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Musclin Regulates Glucose Uptake in Human Skeletal Muscle Cells via Insulin-Independent pathway

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Musclin Regulates Glucose Uptake in Human Skeletal Muscle Cells via Insulin-Independent pathway

Directed by Professor Chul Woo Ahn

The Master's Thesis
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

So Yeon Jo

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This certifies that the Master's Thesis of
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The Graduate School
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December 2021

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ABSTRACT

Musclin Regulates Glucose Uptake in Human Skeletal Muscle Cells via Insulin-Independent pathway

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(Directed by professor Chul Woo Ahn)

Exercise is considered as the most effective therapeutic strategy in the improvement of metabolic diseases. Skeletal muscle is involved in the homeostasis of energy metabolism via its secretory proteins known as myokines, thereby (skeletal muscle is) considered as an endocrine organ. Musclin (osteocrin) is one of the putative myokines, and its roles in lipid metabolism have been previously suggested. However, its physiological mechanism has not been elucidated. Recently, G-protein coupled receptor 1 (GPR1) has been reported to have a binding site for musclin. By considering that GPR1 is coupled to $G\alpha_{q/11}$ and $G\alpha_s$, we hypothesized that musclin exerts its physiological effects via Ca^{2+}- or cyclic AMP-dependent pathways. We investigated the effects of musclin on glucose metabolism in primary human skeletal muscle cells. Musclin increased Akt phosphorylation on Thr308 and Ser473. Besides, it increased phosphorylation on Ser2481 of mTORC2, *thereby*, stimulated glucose transporter type 4 (GLUT4) translocation. Musclin increased intracellular cyclic AMP (cAMP) levels which are responsible for the activation of Akt at Ser473 through mTORC2. These effects remain unclear whether GPR1 was related. In conclusion, our study demonstrated that musclin promotes glucose uptake in primary human skeletal muscle cells via insulin-independent pathway and suggested that musclin, as a myokine, represents exercise-induced improvement of glucose metabolism.

Key words: myokine, glucose uptake, exercise, insulin resistance (IR), human skeletal muscle cell

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I. INTRODUCTION

The incidence of diabetes mellitus (DM) continues to increase worldwide, and drugs such as metformin have been developed to lower the incidence of diabetes, but they are known to be effective in lowering the incidence rate not only through drugs but also through exercises that cause muscle contraction [1]. Although the health effect of these exercises is known to be the result of improving obesity, which is the source of various diseases, studies have shown that overweight and muscular people are less likely to develop diseases such as diabetes and cardiovascular disease than underweight people [2, 3]. According to these preceding studies, it can be expected that the effect of exercise will be correlated with other physiological mechanisms.

When muscle contraction occurs in skeletal muscle through exercise, a hormone called myokine is secreted, and the secreted hormone is known to be involved in regulating energy metabolism [4]. A representative example of Myokine is interleukin

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6 (IL-6), an inflammatory cytokine. IL-6 is a cytokine secreted from immune cells, but is known to be produced during long-term exercise in skeletal muscles, and IL-6 produced in skeletal muscles is secreted into liver and adipose tissue, affecting energy metabolic regulation, and is regulated and secreted by Ca^{2+} level in cells [5]. In addition, in 2012, a research team by Bruce M. Spiegelman revealed a myokine called irisin, and in the case of irisin, it was reported that brown fat made from brown fat by inducing AMPK activation in white fat had a positive effect on energy metabolism[6].

Musclin (a.k.a. osteocrin) is a myocaine secreted by continuous muscle contraction in skeletal muscles, such as Interleukin 6 and irisin [7]. Musclin, first discovered as a factor regulating bone metabolism in bones [8], is encoded by a gene called Ostn and has 30–130 amino acid except for the N-terminal signal sequence [7, 9]. It is also known to have a similar region to that of the natriuretic peptides (NPs) family, except that it does not have two cysteine residues required to form the Ω-like structure[7, 9].

In general, it is known that muscle contraction increases intracellular Ca^{2+} concentration and AMP/ATP ratio, leading to 5′ AMP-activated protein kinase (AMPK) activation [10], but it is not yet known how musclin relates to these mechanisms. In addition, previous studies have shown that musclin is known to have strongly bind with natriuretic peptide receptor C (NPRC) [11], which is responsible for decomposition of NPs, and that co- treatment with ANP, one of the NPs, results in contention inhibition of NPRC-NPs, increasing the concentration of cGMP level in cells [12, 13]. However, it has not yet been revealed what physiological action cGMP increased by musclin alone induces, and experiments using mice that eliminated the musclin-encoding gene Ostn have reported a decrease in physical endurance in Ostn-KO mice compared to wild type, thus suggesting the possibility that muscle is correlated with motor ability. There are other previous studies showing contrary results, which have been reported that glucose uptake and glycogen synthesis

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by insulin are inhibited by musclin. [7, 14]. To prove, the most commonly known Akt activity among insulin signaling pathway was confirmed, and as a result, the Akt activity was suppressed [7, 14]. However, according to the results of other previous studies related to NPs and glucose uptake, glucose uptake was confirmed by ANP and BNP, the types of NPs in adipocytes. In addition, Akt phosphorylation sites at Ser473 and Thr308 and AS160 phosphorylation on Thr642 was confirmed by BNP. Also, activation of Mammalian target of lapamycin complex 1/2 (mTORC1/2), Raptor Ser792, and Rictor Thr1135 was confirmed. Therefore, the glucose uptake by BNP was proved [15]. Since musclin is known to have a similar region to NPs [7, 9], it is possible to induce glucose uptake by musclin, but has not been studied yet.

In addition, it has been reported that musclin binds to GPR1 and GPR68, which are G-protectin coupled receptor [16]. GPR68, a proton-sensitive GPCR, was activated in an acid (pH < 7) environment, and GPR1 was known to be activated by hexose such as glucose. Two types of GPCR are known as Gs family receptors, and when activated, they activate adenylyl cyclase (AC) to increase intracellular concentration of cAMP [17, 18]. However, the results of whether musclin can combine with GPR1 and GPR68 to activate AC and change the cAMP level have not yet been revealed. According to other previous studies, isoproterenol binding to β2-Adrenoceptor in rat skeletal muscle cells activates PKA, resulting in an increase in cAMP level [19]. It was then confirmed that the increased cAMP activated mTORC2 at Ser2481 [19]. In addition, it was proved that GLUT4 movement was confirmed by cAMP to induce glucose uptake [19]. Therefore, musclin is expected to induce phosphorylation of mTORC2 at Ser2481 by activating PKA by binding to GPR1 and GPR68 that induce cAMP generation, but it has not been revealed yet.

Previous studies reported so far are expected to have physiological effects in relation to increased cAMP and cGMP concentrations in cells by independent musclin

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treatment, but studies to prove this have not yet been supported. Therefore, further research is needed on how the independent musclin affects the mechanism related to energy metabolism. As a result, the therapeutic effect of insulin resistance (IR), a disease related to energy metabolism, can be expected.

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II. MATERIALS AND METHODS

1. Cell culture and treatment

Primary human skeletal muscle cell line, hSkMC cell, was purchased from American Type Culture Collection (ATCC, PCS-950-010, Manassas, Virginia, USA). The cells were cultured at 37 ℃ in 5% CO^2 atmosphere in basal medium (ATCC, PCS-500-030) supplemented with 4% FBS (16000-044, GIbco), 10mM L-glutamin (25030081, Gibco), 5ng/ml rh EGF (E9644, Sigma), 5ng/ml rh FGF-b (SRP 3043, Sigma), 10uM Dexamethasone (D4902, Sigma), 25ug/ml rh insulin (91077C, SAFC) and 0.5% antibiotics (15140-122, Gibco). This media becomes growth medium, which induces cell proliferation. The growth medium was replaced every other day until the culture vessel reaches more than 90% confluence.

We induced skeletal muscle differentiation at approximately 90% confluence of the myoblasts by changing the growth medium to differentiation medium (DMEM/F12 + 2% horse serum). The differentiation medium was replaced every other day. After 7 days, the myotubes were used. To examine the effects of musclin, cells were treated with 100, 200, and 500nM musclin.

2. Measurement of cAMP and cGMP productions

To examine the cellular responses in human skeletal muscle cells stimulated by musclin, cAMP and cGMP production were measured by Cyclic AMP ELISA KIT (581001, Cayman Chemical Technology, Ann Arbor, MI, USA) and Cyclic GMP ELISA KIT (581021, Cayman Chemical Technology, Ann Arbor, MI, USA). For this assay, cells were treated with 200nM musclin for 30 min. Then, cells were incubated 0.1M HCl for 20mins and supernatants

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were assayed directly. cAMP or cGMP in the lysates was acetylated to increase sensitivity using potassium hydroxide and acetic anhydride. Assay was performed according to instructions. cAMP or cGMP productions were calculated by reading HRP activity at OD_{405nm} with plate reader (VersaMax, Mplecular Device, Sunnyvale, CA, USA).

3. Immunocytochemistry and confocal microscopy imaging

In order to confirm the translocation of GLUT4 from the cytoplasm to the cell membrane, GLUT4 was analyzed by immunocytochemistry (ICC) followed by imaging laser confocal microscopy. For ICC, cells were cultured on 22x22mm coverslip (0101050, Marienfeld, Lauda-Konigshofen, Germany). Before seeding, the cell culture slide was coated with Poly-D-lysine (P6407, Sigma Aldrich, St. Louis, MS, USA) at concentration of 50μg/ml for overnight at 4 ℃. Prior to ICC, cells were treated with or without 500nM insulin, 200nM Musclin 31-78, 200nM Musclin 83-133 for 30min in 37 ℃ humidified incubator.

For ICC, cells were fixed in containing 4% (*v/v*) paraformaldehyde (PC2031-100-00, Biosesang, Gyeonggi-do, Korea) for 10mins at room temperature, and were washed five times with PBS containing 0.1% Tween 20 (0777, VWR life science, Solon, Ohio, USA) (PBST). To block unspecific binding of the antibodies, cells were incubated in PBST containing 10% (*v/v*) goat serum (S26, EMD Millipore, Billerica, MA, USA) at room temperature for 2hr. Then, cells were incubated in primary antibody for GLUT4 (diluted at 1:250, ab654, abcam, Cambridge, UK) diluted in blocking solution (10% goat serum in PBST) overnight at 4 ℃. After five times of washing for 5mins with PBST, cells were

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incubated in blocking solution containing Alexa Fluor 647 (ab150115, abcam, Carlsbad, CA, USA) to detect GLUT4 with confocal microscopy. For counter staining, cells were incubated with 4`,6-Diamidino-2-Pheylindole, Dilactate (DAPI) solution (D3571, Invitrogen, Carlsbad, CA, USA) diluted blocking solution at 5ug/ml for 10mins. After washing, the cells were mounted with coverslip with fluorescent mounting medium (S3023, Dako, Carpinteria, CA, USA). The cells were imaged at room temperature using confocal microscopy (LSM780, Carl Zeiss, Stuttgart, Germany) with a 20X objective lens; and the images were processed by Zen Blue Edition for Windows (Carl Zeiss, Stuttgart, Germany).

4. Quantitative real time PCR (qRT-PCR) and semi-quantitative PCR

For mRNA extraction to examine transcriptional levels, cells were collected in Tri-RNA Reagent (FATRR 001, Favorgen, Kaohsiung, Taiwan) and extraction protocol, which includes chloroform (UN1888, DUKSAN, Gyeonggi-do, Korea), and iso-propyl alcohol (UN1219, DUKSAN, Gyeonggi-do, Korea) was used. After extraction, cDNA was synthesized from RNA samples of human skeletal muscle cells by reverse transcriptase PCR using M-MLV Reverse Transcriptase kit (M1705, Promega, MA, USA). The PCR protocol followed the kit.

The gene expression levels of differentiation markers, NPRs, and GPR1/68 were tested by quantitative real time PCR (qRT-PCR) using TOPreal™ qPCR2X PreMIX (SYBR Green with low ROX) (RT500M, enzynomics, Daejeon, Korea) on LightCycler 480 System (Roche, Basel, Switzerland). All reactions were performed in triplicate. RNA levels were normalized to the level

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of GAPDH and calculated as delta-delta threshold cycle (ΔΔCT). Primers used for qRT-PCR are listed in Table1. Also, the expression level of differentiation markers, NPRs, and GPR1/68 were tested by semi quantitative real time PCR ($_{semi}$PCR) using TopTaq DNA Polymerase kit (200205, QIAGEN, Hilden, Germany). The PCR protocol was as follows: pre-denaturation at 94 ℃ for 7mins, denaturation at 94 ℃ for 30secs, annealing at 60 ℃ for 30secs, and amplification at 72 ℃ for 30secs.

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Table 1. List of primers

Name	Primer	Product size	T_m (℃)
MYOG: forward	ATCATCTGCTCACGGCTGAC	117	60
MYOG: reverse	TGGGCATGGTTTCATCTGGG		
DES: forward	CCAACAAGAACAACGACGCC	135	60
DES: reverse	ATTCCCGCATCTGCCTCATC		
MYH2: forward	TGGCACTGAGCTGTTCAAGATA	249	60
MYH2: reverse	CGCAGGATCTTTCCCTCTTCA		
NPR1: forward	AAGTCATCCAACTGCGTGGT	123	60
NPR1: reverse	CGTCCACAGCTTTTTGGCAT		
NPR2: forward	CTGCGCATGGAACAGTATGC	228	60
NPR2: reverse	GGGTGCTCTCTGCTGACAAT		
NPR3: forward	TGCCAACGGAGACCGATATG	201	60
NPR3: reverse	TTGCAGGGAGAGCTGTTTGT		
GPR1: forward	TTGGGAGCTCACCATTCACC	203	60
GPR1: reverse	TGCCAGAACAGCTGACTTCC		
GPR68: forward	TCAGCACCGTGGTCATCTTC	179	60
GPR68: reverse	GCTGACGAAGCAGTAGAGCA		

MYOG, myogenin; DES, desmin; MYH2, myosin heavy chain; NPR1, natriuretic peptide receptor 1; NPR2, natriuretic peptide receptor 2; NPR3, natriuretic peptide receptor 3; GPR1, G-protein receptor 1; GPR68, G-protein receptor 68.

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5. Western Blots

Human skeletal muscle cells were grown in 60mm plates and subjected to serum starvation for 4 hours before treatment. After the experimental treatment, the medium was removed, and the cells were washed twice with ice-cold PBS and extracted by RIPA lysis extraction buffer (89,900, Thermo Scientific, Waltham, MA, USA) containing protease/phosphatase inhibitor cocktail (5872 s, cell signaling technology, Danvers, MA, USA) for 5 min on ice. The samples were centrifuged at 13,000 rpm at 4 °C for 15 min. The Proteins were quantified with the BSA assay (23,225, Pierce, Rockford, IL, USA) and were heated at 95 °C for 3 min. The supernatants were resolved on 8-10% sodium dodecyl sulfate-PAGE gels and then transferred to nitrocellulose (NC) membranes (10,401,196, Capitol Scientific, Austin, TX, USA), which were blocked in the Tris-buffered saline containing 50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20, and 5% BSA (w/v ratio) for 2 h at room temperature. All antibody used in current study are listed in Table 2. And the membrane was incubated overnight at 4°C with primary antibodies, then, proved with HRP-conjugated secondary antibodies reacted for 1 h at room temperature. The blots were visualized by chemiluminescence using pierce ECL substrate (32106, Thermo Scientific, Waltham, MA, USA). The intensities of the protein bands developed on medical X-ray films were quantified by using ImageJ software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, Madison, WI, USA). Protein levels were normalized with the levels of the loading control such as GAPDH.

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Table 2. List of primary and secondary antibodies

Primary Antibodies			
Name	**Manufacturer**	**Dilution**	**Gel %**
1. GAPDH	2118, Cell Signaling	1:2000	10, 8
2. $pAkt^{thr308}$	4060, Cell Signaling	1:1000	10
3. $pAkt^{ser473}$	4056, Cell Signaling	1:1000	10
4. $_{(pan)}$ Akt	9272, Cell Signaling	1:1000	10
5. $pmTORC2^{ser2481}$	2974, Cell Signaling	1:1000	8
6. mTORC2	2983, Cell Signaling	1:1000	8
7. Vinculin	SC-5573, Santa Cruz	1:500	8
8. GLUT4	Ab654, Abcam	1:1000	10
Secondary Antibodies			
1. Anti-rabbit IgG, HRP-linked antibody	7074,Cell Signaling	1:3000	All
2. Anti-mouse IgG, HRP-linked antibody	7076,Cell Signaling	1:3000	All

The numbers in manufacturer are catalog number of products. Optimized antibody concentrations are presented at dilution. Percentage of gels which were used in SDS-PAGE is presented. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; Akt, Protein kinase B; mTORC2, mammalian target of rapamycin complex 2; p, phosphorylation, HRP, horseradish peroxidase.

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6. Glucose uptake measurement

Glucose uptake was measured by using glucose uptake cell-based assay kit (Cayman Chemical 600470, Ann Harbor, MI, USA) according to the manufacturer's protocol. Cells were cultured and differentiated in 96-well plate (Nunc, 165305, Roskilde, Denmark). Differentiated human skeletal muscle cells were washed with PBS and then starved in serum-free without-glucose DMEM for 24 h. Cells were treated with test compounds in the same buffer containing fluorescent glucose analog, 2-deoxy-2-[(7-nitro21,1,3-benzoxadiazol-4-yl) amino]-D-glucose) (2-NBDG) at concentration 100μg/ml in 37 °C for 1h. The experimental compounds 200nM human musclin (Phoenix Pharmaceuticals Inc**,** Burlingame, CA 94010, USA**)**; for positive controls, 500nM insulin (91077C, Sigma Aldrich, St. Louis, Ms, USA). The amount of 2-NBDG taken up by cells were calculated based on the measures at 485/535nm (excitation/emission) with a plate reader designed to detect fluorescein (POLARstar Optima, BMG Labtech, Ortenberg, Germany).

7. Statistical analysis

All data was analyzed using SPSS software Version 26.0 for windows (IBM, Armonk, NY, USA). All data was represented as mean±SD and the mean differences between the conditions were assessed by independent *t-test or the Mann-Whitney U-test*. $P<0.05$ was considered statistically significant.

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III. RESULTS

1. Confirmation of GPR1 in differentiated human skeletal muscle cells

When more than three nuclei are seen in a single cell, it is judged that the cell has differentiated. Therefore, we confirmed that myotubule was differentiated from myoblast by polynucleus (Figure 1(a)) and used differentiated cells on the seventh day of the experiment. Expressions of myotubule differentiation markers myogenin, desmin and myosin heavy chain were confirmed by PCR in cells on the fifth and seventh days of differentiation (Figure1(b)).

In addition, the expression of NPR1, NPR2 and NPR3 in differentiated cells used in experiments was confirmed by PCR (Figure 1(c)) because musclin binds to NPR3 which is a natriuretic peptide receptor and induces competitive inhibition. As a result, it was confirmed that NPR3 existed in differentiated human skeletal muscle cells. Furthermore, since musclin binds to G-protein coupled receptors GPR1 and GPR68, PCR was used to confirm the expression of GPR1 and GPR68 in our cells (Figure 1(d)). The gene expression levels of NPRs, GPR1, and GPR68 were analyzed by quantitative real time PCR (Figure 1(e)).

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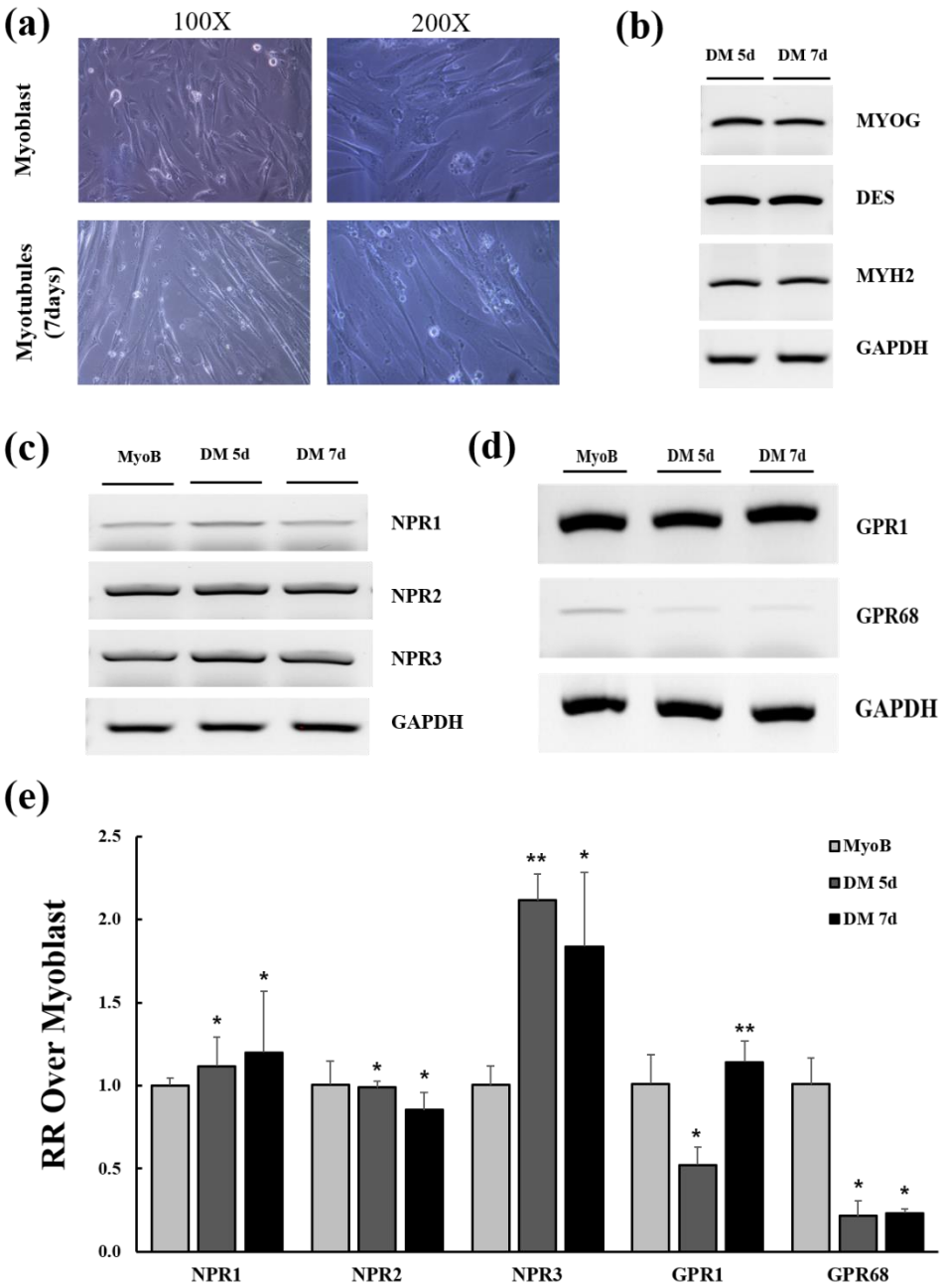

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Figure 1. Confirmation of human skeletal muscle cell differentiation and NPRs, GPR1 and GPR68 expression levels in human skeletal muscle cell. Primary human skeletal muscle cells were differentiated from myoblast to myotubule. Expression of myotubule differentiation markers (MYOG, DES, and MYH2) was detected in myotubules at fifth and seventh day (DM 5d and 7d). And NPR1, NPR2, and NPR3 expression was confirmed in myoblast (MyoB) and DM 5d and 7d. Expression of GPR1 was observed in MyoB and DM 5d and 7d. However, GPR68 expression was not confirmed in MyoB, DM 5d, and DM 7d. Under all conditions, GAPDH was used as a loading control. The graph is presented by relative ratio to myoblast. *P<0.05 and **P<0.001 vs. control. MYOG, myogenin; DES, desmin; MYH2, myosin heavy chain; NPR1, natriuretic peptide receptor 1; NPR2, natriuretic peptide receptor 2; NPR3, natriuretic peptide receptor 3; GPR1, G-protein receptor 1; GPR68, G-protein receptor 68.

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2. The effects of musclin on cGMP production

Musclin is known to bind to NPR3, the natural peptides receptor responsible for decomposing the natriuretic peptides (NPs). Previous studies have confirmed that musclin increases the level of cGMP in COS-7 cells. The reason for this is that musclin binds to NPR3 and other NPs inhibit binding to NPR3 and inhibit the degradation of NPs. Undecomposed NPs are further increased in the binding of NPR1 and NPR2, increasing the level of cGMP in cells.

Musclin was treated separately from our differentiated human skeletal muscle cells, and the cGMP level in the cells remained unchanged (Figure 2(a)), but it was confirmed that CNP, one of the NPs known to induce cGMP, was placed in positive control and that the cGMP level in the cell increases compared to control when musclin and CNP co-treat (Figure 2(b)).

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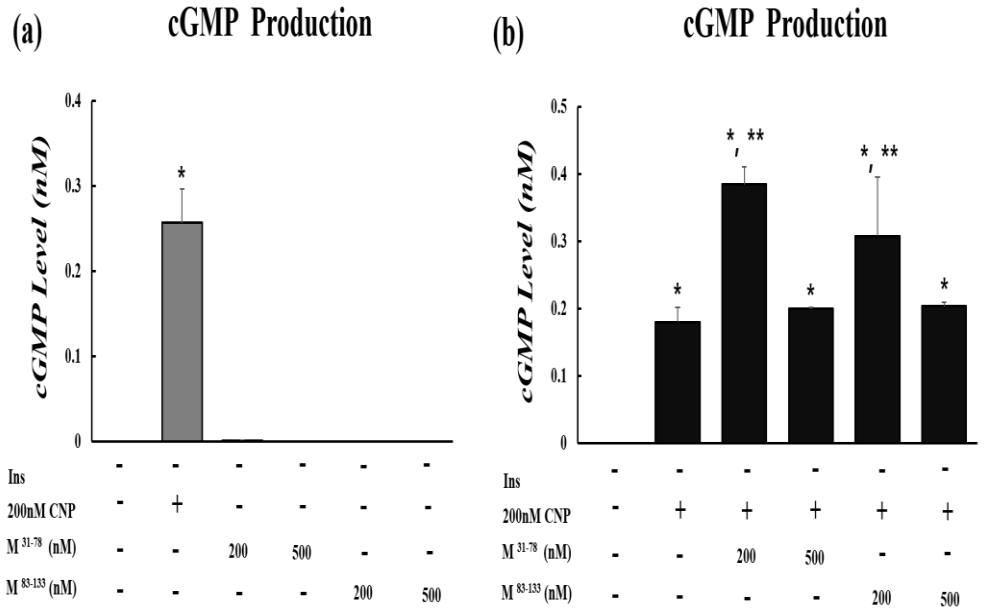

Figure 2. The effects of musclin on cGMP production.

Cells were treated with 200nM CNP and musclins (M^{31-78} and M^{83-133}) at 200nM and 500nM for 30min, respectively (figure 2 (a)). In addition, cells were co-treated with 200nM CNP and musclins (M^{31-78} and M^{83-133}) at 200nM and 500nM for 30min (figure 2 (b)). cGMP production was measured by ELISA assay. Control means that cells were not treated with CNP, M^{31-78}, and M^{83-133}. $^{*}P<0.001$ vs. control, $^{**}P<0.05$ vs. CNP. Ins, insulin; CNP, C-type natriuretic peptide; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; cGMP, cyclic GMP.

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3. Musclin increases the intracellular concentration of cyclic AMP

Musclin is known to bind to GPR1, a G-protein coupled receptor, and it is known that when GPR1, a Gs family, is activated, the concentration of cAMP in cells increases by adenylyl cyclase. Therefore, to confirm whether musclin binds to GPR1 to activate adenylyl cyclase and induce intracellular cAMP concentration changes, we used epinephrine, known to induce cAMP, as the positive control and musclin was treated separately at 200nM and 500nM.

As a result, both concentrations of musclin showed an increase in cAMP levels in differentiated human skeletal muscle cells (Figure 3).

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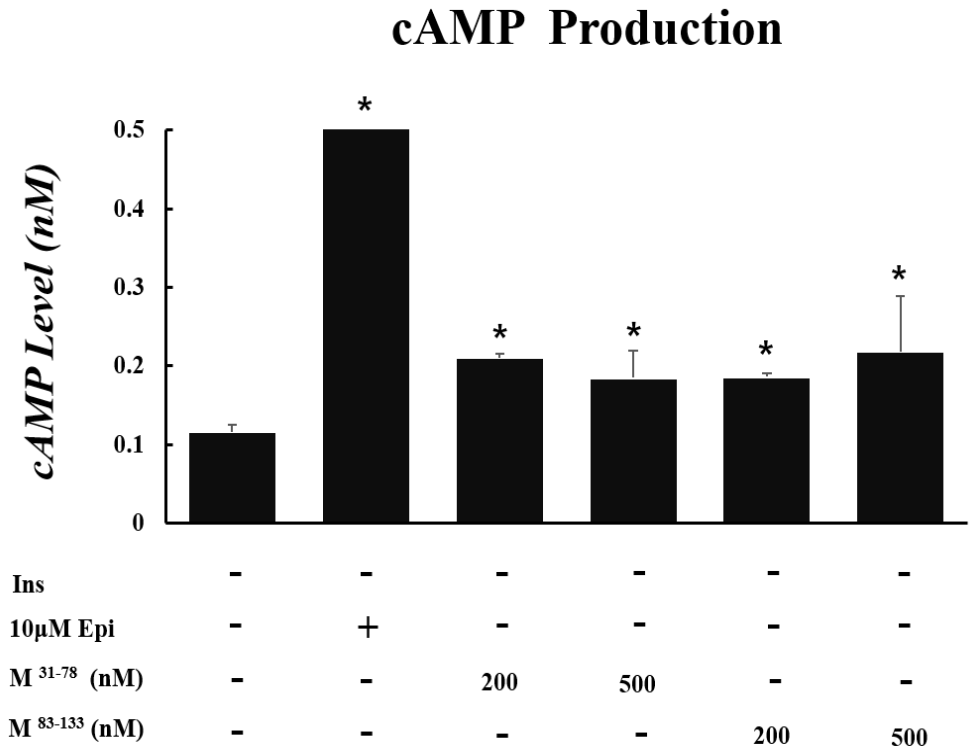

Figure 3. The effects of musclin on cAMP production. Cells were treated with 10μM Epi and musclins (M^{31-78} and M^{83-133}) at 200nM and 500nM for 30min, respectively. cAMP production was measured by ELISA assay. Both M^{31-78} and M^{83-133} increased cGMP production at 200 and 500nM compared to control. Control means that cells were not treated with Epi, M^{31-78}, and M^{83-133}. $^{*}P<0.05$ vs. control. Ins, insulin; Epi, epinephrine; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; cAMP, cyclic AMP.

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4. The Confirmation of mTORC2 activation by musclin

According to other previous studies, isoproterenol binding to β2-Adrenoceptor in rat skeletal muscle cells activates PKA, resulting in an increase in cAMP level. It was then confirmed that the increased cAMP activated mTORC2 at Ser2481. We confirmed that musclin binds to GPR1 and increases intracellular cAMP level by adenyl cyclase. Increased intracellular cAMP activates PKA, but muslin can also activate PKA.

As a result, phosphorylation on Ser2481 of mTORC2 (p-mTORC2^{ser2481}) was confirmed by musclin (Figure 4). Musclin increased concentration of intracellular cAMP level, activated PKA, and activated PKA proved phosphorylation on Ser2481 of mTORC2.

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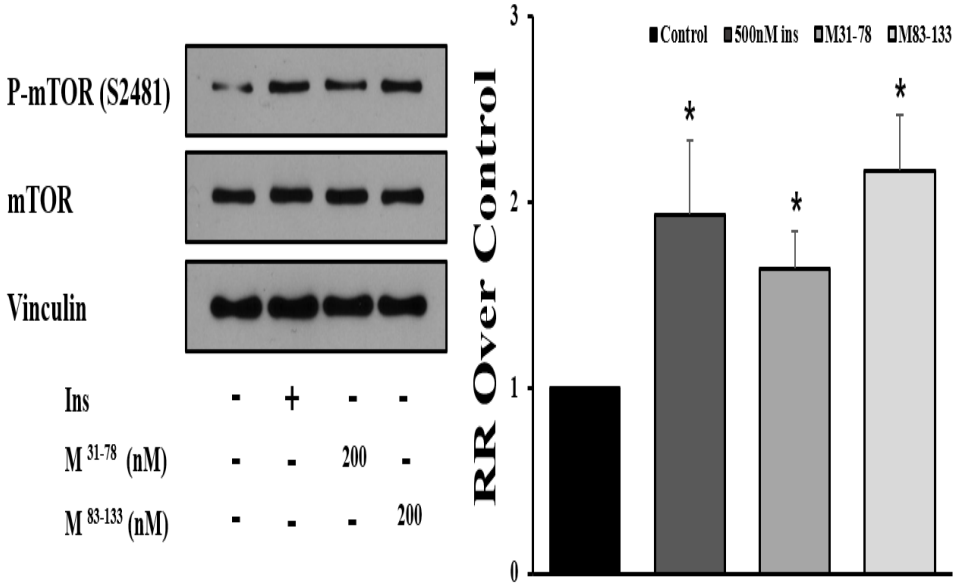

Figure 4. The effects of musclin on mTORC2 phosphorylation. Cells were treated with 500nM Ins and musclins (M^{31-78} and M^{83-133}) at 200nM for 30min, respectively. Ins, M^{31-78}, and M^{83-133} increased phosphorylation level on Ser2481 of mTORC2 in primary human skeletal muscle cells. The relative phosphorylated levels were measured by western blot and normalized to mTORC2. Control means that cells were not treated with ins, M^{31-78}, and M^{83-133}. $^{*}P<0.05$ vs. control. Ins, insulin; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; mTORC2, mammalian target of rapamycin complex 2.

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5. Akt phosphorylation at Ser473 and Thr308 by musclin

To determine the effects of musclin on glucose uptake, insulin-mediated Akt signaling pathway was examined by western blotting (WB). We confirmed that phosphorylation levels on thr308 of Akt ($pAkt^{thr308}$) and phosphorylation on ser473 of Akt ($pAkt^{ser473}$) occurs by insulin as a positive control, and phosphorylation of Akt is also responsive to two types of musclins, which are weaker than insulin (Figure 5). Based on the previous results, it is known that musclin confirmed the phosphorylation on Ser2481 of mTORC2, and that the phosphorylation on Ser473 of Akt was derived by p-$mTORC2^{ser2481}$. These results suggest that musclin may induce glucose uptake by insulin-independent Akt signaling.

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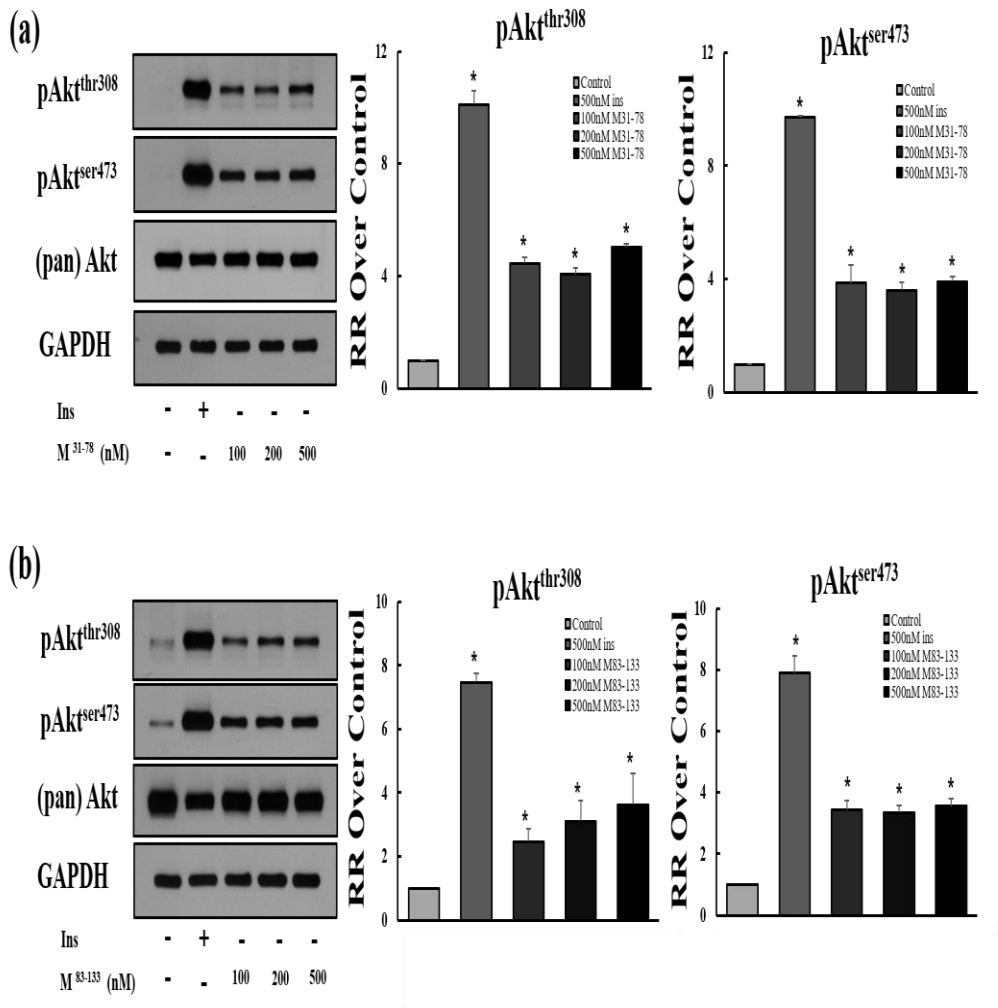

Figure 5. The effects of insulin and musclin on Akt phosphorylation levels. Cells were treated with 500nM Ins as positive control and musclins (M^{31-78} and M^{83-133}) at 100nM, 200nM, and 500nM for 30min, respectively. Phosphorylation on Ser473 and Thr308 of Akt were increased by insulin. (a) M^{31-78} phosphorylated Akt at Thr308 and Ser473. (b) M^{83-133} activates Akt. The relative phosphorylated levels were measured by western blot and normalized to Akt. Control means that cells were not treated with ins, M^{31-78}, and M^{83-133}. $^{*}P<0.05$ vs. control. Ins, insulin; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; Akt, protein kinase B.

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6. Musclin is associated with PI3K pathway by wortmannin

We confirmed the phosphorylation on Thr308 of Akt by musclin. To investigate the connection between PI3K and musclin, known to activate Akt Thr308, we used wortmannin, PI3K's inhibitor. Positive control has insulin, and both types of muslin are treated with wortmannin. Wortmannin inhibited insulin and musclin-induced Akt phosphorylation on Thr308 and Ser473 (Figure 6). Therefore, it can be predicted that musclin is correlated with PI3K pathway.

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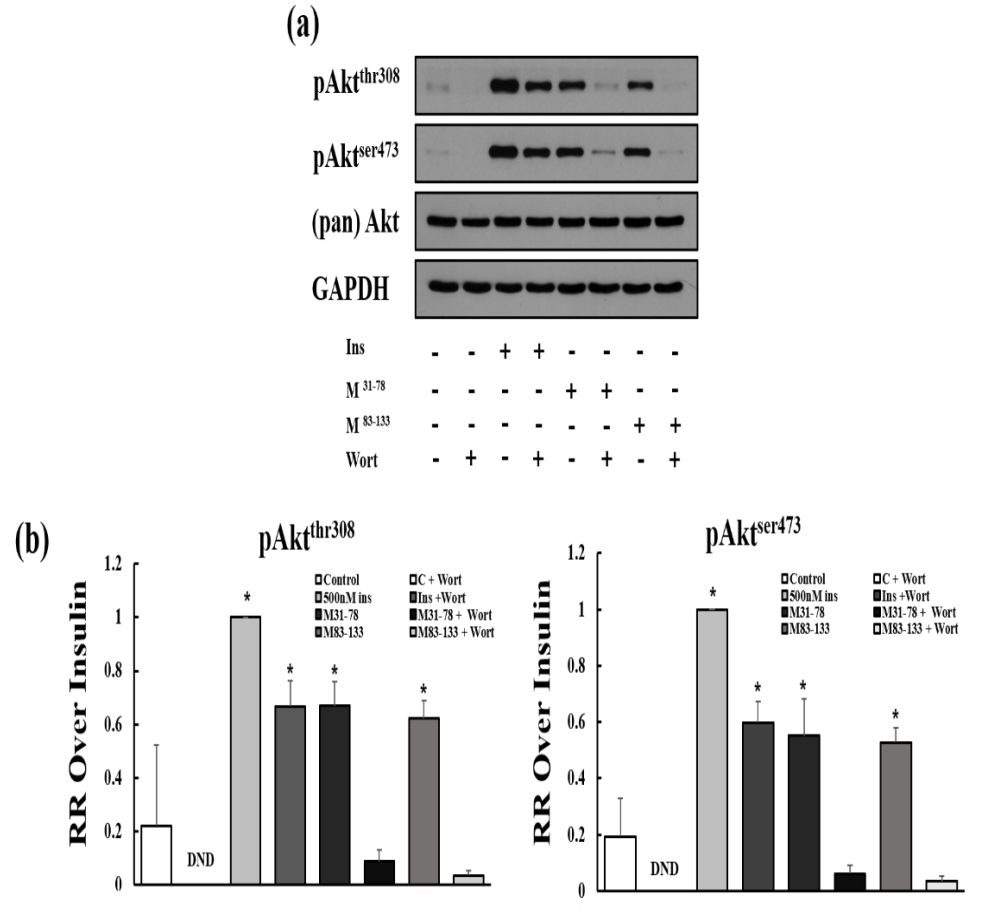

Figure 6. The effects of wortmannin on Akt phosphorylation when musclin is treated. Cells were treated with 500nM Ins as positive control and 200nM musclins (M^{31-78} and M^{83-133}) for 30min after pre-treated 20nM Wort for 15min. Phosphorylation on Ser473 and Thr308 of Akt were increased by Ins, M^{31-78} and M^{83-133}, respectively. However, Ins and musclins (M^{31-78} and M^{83-133}) pre-treated with Wort were attenuated Akt activation. The relative phosphorylated levels were measured by western blot and normalized to Akt. Control means that cells were not treated with ins, M^{31-78}, and M^{83-133}. $^{*}P<0.05$ vs. control. Ins, insulin; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; Akt, protein kinase B; Wort, wortmannin; DND, did not detected.

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7. Conformation of insulin and musclin Co-treat on Akt phosphorylation levels

The results of the previous experiment confirmed that phosphorylation of Akt was induced for insulin and musclin, respectively. Insulin experienced strong Akt activation, while musclin induced Akt activation less strongly than Insulin. To confirm the relationship between the two hormones that induce Akt activation, we treated insulin in musclin$^{31\text{-}78}$ and musclin$^{83\text{-}133}$ respectively to confirm phosphorylation of Akt (Figure 7). When musclin$^{31\text{-}78}$ and musclin$^{83\text{-}133}$ were co-treated with insulin, they became phosphorylation of Akt on Thr308 and Ser473 similar to the insulin, positive control. Therefore, even if the pathways of the two hormones are not the same, they eventually induce Akt activation, which helps the glucose translocation from the blood to the skeletal muscle cells.

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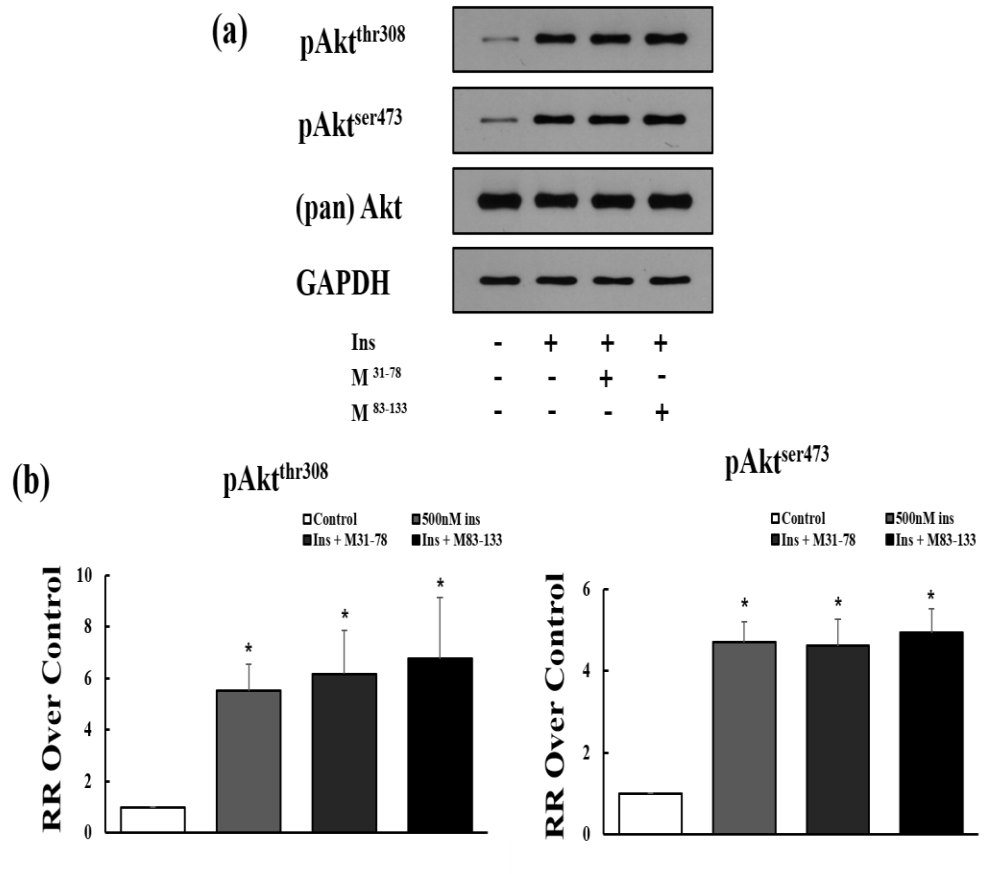

Figure 7. The effects of insulin and musclin Co-treat on Akt phosphorylation levels. Cells were treated with 500nM Ins as positive control and 200nM musclins (M^{31-78} and M^{83-133}) co-treated with Ins for 30min. 200nM musclins (M^{31-78} and M^{83-133}) co-treated with Ins were phosphorylated on Ser473 and Thr308 of Akt similarly to insulin, a positive control. The relative phosphorylated levels were measured by western blot and normalized to Akt. Control means that cells were not treated with ins, M^{31-78}, and M^{83-133}. $^{*}P<0.05$ vs. control. Ins, insulin; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; Akt, protein kinase B.

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8. GLUT4 translocation by musclin

In skeletal muscle cells, glucose enters the cell via facilitated diffusion through the glucose transporter type 4 (GLUT4). GLUT4 is the insulin-regulated glucose transporter found primarily in skeletal muscle. Akt activation causes GLUT4 storage vesicles (GSVs) floating in the cytoplasm to move to the plasma membrane and embed GLUT4 in the cell membrane. We used Immunocytochemistry (ICC) to confirm whether GLUT4 was transferred from the cytoplasm to the plasma membrane by musclin treatment in primary human skeletal muscle cells (Figure 8). GLUT4 translocation was confirmed by musclin$^{31\text{-}78}$ and musclin$^{83\text{-}133}$ which were treated separately, as well as insulin, which is positive control compared to control (Figure 8). GLUT4 translation was also induced when insulin and musclin were co-treated (Figure 8). Therefore, GLUT4 translocation occurs through musclin and glucose in blood can be taken into the skeletal muscle cells.

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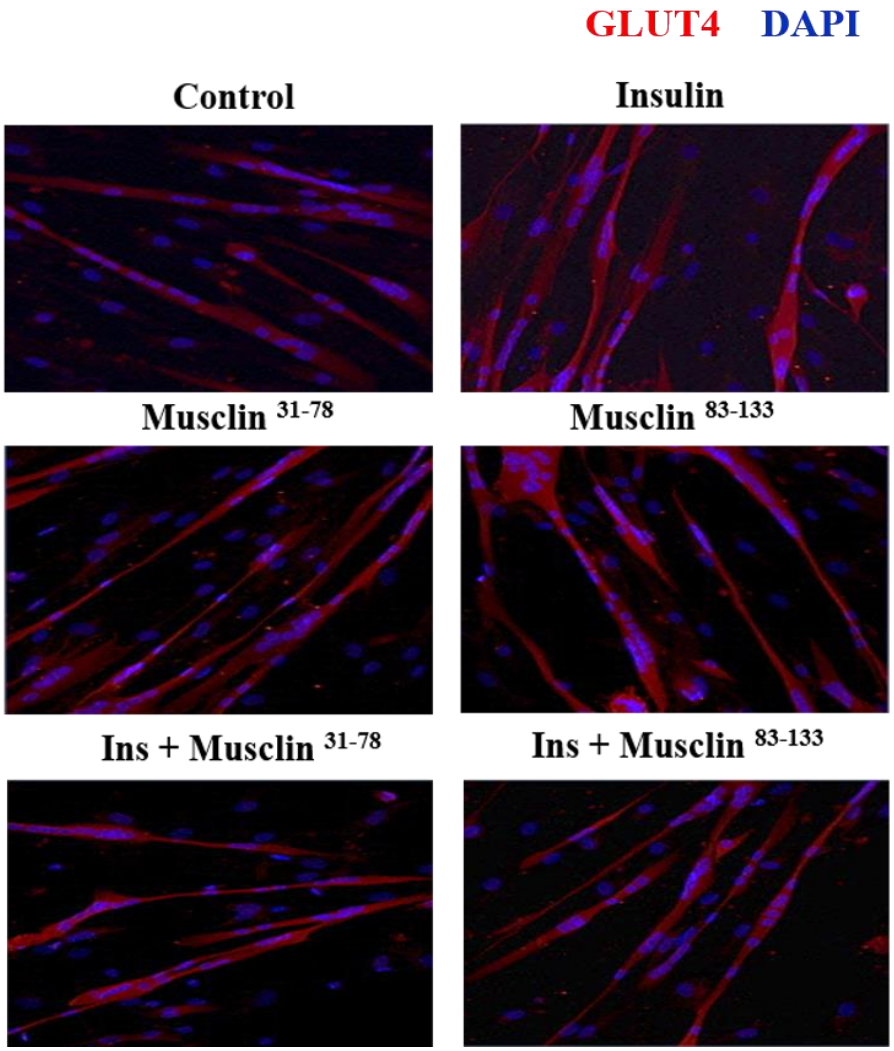

Figure 8. The effect of musclin on glucose transporter type 4 (GLUT4) translocation. Representative images of primary human skeletal muscle cell treated by 500nM insulin and 200nM musclins (M^{31-78} and M^{83-133}), respectively, show that translocation of GLUT4 (Cy3) increased from cytosol to plasma membrane compare with control. 200nM musclins (M^{31-78} and M^{83-133}) co-treated with Ins translocated GLUT4 (Cy3) to cell membrane. Nucleus were stained with DAPI (blue). Control means that cells were not treated with ins, M^{31-78}, and M^{83-133}. Ins, insulin; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; GLUT4, glucose transporter type 4

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9. 2-NBDG uptake in response to Musclin

Musclin$^{31\text{-}78}$ and musclin$^{83\text{-}133}$ activated Akt in primary skeletal muscle cells. Akt phosphorylation on Thr308 and Ser473 were known to regulate the concentration of glucose in the blood by moving it into the cells. To confirm changes in glucose concentration in the blood through musclin, glucose (2-NBDG) uptake in response to musclin in primary human skeletal muscle cells was measured. The procedures of the assay are detailed in subheading II-6. Insulin is commonly known to induce glucose uptake, so insulin is placed as a positive control. Both musclin$^{31\text{-}78}$ and musclin$^{83\text{-}133}$ increased 2-NBDG uptake in primary human skeletal muscle cells (Figure 9). Furthermore, it was confirmed that 2-NBDG uptake increased when insulin and musclin were treated simultaneously (Figure 9).

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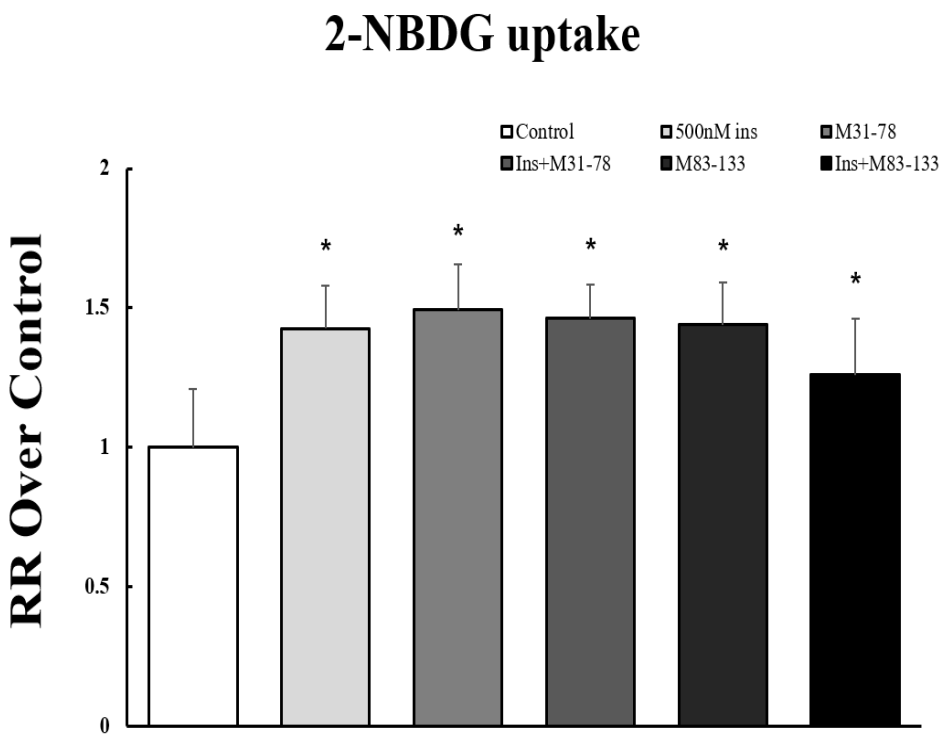

Figure 9. The effect of musclin on glucose uptake.

Cells were treated with 500nM Ins as positive control and 200nM musclins (M^{31-78} and M^{83-133}) for 1hr. Ins and musclins (M^{31-78} and M^{83-133}) significantly increased glucose analog (2-deoxy-2-[(7-nitro21,1,3-benzoxadiazol-4-yl) amino]-D-glucose, 2-NBDG) uptake, respectively. Both of 200nM musclins (M^{31-78} and M^{83-133}) co-treated with Ins also increased 2-NBDG uptake significantly. **significantly higher than control.* Ins, insulin; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; 2NBDG, 2-deoxy-2-[(7-nitro21,1,3-benzoxadiazol-4-yl) amino]-D-glucose.

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IV. DISCUSSION

The key finding of this study was that musclin stimulates glucose uptake via Akt activation mediated GPR1 pathway in primary skeletal muscle cells. Musclin increased Akt phosphorylation, mTORC2 phosphorylation and intracellular concentration of cAMP level, thereby, it is suggested that muslin has glucose uptake effects via translocation of GLUT4 from the cytosol from the plasma membrane. In addition, it was shown that musclin exerts the effects through PI3K pathway, which is known to be activated by G protein beta gamma (Gβγ). To the best our knowledge, this was the first study reporting a specific pathway of muslin on glucose uptake via GPR1 pathway and Akt phosphorylation in primary human skeletal muscle cells.

Musclin was identified as a novel myokine, many studies have focused on the characterization of its mechanism. Musclin can function as an autocrine and paracrine factor in skeletal muscles, and that increased production of musclin is known to be associated with insulin resistance in the skeletal muscle of obese mice [7]. Musclin included a homologous region to the functional 17 amino acids of the NP family, and basic KKKR sequence, a putative serine protease cleavage site [20]. Different from the NP family, the preserved 17 amino acids of musclin are not positioned between two cysteine residues, crucial for physiological protein folding to produce natriuretic activity. Therefore, it is unlikely that musclin has a natriuretic activity [7]. Previous studies have shown that musclin is known to have strongly bind with natriuretic peptide receptor C (NPRC), which is responsible for decomposition of NPs, and that co- treatment with ANP, one of the NPs, results in contention inhibition of NPRC-NPs, increasing the concentration of cGMP level in cells [12, 13]. Musclin was treated separately from our differentiated human skeletal muscle cells, and the cGMP level in the cells remained unchanged (Figure 2(a)), but it was confirmed that CNP, one of the NPs known to induce cGMP, was placed in positive control and that the cGMP level in the cell increases compared to control when musclin and CNP co-treat (Figure

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2(b)).

In addition, previous studies have shown that GPR1 is widely distributed in skeletal muscles [21]. Furthermore, GPR1 was known to be involved in glucose homeostasis because of changes in blood glucose regulation in the absence of GPR1 due to reduced physiological effects in GPR1 KO mice [21]. Recently, musclin has been known to bind to GPR1 as a β-arrestin biased receptor. [16, 22]. To investigate the relationship between musclin and GPR1, we confirmed the expression of GPR1 in primary human skeletal muscle cells (Figure1(d)). In the yeast *Saccharomyces cerevisiae*, two Gα subunits, Gpa1 and Gpa2, have been characterized. Unlike Gpa1, Gpa2 was shown to regulate the intracellular concentration of cAMP level in response to glucose stimulation through interaction with a putative GPCR, Gpr1 [18, 23, 24]. However, it is not clear what signal transduction occurs when musclin binds to GPR1 in primary skeletal muscle cells. Therefore, we treated musclin in skeletal muscle cells to confirm the intracellular cAMP level, and as a result, we confirmed that musclin increased the intracellular concentration of cAMP level in primary skeletal muscle cells (Figure 3). According to previous studies, GPR1 was known as Gs family receptors and it activate adenylyl cyclase to increase intracellular cAMP level [17, 18]. Therefore, it can be predicted that musclin binds to GPR1 to induce intracellular cAMP generation. Recent studies showed that mTORC2 was activated by the increased level of cAMP and it was proved that GLUT4 movement was confirmed by cAMP to induce glucose uptake in rat skeletal muscle cells [19]. We confirmed whether the increased cAMP level by musclin in primary human skeletal muscle cells induced phosphorylation of mTORC2. As a result, phosphorylation on Ser2481 of mTORC2 was confirmed by musclin (Figure 4). Musclin increased concentration of intracellular cAMP level (Figure 3), activated PKA, and activated

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PKA proved phosphorylation on Ser2481 of mTORC2. Both musclins (M31-78 and M83-133) confirmed that mTORC2 activation increased compared to control, and that especially M83-133, when compared to positive control insulin, was activated similar to insulin (Figure 4). Previous studies have shown that mTORC2 plays an important role in glucose homeostasis through studies indicate that ablation of mTORC2 inhibited glucose uptake by insulin in skeletal muscle [25].

The Akt phosphorylation on Thr308 and Ser473 play a key role in insulin signaling, and its activation by insulin has been shown to play a crucial role in GLUT4 translocation to plasma membrane [26]. Also, studies have shown that activation of mTORC2 leads to phosphorylation of Akt on Ser473 [19]. However, other previous studies reported that glucose uptake and glycogen synthesis by insulin are inhibited by musclin. [7, 14]. In addition, the most commonly known Akt activity among insulin signaling pathway was suppressed by musclin treatment [7, 14]. Therefore, in order to prove phosphorylation of Akt by muscln, musclin was treated with primary human skeletal muscle cells to confirm Akt activation. As a result, it was confirmed that musclin (M31-78 and M83-133) induced Akt phosphorylation on Thr308 and Ser473, *respectively* (Figure 5). In addition, it has been confirmed that phosphorylation of Akt occurs not only when treated alone but also when co-treated with insulin, which is a positive control (Figure 7). Accordingly, it is expected that musclin will not inhibit insulin-induced Akt activation. These results suggest that musclin may induce glucose uptake by insulin-independent Akt signaling.

Akt was a crucial signal key downstream of phosphatidylinositide 3-kinase (PI3K) and phosphorylation of Thr308 and Ser473 after plasma membrane recruitment of Akt leads to full activation of Akt [27, 28]. Signaling of PI3K was a key pathway in the regulation of glucose uptake by insulin [29, 30]. Since Akt

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phosphorylation occurred by musclin, Wortmannin, known as PI3K inhibitor [31, 32], was used to confirm its correlation with PI3K pathway. As a result, activation of Akt was shown by musclin alone, but Akt phosphorylation on Thr308 and Ser473 was significantly reduced when musclin and wortmannin were co-treated (figure 6). Through this result, it can be suggested that Akt activation by musclin is not only by intracellular cAMP level, but also PI3K-dependent pathway.

The Akt phosphorylation on Thr308 and Ser473 plays a key role in insulin signaling [26], and its activation by insulin has been shown to play a crucial role in GLUT4 translocation to plasma membrane in rat and human skeletal muscle[33-35]. We identified whether musclin also causes GLUT4 translocation by ICC, as musclin induces Akt activation, in human skeletal muscle cell. As a result, it was confirmed that GLUT4 translocation increased in musclin alone treatment compared to control, and that GLUT4 translocation increased in musclin co-treated with insulin as a positive control (Figure 8). Previous studies on GLUT4 knockout mice have unambiguously shown that GLUT4 is necessary for muscle contractions as well as insulin to increase glucose uptake in skeletal muscle [27, 36-38]. Therefore, GLUT4 in the cytoplasm is translocated to the cell membrane by musclin, so it can be predicted that free glucose in the blood can be transferred into the cell. However, in preliminary experiments, musclin reduced insulin-induced glucose uptake in differentiated rat P3 myocytes. These findings indicate that musclin is correlated with insulin resistance (IR) [14]. IR is a characteristic feature in the pathogenesis of type 2 diabetes (T2D) and obesity [39, 40]. Thus, we used 2NBDG, a fluorescently-labeled deoxyglucose analog, to confirm the relationship between musclin and glucose uptake followed by immunocytochemical detection of fluorescence produced by the primary human skeletal muscle cells. As a result, it was confirmed that glucose

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uptake is induced by musclin (M31-78 and M83-133) similar to insulin, which is a positive control, *respectively* (Figure 9). In addition, it was suggested that simultaneous treatment of insulin and musclin increases glucose uptake (Figure 9). Therefore, it can be suggested that musclin plays a positive role in adjusting glucose levels in the primary human skeletal muscle cells.

Given that it is the first study suggesting that musclin stimulates glucose uptake through cAMP and Akt activation in primary human skeletal muscle cells, musclin could make it a potential treatment for insulin resistance on patients with type 2 diabetes.

Schematic representation of the study is shown in Figure 10.

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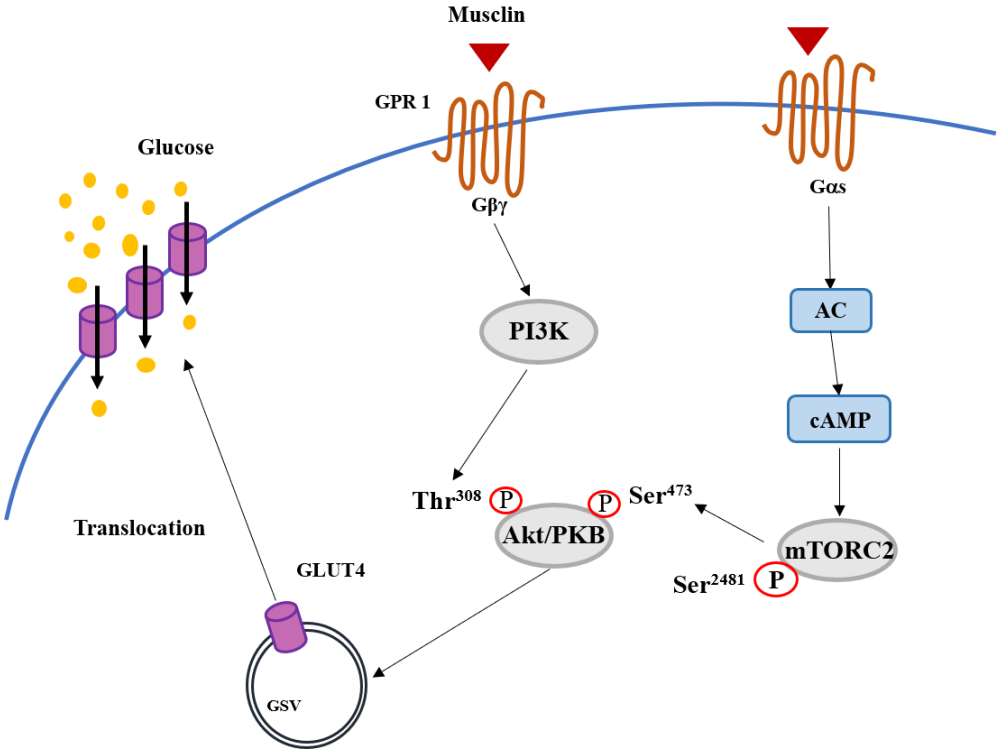

Figure 10. schematic illustration of this study. Musclin may interact with GPR1 in primary human skeletal muscle cells, in turn; it induces an increase in intracellular levels of cAMP. Increased cAMP by musclin (M^{31-78} and M^{83-133}) was activated mTORC2 at Ser2481. In addition, musclin (M^{31-78} and M^{83-133}) phosphorylates on Thr308 and Ser473 of Akt, *respectively*. Furthermore, GLUT4 translocation occurs through musclin (M^{31-78} and M^{83-133}) from the cytoplasm to the plasma membrane. As a result, musclin (M^{31-78} and M^{83-133}) increased 2-NBDG uptake in primary human skeletal muscle cells. GPR1, G-protein receptor 1; AC, adenylyl cyclase; cAMP, cyclic AMP; M^{31-78}, musclin peptide 31-78; M^{83-133}, musclin peptide 83-133; mTORC2, mammalian target of rapamycin complex 2; Akt, protein kinase B; PI3K, phosphatidylinositol-4,5-bisphophatate 3-kinase; GSV, GLUT4 storage vesicles; GLUT4, glucose transporter type 4; 2NBDG, 2-deoxy-2-[(7-nitro21,1,3-benzoxadiazol-4-yl) amino]-D-glucose.

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V. CONCLUSION

We report that musclin increased glucose uptake in primary human skeletal muscle cell through mechanisms involving the phosphorylation of Akt/PKB and increased intracellular cAMP level via insulin-independent pathway. Our results demonstrate that vesicle containing glucose transporter type 4 (GLUT4), known as the glucose uptake pathway in the skeletal muscle cell, was translocated from cytosol to plasma membrane by musclin$^{31\text{-}78}$ and musclin$^{83\text{-}133}$ treatment. Also, we found that phosphorylation at Thr308 and Ser473 of Akt by musclin$^{31\text{-}78}$ and musclin$^{83\text{-}133}$ and co-treat with insulin. In addition, mTORC2 was phosphorylated at Ser2481 and intracellular cAMP level was increased by musclin.

In summary, musclin was shown to stimulate glucose uptake via phosphorylation of Akt/PKB and increased intracellular cAMP level in primary human skeletal muscle cells in a mechanism likely involving GLUT4 translocation. These findings provide insight into the glucose metabolism of musclin, and could potentially become the focus of future research on myokines into the novel therapeutic target for insulin resistance on patients with type 2 diabetes.

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REFERENCES

1. Lee, I.M., et al., *Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy.* Lancet, 2012. **380**(9838): p. 219-29.
2. Adams, K.F., et al., *Overweight, obesity, and mortality in a large prospective cohort of persons 50 to 71 years old.* N Engl J Med, 2006. **355**(8): p. 763-78.
3. Balducci, S., et al., *Changes in physical fitness predict improvements in modifiable cardiovascular risk factors independently of body weight loss in subjects with type 2 diabetes participating in the Italian Diabetes and Exercise Study (IDES).* Diabetes Care, 2012. **35**(6): p. 1347-54.
4. Corcoran, M.P., S. Lamon-Fava, and R.A. Fielding, *Skeletal muscle lipid deposition and insulin resistance: effect of dietary fatty acids and exercise.* Am J Clin Nutr, 2007. **85**(3): p. 662-77.
5. Munoz-Canoves, P., et al., *Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword?* FEBS J, 2013. **280**(17): p. 4131-48.
6. Bostrom, P., et al., *A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis.* Nature, 2012. **481**(7382): p. 463-8.
7. Nishizawa, H., et al., *Musclin, a novel skeletal muscle-derived secretory factor.* J Biol Chem, 2004. **279**(19): p. 19391-5.
8. Thomas, G., et al., *Osteocrin, a novel bone-specific secreted protein that modulates the osteoblast phenotype.* J Biol Chem, 2003. **278**(50): p. 50563-71.
9. Subbotina, E., et al., *Musclin is an activity-stimulated myokine that enhances physical endurance.* Proc Natl Acad Sci U S A, 2015. **112**(52): p. 16042-7.
10. Herzig, S. and R.J. Shaw, *AMPK: guardian of metabolism and mitochondrial homeostasis.* Nat Rev Mol Cell Biol, 2018. **19**(2): p. 121-135.
11. Dickey, D.M., S. Abbey-Hosch, and L.R. Potter, *Natriuretic Peptides, Their Receptors, and Cyclic Guanosine Monophosphate-Dependent Signaling Functions.* Endocrine Reviews, 2006. **27**(1): p. 47-72.
12. Kita, S., et al., *Competitive binding of musclin to natriuretic peptide receptor 3 with atrial natriuretic peptide.* J Endocrinol, 2009. **201**(2): p. 287-95.
13. Koller, K.J., et al., *Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP).* Science, 1991. **252**(5002): p. 120-3.
14. Liu, Y., et al., *Musclin inhibits insulin activation of Akt/protein kinase B in rat skeletal muscle.* J Int Med Res, 2008. **36**(3): p. 496-504.

연세대학교
YONSEI UNIVERSITY

15. Coue, M., et al., *Natriuretic peptides promote glucose uptake in a cGMP-dependent manner in human adipocytes.* Sci Rep, 2018. **8**(1): p. 1097.
16. Foster, S.R., et al., *Discovery of Human Signaling Systems: Pairing Peptides to G Protein-Coupled Receptors.* Cell, 2019. **179**(4): p. 895-908 e21.
17. Huang, X.P., et al., *Allosteric ligands for the pharmacologically dark receptors GPR68 and GPR65.* Nature, 2015. **527**(7579): p. 477-83.
18. Lorenz, M.C., et al., *The G protein-coupled receptor gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in Saccharomyces cerevisiae.* Genetics, 2000. **154**(2): p. 609-22.
19. Sato, M., et al., *Improving type 2 diabetes through a distinct adrenergic signaling pathway involving mTORC2 that mediates glucose uptake in skeletal muscle.* Diabetes, 2014. **63**(12): p. 4115-29.
20. Schmitt, M., J.R. Cockcroft, and M.P. Frenneaux, *Modulation of the natriuretic peptide system in heart failure: from bench to bedside?* Clin Sci (Lond), 2003. **105**(2): p. 141-60.
21. Rourke, J.L., et al., *Gpr1 is an active chemerin receptor influencing glucose homeostasis in obese mice.* J Endocrinol, 2014. **222**(2): p. 201-15.
22. Hauser, A.S., et al., *Novel approaches leading towards peptide GPCR de-orphanisation.* Br J Pharmacol, 2020. **177**(5): p. 961-968.
23. Yun, C.W., et al., *Gpr1p, a putative G-protein coupled receptor, regulates glucose-dependent cellular cAMP level in yeast Saccharomyces cerevisiae.* Biochem Biophys Res Commun, 1998. **252**(1): p. 29-33.
24. Kraakman, L., et al., *A Saccharomyces cerevisiae G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose.* Mol Microbiol, 1999. **32**(5): p. 1002-12.
25. Kumar, A., et al., *Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity.* Mol Cell Biol, 2008. **28**(1): p. 61-70.
26. Chen, A., et al., *Urocortin 2 modulates glucose utilization and insulin sensitivity in skeletal muscle.* Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16580-5.
27. Hanada, M., J. Feng, and B.A. Hemmings, *Structure, regulation and function of PKB/AKT--a major therapeutic target.* Biochim Biophys Acta, 2004. **1697**(1-2): p. 3-16.
28. Scheid, M.P. and J.R. Woodgett, *Unravelling the activation mechanisms of*

연세대학교
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protein kinase B/Akt. FEBS Letters, 2003. **546**(1): p. 108-112.

29. Wong, J.T., et al., *Pten (phosphatase and tensin homologue gene) haploinsufficiency promotes insulin hypersensitivity.* Diabetologia, 2007. **50**(2): p. 395-403.
30. Lizcano, J.M. and D.R. Alessi, *The insulin signalling pathway.* Curr Biol, 2002. **12**(7): p. R236-8.
31. Liu, Y., et al., *Wortmannin, a widely used phosphoinositide 3-kinase inhibitor, also potently inhibits mammalian polo-like kinase.* Chem Biol, 2005. **12**(1): p. 99-107.
32. Kim, S.H., et al., *The reno-protective effect of a phosphoinositide 3-kinase inhibitor wortmannin on streptozotocin-induced proteinuric renal disease rats.* Exp Mol Med, 2012. **44**(1): p. 45-51.
33. Karlsson, H.K., et al., *Kinetics of GLUT4 trafficking in rat and human skeletal muscle.* Diabetes, 2009. **58**(4): p. 847-54.
34. Ryder, J.W., et al., *Use of a novel impermeable biotinylated photolabeling reagent to assess insulin- and hypoxia-stimulated cell surface GLUT4 content in skeletal muscle from type 2 diabetic patients.* Diabetes, 2000. **49**(4): p. 647-54.
35. Koistinen, H.A., et al., *5-amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes.* Diabetes, 2003. **52**(5): p. 1066-72.
36. Zisman, A., et al., *Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance.* Nat Med, 2000. **6**(8): p. 924-8.
37. Howlett, K.F., et al., *Exercise-induced muscle glucose uptake in mice with graded, muscle-specific GLUT-4 deletion.* Physiol Rep, 2013. **1**(3): p. e00065.
38. Ryder, J.W., et al., *Postexercise glucose uptake and glycogen synthesis in skeletal muscle from GLUT4-deficient mice.* FASEB J, 1999. **13**(15): p. 2246-56.
39. Lebovitz, H.E., *Type 2 diabetes: an overview.* Clin Chem, 1999. **45**(8 Pt 2): p. 1339-45.
40. Bhatia, V. and P. Viswanathan, *Insulin resistance and PPAR insulin sensitizers.* Curr Opin Investig Drugs, 2006. **7**(10): p. 891-7.

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ABSTRACT (IN KOREAN)

분화된 인간 근육 세포에서 Musclin이 인슐린과 다른 경로를 통해 포도당 섭취에 미치는 효과 검증

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조 소 연

운동 시 근수축이 발생하여 마이오카인이 분비된다. 분비되는 여러 마이오카인 중 뼈에서 분비된다고 알려진 Musclin (*a.k.a.* osteocrin)이 지속적인 근 수축을 통해 골격근에서도 분비된다고 밝혀졌다. Musclin은 NPs의 분해를 담당하는 natriuretic peptide receptor C (NPRC)와 강한 결합력을 가져 다른 NPs들이 NPRC와의 결합을 억제하여 세포 내 cGMP 농도 증가를 유도한다고 보고된 바 있다. 또한 musclin이 운동능력에 긍정적인 상관성을 갖는다고 제시된 반면, musclin이 insulin에 의한 glucose uptake와 glycogen synthesis를 억제한다고 보고되었다. 하지만 본 연구에서 musclin 독립 처치에 의해 Akt^{Thr308} 과 Akt^{Ser473} 활성이 증가되고 $mTORC2^{Ser2481}$ 활성 또한 증가됨을 확인하였다. 뿐만 아니라, 최근 musclin이 G-protein coupled receptors (GPCRs) 중 G_s계열 수용체인 GPR1과 결합한다고 알려졌으며 그 결과 adenylyl cyclase (AC)를 활성화시켜 세포 내 cyclic AMP 농도를 증가시킨다고 예상 가능하지만 이에 대

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해 단독적인 musclin의 생리학적 매커니즘에 대해 연구된 바 없다. 따라서 musclin에 대한 cGMP 및 cAMP와 Akt의 상관성에 관해 추가 연구가 필요하며, musclin이 에너지대사에 대해 어떠한 생리학적 메커니즘에 영향을 미치는지 규명하여 에너지대사 질병 중 하나인 insulin 저항성(*insulin resistance*, IR)의 치료 효과를 제시한다.

핵심되는 말: 마이오카인, 대사작용, 운동, 인슐린 저항성, 인간 골격근 세포