Amelioration of muscle wasting by glucagon-like peptide-1 receptor agonist in muscle atrophy

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Abstract

Background  Skeletal muscle atrophy is defined as a reduction of muscle mass caused by excessive protein degradation. However, the development of therapeutic interventions is still in an early stage. Although glucagon-like peptide-1 receptor (GLP-1R) agonists, such as exendin-4 (Ex-4) and dulaglutide, are widely used for the treatment of diabetes, their effects on muscle pathology are unknown. In this study, we investigated the therapeutic potential of GLP-1R agonist for muscle wasting and the mechanisms involved.

Methods  Mouse C2C12 myotubes were used to evaluate the in vitro effects of Ex-4 in the presence or absence of dexamethasone (Dex) on the expression of muscle atrophic factors and the underlying mechanisms using various pharmacological inhibitors. In addition, we investigated the in vivo therapeutic effect of Ex-4 in a Dex-induced mouse muscle atrophy model (20 mg/kg/day i.p.) followed by injection of Ex-4 (100 ng/day i.p.) for 12 days and chronic kidney disease (CKD)-induced muscle atrophy model. Furthermore, we evaluated the effect of a long-acting GLP-1R agonist by treatment of dulaglutide (1 mg/kg/week s.c.) for 3 weeks, in DBA/2J-mdx mice, a Duchenne muscular dystrophy model.

Results  Ex-4 suppressed the expression of myostatin (MSTN) and muscle atrophic factors such as F-box only protein 4 (FBXO4) and muscle RING-finger protein-1 (MURF-1) in Dex-treated C2C12 myotubes. The suppression effect was via protein kinase A and protein kinase B signalling pathways through GLP-1R. In addition, Ex-4 treatment inhibited glucocorticoid receptor (GR) translocation by up-regulating the proteins of GR inhibitory complexes. In a Dex-induced muscle atrophy model, Ex-4 ameliorated muscle atrophy by suppressing muscle atrophic factors and enhancing myogenic factors (MyoG and MyoD), leading to increased muscle mass and function. In the CKD muscle atrophy model, Ex-4 also increased muscle mass, myofiber size, and muscle function. In addition, treatment with a long-acting GLP-1R agonist, dulaglutide, recovered muscle mass and function in DBA/2J-mdx mice.

Conclusions  GLP-1R agonists ameliorate muscle wasting by suppressing MSTN and muscle atrophic factors and enhancing myogenic factors through GLP-1R-mediated signalling pathways. These novel findings suggest that activating GLP-1R signalling may be useful for the treatment of atrophy-related muscular diseases.

Keywords  Skeletal muscle atrophy; GLP-1R agonists; Dexamethasone; Glucocorticoid receptor; Chronic kidney disease; Duchenne muscular dystrophy

Introduction

Skeletal muscle atrophy is a pathological condition caused by excessive muscle protein degradation by diverse factors: genetic factors, various diseases, disuse, and aging. Patients either with chronic kidney disease (CKD) or cancer or who are getting older are highly susceptible to the development of skeletal muscle atrophy.¹⁻⁵ It disrupts the quality of life...
and increases mortality and morbidity.\textsuperscript{6} Thus, the demand for the development of specialized medicine for muscle atrophy has been increasing and becoming an important research topic worldwide. The understanding of molecular mechanisms implicated in muscle atrophy has substantially progressed for the last few decades; however, there are still no effective pharmacological and therapeutic approaches up to now, except for exercise regimens.\textsuperscript{1} In addition, the use of exercise regimen is severely limited in the elderly or bedridden population or those with acute illnesses.\textsuperscript{3} Currently, myostatin (MSTN) and activin A antagonists are considered the most promising candidates for the treatment of muscle atrophy, but they still have obstacles (such as off-targeting to other transforming growth factor family members) to overcome for use on human patients.\textsuperscript{1,7,8}

Glucocorticoid (GC) is implicated in protein and glucose metabolism in skeletal muscle.\textsuperscript{9–11} Circulating GC levels increase during fasting/starvation (catabolic conditions) or in various diseases (e.g. CKD, Cushing’s syndrome, and cancer) and in the elderly. It is a known risk factor for the development of muscle atrophy.\textsuperscript{1,11} Chronic or excessive exposure to GC induces muscle atrophy by up-regulating the expression of MSTN and muscle-specific E3 ubiquitin ligases such as F-box only protein 32 (atrogin-1) and muscle RING-finger protein-1 (MuRF-1).\textsuperscript{11}

Glucagon-like peptide-1 (GLP-1) is a 30-amino-acid peptide hormone synthesized and secreted from the intestinal endocrine L-cells. It has numerous physiological actions through its receptor, GLP-1R, including promoting glucose-induced insulin secretion, elevating β-cell survival, inhibiting glucagon production, and delaying gastric emptying.\textsuperscript{12–14} Therefore, GLP-1-based drugs have been developed as an anti-diabetic therapy. Although some studies have implicated the possible effects of GLP-1-based drugs on muscle mass, metabolism, and function, little is known about the precise mechanisms underlying its effects.\textsuperscript{15–17} Therefore, we investigated whether GLP-1-based drugs have a therapeutic effect in muscle wasting.

In the current study, we examined whether a GLP-1R agonist, exendin-4 (Ex-4), regulates muscle atrophic and myogenic factors and, if so, what mechanisms are involved. In addition, we also investigated whether GLP-1R agonists attenuate muscle atrophy in different types of in vivo models of muscle atrophy.

**Materials and methods**

**Animals**

In order to generate a dexamethasone (Dex)-induced muscle atrophy mouse model, we administered Dex (D4902, Sigma-Aldrich, MO, USA) intraperitoneally at 20 mg/kg/day to 10-week-old C57BL/6 male mice ( Orient Bio Inc, Gyeonggi, Korea) for 8 days followed by injection of Ex-4 (1933, Tocris Bioscience, Bristol, UK) at 100 ng/day for 12 days. To generate a CKD-induced muscle atrophy model, 10-week-old C57BL/6 mice with similar body weights underwent subtotal nephrectomy in two stages, as described previously.\textsuperscript{18} Briefly, in the first stage, ~70% of the right kidney was removed. Seven days later, the entire left kidney was removed. CKD developed in 2 weeks, as shown by the increased urea nitrogen and creatinine in blood, indicating kidney dysfunction. Sham mice underwent surgery without damaging the kidneys. Ex-4 (100 ng) or an equal amount of phosphate-buffered saline (PBS) was injected intraperitoneally every day for 8 weeks. DBA/2-mdx mice (Duchenne muscular dystrophy model) were purchased from the Jackson Laboratory (MA, USA). Dulaglutide (Trulicity®, Eli Lilly and Company, IN, USA) was injected subcutaneously at 1 mg/kg into 7-week-old male DBA/2-mdx mice once a week for 3 weeks. Body weight and food intake were assessed. All mice were housed at ~23 ± 1°C with 12 h light/dark cycles with free access to water and a normal diet. All animal experiments were performed according to the animal protocols approved by the Institutional Animal Care and Use Committee at Gachon University.

To verify the expression of GLP-1R in muscle tissue, we purchased floxed Glp1r mice (#09030) on a C57BL/6 background from the European Mouse Mutant Archive (EMMA), which allowed Cre-mediated recombination of GLP-1R. We crossed the floxed Glp1r mice with the Cre-expressing mouse line, β-actin Cre, to obtain wild-type (Glp1r\(^{+/+}\)Cre\(^{-}\)) and heterozygous (Glp1r\(^{lox/+}\)Cre\(^{-}\)) mice. These heterozygous (Glp1r\(^{lox/+}\)Cre\(^{-}\)) mice were mated to obtain homozygous (Glp1r\(^{lox/lox}\)Cre\(^{-}\)) mice.

**Cell culture**

C2C12 cells (CRL-1772, ATCC®, VA, USA) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Welgene, Daegu, Korea) supplemented with 10% foetal bovine serum (S001-07, Welgene, Daegu, Korea), 0.2 mM glutamine, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. To differentiate myoblasts from myotubes, C2C12 cells were seeded at 1 × 10\(^{6}\) cells per 6-well plate, and then the medium was replaced with a differential medium containing 2% horse serum (16050-122, Gibco, UK), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin for 5 days. In order to determine whether Ex-4 acts through GLP-1R, the C2C12 myotubes were pretreated with 20 nM exendin-9 (Ex-9), GLP-1R antagonist (E7269, Sigma-Aldrich, MO, USA) for 10 min and then treated with 20 nM Ex-4 for 30 min. To further investigate the molecular mechanism of Ex-4 action, C2C12 myotubes were first pretreated with 1 μM of Dex 6 h before treatment with 20 nM Ex-4, 20 nM MG132 (C2211, Sigma-Aldrich, MO, USA),
or 20 nM RU486 (M8046, Sigma-Aldrich, MO, USA) for further 6 h.

**Transfection**

C2C12 cells were seeded in 6-well plates with $1 \times 10^5$ cells per well and then differentiated into myotubes for 5 days using a differentiation medium. At 4 days during differentiation, the cells were transiently transfected with siRNA control (Bioneer, Daejeon, Korea) or siRNA GLP-1R (Bioneer, Daejeon, Korea) using Lipofectamine® RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After 6 h of transfection, the medium was replaced with differentiation medium, and then the cells were kept until 5 days. The cells were treated with 20 nM Ex-4 for 30 min at 5 days.

**Reverse transcriptase-quantitative polymerase chain reaction**

Total RNA was isolated from muscle tissue and C2C12 myotubes with RNeasy (9109, Takara, Japan) reagent according to the manufacturer’s protocol. The cDNA was synthesized from 2 μg total RNA using the PrimeScript 1st strand cDNA synthesis kit (6110A, Takara, Japan). Quantitative real-time PCR (qRT-PCR) was performed using CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The relative gene expression levels were normalized by cyclophilin RNA expression. The primer sequences used are shown in Table 1.

### Western blotting

Total protein was isolated using mammalian protein extract buffer (28-9712-79, GE Life Sciences, NY, USA) containing a protease inhibitor cocktail (P8340, Sigma-Aldrich, MO, USA). The cytosolic extract (CE) and nuclear extract (NE) were isolated from treated C2C12 myotubes as previously described. Western blotting was conducted with equal amounts of protein and reacted with the following antibodies: anti-MSTN (ab203076, Abcam, MA, USA), anti-atrogin-1 (ab74023, Abcam, MA, USA), anti-MuRF-1 (ab172479, Abcam, MA, USA), anti-actin (#37926, Abcam, MA, USA), and anti-desmin (sc-8046, Santa Cruz Biotechnology, CA, USA). The cytosolic extract (CE) and nuclear extract (NE) were isolated from treated C2C12 myotubes as previously described.

### Table 1 The list of primers sequences

| No. | Gene symbol   | Sense     | Sequences |
|-----|---------------|-----------|-----------|
| 1   | Mouse cyclophilin | Sense     | 5’ TGGAGAGCAGCACAAGAGACAGA 3’  |
|     |                | Anti-sense | 3’ TGGCGGAGTCAGCAATGAT 5’  |
| 2   | Mouse MSTN    | Sense     | 5’ GCCATGACTTGTGCAGAC 3’  |
|     |                | Anti-sense | 3’ TGGGTCGATAATCAGTC 5’  |
| 3   | Mouse atrogin-1 | Sense     | 5’ GAAACATCGCCACACTCTG 3’  |
|     |                | Anti-sense | 3’ CTGAGGGAAAGTCAAGC 5’  |
| 4   | Mouse MuRF-1  | Sense     | 5’ TGGACACAGAGGTAAAG 3’  |
|     |                | Anti-sense | 3’ TGTCTACCTACATTCTCTC 5’  |
| 5   | Mouse MyoD    | Sense     | 5’ TTCTATGC GCCACACT 3’  |
|     |                | Anti-sense | 3’ AAGTCGTCGCTGCTCAA 5’  |
| 6   | Mouse MyoG    | Sense     | 5’ CAAACCCAGGAGATCTTG 3’  |
|     |                | Anti-sense | 3’ AGATAGGACGTAAGGAGTG 5’  |
| 7   | Mouse KLF15   | Sense     | 5’ CACAAATGC ACTT CCTGAGG 3’  |
|     |                | Anti-sense | 3’ TGGACAACTCATCGAGGGG 5’  |
| 8   | Mouse Sesn1   | Sense     | 5’ TATGGCCATGC AAAAAATG 3’  |
|     |                | Anti-sense | 3’ TTCAAACATGCAGTGTAT 5’  |
| 9   | Mouse REDD1   | Sense     | 5’ CAGAGAGGGGGGCTG 3’  |
|     |                | Anti-sense | 3’ CCTATCCAGGAGGAGCTT 5’  |
| 10  | Mouse p85α    | Sense     | 5’ CAAAGCCAGGAGAATGCT 3’  |
|     |                | Anti-sense | 3’ ATAGCAGCCCC GCCTACTC 5’  |
| 11  | Mouse FoxO3α  | Sense     | 5’ GGAATGGGCAAAGAGCA 3’  |
|     |                | Anti-sense | 3’ AAACGGATCACTGCCCACCTG 5’  |

Journal of Cachexia, Sarcopenia and Muscle 2019: 10: 903–918
DOI: 10.1002/jcsm.12434
CA, USA). The target binding was detected using the Chemidoc™ XRS+ system with Image Lab™ Software (#1708265, Bio-Rad, CA, USA), and band density was quantified by the Image J program.

Measurement of protein synthesis

Protein synthesis was measured using the surface sensing of translation technique, as previously described. In brief, C2C12 cells were differentiated for 5 days. At 5 days, the cells were treated with or without 1 μM Dex for 6 h. After 6 h, 20 nM Ex-4 was added and incubated for further 6 h, followed by 1 μM puromycin (P8933-10MG; Sigma-Aldrich, St Louis, MO, USA) incubation. Cycloheximide, protein synthesis inhibitor (CHX, C7698-1G, Sigma-Aldrich), was used as a negative control treatment. Puromycin-labelled proteins were assessed by immunoblotting with an anti-puromycin antibody (MABE343; Millipore, Burlington, MA, USA) using an equal amount of total protein per sample.

Immunofluorescent staining

Immunofluorescent (IF) staining was performed as previously described. Briefly, the cells were fixed in 100% methanol or 10% neutral buffered formalin (NBF) for 20 min at RT and washed with PBS. The fixed cells were permeabilized at 25°C with PBS containing 0.27% Triton X-100 for all staining except GLP-1R staining. Cells were incubated with protein blocking solution (Dako, CA, USA) at 25°C for 1 h. The cells were exposed to anti-GR antibodies overnight at 4°C and then incubated with fluorescein isothiocyanate-conjugated secondary antibody (sc-2012, Santa Cruz Biotechnology, CA, USA) followed by 4′,6-diamidino-2-phenylindole staining. Fluorescent images of the cells were taken using laser scanning confocal microscopy (A1 plus, Nikon, Tokyo, Japan).

Measurement of cyclic adenosine monophosphate production

C2C12 myotubes were pretreated with 20 nM Ex-9 for 10 min and then treated with 20 nM Ex-4 for 30 min. The cells were washed and incubated with 0.1 M HCl for 30 min at 25°C, and then the supernatant was harvested after centrifugation. The cyclic adenosine monophosphate (cAMP) levels were determined using a Direct cAMP ELISA kit (ADI-901-006, Enzo Life Sciences, Lausen, Switzerland) according to the manufacturer’s protocol.

Immunoprecipitation assay

In order to perform the immunoprecipitation (IP) assay, cell lysates were first pre-cleaned, and then 400 μg of protein was incubated with 2 μg of primary antibodies (anti-GR antibody, anti-HSP70 antibody, anti-HSP90 antibody, anti-FKBP52 antibody, or anti-p23 antibody) overnight at 4°C. Subsequently, the cell lysates were further incubated with protein A/G-plus agarose beads (sc-2003, Santa Cruz Biotechnology, CA, USA) for 4 h at 4°C and then washed five times with mammalian protein extract buffer. The samples were stored at −80°C until use.

Grip strength/four-limb hanging test

Limb grip strength was measured in mice using a grip strength meter (BIO-G53, BIOSEB, FL, USA). Mice were lifted and held by their tail so that their limbs could grasp a wire grid. The mice were then gently pulled backward by the tail with their posture parallel to the surface of the table until they released the grid. The peak force applied by the limbs of the mouse was recorded in grams (g). Each mouse was tested three times, and the average value was used for statistical analysis. Four-limb hanging tests were performed as previously described. Briefly, each mouse was placed on a grid so that it grasped the grid with its four paws. The grid was inverted, and then the time the mouse spent hanging was measured for up to 10 min. The longer trial was used for analysis.

Tissue collection

At the end of each experiment, mice were weighed and humanely sacrificed. Blood samples were collected immediately before sacrifice after 4 h of fasting. Subsequently, the serum was separated and stored at −80°C until use. Skeletal muscles and other tissues were weighed and stored at −80°C for further analysis. For histological analysis, some parts of muscle tissues were embedded in optimal cutting temperature compound (4583, Tissue-Tek, CA, USA) or were fixed in 10% NBF.

Serum analysis

The levels of blood urea nitrogen (BUN) and creatinine in the serum were measured using the AU680 chemistry system (AU680, Beckman, CA, USA) as described in the creatinine (OSR6178, Beckman, CA, USA) and BUN (OSR6234, Beckman, CA, USA) kit instructions.
**Immunohistochemistry**

Frozen serial transverse cryosections (7 μm) from the tibialis anterior (TA) muscle of Dex and CKD mice were mounted on glass slides. Slides were fixed with pre-cooled 10% NBF for 20 min at 4°C and then washed three times with PBS. The slides were stained with haematoxylin and eosin (H&E) and observed under the light microscopy. Paraffin serial transverse sections (7 μm) of TA muscle of DBA/2J-mdx mice were also stained with H&E. The cross-sectional area (CSA) was assessed using the Image J program.

Frozen sections (7 μm) of muscle tissue were immunohistochemically stained for the expression of MSTN. The slides were fixed with pre-cooled 10% NBF for 20 min at 4°C and washed three times with PBS. To block endogenous peroxidase activity, the slides were dipped in 0.3% H2O2 solution in PBS and then incubated with a protein blocking solution (X0909, Dako, CA, USA) for 1 h at 25°C. Subsequently, the slides were exposed to anti-MSTN (ab996, Abcam, MA, USA) antibody overnight at 4°C followed by incubation for 30 min with biotinylated antibodies. The bound antibodies were detected with the DAB substrate chromogen system (K346811, Dako, CA, USA) and observed under the light microscope.

**Statistical analysis**

All values are expressed as the mean ± standard error. Statistical analysis was performed using one-way or two-way analysis of variance test of the IBM SPSS Statistics 19 program (IBM, NY, USA). Statistical significance was set at P < 0.05. All in vitro experiments were conducted at least three times independently, unless otherwise stated.

**Results**

**Ex-4 down-regulates the expression of myostatin and muscle atrophic factors in C2C12 myotubes**

MSTN is a negative regulator of muscle mass by up-regulating muscle atrophy-related factors such as Murf-1 and atrogin-1. Therefore, we first determined whether Ex-4 regulates MSTN expression in C2C12 myotubes after treatment with various concentrations of Ex-4 for 6 h. MSTN mRNA levels decreased in a dose-dependent manner up to 20 nM Ex-4 (Figure S1A). In a time course study using 20 nM Ex-4, the reduction of MSTN mRNA expression reached a maximum level at 6 h and gradually increased thereafter (Figure S1B). We then investigated whether Dex-induced MSTN expression is inhibited by Ex-4 in C2C12 myotubes. Dex treatment significantly up-regulated MSTN mRNA and protein expression, which was reduced by Ex-4 treatment (Figure 1A, B). Similarly, the expression pattern of atrogin-1 and Murf-1 was correlated with MSTN expression at both the mRNA and protein levels (Figure 1C, D). In contrast, myogenic factors including MyoD and myogenin (MyoG) were significantly reduced in Dex-treated condition, but recovered by Ex-4 treatment. Ex-4 treatment alone also increased the expression of myogenic factors per se as shown in Figure 1E. We also measured protein synthesis in C2C12 myotubes by quantifying puromycin-labelled proteins. Ex-4 treatment significantly increased protein synthesis compared with that in the control without Ex-4 treatment. Dex treatment significantly reduced protein synthesis compared with that in the control, but this was reversed by Ex-4 treatment (Figure 1F).

**Ex-4 regulates the expression of myostatin through glucagon-like peptide-1 receptor-mediated protein kinase A and AKT signalling pathways**

GLP-1R expression in muscle tissues and muscle cells is controversial. Therefore, we first investigated whether GLP-1R is expressed in muscle tissue and C2C12 myotubes. GLP-1R was abundantly expressed in the pancreas and at lower levels in the TA muscle of WT mice. The GLP-1R expression level decreased by around 50% in heterozygous (GLP1R<sup>lox/cre</sup>) mice compared with WT (GLP1R<sup>lox/cre</sup>) and was barely detectable in homozygous (GLP1R<sup>lox/lox</sup>cre<sup>-/+</sup>) mice (Figure 2A). GLP-1R expression was also detected in C2C12 myotubes, as confirmed by the results for both IF staining and western blotting, although the expression level was lower than that of INS-1 cells (Figure S2A, S2B). Next, we determined whether GLP-1R-mediated cAMP production is actually activated by Ex-4 in C2C12 myotubes. Ex-4 increased cAMP production by 50% compared with control vehicle but reduced in the presence of the GLP-1R antagonist Ex-9, suggesting that Ex-4 acts through GLP-1R in C2C12 myotubes (Figure 2B). Increased cAMP production subsequently activated two downstream signalling levels of PKA and AKT signalling as shown by increased phosphorylated PKA (Thr197) and AKT (Ser473) (Figure 2C, D, Figure S3A, S3B). As expected, the activated PKA signalling increased its target protein expression of HSF-1, whereas the elevated AKT signalling suppressed the activation of NF-κB (Ser536) (Figure 2C, D, Figure S3A, S3B). In contrast, this activity was attenuated by pretreatment with Ex-9 (Figure 2C, D; Figure S3A, S3B). It is well known that NF-κB binds to the promoter region of MSTN, which leads to the induction of its expression. Consistent with the reduction of NF-κB activity by Ex-4 treatment, MSTN expression was decreased by Ex-4 treatment, and this decrease was inhibited by Ex-9 treatment (Figure 2E, Figure S3C). Similarly, GLP-1R knockdown (Figure 2F, Figure S3D) inhibited the up-regulation of upstream anabolic signalling pathways (p-PKA/
PKA, p-AKT/AKT) and its target gene (HSF-1/actin) and blunted the decrease in p-NF-κB signalling pathway activation induced by Ex-4 (Figure 2G, H, Figure S3E, S3F). In addition, GLP-1R knockdown abolished the down-regulation of MSTN expression induced by Ex-4 (Figure 2I, Figure S3G). Collectively, these results suggest that the effects of Ex-4 are mediated by the GLP-1R signalling pathway.

**Ex-4 inhibits the translocation of glucocorticoid receptor from the cytosol into the nucleus**

GC degrades skeletal muscle proteins through its receptor, GR, by regulating its target genes involved in the ubiquitin proteasome system (UPS). To examine whether Ex-4 affects GR translocation, we performed IF staining in C2C12 myotubes. The GR expression in the nucleus was increased by Dex but decreased with Ex-4 treatment (Figure 3A). GR protein expression decreased in the CE of Dex-treated C2C12 myotubes but increased in NE. In contrast, Ex-4 treatment resulted in a totally opposite trend as shown in Figure 3B and Figure S4A. We further investigated the expression level of GR target genes such as Krüppel-like factor 15 (KLF15), sestrin 1 (Sesn1), regulated in development and DNA damage responses 1 (REDD1), phosphatidylinositol 3-kinase regulatory subunit α (p85α), and Forkhead box O3a (FoxO3a) in C2C12 myotubes treated with Ex-4. The KLF15, Sesn1, REDD1, p85α, and FoxO3a mRNA expression levels were up-regulated in Dex-treated C2C12 myotubes, whereas they were decreased by Ex-4 or RU486, a GR inhibitor (Figure 3C), indicating that Ex-4 inhibits GR translocation from the cytosol into the nucleus, thereby down-regulating the expression of GR target genes.

**Ex-4 up-regulates the proteins of glucocorticoid receptor inhibitory complexes in C2C12 myotubes**

GR is a ligand-dependent transcription factor whose inactive form is associated with chaperone complexes, including...
HSP90, HSP70, FKBP52, and p23. Once GC binds to GR within GR inhibitory complexes, the inhibitory complexes are separated from GR and then degraded by 26S proteasome in the cytoplasm, allowing GR translocation to nucleus. To investigate whether Ex-4 affects the interaction of GR and GR inhibitory complex proteins, the affinity of GR to GR inhibitory complex proteins was assessed using the IP assay. The amount of proteins (HSP90, HSP70, FKBP52, or p23) bound to GR was decreased in Dex-treated C2C12 myotubes but was increased by Ex-4 (Figure 3D). Examination of the expression of GR inhibitory complex proteins in cytoplasm showed that Dex treatment significantly reduced the expression of HSP90, HSP70, FKBP52, and p23 in cytoplasm. However, this reduction was inhibited by Ex-4 treatment. Treatment with RU486, a GR inhibitor, yielded similar results (Figure 3E, Figure S4B), suggesting that Ex-4 up-regulates the expression of GR inhibitory complex proteins and increases the interaction between GR and GR inhibitory complex proteins, thereby inhibiting GR translocation into the nucleus. 26S proteasome expression was not changed by Ex-4 treatment (Figure 3F).
**Ex-4 ameliorates muscle wasting in Dex-induced muscle atrophy mice**

To determine whether Ex-4 affects muscle atrophy in vivo, we treated Dex-induced muscle atrophy mice with Ex-4. The body weight decreased after Dex administration, but the Ex-4 treatment gradually recovered the loss of body weight in Dex-administered mice (*Figure 4A*). The Ex-4 alone injected group had reduced body weight as compared with that of the control vehicle group (*Figure 4A*). Food intake decreased in all three groups except for the control vehicle group (*Figure S5A*). Total muscle weight decreased in the Dex-administered mice but significantly increased with Ex-4 treatment (*Figure 4B*). Consistent with this, weights of the gastrocnemius, TA, quadriceps, and extensor digitorum longus were reduced in Dex-administered mice, but Ex-4 treatment recovered to the levels of control group. However, the weight of soleus (SOL) was identical among all groups (*Figure 4C*). In addition, the weight of white adipose tissue (WAT), including epididymal and inguinal adipose tissues, was lower in other three groups (Con + Ex-4, Dex + Vehicle, and Dex + Ex-4) than in the control group but comparable within three groups (*Figure S5B*). The CSA of TA muscle was reduced by ~40% by Dex treatment compared with the control vehicle group. However, this decrease was significantly recovered by as much as the control level by Ex-4 treatment. Treatment with Ex-4 alone also significantly increased CSA, compared with the control group (*Figure 5A, B*). Moreover, the reduced grip strength in Dex-treated mice was recovered by Ex-4 treatment, which

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*Figure 3* Ex-4 inhibits the translocation of GR from cytosol into nucleus by up-regulating the proteins of GR inhibitory complexes. (A) C2C12 myotubes treated with 1 μM Dex at 6 h prior to treatment of with or without 20 nM Ex-4 for 6 h. GR expression was determined by immunostaining with anti-GR (green). The nucleus was detected by 4′,6-diamidino-2-phenylindole (DAPI) staining (blue) (magnification ×400). (B) The treated C2C12 myotubes were separated into cytosolic extract (CE) and nuclear extract (NE). The protein level of GR was measured in CE and NE using western blotting. (C) The differentiated C2C12 myotubes were pretreated with 1 μM Dex and then 6 h later incubated with 20 nM Ex-4 or RU486 until 12 h. The CE were isolated and then subjected to western blotting and probing with appropriate antibodies. (D) 26S proteasome level was assessed by western blotting. All value expressed as the mean ± standard error. Significant differences are indicated as **P < 0.01, *P < 0.05** compared with Con + vehicle or Con + Dex. n = 3. Con, control; Dex, dexamethasone; Ex-4, exendin-4.
was positively correlated to the CSA of the TA muscle (Figure 5C). Taken together, these effects of Ex-4 result in increasing muscle strength and function. In line with aforementioned data, the level of BUN, a catabolism marker in the body, was significantly reduced in the Ex-4 treatment groups compared with Dex-administered group (Figure 5C).

Mechanistically, Dex up-regulated the expression of MSTN, atrogin-1, and MuRF-1 (Figure 5D, E, F) but suppressed that of myogenic factors including MyoD and MyoG (Figure 5G). In contrast, Ex-4 administration prevented the atrophic effect of Dex in TA muscle by directly reducing the expression of muscle atrophy factors and increasing the expression of myogenic factors (Figure 5D, E, F, G). In agreement with the results for myogenic factors expression, Ex-4 restored the expression levels of myofibrillar proteins, such as tropomyosin, MHC, and desmin, which were decreased by Dex treatment in the TA muscle of mice (Figure 5H).

**Glucagon-like peptide-1 receptor agonists increase muscle mass and improves muscle functions in chronic kidney disease and in DBA/2j-mdx mice**

Because the muscle atrophic phenotype is often observed in patients and animal models with CKD,1,2,18 we further investigated whether Ex-4 ameliorates muscle atrophy using mice with partially removed kidney through subtotal nephrectomy. We first verified the CKD mouse model having increased BUN and creatinine levels in the serum because these are known indicators of kidney function and catabolism. We found that treatment with Ex-4 reduced these levels (Figure 6A). The body weight decreased in CKD mice compared with sham mice; however, Ex-4 administration to CKD mice, compared with the vehicle-treated mice, significantly increased body weight (Figure 6B). Body weight was continuously increased in sham + vehicle group and sham + Ex-4 groups, but the level...
of increase was lower in sham + Ex-4 group probably owing to the lower food intake. Total muscle mass was increased by Ex-4 treatment in CKD mice compared with vehicle-treated mice (Figure 6C). In addition, Ex-4 treatment also increased muscle mass in sham-operated, vehicle-treated mice. The CSA of TA muscle was significantly decreased in CKD mice but was significantly increased by Ex-4 treatment (Figure 6D, E). The Ex-4 treatment also enhanced grip strength in CKD mice compared with vehicle treatment group (Figure 6F), suggesting that Ex-4 improves muscle function in a mouse model of CKD.

We then examined the therapeutic effect of GLP-1R agonist in a neuromuscular disease caused by dystrophin deficiency. Administration of a long-acting GLP-1R agonist, dulaglutide (1 mg/kg/week) for 3 weeks, to DBA/2J-mdx mice did not show the differences in body weight between vehicle and dulaglutide treatment group (not shown data). However, dulaglutide treatment increased the CSA of TA muscle (Figure 7A, B) and grip strength than did vehicle treatment (Figure 7C, D). Dulaglutide also substantially increased the four-limb hanging time in DBA/2J-mdx mice (Figure 7E).

**Discussion**

GLP-1 and GLP-1R agonists have been commonly used as anti-diabetic drugs to lower blood glucose levels. In skeletal muscles, Ex-4 regulates glucose uptake and oxygen consumption, as well as increases insulin sensitivity. However,
Recent studies have shown that GLP-1R agonists have therapeutic effects on muscle atrophy in various models, including a dex-induced muscle atrophy mouse model and a CKD-derived muscle atrophy model. The GLP-1R agonist Ex-4 was found to down-regulate MSTN mRNA and protein expression in C2C12 myotubes in the presence or absence of Dex. The up-regulation of muscle atrophic factors and down-regulation of myogenic factors (MyoD and MyoG) by Dex in C2C12 myotubes was reversed by Ex-4 treatment, indicating that Ex-4 may regulate muscle atrophic factors, at least partly, by regulating MSTN expression. The expression of GLP-1R in muscle is controversial, because both expression and non-expression have been reported. In order to carefully address this debatable issue, we first tested several commercially available antibodies from different companies and also used muscle tissues from Glp1r−/− mice to check the specificity of the tested antibodies. Our data showed that GLP-1R is highly expressed in the pancreas and lower expression in muscle tissues. However, it was barely detectible in homozygous Glp1r−/− mice. These data strongly indicate that GLP-1R is expressed in muscle.

Figure 6 Ex-4 increases total muscle mass and improves muscle functions in CKD mice. Ten-week-old C57BL/6 male mice underwent two-step subtotal nephrectomy to induce muscle atrophy. (A) The serum level of blood urea nitrogen (BUN) and creatinine. (B) The body weight change during treatment. Black arrow indicates the start of Ex-4 injection. Significant differences are indicated as **P < 0.01, *P < 0.05 compared with sham + vehicle; ###P < 0.01, ##P < 0.05 compared with CKD + vehicle. (C) The weight of total muscle tissue. Total muscle weight was normalized to final body weight (g). (D) Frozen serial transverse cryosections (7 μm) of TA muscle were stained with H&E and examined under a microscope (magnification ×200). (E) The CSA of muscle fibre was measured using Image J program. (F) The grip strength was normalized to the final body weight (g). All values are expressed as the mean ± standard error. Significant differences are indicated as **P < 0.01, *P < 0.05 compared with sham + vehicle or CKD + vehicle. n = 5–7/group.
tissue and C2C12 myotubes, as observed in human muscle satellite cells and cardiomyocytes.\(^{25,45}\)

Silveira et al. reported that activated cAMP/PKA signalling in skeletal muscle suppresses the UPS\(^{46}\) and activated AKT signalling induces protein synthesis in skeletal muscle.\(^{47-49}\)

Our data showed that Ex-4 treatment activates both PKA and AKT signalling pathways and inhibits phosphorylated NF-κB protein expression. Activated AKT signalling can directly suppress the phosphorylated protein level of NF-κB in C2C12 myotubes, which may in turn reduce the binding of NF-κB to promoter regions of MSTN.\(^{50}\) As expected, Ex-4 treatment decreased MSTN protein expression and Ex-9 or GLP-1R knockdown inhibited this effect, suggesting that the GLP-1R-mediated signalling pathway might be involved in regulation of MSTN expression. PKA is known to activate HSF-1,\(^ {51}\) which in turn regulates HSP70 expression through the regulatory heat-shock elements on its promoter.\(^ {52}\) Consistent with these data, our data also showed that Ex-4 up-regulated HSF-1 expression through GLP-1R signalling, suggesting that Ex-4 can increase the expression of HSP70, a component of the GR inhibitory complex.

In the cytosol, the increased GCs form complex with GR and then move to the nucleus for binding to the GC response element on the promoter of GR target genes (MSTN, KLF15, and FoxO3a).\(^ {29,53-55}\) They are well known to activate UPS through E3 ligase that degrades muscle proteins.\(^ {26,55,56}\) Our data showed that Ex-4 inhibited GR translocation into the nucleus and down-regulated GR-targeted genes (KLF15, FoxO3a, REDD1, p85α, Sesn1), which was similar to the effects of the GR inhibitor, RU486.

**Figure 7** Dulaglutide improves muscle strength in DBA/2J-mdx mice. Seven-week-old DBA/2J-mdx male mice were subcutaneously administered with 1 mg/kg of dulaglutide once a week for 3 weeks. (A) Sections of TA muscle were stained with H&E and examined under a microscope (magnification ×200). (B) The CSA of muscle fibre was measured using Image J program. (C) The grip strength before administration of dulaglutide. (D) The grip strength after administration of dulaglutide. The grip strength was normalized to the final body weight (g). (E) Four-limb hanging test after administration of dulaglutide. The left bar graph shows the average of total hanging time of each group; the right dot graph shows the distribution of individual mice for hanging time. All values are expressed as the mean ± standard error. Significant differences are indicated as **P < 0.01, *P < 0.05 compared with vehicle. n = 9–10/group.
GR interacts with chaperone proteins (HSP70, HSP90, FKBP52, and p23) in the cytoplasm, which functionally inhibits GR translocation to the nucleus. 56 Once GCs bind to GR; these interaction proteins are separated from the GR and then are degraded by the 26S proteasome. 57 HSP70, a member of the heat-shock protein family, is elevated during cellular stress conditions in skeletal muscle. Previous studies report that the overexpression of HSP70 in mouse skeletal muscle protects against muscle damage and age-related muscle dysfunction 58 and reduces MuRF-1 and atrogin-1 promoter activity in a rodent model of disused muscle atrophy. 59 Ex-4 treatment increased the affinity of GR and GR inhibitory complexes by increasing the levels of HSP70, HSP90, FKBP52, and p23 in Dex-treated C2C12 myotubes. However, Ex-4 per se did not increase the expression of 26S proteasomes, indicating that the accumulation of GR inhibitory complex is not due to a reduction in the level of the 26S proteasome. Collectively, these data suggest that inhibition of the GCs–GR complex translocation to the nucleus is at least partially due to the up-regulation of inhibitory complexes of proteins.

Inflammation is a risk factor for inducing muscle atrophy by regulating the NF-κB signalling pathway 60,61 and the suppression of inflammation reverses muscle atrophy. 61,62 In fact, GLP-1R agonists have shown anti-inflammatory effects in many different diseases. 63 Our result showed that Ex-4 treatment inhibited the activation of NF-κB, a major transcription factor involved in the inflammatory response. Therefore, it is quite possible that the anti-inflammatory effects of Ex-4 may also contribute to the amelioration of muscle atrophy. Furthermore, GLP-1R agonists increase the expression of MyoD and MyoG, which are known as major transcription factors involved in the muscle tissue repair of satellite cells. 64,65 The PKA-mediated CAMP response element binding protein is activated by muscle injury and promotes muscle regeneration, 56–68 suggesting that CAMP/PKA signalling mediated by GLP-1R might give beneficial effects on recovery from muscle atrophy. Further studies are required to clearly identify these mechanisms.

We then investigated whether GLP-1R agonists have therapeutic effect on pharmacologically, surgically, or genetically induced muscle atrophy in vivo. Dex administration in mice reduced the weight of all muscle types assessed in the current study, but these were statistically recovered to the level of control group by Ex-4 treatment. The exception was the SOL muscle, and this may be due to the lower expression level of MSTN in SOL than other types of muscle, suggesting that MSTN is a potential mediator of Ex-4 effects. 69,70 The beneficial effects of Ex-4 were established by the down-regulation of muscle-specific E3 ligase proteins such as atrogin-1 and MuRF-1, while up-regulating myogenic factors including MyoD and MyoG. As a consequence, muscle mass and muscle function improved in Dex-induced muscle atrophy mice. In support of our results, recent studies reported that the inhibitors of DPP4, a GLP-1 degradation enzyme, are associated with improvement of reduced muscle mass in diabetic and elderly diabetic patients15,71 and with improvement of mitochondrial biogenesis in a heart failure mouse model.16

CKD is a model of cachexia that is mainly induced by inflammatory cytokines in the human pathological state. 18 Similar to the observations in Dex-treated mice, Ex-4 significantly increased total muscle mass, CSA of the TA muscle, and muscle function in CKD mice. Ex-4 and sitagliptin (DPP4 inhibitor) are known to improve kidney function by attenuating inflammation in chronic or diabetic kidney disease models. 17,72 Therefore, it is unclear whether the beneficial effect of Ex-4 is due to its role in improving kidney function, its direct effect on muscle, or both in the current study. Further studies are needed to clarify this. Duchenne muscular disease is a neuromuscular disease caused by dystrophin deficiency and is the most common and severe form of muscular dystrophy. 73 Along with highlighting high clinical potential, our data also showed that a long-acting GLP-1R agonist, dulaglutide, substantially improved muscle atrophy in DBA/2J-mdx mice. Collectively, our findings strongly suggest that GLP-1R agonists may have high translational value for treating diverse types of muscle atrophy.

The reduction in fat deposits and reduction in food intake, which are known effects of GLP-1R agonists, are not desirable effects in chronic wasting conditions. However, our data showed that Ex-4 treatment did not affect food intake in Dex-induced (Figure S5) or CKD-induced muscle atrophy models (data not shown). In addition, body weight increased after Ex-4 treatment, although the changes were very modest in both models. Liver weights were comparable between the sham and CKD groups, regardless of Ex-4 treatment (data not shown). These data suggest that Ex-4 plays different roles in pathological conditions and that the beneficial effect of Ex-4 appears to override the negative effect of reduced food intake. However, appropriate precautions are needed for clinical application of Ex-4.

In conclusion, we found that the GLP-1R agonist has therapeutic effects on muscle atrophy by suppressing MSTN and muscle atrophic factors and enhancing myogenic factors via GLP-1R signalling-mediated regulation and suppression of GR translocation. Our data provide proof-of-concept evidence for the therapeutic effects of GLP-1R agonists on muscle atrophy. These findings highlight the potential application of GLP-1R agonists for the treatment of muscle wasting diseases.

Acknowledgements

This study was supported by grants from the Korea Health Technology R&D Project through the Korea Health Industry
Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea (grant number HI14C1135), and the National Research Foundation of Korea (NRF 2017R1D1A1B03036210). The authors certify that they comply with the ethical guidelines for authorship and publishing of the *Journal of Cachexia, Sarcopenia and Muscle.*

**Online supplementary material**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Ex-4 downregulates MSTN mRNA expression. A-B. C2C12 myoblasts were differentiated into C2C12 myotubes for 5 days. C2C12 myotubes were treated with A. the indicated concentrations of Ex-4 for 6 h and B. for the indicated times with 20 nM of Ex-4. mRNA levels of MSTN were measured using RT-QPCR. All values are expressed as the mean ± SE. Significant differences are indicated as **p<0.01, *p<0.05 compared with Ex-4 (0 nM) or Ex-4 (0 h).** n=3.

**Figure S2.** GLP-1R is expressed in muscle tissue and C2C12 cells. A. INS-1 and C2C12 myotubes were immunostained with anti-GLP-1R antibody (green) and DAPI (blue for the nucleus). Cells were observed under a confocal microscope (magnification 200×). B. The protein expression levels of GLP-1R were determined among INS-1 cells, C2C12 myoblast and myotubes using western blotting.

**Figure S3.** Ex-4 regulates the expression of MSTN through GLP-1R mediated PKA and AKT signaling pathways. A-C. The quantitative data for the expression of GLP-1R downstream mediators such as PKA and HSF-1 (A), AKT and NF-κB (B) by Ex-9. C. Quantitative graph of MSTN protein levels. D-G. GLP-1R siRNA-transfected C2C12 myotubes were treated with 20 nM Ex-4 for 30 min. D. The quantitative data for the protein levels of GLP-1R and GLP-1R downstream mediators including PKA and HSF-1 (E), AKT and NF-κB (F) by GLP-1R siRNA. G. Quantitative graph of MSTN protein levels. All values are expressed as the mean ± SE. Significant differences are indicated as **p<0.01, *p<0.05 compared with Con+Vehicle or Con+Ex-4, compared with Con siRNA+Vehicle or Con siRNA+Ex-4.** n=3. Con, Control; Ex-4, Exendin-4.

**Figure S4.** Ex-4 inhibits the translocation of GR from cytosol into nucleus by upregulating the proteins of GR inhibitory complexes. A. The quantitative graph for GR protein level in CE and NE using western blotting. B. The proteins level of GR inhibitory complexes. C2C12 myotubes were pretreated with 1 μM Dex, and then 6 h later, incubated with 20 nM Ex-4 or RU486 until 12 h. The CE were isolated and then subjected to western blotting and probing with appropriate antibodies (HSP70, HSP90, FKBP52 and p23). All values are expressed as the mean ± SE. Significant differences are indicated as **p<0.01, *p<0.05 compared with Con+Vehicle or Con+Dex.** n=3. Con, Control; Dex, Dexamethasone; Ex-4, Exendin-4.

**Figure S5.** Ex-4 reduced food intake and catabolic effects in Dex-administered mice. A. Food intake. B. Total weight of white adipose tissue (WAT). C. The serum levels of BUN. All values are expressed as the mean ± SE. Significant differences are indicated as **p<0.01, *p<0.05 compared with Con+Vehicle or Con+Dex.** n=5-8/group. Con, Control; Dex, Dexamethasone; Ex-4, Exendin-4. 

Supplementary Figure legends

**Conflict of interest**

Y.H.H., J.H.L., K.W.J., C.S.C., and H.-S.J. declare that they have no conflict of interest.

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