Bioorganic & Medicinal Chemistry Letters 26 (2016) 3978–3983

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Syringaresinol induces mitochondrial biogenesis through activation of PPARβ pathway in skeletal muscle cells

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ARTICLE INFO

Article history:
Received 8 March 2016
Revised 20 June 2016
Accepted 1 July 2016
Available online 1 July 2016

Keywords:
Syringaresinol
PPARβ
Mitochondrial biogenesis
Skeletal muscle

ABSTRACT

Activation of peroxisome proliferator-activated receptors (PPARs) plays a crucial role in cellular energy metabolism that directly impacts mitochondrial biogenesis. In this study, we demonstrate that syringaresinol, a pharmacological lignan extracted from *Panax ginseng* berry, moderately binds to and activates PPARβ with K_D and EC_{50} values of 27.62 ± 15.76 μM and 18.11 ± 4.77 μM, respectively. Subsequently, the expression of peroxisome proliferator-activated receptor γ coactivator-1α together with PPARβ transcriptional targets, mitochondrial carnitine palmitoyltransferase 1 and uncoupling protein 2, was also enhanced in terms of both mRNA and protein levels. The activation of these proteins induced mitochondrial biogenesis by enrichment of mitochondrial replication and density within C2C12 myotubes. Importantly, knockdown of PPARβ reduced the syringaresinol-induced protein expression followed by the significant reduction of mitochondrial biogenesis. Taken together, our results indicate that syringaresinol induces mitochondrial biogenesis by activating PPARβ pathway.

Mitochondrial biogenesis can be defined as an increase of number, size, and mass of mitochondria that enhances the function.[1] Mitochondrial function plays an essential role in cellular energy and homeostasis.[2] Perturbation in regulation of mitochondrial biogenesis has been determined to be associated with many pathologies associated with heart failure, Type 2 diabetes, Alzheimer's disease, and aging.[3–5] Mitochondrial proteins and enzymes are encoded by both nuclear and mitochondrial genomes. The mitochondrial gene complement encodes for 13 proteins, while the rest of over 1000 proteins encoded by nuclear genome is synthesized on cytosolic ribosomes.[6–8]

The peroxisome proliferator-activate receptors (PPARs), the ligand-activated transcription receptors, belong to the nuclear receptor family.[9,10] PPARs play a major role in governing the transcriptional machinery that controls the expression of genes and proteins in response to the cellular metabolism, growth, and differentiation.[11–13] Among the PPARs, PPARβ (also called as PPARδ) is well-known to regulate coordinately nuclear genes encoding key mitochondrial proteins in skeletal muscle such as carnitine palmitoyltransferase 1 (CPT1) and the uncoupling proteins family (UCPs).[14–16] CPT1 regulates the entry of long-chain fatty acids into mitochondria for oxidation,[17] whereas UCPs provide the energy for oxidative phosphorylation.[18] Additionally, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is known as an up-regulator of mitochondrial biogenesis and functions by improving the expression of mitochondrial proteins.[19,20]

Syringaresinol (Syr), a pharmacological lignan, was originally discovered as a prolonging effect on exercise time.[21] Syr is also known as an inducer for vasorelaxation through the phosphorylation and dimerization of endothelial nitric oxide synthase.[22] Recently, (+)-syringaresinol, isolated from *Panax ginseng* berry, was shown to delay cellular senescence in human umbilical vein endothelial cells (HUVECs) against apoptosis induced by hypoxia/oxygenation in H9c2 cells by preventing mitochondrial dysfunction.[23–25] These results raised a possibility that Syr induces mitochondrial biogenesis. However, the molecular mechanism remains elusive. In this study, we demonstrate, for the first time, that Syr induces mitochondrial biogenesis through activation of PPARβ and its transcriptional targets in skeletal muscle cells.

Abbreviations: PPAR, peroxisome proliferator-activated receptor; Syr, syringaresinol; LBD, ligand binding domain; CPT1, carnitine palmitoyltransferase 1; UCP2, uncoupling protein 2; ITC, isothermal titration calorimetry; TR-FRET, time-resolved fluorescence resonance energy transfer; siRNA, small interfering RNA.

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http://dx.doi.org/10.1016/j.bmcl.2016.07.001
0960-894X/

Firstly, we investigated whether Syr could directly interact with the ligand binding domain (LBD) of PPARβ. We used the X-ray complex structure of PPARβ-LBD with 3-[5-Methoxy-1-(4-methoxybenzenesulfonyl)-1*H*-indol-3-yl]-propionic acid as a template (PDBid: 3ET2) for in silico docking.[26] The docking was performed using *SwissDock*.[27] Our complex model displayed that Syr suitably locates in the PPARβ-LBD pocket. H413 residue on Helix10 forms a hydrogen bond with Syr with a distance value of 3.4 Å (Fig. 1A). This localization is consistent with the pattern of ligand/PPARβ-LBD interaction in which H413 is a key residue binding with the ligand through a hydrogen bond.[28] To verify the interaction, we purified suitable amount of PPARβ-LBD and conducted isothermal titration calorimetry (ITC) experiments with Syr.[29] The purified PPARβ-LBD showed a single band of 36.5 kDa on Pro260 chip (Fig. 1B). The interaction of Syr and PPARβ-LBD was characterized by ITC with the best fit of one-site model ($\Delta H = -370 \pm 125$ kcal mol^{-1}). This finding supports the one binding site between Syr and PPARβ-LBD. The binding affinity is 27.62 ± 15.76 μM for K_D (Fig. 1C). Interaction between the ligand and PPARβ-LBD is often believed to be crucial for PPARβ-LBD activation. Expectedly, further time-resolved fluorescence resonance energy transfer (TR-FRET) experiments indicate that Syr activated PPARβ-LBD by recruitment of a fluorescein labeled coactivator C33 in a dose-dependent manner with an EC_{50} value of 18.11 ± 4.77 μM (Fig. 1D). Collectively, these results suggest that syringaresinol is a potential natural agonist of PPARβ.

We next examined the capacity of Syr to activate the expression of PPARβ and its transcriptional targets in mammalian muscle cells. Mouse skeletal muscle C2C12 cells were fully differentiated into myotubes for 7 days and later stimulated with several concentrations of Syr.[30] Based on K_D and EC_{50} values, we chose the concentrations of Syr at 10, 25, and 50 μM for the simulation. DMSO (0.1%) and PPARβ agonist, GW0742 (1 μM), were used as negative and positive controls, respectively. Real-time qPCR and immunoblot experiments were performed as described elsewhere.[31,32] Our data indicate that Syr significantly increased the expression of PPARβ at concentration of 50 μM but not at lower concentrations. Syr induced expression of PPARβ mRNA by more than 2.0-

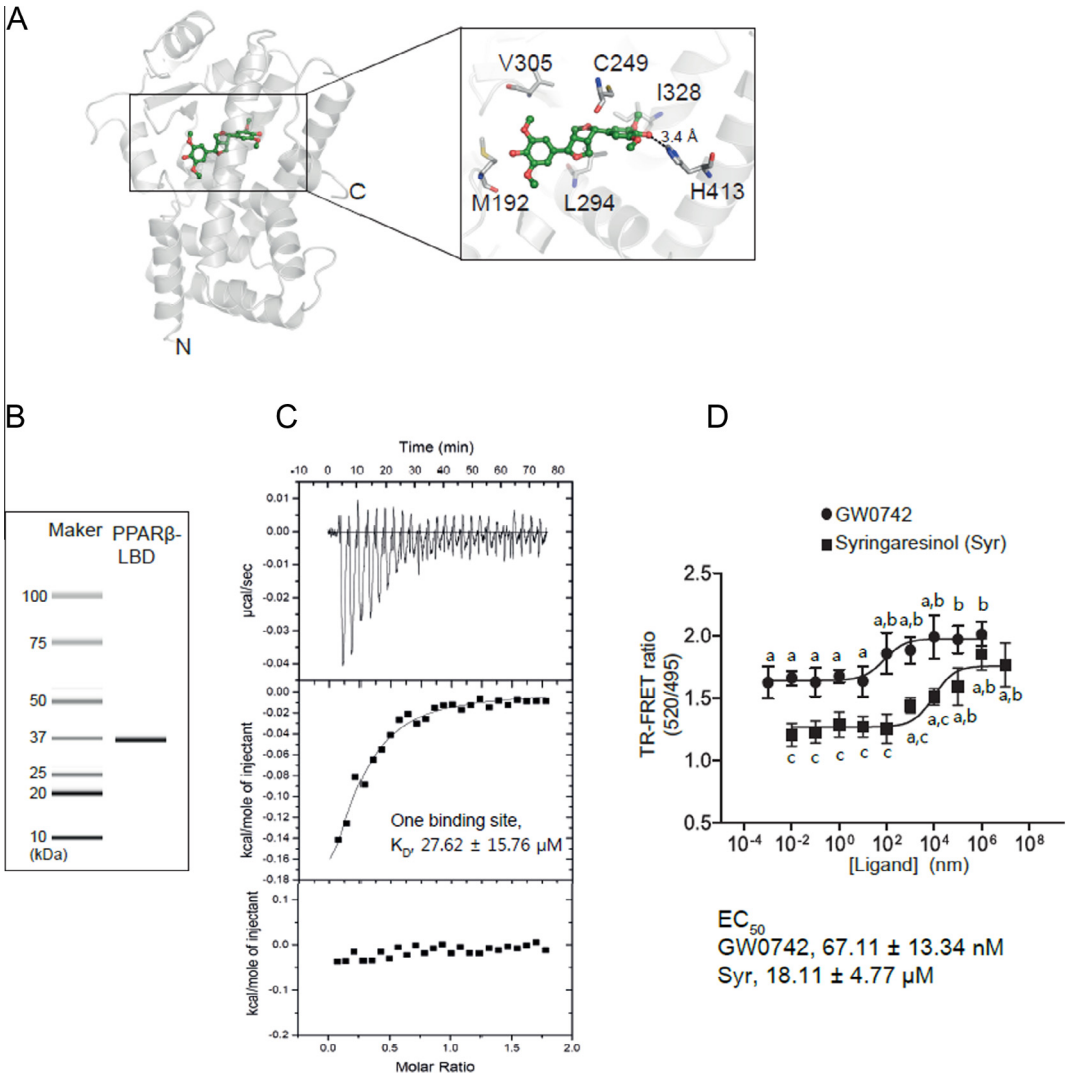

Figure 1. Syringaresinol binds to and activates PPARβ-LBD. (A) Docking of PPARβ-LBD/Syr complex. Complex model displays the localization of Syr in ligand binding pocket of PPARβ. The C-backbone of Syr is shown in forest color and Oxygen in red. Hydrogen bond formed between H413 residue and Syr is shown as a dashed line. (B) The purified PPARβ-LBD showed a single purified band assessed by Experion Pro260 chip. (C) The interaction between PPARβ-LBD and Syr was characterized by ITC. ITC data were best fitted with a one binding-site model. (D) TR-FRET assays with Syr compared to PPARβ agonist, GW0742. Values that are denoted by different letters are significantly different ($P \leqslant 0.05$).

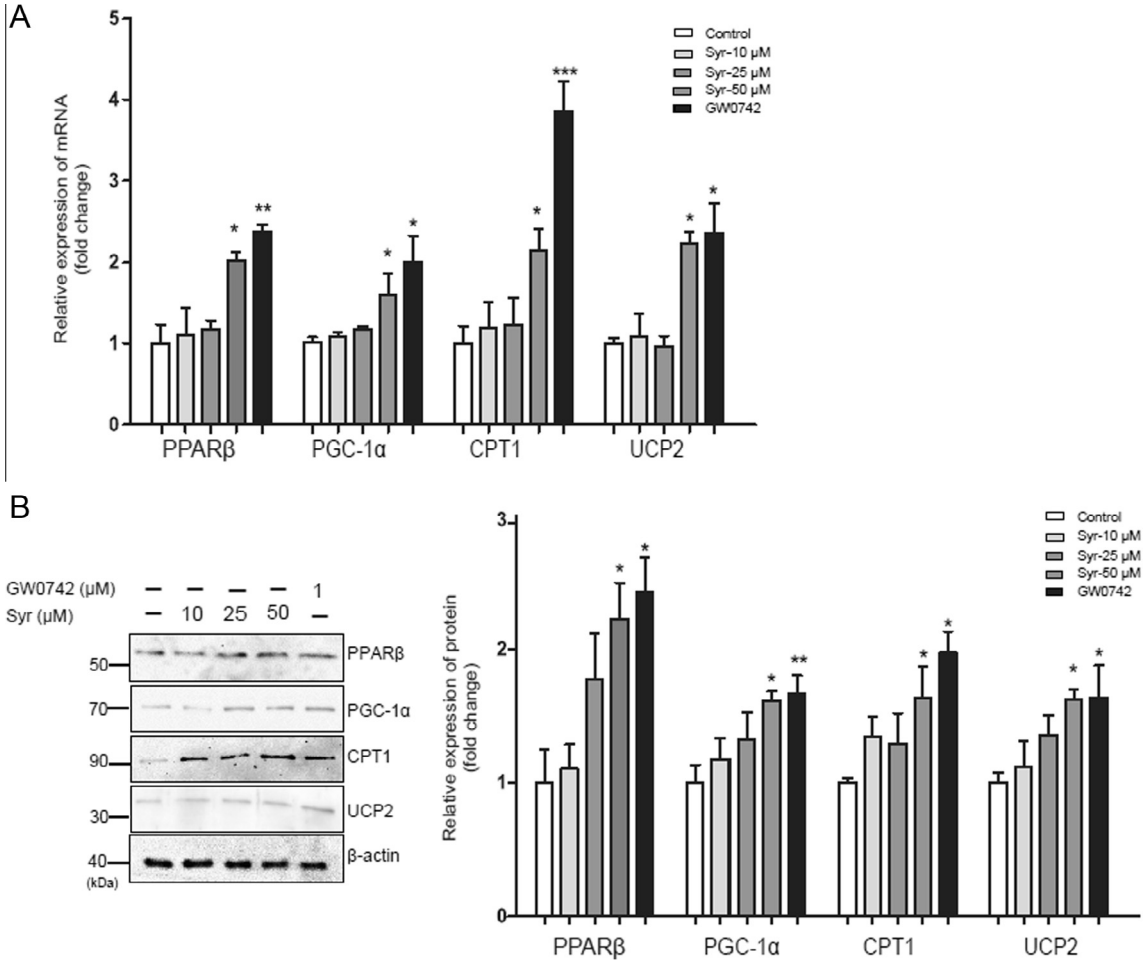

Figure 2. Syringaresinol activates the expression of PPARβ, its transcriptional coactivator, PGC-1α, and their target genes, CPT1 and UCP2, in C2C12 myotubes. The expression levels of mRNA and protein were quantified by real-time qPCR (A) and immunoblot (B), respectively. Data are from triplicate experiments. Results are shown by the mean ± SEM. *P* values compared to control are denoted as *, **, *** with $P \leqslant 0.05$, $P \leqslant 0.01$ and $P \leqslant 0.001$, respectively (two-tail Student's *t* test).

fold higher compared to controls. Similar trends were detected in protein expression levels (Fig. 2A & B). Subsequently, we explored the capacity of Syr to activate the expression of the transcriptional cofactor, PGC-1α. PPARβ agonist and Syr enhanced the expression of PGC-1α similarly. Stimulation of C2C12 myotubes with 50 μM Syr increased approximately 1.5-fold of PGC-1α expression in both mRNA and protein levels (Fig. 2). We also observed Syr dramatically enhanced the expression of PPARβ target genes, including CPT1 and UCP2, key enzymes in the regulation of mitochondrial biogenesis. At concentration of 50 μM, Syr enhanced approximately 2.0-fold the protein level (Fig. 2B), while mRNA levels were activated to 4.0 and 3.0-fold for CPT1 and UCP2, respectively (Fig. 2A). Such activation of PGC-1α, CPT1 and UCP2 in skeletal muscle cells have been demonstrated to induce mitochondrial biogenesis, oxidative metabolism.[1,6] Hence, we further investigated the capacity of Syr to induce mitochondrial biogenesis in C2C12 myotubes.

To explore the potential of Syr to induce mitochondrial biogenesis, C2C12 myotubes were stimulated with Syr. Mitochondrial DNA (mtDNA) content and mitochondrial density were later quantified.[33] The mtDNA content, determined by the ratio of mtDNA to nuclear DNA, indicates the mitochondrial replication. Real-time qPCR results reveal that mtDNA content was slightly increased by Syr at 25 μM and 50 μM (Fig. 3A). Subsequently, C2C12 myotubes were probed with Mitotracker Green.[33] This reagent accurately probes mitochondrial density in living cells by binding to mitochondria. The results showed that Syr significantly increased mitochondrial density at both 25 μM and 50 μM by 14% and 18%, respectively (Fig. 3B). To unravel the induction of mitochondrial biogenesis by Syr, mitotracker-probed images were obtained using fluorescence microscope at a constant exposure time.[33] We observed that Syr enhanced the mitochondrial density substantially, suggesting that Syr induced mitochondrial biogenesis in skeletal muscle cells (Fig. 3C).

To examine whether PPARβ plays crucial role in mediating the induction of mitochondrial biogenesis by Syr, we knocked down the expression of PPARβ by transfecting PPARβ-specific small interfering RNA (siRNA) into C2C12 myotubes. Next, cells were stimulated with Syr (50 μM).[34] Immunoblot results indicated the reduction of 40% PPARβ protein level in PPARβ knockdown cells compared to that in control (Fig. 4A). Knockout of PPARβ abolished the significant induction of PPARβ expression stimulated with either Syr or GW0742 (Fig. 4A). Although, not significant, we also observed reducing trends in the expression of PGC-1α, CPT1, and UCP2 proteins in the PPARβ knockdown cells (Fig. 4A). Consequently, knocking down of PPARβ suppressed the increased level of mtDNA content and density within the C2C12 myotubes. Knockdown of PPARβ decreased 50% of mtDNA content and 10% of mito-

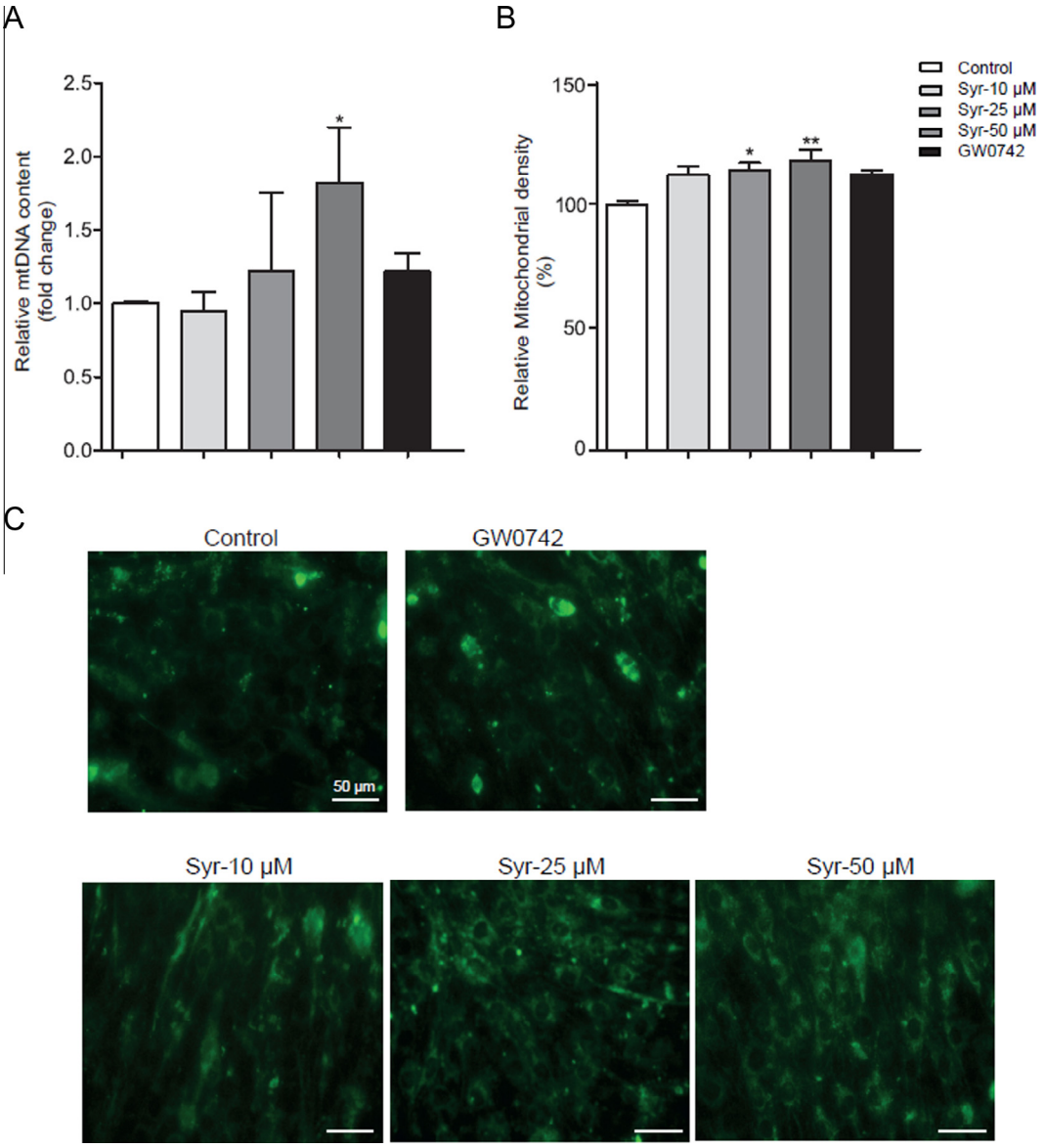

Figure 3. Syringaresinol induces mitochondrial biogenesis in C2C12 myotubes. (A) Dose-dependent induction in mtDNA content by Syr was measured using real-time qPCR. (B) Mitochondrial density was determined by using Mitotracker Green Probes. (C) Mitochondrial density was indicated by green fluorescence. Representative figures are from triplicate experiments that gave similar results. Data are represented as the mean ± SEM. *P* values compared to controls are denoted as *, ** with $P \leqslant 0.05$ and $P \leqslant 0.01$, respectively (two-tail Student's *t* test).

chondrial density, respectively (Fig. 4B & C). While the significant induction of mtDNA content and density were determined in normal cells stimulated with Syr, we observed a similar reduction trend in PPARβ knockdown cells regardless of simulation (Fig. 4B & C). Consistent with these findings, mitotracker-probed images substantially showed the enrichment of mitochondrial density by Syr simulation in normal cells, but not in PPARβ knockdown cells (Fig. 4D). Taken together, these data suggest that normal expression of PPARβ is required for Syr-induced mitochondrial biogenesis.

Additionally, we examined whether Syr could activate AMP-activated protein kinase (AMPK) and/or silent information regulator gene homologue 1 (SIRT1), which are also known as important regulators involved in mitochondrial biogenesis.[1,35] Activation of SIRT1 and/or AMPK is evidenced to enhance mitochondrial biogenesis by induction of a subset of PPARβ target genes.[1,15,35] It has reported that Syr upregulates SIRT1 and AMPK expression in human umbilical vein endothelial cells.[15,25] However, in our experiments on skeletal muscle cells, although there was a slight induction, we did not observe the significantly increased expression of SIRT1 protein and the phosphorylation activation of AMPK (pThr172-AMPK) by Syr compared to those in control and GW0742, respectively (Fig. 5). These results therefore suggest that Syr induced mitochondrial biogenesis through PPARβ activation, but not SIRT1 or AMPK, in skeletal muscle cells.

In summary, our results reveal that syringaresinol directly interacts with and activates PPARβ-LBD followed by induction of mitochondrial biogenesis in skeletal muscle cells. Based on ligand–protein interaction, together with cellular simulation experiments, we determined the pharmacological effects of Syr on mitochondrial biogenesis at 50 μM. Importantly, knockdown of PPARβ lessens the effects of Syr, indicating that Syr induces mitochondrial biogenesis through activation of PPARβ pathway.

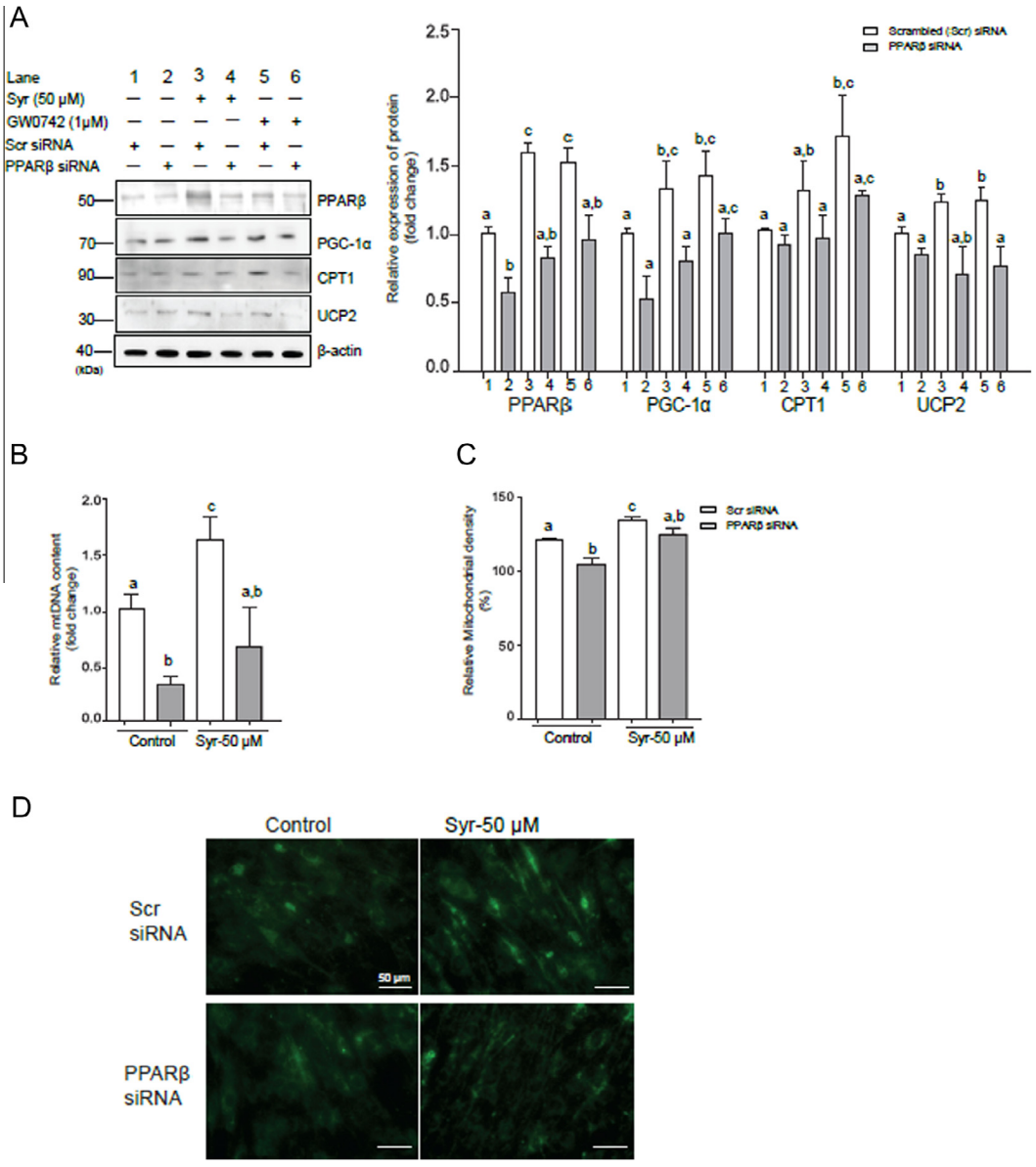

Figure 4. Knockdown of PPARβ lessens the effect on mitochondrial biogenesis by syringaresinol. (A) Protein expression levels of PPARβ, PGC-1α, CPT1, and UCP2 in PPARβ knockdown cells compared to controls. (B and C) The induction in mtDNA content and mitochondrial density of Syr in PPARβ knockdown cells. (D) Mitotracker Green-probed images indicate mitochondrial density. Values that are denoted by the different letters are significantly different ($P \leqslant 0.05$).

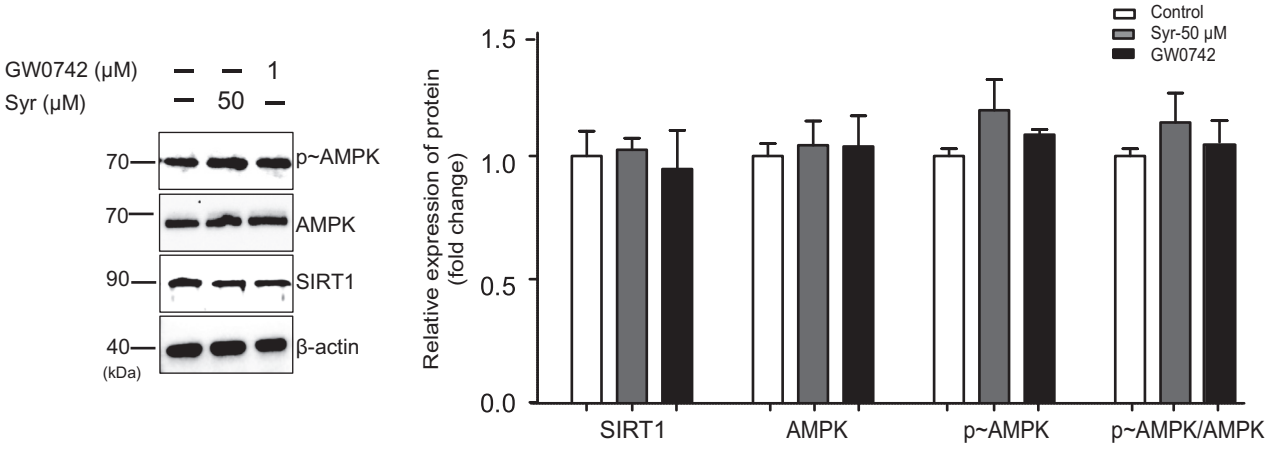

Figure 5. Protein expression levels of SIRT1, AMPK and pThr172-AMPK in syringaresinol-stimulated cells compared to controls.

Acknowledgments

This work was carried out with the support of the 'Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01052801 and PJ010090032015)', Rural Development Administration (RDA), Republic of Korea, and was supported by a grant (14162MFDS973) from the Korean Ministry of Food and Drug Safety, and Korea University Grant, Republic of Korea.

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29. Recombinant protein purification. PPARβ-LBD construct ranging from 254 to 441 was cloned into pET32a vector (Invitrogen) between *Bam*HI and *Eco*RI restriction enzyme sites. Protein was overexpressed in *E. coli* Rosetta2 strain and purified by affinity chromatography on Ni-NTA resin column (Qiagen). Protein was dialyzed against buffer A (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 50 mM EDTA) overnight at 4 °C. Further purification was conducted on Anion exchange Hitrap Q column (GE HealthCare) equilibrated with buffer A and eluted with gradient from 10% to 80% of buffer B (50 mM Tris–HCl pH 7.4, 1 M NaCl, 50 mM EDTA). Fractions containing pure protein were confirmed by using Experion Pro260 chip (Bio-Rad) and followed by concentrating by 10 kDa-centrifugal filters (Merck).
Syringaresinol sample. (8*R*,8′*R*)-(+)-Syringaresinol was synthesized at Hanchem company with the high purity (>99.7%).
To determine the binding affinity between PPARβ-LBD and Syr, Isothermal titration calorimetry (ITC) experiments were performed on a VP-ITC instrument (MicroCal). All buffer, protein and ligand solutions were centrifuged and degassed well before used. 2.0 mM Syringaresinol in buffer supplemented with 5% DMSO was titrated to sample cell containing 0.2 mM PPARβ-LBD in the same buffer at 20 °C. Each of injection is 10 μL among 25 of the total number of injections. In the reference experiment, the buffer was injected into protein.
30. Cell culture, differentiation and treatment. Mouse skeletal muscle C2C12 cells were obtained from American Type Culture Collection (USA). Cells were maintained and grown in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 20% fetal bovine serum (FBS, Hyclone) and 100 units/ml of penicillin together with 100 mg/ml streptomycin (PEST, Sigma), 5% CO_2 (v/v) at 37 °C. When cells reached to approximately 70% confluent, medium was switched to DMEM supplemented with 2% horse serum (Hyclone) for differentiation. During differentiation, medium was change daily for 7 days. After 7-day differentiation, cells were washed with phosphate saline buffer (PBS, pH 7.4) and stimulated for 24 h in serum-free DMEM medium containing either of DMSO (0.1%)-negative control, GW0742 (1 μM)-positive control or several concentrations of Syr.
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33. Relative mtDNA was assayed through qPCR from total DNA and normalized with nuclear gene encoding GAPDH. Set of primers specifically amplifies an 118 bp amplicon on mitochondrial NADH dehydrogenase subunit 6 (ND6) (spreading from 13601 to 13718 in *Mus musculus* mitochondrion genome). Mitochondrial density was indicated by using Mitotracker Green probe (Molecular Probes) followed the manufacturer's instructions. Briefly, after treatment myotubes for 24 h. Cells were washed with PBS pH 7.4 and incubated with Green probes (200 nM) for 30 min at 37 °C. Subsequently, cells were washed again with PBS. Green fluorescence intensity was measured with excitation and emission wavelength of 490 and 516 nm, respectively by using SpectraMAX (Molecular Devices Cor.). Images were obtained with ×40 fluorescence microscopy (Nikon) after 800 ms of exposure. Representative figures are from at least triplicate experiments that gave similar results.
34. Transient transfection. Differentiated C2C12 cells were transfected with scramble or PPARβ siRNA (SantaCruz). siRNA duplex (200 pmol) was transfected to cells using Lipofectamine 2000 reagent (Invitrogen) followed the manufacturer's instructions. After 6 h of initial transfection, the cells were transfected again. Subsequently, transfected cells were treated with compounds for 24 h before lysed for protein expression level determination.
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