Cuban Policosanol Prevents the Apoptosis and the Mitochondrial Dysfunction Induced by Lipopolysaccharide in C2C12 Myoblast via Activation of Akt and Erk Pathways

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Summary Skeletal muscle plays crucial roles in locomotion, protein reservoir, and maintenance of metabolic homeostasis. Loss of muscle, known as muscle atrophy, causes the metabolic diseases such as type 2 diabetes mellitus, hypertension, and so on. Therefore, great efforts have been devoted to prevent the muscle atrophy. Policosanols are a mixture of long chain fatty alcohols extracted from various natural sources. They have long been used as functional foods to lower the level of serum lipids, including triacylglycerol and cholesterol, and to protect against inflammatory stress. In this study, we examine the protective effect and molecular mechanism of Cuban policosanol on skeletal muscle cell death and mitochondrial dysfunction using lipopolysaccharide-treated C2C12 cells. Our results demonstrated that policosanol significantly rescued cell survival (40% vs. 88%; LPS vs. LPS+policosanol) via activation of the Akt pathway, resulting in inhibition of apoptosis ($p<0.05$). Moreover, policosanol restored the LPS-induced repression of collagen by two fold (0.33 ± 0.04 vs. 0.67 ± 0.03 compared to that of control; LPS vs. LPS+policosanol) via activation of ERK-mTOR-p70S6K pathways. In addition, policosanol increased the mitochondrial fusion by regulating the activities of DRP1 and Mfn2, leading to ameliorate the mitochondrial dysfunction induced by LPS. Improved mitochondria function increased the oxygen consumption rate with glucose as fuel source, indicating that policosanol could shift the glucose metabolism from lactate fermentation, induced by lipopolysaccharide, to oxidative phosphorylation. Thus, policosanol is a promising agent for preventing the inflammation-induced muscle cell death and mitochondrial dysfunction.

Key Words policosanol, inflammation, muscle cell death, mitochondrial dynamics, ERK-mTOR signaling

Skeletal muscles are the most abundant tissues in humans and other mammals. They play a pivotal role in regulating mammalian locomotion and protein reservoir. In addition, they also regulate glucose, lipid, and protein homeostasis to prevent the development of metabolic diseases, such as obesity, hypertension, and type 2 diabetes (*1*). Therefore, skeletal muscle health is a crucial marker of underlying human health conditions.

The skeletal muscle mass is maintained by a balance between the anabolic and catabolic metabolisms of muscle proteins (*2*). Muscle growth is mainly promoted by hormonal signaling and nutritional supplementation (*3*, *4*); insulin, growth hormone, and insulin-like growth factor 1 (IGF1) are the main hormones that promote muscle growth (*4*). These anabolic hormones activate the PI3K/Akt signaling pathway to induce protein synthesis during the development and regeneration of skeletal muscles (*3*). Several pathological conditions, including cancer, infection, diabetes, physical inactivity, aging, and malnutrition, induce muscle loss, which is defined as muscle atrophy (*5*). Skeletal muscle atrophy is a complex process that changes the muscle fiber type (*6*), impairs muscle strength (*7*), and promotes protein break-down (*8*). Muscle atrophy is mainly induced by an impaired insulin signaling pathway and occurs via cell death and protein degradation processes, such as activation of ubiquitin-proteasome and autophagy-lysosome systems (*5*). Atrogin-1/MAFbx and MuRF1 are two major ubiquitin E3 ligases involved in ubiquitin-proteasome-mediated muscle atrophy (*9*). Expression of these E3 ligases are up-regulated by the FOXO family of transcription factors via impairment of the PI3K/Akt pathway in diabetic patients and inflammation (*10*, *11*).

Mitochondrial quality control in cells is essential for adaptation to cellular energy requirement, apoptosis, and metabolic regulation in response to physiological stress. The mitochondrial length is dynamically controlled between elongation and fragmentation by fusion and fission reactions. For example, mitochondria damaged by reactive oxygen species or inflammation are fragmented by fission and subsequently removed by

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autophagy or mitophagy (*12*). Thus, the mitochondrial dynamics are tightly regulated by a mitochondrial quality control system comprising of the dynamin-related protein 1 (DRP1), optic atrophy protein-1 (Opa-1), and mitofusin 1/2 (Mfn1/2) (*12*). Parra et al. demonstrated that insulin induces mitochondrial fusion via over-expression of Mfn2 and Opa-1 and activation of the Akt-mTOR signaling pathway (*13*).

Policosanol is a mixture of long-chain (C20–36) fatty alcohols obtained from natural sources such as rice bran, wheat, sugarcane, and beeswax (*14*). Generally, it has been used as a functional food because of its various health benefits such as prevention of hyperlipidemia, hypercholesterolemia, hyperglycemia, microbial growth, and inflammation (*15*). Recently, it has been reported that Cuban policosanol shows anti-aging and tissue regeneration effect in hyperlipidemic zebrafish (*16*) and anti-hepatic inflammation by prevention of reactive oxygen species (ROS) in spontaneous hypertensive rats (*17*). However, the anti-inflammatory mechanism of policosanol remains yet to be elucidated. Thus, we hypothesized that the anti-inflammatory properties of policosanols could prevent skeletal muscle cell death and mitochondrial dysfunction in inflammatory conditions such as sepsis and insulin resistance.

Although it has been reported that octacosanol increases running endurance time in trained rats (*18*), there is no further report that policosanol has a preventive effect on the skeletal muscle impairment induced by aging and diseases. In this study, we therefore explored whether Cuban policosanol has protective effect on skeletal muscle cell dysfunction induced by lipopolysaccharide (LPS) using C2C12 mice skeletal cells. We also examined the effects of policosanol on regulating mitochondrial dynamics and the Akt and mTOR signaling pathway and revealed the possible mechanism underlying the function of policosanol in preventing skeletal muscle dysfunction.

MATERIALS AND METHODS

Materials. Cuban policosanol was obtained from Rainbow & Nature Pty, Ltd (Thornleigh, NSW, Australia) (*19*). The policosanol is composed as followed: 1-tetracosanol ($C_{24}H_{49}OH$, 0.1–20 mg/g); 1-hexacosanol ($C_{26}H_{53}OH$, 30.0–100.0 mg/g); 1-heptacosanol ($C_{27}H_{55}OH$, 1.0–30.0 mg/g); 1-octacosanol ($C_{28}H_{57}OH$, 600.0–700.0 mg/g); 1-nonacosanol ($C_{29}H_{59}OH$, 1.0–20.0 mg/g); 1-triacontanol ($C_{30}H_{61}OH$, 100.0–150.0); 1-dotriacontanol ($C_{32}H_{65}OH$, 50.0–100.0 mg/g); 1-tetratriacontanol ($C_{34}H_{69}OH$, 1.0–50.0 mg/g). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT, USA). Bacterial lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), PI3K inhibitor (Ly294002), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Inhibitors of p38 (SB202190) and ERK (PD98059) were purchased from Abcam (Cambridge, UK). Antibodies against p-Akt (Ser473), Akt, BAX, Caspase-3, DRP1, p-DRP1 (Ser616), p-DRP1 (Ser637), p-JNK (Thr183/Tyr185), JNK, phospho-p70 S6 kinase (Thr389), p70 S6 kinase, p-ERK (Thr202/Tyr204), ERK, p-p38 (Thr180/Tyr182), p38, Bcl-2, mitofusin-2, p-mTOR (Ser2448), mTOR, ATF4, MyoD, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA); mouse-MuRF1 and anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture conditions. We cultured the C2C12 mouse myoblast cell line in high glucose (4.5 g/L) DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco, Grand Island, NY, USA) in a 5% CO_2 humidified incubator at 37˚C. Cells were sub-cultured before they reached 70% confluence.

Cell viability analysis. We evaluated cell viability by the MTT assay. To this end, we seeded 3×10^3 cells/well in 96-well plates and incubated them for 24 h. Subsequently, we treated the cells with LPS and policosanol and further incubated them for 24 h. Following this, the cells were washed with serum-free medium and stained with MTT by the manufacturer's instruction. After 2 h incubation, the MTT staining solution was removed and formazan was dissolved in 150 μL of extraction solution (4 mM HCl, 10% Triton-X100, and 90% isopropanol) for 15 min. Cell viability was determined by measuring the absorbance of the formazan crystals at 590 nm using VersaMax microplate reader from Molecular Devices, LLC (San Jose, CA, USA).

Cell apoptosis measurement. We evaluated LPS-induced cellular apoptosis using the Muse® Cell Analyzer (Luminex Corp., Austin, TX, USA). We seeded 1×10^5 cells/well in 6-well plates and incubated them for 24 h. Post incubation, the cells were treated with LPS and policosanol or DMSO and were further incubated for 24 h. Apoptotic cells were detected using Muse® Annexin V and Dead Cell assay kit (Luminex Corp.).

Immunofluorescence assay. We incubated 2×10^5 cells on glass in a 6-well plate. Upon reaching the 80% confluence, the cells were treated with LPS and policosanol or DMSO and incubated for 24 h. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for another 15 min. Following this, the cells were immunostained overnight with TOM20 antibodies at 4˚C and were subsequently incubated with Alexa Fluor-568-conjugated secondary antibodies for 3 h at room temperature. Cell nuclei were stained with DAPI, and cell images were visualized using the Olympus IX-81 fluorescence microscope (Olympus Imaging, Center Valley, CA, USA).

Western blotting. To isolate proteins for western blotting, we seeded 5×10^5 cells/dish in 100 mm culture dishes. Once 80% cell confluence was reached, cells were pretreated with policosanol for 1 h before LPS treatment and further incubated for 24 h. Subsequently, the cells were lysed with RIPA buffer (Thermo Scientific, Waltham, MA, USA) containing 10 mM β-glycerophosphate, 50 mM potassium fluoride, 1 mM sodium orthovanadate, 0.5 mM EDTA, 1×Xpert protease inhibitor, and 1×phosphatase inhibitors. Protein concentrations were determined by the BCA protein test kit. Proteins

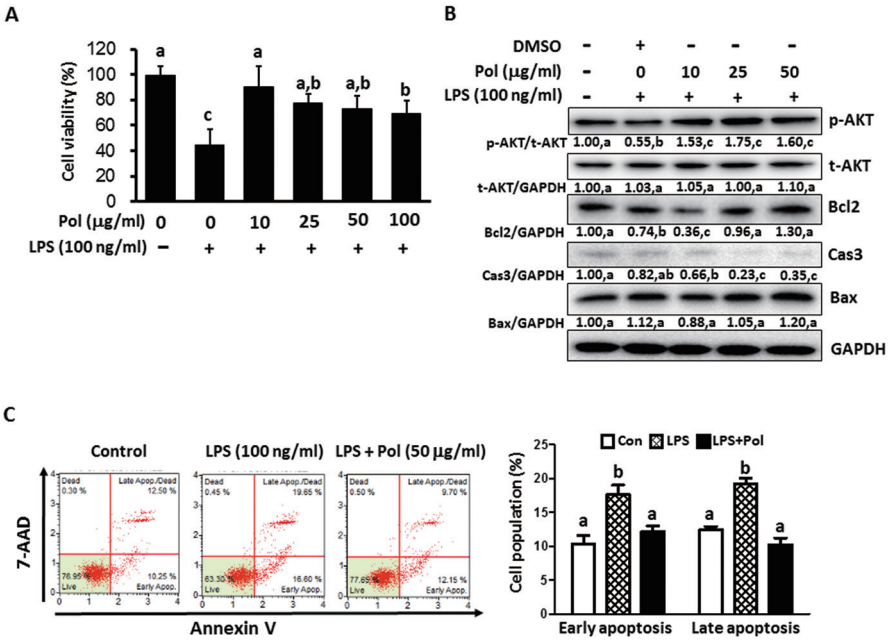

Fig. 1. Preventive effect of policosanol on LPS-induced cell apoptosis in C2C12 cells. (A) MTT analysis. C2C12 cells were cultured in 96-well plates for 24 h. The cells were treated with LPS with/without policosanol at indicated concentration for 24 h and MTT analysis was carried out. Precipitated formazan was dissolved and its concentration was determined by spectrophotometry. (B) Western blot analysis. Extracted proteins (20 μg) isolated from C2C12 cells were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Western blotting was carried out and protein bands were detected using ECL. Numbers are the mean fold-intensity ($n=3$) of each band compared to control. (C) Flow cytometry analysis for cellular apoptosis. LPS-induced cellular apoptosis was evaluated using Muse® Cell Analyzer. The data for cell viability and apoptosis are represented as the mean±SE ($n=3$). Statistical analysis was performed by One-way ANOVA test and $p\leq0.05$ was considered as statistically significant. The character of a, b, and c means the statistically different group.

(20 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes using submerged electro-transfer method. These membranes were blocked with a 5% skimmed milk in tris-buffered saline (TBST) solution, containing 0.05% Tween-20, for 1 h. Thereafter they were incubated overnight with a specific primary antibody at 4°C. On the following day, the membranes were washed with TBST solution and incubated with horseradish-conjugated secondary anti-rabbit IgG or anti-mouse IgG antibodies for 1 h at room temperature followed by washing the membrane with TBST. Protein bands were visualized using the ECL reagent and band intensity was analyzed using ImageJ software (Ver. 1.53; NIH, Bethesda, MD, USA).

Measurement of the oxygen consumption rate. Oxygen consumption rate (OCR) of intact cells was measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Santa Clara, CA, USA). In brief, 2×10^3 cells/well were seeded in an XF24 cell culture plate and incubated for 24 h. Cells were incubated with LPS and policosanol or DMSO for 24 h. Prior to the measurement, the cells were kept in DMEM containing 25 mM glucose and 1 mM pyruvate. After equilibration, OCR measurement was carried out by injecting electron transporter chain inhibitors in the cells; 1 μM Oligomycin, 5 μM CCCP, and mixture of Rotenone (1 μM)+Antimycin (5 μM). OCR data were normalized by cell number after DAPI staining.

Measurement of the lactate concentration in cells. To measure lactate production in C2C12 cells, we cultured the cells in complete DMEM till 80% confluence was reached. Subsequently, cells were washed with Hank's balanced salt solution (HBSS) and kept in DMEM without pyruvate and serum. These cells were pretreated with 50 μg/mL of policosanol or DMSO for 1 h, following which the cells were treated with 100 ng/mL of LPS. Post 24 h of incubation, the culture media were collected and deproteinized using perchloric acid (6% final concentration). Following this, cell lysates were centrifuged at 10,000 $\times g$ for 10 min and the supernatants were neutralized using 30% KOH solution. The neutralized solutions were centrifuged for 10 min at 10,000 $\times g$. The lactate concentration was measured enzymatically using a spectrophotometer (*20*).

Statistical analysis. Obtained data were expressed as mean±SE. Statistical analysis was performed by One-way ANOVA test and a value of $p\leq0.05$ was considered as statistically significant.

RESULTS

Policosanol prevents LPS-induced apoptosis in C2C12 cells

To investigate the anti-inflammatory effect of policosanol, we first tested cell viability by the MTT assay. We observed that policosanol inhibited LPS-induced cell death in a dose-dependent manner (Fig. 1A). Moreover,

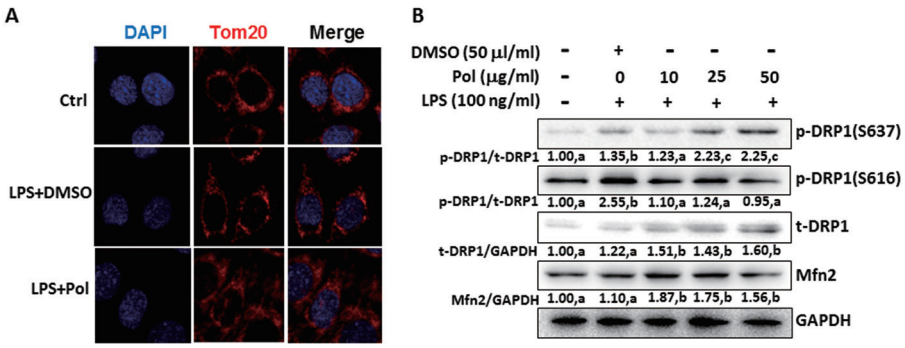

Fig. 2. Effect of policosanol on mitochondrial dynamics. (A) Change in mitochondrial morphology. C2C12 cells were treated with LPS and policosanol for 24 h, fixed with 4% paraformaldehyde, and immunostained with TOM20 antibody. Fluorescence was detected using a fluorescence microscope. (B) Western blot analysis of p-DRP1(S637), p-DRP1(S616), and Mfn2. Extracted proteins were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. Western blotting and protein bands were detected using ECL. Numbers are the mean fold-intensity ($n=3$) of each band compared to control. Statistical analysis was performed by One-way ANOVA test and $p \leq 0.05$ was considered as statistically significant. The character of a, b, and c means the statistically different group.

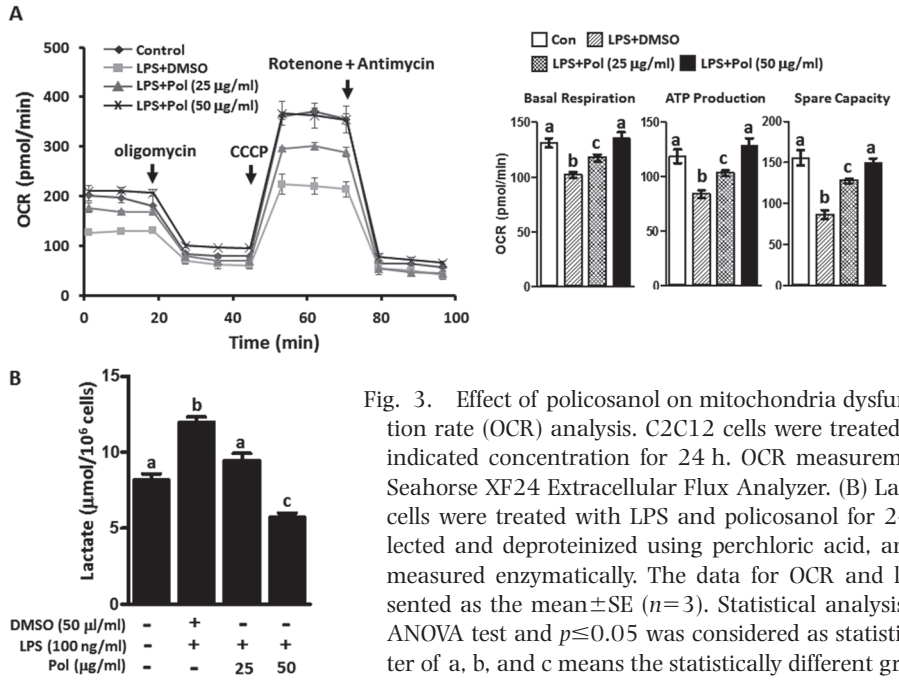

Fig. 3. Effect of policosanol on mitochondria dysfunction. (A) Oxygen consumption rate (OCR) analysis. C2C12 cells were treated with LPS and policosanol at indicated concentration for 24 h. OCR measurement was carried out using a Seahorse XF24 Extracellular Flux Analyzer. (B) Lactate production rate. C2C12 cells were treated with LPS and policosanol for 24 h. Culture media were collected and deproteinized using perchloric acid, and lactate concentration was measured enzymatically. The data for OCR and lactate production are represented as the mean±SE ($n=3$). Statistical analysis was performed by One-way ANOVA test and $p \leq 0.05$ was considered as statistically significant. The character of a, b, and c means the statistically different group.

policosanol treatment significantly increased the phosphorylation of Akt and expression level of Bcl2 (an anti-apoptotic protein). This led to a reduction in caspase 3 expression (a marker of apoptosis) in a dose-dependent manner. Thus, we inferred that the protective effect of policosanol might be because of the activation of the Akt signaling pathway (Fig. 1B). Additionally, we validated the anti-apoptotic effect of policosanol by fluorescence-activated cell sorting using Annexin V and 7-AAD. Consequently, we found that policosanol inhibited LPS-induced early- and late-apoptosis in C2C12 cells (Fig. 1C). These observations strongly indicated that policosanol had a preventive effect on LPS-induced cell death.

Policosanol prevents the mitochondrial fragmentation induced by LPS in C2C12 cells

Mitochondria is fragmented when inflammatory conditions, such as type 2 diabetes, cancer, sepsis, and muscle atrophy, induce endoplasmic reticulum (ER) stress (*21*). Therefore, we examined the policosanol effect on LPS-induced mitochondrial dynamics in C2C12 cells. In this regard, we performed immunofluorescence staining of the mitochondrial outer membrane protein, TOM20. As shown in Fig. 2A, policosanol prevented the LPS-induced mitochondrial fragmentation in C2C12 cells via inhibition of phosphorylation of DRP1 at Ser616, a mitochondrial fission-promoting phosphorylation site (Fig. 2B). In addition, policosanol increased the phosphorylation of DRP1 at Ser637, an inhibitory phosphorylation site for mitochondrial fission, and increased the expression of Mfn2 in a dose-dependent manner. These observations suggested that Cuban policosanol ameliorates the mitochondrial dysfunction upon LPS treatment cells.

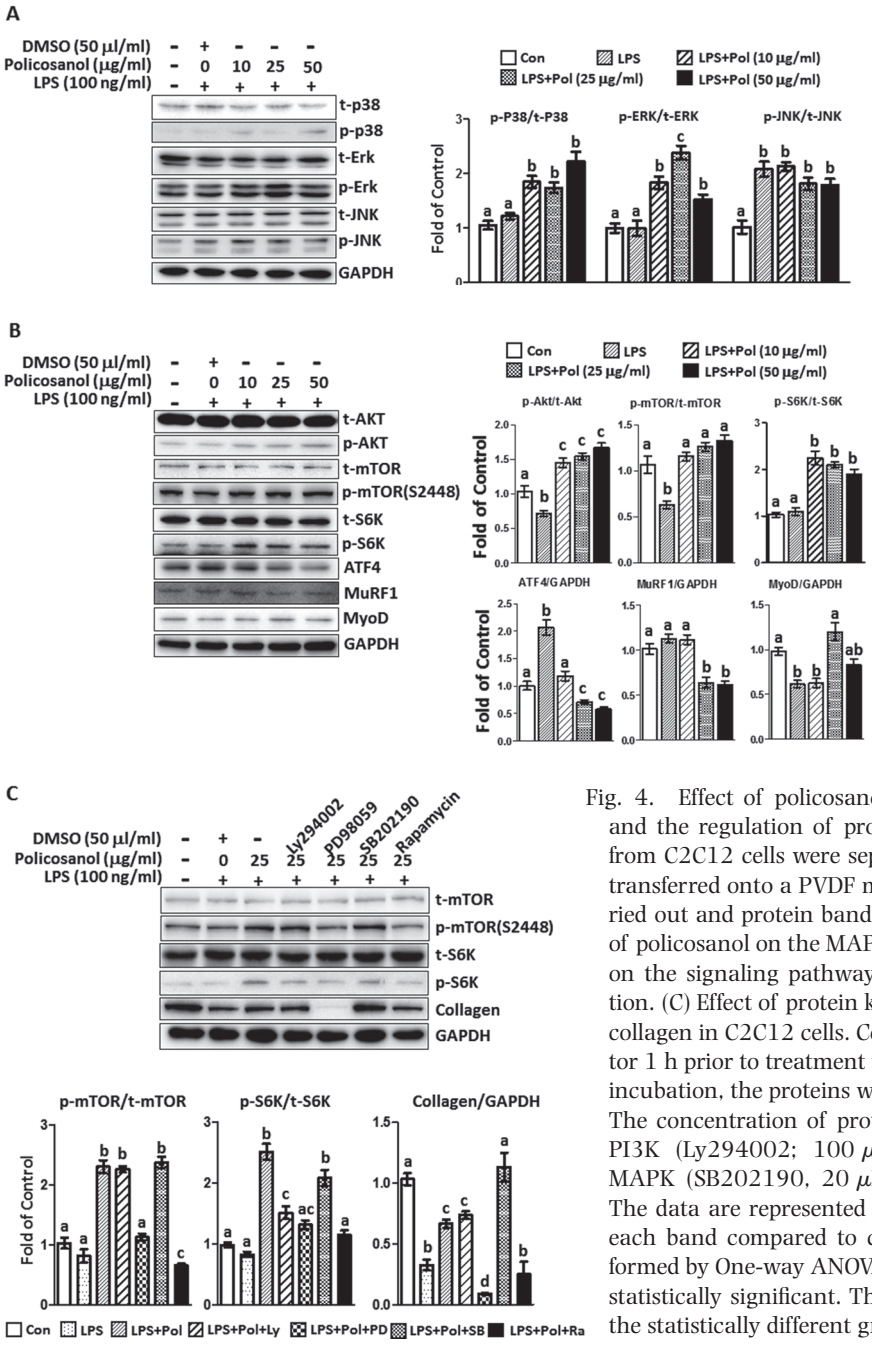

Fig. 4. Effect of policosanol on the Akt and MAPK pathways and the regulation of protein metabolism. Proteins extracted from C2C12 cells were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. Western blotting was carried out and protein bands were detected using ECL. (A) Effect of policosanol on the MAPK pathways. (B) Effect of policosanol on the signaling pathway for protein synthesis and degradation. (C) Effect of protein kinase inhibitors on the expression of collagen in C2C12 cells. Cells were pretreated with each inhibitor 1 h prior to treatment with policosanol and LPS. After 24 h incubation, the proteins were isolated for western blot analysis. The concentration of protein kinase inhibitor was as follows; PI3K (Ly294002; 100 μM), Erk (PD98059, 20 μM), p38 MAPK (SB202190, 20 μM), and mTOR (rapamycin, 5 μM). The data are represented as the mean fold-intensity ($n=3$) of each band compared to control. Statistical analysis was performed by One-way ANOVA test and $p \leq 0.05$ was considered as statistically significant. The character of a, b, c, and d means the statistically different group.

Policosanol increased the oxidative phosphorylation of glucose metabolism in the LPS-treated C2C12 cells

Subsequently, we evaluated the OCR in C2C12 cells to determine whether policosanol ameliorated mitochondrial dysfunction in LPS-induced C2C12 cells. With glucose as an energy source, policosanol administration significantly increased the basal OCR and the maximal OCR, both of which were lower in LPS-treated cells than those in control cells (Fig. 3A). In addition, we observed that ATP generating OCR increased in policosanol-treated cells compared to that in LPS-treated cells by dose-dependently. This observation indicated that policosanol rescued mitochondrial dysfunction induced by LPS. Mitochondrial dysfunction could alter the glucose metabolism from oxidative phosphorylation (OxPhos) to lactate production (*22*, *23*). Policosanol significantly reduced the LPS-induced lactate production in C2C12 cells by dose-dependent manner (Fig. 3B) which due to increase the OxPhos, indicating that policosanol improves the mitochondrial function.

Policosanol increases the expression level of collagen in LPS-treated C2C12 cells via activation of the ERK-mTOR-p70S6K signaling pathway

In order to reveal the molecular mechanism underlying the preventive effect of policosanol on LPS-induced inflammation, we monitored protein metabolism (synthesis and breakdown) and the MAPK signaling pathways, which are the inflammatory signaling pathways (*8*, *23*). Among the MAPK proteins, only JNK was phosphorylated upon LPS treatment, whereas phosphorylation status of p38 and ERK were unchanged (Fig. 4A). However, we observed that phosphorylation of p38 and

ERK was increased in a dose-dependent manner upon policosanol treatment. On the other hand phosphorylation of JNK was unchanged upon policosanol treatment (Fig. 4A). Additionally, policosanol up-regulated the Akt and the mTOR signaling pathways, leading to increase in the phosphorylation of p70S6K and the expression of MyoD, a key regulator of muscle differentiation and protein synthesis (*24*) (Fig. 4B). Additionally, policosanol down-regulated the ATF4, an ER stress maker, and the MuRF1, an E3 ligase, indicating that policosanol prevents the LPS-induced ER stress and the ubiquitin-dependent protein degradation (Fig. 4B).

Next, we investigated the impact of kinase inhibitors on the beneficial effect of policosanol on the protein metabolism in LPS-treated C2C12 cells. ERK inhibitor (PD98059) and rapamycin, but not the p38 inhibitor (SB202190), significantly inhibited the policosanol-induced mTOR/p70S6K signaling pathway in LPS-treated C2C12 cells, leading to down-regulate the expression of collagen A1 (Fig. 4C). Surprisingly, the PI3K inhibitor could not inhibit policosanol-induced mTOR activation (Fig. 4C), suggesting that policosanol activates the mTOR by the PI3K-independent manner. These observations suggested that policosanol may exhibit multiple functions to regulate cell survival and protein metabolism in C2C12 cells.

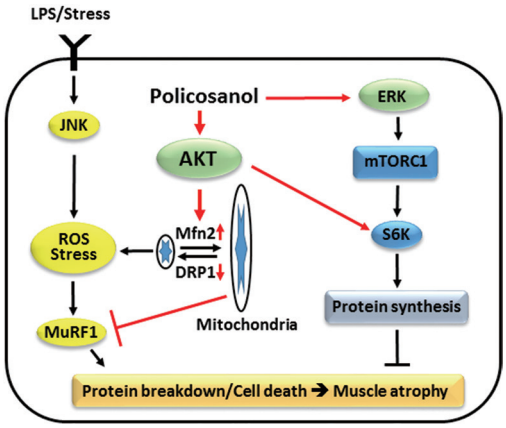

Fig. 5. Proposed model for the protective effect policosanol on the apoptosis and the dysregulation of protein metabolism induced by LPS in the skeletal muscle cells. Cuban policosanol activates the Akt-p70S6K signaling pathway and the Erk-mTOR-p70S6K signaling pathway, leading to protect the cell death, the mitochondrial dysfunction, and the dysregulation of protein metabolism induced by LPS in C2C12 cells.

DISCUSSION

This study aimed to determine whether policosanol has a protective effect on the deteriorative cellular functions induced by LPS using skeletal myoblast C2C12 cells. Previously, we had reported that LPS induces the apoptosis in myoblast by the ROS dependent manner (*23*). In this study, we showed that policosanol significantly ameliorated LPS-induced cellular dysfunction via activation of the Akt-dependent anti-apoptosis pathway and restoration of mitochondrial functions, which presumably resulted in reducing of the ROS stress (Fig. 1B, Fig. 2B, and Fig. 5). Furthermore, policosanol improved the LPS-induced impairment of protein metabolism in C2C12 cells via activation of the Akt-p70S6K and ERK-mTOR-p70S6K and inhibition of ROS induced the expression of E3 ligases (Fig. 4 and Fig. 5).

Previously, several reports have shown that policosanol exhibits anti-diabetic effects by increasing insulin sensitivity and improving metabolism (*25*, *26*). In this study, we also observed that policosanol increased phosphorylation of Akt in LPS-induced insulin-resistant C2C12 cells, leading to increased cell survival. Since insulin resistance is associated with accelerated muscle loss in type 2 diabetes mellitus (*27*, *28*), activating insulin signaling by policosanol presumably activates cell proliferation in skeletal muscle. However, our findings and previous study (*26*) could not reveal the exact molecular mechanism underlying policosanol-mediated Akt phosphorylation. In Fig. 4C, we observed that PI3K inhibitors could not inhibit the activation of mTOR induced by policosanol treatment in C2C12 cells, indicating that policosanol may phosphorylate Akt (Ser437) in a PI3K-independent manner. IKKϵ, TBK1, and DNA-PK also phosphorylate the Akt (Ser437), which are not inhibited by PI3K inhibitor (*29*). Therefore, further study is required to elucidate how policosanol activate Akt signaling pathway.

Since mitochondria play pivotal roles in multiple biological processes, including cellular bioenergetics, supplying building materials, and apoptosis, it is crucial to maintain these mitochondrial functions. For example, impaired insulin signaling and oxidative stress translocates DRP1 onto the mitochondria and inhibits Mfn2, causing mitochondrial fragmentation and cellular dysfunction (*30*, *31*). In this study, we found that policosanol prevented LPS-induced mitochondrial fragmentation by down-regulating fission-promoting and up-regulating fusion-promoting phosphorylations on DRP1. Additionally, policosanol increased the mitochondrial fusion by up-regulating Mfn2 expression. Moreover, up-regulation of the insulin signaling pathway may induce mitochondrial elongation; a number of studies have reported that change in mitochondrial morphology is closely associated with mitochondrial function (*30*, *32*). Indeed, we found that policosanol significantly improved the OCR in LPS-treated C2C12 cells. Furthermore, impaired mitochondrial function shifts glucose metabolism from oxidative phosphorylation to lactate production (*23*, *33*). Our study indicated that policosanol increases oxidative phosphorylation, resulting in decrease of lactate production and improvement of mitochondrial dysfunction.

The MAPK cascades play crucial roles in the regulation of most cellular processes, including cell proliferation, differentiation, stress responses, and metabolic adaption (*34*). Three classes of MAPKs, such as ERK, JNK, and p38 are identified in mammals. Each MAPK is differentially involved in the regulation of cell cycle, apoptosis, gene expression, and enzyme activity. More-

over, studies have reported that lipopolysaccharides induce tissue inflammation via the TLR4-MAPK signaling pathway (*35*, *36*). For instance, LPS significantly induces the JNK pathway, inhibiting the Akt-mTOR signaling pathway, which presumably induces muscle atrophy. Contrastingly, activation of the ERK stimulates cell growth and differentiation (*34*). In the present study, we demonstrated that policosanol activated the ERK, Akt, and mTOR signaling pathways, suggesting that policosanol prevented LPS-induced apoptosis in skeletal muscles and increased cell survival (Fig. 1).

Skeletal muscle mass is regulated by a dynamic balance between anabolic and catabolic protein metabolism. Insulin and IGF1 are known to activate both the ERK and the PI3K-Akt-mTOR pathways to increase muscle mass. Impairment of these pathways induces muscle atrophy by activation of ubiquitin- and autophagy-dependent protein degradation (5). Numerous studies have reported that activation of ERK increases collagen synthesis in fibroblast cells (*37–39*). In this study, we also demonstrated that policosanol activated the ERK-mTOR-p70S6K and Akt-p70S6K pathways, which in turn restored the protein metabolism in C2C12 cells. We confirmed this result by using an ERK inhibitor, which inhibited the policosanol-induced collagen synthesis (Fig. 4C). In addition, policosanol prevented the LPS-induced expression of E3 ligases, well agreed to other study (*10*). Numerous studies have reported that activation of p38 MAPK increases protein synthesis by up-regulating translation initiation (*40*) and rRNA processing (*41*). In contrast those reports (*40*, *41*), our observation was that activation of the p38 MAPK pathway by policosanol might not be involved in promoting protein synthesis because a p38 MAPK inhibitor could not inhibit collagen synthesis in C2C12 cells (Fig. 4C).

In conclusion, Cuban policosanol stimulates the Akt signaling pathway to prevent the LPS-induced apoptosis and the Erk-mTOR-p70S6K pathway to prevent the LPS-induced protein degradation in C2C12 cells, which presumably due to ameliorating mitochondrial dysfunction. Although precise molecular mechanisms underlying the involvement of Cuban policosanol in the protection on skeletal muscle remains to be determined, our data showed Cuban policosanol are potent anti-inflammatory molecules, which protect the skeletal muscle damage induced by sepsis, aging, and metabolic diseases. However, further study is required to examine the beneficial effect of policosanol on muscle atrophy using in vivo models.

Authorship

Research conception and design: NHJ, K-HC, and ALJ; experiments: ALJ, JWH, JIA, and NHJ; statistical analysis of data: ALJ, NHJ, and K-HC; writing of the manuscript: NHJ and K-HC. All authors read and approved the final manuscript.

Disclosure of state of COI

The authors declare no conflicts of interest concerning this article.

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