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A Thesis for the Degree of Master of Science

The Novel Effects of 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone, a major Metabolite of Procyanidins, on C2C12 Murine Myoblasts Myogenesis

DHPV 의 노화성 근감소 억제 및 근육분화 촉진 효능

Feburary, 2018

By Seunghee Yang

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ABSTRACT

Procyanidins have many health benefits. After intake, procyanidins are degraded into small metabolites by gastrointestinal microorganisms. According to a previous study, 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone is the most abundant among these metabolites. The object of this study is to determine the effects of DHPV on muscle and whether DHPV could recover aged muscle to normal. TGF-β is connected to muscle aging and attenuates the muscle differentiation and inhibits myogenesis.

In this study, I measured myoD, myogenin, and the myosin heavy chain expression level to determine whether DHPV improved the myogenesis of C2C12, which had been inhibited by a TGF-β treatment. The results show that the RNA and protein expression level of these 3 biomarkers increases in

the DHPV treated group in comparison to TGF-β only treated. In addition, DHPV modulated the TGF-β signaling pathway and C2C12 myogenesis by regulating p-smad2/3, p-JNK, p-ERK and p-p38 expression. Taken together, it may be suggested that DHPV is an ideal therapeutic candidate for recovering myogenesis and muscle differentiation evoked by aging.

Keywords: DHPV; myogenesis; procyanidins; TGF-β; sarcopenia

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CONTENTS

I. Introduction

The aging population is growing rapidly worldwide. According to the United Nations, the number of people over 60 will rise to more than 2 billion by 2050 [1]. Decline of muscle mass and strength is one of the physical changes associated with aging and gradually begins at age 40 [2]. Sarcopenia, decrease of muscle mass and loss of function, is an age-related senile disease. In 2000, the estimated health care costs of sarcopenia in the U. S. was 18.5 billion dollars [3]. Since impaired physical function lowers the quality of life, the importance of studying sarcopenia is growing [4].

Skeletal muscle occupies approximately half of the entire body weight [5]. Myogenesis is a process to proliferate and differentiate myogenic cells

into skeletal muscle as well as to form myotubes and myofibers. [6, 7]. There are several biomarkers verified during myogenesis, including myoD, myogenin and myosin heavy chain [8].

Meanwhile, according to previous research, TGF-β is markedly increased in the old muscle cells compared to young ones [9]. TGF-β is a well-known muscle differentiation inhibitor [13-20], that mediates the expression of myogenic biomarkers by regulating p-smad2/3 and non smad2/3 pathways [10, 11]. During muscle differentiation, TGF-β signaling activates smad2/3 by up-regulating p-smad2/3, which acts on muscle regulatory factors [13, 14], and regulates non-smad pathways including the ERK, JNK and p38 pathways [11].

5-(3',4'-Dihydroxyphenyl)-γ-valerolactone is a main metabolite

of procyanidins, which are plentiful in the husks of Yak-Kong, and cocoa. Previous study has shown that DHPV was the highest detected metabolite after the uptake of cocoa polyphenol in human plasma [12]. According to the kinase array conducted in our lab, DHPV attenuates the activity of TGF-β receptor 1 by half. In our lab, we have studied DHPV's beneficial effects such as its anti-obesity effects and anti-oxidant effects in neurons, but there are no studies of DHPV's myogenic role. Based on the result of DHPV's kinase array and several health benefits, this study researches the muscle myogenic effects of DHPV and investigates the effects of DHPV to indentify the molecular mechanisms underlying the myogenic role of DHPV on C2C12 murine myoblasts.

Ⅱ. Materials and methods

1. Chemical and reagents

Dulbecco's modified eagle medium (DMEM), horse serum and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone (DHPV) was purchased from Chemieliva pharmaceutical company (China). All-*trans* retinoic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). TGF-β was purchased from R&D Systems (Minneapolis, MN, USA). The sulforhodamine B assay kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-myosin heavy chain, anti-myogenin, anti-myoD, anti-b-actin, anti-lamin B, anti-smad2/3 antibodies were obtained from Santa

Cruz Biotechnology (Santa Cruz, CA, USA). The anti-p-smad2 antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The anti-p-smad3 antibody was obtained from Ab cam (Bristol, UK).

2. Cell culture and treatments

C2C12 murine myoblasts (ATCC, Manassas, VA, USA) were cultured in DMEM containing high glucose with FBS. Three days after being seeded, the myoblasts were replenished to low glucose DMEM with horse serum and each began differentiation. They were replenished everyday. 5 ng/ml TGF-β, 1 μM DHPV were used to lead to inhibit myogenesis and promote myogenesis respectively. And All-*trans* retinoic acid was used as a DHPV's positive control to promote myogenesis.

3. Sulforhodamine B assay (Cell viability)

C2C12 cells were cultured in the 96-well plate at a density of 2.5×10^5 cells/ml in the presence of DMEM that contained low glucose with horse serum for 24 h. They were then analyzed using a Sulforhodamine B assay kit according to manufacturer's instruction. This was done to measure cell viability in the presence of TGF-β, DHPV and retinoic acid.

4. Hematoxylin and Eosin staining (H&Estaining)

After four days of differentiation, each sample was carefully washed twice using PBS. To visualize myotubes and nuclei, hematoxylin and eosin (H&E) were used. The hematoxylin was to stain the cytoplasm, and the eosin

was for nucleus staining. These myotubes were examined at 100 times magnification.

5. Western blot assay

Protein extracts were harvested by using RIPA buffer on ice, then centrifuged at 13000 g for 10 min. Protein assay reagent kits (Bio-Rad Laboratories, Hercules, CA, USA) were used to measure the protein concentration, and then the proteins were electrically divided in SDS-polyacrylamide gel. A transfer was conducted onto a Nitrocellulose blotting membrane (GE Healthcare Life Science, Amersham, UK), which was blocked in 5% skimmed milk for 1 h, followed by incubation with the primary antibodies in a 4 ℃ refrigerator for 16 h. The antibodies were myoD,

myogenin, myosin heavy chain, smad2/3, p-smad2, p-smad3, b-actin and lamin B, as indicated. After incubation, the membranes were washed 3 times with TBS-T for 10 min, and then incubated with secondary antibodies for 1 h in the presence of 5% skimmed milk. Following this, the membranes were washed five times using TBS-T buffer for 8 min. The protein bands were discovered through the use of an ECL detection kit (GE healthcare, St, Giles, UK) in Chemidoc. Protein quantitative analysis was conducted by Image J software (National Institutes of Health, Bethesda, MD, USA).

6. Real-time quantitative PCR

The RNA of C2C12 cells were collected and isolated in the presence of RNA iso Plus (Takara Bio Inc., Shiga, Japan), and then quantified using NanoDrop

ND-2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). In addition, cDNA synthesis was conducted using a PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Kyoto, Japan). A real-time quantitative polymerase chain reaction (RT-qPCR) was conducted using a Bio-Rad CFX 96real-time PCR detection system (Bio-Rad) with their respective primers and SYBR Green Master Mix (Bio-Rad). The primers used were as follows: mouse myoD forward (FW) 5'-GTG GCA GCG AGC ACT ACA GT-3' and mouse myoD reverse (RV) 5'-CTT GCA AAG GAA CTT GGG CTT-3'; mouse myogenin forward (FW) 5'-GCA CTG GAG TTC GGT CCC AA-3' and mouse myogenin reverse (RV) 5'-TAT CCT CCA CCG TGA TGC TG-3'; mouse myosin heavy chain forward (FW) 5'-AAG CGA AGA GTA AGG CTG TC-3' and mouse myosin heavy chain reverse (RV) 5'-CTT GCA AAG

GAA CTT GGG CTT-3'; and mouse GAPDH forward (FW) 5'-CAA GGA GTA AGA AAC CCT GGA CC-3' and mouse GAPDH reverse (RV) 5'-CGA GTT GGG ATA GGG CCT CT-3'. The GAPDH was used as a reference gene.

7. Statistical analysis

The data was specified as mean ± standard deviation. One-way ANOVA test was used for comparative study between each groups, and Student' *t*-test was used to consider significant differences.

Ⅲ. RESULTS

1. DHPV promotes myotube formation and has no toxic effect on C2C12 murine myoblasts.

To identify the cytotoxicity of DHPV, TGF-β and Retinoic acid on C2C12 murine myoblasts, the cell viability was tested using a Sulforhodamine B assay. C2C12 myoblasts were treated with 1 μM DHPV, 5 ng/ml TGF-β and 1 μM Retinoic acid for 24 h in a 96 well plate. The four groups were the control, TGF-β only, DHPV with TGF-β and Retinoic acid with TGF-β. The results showed that they are not toxic to C2C12 myoblasts at each concentration (Fig. 1A). The cell viability was indicated as a relative ratio to control.

To visualize C2C12 myotube formation, I conducted hematoxylin and eosin staining. TGF-β is a well-known myogenesis inhibitor [13-20], that diminishes myotube formation in C2C12. According to this result, TGF-β treatment inhibited cell differentiation and C2C12 myotube formation (Fig. 1B). A lower density of myotubes was observed in the TGF-β only group compared with the control, DHPV and retinoic acid treated groups.

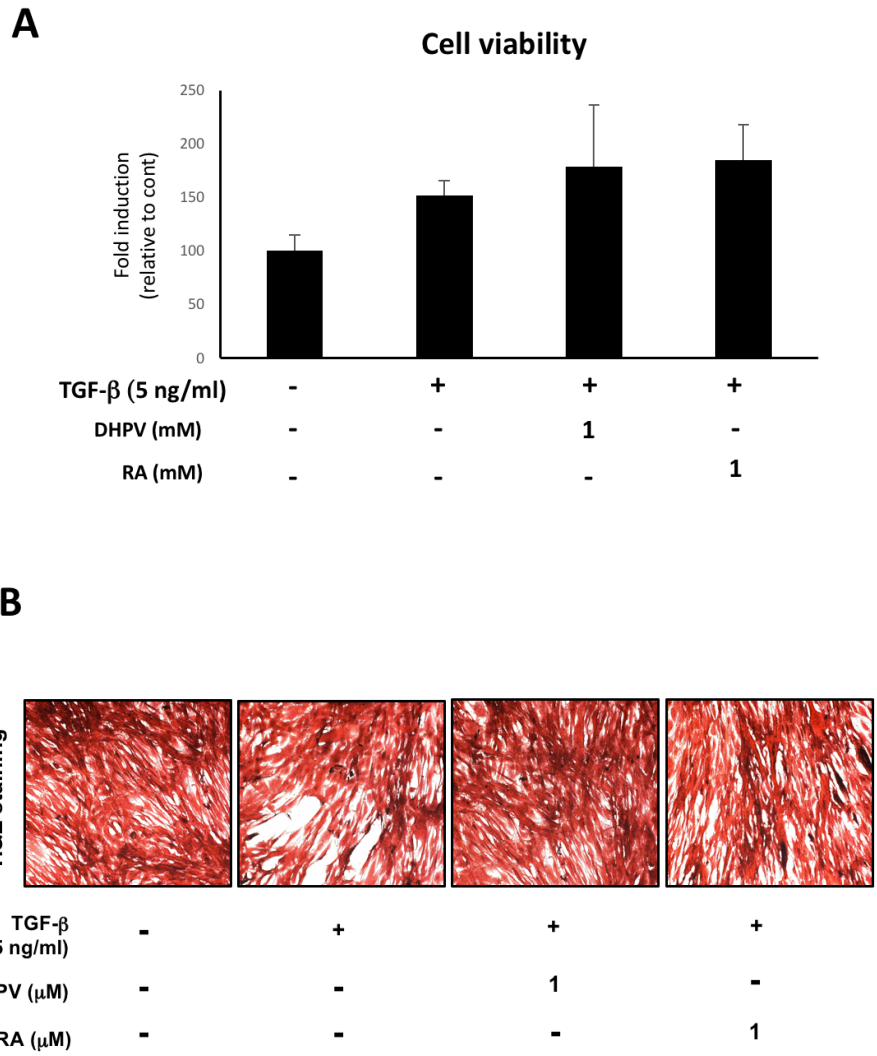

Figure 1. The cytotoxicity of DHPV and its effect on C2C12 myotube formation. (A) The data used to determine whether 1 μM DHPV, Retinoic

acid and 5 ng/ml TGF-β have a cytotoxic effect on C2C12 cells (A). The cells were incubated after treatment for 24 h. The cell viability of the C2C12 cells was measured using a Sulforhodamine B assay, and marked according to the relatively fold induction compared to the control. Each group was triplicated and expressed as means ± standard deviation. (B) Representative images of the C2C12 myoblasts which is differentiated in each sample for 96 h.

2. DHPV improves the myoD expression compared to TGF-β treatment.

The expression of the muscle differentiation biomarker myoD was investigated to explore the role of DHPV on myogenesis to counteract TGF-β. Since myoD usually manifests in the early stage of muscle differentiation, the cells were incubated in a differentiation medium containing each sample for 24 h. The results showed that TGF-β significantly decreased the myoD protein expression level in comparison to the control (Fig. 2A, B). However, DHPV and retinoic acid significantly enhanced myoD expression compared to TGF-β (Fig. 2A, B). For further study, the mRNA expression level in each group was investigated. 24 h after DM, RT-qPCR was conducted. As a result, TGF-β significantly down-regulated *myoD* expression, but DHPV effectively

counteracted *myoD* expression (Fig. 2C). Retinoic acid made *myoD* expression increase slightly, but not significantly (Fig. 2C). Each group was triplicated and expressed as means ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

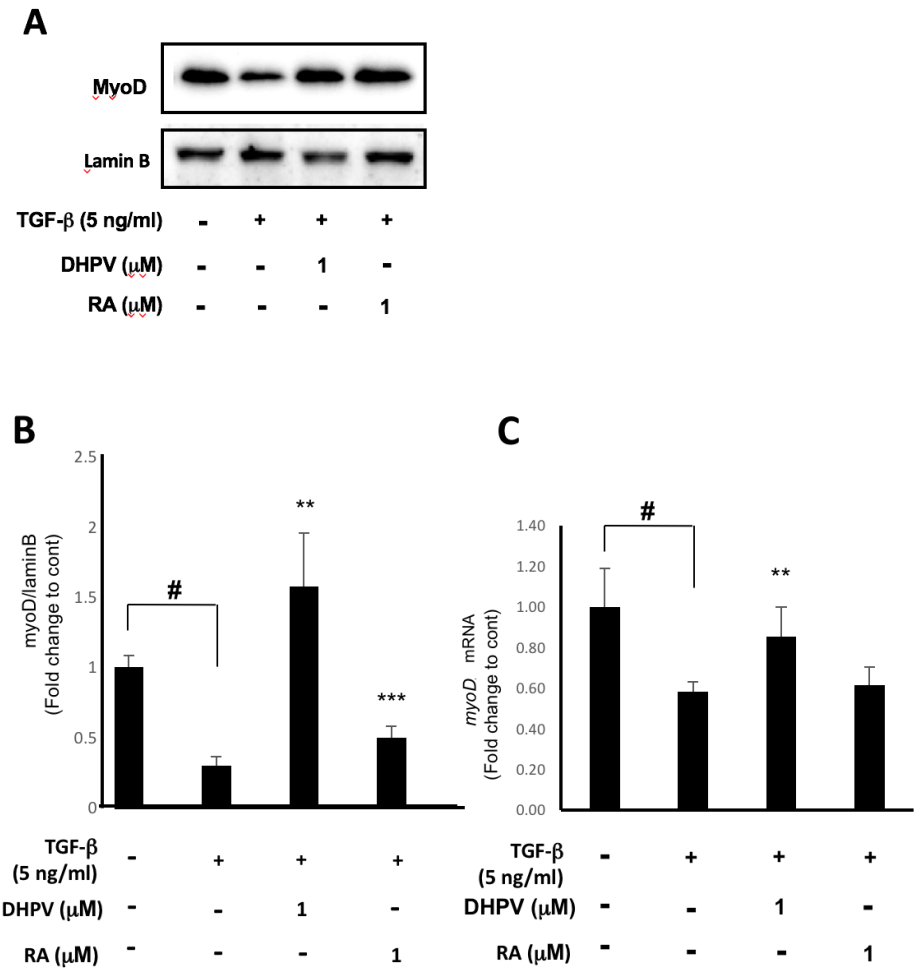

Figure 2. DHPV treatment stimulates myoD expression against TGF-β. (A) Western blot analysis of the myoD expression cultured in a differentiation medium containing each samples for 24 h. Lamin B was used as the loading

control. (B) The quantification of the protein bands from (A) (by Image J) expressed relative to the control. (C) The *myoD* mRNA expression level in C2C12 cells in each group after 24 h.

3. DHPV up-regulates the expression of myogenin in spite of TGF-β treatment on C2C12 myoblasts.

Myogenin is usually expressed in the middle of muscle differentiation. Thus, after each sample treatment was treated for 72 h, the myogenin expression level was measured. It was found that TGF-β treatment significantly attenuated myogenic protein expression in C2C12 myoblasts and DHPV and Retinoic acid enhanced myogenin protein expression (Fig. 3A, B). RT-qPCR was also conducted after 72 h of sample treatment, showing that TGF-β reduced the *myogenin* mRNA level, and in contrast, DHPV enhanced *myogenin* expression (Fig. 3C).

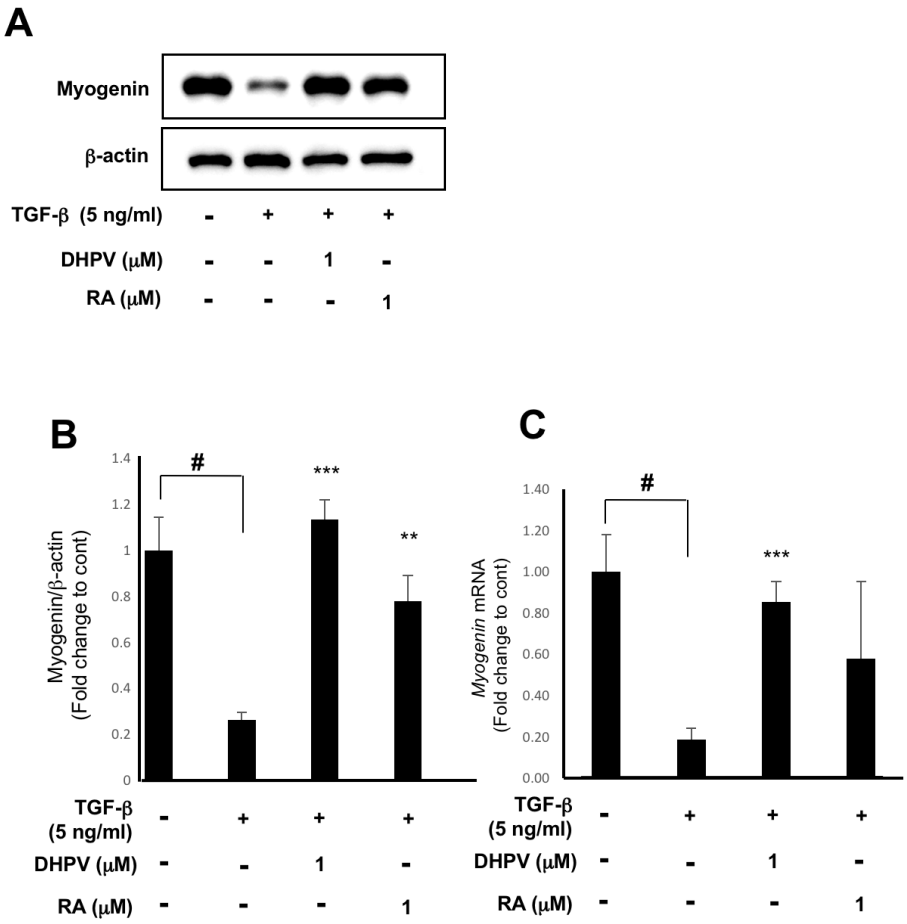

Figure 3. The inhibition of myogenin expression is counteracted by DHPV treatment. (A) Myogenin protein expression in C2C12 myoblasts after 72 h of DHPV, retinoic acid and TGF-β treatment (n=3). β-actin was used as a

loading control. (B) Quantification of myogenin protein expression by Image J. (C) *Myogenin* expression in C2C12 myoblasts after 72 h of DHPV, Retinoic acid and TGF-β treatment (n=7). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4. DHPV treatment couteracts the inhibition of myosin heavy chain expression induced by TGF-β.

Myosin heavy chain is a biomarker that appears in the last stage of muscle differentiation. To determine the myosin heavy chain expression level, western blot analysis and RT-qPCR were conducted for each sample after 96 h of treatment. As with the previous two biomarkers, myosin heavy chain protein expression was dramatically attenuated when treated with TGF-β only (Fig. 4.A-C). According to the quantification of protein bands, treatment with DHPV and Retinoic acid enhanced the myosin heavy chain level to higher levels than in the control or similar, which was significant (Fig. 4A, B). Even so, *myosin heavy chain* expression was slightly different from the protein level. TGF-β did not reduce the *myosin heavy chain* level

and showed no significance. However, the DHPV and Retinoic acid treatment significantly elevated the *myosin heavy chain* level compared to the control group (Fig. 4C)

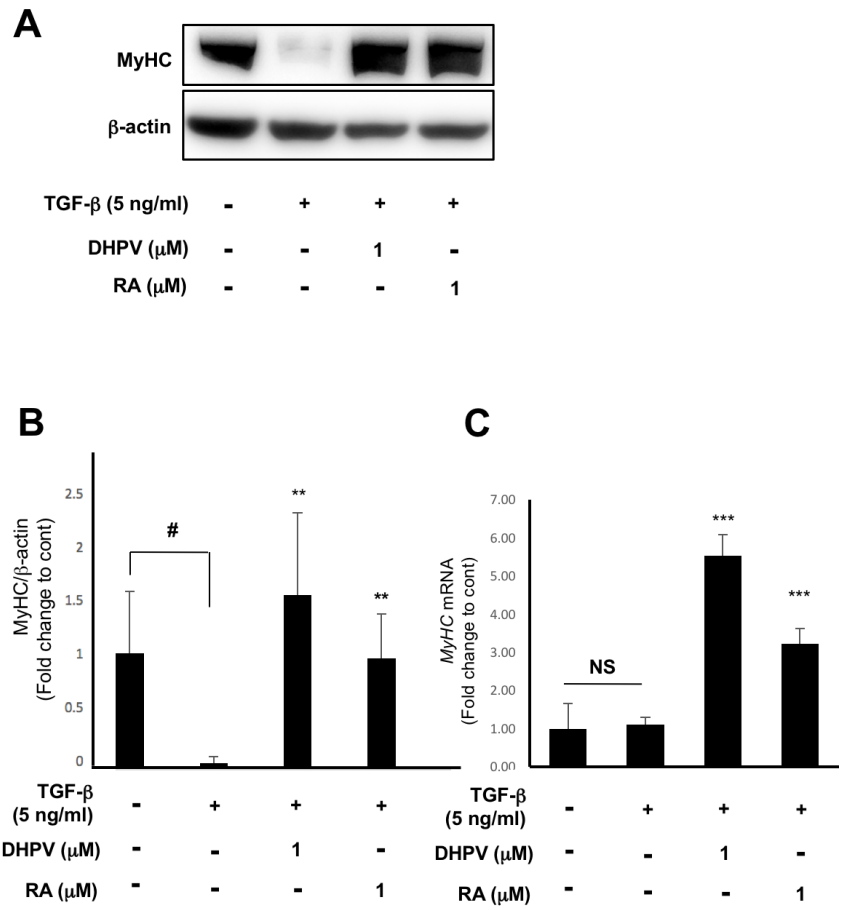

Figure 4. DHPV treatment elevates myosin heavy chain expression even in TGF-β treated C2C12 myoblasts. (A) Myosin heavy chain protein expression

was differentiated for 96 h in C2C12 myoblasts. (B) Quantification of the western blot analysis from (A) (n=3). (C) *Myosin heavy chain* expression in C2C12 myoblasts (n=6). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5. DHPV promotes C2C12 myogenesis by regulating both smad 2/3 signaling and non-smad signaling pathways.

TGF-β induces the TGF-β signaling pathway and plays a key role in inhibiting myogenesis in C2C12 myoblasts [13-20]. Therefore, I examined the expression of phosphorylated smad2/3 which is a well-known downstream protein of the TGF-β signaling pathway. The expression of p-smad2/3 protein decreased when treated with DHPV in contradiction to treatment with TGF-β only (Fig. 5A). MAPK singling pathway is also related with TGF-β, which is consist of ERK, JNK and p38 signaling pathways [28]. According to this data, DHPV down-regulated phosphorylated-JNK and phosphorylated-p38 protein expression against the TGF-β treatment. Phosphorylated-ERK slightly increased when DHPV

treated compared to TGF-β treatment (Fig. 5B). From these results, it was found that DHPV diminished myogenesis inhibition caused by TGF-β signaling pathways.

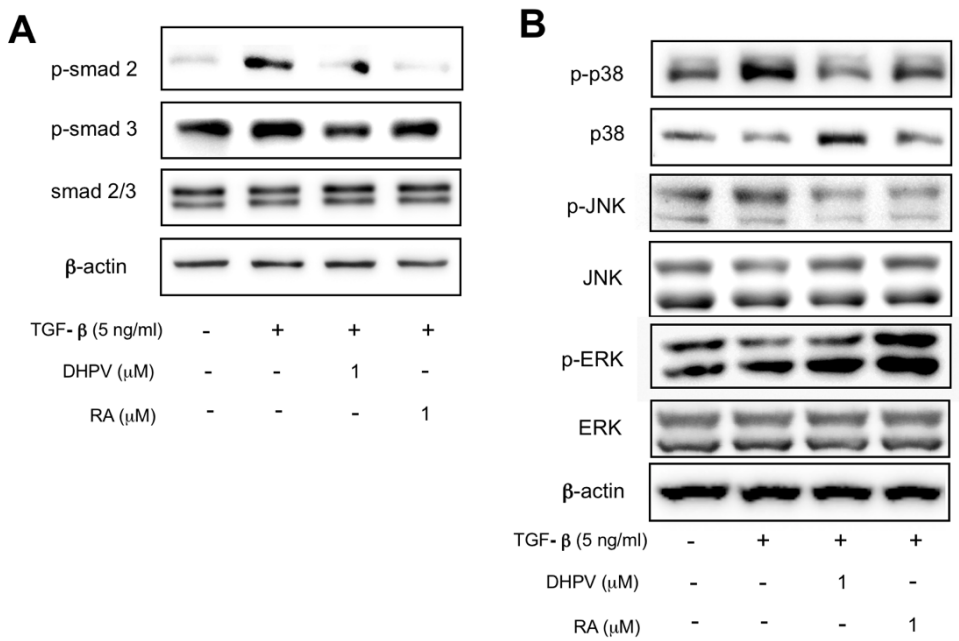

Figure 5. DHPV mediates both smad2/3 signaling pathway and MAPK signaling pathways. (A) DHPV decreased p-smad2/3 protein expression which was increased by the TGF-β. β-actin was used as a loading control. (B) The protein expression of three kinds of MAPK was examined. DHPV mediated C2C12 myogenesis via MAPK signaling pathways. β-actin was used as a loading control.

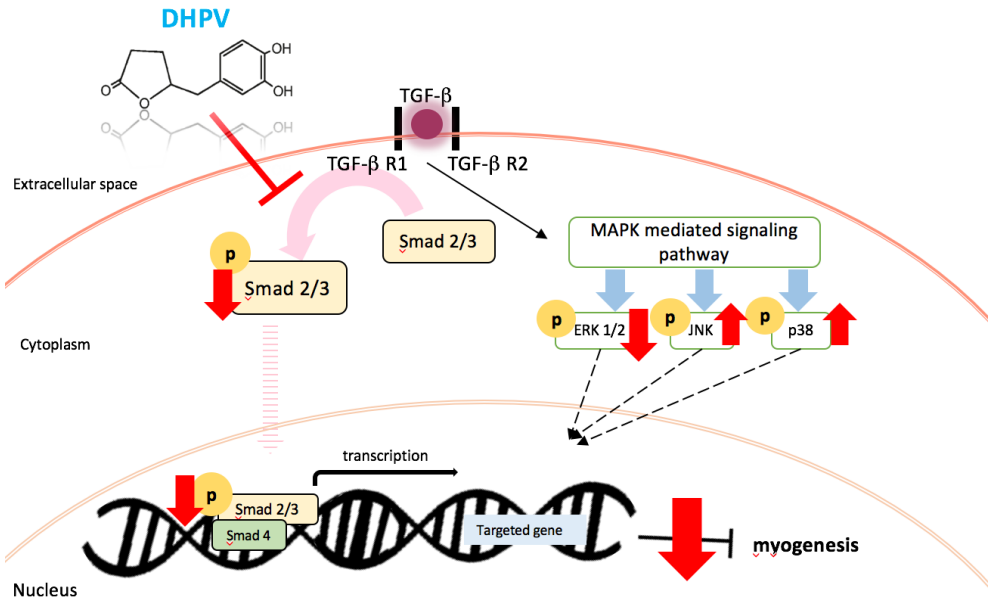

Figure 6. The summary of the myogenic role of DHPV on C2C12 myogenesis against TGF-β.

Ⅳ. Discussion

There are several previous studies regarding the health benefits of procyanidins such as their neuroprotective effects and anti-atherosclerotic ability [23-26]. Furthermore, procyanidins were previously found to have antioxidant effects and anti-obesity effects [21, 22]. At this point, the question arises as to what other novel health benefits of DHPV may have and what type of progress could be made by deactivating TGF-β receptor 1. As I metioned earlier, according to the kinase array results, DHPV lowered the TGF-β receptor 1 activity by half. Since TGF-β is deeply relevant to old muscle [9], DHPV's role muscle protection against TGF-β was studied.

This study explores the myogenic role of DHPV against TGF-β using the muscle differentiation facilitator all-*trans* retinoic acid as a positive

control [27]. There are two types of pathways in TGF-β signaling pathway, the smad2/3 pathway and non-smad pathways. The results show that DHPV promotes the protein expression of three biomarkers of muscle differentiation as well as RNA expression, and is usually more effective than retinoic acid. DHPV was also confirmed to promote C2C12 myotubes formation more than TGF-β inhibits it, showing a high density of differentiated muscle. In the signaling pathway, the results show that DHPV regulates TGF-β signaling by down-regulating smad2/3 phosphorylation as well as JNK and p38 phosphorylation and upregulating ERK phosphorylation. In conclusion, DHPV promotes C2C12 myogenesis (Fig. 6).

This study, which is just the beginning of this researcher's work dealing with DHPV's myogenic function on muscle cells by inhibiting TGF-

β because only two pathways were studied. As such, further study on other TGF-β signaling pathways should be conducted. Since there are no previous studies regarding DHPV or procyanidins and myogenesis promotion, these results are novel in their discovery of new features of the metabolites of natural substances.

It was observed that DHPV has myogenic effects on C2C12 murine myoblasts, and as such, its precursor procyanidins have the possibility to have similar effects as DHPV. Therefore, for further study, it is necessary that the research into the effects of procyanidins on C2C12 myoblasts should be conducted. Whether DHPV directly binds to TGF-β receptors or not should be also determined.

Administrating DHPV treatment to C2C12 murine myoblasts recovers the muscle differentiation suppressed by TGF-β to normal levels and promotes myogenesis. This new approach suggests that DHPV may be used therapeutically for the prevention of sarcopenia.

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Ⅵ. 국문초록

프로시아니딘은 약콩 껍질에 풍부한 폴리페놀류의 물질로, 건강에 유익한 효능이 많이 밝혀져있다. 섭취 후, 프로시아니딘은 장내 균총에 의해 작은 대사체들로 분해되는데, 선행 연구에 따르면 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone 은 프로시아니딘에 의해 생성된 대사체들 중 가장 풍부한 양을 차지한다. 또한 DHPV 는 노인성 근감소증을 일으키는 주요한 원인으로 꼽히는 TGF-β 의 리셉터의 활성을 절반가량으로 떨어뜨렸는데, 이러한 kinase array 결과에 기반하여 이 연구가 시작되었다. 이 연구의 목적은 DHPV 의 TGF-beta 에 의해 분화 억제된 근육세포에서 분화 회복여부를 관찰히고, DHPV 가 노인성 근 감소증을 진행시키는 분자적 매커니즘을 조절하여 노인성 근 감소증을 억제하는 것을 밝혀내는데에 있다.

이 연구는 TGF-β에 의해 분화억제된 C2C12 cell에서 DHPV가 근육분화의 바이오마커인 myoD, myogenic, myosin heavy chain의 발현을 정상상태만큼 증가시키는 것을 확인하였다. 더 나아가 TGF-β의 하위 시그널의 단백질의 발현을 조절하며 억제된 myogenesis를 회복하는 것을 확인하였다.

본 연구를 통해, 프로시아니딘 대사체 DHPV 가 노화성 근감소를 억제하는 약리적인 기능을 할 수 있는 가능성을 확인하였다.