Protective effects of delta-like 1 homolog against dexamethasone induced muscular atrophy

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Directed by Professor Bong-Soo Cha

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I am grateful to my family for always supporting me. Thank you.

Ji Young Lee
Yonsei University, College of Medicine
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ABSTRACT

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Skeletal muscle atrophy is accelerated in elderly patients with diabetes and caused by the imbalance between muscle growth and wasting. To identify whether delta-like 1 homolog (DLK1) treatment can prevent muscle wasting, we assessed muscle mass as well as muscle differentiation and atrophy markers in dexamethasone-induced atrophy models: mice were randomly divided into three groups; (1) control group, (2) atrophy group by dexamethasone (Dex group), and (3) DLK1 administration to atrophy mice (DLK1 group). The expressions of genes related to muscle differentiation and atrophy were determined using RT-PCR. We observed that dexamethasone reduced muscle mass and markedly increased atrophy makers including atrogin1 and MuRF1. DLK1 treatment attenuated these degenerative changes. Furthermore, the level of myostatin, which inhibits muscle cell growth, was reduced in DLK1 treatment group compared with Dex group. Cell experiments were performed using C2C12 myotubes. DLK1 treatment significantly attenuated dexamethasone-induced destructions of myotube cells. In adddition, DLK1 treatment suppressed the signaling pathway in which endogenous myostatin activated during atrophy progress. These results

suggest that DLK1 attenuates dexamethasone-induced muscle atrophy in mice potentially by suppressing the downstream signaling of myostatin/myogenin/atrogin1/MuRF1 pathway. Interestingly, in the analysis of electron microscope, therapeutic effect of DLK1 did not only affect muscle atrophy, but it improved intracellular mitochondria as well. Furthermore, we found that muscular atrophy could result in mitochondrial biogenesis impairment and that the DLK1 treatment could supplement mitochondrial biogenesis.

Our study implies that DLK1 could be a promising candidate in the treatment of aging or diabetes-related sarcopenia, characterized by muscle atrophy and dysfunction.

Key words: delta-like 1 homolog, muscular atrophy, myostatin, myogenin, atrogin1, MuRF1.

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I. INTRODUCTION

Muscle atrophy is induced by various conditions such as fasting, disuse, injury, side effects of pharmaceutical therapy, and aging. Muscular atrophy can cause falls which leads to a serious social problems, particularly in an aging society.[1] Muscular atrophy is the loss of skeletal muscle mass as a consequence of increased myofibrillar protein degradation and its decreased protein synthesis.[2] This study investigated whether DLK1 could be a potential therapeutic substances to improve muscular atrophy and clarified their mechanism of action.

In this study, DLK1 protein was used as a candidate molecule for treatment of muscular atrophy. To investigate the therapeutic effect of the DLK1 on the animal model of atrophy, soluble DLK1 peptide consisted of the N-terminal extra cellular domain of DLK1 fused with a human Fc fragment, was generated. This recombinant protein contains six epidermal growth factor-like domains and the juxtamembrane portion, which corresponds to residues 25–302 of the DLK1 (P80370, UniProt Knowledge base). Delta-like 1 homolog (DLK1) is a transmembrane protein belonging to the epidermal growth factor-like

repeat-containing family. Waddell *et.al*, reported that DLK1 is involved in muscle regeneration as well as differentiation into muscle tissue and skeletal muscle formation.[3]

In the current study, muscle atrophy C57BL/6J mouse model experiments were conducted. High concentrations of dexamethasone were administered to C57BL/6J mice to induce muscular atrophy.[4] Increased cytokines such as myostatin after dexamethasone treatment was associated with a decreased muscle mass, and dexamethasone treatment caused typical atrophy conditions. Dexamethasone-treated C2C12 myotubes revealed atrophied features along with increased endogenous myostatin expression and other metabolic changes, which were previously found in experimental model of the atrophied muscles.[5] The *in vitro* dexamethasone-induced C2C12 cell atrophy model is a convenient and well established model to clarify the effects and mechanisms of active ingredients such as DLK1.

Here, we presented dexamethasone-induced muscle wasting and degeneration as a representative model of atrophy. High doses or long-term usage of dexamethasone have serious side effects including muscular atrophy.[6] Muscular atrophy in mice can be induced by continuously injected dexamethasone at high concentrations.[7] Among the various muscular atrophy models, high dose dexamethasone was mainly used this study to establish a muscular atrophy model. Previous studies of glucocorticoid's effects on C2C12 myoblasts demonstrated that high doses of the glucocorticoids (dexamethasone or prednisolone) induced cell death and myogenic differentiation factor (Myod1) degradation via the ubiquitin proteasome pathway.[8]

Glucocorticoids stimulate the production of myostatin. Myostatin acts as a negative regulator of muscle mass gain by inhibiting the proliferation of myoblasts. Myostatin is an important regulator of muscular atrophy. Increased expression of myostatin may have been observed in the blood and tissues of mice with muscle atrophy. In addition, the mice muscle was overdeveloped when the expression of myostatin gene is knocked out[9].

Myostatin pathway has pleiotrophic roles in many fundamental processes during

early development and mature tissue homeostasis by regulating cell fate determination, proliferation, differentiation and death. Myostatin, also known as growth differentiation factor 8, is a secreted growth differentiation factor that is a member of the TGF-β protein family.[10] At the same time, myostatin is a myokine which is produced and released by myocytes. It acts on muscle cells in an autocrine fashion to inhibit myogenesis.

Recent studies show that muscle-specific E3 ubiquitin ligases, atrogin1/muscle atrophy F-box (atrogin1) and muscle RING-finger protein-1 (MuRF1), the so-called ‘atrogenes', have important functions in muscular atrophy associated with dexamethasone treatment.[11]

Myogenin, which is essential during differentiation, is expressed upon the differentiation of myoblasts to multinucleated myotubes. Mice that lack the myogenin gene, died at birth due to severe skeletal muscle deficiency, as myoblasts are unable to fuse into multinucleated myofibers in those mice.[12]

We further investigated whether muscular atrophy could result in mitochondrial biogenesis impairment and demonstrated the beneficial effect of DLK1 on mitochondrial biogenesis. Mitochondrial biogenesis as a process by which new and functional mitochondria are generated in the cells, plays a pivotal role in the replacement of impaired mitochondria.

This study had the following four research goals and investigated the goals using animal and cell experiments: quantitative changes in muscle fibers, qualitative changes in muscle fibers, changes in mitochondrial biogenesis, and mechanism of action of DLK1 protein, related to muscular atrophy. Therefore, we evaluated the therapeutic effects of recombinant DLK1 protein in a muscle atrophy model.

II. MATERIALS AND METHODS

1. Experimental animals and study design

Eight-week-old male C57BL/6J mice were purchased (Joong-ang Experimental Animal Co., Seoul, Korea) and acclimatized for 2 weeks. Mice were housed in an animal facility maintained at a temperature of 23 ± 2°C and a humidity of 55 ±

5%. Mice were exposed to a 12 h light, 12 h dark cycle and fed an unrestricted standard chow diet. All animals had free access to drinking water. Dexamethasone (Dex) or distilled water (DW) was administrated daily by oral gavage (PO). Delta-like 1 homolog (DLK1) or phosphate buffered saline *(*PBS) was injected daily by intraperitoneal (IP) into mice**.** Ten-week-old mice were divided into three groups: (1) control group (PBS for IP and DW for PO; n = 6), (2) Dex group (PBS for IP and 1 mg/Kg Dex for PO; n = 8), and (3) DLK1 group (0.8mg/Kg DLK1 for IP and 1mg/Kg Dex for PO; n = 8). PBS and DW were purchased from DaiHanPharm (Ansan, Korea), and Dex was purchased from Sigma Aldrich (St. Louis. MO, USA). Body weight was measured every two days. Following 2 weeks of treatment, mice were fasted for 6 h, then sacrificed. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System.

2. Atrophy model

To generate a dexamethasone-induced muscle atrophy mouse model, we administered dexamethasone (D2915, Sigma-Aldrich, saint louis, MO, USA) by oral gavage at 1 mg/kg/day to 10-week-old C57BL/6J male mice for 2 weeks. Dexamethasone induced atrophy was produced by treating C2C12 cells on the fourth day of differentiation with 10 μM dexamethasone (D4902) for 24 h. Control cells were incubated with the same volume of ethanol.

3. Tissue collection and histological analysis

Tibialis aterious muscle (TA) tissue was fixed using 10% PBS buffered formaldehyde for 48 hrs, paraffin-embedded and sectioned into 5-μm thick slices for hematoxylin and eosin (H&E) or Masson's trichrome staining. After sealing the slides with neutral gum, the stained tissue slices were examined at 100× magnification. Histological images were analyzed using ImageJ software (NIH Image, Bethesda, MA, USA).

4. Biochemical measurements

Blood samples were obtained with syringe by puncture into the heart and transferred to a Microtainer blood collection tube (Becton Dickinson, Franklin Lakes, NJ, USA) for analysis. Blood was immediately centrifuged at 13,000 *g* for 3 min at 4 ℃. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and triglyceride (TG) were measured by Fuji Drichem clinical chemistry analyzer (Fuji film, Tokyo, Japan).

5. Body composition analysis using dual energy X-ray absorptiometry

On the day before the experiment was completed, four mice from each group were selected and subjected to dual energy X-ray absorptiometry (DXA, InAlyzer Medikors, Seongnam, Gyeonggi, Korea) for whole body composition analysis. DXA uses two separate low-dose X-ray exposures to read bone and soft tissue mass with a high degree of precision. Prior to the start of the experiment, the system was calibrated according to the manufacturer's instructions. Software integrated to the scan was used for data analysis. The mice for which the experiment was completed were re-rearing.

6. Transmission electron microscopy (TEM)

Two mice with a median body weight in each group were selected as representative mice and sampled. A minimum of 5 tissue sites was randomly observed and a representative image was presented. Samples of tibialis anterior (TA) muscle were fixed with 2% glutaraldehyde in paraformaldehyde (Merck & Co., Inc., Kenilworth, NJ, USA) overnight at 4℃ and post-fixed for one hour in 1% OsO4 resin (Polysciences Inc., Warrington, PA, USA). Samples were then dehydrated in ethanol (Merck & Co.), embedded in rubber molds with epoxy resin (Polysciences Inc.), and polymerized in an oven at 60℃ for 20 hrs. Semithin sections (1 μm thick) were cut using a Leica Ultracut UCT (Leica Microsystems Inc. Buffalo Grove, IL USA) and stained with toluidine blue (Merck). Ultrathin sections (70 nm thick) were cut and mounted on coated copper grids (Nisshin EM, Tokyo, Japan) and double stained with 6% uranyl acetate (Ted Pella Inc, Redding, CA, USA) and lead citrate (Wako, Osaka, Japan). The

ultrastructure of tissue sections was observed with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 80Kv. Images were viewed with Camera-Megaview III software (EMSIS Muenster, Germany).

7. Quantitative real-time PCR

Total RNA was extracted using a Hybrid-R RNA purification kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. cDNA synthesis was performed on 1.0 μg total RNA per sample in a 20 μL reaction using high-capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA) and 2.5 μM random primers. The reaction conditions were: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min. cDNA samples were then stored at −20°C until processing. Quantitative real-time PCR (qPCR) was performed in 10 μl reactions containing 1.0 μl cDNA, 5 pmol of each oligonucleotide primer and 5.0 μL of Power SYBR Green PCR Master Mix (Life Technologies, Warrington, UK). qPCR reactions were performed in a StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA,USA) in a 96-well plate. The reaction conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Expression levels were determined by comparing the cT of each sample with the cT of the corresponding sample at the 1-cell stage normalized to the cT of 18S ribosomal RNA(18S). All primers sets were validated by the presence of a single peak when a melt-curve analysis was performed. All reactions were performed in triplicate and repeated in two independent experiments for TA muscle tissue and C2C12 myotube. Expression of 18S ribosomal RNA(18S), Myostatin, muscle atrophy F-box protein (atrogin1), muscle RING-finger protine-1(MuRF1), Myod1, myogenin, GTPase mitochondrial dynamin like GTPase (Opa1), and dynamin-related protein-1 (Drp1). The mouse cDNA sequences were obtained from GenBank (Table 1).

Table 1. Primers used for real-time quantitative PCR

Mouse primers	Sequences (5'→ 3')
18S ribosomal RNA (accession No. NR_003278.3)	
Forward	GATGTGAAGGATGGGAAGTACAG
Reverse	CTTCTTGGATACACCCACAGTTC
myostatin(accession No. NM_010834.3)	
Forward	ACGCTACCAGGGAAACAATC
Reverse	AAAGCAACATTTGGGCTTTC
atrogin1(accession No. NM_026346.3)	
Forward	ATGCACACTGGTGCAGAGAG
Reverse	TGTAAGCACACAGGCAGGTC
MuRF1(accession No. DQ229108.1)	
Forward	TGAGGTGCCTACTTGCTCCT
Reverse	TCACCTGGTGGCTATTCTCC
Myod1 (accession No. NM_010866.2)	
Forward	GCTGCCTTCTACGCACCTG
Reverse	GCCGCTGTAATCCATCATGC
myogenin (accession No. NM_031189.2)	
Forward	CAGTGAATGCAACTCCCACAG
Reverse	TGGACGTAAGGGAGTGCAGA
Opa1 (accession No. XM_006522656.4)	
Forward	CTTGCCAGTTTAGCTCCCGA
Reverse	CCATTTGGGACCTGCAGTGAA
Drp1 (accession No. NM_001276340.1)	
Forward	GCCTCAGATCGTCGTAGTGG
Reverse	TGCTTCAACTCCATTTTCTTCTCC

MuRF1: muscle RING finger protein 1; Myod1: myogenic differentiation 1; Opa1: mitochondrial dynamin like GTPase; Drp1: dynamin-related protein 1.

9. Cell culture

Mouse myoblast cell line C2C12 was purchased from the American Type Culture Collection (Manassas, USA) and grown in Dulbecos Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen-Gibco, Canada) and 1% penicillin/streptomycin at 37℃ in 5 % CO_2. After growing to 100% confluence, cells were further cultured in DMEM containing 2% horse serum (differentiation medium; DM) for 5 days to induce differentiation of myotubes. The first day of incubation in differentiation medium was considered day 0 of differentiation. DM was changed every 24 hours. C2C12 myotubes were grown as described above and on differentiation day 4 were treated with different media (1) control group, 0.02% v/v ethanol; (2) Dex group, 10 μmol dexamethasone; (3) Dex + DLK1 group, 10 μmol dexamethasone and 5.06 μg/ml) added to culture medium (DM) in triplicate wells. After 24 h growth, cells were harvested for extraction of total RNA, for analysis by reverse transcription polymerase chain reaction (RT-PCR). Experiments were repeated three times to confirm the accuracy of the results.

10. Myostatin, siRNA knock down test

For siRNA silencing, C2C12 cells were transfected with siRNAs the complexed with Lipofectamine 2000 reagent (Invitrogen, CA, USA), according to manufacturer instructions. Double-stranded small interfering RNAs (siRNAs) targeting myostatin (20 nM) were purchased from QIAGEN (Cambridge, USA). C2C12 cells were transfected with negative control siRNA or myostatin siRNAs according to the manufacturer's instructions.

Myostatin siRNA transfection was performed two times on myotube cells of day 0 and day 3 of differentiation and tested on the transfected cell line with 70% myostatin gene knockdown expression. After sufficient transfection, C2C12 cells were washed with PBS, and growth medium with added DLK1 or DLK1 dexamethasone was changed on cell differentiation day 4. After 24 hrs, cells were harvested for western blot analysis.

11. Western Blot Analysis

C2C12s were grown for 4 days, with differentiation medium changed every 24 hrs. On the day of the experiment, fresh medium was added, and treatments were performed as follows. Cells were washed with PBS, scraped off in ice-cold PhosphoSafe buffer (Novagen), and centrifuged for 5 min at 16,000 ×g (4°C). Protein concentration was determined from clear supernatants by Bradford reagent (Sigma Aldrich) using BSA as a control. Sample aliquots were boiled for 5 min, and equal amounts (20 μg) of total protein were electrophoresed in SDS/PAGE 10% gradient gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) and assessed for equal loading/transference by Ponceau s red stains. Samples were immunoblotted overnight with the indicated primary antibodies (typically 1:1,000 dilution) followed by secondary antibody conjugated with horseradish peroxidase (1:5,000 dilution). The supersignal west pico plus kit (Thermo) was used for detection. Proteins of interest were detected with the following specific antibodies: myogenin (F5D, Abcam, Boston, USA), GDF8/myostatin (Abcam), and GAPDH (6C5, santacruz biotechnology, INC., Texas, USA).

12. Statistical analysis

Results are presented as means ± standard error of the mean. Statistical significance was calculated using one-way analysis of variance (ANOVA) to assess differences between the groups. Additionally, the student's t-test and the F-test were used to compare DXA analyses. A p value <0.05 was considered statistically significant. Experiments were repeated three times, until the accuracy of the results were confirmed. All statistical procedures were conducted using GraphPad Prism statistical software (version 5, GraphPad Software, San Diego, USA).

III. RESULTS

1. Biochemical characterization

Dexamethasone at oral dose 1.0 mg/kg was used as an atrophy mice inducer.

Dexamethasone induced atrophy mice treated with DLK1 by intraperitoneal route with a dose of 0.8 mg/kg for 14 days. The same volume of PBS was injected intraperitoneally into the control group and Dex group. Body weight changes in all three groups were gradually increased during the study period (Figure 1A). There was no difference in serum TG levels between the Dex group and the control group. The serum TG levels were significantly decreased in the DLK1 group compared with the Dex group ($p < 0.05$), (Figure 1 B). Serum levels of ALT were significantly increased in the Dex group compared with the control group (Figure 1C). Serum levels of ALT were significantly increased in the Dex group compared with the control group ($p < 0.05$). There was no difference in serum AST levels between the the Dex group and the DLK1 group (Figure 1D).

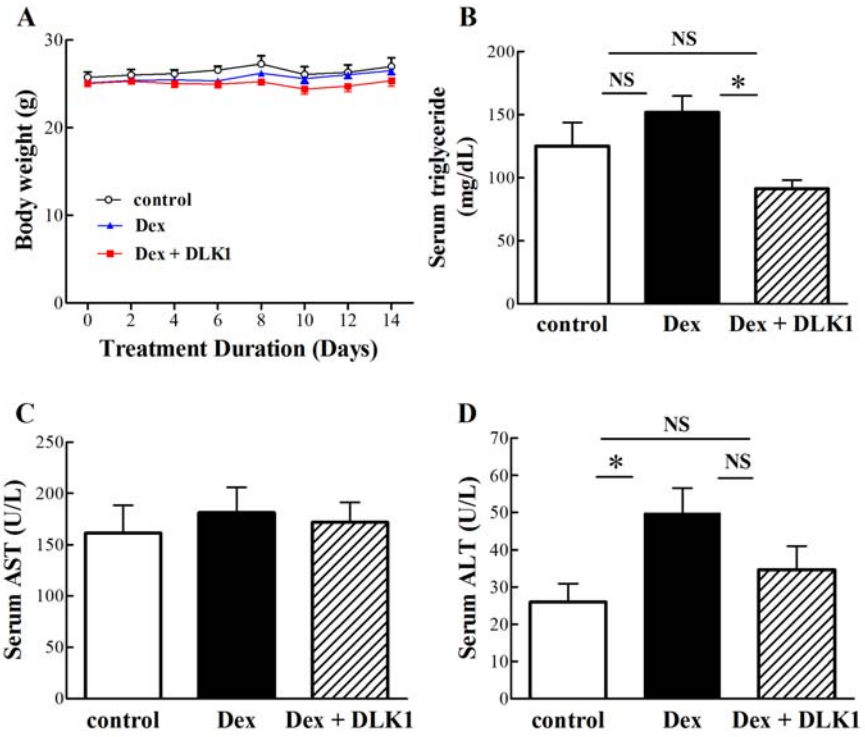

Figure 1. Body weight changes and chemical characteristics in C57BL/6J mice according to dexamethasone-induced muscle atrophy and DLK1 treatment. (A)

body weight changes observed after 2weeks of daily intraperitoneal DLK1 treatment and daily oral dexamethasone treated C57BL/6J mice. Concentration of (B) serum triglyceride (mg/dL). (C) serum aspartate transaminase (AST, U/L) and (D) serum alanine aminotransferase (ALT, U/L). control group (Sham operation, n = 6), Dex group (1 mg/Kg dexamethasone PO and PBS IP daily, n =8), DLK1 group (1 mg/Kg dexamethasone PO daily + 0.8 mg/Kg DLK1 protein IP daily, n = 8). Results are presented as mean ± SEM. ∗ p value <0.05 was considered statistically significant.

2. Effect of DLK1 on lean body mass

We utilized DXA analysis to measure lean body mass following 2 wks of dexamethasone and DLK1 treatment. Four mice with an average body weight of 24 g from each group were selected and used for whole body scan experiments. Body weight, bone volume, and fat mass were measured in all three groups. Percentage of fat in tissue was significantly increased in the Dex group, compared with the control group (p <0.05). A statistically significant decrease in lean body mass was observed in the Dex group, compared with the control group. In contrast, a statistically significant increase in lean body mass was observed in the DLK1 group, compared with the Dex group (p <0.001).

Table 2. Effects of DLK1 on body composition in dexamethasone induced skeletal muscle atrophy mice using dual energy X-ray absorptiometry

	Control	Dex	Dex + DLK1
body weight (g)	23.7± 0.1	23.8 ± 0.2	24.1 ± 0.1
Bone volume (cm^3)	0.40 ± 0.0	0.40 ± 0.0	0.40 ± 0.0
fat mass (g)	3.86 ± 0.2	4.90 ± 0.2	4.52 ± 0.3
fat in tissue (%)	16.4 ± 0.8	20.6 ± 0.8*	19.4 ± 1.3

lean body mass (g)	19.3 ± 0.1	18.4 ± 0.0*	18.9 ± 0.2†

Data are presented as mean ± SEM. control group (Sham operation), Dex group (1 mg/Kg dexamethasone PO and PBS IP daily), DLK1 group (1 mg/Kg dexamethasone PO daily + 0.8 mg/Kg DLK1 protein IP daily). n=4 For each group; * $p < 0.05$ vs control by t-test, † $p < 0.001$ vs Dex by F-test.

3. Effect of DLK1 on tibialis anterior muscle

We compared the TA muscle weight of mice between the three groups. After 2 wks of dexamethasone and DLK1 treatment, TA muscle weight was significantly lower in the Dex group, compared with the control group (p <0.05), and there was no difference in TA muscle weight between the DLK1 group and the control group (p <0.05) (Figures 3A, 3B). H&E staining revealed decreased muscle fibrils size and atrophy in the Dex group. The extent of fibrosis was further assessed by analyzing collagen deposition using trichrome staining (Figure 3C). Histology studies based on the cross sectional area of myofibril, revealed a decreased muscle fibrils' area in the Dex group, compared with the control group (22.3 % reduction, p <0.05). DLK1 group showed the similar extent of the TA muscle fibrils' area with the control group (Figures 3D).

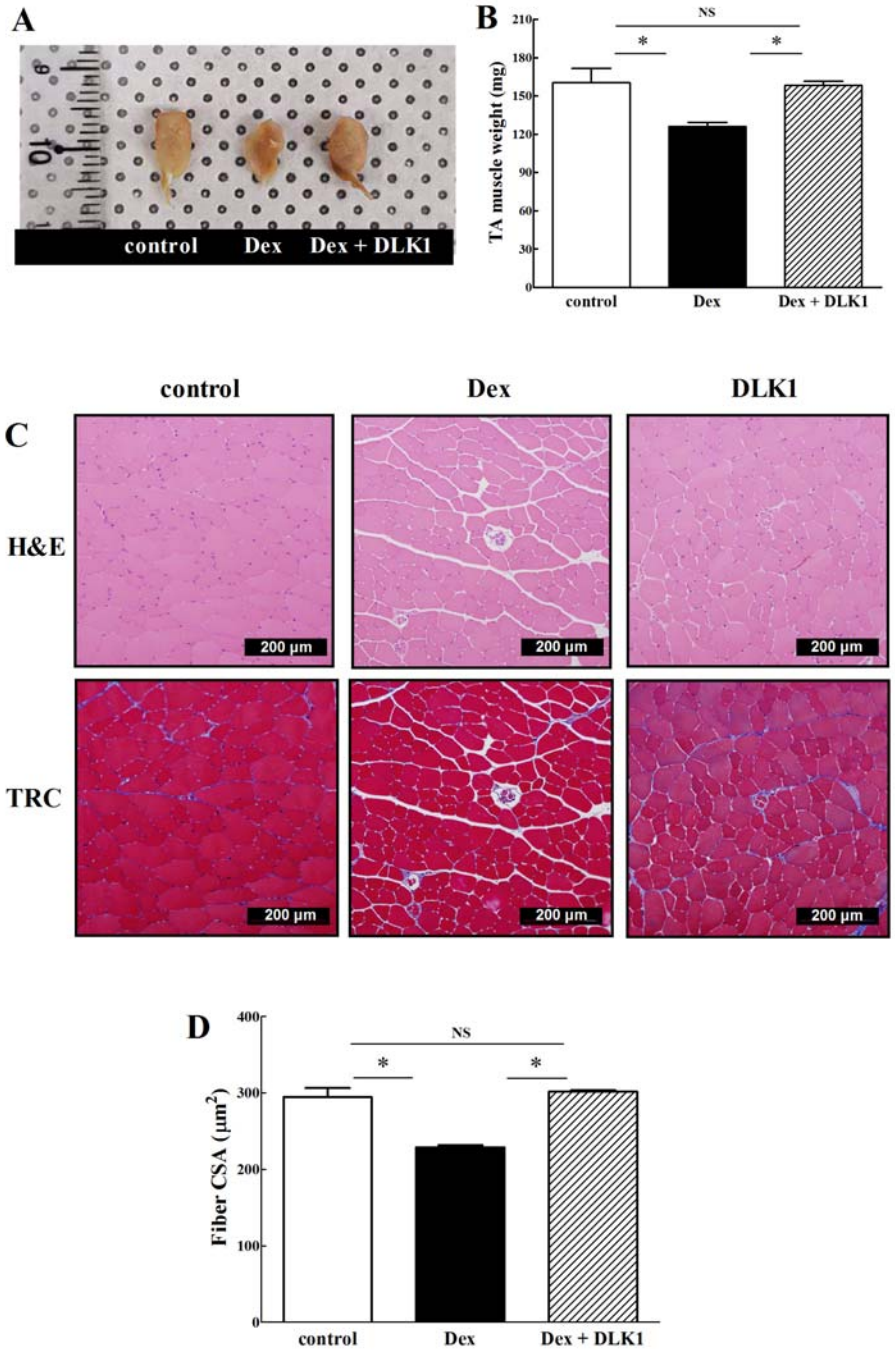

Figure 2. Comparison of the DLK1 effect through the dissection of tibialis

anterior (TA) muscles. (A) TA muscles from a mouse hindlimb are shown for size comparison. (B) TA muscle weight, n = 6 for each group (mg). (C) TA muscle histology (×100, magnification), hematoxylin and eosin (H&E) stain and Masson's trichrome stain (TRC). The black scale bar means 200 μm. (D) mean cross-sectional area (CSA; μm^2) of the TA muscle. control group (Sham operation, n = 6), Dex group (1 mg/Kg dexamethasone PO and PBS IP daily, n =8), DLK1 group (1 mg/Kg dexamethasone PO daily + 0.8 mg/Kg DLK1 protein IP daily, n = 8). $* p < 0.05$ was considered statistically significant. Results are presented as mean ± SEM.

4. DLK1 treatment down-regulates the expression of myostatin and muscle atrophic factors

Myostatin is a negative regulator of muscle mass by upregulating muscle atrophy related factors such as atrogin1 and MuRF1. We first determined whether dexamethasone regulates myostatin expression in C57BL/6J mice after treatment with dexamethasone at different concentrations-(0.2 mg/kg or 1 mg/kg) for 2 wks. At a dose of 1 mg/Kg dexamethasone, myostatin mRNA expression was up to 2-fold increased, compared with the control. Based on the preliminary results, these subsequent experiments were conducted using dexamethasone at a dose of 1 mg/Kg. We then investigated whether dexamethasone-induced myostatin expression in TA muscle was attenuated by DLK1. Dexamethasone treatment significantly upregulated myostatin mRNA expression, compared with the control. DLK1 treatment significantly reversed the dexamethasone-induced upregulation of myostatin mRNA expression. The anti-atrophic effects of DLK1 were investigated by examining mRNA expression levels of the muscular atrophy markers atrogin1 and Murf1 in TA muscle. Similarly, the expression patterns of atrogin1 and MuRF1 was correlated with myostatin expression at the mRNA level. In contrast, myogenic factor Myod1 was significantly reduced in the Dex group, but recovered in the DLK1 group. Similarly, myogenic factor myogenin was significantly reduced in the Dex group, but recovered in the DLK1 group (Figure

4).

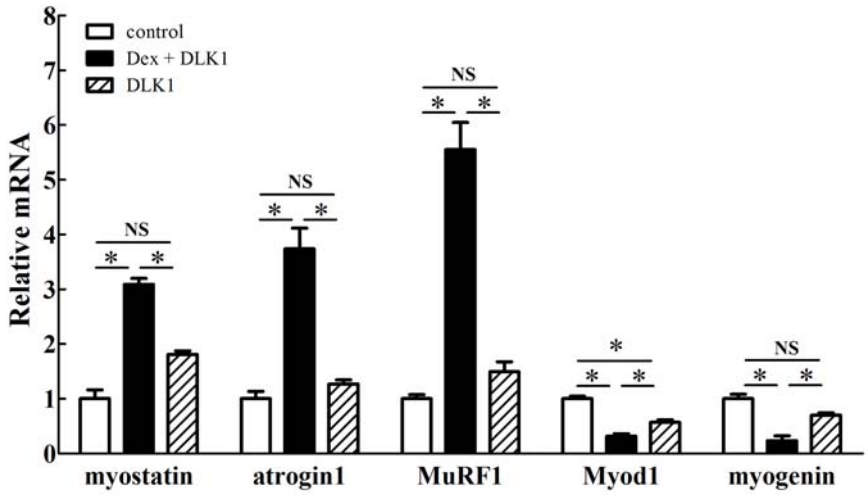

Figure 3. Comparison of DLK1's inhibitory effects on myostatin mediated-mus cle atrophic factors. The mRNA level of muscle atrophic factors (myostatin, atrogin1, and MuRF1), myogenic factors (Myod1, myogenin) in each group was compared and 18s mRNA was used as loading controls. Control group (Sham operation, n = 6), Dex group (1 mg/Kg dexamethasone PO and PBS IP daily, n =8), DLK1 group (1 mg/Kg dexamethasone PO daily + 0.8 mg/Kg DLK1 protein IP daily, n = 8). Independent t-test was performed for statistical analysis. *p* value < 0.05 was considered statistically significant.

5. Morphological characteristics of myofibril evaluated using TEM

Using TEM, we analyze the effects of DLK1 on intermyofibrillar morphological alterations. Other important organelles of the TA muscle (myofibril, Z line, and mitochondria) were clearly shown in the electron microscopy images. A significant increase in damaged mitochondria was observed in the TA muscle cells of dexamethasone-treated mice. In the most severely damaged mitochondria, almost complete dissolution of the internal architecture of both Z line and myofibril was evident. TEM images after dexamethasone treatment showed a loss of mitochondrial membrane structure including cristae and double outer

membrane. The size of mitochondria in TA muscles of the Dex group was considerably larger than the control group, but smaller than the DLK1 group (Figure 5A).

6. DLK1 regulates mitochondrial biogenesis by Opa1 and Drp1

Representative TEM micrographic images of mitochondria in tibialis aterious muscle cells (20,000× magnification) were presented in Figure 4A. In control group, the structure of mitochondria and its cristae was intact. Swollen mitochondria with disorganized and fragmented cristae were observed in Dex group. Mitochondrial swelling and cristae disarray were markedly attenuated in DLK1 group. Mitochondria are dynamic organelles that undergo fusion and fission processes which are regulated by mitochondria-shaping mRNA like Drp1 and Opa1. Thus, we tested the mitochondria-shaping factors in the three groups. A decline of Opa1 transcripts was found in dexamethasone-treated TA muscle. Moreover, dexamethasone induced a significant decrease in Opa1 levels as well as an increase in the expression of fission regulator, Drp1, compared with the control. In contrast, DLK1 treatment maintained mitochondrial fusion-fission machinery (Figure 4B).

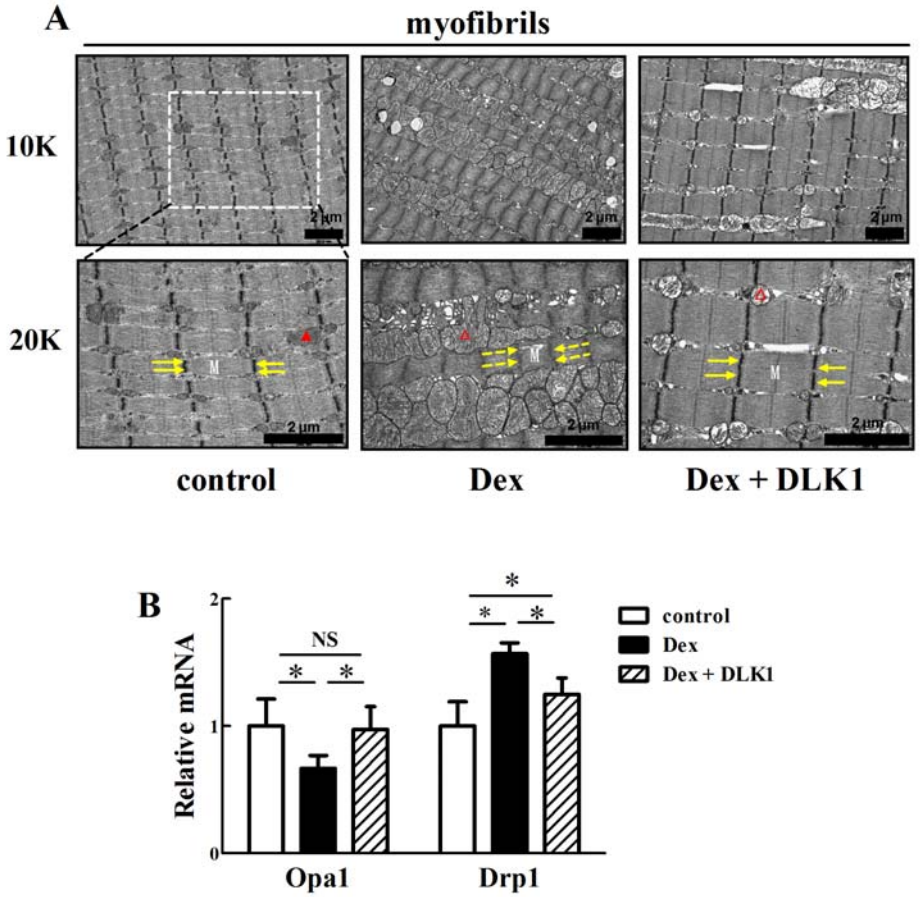

Figure 4. Tibialis anterior muscle morphology imaged by electron microscope and evaluation of DLK1's effects using the mitochondrial biogenesis marker (A) Electron microscopy images of TA muscle in control, Dex, and DLK1 groups after 2 weeks of dexamethasone and DLK1 treatment (×10,000 magnification and ×20,000 magnification). TEM images of TA myofibrils are shown (scale bar: 2 μm). M indicates myofilament. Z line (yellow arrow), and mitochondria (red arrowhead) are represented. Dashed arrow indicates a broken structure, and hollow arrowhead indicates a swollen structure. (B) Folds of increase in mitochondrial biogenesis marker: fusion (Opa1) and fission (Drp1). Data are means ± SEM. control group (Sham operation, n = 6), Dex group (1 mg/Kg dexamethasone PO and PBS IP daily, n =8), DLK1 group (1 mg/Kg

dexamethasone PO daily + 0.8 mg/Kg DLK1 protein IP daily, n = 8). ∗ *p* value <0.05 was considered statistically significant.

7. Atrophy model on C2C12 cells

We treated differentiated C2C12 myotubes with dexamethasone transiently for 24 hrs in DM on differentiation day 4, and this treatment resulted in significantly enhanced muscular atrophy. In contrast, myotube thickness was increased by DLK1 treatment. Myotubes treated with 5 μg/ml DLK1 had a 23% increase in myotube thickness compared with that of dexamethasone treated myotubes (Figure 5).

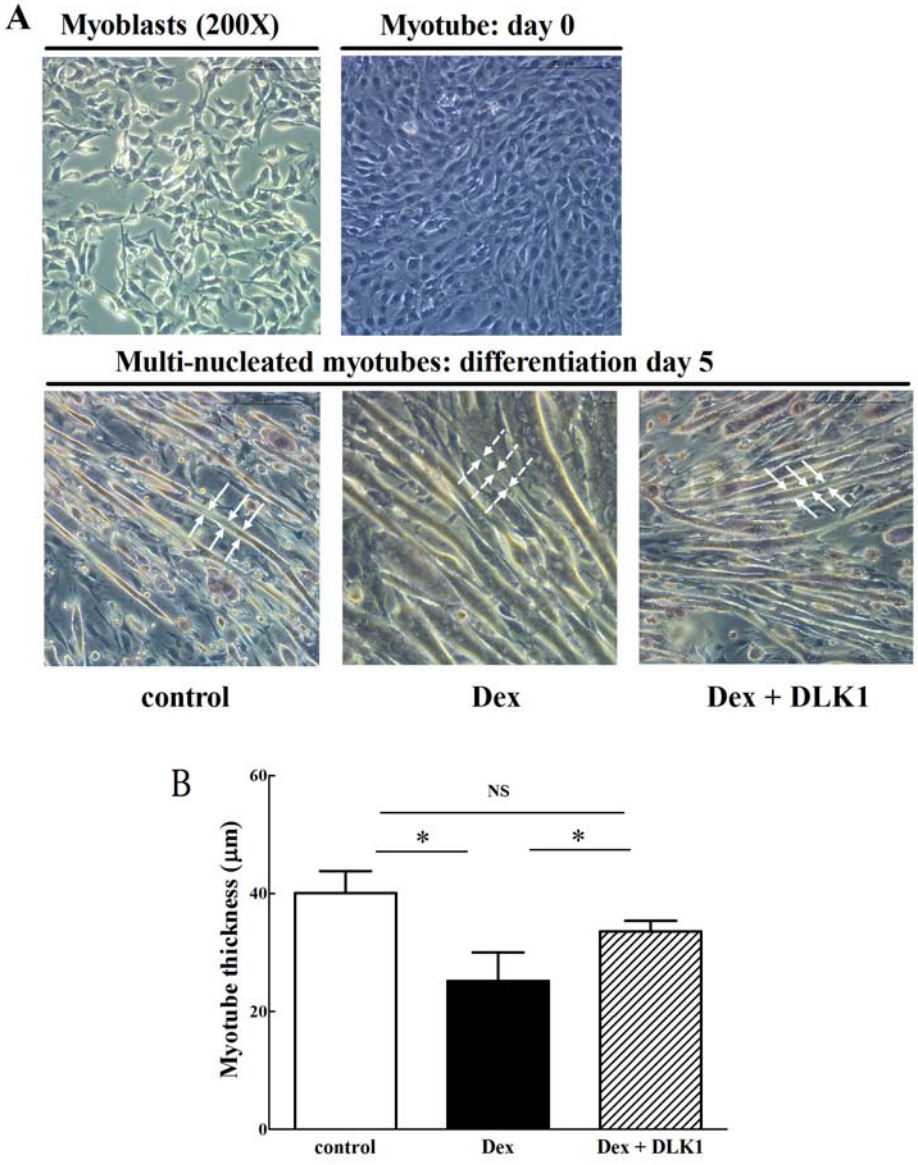

Figure 5. Effect of DLK1 in the atrophy model on C2C12 cells. Dexamethasone treatment after differentiation induced myotube atrophy. (A) H&E stain showing C2C12 myoblast, phenotypic change of C2C12 myotubes treated with differentiation medium (2% horse serum, DMEM medium). Differentiated C2C12 cell treated with mixture for 24 hrs on differentiation day 4: control group, Dex

group (dexamethasone 10 μM), DLK1 group (Dex 10 μM + DLK1 5.0 μg/mL). (B) Myotube thickness of C2C12 myotubes (μm). Results are presented as mean ± SEM. *p* value <0.05 was considered statistically significant.

8. Effect of DLK1 treatments on atrophic C2C12 cells

In this study, dexamethasone-treated C2C12 myotube showed a significant increase of myostatin mRNA level compare to control, while DLK1 treatment normalized myostatin mRNA expression level. Mechanistically, dexamethasone upregulated the expressions of atrogin1 and MuRF1, but suppressed those of myogenic factors including Myod1 and myogenin. In contrast, DLK1 treatment prevented the atrophic effect of dexamethasone in C2C12 myotubes by directly reducing the expression of muscle atrophy factors and increasing the expression of myogenic factors (Figure 6).

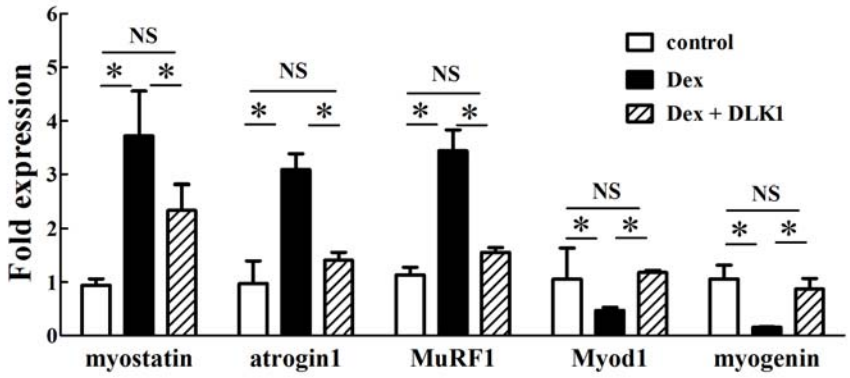

Figure 6. Comparison of DLK1 effects through myostatin mediated muscle atrophic factors in C2C12 myotubes. mRNA level of muscle atrophic factors (myostatin, atrogin1, and MuRF1), and myogenic factors (Myod1, myogenin), mRNA levels were assessed using RT-PCR in C2C12 myotubes. Differentiated C2C12 cell treated with mixture for 24 hrs on differentiation day 4: control, Dex (dexamethasone 10 μM), and DLK1 (dexamethasone 10 μM + DLK1 protein 5.0 μg/mL). Results are presented as the mean ± SEM. *p* value <0.05 was considered

statistically significant.

9. Effect of DLK1 treatments on mitochondria

Dexamethasone induced significant structural and morphological changes of mitochondria, characterized as mitochondrial elongation and swollen mitochondria, and these changes were efficiently reversed by DLK1 treatment (Figure 7A). Dexamethasone induced a significant decrease in Opa1 levels as well as an increase in the expression of fission regulator Drp1, compared with the control. In contrast, DLK1 treatment maintained mitochondrial fusion-fission machinery (Figure 7B).

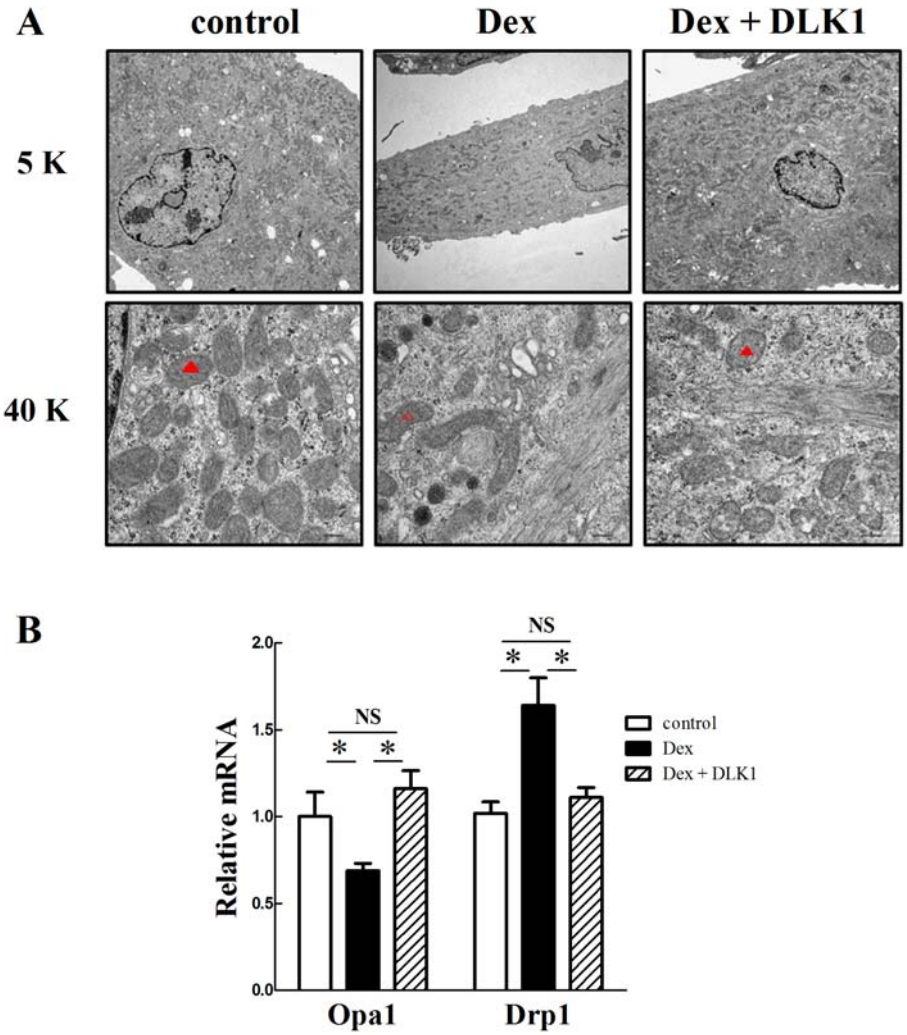

Figure 7. DLK1 regulate mitochondrial biogenesis by Opa1 and Drp1 on C2C12 cells. (A) Myotube morphology imaged by electron microscope (×5,000 magnification and ×40,000 magnification). TEM images of TA myofibrils (scale bar: 2 μm) are shown. Mitochondria (red arrowhead) are represented. Hollow arrowhead indicates a swollen structure. (B) Folds of increase in mitochondrial biogenesis marker: fusion (Opa1) and fission (Drp1). Differentiated C2C12 cells treated with normal saline, Dex (dexamethasone 10μM), DLK1 (Dex 10 μM +

DLK1 5.0 μg/mL) for 24 hrs on differentiation day 4. Results are presented as the mean ± SEM. p value <0.05 was considered statistically significant.

10. Myostatin siRNA knockdown test on C2C12 cells

C2C12 cells transfected with negative control siRNA were collected and western blot analysis was performed. Myostatin expression was evaluated by western blot to confirm negative control of siRNA. Dexamethasone-treated mice showed significantly increased expression of myostatin and significantly decreased levels of myogenin on C2C12 transfected with negative control siRNA. Decreased levels of myogenin were recovered by the DLK1 treatment even up to the similar level with the control group. To confirm the DLK1 protein signaling pathway, we preformed knockdown of endogenous myostatin expression. After transfection with myostatin siRNA, no obvious myogenin down-regulation was observed in the Dex or DLK1 groups, compared with the control group. This implies that dexamethasone may enhance the atrophy progress by regulating the myostatin signaling pathway via the myostatin receptor, activin type IIB. Thus, DLK1 protein may have the potential to directly inhibit atrophic progression via the regulatory myostatin signaling pathway (Figure 8).

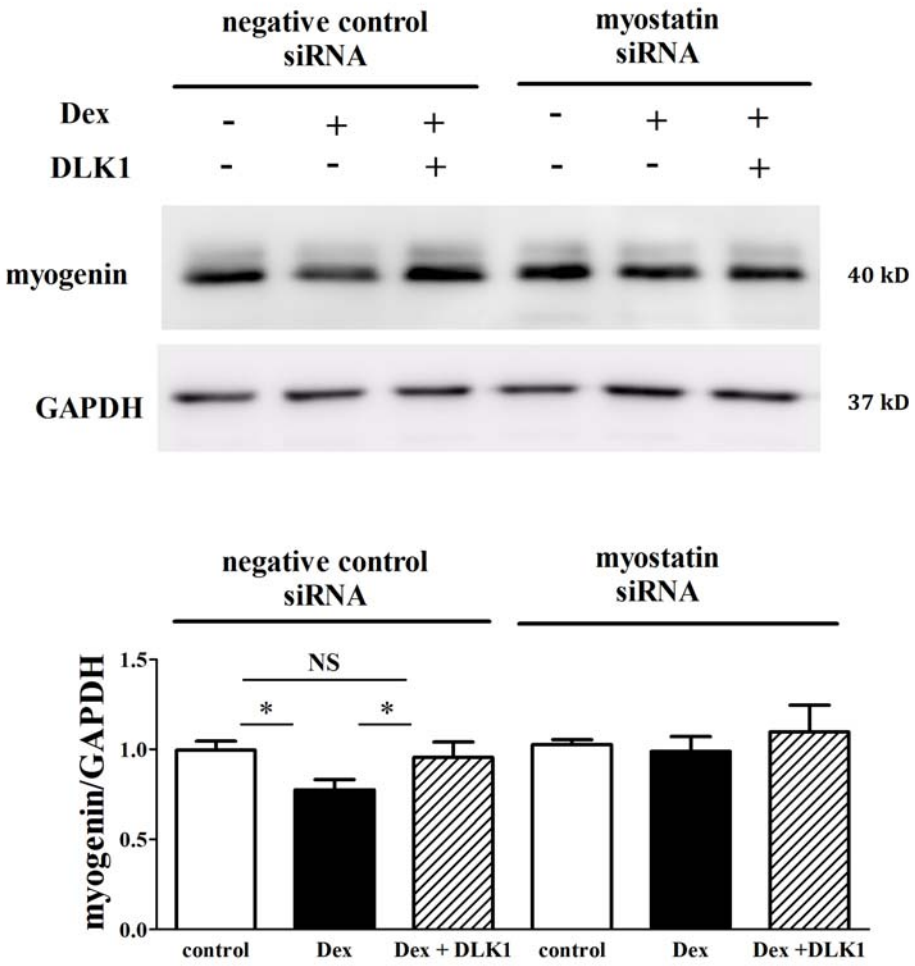

Figure 8. Myostatin siRNA test on C2C12 cells. Western blot analysis of expression of myogenin after transfection with (A) negative control siRNA only; (B) myostatin siRNA. The band intensity ratios of myogenin were normalized to GAPDH. Transfected C2C12 cells treated with normal saline, Dex (dexamethasone 10 μM), DLK1 (Dex 10 μM + DLK1 5.0 μg/mL), for 24 h on differentiation day 4: Results are presented as the mean ± SEM. *p* value <0.05 was considered statistically significant. *p* value <0.05 was considered statistically significant.

IV. DISCUSSION

The aim of this study was to investigate the effect of DLK1 protein on skeletal muscle atrophy. The balance between myofibrillar protein degradation and synthesis determines muscle mass.[13] In this study, we investigated the protective action of DLK1 against the muscle atrophy along with increased myogenin via modulating myostatin expression.

Dexamethasone increases muscle degradation, suppress muscle synthesis, and provoke muscular atrophy.[14] Muscle proteolysis is caused by stimulation if the ubiquitin proteosome system. In the ubiquitin proteasome system, there are muscle specific proteins called atrogenes named atrogin1 and MuRF1, which have a critical role in dexamethasone-induced muscular atrophy.[15] In the current dexamethasone-induced muscular atrophy model, the expression of atrogin1 and MuRF1 was significantly increased on the 14th day after dexamethasone treatment. Muscular atrophy related factors including atrogin1 and MuRF-1 were recovered to normal levels when DLK1 protein was administered in the current study. We also confirmed that DLK1 suppressed the expression of atrogin1 and MuRF1 in C2C12 cells.

Myostatin, a member of the transforming growth factor β (TGF-β) family, is essential for the negative regulation of skeletal muscle growth. Hence, we tested the association of myostatin with the dexamethasone-induced catabolic effect on skeletal muscle.[16] In a line with the previous studies, glucocorticoids upregulated the levels of both mRNA and protein of myostatin.[17] In the present study, the mRNA level of myostatin was increased by dexamethasone, suggesting that myostatin may be associated with the catabolic effect of glucocorticoid. In this study, dexamethasone treated C2C12 myotube showed significantly increased mRNA level of myostatin mRNA compare to control, while DLK1 treatment normalized its level.

The balance between muscle proteolysis and protein synthesis is also regulated by myokines, the cytokines secreted by the muscle itself.[18] Myostatin is a myokine that has an important function as a negative regulator in muscle hypertrophy.[19] In our study, administration of DLK1 completely inhibited the

dexamethasone-induced increase in myostatin expression. Another study reported that the inhibition of myostatin in adult and older animals increased muscle mass.[20] Additionally, mutation of myostatin lead to increases in muscle mass in mice.[21] Therefore, myostatin has attracted attention as a potential molecular target for suppressing the loss of muscle weight associated with aging and sarcopenia.

Glucocorticoids selectively decreased Myod1 expression levels in differentiated C2C12 myotubes caused by accelerated Myod1 degradation by the ubiquitin system. Taken together, these results suggest that, a reduction in Myod1 expression in muscle cells may be a common catabolic consequence. Activated myogenin is related to the enhancement of biosynthetic pathways.[22] In our study, DLK1 treatment protected mice or cells against atrophic process by Myod1 and myogenin activation. Our data suggests that DLK1 treatment could influence muscle regeneration.

Myogenin is a key regulator for expressed upon the differentiation of myoblasts to multinucleated myotubes, under atrophy conditions. Myogenin is essential during differentiation. As myoblasts are unable to fuse into multinucleated myofibers, mice lacking the myogenin gene die at birth due to severe skeletal muscle deficiency.[23]

When we co-administered DLK1 and dexamethasone, the diameter of myotubes was increased more dramatically, compared with dexamethasone treatment alone. Dexamethasone induced muscular atrophy is characterized by decreased muscle size and protein content, loss of cellular organelles, reduced muscle strength, and fatigue resistance.[24] In this study, intraperitoneal administration of DLK1 increased TA muscle weight in dexamethasone-treated C57BL/6J mice. In particular, DLK1 supplementation counteracted the dexamethasone-induced reductions in TA muscle weight. However, no significant changes in gastrocnemius muscle weight were observed.

In the current study, histological changes of muscle fibers were assessed using electron microscopy. Muscle changes during atrophic conditions are accompanied by changes in muscle fibers, including the aging of myofilament and changes in mitochondria.

As a result of observing the tibialis anterior muscle of a mouse with muscular atrophy with an electron microscope, it was possible to observe the swollen features of mitochondria constituting muscle cells.[25] Furthermore, TEM showed that the TA muscles of the DLK1 group had decreased mitochondria density, in comparison with the control group. Understanding of these defects in the skeletal muscle of mice provides valuable insights about the therapeutic potential of DLK1.

Previous studies have identified disruption of homeostatic regulation by two transcription factors, Opa1 and Drp1, that regulated mitochondrial growth in a muscle wasting mouse model[26]. In the present study, exposure to dexamethasone led to mitochondrial dysfunction, as indicated by a decrease in mitochondrial fission protein, Drp1.[27] These results confirmed that dexamethasone decreased Drp1 mRNA expression. A number of studies have shown that mitochondrial fission is an early event that takes place during the muscle cell degeneration process.[28] The loss of mitochondrial fission may impair the energy generation needed for metabolism and also induces muscular degeneration. Furthermore, we evaluated the expression of the key mitochondrial fission factor, Drp1. Our results showed that dexamethasone increased Drp1 levels *in vitro* and *in vivo*. DLK1 blocked dexamethasone-mediated increase in Drp1 expression *in vitro* and *in vivo*. These results suggest that the DLK1 treatment attenuated dexamethasone-mediated upregulation of mitochondrial fission through the inhibition of Drp1 recruitment to the mitochondria.

Mitochondrial fusion is governed by Opa1 at the IMM. Previous studies have shown that Opa1 maintains its IMM morphology.[29] While Drp1 is the fission master regulator, Opa1 is required for outer-mitochondrial membrane fusion. Distinctly, Opa1 is also crucial for inner mitochondrial membrane (IMM) fusion and the maintenance of proper mitochondrial cristae architecture. Increasing evidence have supported the correlation between mitochondrial dynamics disruption and muscle atrophy. In this study, we demonstrated that dexamethasone treated cells and mice showed decreased expression of Opa1. DLK1 treated cell and mice showed normalized levels of Opa1.

Our work with myostatin RNA interference has revealed that the down regulation of endogenous myogenin gene expression in muscle cells can lead to atrophy. No obvious myogenin down regulation was noted after treatment in the Dex or DLK1 groups, compared with the control group. This indicates that DLK1 may protect against the progression of muscular atrophy by regulating the myostatin signaling pathway via the myostatin receptor activin type IIB. These studies indicate that DLK1 may be a preventive or therapeutic target for muscular atrophy and metabolic dysfunction by inhibiting myostatin signaling.

In summary, our study determined that the DLK1 protein has a therapeutic effect on muscular atrophy. According to our experimental results, it was revealed that the action of DLK1 suppresses the activation of the myostatin-driven signal transduction system. Our study implies that DLK1 could be a promising candidate in the treatment of aging or diabetes-related sarcopenia, characterized by muscle atrophy and dysfunction.

V. CONCLUSION

The present study demonstrated that the exogenous administration of DLK1 markedly reduced muscular atrophy in mice by enhancing muscle regeneration. Furthermore, DLK1 significantly protected against the progression of muscular atrophy in mice by inhibiting myostatin signaling following atrogen downregulation. In addition, the present suggest that the anti-atrophic effects of DLK1 attribute to the downregulated expression of the muscle specific E3 ubiquitin ligases atrogin1 and MuRF1. Expression of mitochondrial regulation factors (Opa1 and Drp1) was normalized by DLK1 treatment in dexamethasone-treated mice. This study provides important novel information about the influence of DLK1 on dexamethasone-induced muscular atrophy. Taken together, these findings suggest that DLK1 may be a novel therapeutic option for the treatment of muscular atrophy.

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ABSTRACT(IN KOREAN)

덱사메타손이 유도한 근위축증에 대한 DLK1 투여의 보호 효과

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이 지 영

근위축증은 노령의 당뇨환자에서 근육의 유지와 근육량 감소의 불균형 가속화로 발생한다. 근위축증 치료를 위해, 본 연구는 덱사메타손이 유도한 근위축증 모델에서 Delta-like 1 homolog (DLK1)의 보호 효과를 연구한다.

10주령 수컷 C57BL/6J 마우스 모델을 세 그룹으로 나누었다: (1) 정상군 (2) 1 mg/Kg 덱사메타손 경구 투여 대조군 (3) 0.8 mg/Kg 융합 DLK1 단백질 투여군. 대조군에서는 근육량이 유의미하게 감소되었으며 근감소증 인자인 atrogin1과 MuRF1의 발현이 증가되었다. DLK1 투여군에서는 근육량이 정상군 수준으로 유지 되었으며 대조군과 비교하여 근위축증 인자가 유의미하게 증가하였다($p<0.05$). 근육세포성장 억제인자인 myostatin의 발현은 근위축증 대조군에서 증가하였으며 DLK1 투여군에서 유의미하게 감소하였다($p<0.05$). 세포실험은 근육세포인 C2C12 세포주에서 실시 하였다. 분석결과, 덱사메타손 단독 처리로 증가한 근위축증 인자들이 덱사메타손과 DLK1 동시 투여 시에 정상 대조군 수준으로 회복됨을 확인하였다. Myostatin siRNA test를 실시, myostatin 발현을 억제한 C2C1 근육세포에서는 DLK1 치료효과가 관찰되지 않았다. 이는 DLK1이 myostatin/myogenin 경로의

신호 전달에 관여하여 근육 위축을 완화함을 추정하게 한다. 근육세포의 전자현미경 관찰결과 DLK1의 투여군에서 근위축증의 완화뿐만 아니라 근위축증에서 관찰 되는 근육세포내에 존재하는 마이토콘드리아의 항상성유지에도 효과적인 것으로 관찰되었다.

본 연구는 근육 위축과 기능장애를 특징으로 하는 노령환자 또는 당뇨병환자에서 DLK1이 근위축증의 치료에 관한 새로운 약물 개발 가능성을 시사할수 있다.

핵심되는 말: delta-like 1 homolog, 근위축증, 미오스타틴, 미오게닌, atrogin1, MuRF1