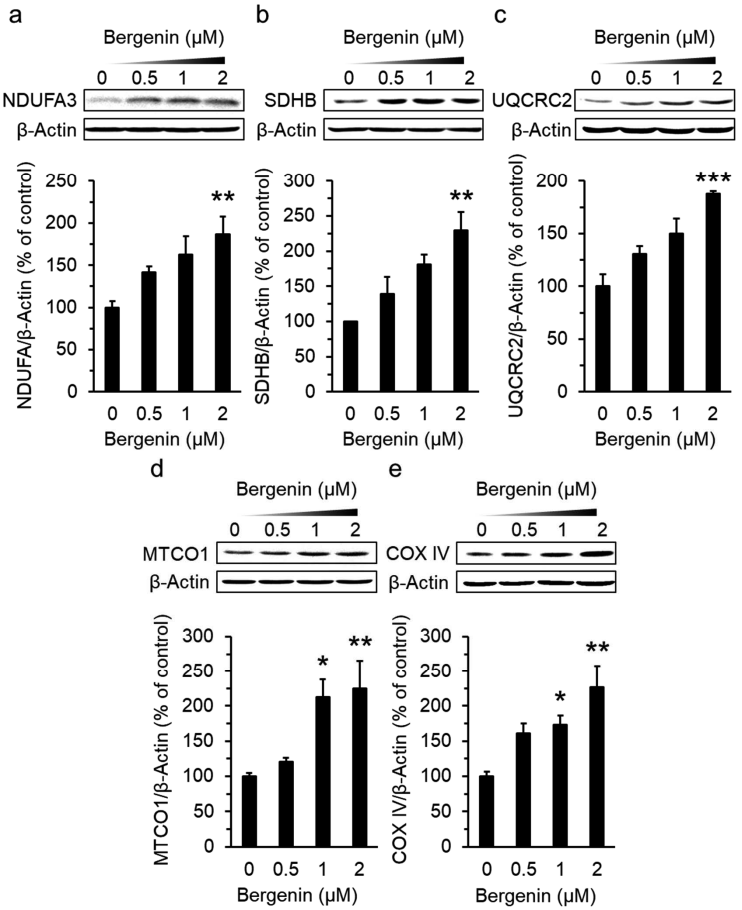

Figure 3. Bergenin increases the expression of electron transport chain proteins in HepG2 hepatocytes
HepG2 hepatocytes were treated with 0–2 μM bergenin for 24 h. Levels of electron transport proteins were analyzed using immunoblotting with antibodies specific for (a) NDUFA3, (b) SDHB, (c) UQCRC2, (d) MTCO1, and (e) COX IV. β-actin was used as the loading control. ImageJ software was used to analyze the intensity of the immunoblotting bands. The results are presented as the mean ± S.E. ($n = 3$). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, comparison with the vehicle-treated control (analysis of variance, Dunnett's multiple comparison test).

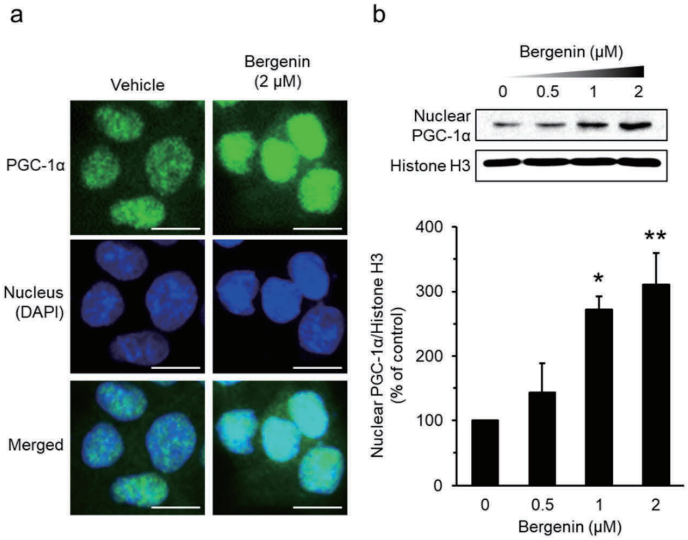

Figure 4. Bergenin increases PGC-1α nuclear translocation in HepG2 hepatocytes
HepG2 hepatocytes were treated with bergenin at the indicated concentrations for 15 h. (a) Cells were immunostained with an anti-PGC-1α antibody and DAPI. Cell images were obtained using fluorescence microscopy. Scale bar, 10 μm. (b) After extracting the nuclear fraction, the protein levels of PGC-1α in the nucleus were analyzed using immunoblotting. ImageJ software was used to analyze the intensity of the immunoblotting bands. Histone H3 was used as the loading control. The results are presented as the mean ± S.E. ($n = 3$). *$p < 0.05$ and **$p < 0.01$, comparison with the vehicle-treated control (analysis of variance, Dunnett's multiple comparison test).

bergenin-induced SIRT1 expression in HepG2 hepatocytes (Fig. 6b). In addition, compound C significantly inhibited the effect of bergenin on mitochondrial biogenesis (Fig. 6c). These data suggest that AMPK activation is required for bergenin-induced SIRT1 expression and mitochondrial biogenesis.

DISCUSSION

Mitochondria are particularly abundant in the liver and regulate energy metabolism. Aging and obesity attenuate mitochondrial function in the liver. Therefore, promoting mitochondrial biogenesis contributes to the maintenance and recovery of cellular functions, including energy production, by increasing mitochondrial content. This study showed that bergenin activates mitochondrial biogenesis and increases protein levels of mitochondrial constituents in HepG2 hepatocytes (Figs. 2 and 3). The 4-*O*-methyl gallic acid moiety in the structure of bergenin may partially contribute to bergenin activity, as suggested by preliminary experiments with 47 food components which showed that gallic acid also promoted mitochondrial biogenesis, although to a lesser extent than bergenin (Supplementary Fig. 1). These compounds were compared with a positive control, resveratrol, a compound known to promote mitochondrial biogenesis (19). Bergenin seemed to be as or more effective than resveratrol in promoting mitochondrial biogenesis, at least under our testing conditions, and may, therefore, have additional biological values when used in combination with resveratrol. In addition to its promoting effect on mitochondrial biogenesis, bergenin also enhanced the nuclear translocation of PGC-1α (Fig. 4). These findings suggest that bergenin activates the transcriptional activity of PGC-1α, inducing the expression of mitochondria-associated

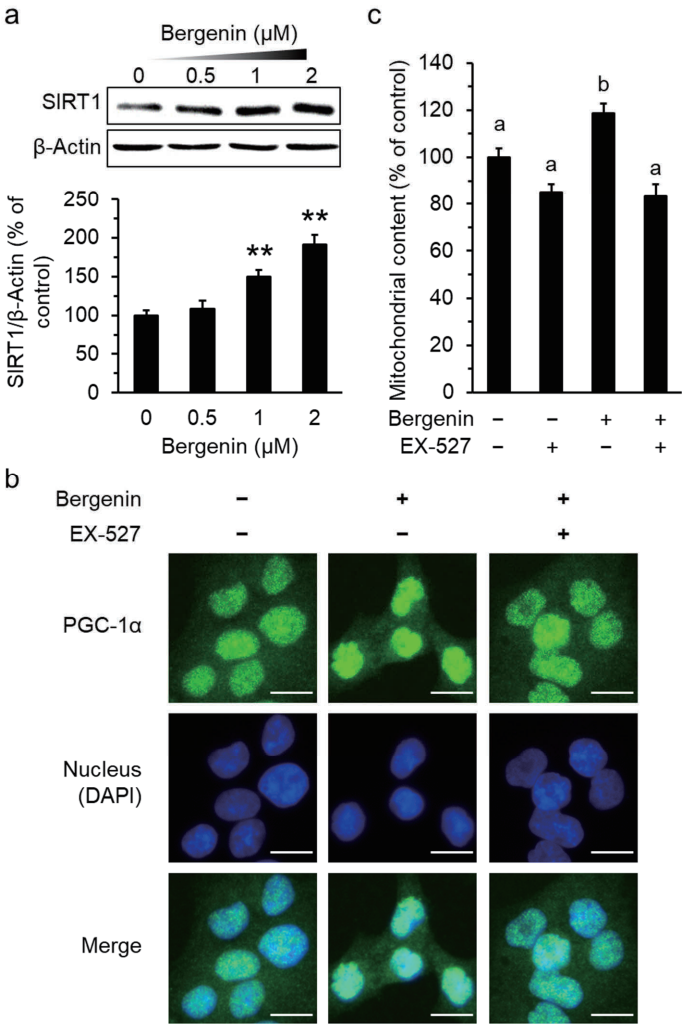

Figure 5. Bergenin enhances mitochondrial biogenesis by inducing SIRT1 expression in HepG2 cells
(a) HepG2 hepatocytes were treated with 0–2 μM bergenin for 15 h. Protein levels of SIRT1 were analyzed using immunoblotting. ImageJ was used to analyze the intensity of the immunoblotting bands. β-actin was used as the loading control. The results are presented as the mean ± S.E. ($n = 3$). $^{**}p < 0.01$, comparison with the vehicle-treated control (analysis of variance ; ANOVA, Dunnett's multiple comparison test). (b-c) HepG2 hepatocytes were cultured with or without EX-527 (20 μM). After 15 h, the cells were treated with bergenin (2 μM) for 24 h. (b) Cells were immunostained with anti-PGC-1α antibody and DAPI. Cell images were obtained using fluorescence microscopy. Scale bar, 10 μm. (c) Cells were stained with MitoTracker Green FM, and the fluorescence intensity was measured using a fluorescent microplate reader with excitation at 485 nm and emission at 520 nm. The results are presented as the mean ± S.E. ($n = 3$). Values without a common letter differ significantly among the groups ($p < 0.05$, ANOVA, Tukey–Kramer test).

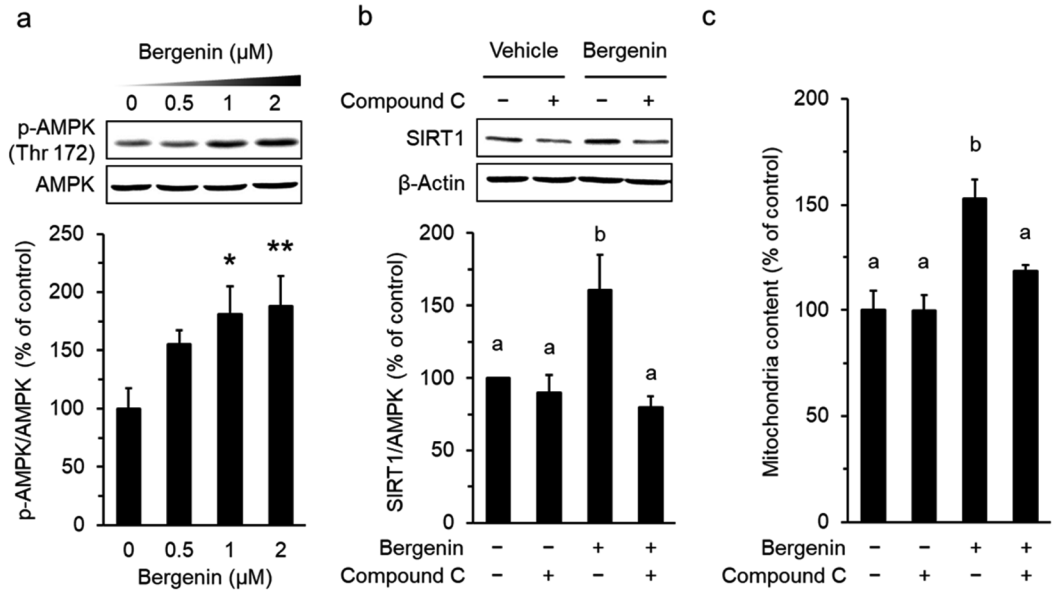

Figure 6. Bergenin enhances SIRT1 expression and mitochondrial biogenesis by activating AMPK in HepG2 hepatocytes
(a) HepG2 hepatocytes were exposed to bergenin (0–2 μM) for 6 h. Protein levels of phosphorylated AMPK (Thr 152) were analyzed using immunoblotting. Total AMPK was used as the loading control. The results are presented as the mean ± S.E. ($n = 3$). *$p < 0.05$ and **$p < 0.01$, comparison with the vehicle-treated control (analysis of variance ; ANOVA, Dunnett's multiple comparison test). (b–c) HepG2 hepatocytes were cultured with or without compound C (5 μM). After 8 h, the cells were treated with bergenin (2 μM) for 15 h. (a) The protein levels of SIRT1 were analyzed using immunoblotting. β-actin was used as the loading control. ImageJ software was used to analyze the intensity of the immunoblotting bands. The results are presented as the mean ± S.E. ($n = 3$). Values without a common letter differ significantly among the groups ($p < 0.05$, ANOVA, Tukey–Kramer test).

genes encoded by nDNA and mtDNA. Bergenin also enhanced SIRT1 expression, whereas the SIRT1 inhibitor abrogated the PGC-1α nuclear translocation induced by bergenin (Fig. 5), suggesting that bergenin enhances mitochondrial biosynthesis through upregulation of PGC-1α deacetylation by SIRT1. The expression of PGC-1α decreases with aging and obesity, which leads to a decrease in mitochondrial mass (20, 21). In contrast, overexpression of PGC-1α increases mitochondrial abundance and functional health in liver cells (12). Thus, the activation of hepatic PGC-1α by bergenin may help to recover the decline in metabolic function due to aging and obesity through the maintenance of mitochondrial function. In addition, the fact that bergenin promotes mitochondrial biogenesis may partially explain the mechanism underlying the hepatoprotective effects of bergenin. The livers of patients with non-alcoholic fatty liver disease (NAFLD) or obesity exhibit ATP depletion and increased reactive oxygen species production, which are characteristic of mitochondrial disorders (22). A previous report showed that the activation of mitochondrial biogenesis ameliorates fatty liver disease, suggesting that the activation of mitochondrial biogenesis leads to the suppression of metabolic dysfunction (23). Therefore, bergenin promotes hepatic mitochondrial biogenesis, which may reduce the risk of developing serious diseases, such as obesity and fatty liver.

Understanding the pharmacokinetic properties of food ingredients is crucial in maximizing their bioavailabilities. Bergenin is absorbed from the intestinal tract, transported to the liver via the portal vein, and finally excreted in the urine. Metabolite analyses have detected orally administered bergenin in the bile and urine as glucuronide metabolites in addition to the unchanged form, suggesting that a part of bergenin is excreted via glucuronidation (24). The distribution of bergenin in six tissues (liver, kidney, lung, heart, spleen, and brain) after oral ingestion in rats has been investigated, and the highest bergenin concentration and longest retention time was found in the kidney, while the lowest distribution was found in the brain (25). Bergenin is classified as Class IV in the Biopharmaceutics Classification System due to its low solubility and permeability (26). Two pharmacokinetic studies of bergenin in humans have been reported, involving a single dose of 250 mg bergenin (27, 28) : peak plasma concentrations of bergenin were observed approximately 2 h after oral administration, and ranged from 15-67 ng/ml (0.049-0.20 uM) ; the terminal elimination half-life was approximately 4 h. Therefore, while various medicinal effects of bergenin have been demonstrated, the efficiency of its bioavailability remains an issue. Meanwhile, various studies have been conducted on enhancers aimed at improving the bioavailability of bergenin. Xuan *et al*. showed that coadministration of borneol or poloxamer 188 improved the gastrointestinal absorption of bergenin, increasing its bioavailability by 4.4-fold and 1.8-fold, respectively, compared with bergenin alone (29). Enhancing the solubility of bergenin by forming a complex with phospholipids is reported to increase the maximum plasma concentration of bergenin and improve its bioavailability (26, 30). Therefore, future *in vivo* studies and human clinical trials will benefit from considering improvements in absorption efficiency in order to evaluate the efficacy of bergenin.

Our findings demonstrated that bergenin activates AMPK in hepatocytes (Fig. 6). Jie *et al*. reported that bergenin exerted a protective effect against D-galactosamine-induced acute liver injury in rats and indicated that the mechanism of action was the upregulation of AMPK (31). Gracia *et al*. developed a genetically engineered mouse model that showed liver-specific activation of AMPK upon administration of doxycycline ; they demonstrated that hepatic AMPK activation could be a preventive and therapeutic target for NAFLD (32). Furthermore, in their study,

liver-specific AMPK activation decreased the expression of hepatic inflammation- and fibrosis-related genes and increased the expression of lipid metabolism-related genes, suggesting that hepatic AMPK activation leads to the inhibition of hepatic inflammation and fibrosis. Bergenin has been shown to suppress inflammation and fibrosis in hepatitis models induced by various drugs, such as carbon tetrachloride, D-galactosamine, and iron (31, 33-36). In human clinical trials, natural ingredients, such as berberine, caffeine, and resveratrol, have been reported to have preventive effects against NAFLD by activating AMPK (37). Therefore, in the future, it would be beneficial to verify whether bergenin prevents NAFLD in human clinical trials.

In conclusion, this study showed that bergenin stimulated mitochondrial biogenesis by activating the AMPK/SIRT1 axis in HepG2 hepatocytes. The activation of mitochondrial biogenesis has been reported to be effective in reversing mitochondrial dysfunction (4). Therefore, the promoting effect of bergenin may lead to the development of methodologies to prevent and improve treatments for serious diseases, such as diabetes, caused by aging and obesity.

CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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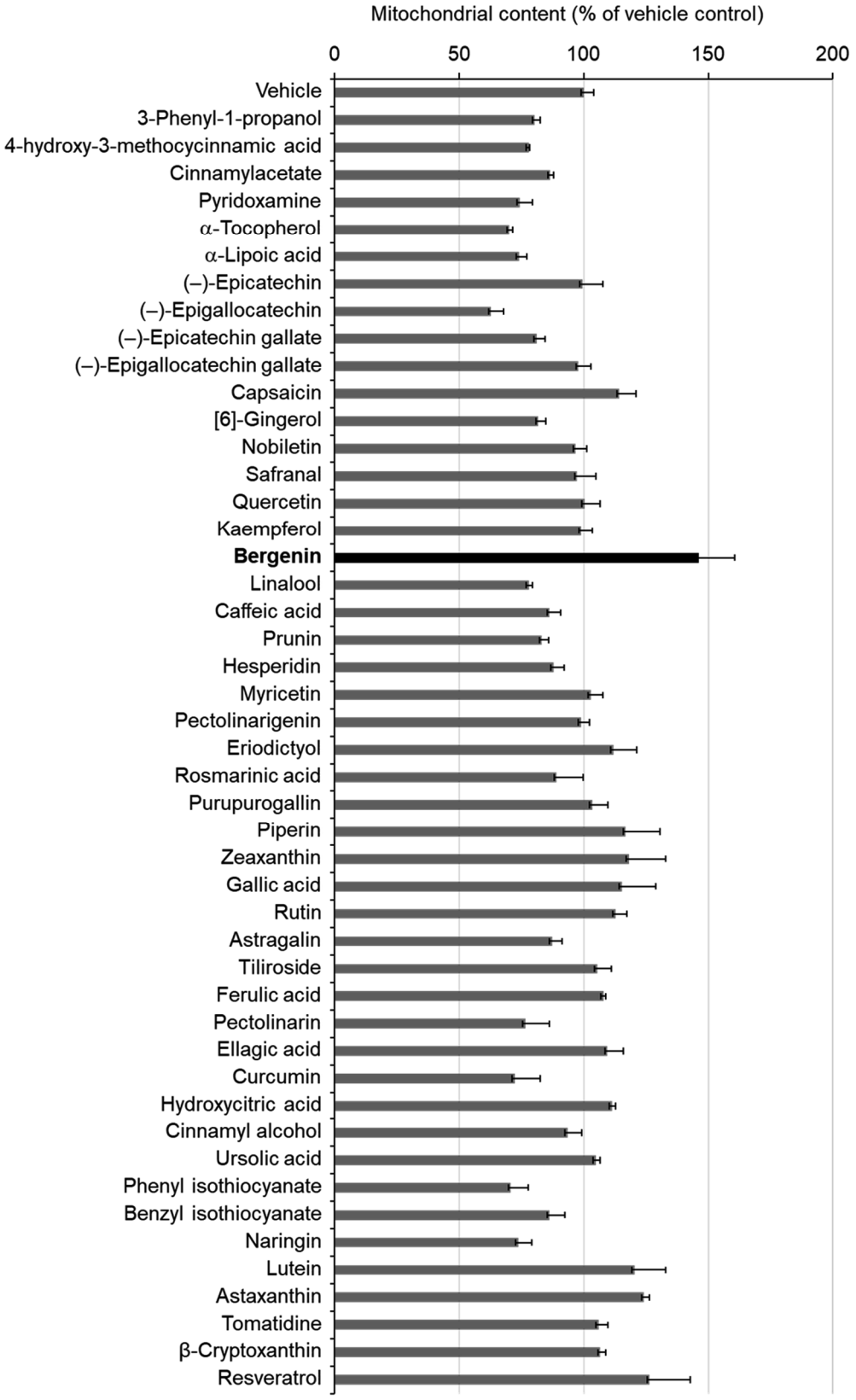

Supplementary Figure 1